Universidade de Aveiro Departamento de Biologia
2020

Rita Carina Saraiva Silva Bicho

longINg_nano: Efeitos de exposição longa e multigeracionais de nanomateriais em invertebrados terrestres

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Mónica Amorim, Investigadora Principal do Departamento de Biologia da Universidade de Aveiro, e co-orientação do Doutor Janeck James Scott-Fordsmand (Investigador Sénior da Universidade de Aarhus, Dinamarca).

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Dedico este trabalho ao Francisco, á minha filha Leonor e à minha família pelo apoio.

o júri

Presidente

Prof. Doutor João Carlos de Oliveira Matias
Professor catedrático do Departamento de Departamento de Economia, Gestão, Engenharia Industrial e Turismo da Universidade de Aveiro

vogais

Prof. Doutor Augusto Manuel Rodrigues Faustino
Professor associado do Instituto de Ciências Biomédicas Abel Salazar (ICBAS) da Universidade do Porto

Prof. Doutor Tiago Manuel Ferreira Natal-da-Luz
Investigador Doutorado no Departamento de Ciências da Vida da Universidade de Coimbra

Prof. Doutora Isabel da Silva Henriques
Professora auxiliar no Departamento de Ciências da Vida da Universidade de Coimbra

Prof. Doutor Amadeu Mortágua Velho da Maia Soares
Professor catedrático do Departamento de Biologia da Universidade e Centro de Estudos do Ambiente e do Mar (CESAM) de Aveiro

Doutora Mónica João de Barros Amorim (orientadora)
Investigadora principal com agregação do Departamento de Biologia e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro

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palavras-chave

Nanomateriais, exposição de longa duração, ciclo de vida completo, estádios de vida, resposta multigeracional e transgeracional, epigenética, via metabólica adversa, ecotoxicologia de solo, enquitraídeos, cobre, prata.

resumo

Ainda nos dias de hoje as expectativas para o mercado internacional de nanomateriais (NMs) projectados é de um crescimento exponencial contínuo. Isto é em parte devido ao facto de que o número de aplicações para estes NMs continua a crescer. Inevitavelmente este uso acarreta risco para o ser humano e para o ambiente. Para o ambiente terrestre existe um risco acrescido devido á presença destes materiais em biossólidos, que permanecem como uma fonte de NMs. Nas últimas décadas tem-se feito progressos no sentido de melhorar o conhecimento sobre o perigo dos NMs e a avaliação do risco ambiental. Sobre a investigação realizada até então ficou claro que os resultados fornecidos pelos testes padrão, que incluíam efeitos de curta duração, não eram suficientes para avaliar os efeitos dos NMs. Assim sendo, dados sobre exposições de longa duração eram necessários urgentemente. Exposições multigeracionais eram particularmente recomendadas, uma vez que fornecem informação sobre os efeitos nas pós-gerações e efeitos transgeracionais. Por outro lado, existia um aumento contínuo sobre o número de evidências que reportavam que os NMs podem afectar mecanismos de epigenética, ao mesmo tempo, a epigenética poderia ajudar a explicar os efeitos multigeracionais. Assim, a epigenética tornou-se uma ferramenta importante a incluir na avaliação do perigo e do risco ambiental dos NMs.

O objectivo desta tese foi desenvolver novos ensaios de ecotoxicidade para o invertebrado de solo *Enchytraeus crypticus*, que é uma espécie modelo padronizada, para avaliar efeitos de longa duração dos NMs. Para além disso, tinha-se como objectivo investigar efeitos de epigenética e outras respostas gerais de stresse, nos diferentes níveis de organização biológica, que decorriam destas exposições de longa duração aos NMs. Finalmente pretendia-se também integrar todos os dados no formato de via metabólica adversa (VMA).

Para a inclusão de efeitos de longa duração, testes de ciclo de vida completo (CVC) e multigeracionais (MG) foram desenvolvidos para o *E. crypticus*. Os organismos foram expostos a NMs de prata (Ag), NMs de óxido de cobre (CuO), carboneto de tungsténio e cobalto (WCCo) nanoestruturado e os correspondentes compostos na forma iónica (para comparação). As exposições de CVC mostraram efeitos específicos para os NMs em relação á sua forma iónica. As exposições MG mostraram diferentes respostas dos organismos para os NMs em relação á sua forma iónica, contudo para ambas as formas houve uma transferência de efeitos para as subsequentes gerações expostas e gerações não expostas, estas indicando efeitos transgeracionais. Para além disso, a avaliação da toxicidade epigenética nestas exposições MG foi positiva para os NMs e respectiva forma iónica. Finalmente a integração dos dados de (eco)toxicoepigenética, nos diferentes níveis de organização biológica, no formato de VMA, provou ser muito útil para distinguir entre NMs e a forma iónica, eventos moleculares e eventos chave.

Em suma a investigação feita durante esta tese providenciou um suporte importante para a avaliação de efeitos de longa duração dos NMs no solo e, para a inclusão da avaliação epigenética na ecotoxicologia de solo.

keywords

Nanomaterials, long term exposure, full life cycle, life stages, multigenerational and transgenerational response, epigenetics, adverse outcome pathway, soil ecotoxicology, enquitraeids, copper, silver.

abstract

Till today, the expectations for the international market of engineered nanomaterials (NMs) are a continuous exponential grow. This is partly due to the number of applications that continues to increase. Inevitably this usage poses a risk for humans and the environment. The terrestrial compartment is at particular risk due to the presence of these materials in biosolids, remaining as a sink. Over the last decades progress has been made to improve the knowledge of NMs hazard and environmental risk assessment (ERA). Overall from the research made it was clear that results provided by most standard tests, that covered short term effects were not enough to evaluate effects of NMs. Hence data from long term exposures was urgently needed. Multigenerational exposures were highly recommended once they can provide information on post-generational and transgenerational effects. On the other hand, there was a continuously increase in the number of evidences that NMs can affect epigenetic mechanisms, and that epigenetics could help to explain multigenerational effects. So, epigenetics came as an important tool to include on the evaluation of NMs hazard and ERA.

The aim of this thesis was to develop new ecotoxicity assays for the soil invertebrate standard model species *Enchytraeus crypticus*, to evaluate long term effects of NMs. Further it was aimed to investigate epigenetic effects, and other stress responses at different levels of biological organization, incurring from the long term exposures to NMs. Finally, it was intended to integrate all the data and build onto an adverse outcome pathway (AOP) framework.

To cover long term effects, full life cycle (FLC) and multigenerational (MG) tests were developed for *E. crypticus*. Organisms were exposed to silver (Ag) NMs, coper oxide (CuO) NMs, Nanostructured Tungsten Carbide Cobalt (WCCo) and the corresponding ionic forms (for comparison). FLC exposures showed life stage effects which were specific for the NMs in relation to the ionic form. MG exposures showed different organisms´ responses between NMs and the ionic form, however for both forms there was transference of effects to subsequent exposed generations and unexposed generations, indicating transgenerational effects. Moreover, evaluation of epigenetic toxicity from these MG exposures was positive for both NMs and ionic form. Finally the integration of (eco)toxicoepigenetics data, at the several levels of biological organization, into the AOP framework proved to be very useful to distinguish different molecular initiating and key events, between NMs and the ionic form.

In sum the research done during this thesis provided a stepping stone for the long term assessment effects of NMs in soil, and for the inclusion of evaluation of epigenetics in soil ecotoxicology.

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Chapter I - General Introduction

1.1 Nanomaterials

The world-wide market of engineered nanomaterials (NMs) is expected to continue to grow at an exponential rate and presently at 2020 estimations for the market value are about 11.3 billion USD (Inshakova and Inshakov, 2017). This is due to the continuous growth in number of applications. Examples of NMs usage include paints and coatings for the aerospace, automotive and construction industries, healthcare, sport goods, cosmetics, food and beverages, home and garden, electronics and computing, among many others (Inshakova and Inshakov, 2017; Jeevanandam et al., 2018). Inevitably all these applications and usage poses a risk for humans and the environment. Particularly for the terrestrial environment following the actual trends of NMs usage, titanium dioxide (TiO_2), zin oxide (ZnO) and silver (Ag) NMs and their transformed by-products are expected to be present in considerable quantities as a consequence of land application of biosolids (Lead et al., 2018). Also copper (Cu) NMs are extensively used as wood-preservatives e.g. estimated as 50% of the Cu containing of these products on the North American market (valued at 4.9 billion USD) (Evans et al., 2008). Further, copper oxide (CuO) NMs are also expected to be present in soils since they are used in several applications, e.g. in fungicides (Gogos et al., 2012; Weitz et al., 2015), fertilizers, additives for soil remediation, and as growth regulators in plants (Zhu et al., 2012). So, over the last 20 years this topic has caught the attention of scientists, regulators, policy makers and the general public and a big effort has been made by OECD to improve NMs toxicity testing guidelines (Rasmussen et al., 2019). Nevertheless, still to today there is no international regulation for production, handling or labelling or defined protocols to evaluate (eco)toxicological effects of NMs (Jeevanandam et al., 2018).

1.2 Nanomaterials transformations and long term exposure assessment

For a proper hazard and risk assessment, to know about NMs properties and transformations in the environmental media is crucial, since these will affect their fate, behaviour, and ecotoxicity (Louie et al., 2014). Among some of the major transformations for NMs in different media are related to ligation, dissolution, aggregation/agglomeration and adsorption. Specifically for metallic engineered NMs like Zn, Cu, cadmium (Cd) or Ag, their solubility is high, so to understand their dissolution rate in the different media is a very important property to evaluate their toxicity (Misra et al., 2012). Nevertheless, the literature shows that effects of NMs can be attributed to nanoparticulate specific effects, the ionic fraction or the

combination of both (Lead et al., 2018). For example for ZnO NMs mostly effects are explained by the ionic fraction (Franklin et al., 2007). Whereas for Ag NMs (Leclerc and Wilkinson, 2014) and CuO NMs (Hartmann et al., 2014) effects were attributed to both ions and nanoparticles (NPs). Hence for regulatory purposes there is still the debate if the current legislation for the ionic counterpart is sufficient to ensure safety of these NMs (Hund-Rinke et al., 2016; Pettitt and Lead, 2013). In complex media like soil, NMs dissolution rate can be slower. For example for Ag NMs it was shown slow dissolution rates in soil, providing a constant source of released ions so over time the toxicity of these NMs increased (Diez-Ortiz et al., 2015). Hence long term studies are most needed to evaluate effects of NMs, but in soil compared to aquatic studies they are still scarce (Diez-Ortiz et al., 2015; Lead et al., 2018). Further, many times ecotoxicity studies with terrestrial organisms are performed in artificial scenarios like hydroponics (Lead et al., 2018). Above all chronic toxicity data and long term exposure effects are amongst the top recommendations for NMs risk assessment for obvious reasons, the increase in relevancy and decrease in uncertainty (Dekkers et al., 2016; Gedda et al., 2019; Oomen et al., 2018; Scott-fordsmand et al., 2018).

1.3 Mechanistic data and grouping of nanomaterials

Further on the improvement of nano-risk assessment, NMs grouping and prioritization is another urgent recommendation, but for this to happen, to understand the mechanisms of NMs toxicity is essential (Dekkers et al., 2016; Scott-fordsmand et al., 2018). Mechanistic based knowledge can be provided i.e. beyond information of apical endpoints (survival, reproduction) as covered by most standard guidelines, for example with the application of multi-omic tools that can to provide information of effects at lower levels of biological organization. This can help to identify the molecular initiating event(s) (MIE) for the affected pathway leading to an adverse outcome, like in the principle of the Adverse Outcome Pathway (AOP) (Ankley et al., 2010) framework. Several stressors, including NMs, can have similar key events (KEs) at the commonly measured higher levels of biological organization, e.g. reproduction, the main difference being the MIE (Gerloff et al., 2017). Hence, mechanistic based knowledge can be used to evidence possible initiating differences and if these may lead to longer term consequences that differ.

1.4 Multigenerational exposure and epigenetic effects of nanomaterials

Included in the evaluation of long term exposure effects, in particular multigenerational studies can provide information on post-generational and transgenerational effects. Indeed the

literature shows increase evidence that NMs cause multigenerational effects. For example, for the aquatic invertebrate *Daphnia* (various species) exposure to carbon and Ag NMs showed an increased sensitivity or tolerance in subsequent generations (Arndt et al., 2014; Völker et al., 2013). For the nematode *Caenorhabditis elegans* exposure to gold (Au) NMs cause increased toxicity in second (unexposed) generation (Kim et al., 2013) and exposure to Ag NMs an increased sensitivity in second generation, and the effect remained during later generations and unexposed generations (Schultz et al., 2016). So both studies showed evidences of transgenerational effects. One of the possible causalities for this transference of effects to subsequent generations is that NMs may have affected epigenetic mechanisms. In human toxicology there is indubitably evidence that NMs cause changes in epigenetic mechanisms (Smolkova et al., 2019). Therefore, the inclusion of epigenetic studies is highly recommended for a proper nano-risk assessment (Gedda et al., 2019). However, epigenetic studies with environmental relevant species are very scarce in the literature (Chatterjee et al., 2018) and even more rare on the effects of NMs. Several studies shown that for vertebrates (Dolinoy, 2008) and invertebrates (Kucharski et al., 2008) exposed to environmental stressors these presented phenotypic plasticity regulated by epigenetic mechanisms. Additionally it was observed for several environmental species epigenetic changes after exposure to chemicals (Vandegehuchte and Janssen, 2014). For example, studies with invertebrates have shown epigenetic changes caused by exposure to metals and metalloids (Kille et al., 2013; Santoyo et al., 2011; Šrut et al., 2017; Sussarellu et al., 2018; Vandegehuchte et al., 2009) and endocrine disruptors (Lee et al., 2018; Novo et al., 2018). Also, studies with fish showed effects with endocrine disruptors (González-Rojo et al., 2019; Olsvik et al., 2014) and pesticides (Bachère et al., 2017). Epigenetic studies are becoming very important in the field of ecotoxicology since they can provide important knowledge on organisms responses to stressors and possible adaptation or not to these environmental challenges over long periods of time (Chatterjee et al., 2018). It has even been suggested that the epigenetic status of an organism could serve as a tool to identify former exposures to contaminants (Mirbahai and Chipman, 2014). Further, several authors have highlighted the importance of combining the knowledge of epigenetic effects with effects at the several levels of biological organization, like provided in the AOP framework for the improvement of risk assessment (Angrish et al., 2018; Chatterjee et al., 2018; Goodman, 2017; Vandegehuchte and Janssen, 2014).

1.5 Aims and thesis structure

The main aim of this thesis was to develop and optimize ecotoxicity assays for the hazard assessment of nanomaterials (NMs), using the soil invertebrate standard model species *Enchytraeus crypticus*. These new assays were designed to cover longer term exposures, namely a full life cycle exposure (FLC) and a multigenerational (MG) exposure. The developed tests should be validated exposing *E. crypticus* to selected metallic NMs (and the ionic form for comparison). Further it was aimed to investigate epigenetic effects, and other stress responses at different levels of biological organization, incurring from the long term exposures to NMs. Finally, it was intended to integrate the data from the various levels and possibly to identify different KE between compounds (NM and ionic form) and build onto the adverse outcome pathway (AOP) framework.

This thesis was structured in xx chapters as follows:

Chapter I: General introduction and aims of the thesis.

Chapter II: Bicho R.C., Santos F.C.F., Gonçalves M.F.M., Soares A.M.V.M., Amorim M.J.B. (2015) Enchytraeid Reproduction Test (PLUS): hatching, growth and full life cycle test-an optional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology. 24, 5, 1053-1063.

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

Chapter IV: Bicho R.C., Santos F.C.F., Scott-Fordsmand J.J., Amorim M.J.B. (2017) Effects of copper oxide nanomaterials (CuONMs) are life stage dependent - full life cycle in *Enchytraeus crypticus*. Environmental Pollution, 224, 117-124.

Chapter V: Bicho R.C., Santos F.C.F., Scott-Fordsmand J.J., Amorim M.J.B. (2017) Multigenerational effects of copper nanomaterials (CuONMs) are different of those of $CuCl_2$: exposure in the soil invertebrate *Enchytraeus crypticus*. Scientific Reports, 7.

Chapter VI: Bicho R.C., Dick R., Mariën J., Scott-Fordsmand J.J., Amorim M.J.B. (2020) Epigenetic effects of (nano)materials in environmental species – Cu case study in *Enchytraeus crypticus*. Environment International, 136, 105447.

Chapter VII: Bicho R.C., Faustino A.M.R., Rêma A., Scott-Fordsmand J.J., Amorim M.J.B. (2020) Impact of copper materials (Cu NMs and $CuCl_2$) exposure on soil invertebrates – histology and immunohistochemistry as confirmatory assays for transient changes in *Enchytraeus crypticus*. *Submitted.*

Chapter VIII: Bicho R.C., Scott-Fordsmand J.J., Amorim M.J.B. Multigenerational exposure to WCCo nanomaterials - epigenetics in the soil invertebrate *Enchytraeus crypticus. Submitted.*

Chapter IX: Bicho R.C., Scott-Fordsmand J.J., Amorim M.J.B. Life cycle methylation profile of *Enchytraeus crypticus* (Oligochaete): from blastula to mature adult. *Submitted*

Chapter X: General discussion.

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Chapter II - Enchytraeid Reproduction Test^PLUS - hatching, growth and full life cycle test – an optional multi-endpoint test with *Enchytraeus crypticus*

Rita C. Bicho*, Fátima C. F. Santos, Micael F. M. Gonçalves, Amadeu M.V. M. Soares, Mónica J.B. Amorim

Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

*Corresponding author: ritabicho@ua.pt

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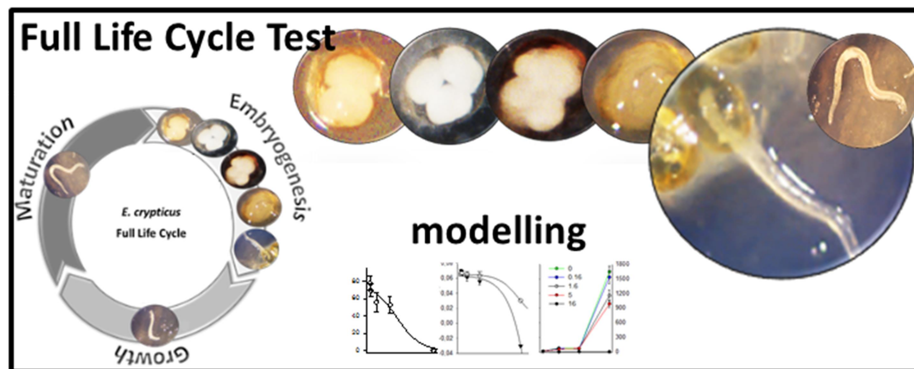
Abstract

Soil ecotoxicity standard tests for invertebrates are usually limited to the assessment of endpoints like survival and reproduction. Adverse effects may occur at other developmental stages, e.g. embryo development, hatching or growth. The species *Enchytraeus crypticus* is a model organism in the standard soil ecotoxicology test, where survival and reproduction are assessed. In the present study we optimized the test method to include additional endpoints. The proposed test start with synchronized age organisms', and included additionally hatching success, growth, maturation status and full life cycle. This allows for the calculation of cocoon production and population growth rate. Results indicated that Cd is embryotoxic, main effect occurs on the embryo developmental stage and maturity. Further, the full life cycle test can discriminate between pre- and post-embryo formation. The increased sensitivity and full life cycle detail level makes it potentially useful for novel materials e.g. nanomaterials where the mode of action and hence effect target is unknown.

Graphical abstract



Keywords: life stages; hatching; growth; full life cycle; population growth rate; soil ecotoxicology.

Introduction

In ecotoxicology e.g. for soil invertebrates, standardized tests are focused on the assessment of effects in terms of survival and reproduction of the organisms (van Gestel 2012), with endpoints like growth or hatching success only indirectly covered.

The assessment of effects at early life stages is generally time efficient and also very sensitive (Truong et al. 2011). The zebrafish (*Danio rerio*) embryo standard toxicity test (OECD 2013) provides a good example where such a tool and endpoints have been explored in depth (Truong et al. 2011). Embryo toxicity test methods have been developed mostly for aquatic organisms (Druart et al. 2012). Snails (*Helix aspersa* and *Monacha obstructa*) and slugs (*Deroceras reticulatum*) are among the few soil invertebrate species with reported studies on embryotoxicity, having showed high sensitivity of eggs to metals and pesticides (Druart et al. 2010; Iglesias et al. 2002; Iglesias et al. 2000; Shoaib et al. 2010). Studies on aquatic invertebrates (*Lymnaea stagnalis, Crassostrea gigas* and *Marisa cornuarietis*) have also

shown that embryos can be more sensitive to contaminants than adults (Geffard et al. 2002; Gomot 1998; Schirling et al. 2006). Growth, another important endpoint, is only included in *Helix aspersa (ISO 2006)* and *Caenorhabditis elegans* (ISO 2010). Despite that there are several examples showing that body size can be affected in collembolans (Broerse and van Gestel 2010; Bur et al. 2012; Bur et al. 2010) or body weight in earthworms (Khalil et al. 1996; van Gestel et al. 1991; Zhou et al. 2008; Zhou et al. 2007).

Recently, e.g. in the regulatory context of specific compounds like endocrine disruptors, the need to develop full life cycle tests has been highlighted (Crane et al. 2010). One of the reasons is that effects can be discriminated between developmental or mature stages (Ingersoll et al. 1999), a key point for e.g. sex differentiation. In the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters (OCDE 2002), the fish life cycle toxicity test is included. Another important aspect lays on the fact that a full life cycle test can provide life-history information useful to model population (Walthall and Stark 1997; Brinke et al. 2013). There is currently only one standardized full life cycle test, the Chironomid life-cycle toxicity test (OECD 2010a). The lack of standardization of full life cycle tests for other species is not surprising considering the required dedication in time and associated cost (Crane et al. 2010). Soil invertebrates with short life-cycle like *Enchytraeus crypticus* represent potential model species for full life cycle optimization and standardization, as we hereby propose. Enchytraeids have an ecotoxicity test standardized for *Enchytraeus albidus* (42 days) or *E. crypticus* (21 days) (ISO 2004; OECD 2004; OECD 2010b) where survival and reproduction or bioaccumulation (14 days for uptake) are assessed. In the present study we focused on the optimization of the procedures for a full life cycle test and additional endpoints (hatching success, growth, maturity status) using *E. crypticus*. Further, the results were validated and exemplified using Cadmium (Cd) as test substance.

Experimental

Test organism

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. Cultures are kept in agar plates prepared with a salt solution of $CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$, fed *ad libitum* with oatmeal and maintained in laboratory under controlled conditions at 18ºC and a photoperiod of 16:8 (light: dark).

Synchronized cultures of Enchytraeids are prepared by transferring adults with well-developed clitellum into fresh agar plates to lay cocoons. The number of adults to transfer should be two and a half the number of cocoons required, e.g. per petri dish (100x15 mm size) per 125 adults ca. 50 cocoons are obtained 1-2 days after.

These synchronized 1-2 days old cocoons are used for hatching and full life cycle test. Results of a hatching test comparing performance between 1-2 days with 2-3 days are given and discussed. For the growth test, which starts with juveniles, obtained 11 days after transferring the cocoons to the agar plates, the juveniles are allowed 11 days of growth.

Test soil

The standard natural soil LUFA 2.2 (Speyer, Germany) was used. Main characteristics of the soil can be summarized as follows: pH ($CaCl_2$) of 5.5, 46% of maximum water-holding capacity (WHCmax), 4% organic matter content, and a grain size distribution of 6% clay, 14% silt and 80% sand.

Test chemical and spiking

Cadmium chloride hemi-pentahydrate ($CdCl_2 \cdot 2^{1/2}H_2O$) Fluka, Sigma-Aldrich, 98% purity) was used. The tested concentration range was 0-0.16-0.5-1.6-5-16-50-160 mg Cd/kg soil (DW), selected based on a sub-lethal range (Castro-Ferreira et al. 2012). Test chemical was spiked onto the pre-moistened soil as aqueous solution. Stock solution was prepared and

serially diluted. Soil batches per concentration were homogeneously mixed and allowed to equilibrate for 3 days prior test start. Soil moisture was adjusted to 50% of the WHC_{max}.

Test procedures

Test followed the standard guideline (ISO 2004; OECD 2004) procedures with adaptations for: a) hatching, b) growth, c) full life cycle as described in detail in the next sections. Each replicate consisted of a test vessel 8x4ø cm with 10 g of soil (DW).

The schedule, test designs and sampling days were optimized and are represented in Fig. 1A and Fig. S1 (supplementary material).

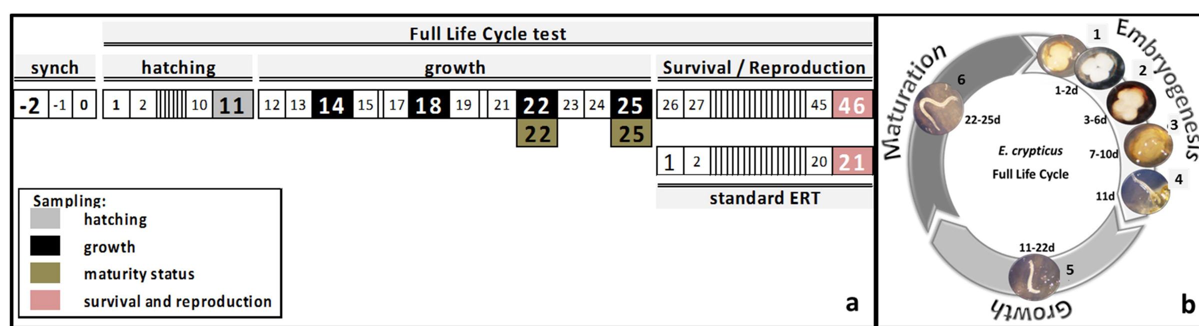


Fig. 1 Schematic representation of *Enchytraeus crypticus* **a:** Proposed test design, including the synchronizing period (synch) and the sampling days required per endpoint: hatching, growth, maturity status, reproduction and survival. **b:** Full life cycle stages. The figure includes the 3 main stages: embryogenesis, growth and maturation and respective time in days (d) per event. **1**: first cell divisions; **2**: gastrula; **3**: organogenesis; **4**: hatching juvenile; **5**: juvenile; **6**: adult with developed clitellum.

Hatching test (Ht)

Cocoons (n=15 per replicate) were used. The process consisted of gently picking 2 cocoons at a time from the agar plates using a brush. During the 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, ensure exposure and hatching success. Five replicates were used. Total test duration is 11 days and the endpoint is the number of juveniles hatched.

Test duration was selected based on preliminary tests performed using agar media, ISO water (and soil). Tests were performed on 24 wells plates containing 1 ml of ISO water or agar and 15 cocoons per well. The number of hatched juveniles was compared between media. In water it was possible to observe cocoons development daily. Observations were made using a stereo microscope and cocoon development was captured in pictures (Dinocapture 2.0) (Fig. 1B).

In the test soil, observations were made at day 11. To extract organisms from soil, replicates were fixated with 96% ethanol and Bengal red (1% solution in ethanol). After some hours, soil samples were sieved through a mesh (500 μm) to separate individuals from most of the soil and facilitate counting using stereo microscope.

For the test validation with Cd (0-1.6-5-16-50-160 mg/kg), 5 replicates per treatment were used.

Growth test (Gt)

For the preliminary test, juveniles (n=20) (with 11 days after cocoon laying) were used. Total test duration was 22 days (11+11) (Fig. 1A). Sampling for juvenile's length measurements included days: 11, 11.5, 12, 12.5, 13, 14, 15, 16, 18, 21, 22 days. Food supply (6mg) was added at test start and water content was replenished weekly. Three replicates were used per sampling point. Length measurement was performed with the help of a magnifying glass, carefully transferring each individual with a paintbrush to a millimeter paper, and its length was recorded. Further, the observation of developed clitellum is noted. Organisms were extracted from soil with alcohol and Bengal rose solution.

For the test validation with Cd (0-1.6-5-16-50-160 mg/Kg), 4 replicates per treatment were used. Total test duration was 14 days (11+14). Juveniles (n= 20 per replicate) were used. Sampling points included the selection of days: 14, 18, 22 and 25 (post-hatching days). The presence of clitellum was recorded at days 22 and 25 to estimate maturity status. Four extra

replicates were left for 21 additional days and assessed for reproduction (total 35 test days) as pilot study.

Full life cycle test (FLCt)

For the preliminary test, cocoons (n=15 per replicate) were used. Total test duration was 50 days (11+18+21). Sampling for juvenile's counting and length measurements included days 14, 18, 22, 25, 29 and 50. The presence of clitellum was recorded at days 25 and 29. Length and maturity status were assessed using a sub-sample of 20 organisms from the total number. Food supply (6mg) is added at day 11 and food and water content were replenished weekly. Three replicates were used per sampling time.

For the test validation with Cd (0-0.16-1.6-5-16 mg/Kg), cocoons (n=15 per replicate) and 4 replicates per treatment were used. Total test duration was 46 days, with samplings at days 11, 25 and 46. The presence of clitellum was recorded at day 25. Cocoon production is calculated (see data analysis for details)

Data analysis

One-way analysis of variance (ANOVA) followed by Dunnett´s or Dunn's multiple comparison post-hoc test ($\alpha \leq 0.05$) was used to assess differences between controls and Cd treatments (SigmaPlot 1997). Effect Concentrations (EC) calculations were performed for the various endpoints modelling data to logistic or threshold sigmoid 2 or 3 parameters regression models, as indicated in the Table 1, using the Toxicity Relationship Analysis Program (TRAP) software. Growth data was modeled and fitted to a sigmoid curve (logistic 3 parameters) using regression analysis (SigmaPlot 1997).

Population growth data were modeled and fitted to a sigmoid curve (logistic or threshold sigmoid 2 parameters) using regression analysis (SigmaPlot 1997).

Cocoon production

Based on the FLCt results the cocoon production (CoP) was calculated as follows:

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

where Jt is the number of juveniles at test end (day 46), Jh is the number of juveniles hatched (day 11), Js is the number of juveniles hatched that survived (day 25), and Coi is the initial number of cocoons.

Population growth

The overall instantaneous population growth rate was calculated both as the cocoon population growth rate and as the juvenile population growth rate, as these directly reflect the population increase. Hence, the number of starting cocoons (n=15) was related to the number of produced cocoons in the next generation (likewise the juveniles produced were related to juveniles in next generation i.e. after 46 days). The growth rate was calculated as the instantaneous population rate (ri): ri= ln (Nf / No) / t, where Nf and No is respectively the final and initial numbers (of cocoons or juveniles), and t is the time (total number of days of the test) (Sibly 1999). This measure was chosen because it has been presented a good link of effects from individuals to population levels (Forbes and Callow 1999)

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

Results

Control conditions: optimization

No significant changes occurred in soil pH due to test duration.

Hatching test

Results showed that the number of juveniles per cocoon was 2.8 ± 0.13 (AV ± SE) and that 11 days are sufficient for hatching of the total number of juveniles (Fig. 2A). Test performed

in ISO water allowed visualization along time showing that the majority of the embryos hatch between day 9 and 11.

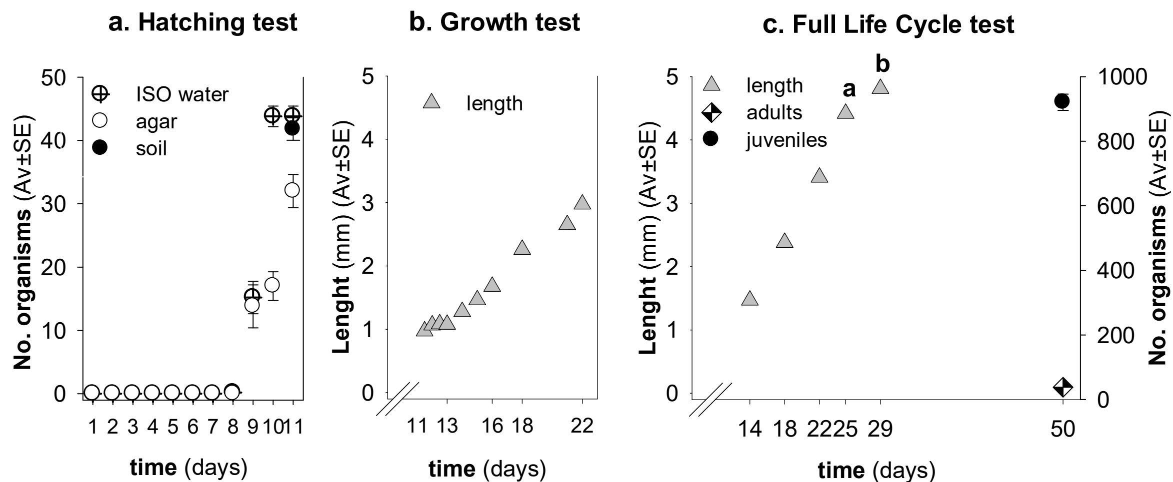


Fig. 2 Results from the optimization tests in control conditions for hatching, growth and life cycle, using *Enchytraeus crypticus* in LUFA 2.2 standard natural soil. All values are expressed in average ± standard error (Av ± SE). **A:** <u>hatching test</u> using 3 exposure media: ISO water, agar and soil. **B**: <u>growth test</u> in soil. **C:** <u>full life cycle test</u> in soil. **a**: ≈43% mature; **b**: ≈86% mature.

Observations of cocoon development showed the following sequence (Fig. 1B), with the first cell divisions being observed in the first 2 days, gastrula stage between the 3rd and 6th day and organogenesis between days 7 and 10, after which embryos hatched.

Growth test (Gt)

Results indicated that growth is minor between days 11-13 with the exponential growth phase occurring between days 14 and 22 (Fig. 2B). Observations showed that the organisms did not reach the maximum length and maturity status (no visible clitellum) at day 22.

Full life cycle test (FLCt)

Results showed that the selected sampling points to assess the various endpoints were adequate. Organisms' growth follows a sigmoid curve (Fig. S2A). The exponential growth

phase occurred between days 14 and 25, with individuals reaching maturity between days 25 (ca. 43%) and 29 (ca. 86%) (Fig. 2C). It was also observed that size was similar at day 50, i.e., the maximum growth is reached with 25 days. At day 50, survival and reproduction were assessed: the number of adults was 39±2 and of juveniles was 925±35 (Av±SE) per 15 initial cocoons. Length measurements of the second generation of organisms showed that juveniles had a length of 1.85mm±0.04 (Av±SE). The numbers of individuals counted and measured in this test are summarized in Table S1 (supplementary information). The validity criteria within the standard guideline was fulfilled (CV<20%, n≥25 juveniles).

Cadmium test: validation

No significant changes occurred in soil pH due to spiking or test duration. *Hatching test (Ht)* Results can be observed in Fig. 3A. In regard to the validity criteria, CV<20% and n≥25 juveniles.

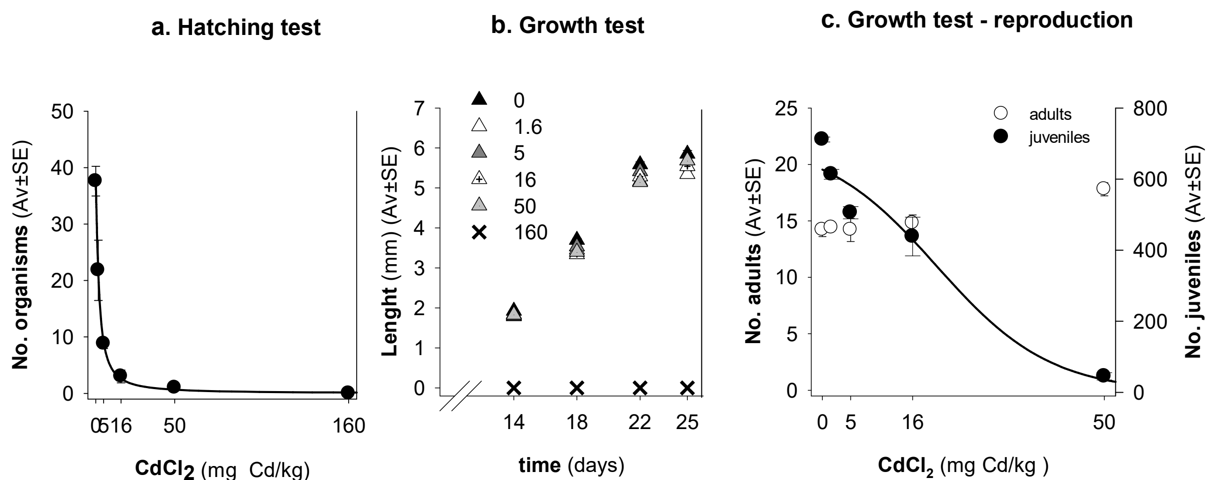


Fig. 3 Results in terms of hatching, growth and reproduction for *Enchytraeus crypticus* when exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil. All values are expressed as average ± standard error (Av±SE). A: hatching test; **B**: growth test; **C**: growth test – reproduction. The solid lines represent the model fit to data.

The number of hatched juveniles was significantly reduced within the concentration range and in a dose-response pattern. Concentrations ≥ 50 mg/Kg were lethal so virtually no embryos hatched. Effect concentrations are summarized in Table 1. During counting of the juveniles, cocoons were perforated and checked for presence of unhatched embryos inside: in controls all juveniles were hatched and in the treatments the unhatched cocoons had no embryos inside.

Table 1 Summary of the effect concentrations, for *Enchytraeus crypticus* when exposed to $CdCl_2$ in LUFA 2.2 soil. Results show ECx (Effect Concentration) estimates and the 95% confidence intervals (in brackets) and the respective models used to fit data including the values for slope (S) and intercept (Y0). EC values are given per endpoint and test type. Ht: Hatching test; Gt: growth test. FLCt: Full life cycle test; ERT: Standard Enchytraeid Reproduction Test; n.e.: no effect; n.d.: not determined. [a](Castro et al. 2012)

Test	Endpoint	EC_{10} (mg/kg)	EC_{20} (mg/kg)	EC_{50} (mg/kg)	EC_{80} (mg/kg)	Model (parameters)
Ht	hatching	0.4 (0.1-1.0)	0.7 (0.3-1.4)	2.0 (1.4-2.8)	6.1 (3.7-10.2)	Logistic 2 param (S:0.73; Y0:37)
Gt	growth	n.e.	n.e.	n.e.	n.e.	
	reproduction	n.d.	6.2 (2.7-9.8)	20.2 (15.7-24.8)	34.3 (25.4-43.1)	Logistic 2 param (S:0.02; Y0:711)
FLCt	hatching	n.d.	2.6 (0.1-5.0)	6.7 (3.3-10.1)	10.8 (4.3-17.2)	Logistic 2 param (S:0.08; Y0:77)
	growth	n.e.	n.e.	n.e.	n.e.	Logistic 2 param (S:0.06; Y0:5.2)

	Survival (25d)	11.6 (10.4-12.8)	12.7 (11.8-13.6)	14.5 (14.1-14.9)	16.3 (16.2-16.4)	Logistic 2 param (S:0.19; Y0:66)
	Maturity status	n.d.	1.8 (0.2-3.4)	4.7 (3.1-6.4)	6.9 (3.9-9.8)	Logistic 2 param (S:0.12; Y0:82)
	reproduction	n.d.	2 (0.7-3.4)	5.6 (3.8-7.5)	8.3 (5.1-11.4)	Threshold sigmoid 2 param (S:0.11; Y0:1561)
	Survival (46d)	3.6	5.3 (1.4-9.2)	8.6 (3.5-13.7)	11 (n.d.)	Threshold sigmoid 2 param (S:0.11; Y0:77)
	Cocoon production	0.3	2	5.4 (2.8-8.1)	7.8 (3.4-12.3)	Threshold sigmoid 2 param (S:0.11; Y0:378)
	population growth rate (Cocoon)	3.1 (0.9-5.3)	5.1 (3.2-6.9)	8.4 (3.5-13.4)	11.8 (3.2-20.4)	Logistic 2 param (S:0.1; Y0:0.07)
	population growth rate (Juveniles)	9.7 (0.8-18.5	11.9 (5.8-17.8)	15.6 (13.1-18.1)	19.3 (13.6-25)	Logistic 2 param (S:09; Y0:0.06)
ERT[a]	reproduction	15 (11-18)	-	35 (31-38)	-	Logistic
	survival	-	-	>320	-	-

Growth test (Gt)

Results showed that juveniles' growth was not affected significantly (Fig. 3B). Independently of the treatment growth was described by a sigmoid curve as in control (Fig. S2B). For 160 mg Cd /kg the juveniles died. Cd exposure did not affect time to reach maturity of juveniles,

where the following % of individuals with clitellum at day 25 was: 89%, 78%, 91%, 92% and 92% for 0, 1.6, 5, 16 and 50 mg/kg the respectively. The assessment of reproduction from juveniles showed a clear effect on reproduction with Cd concentration (Fig. 3C, Table 1).

Full life cycle test (FLCt)

Results showed a decrease in a dose-response manner in the number of hatched juveniles within the tested range (Fig. 4A).

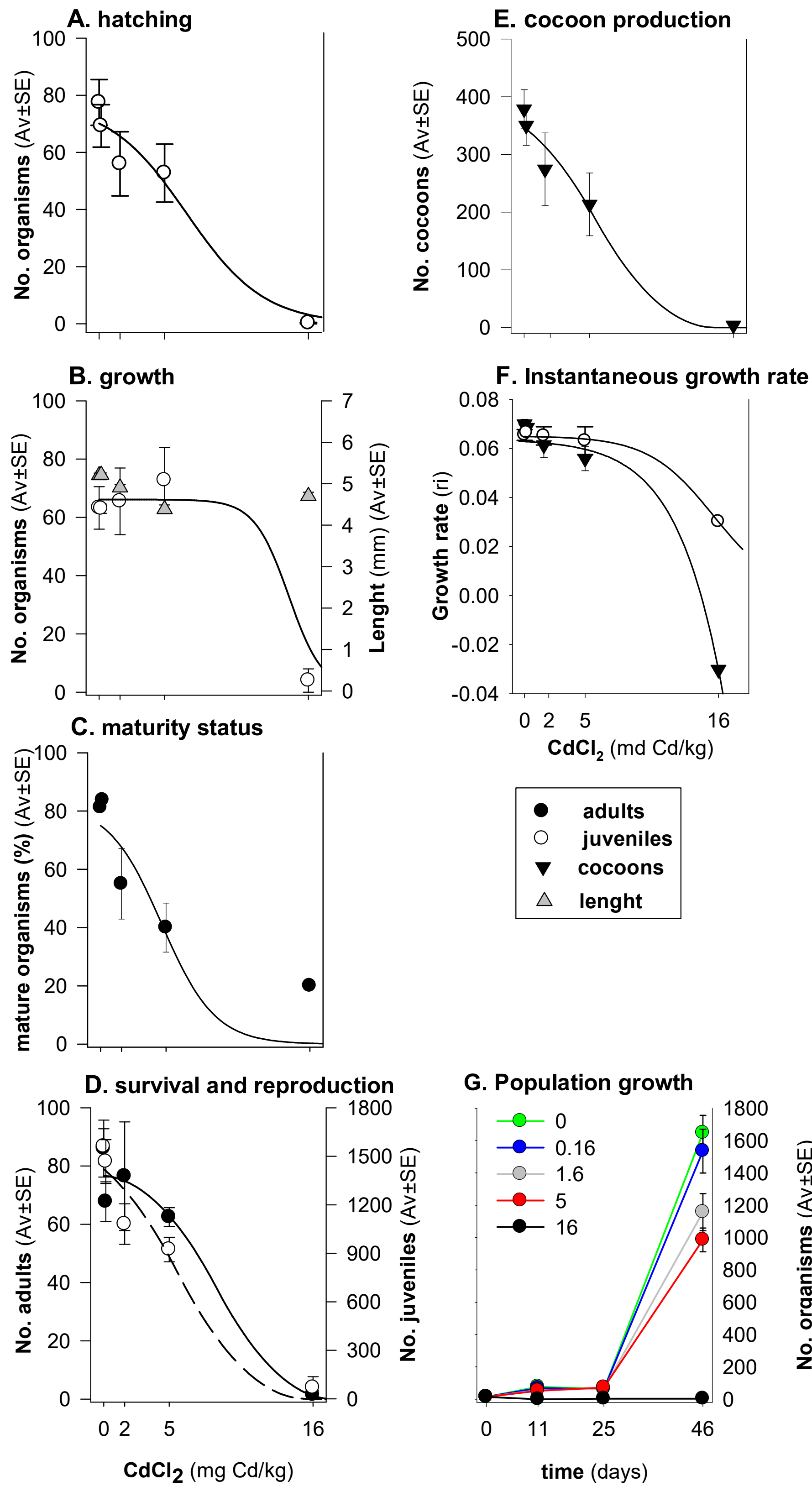


Fig. 4 Results of the full life cycle test for *Enchytraeus crypticus* when exposed to $CdCl_2$ (mg Cd /kg DW soil) in LUFA 2.2 standard natural soil including the various endpoints: hatching

(11d), growth, survival and maturity (25d), survival/reproduction (46d). Moreover, the calculated cocoon production (46d), instantaneous population growth rate and the population growth. All values are expressed in average ± standard error (Av±SE). The solid and dashed lines represent the model fit to data. **A**: hatching; **B**: growth; **C**: maturity status; **D**: survival and reproduction; **E**: cocoon production; **F**: instantaneous population growth rate (ri); **G**: population growth.

There was no effect of Cd on length for juveniles at day 11 and 25 (Table S2, supplementary material). At day 46, for the highest tested concentration (16 mg Cd/kg) there was high mortality and the surviving organisms were larger.

In terms of maturity, the percentage of clitellate animals decreased with Cd concentration in a dose related manner (Fig. 4C). In terms of survival/reproduction for the FLCt (46 days) (Fig. 4D) (CV<20%, No juveniles>25), toxic effects were higher than the standard ERT (Table 1). Also the effect as cocoon production from surviving adults decreased in a dose related manner (Fig. 4E).

Population growth

Results show that the population growth decreases with increasing Cd concentration (Fig. 4G). Also the instantaneous growth rate (ri) decreases in a dose related manner and e.g. at 16 mg Cd/kg ri is negative when based on cocoons (Fig. 4F). With this model it is possible to derive ECx values or predict population responses at any given concentration e.g. national or international soil quality criteria levels.

Discussion

The measurements under control conditions, as performed along the various sampling points in time, allowed the test optimization for *E. crypticus* full life cycle. This has been studied and implemented in an ecotoxicity test for the first time for an enchytraeid.. The proposed

multi-endpoint test contains many advantages compared to the standard test, with limited additional work is required. Overall, the new test includes few additional steps which are easily performed. One important aspect is the synchronization of cocoons, a variation of 1 day produces significant differences in the outcome of the test (Table S3, supplementary information), hence we recommend the test should be started with cocoons laid between 1-2 days old maximum.

In terms of coefficient of variation, the results were always within the standard ERT validity criteria (CV<20%) which seems to be a good indication of reproducibility. Also in terms of numbers of juveniles obtained per cocoon, the average of ca. 3 to 5 occurred in all tests performed; hence also here we confirm the reproducibility. We propose that for the Ht the minimum number of juveniles obtained per 10 cocoons should be ≥ 25 which resembles the standard ERT.

Growth followed a sigmoid curve, this had never been reported for Enchytraeids to the best of our knowledge. This is similar to the growth curves observed for other soil invertebrates, e.g earthworms, collembolans and mites (Eriksen-Hamel and Whalen 2006; Folker-Hansen et al. 1996).

The fact that cultures and organisms age are synchronized should be highlighted. This is a very important aspect in ecotoxicity testing due to the assumed differences in sensitivity of varying age as also illustrated here. This will reduce the overall variance hence the ability to detect lower level effects. The fact that this has been developed here and now optimized offers a new potential for this species, e.g. this is a key point for genomic (transcriptomic) studies.

To summarize, the results showed that the following time is required for the multiple endpoints (Table 2):

Table 2 Overview of updated test proposal, indicating the life stage at test start, endpoints measured, exposure time (and sampling dates within), test designations and attributed tiered level approach.

Start life stage (days)	**endpoint**	**Exposure Time** (days)	**Test designation**	**Level /Tier**
Cocoon (1-2d synchronized)	Hatching	11	Hatching test (Ht)	**1**
Juveniles (10-11d synchronized)	Growth	14 (3-7-11-14)	Growth test (Gt)	**2**
	Maturation	14 (11-14)	Maturation test (Mt)	
	Survival	14	Juvenile survival	
	Survival	35	Juvenile survival	
	Reproduction	35	Juvenile reproduction	
Cocoon (1-2d synchronized)	Hatching	11	Full Life Cycle test (FLCt)	**3**
	Growth	25 (11-14-18-22-25)		
	Maturation	25 (22-25)		
	Survival	46		
	Reproduction	46		
	Population growth rate	46		
Mature adults (not synchronized)	Survival	21	Standard Enchytraeid Reproduction Test (ERT)	**0**
	Reproduction	21		

The test set-up can be built up to include more or less endpoints (and respective sampling times) as foreseen relevant, being also possible to add more levels in a stepwise approach, i.e., from 1 to 2 and 3. The level of detail to select will depend on the objective of the study or type of chemical. For instance, additional endpoints like maturity status can be very relevant when studying the potential for endocrine disruption of chemicals; level 3 will be recommended to assess the mechanisms of action of chemicals, allowing to investigate for instance when an effect is observed at the reproduction at which stage it occurs: can be detected during hatching, juvenile survival, adult mortality or maturity status.

The test validation using Cd as test substance was bringing information at various levels as mentioned. The direct comparison at the reproduction level between EC50 values from the ERT and FLCt showed the differences in sensitivity of the organisms, i.e., the exposure of cocoons to $CdCl_2$ is a 6 fold more toxic than the exposure of mature adults. While the cocoons´ exposure is also implicitly incorporated in the traditional design (as cocoons are laid and hatch), it does not provide a specific knowledge regarding this and hence no further understanding of e.g. mode of action. The effect observed at the individual Ht and Gt (levels 1 & 2) shows that Cd acts at the embryo developmental stage, confirmed by the decrease in the hatching success (Ht) and no effect observed in terms of growth and survival of hatched juveniles. Moreover, the no effect on % maturity in the Gt, which starts from juveniles, versus the effect on % maturity observed on the FLCt, clearly shows that the main effect occurs during embryogenesis.

One interesting observation was that growth seemed to be affected with dose from 0-5mg/kg, whereas for the highest concentration (16 mg Cd/kg) there was high mortality and the surviving organisms were comparatively larger, both as juveniles (25d) and as adults (46d) and second generation juveniles (46d). This could indicate that the most resistant organisms were selected and survived. On the other hand it could also be a density effect where less organisms grow faster. Studies in other species such as *Eisenia fetida*, *E. andrei*, and *Folsomia candida* (Bur et al. 2010; Spurgeon et al. 1994; van Gestel et al. 1991) also showed an effect a reduction on growth due to exposure to $CdCl_2$. The differences in life cycle stages duration for the different species can mask the effect, i.e., for *E. crypticus* the apparent trend to reduce growth observed at day 25 was already compensated by day 46.

The Gt also showed that 160 mg Cd/kg caused 100% mortality of juveniles as opposed to 100% survival of adults in the standard ERT (Castro-Ferreira et al. 2012). Again this highlights the differences in sensitivity between life stages. The effects on reproduction in the

Gt juveniles (35d) again confirm a gradient of sensitivity to Cd, this being cocoons>>juveniles≥adults.

Regarding the Ht it was possible to observe that in exposed unhatched cocoons there was no formation of embryos, indicating that Cd can be embryotoxic to *E. crypticus*. This is not surprising as Cd is well known for being a developmental toxicant (Blechinger et al. 2002; Domingo 1994; Webster 1990). Several studies have shown that Cd can induce embryotoxicity to different vertebrates: chicks, rats, frogs, zebrafish and including humans (Chen and Hales 1994; Chisolm 1974; Chow et al. 2008; Gilani and Alibhai 1990; Sunderman et al. 1991). The test comparing 1-2d with 2-3d old cocoons (Table S3) confirmed that effects of Cd occurred only when using 1-2d old cocoons. This indicates that Cd must act by disrupting embryogenesis during the first 2 days. Studies with Cd in invertebrate species *Lymnaea stagnalis* and *Helix aspersa* (Druart et al. 2010; Gomot 1998) corroborate the embryotoxicity and confirm the time dependency with the younger exposed embryos more affected (Druart et al. 2010). Our results seem to be in line with what is observed in fish where embryotoxicity results can predict effects at later stages in the life cycle (Shin et al. 2014; Truong et al. 2012).

In sum, the FLCt can be potentially useful to assess the effects of new materials e.g. nanomaterials, where the mode of action and hence effect target is unknown. Further, the exposure time frame of most standard tests is below the time for uptake and effect (Gomes et al. 2013; Mrakovcic et al. 2013). Aging and long term toxicity tests are among some of the most common recommendations, particularly within nanotoxicology (Kumar et al. 2014). Furthermore, an additional generation can be included and explore the generational effects.

Finally, the combined results from FLCt provided life-history information that could be used to predict population increase and population growth rate (Sibly 1999; Brinke et al. 2013), and their dependency of the exposure to Cd e.g. related to national or international soil

quality criteria levels. The effects on hatching success and maturity status were in particular explaining most of the reduction in ri, i.e. the relative change with increase Cd exposure was largest for these. Survivors that matured were not able to fully compensate for the loss of individuals by increasing reproduction, on the contrary, reproduction also decreased with increase of Cd. Despite the effect on hatching success on first generation, this is probably also a delay effect as reflected by the higher number of surviving adults. Population growth rate is suggested as a very important endpoint to be included in ecotoxicological studies by several authors (Bechmann 1994; Menezes-Oliveira et al. 2013; Menezes-Oliveira et al. 2011; Stark et al. 1997; Widarto et al. 2007). This study showed that for Cd exposure enchytraeids' population growth rate the hatchability was of primary importance. Also in our study, population growth rate showed to have increased sensitivity and prediction power. Results from the standard ERT test show EC50 ca. 35 mg Cd/kg, whereas our results allowed to predict that already for concentrations of 16 mg Cd/kg the ri value is negative, hence predicts population decline earlier.

Conclusions

For the first time a full life cycle test has been developed for an enchytraeid. The level of additional endpoints can be selected within a multiplex endpoint system, from level 1-3 depending of the objective or in-depth of the study. The full set includes hatching, growth, maturity, survival and reproduction and population growth rate (level 3). Moreover, the synchronization of *E. crypticus* cultures provides a large range of applications that require increased accuracy. This includes from studies at genomic level, epigenetic, mode of action (e.g. endocrine disruption) and exploring effects of nanomaterials.

Results with Cd validation indicate that this novel assay is more sensitive than the standard ERT test. Moreover, the performance of FLCt provided information on specific life stage effects. Evidences were that the main effect occurred during embryogenesis. Additionally, the

proposed FLCt provided life history information that could be used to model population dynamics and growth rate, thus enhancing ecological relevance.

These additional endpoints can be included in a revised version of the current OECD guideline

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Supplementary material

Enchytraeid Reproduction Test^PLUS - hatching, growth and full life cycle test – an optional multi-endpoint test with *Enchytraeus crypticus*

Rita C. Bicho*, Fátima C. F. Santos, Micael F. M. Gonçalves, Amadeu M.V. M. Soares, Mónica J.B. Amorim

Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

*Corresponding author: ritabicho@ua.pt

Table S1: Results in terms of number of organisms (average ± standard error) and length measurements from the full life cycle test in control conditions samples during growth (days 14-18-22), maturity status (days 25-29) and reproduction (day 50), indicating survival and reproductive output.

Time (days)	No. juveniles	No. adults	length (mm)	N
14	32.3 ± 5.1	-	1.5 ± 0.02	94
18	32.0 ± 5.2	-	2.4 ± 0.05	98
22	35.0 ± 1.7	-	3.4 ± 0.06	104
25	-	29.3 ± 1.2	4.4 ± 0.05	90
29	-	32 ± 1.4	4.8 ± 0.05	98
50	924.6 ± 35 (from reproduction)	39 ± 2	5.19 ± 0.04	44

Table S2 Results in terms of length of organisms from the full life cycle test with Cd (average ± standard error;), including days 11, 25 and 46 days after cocoon deposition. From each replicate a subsample of 20 organisms were measured (N=20 per replicate).

	Treatment (mg Cd /kg)	juvenile (mm)	N	adult (mm)	N	juvenile (mm)	adult (mm)	N
days		11		25		46	46	
	0	1.2±0.01	80	5.2±0.48	80	1.5±0.03	4.9±0.05	80
	0.16	1.3±0.01	80	5.2±0.41	80	1.5±0.04	5.0±0.05	80
	1.6	1.3±0.01	80	4.9±0.63	80	1.5±0.03	4.4±0.07	80
	5	1.2±0.12	80	4.4±0.93	80	1.5±0.03	4.7±0.05	80
	16	1.3	1	4.7±0.37	13	1.9±0.11	7.5±0.37	6a+27j

The results of the hatching test performed to compare cocoons with starting age between 1-2 and 1-3 days old in a limit test with 5 mg Cd/kg can be observed in table S3. Significant differences were observed in terms of reduction in the number of juveniles hatched from cocoons with 1-2 days old compared to no effects for the 1-3 days old cocoons.

Table S3 Results in terms of number of juveniles (average ± standard error) for hatching test with Cd, comparing hatchability success of 1-2 and 1-3 days old cocoons. Dunn´s *p < 0.001.

Cadmium (mg Cd /kg)	No. juveniles 1-2 days	1-3days
0	37.2 ± 1.9	41.8 ± 3.7
5	16 ± 3.2*	41.8 ± 2.1

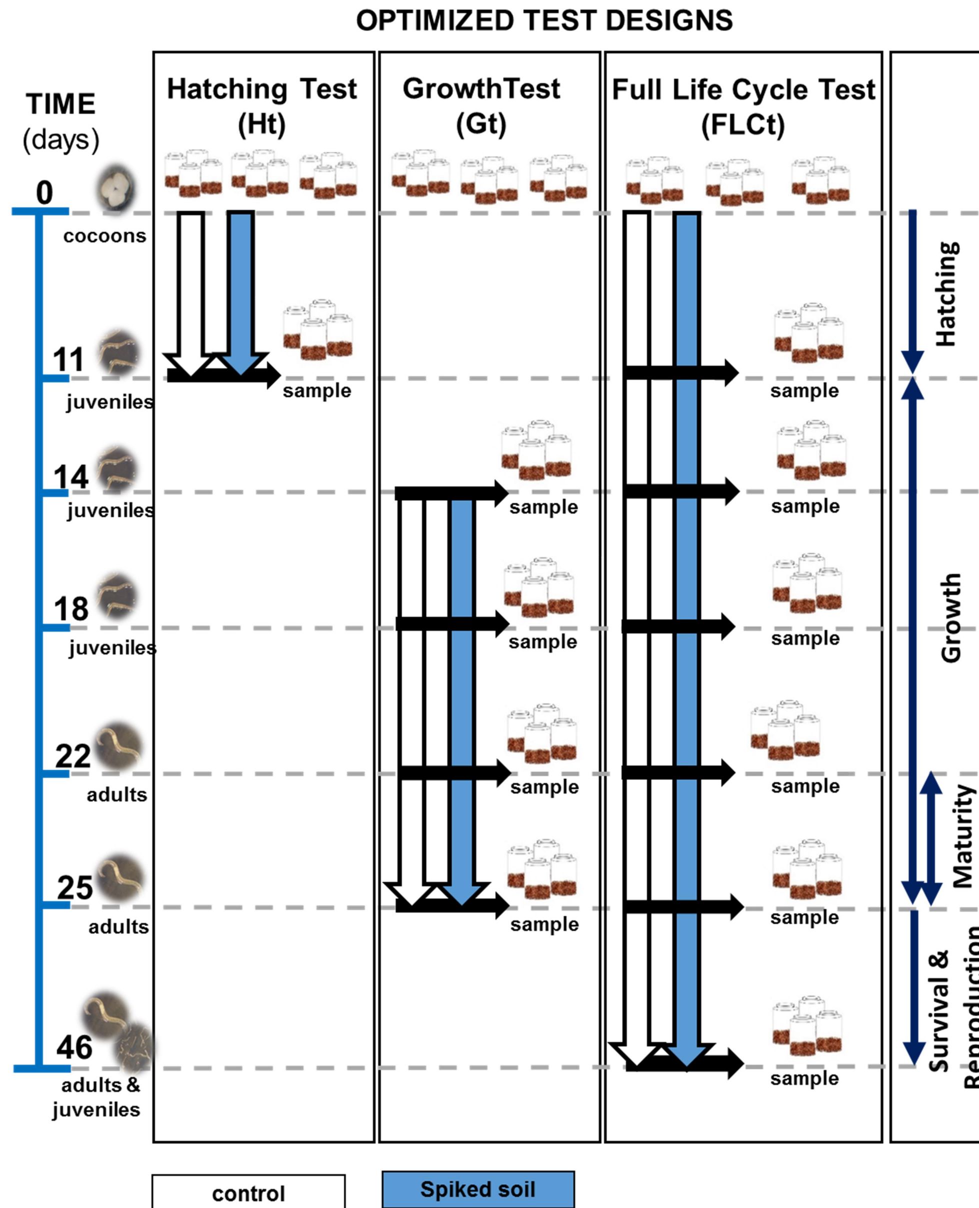

Fig. S1 Schematic representation of the proposed test design for Hatching, Growth and Full Life Cycle Test for *Enchytraeus crypticus*. Scheme includes the sampling days (signed by black arrows) required per endpoint (hatching success, growth, maturity status, survival and reproduction), proposal of minimum replicates/treatment.

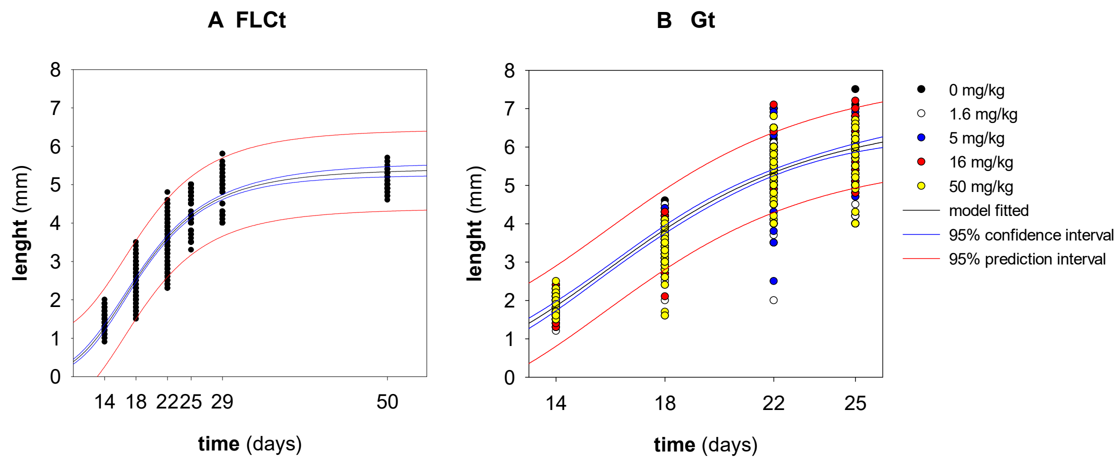

Fig. S2 Results of growth (length) for *Enchytraeus crypticus* in LUFA 2.2 standard natural soil from the **A**: full life cycle test (FLCt) in control conditions and **B**: growth test (Gt) when exposed to $CdCl_2$ (0-1.6-5-16-50mg Cd/kg soil DW). All data points are plotted and black line represents the fit to sigmoid model (f= a/(1+exp(-(x-x0)/b))). Model parameters for A: a=5.3; b= 4.5; x0=18.8. Model parameters for B: a=6.4; b=3.1; x0=16.7.

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

Rita C. Bicho[1*], Tânia Ribeiro[1], Natália P. Rodrigues[1], Janeck J. Scott-Fordsmand[2], Mónica J.B. Amorim[1]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[2]Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark
*Corresponding author: ritabicho@ua.pt

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Highlights

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

Abstract

Information on effects of silver nanoparticles on soil invertebrates, especially using long-term exposures, is scarce. In this study we investigated the effects of the reference Ag (NM300K) (compared to $AgNO_3$) using the full life cycle test (FLCt) of the soil invertebrate *Enchytraeus crypticus*. Results showed that effects were higher compared to the standard reproduction test, which is shorter and does not cover the FLC. Both Ag forms caused a reduction on hatching success, juvenile and adult survival and reproduction with similar ECx. Differences between $AgNO_3$ and Ag NM300K could be discriminated using the FLCt: $AgNO_3$ decreased hatching success was shown to be a delay in the process, whereas Ag NM300K caused irreversible effects during the same time frame. These effects may have occurred during embryo development, hatching (inhibition) or survival of hatched juveniles. Ag NM300K caused non-monotonic concentration-response effect as observed by the high effect of the

lowest concentration (20mg kg-1). It is known that dispersion is higher at lower concentrations - this could explain the increased effect at low concentration. Non monotonic responses are well described in the literature, where effects of high cannot predict for low concentrations, hence special attention should be given for NMs low concentration effects.

Keywords:
Hazard assessment; oligochaete; terrestrial compartment; life stages; long term.

1. Introduction

Worldwide there has been a fast increase in production of nanomaterials (NMs) due to their exceptional physico-chemical properties. Silver nanomaterials (AgNMs) are among the most widely used in consumer products applications [1, 2], such has electronics, medical devices, clothing, personal care products, cosmetics, detergents, among others [3, 4]. With the increase use of these products there is a particular concern for environmental exposure. The terrestrial environment is one of the primary receivers of NMs [5-7] through the application of sewage sludge as fertilizer [7-10]. For example, during clothing laundry AgNMs can outflow to wastewater and end up in soils via sewage sludge [4, 5, 9]. Additional sources include landfills and waste incineration [5, 11]. In Europe predictions indicate an annual increase for AgNMs of 1µg/kg dry soil [12] and calculations showed predicted environmental concentrations (PECs) of 1.5 to 1.6 µg/kg for soil receiving sewage sludge [10, 12] ; for the U.S. predictions reach up to 13 µg/kg in the soil via sewage sludge [13]. A recent study [7] shows maximum PECs for AgNMs in different soils areas, 0.5 µg /kg for sludge treated soil; 0.08 µg/kg for urban soils; 0.06 µg/kg for natural soils and 0.02 µg/kg for agricultural soils.
Despite the increasing number of studies currently available to assess the biological effects of AgNMs, these are still unclear and particularly scarce in the terrestrial environment. From available studies, indications are that current ISO and OECD test guidelines may underestimate effects of NMs and that e.g. longer-term exposures are required to assess the hazard of NMs [14-19]. For instance, standard reproduction tests (28 days) with earthworms (*Eisenia fetida*) and springtails (*Folsomia candida*) indicated that silver nitrate ($AgNO_3$) was more toxic than AgNMs. However, long-term exposures (52 and 310 days) with earthworms (*Lumbricus rubellus* and *Eisenia fetida*) showed that AgNMs toxicity increased with exposure time, whereas $AgNO_3$ toxicity decreased [14, 17]. The need for more long-term relevant endpoints and more mechanistic studies has been highlighted [20-22]. In line with this, Bicho et al. [23] developed the *Enchytraeus crypticus* full life cycle test (FLCt) which

has additional endpoints, e.g. hatching success and growth compared to the common 1-2 endpoint standard tests (survival, reproduction) hence being more comprehensive and allowing to discriminate effects between different life stages [24] while also providing life history information to model population dynamics [25, 26]. Full life cycle studies with soil invertebrates are scarce, this being inexistent for nanoparticles. There is an example where a FLC study was performed with *Caenorhabditis elegans* exposed to AgNMs although exposure was performed in "Simulated Soil Pore Water" media rather than soil [27].
In the present study we investigated the effects of AgNMs on the various stages of the FLCt of *E. crypticus* (46 days). Further, $AgNO_3$ is tested for comparison purpose. Moreover, results are compared to the standard Enchytraeid Reproduction Test (ERT) (21 days) [28, 29].

2. Material and Methods

2.1 Test organisms

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

2.2 Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics can be described as follows: pH (0.01 M CaCl2) = 5.5, organic matter = 1.77%, CEC (cation exchange capacity) = 10.1 meq/100g, WHC (water holding capacity) = 41.8%, grain size distribution of 7.3% clay, 13.8% silt, and 78.9% sand.

2.3 Test materials and spiking

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

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

2.4 Test procedures

2.4.1 Enchytraeid Reproduction Test (ERT)

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

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

2.4.2 Full Life Cycle test (FLCt)

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

2.5 Data analysis

One-way analysis of variance (ANOVA) followed by Dunnett´s comparison post-hoc test ($p \leq 0.05$) was used to assess differences between controls and treatments with SigmaPlot 11.0 ed. Effect Concentrations (ECx) calculations were performed for the various endpoints

modelling data to logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 1, using the Toxicity Relationship Analysis Program (TRAP v1.22) software.

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

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

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

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

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

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

3. Results

3.1 Biological characterization

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

3.1.1 Enchytraeid Reproduction Test (ERT)

Results can be observed in Figure 1.

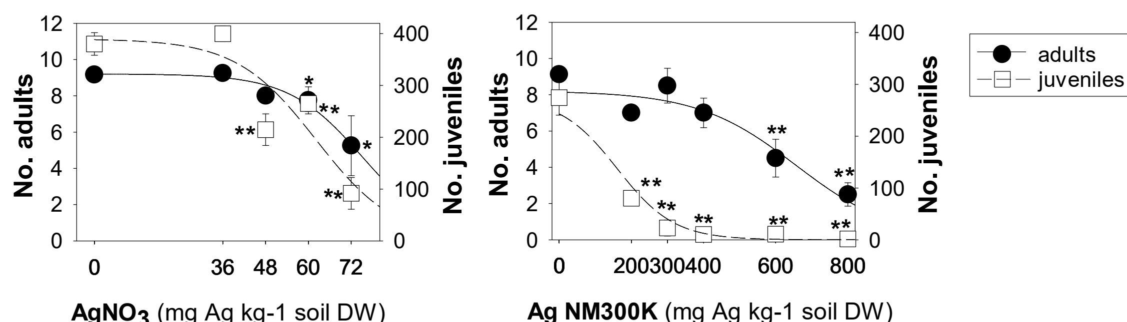


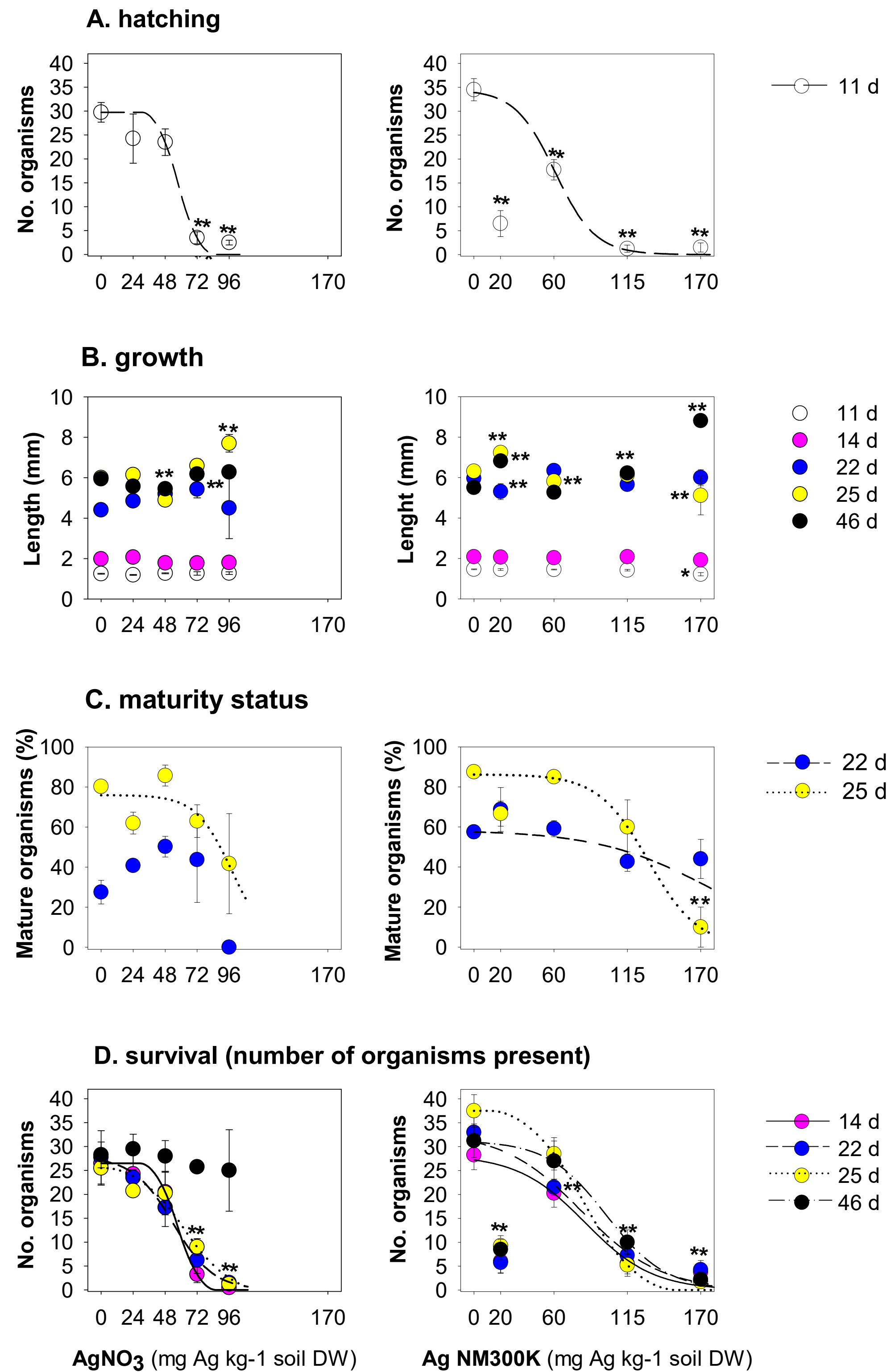
Fig. 1. Results of the Enchytraeid Reproduction Test (ERT) standard test in terms of survival and reproduction of *Enchytraeus crypticus* when exposed to $AgNO_3$ and Ag NM300K (mg

Ag kg-1 DW soil) in LUFA 2.2 soil. All values are expressed as average ± standard error (Av±SE). The lines represent the model fit to data. * p<0.05 and **p<0.001, Dunnets'.

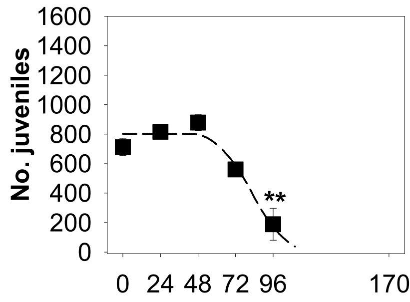
Effects occurred for both survival and reproduction in a concentration-response manner within the tested range for both Ag forms. $AgNO_3$ significantly reduced the number of adults at concentrations ≥60 mg Ag kg-1 (d.f.=4 and 20; F=4.4; p=0.014). The number of juveniles was significantly lower at concentrations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

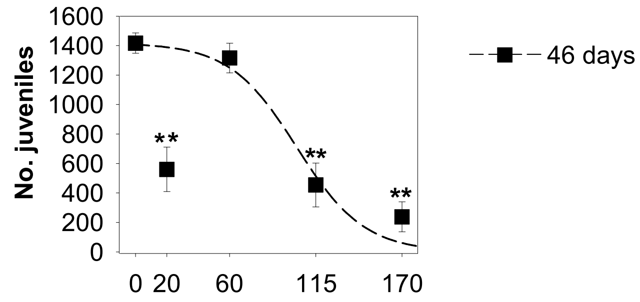
3.1.2 Full Life Cycle test (FLCt)

Results can be observed in Figure 2.

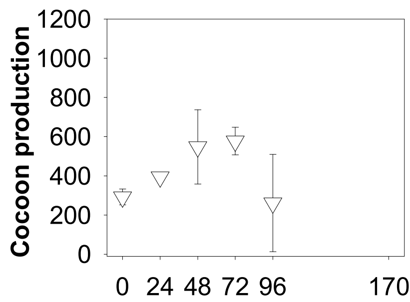

A. hatching
No. organisms
11 d
B. growth
Length (mm)
Lenght (mm)
11 d
14 d
22 d
25 d
46 d
C. maturity status
Mature organisms (%)
22 d
25 d
D. survival (number of organisms present)
No. organisms
14 d
22 d
25 d
46 d
AgNO3 (mg Ag kg-1 soil DW)
Ag NM300K (mg Ag kg-1 soil DW)

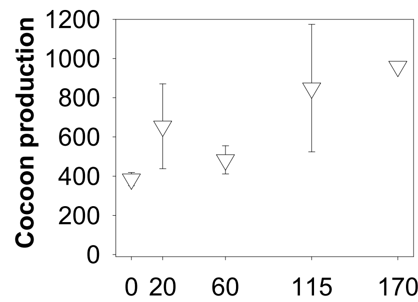
E. reproduction



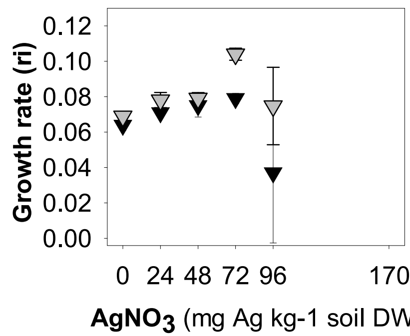


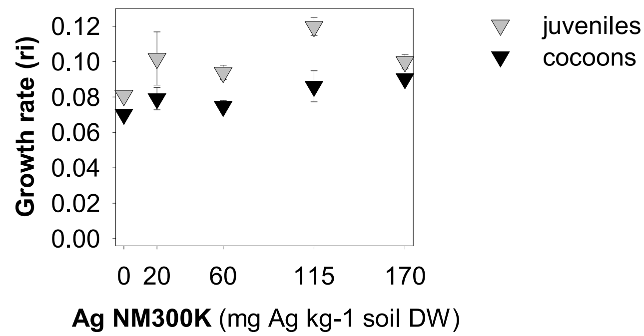
F. cocoon production



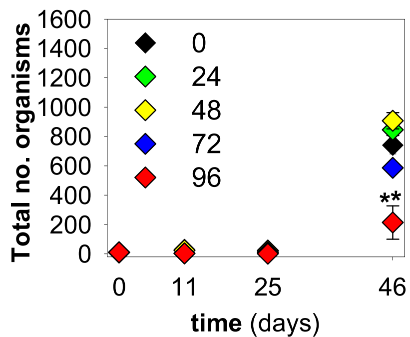


G. instantaneous growth rate





H. population growth



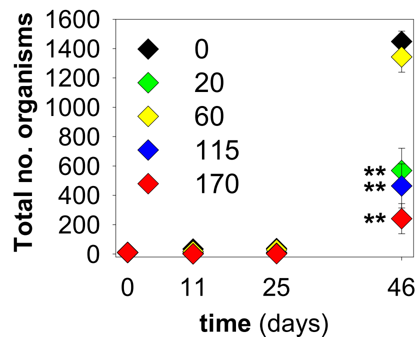


Fig. 2. Results of the Full Life Cycle test (FLCt) for *Enchytraeus crypticus* when exposed to $AgNO_3$ and Ag NM300K (mg Ag kg-1 DW soil) in LUFA 2.2 soil including the various endpoints: **A**: hatching ; **B:** growth; **C:** maturity status; **D:** survival (number of organisms present) **E**: reproduction;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

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

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

$AgNO_3$ significantly reduced the number of hatched juveniles at concentrations ≥72 mg Ag kg-1 (d.f.=4 and 19; F=20.2; p<0.001). The number of survived juveniles at 14 days was significantly lower at concentrations ≥72 mg Ag kg-1 (d.f.=4 and 19; F=16.3; p<0.001). Whereas reproduction was significantly decreased at concentrations ≥96 mg Ag kg-1 (d.f.=4 and 19; F=18.7; p<0.001). Results for Ag NM300K showed a significant reduction for the number of hatched juveniles at concentrations ≥20 mg Ag kg-1 (d.f.=4 and 19; F=52.6; p<0.001). The number of survived juveniles at 14 days was significantly lower at concentrations ≥72 mg Ag kg-1 (d.f.=4 and 19; F=20.3; p<0.001). Whereas reproduction was significantly decreased at concentrations ≥96 mg Ag kg-1 (d.f.=4 and 19; F=20.3; p<0.001).

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

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

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

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

Test	Endpoint	Time (days)	EC10	EC20	EC50	EC80	NOEC/ LOEC	Model and parameters
	$AgNO_3$							
FLC	Hatching	11	**42** (30-54)	**48** (38-57)	**58** (51-65)	**65** (54-76)	24/ <48	Threshold 2 par (S: 0.04; Y0: 29.8)
	Growth		n.e.	n.e.	n.e.	n.e.	-	-
	Maturity status	25	**69** (25-112)	**79** (53-106)	**98** (78-118)	**117** (71-163)	24/ <48	Log 2 par (S: 0.02; Y0: 76)
	Survival	14	**41** (28-55)	**47** (37-57)	**57** (50-65)	**65** (53-77)	24/ <48	Threshold 2 par (S: 0.03; Y0: 26.5)
		22	**21**	**33** (16-51)	**54** (43-65)	**75** (57-93)	<24/ <24	Log 2 par (S: 0.02; Y0: 27.8)
		25	**29** (6-51)	**40** (24-56)	**62** (52-73)	**78** (62-95)	<24/ <24	Threshold 2 par (S: 0.02; Y0: 25.5)
		46	**85** (20-151)	**109**	**149**	**189**	<24/ <24	Log 2 par (S: 0.01; Y0: 29)
	Reprod	46	**61** (47-75)	**68** (58-78)	**83** (76-89)	**93** (82-103)	24/<48	Thresh 2 par (S: 0.02; Y0: 802.5)
ERT	Survival	21	**52** (38-67)	**61** (52-69)	**75** (66-84)	**90** (70-110)	36/ <48	Log 2 par (S: 0.02; Y0: 9.2)
	Reprod	21	**38** (24-51)	**47** (37-56)	**62** (57-68)	**78** (67-88)	<36/ <36	Log 2 par (S: 0.02; Y0: 389.8)
	Ag NM300K							
FLC	Hatching	11	**28**	**40** (22-59)	**61** (55-67)	**82** (62-101)	20/ <60	Log 2 par (S: 0.02; Y0: 34.5)
	Growth		n.e.	n.e.	n.e.	n.e.	-	-
	Maturity status	22	**85** (10-160)	**119** (73-165)	**178** (131-225)	**237** (136-338)	<20/ <20	Log 2 par (S: 0.006; Y0: 58.4)
		25	**89** (57-121)	**105** (84-126)	**131** (112-149)	**157** (120-194)	20/ <60	Log 2 par (S: 0.02; Y0: 76)
	Survival	14	**28**	**48** (22-74)	**83** (66-100)	**118** (92-145)	20/ <60	Log 2 par (S: 0.01; Y0: 28.2)
		22	**16**	**40**	**81**	**122**	<20/	Log 2 par

				(17-62)	(67-95)	(99-144)	<20	(S: 0.008; Y0: 33)
		25	**43** (20-65)	**56** (39-72)	**82** (70-94)	**100** (82-119)	<20/ 20	Log 2 par (S: 0.01; Y0: 37.5)
		46	**51** (12-90)	**69** (40-97)	**99** (82-117)	**130** (105-155)	<20/ <20	Log 2 par (S: 0.01; Y0: 31.2)
	Reprod	46	**56** (21-91)	**73** (48-99)	**103** (88-118)	**133** (111-154)	20/ <60	Log 2 par (S: 0.01; Y0: 1417)
ERT	Survival	21	**356** (195-517)	**467** (353-580)	**657** (578-735)	**846** (709-984)	<200/ <200	Log 2 par (S: 0.018; Y0: 8.2)
	Reprod	21	n.d.	**52** (35-139)	**161** (102-220)	**270** (192-348)	<200/ <200	Log 2 par (S: 0.003; Y0: 274.5)

4. Discussion

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

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

Curiously, for $AgNO_3$ the FLCt showed lower effect. One hypothesis could be that organisms exposed to $AgNO_3$ from cocoon stage develop some sort of resistance and hence survive higher levels of Ag, although, this was not the case in Ag NM300K exposure. Therefore, maybe this illustrates differences in mechanisms of action between the two Ag forms, i.e.

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

Since the level of detail of the FLCt is considerably higher, this allows increased interpretation of results. The differences observed in terms of adults survival between Ag forms seems to indicate that Ag NM300K caused a) embryotoxic effect or inability to hatch, hence decreased hatching success, or b) the juveniles hatch later (25<days<46) and their survival is affected, hence the decreased number of adults at test end. On the other hand, the effects observed for $AgNO_3$ in terms of reduced hatching (11-25 days) reflected a delay in hatching since longer exposure (46 days) showed that adults were present and at similar numbers to controls. Again this indicates that there are differences in the mechanisms of action between $AgNO_3$ and Ag NM300K and highlights the importance of the additional test endpoints and longer term exposures when testing NMs such as Ag, as also referred by other authors [14, 15, 17, 18]. As documented [14, 17, 30] AgNMs tend to oxidise and Ag ions are released, with AgNMs possibly providing a continuous source of ions [33]. Nevertheless, the released Ag ions in soil media are immediately bound to its constituents, such as organic matter (OM) and clays [33-35]. Coutris et al. [33] compared the "bioaccessible" fraction of AgNMs and $AgNO_3$ along 70 days in two soils: an organic soil with 14% OM and, a mineral soil with 1.5% OM [LUFA 2.2 used in our experiment has 2% OM and 7% Clay]. Ag ions bind slower to soil components in mineral soil. For AgNMs it was observed that "bioaccessible" fraction increases with time, but this process is slower for the mineral soi [33]. This could partly justify the observed differences between Ag forms, i.e., longer time required for AgNMs effects as caused by Ag ions. The results by Gomes et al. [36] with *E. crypticus* also suggest slower oxidation rate effect of AgNMs compared to $AgNO_3$. Li et al. [37] also studied the uptake of AgNM, over 96 hours in artificial soil solution; they observed

that in artificial soil solution the hydrodynamic size of AgNMs increased probably due to higher strength, and they further found evidence that AgNMs toxicity cannot be completely attributed only to the dissolution of the AgNMs and release of Ag ions.

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

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

One particular aspect that requires attention is the high effect caused by the lowest tested concentration of Ag NM300K (20 mg Ag kg-1). In line with these results, and for the same material, van der Ploeg at al. [14] observed that for 15 mg Ag kg-1 the worms had higher tissue concentration than at the highest concentration (154 mg Ag kg-1), and this could not be directly linked to pore-water measurements. It is described that aggregation and agglomeration can increase with higher concentrations in different media, including in soil [5, 43, 44]. These processes interfere with NMs dissolution, e.g. lower agglomeration/aggregation, higher dissolution of NMs [5, 43]. Additionally, lower agglomeration/aggregation, higher amount of single NMs [5]. In our study it is possible that

at low dose (20 mg Ag kg-1), Ag dissolution and the amount of single NMs was higher. This could explain that the increased effect measured was a combined increased effect of Ag ions and nanoparticulate specific effects. Such results could be compared to the low concentration effect or non-monotonic concentration-response curves as observed in the context of endocrine disrupting compounds. Non monotonic responses are well described in the literature where the slope of the curve changes sign within the range of tested concentrations, hence effects of high doses cannot predict for low doses [45-47].

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

5. Conclusions

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

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

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

Chapter IV - Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in *Enchytraeus crypticus*

Rita C. Bicho[1*], Fátima C.F.Santos[1], Janeck J. Scott-Fordsmand[2], Mónica J.B. Amorim[1]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[2]Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark
*Corresponding author: ritabicho@ua.pt

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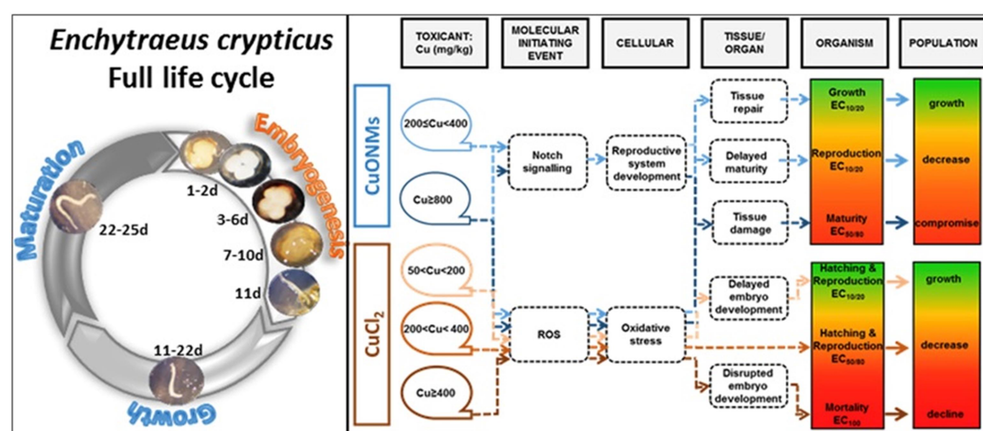
Highlights

- Life stage related effects for CuO NMs using *E. crypticus*.
- Effects were discriminated between Cu forms.
- CuONMs mainly affected juveniles' growth and maturity.
- $CuCl_2$ primary effect was during embryo development and/or hatching success.
- Full life cycle test showed higher sensitivity compared to the standard test.

Abstract

Copper oxide nanomaterials (CuONMs) have various applications in industry and enter the terrestrial environment, e.g. via sewage sludge. The effects of CuONMs and copper chloride ($CuCl_2$) were studied comparing the standard enchytraeid reproduction test (ERT) and the full life cycle test (FLCt) with *Enchytraeus crypticus*. CuONMs mainly affected growth or juveniles' development, whereas $CuCl_2$ mainly affected embryo development and/or hatching success and adults survival. Compared to the ERT, the FLCt allowed discrimination of effects between life stages and provided indication of the underlying mechanisms; further, the FLCt showed increased sensitivity, e.g. reproductive effects for CuONMs: EC_{10} = 8 mg Cu/kg and EC_{10} = 421 mg Cu/kg for the FLCt and the ERT respectively. The performance of the FLCt is preferred to the ERT and we recommend it as a good alternative to assess hazard of NMs. Effects of CuONMs and $CuCl_2$ are life stage dependent and are different between Cu forms.

Graphical abstract



Keywords: Nanoecotoxicology; oligochaete; full life cycle; development; high relevancy.

1. Introduction

The number of consumer products containing nanomaterials and their applications continues to increase in our society (Amorim 2016; Vance et al. 2015). Particularly, copper oxide nanomaterials (CuONMs) have broad usage e.g.: in antimicrobial coatings, antifouling paints, catalysis, superconducting, thermoelectric and sensing materials (Pang et al., 2012; Ramskov et al., 2014). The increased use of nanomaterials (NMs) makes them a significant portion in waste activated sludge (Unsar et al. 2016). This poses a concern especially for the terrestrial compartment given that e.g. in Europe around 53% of the sludge is used in agriculture fields and around 40% in the USA and Canada (Unsar et al. 2016). The toxic effects of CuONMs have been investigated in several taxa, e.g. in aquatic organisms such as fish (Ganesan et al. 2016; Zhao et al. 2011), invertebrates (Croteau et al. 2014; Gomes et al. 2011; Pang et al. 2012) and algae (Aruoja et al. 2009). Regarding soil invertebrates, effects of CuNMs have been shown at the level of survival and reproduction with enchytraeids (*Enchytraeus crypticus* and *Enchytraeus albidus*) (Amorim and Scott-Fordsmand 2012; Gomes et al. 2015a) and earthworms (*Eisenia fetida*) (Heckmann et al. 2010), avoidance behaviour with *E. albidus* (Amorim and Scott-Fordsmand 2012), cellular energy allocation in *E. crypticus* (Gomes et al. 2015b) and oxidative stress in *E. albidus* (Gomes et al. 2012). Moreover, it has been emphasized that for NMs, long-term studies and more mechanistic data and relevant endpoints should be developed and provided (Amorim 2016; Schultz et al. 2015; Scott-

Fordsmand et al. 2014). For instance full life cycle tests are specially recommended (Amorim 2016a; Amorim et al.2016b) because a larger set of endpoints and more information is provided to potentiate the discrimination of unique nano-specific effects (Bicho et al. 2016). At present, the developed full life cycle test (FLCt) with the soil invertebrate *E. crypticus* (Bicho et al. 2015), includes additional endpoints: hatching success and growth besides the common standard test endpoints, survival and reproduction, and hence meeting the aimed goals and criteria.

In this study the effects of CuONMs and copper chloride ($CuCl_2$) via a FLCt with *E. crypticus* (46 days) were evaluated and results were compared to the standard Enchytraeid Reproduction Test (ERT) (21 days), ISO 16387 and OECD 220 (ISO 2004; OECD 2004).

2. Material and Methods

2.1 Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. Cultures are kept in agar plates for several years at the University of Aveiro. For details please see (Bicho et al. 2015). Synchronized cultures were prepared as described in (Bicho et al. 2015). Briefly, mature adults are moved to agar plates to lay cocoons. For the FLC test 1–2 days old cocoons are used, for ERT test 17-18 days juveniles are used. No acclimation period was allowed in the test soil due to practicability issues. The implemented method is comparable across publications.

2.2 Test soil

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

2.3 Test materials and spiking

Copper oxide nanomaterials (CuONMs) (for details see Table 1) and copper (II) chloride dihydrate ($CuCl_2·2H_2O$ > 99.9 % purity, Sigma-Aldrich) were used.

Table 1 Characteristics of the tested CuONMs (Source: FP7-SUN project).

Characteristics	**CuONMs**
Manufacturer	Plasma Chem
CAS number	1317-38-0

Primary size distribution (average)	3-35 (12)
Mode (1st quartile - 3rd quartile)[nm]	10 (9.2-14)
Shape	Semi-spherical
Average crystallite size [nm]	9.3
Crystallite phases (%)	Tenorite 100%
Dispersability in water: D50 [nm]; average agglomeration number (AAN)	139.5 ± 4.6; 346
Dispersability in modified MEM: D50 [nm]; average agglomeration number (AAN)	85.2 ± 2.7; 77
Z-potential in UP water [mV]	+ 28.1 ± 0.6
Isoelectric point [pH]	10.3
Photocatalysis: photon efficiency [unitless]	1.5 x 10-4
Specific Surface Area [m2 g/1]	47.0 ± 1.7
Pore sizes [nm]	13.5 ± 1.6 (BJH) 23.0 ± 0.9 (AVG)
Surface chemistry [atomic fraction]	Cu = 0.46 ± 0.05; O = 0.47 ± 0.05 C = 0.07 ± 0.01
Chemical impurities [mg kg/1]	Na: 505 ± 30; Pb: 36 ± 2 Ag: 13 ± 4

The tested concentrations were 0-200-400-600-800-1600-3200 mg Cu/kg soil dry weight (DW) for CuONMs, and 0-50-100-200-400-800 mg Cu/kg soil (DW) for $CuCl_2$. For CuONMs spiking followed the recommendations for nanomaterials (OECD 2012). Briefly, 5 g of soil per replicate were mixed with the corresponding quantity of the test materials (as dry powder) to reach the nominal concentration. The spiked soil was homogeneously mixed with the rest of the soil (15 g) after which moisture was adjusted to 50 % of the maximum water holding capacity (maxWHC). The spiking of the soil was done per individual replicate to ensure total raw amounts per replicate. The test chemical $CuCl_2$ was prepared as stock aqueous solution, diluted and spiked onto the pre-moistened soil. The amount of soil per concentration were homogeneously mixed and split onto replicates. Soil was allowed to equilibrate for 1 day prior test start.

2.4. In situ characterisation.

The amount of Cu was measured in the test soil and in soil solution (for methodological details see Gomes et al. (2015a)) in a concurrent experiment over 28 days. In the soil the total Cu was measured (measured by Graphite Furnace Atomic Absorption Spectroscopy: AAS-GF) and in soil solution both the total Cu (measured by AAS-GF) and free active form (measured by ion-selective electrode) was measured. The CuO present as nanomaterials was not determined in the soil, due to the technical difficulties e.g. that the particle size is below the theoretical detection limit of 15 nm (Navratilova et al. 2015).

2.4 Test procedures

2.4.1 Enchytraeid Reproduction Test (ERT)

The standard guideline (ISO 2004; OECD 2004) was followed, but instead of using adults from cultures, 10 synchronized age (17-18 days) organisms were used. Organisms were placed in each test container with 20 g of moist soil and food supply (24 ± 1 mg, autoclaved rolled oats). Test ran at 20 ºC and 16:8h photoperiod during a period of 28 days. Food (12 ± 1 mg) and water were replenished every week. Five replicates per treatment were used.
Organisms extraction from soil was performed as described (Bicho et al. 2016).

2.4.2 Full Life Cycle test (FLCt)

A Full Life Cycle test (FLCt) was performed as described in (Bicho et al. 2015; Bicho et al. 2016). Briefly, test starts with synchronized (1-2 days old) cocoons (n=10 per replicate) introduced in each test container with 10 g of moist soil. Four replicates per treatment were used. Sampling points were at 11, 14, 25 and 46 days, where organisms were counted and measured (length) and the presence of clitellum (maturity status) was made at day 25.

2.5 Data analysis

One-way analysis of variance (ANOVA) followed by Dunnett´s comparison post-hoc test ($p \leq 0.05$) was applied to evaluate differences between controls and treatments (SigmaPlot 1997). Effect Concentrations (ECx) calculations were done for all endpoints modelling data to logistic, threshold sigmoid and piecewise linear 2 parameters regression models, as indicated in Table 1, using the Toxicity Relationship Analysis Program (TRAP v1.3) software.

Further, population endpoints were calculated as in (Bicho et al. 2015; Bicho et al. 2016): Cocoon production (CoP) and Population growth rate (r_i).

3. Results

3.1 In situ characterisation

The total Cu measured in the soil ranged between 96-106 % of the added total concentration for both CuONMs and $CuCl_2$, no concentration dependent pattern observed. The total Cu in soil solution was less than 1 % of the total for CuONMs and less than 3 % for $CuCl_2$. The free active Cu was less than 0.001 % for both Cu forms exposure. For controls the total Cu in soil solution was 0.07 % and the active Cu was 0.004 %.

3.2. Biological effects

The validity criteria as in the ERT were achieved, i.e. for juveniles coefficient of variation was < 20 % and the number of juveniles was ≥ 50, for adults mortality was ≤ 20 %. Soil pH (6.3±0.2), did not vary within concentrations and during the test.

3.2.1 Enchytraeid Reproduction Test (ERT)

Results can be observed in Figure 1.

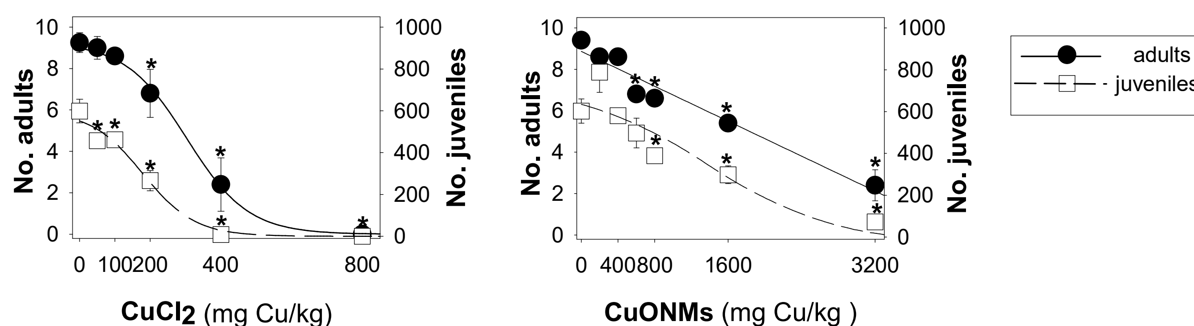


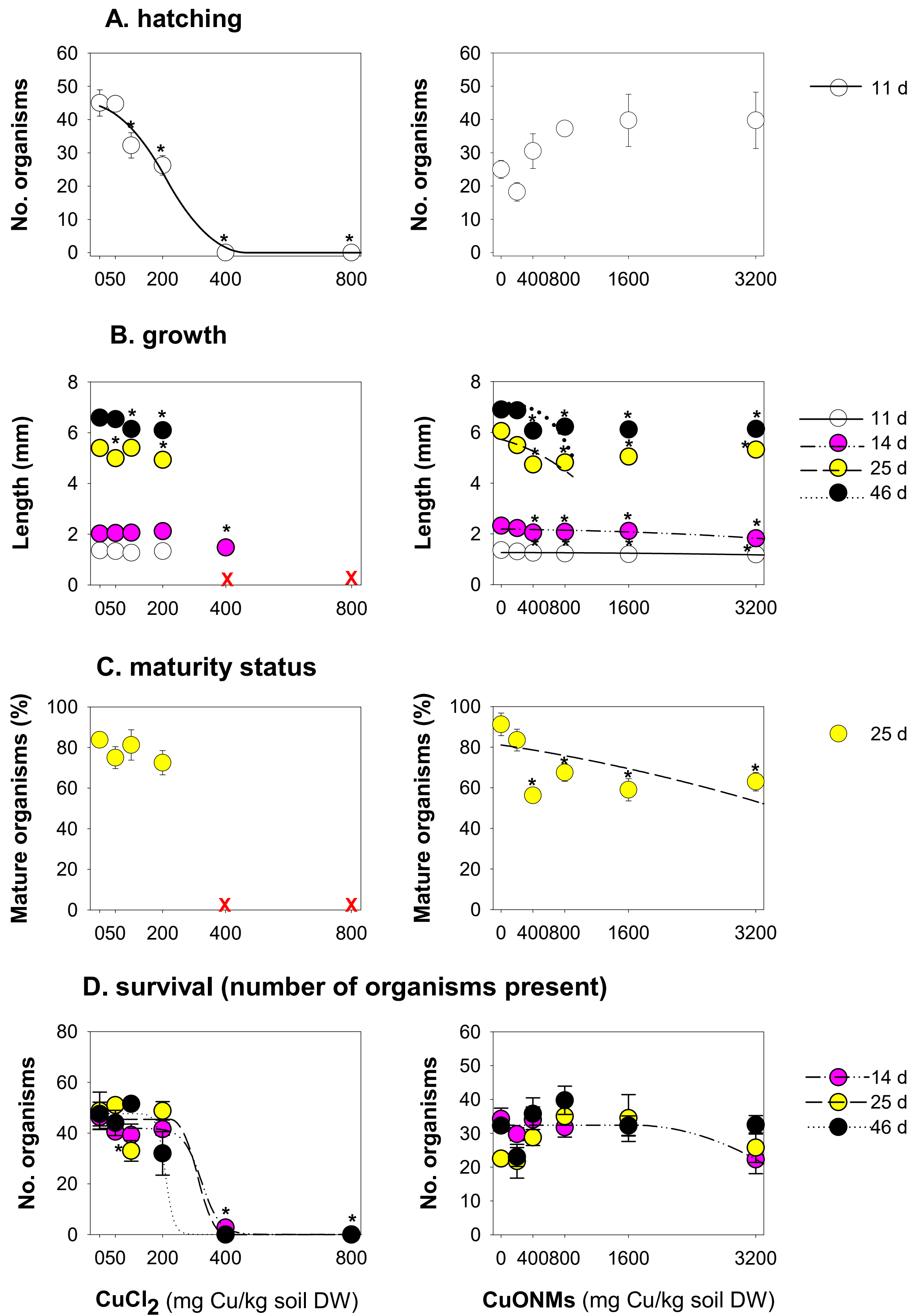
Fig. 1. Results of the standard ERT in terms of survival and reproduction of *Enchytraeus crypticus* exposed to $CuCl_2$ and CuONMs (mg Cu/kg DW soil) in LUFA 2.2 soil**.** All values are expressed as average ± standard error (Av ± SE). The lines represent the model fit to data. *p< 0.001, Dunnets'.

Effects occurred in a concentration-response decrease for both survival and reproduction within the tested range for both Cu forms. $CuCl_2$ was more toxic, showing severe effects at 400 mg Cu/kg whereas for CuONMs the similar toxicity level was observed at 3200 mg Cu/kg.

Results for the concentration-response models are shown in Table 2.

3.2.2 Full Life Cycle test (FLCt)

Results can be observed in Figure 2 and table 2.


A. hatching
No. organisms
11 d
B. growth
Length (mm)
11 d
14 d
25 d
46 d
C. maturity status
Mature organisms (%)
25 d
D. survival (number of organisms present)
No. organisms
14 d
25 d
46 d
CuCl2 (mg Cu/kg soil DW)
CuONMs (mg Cu/kg soil DW)

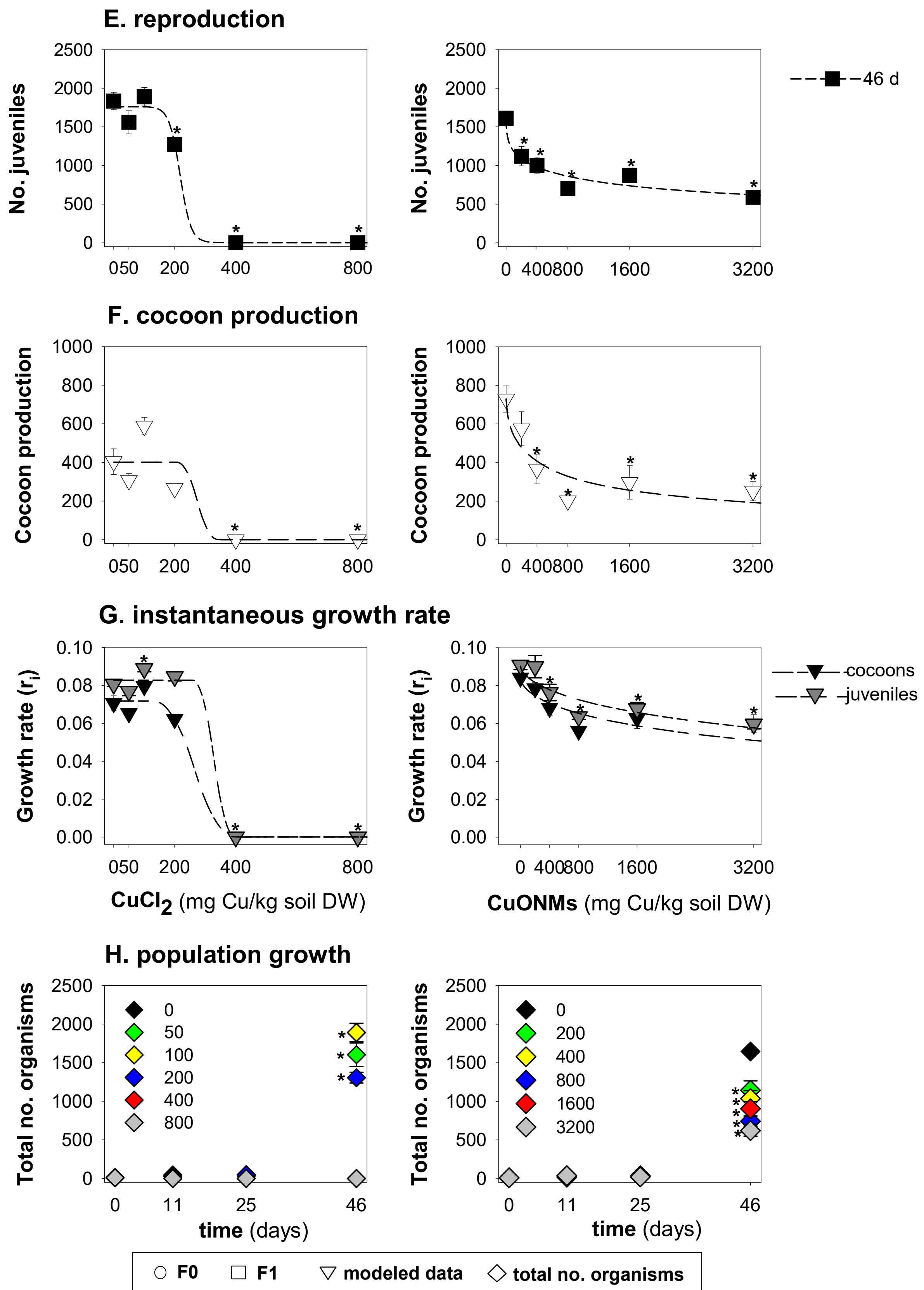

E. reproduction
No. juveniles
46 d
F. cocoon production
Cocoon production
G. instantaneous growth rate
Growth rate (r_i)
cocoons
juveniles
CuCl2 (mg Cu/kg soil DW)
CuONMs (mg Cu/kg soil DW)
H. population growth
Total no. organisms
time (days)
F0
F1
modeled data
total no. organisms

Fig. 2. Results of the Full Life Cycle test (FLCt) for *Enchytraeus crypticus* exposed to $CuCl_2$ and CuONMs (mg Cu/kg DW soil) in LUFA 2.2 soil including the various endpoints: **A**: hatching; **B:** growth; **C:** maturity status; **D**: survival (number of organisms present); E: reproduction; **F**: cocoon production; **G:** instantaneous growth rate (r_i) and **H**: population growth. All values are expressed as average ± standard error (Av±SE). The lines represent the model fit to data. * p <0.001, Dunnets'. d = days, X = absence of organisms.

For $CuCl_2$ there was a decrease in a concentration-response manner in terms of hatching success, survival and reproduction within the tested range (Fig. 2A, D, E). Significant effects ($p < 0.001$, Dunnets') are noted in the respective figure. No effect was observed on maturity status. In terms of growth there was no concentration-response effect, except for significant decreases at particular concentrations, e.g. at 46 days there was a significant decrease in length at 100 mg Cu/kg ($F_{3,318} = 7.76$, $p < 0.001$).

Results for CuONMs showed no effect in terms of hatching success and survival (Fig. 2A, D). Maturity status and reproduction were reduced in a concentration-response manner, although for both there seems to be a stabilization around 400-800 mg Cu/kg and beyond.

In terms of growth there was a growth inhibition with concentration increase, mostly up to 800 mg Cu/kg.

Calculations of cocoon production and instantaneous population growth rate (r_i) show a concentration-response decrease for both Cu forms, although to a higher extent for $CuCl_2$. The instantaneous population growth rate (r_i) at e.g. 400 mg Cu/kg is $r_i = 0$ and $r_i = 0.068$ for $CuCl_2$ and CuONMs respectively.

Table 2 Summary of the effect concentrations, for *Enchytraeus crypticus* when exposed to $CuCl_2$ and CuONMs in LUFA 2.2 soil. Results show ECx (Effect Concentration) estimates including LCx (Lethal Concentration) depending on the endpoint, and the 95 % confidence intervals (in brackets). Model and parameters include the values for slope (S) and intercept (Y0). EC values (mg/kg) are given per endpoint and test material. n.d.: not determined; n.e.: no effect. "par" = parameter. NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration.

Test	Endpoint	Time (days)	EC_{10}	EC_{20}	EC_{50}	EC_{80}	NOEC / LOEC	Model and parameters
	$CuCl_2$							
FLC	Hatching	11	**67** (19-115)	**115** (82-147)	**210** (180-240)	**279** (225-332)	<50/ <50	Threshold 2 par (S: 0.04; Y0: 44.9)
	Growth	11-46	n.e.	n.e.	n.e.	n.e.	-	-
	Maturity status	25	n.e.	n.e.	n.e.	n.e.	-	-
	Survival	14	**260** (43-477)	**284** (102-466)	**324** (199-449)	**364** (285-442)	<50/ <50	Log 2 par (S: 0.09; Y0: 41.9)
		25	**264**	**280**	**312**	**335**	200/ <400	Threshold 2 par (S: 0.01; Y0: 45.4)
		46	**181**	**191**	**209**	**227**	100/ <200	Log 2 par (S: 0.02; Y0: 47.7)
	Reprod	46	**179**	**193**	**218**	**242**	100/ <200	Log 2 par (S: 0.01; Y0: 1761.7)
	Cocoon production	46	**237**	**250**	**274**	**292**	200/ <400	Threshold 2 par (S: 0.01; Y0: 400.9)
	Inst. growth rate (r_i) (cocoons)	46	**190** (167-214)	**215** (193-238)	**265**	**301** (243-359)	100/ <200	Threshold 2 par (S: 0.007; Y0: 0.07)
	Inst. growth rate (r_i) (juveniles)	46	**290**	**303**	**329**	**347**	200/ <400	Threshold 2 par (S: 0.01; Y0: 0.08)
ERT	Survival	28	**112** (26-198)	**183** (118-247)	**303** (251-355)	**424** (344-503)	<50/ <50	Log 2 par (S: 0.003; Y0: 9.3)
	Reprod	28	**18**	**78** (45-111)	**179** (153-206)	**281** (230-332)	<50/ <50	Log 2 par (S: 0.003; Y0: 598.7)
	CuONMs							
FLC	Hatching	11	n.e.	n.e.	n.e.	n.e.	-	-
	Growth	11	**920** (663-1178)	**1121** (604-1638)	**1465** (470-2460)	n.d.	400/ <800	Threshold 2 par (S: 0.001; Y0: 1.29)

		14	**636** (535-738)	**892** (769-1015)	**1329** (1008-1651)	n.d.	400/ <800	Log 2 par (S: 0.0008; Y0: 2.32)
		25-46	n.e.	n.e.	n.e.	n.e.	-	-
	Maturity status	25	n.d.	**1164** (329-2000)	**3833** (2383-5282)	**5756**	800/ <1600	
	Survival	14	**2521**	**2886**	**3611**	**4133**	1600/ <3200	Threshold 2 par (S: 0.0005; Y0: 32.4)
		25-46	n.e.	n.e.	n.e.	n.e.	-	-
	Reprod	46	**8** (0-171)	**42** (6-281)	**1075** (612-1888)	**11108** (1929-63973)	<200/ <200	Log 2 par (S: 0.0001; Y0: 1613.5)
	Cocoon production	46	**18** (1-439)	**59** (7-468)	**582** (289-1172)	**3045** (722-12845)	<200/ <200	Threshold 2 par (S: 0.368; Y0: 729)
	Inst. growth rate (r_i) (cocoons)	46	**155** (41-584)	**622** (322-1198)	**6690** (2364-18933)	**72005** (7010-739630)	<200/ <200	Log 2 par (S: 0.336; Y0: 0.08)
	Inst. growth rate (r_i) (juveniles)	46	**211** (71-626)	**816** (484-1375)	**8215** (2982-22634)	**82715** (9491-720890)	<200/ <200	Log 2 par (S: 0.345; Y0: 0.09)
ERT	Survival	28	**421** (210-631)	**841** (660-1023)	**2103** (1855-2352)	**3365** (2936-3795)	200/ <400	Pcw L 2 par (S: 0.0002; Y0: 8.87)
	Reprod	28	**438** (312-616)	**643** (504-820)	**1377** (1157-1638)	**2383** (1773-3202)	200/ <400	Threshold 2 par (S: 0.0004; Y0: 695.7)

4. Discussion

The results clearly showed that CuONMs and $CuCl_2$ have different effects over the various stages of *Enchytraeus crypticus'* full life cycle, although similarities were also observed.

In both test systems, ERT and FLCt, $CuCl_2$ and CuONMs affect reproduction more than it affected survival, e.g. for CuCl2 the ERT-$EC_{10/50}$= 18/179 mg Cu/kg and ERT-$LC_{10/50}$= 112/303 mg Cu/kg. The results from the FLCt allowed further understanding such as that the $CuCl_2$ effect occurred during embryo development and/or hatching success, and is not related with juvenile mortality or development (obviously not possible to derive this conclusion via the ERT). Available studies for fish species confirm such embryotoxic effects of Cu and

decreased hatching success (Dave and Xiu 1991; Johnson et al. 2007; Ozoh 1979; Palmer et al. 1998). Moreover, we could conclude that for concentrations ≤ 200 mg Cu/kg the effect represented a hatching delay (later samplings, at 14 and 25 days showed an increase in the number of juveniles) and only at concentrations ≥ 400 mg Cu/kg was the effect irreversible because at this concentration there was no hatching. In this case the 11 days hatching ECx was similar to the 46 days LCx, which shows effect predictability for $CuCl_2$. Another important detail to note is related with the comparison between the ERT and FLCt, where the mortality was higher at the highest concentrations when determined via the FLCt. In terms of reproduction the effect is similar at higher concentrations probably because Cu is mostly toxic in the post-cocoon laying stage. Effect on growth was visible on adults (at 46 days). This effect has also been shown before in springtails (*Sinella curviseta*) exposed to $CuCl_2$ (Xu et al. 2009), where a reduction in body length of adult organisms after 21 and 28 days of exposure was observed (but not at 14 days). Further, Ma (1984) observed an effect on juvenile growth and development for *Lumbricus rubellus* when exposed to $CuCl_2$. Here, adult worms were exposed and the growth and development of their offspring juveniles was monitored, showing reduced body weight gain in all tested concentrations (60 – 362 mg Cu/kg soil) and in the highest concentration juveniles never matured (no clitellum development). Also van Gestel et al. (1991) observed a similar effect on growth (body weight) and development of the earthworm *Eisenia andrei*; Here exposure from juvenile stage at concentrations up to 32 mg Cu/kg soil significantly increased body weight and the number of mature organisms, whereas from 56 mg to 100 mg Cu /kg it was significantly decreased.

The population growth rate (r_i) shows that cocoon stage was more sensitive than juvenile. Further, our study showed that e.g. at ≥ 400 mg Cu/kg the population was stable/not growing, since r_i was zero. This has also been observed in springtails (*Folsomia candida*) exposed to $CuCl_2$, where ri was ca. 0 at 3200 mg Cu/kg and negative at 12800 mg Cu/kg (Herbert et al. 2004).

Results of CuONMs exposure show clear differences from $CuCl_2$, i.e. no effect at hatching and survival although juveniles´ growth, maturation and reproduction were compromised. In support of this, Bacchetta et al. (2012) also observed that CuONMs caused no lethal effects (up to 500 mg Cu/L) in *Xenopus laevis* larvae but severely affected their development and caused tissue damage (mostly in the gut). In addition, studies on aquatic species show that CuONMs can inhibit growth and or development of fish, aquatic invertebrate and

amphibian's species (Nations et al. 2011; Pradhan et al. 2012; Ramskov et al. 2014; Zhao et al. 2011).

The estimates for instantaneous growth rate (r_i) for juveniles and cocoon production allowed good prediction, note this is only possible to derive in the FLC test, and hence are very useful for CuONMs hazard and risk assessment. The ECx are particularly relevant for the lower range (EC_{10-20}) because the effects flattened at higher concentrations of CuONMs. As observed, concentration response occurs up to 800 mg Cu/kg after which effects are more or less maintained up to 3200 mg Cu/kg. A logical explanation may be the consequent increased agglomeration and or aggregation with increase in nanoparticle concentration and, following this, a decrease of bioavailability and effect. As shown by Dimkpa et al. (2012) using dynamic light scattering, CuONMs can form aggregates up to 300 nm.

The effect in reproduction observed in *E. crypticus* exposed to CuONMs seems to be due to both a delay and decrease in the juvenile development, i.e. growth and maturation (as opposed to the embryo level for $CuCl_2$).

Additionally, $CuCl_2$ toxicity (reproduction) was higher via the ERT compared to the FLCt; this is less expected as earlier life stages (e.g. cocoons) are often more sensitive than later (e.g. juveniles). This lower toxicity in the FLC seems to indicate some kind of increased tolerance when organisms' exposure starts from cocoons compared to juveniles. This is somewhat confirmed by the results obtained in a multigenerational exposure to $CuCl_2$, where F1 to F4 generations were observed to have decreased toxicity (Bicho et al. *submitted*) compared to F0 (when exposed to the EC_{50}); for CuONMs toxicity is maintained across generations.

Overall, results indicate different response mechanisms to Cu salt and Cu nano. Gomes et al. (2011) study on the toxicity of CuONMs and ionic Cu in the gills of the mussel *Mytilus galloprovincialis* supports this observation as CuONMs effects are a combination of particle effects on cell membranes and a high release of ions inside the cell "*trojan horse effect*". This combination would also cause an activation of the antioxidant defence system. Sincealthough ionic Cu has also been shown to cause oxidative stress, e.g. to springtails (*Folsomia candida*) (Maria et al. 2014) to earthworms (*Eisenia fetida*) (Gaete et al. 2010) and to enchytraeids (*Enchytraeus albidus*) both in the salt and nano form (Gomes et al. 2012), oxidation effects does not seem to provide the full explanation for the differences. Effects at the energy level, showed that both $CuCl_2$ and CuNMs significantly increased energy consumption and cellular energy allocation in enchytraeids (*E. crypticus*) (Gomes et al. 2015b), which probably reflects

energy shifts to deal with stress, e.g. detoxification processes. Such energy reallocations are known to further compromise normal functions like growth/development and reproduction (Calow 1991), like we observe here.

Last, dissimilarities between Cu forms in terms of Cu availability uptake cannot be excluded. Croteau et al. (2014) showed that bioaccumulation of Cu in new waterborne snails (*Lymnaea stagnalis*) was mostly in the form of CuONMs (80-90 %). Also another study with the snail *Potamopyrgus antipodarum* showed that organisms accumulated more Cu from the CuONMs treatment comparing with $CuCl_2$ (Pang et al. 2012). Dimkpa et al. (2012) showed that Cu was solubilized from CuONMs in a sand matrix, but also that NM remain in the media: for 500 mg Cu/kg soluble Cu was 3 mg/L just after spiking, 2.5 mg/L after 1 day and 1 mg/L after 7 and 14 days. This shows the potential solubility of CuONMs with a stabilization or equilibrium point after a certain time, although the sand matrix is a rather simple version compared to soil. As from soil measurements in our study, the free active Cu was less than 0.001 % for both Cu forms exposure, so this does not seem to be the source for differences. On the other hand, the total Cu in soil solution for $CuCl_2$ was similar or up to 3 times higher than for CuONMs at total concentrations between 200-400 mg Cu/kg, which could add on the increased toxicity given potential higher bioavailable fraction, although this cannot account for all differences.

Navratilova et al. (2015) showed that it was possible to detect larger CuONMs by Single Particle ICP-MS, but due to the interaction with soil components it was not possible to separate Cu ions bound to small natural particles from CuONMs present in the sample (the CuONMs used in the present study are below the 15 nm theoretical detection limit). However, our observations are that the nano-form persists (even though in the form of agglomerates) and do not completely solubilize in the presence of soil components, i.e. organic matter, as previously observed via XAS studies by Gomes et al (2015a).

For an overview of the various events and integrated knowledge, a draft AOP was framed (Figure 3).

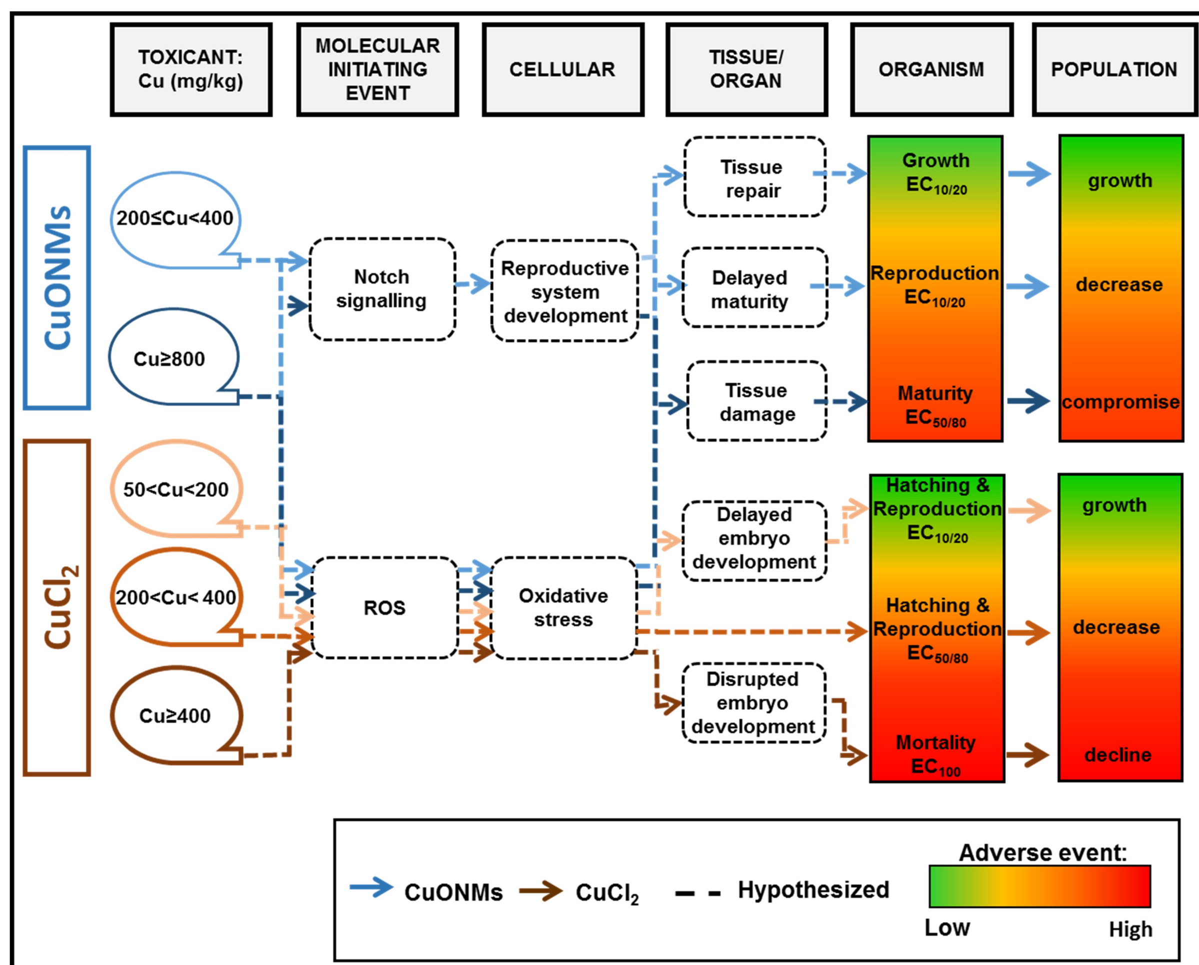


Fig. 3. Adverse Outcome Pathway (AOP) for *Enchytraeus crypticus* when exposed to CuONMs and $CuCl_2$ in LUFA 2.2 soil. Red: adverse effect; Orange: semi-adverse effect; Green: = no adverse effect; Square boxes represent final states for the organism and rounded boxes represent intermediate states. Dashed line represents relationships hypothesized.

The differences between AOPs showed that $CuCl_2$ and CuONMs cause different response patterns in the organism, and with more evidence (further studies) such AOP differences may be used to identify exposure forms and furthermore find mechanistic understanding for population responses.

5. Conclusions

The FLCt represents a clear improvement to the current ERT, and hence a recommended alternative for the hazard and risk assessment of nanomaterials. CuONMs caused toxicity during the juvenile stage, showing reduced growth, maturation and hence reproductive output. $CuCl_2$ caused toxicity during the embryo development and/or hatching success which

is reflected in their survival when adults and consequent decreased reproduction. The differences in the hazard seem to be a combination of differences in fate and effect, as imposed by the NM and organism life cycle stage.

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Chapter V - Multigenerational effects of copper nanomaterials (CuONMs) are different of those of $CuCl_2$: exposure in the soil invertebrate *Enchytraeus crypticus*

Rita C. Bicho[a], Fátima C.F. Santos[a], Janeck J. Scott-Fordsmand[b], Mónica J.B. Amorim[a*]
[a]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[b]Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark
*Corresponding author: mjamorim@ua.pt

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Abstract

Nanomaterials (NMs) are recommended to be tested in longer term exposures. Multigenerational (MG) studies are scarce and particularly important because effects can be transferred to the next generation. The current risk assessment framework does not include MG effects and this is a caveat for persistent materials. Here, the effects of copper NMs (CuONMs) and copper salt ($CuCl_2$) were assessed in a MG exposure (4 generations in spiked soil + 2 generations in clean soil, F1 to F7 generations in total), with the standard soil model *Enchytraeus crypticus*, using relevant reproduction test effect concentrations (EC_{10}, EC_{50}), monitoring survival and reproduction. This represented ca. 1 year continuous exposure tests. MG effects varied with effect concentration and test materials: CuONMs caused increased toxicity for EC_{10} exposed organisms (EC_{50} did not change), and transfer to clean media reset effects, whereas $CuCl_2$ reduced toxicity for EC_{10} and EC_{50}, but the transfer to clean media "revived" the initial effects, i.e. close to EC_{50} levels in F7. Clearly CuONMs and $CuCl_2$ cause different mechanisms of toxicity or response in the long term, not predictable based on short term or one generation studies. The present results can contribute for the improvement of chemicals risk assessment, adding important information for the long term exposure and effects of Cu NMs.

Keywords: environment; oligochaete; generational; transgenerational; epigenetics;

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Introduction

Continuous multigenerational (MG) exposure to chemicals may induce physiological adaptations such as increased tolerance[1, 2]. Physiological adaptation processes can be related to organisms' phenotypic plasticity. This is the ability of a genotype to display several phenotypes that can present variations in biochemistry, physiology, morphology or life traits, among others, in response to environmental changes[3] or stressors like chemicals. Phenotypic plasticity can involve epigenetic mechanisms[3-5] i.e. changes in gene function without altering DNA sequence and that are transferred to the next generation. Further, if gene function effects prevail in non-exposed next generations, then these are named transgenerational epigenetic effects (maternal effects)[4, 6]. Adaptation can involve change in organisms´ genetic material, where e.g. most tolerant genes can be selected[4, 7]. This change in population genetics can be preserved along generations even when the stressor is removed[4]. On the other hand, MG exposure can induce an increase in organisms' sensitivity as shown by e.g. Yu et al[8]. Other studies, where only the parental generation was exposed, show transgenerational transfer of sensitivity[9].

For nanomaterials (NMs), long term studies are one of the key recommendations to ensure a sustainable environmental development[6, 10, 11]. However, few long term studies are available and less than a handful of MG studies are published (see Table S1 for a summary), and none in soils.

For example, for the aquatic invertebrate *Daphnia* (various species) exposure to carbon and silver NMs showed an increased sensitivity or tolerance depending on the test material and test concentrations[12, 13]. There are also studies with *Caenorhabditis elegans*[14] performed in simulated pore water (SPW), agar or agar media (instead of soil) studying the effects of gold NMs, the parental generation was exposed, showing increased toxicity in second (unexposed) generation, hence transgenerational effects. Additionally, Schultz et al.[15] investigated the effects of AgNMs, showing increased sensitivity in second generation, and the effect remained during later generations and unexposed generations, also indicating transgenerational effects.

The effects of CuNMs in enchytraeids are well reported in the literature, assessing various endpoints, covering survival, reproduction[16, 17], avoidance behaviour[16], cellular energy allocation[18] and oxidative stress[19]. Recently a novel study analysed the effects along the full life span of *E. crypticus*[20], showing that organisms exposed to CuONMs EC_{50} lived shorter than when exposed to $CuCl_2$. Although, there are no MG effect studies performed for NMs.

In the present study we aimed to investigate the effects of MG exposure to copper oxide NMs (CuONMs) and copper salt ($CuCl_2$), using *E. crypticus*, a soil model representative[21-23]. Survival and reproduction effects were assessed along four generations of continuously exposed organisms to Cu plus 2 generations in clean media, hence the transgenerational potential was also assessed (6 generations in total).

Methods

Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. *E. crypticus* cultures have been kept for many years at the University of Aveiro. Synchronized cultures were prepared as described in Bicho et al.[23], using juveniles with 17-18 days after cocoon laying. Mature adults with well-developed clitellum are transferred to agar plates to lay cocoons; synchronized 1-2 days old cocoons are placed in new agar plates and left to grow.

Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. Soil characteristics are: pH = 5.5, organic matter = 1.77 %, CEC (cation exchange capacity) = 10.1 meq/100 g, WHC (water holding capacity) = 41.8 %, grain size distribution of 7.3 % clay, 13.8 % silt, and 78.9 % sand.

Test materials and spiking

Copper (II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$ > 99.9 % purity, Sigma-Aldrich, CAS number 10125-13-0) and copper oxide nanomaterials, CuONMs (PlasmaChem GmbH) were used. For details see table 1.

Table 1. Characteristics of the tested CuONMs (Source: FP7-SUN (Sustainable Nanotechnologies European Commission funded project).

Characteristics	**CuONMs**
Manufacturer	Plasma Chem
CAS number	1317-38-0
Primary size distribution (average)	3-35 (12)
Mode (1st quartile - 3rd quartile) [nm]	10 (9.2-14)
Shape	Semi-spherical
Average crystallite size [nm]	9.3
Crystallite phases (%)	Tenorite 100 %
Dispersability in water: D50 [nm]; average agglomeration number (AAN)	139.5 ± 4.6; 346
Dispersability in modified MEM: D50 [nm]; average agglomeration number (AAN)	85.2 ± 2.7; 77
Z-potential in UP water [mV]	+ 28.1 ± 0.6

Isoelectric point [pH]	10.3
Photocatalysis: photon efficiency [unitless]	1.5 x 10-4
Specific Surface Area [m2 g-1]	47.0 ± 1.7
Pore sizes [nm]	13.5 ± 1.6 (BJH) 23.0 ± 0.9 (AVG)
Surface chemistry [atomic fraction]	Cu = 0.46 ± 0.05; O = 0.47 ± 0.05 C = 0.07 ± 0.01

The tested concentrations were 0-500-1400 mg Cu/kg soil (DW) for CuONMs and 0-20-180 mg Cu/kg soil (DW) for $CuCl_2$. Concentrations were selected based on the reproduction effect concentrations EC_{10} and EC_{50}[26] as shown in the table 2 for reference:

Table 2. Estimated effect concentration (EC) values for the Enchytraeid Reproduction Test (ERT) performed with *Enchytraeus crypticus* when exposed to CuONMs and $CuCl_2$ in LUFA 2.2 soil. Confidence intervals are shown in brackets. n.d.: not determined.

	Reproduction		Survival	
	EC_{10}	EC_{50}	LC_{10}	LC_{50}
CuONMs	**438** (312-616)	**1377** (1157-1638)	**421** (210-631)	**2103** (1855-2352)
$CuCl_2$	**18** (n.d.)	**179** (153-206)	**112** (26-198)	**303** (251-355)

For CuONMs spiking followed the recommendations for nanomaterials[31]. Spiking was performed individually, in 5 g of soil per replicate, and mixed with the corresponding amount of the test materials (as dry powders) to the nominal concentration. The spiked soil was added to the remaining soil (35 g) and homogeneously mixed, per individual replicate, to ensure total raw amounts. Soil was allowed to equilibrate for 1 day prior test start. Moisture was adjusted to 50 % of the WHC_{max}. For $CuCl_2$ a stock aqueous solution was done and sequentially diluted; spiking was done onto the pre-moistened soil amounts per concentration, this being homogeneously mixed and split onto replicates. Soil was freshly spiked 1 day prior each of the generations.

In situ characterisation

The amount of Cu was measured in the test soil and in soil solution (for method details see Gomes et al.[17]) in a concurrent experiment over the test duration. In the soil the total Cu was

measured (by Graphite Furnace Atomic Absorption Spectroscopy: AAS-GF) and in soil solution both the total Cu (AAS-GF) and free active form (ion-selective electrode) were measured. The CuO present as nanomaterials was not determined in the soil, due to the technical difficulties, e.g. the particle size is below the theoretical detection limit of 15 nm[28].

Test procedures

A first set of preliminary tests were performed to optimize sampling days, number of organisms, etc (data not shown). The final test design is illustrated in Fig. 1. Test duration per generation is 32 days, this ensures that juveniles are as large size as possible but still allowing to discriminate from adults, i.e. just before being fully grown, mature and releasing cocoons of next generation.

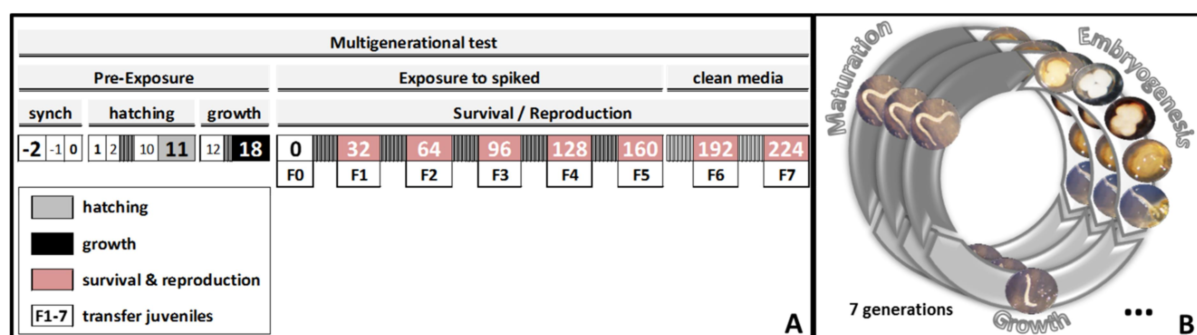

Figure 1. Schematic representation of *Enchytraeus crypticus* multigenerational test. The figure includes **A:** life stages from cocoon to mature adults and the following generations and associated time (days) and **B**: Multigenerational test design including sampling days for synchronization and transfer between generations.

Multigenerational test (MGt)

Test followed the standard guideline[21] with adaptations as follows. Forty (40) juveniles (17-18 days' age) per replicate were used. Organisms were collected and introduced in test vessels, containing 40 g of moist soil and food supply. For each generation tests ran during a period of 32 days at 20 ºC and 16:8 h photoperiod. In total six generations were exposed, hence the total test duration was 224 days. Test design involved 4 + 2 generations, 4 in spiked soil (F0-F4) plus 2 in clean soil (F5-F6) to assess organisms' recovery. Food and water was replenished weekly. For control and the EC_{10} six replicates per treatment were used, for the EC_{50} ten replicates were used to ensure enough number of organisms for next generations and analysis. At the end of each generation, deionized water was added to each replicate and soil was left to deposit for 20 min, after this organisms (adults and juveniles) were carefully transferred to freshly made reconstituted ISO water[32]. From each replicate adults (n = 20) and

juveniles of large and medium size (n = 400) were sampled, snap-freeze and kept at -80 ºC for further analysis. Juveniles of medium size (n = 40) were collected and transferred to freshly spiked test soil for another generation and so forth.

The soil and remaining organisms were counted, replicates were fixated with 96 % ethanol and Bengal red (1 % solution in ethanol) as described[23].

Data analysis

One-way analysis of variance (ANOVA) followed by Dunnett´s comparison post-hoc test ($p \leq 0.05$) was used to assess differences between generation F0 and the other generations within each treatment[33].

Results

Multigeneration (MG) test

The validity criteria from the standard test[21] were fulfilled, i.e. for juveniles the coefficient of variation was < 20 %, the number of juveniles was ≥ 50 and adults' mortality was ≤ 20 %. Values for soil pH did not change significantly within concentrations and during all multigenerational tests.

Results of the MG test in terms of survival and reproduction can be depicted in Fig. 2.

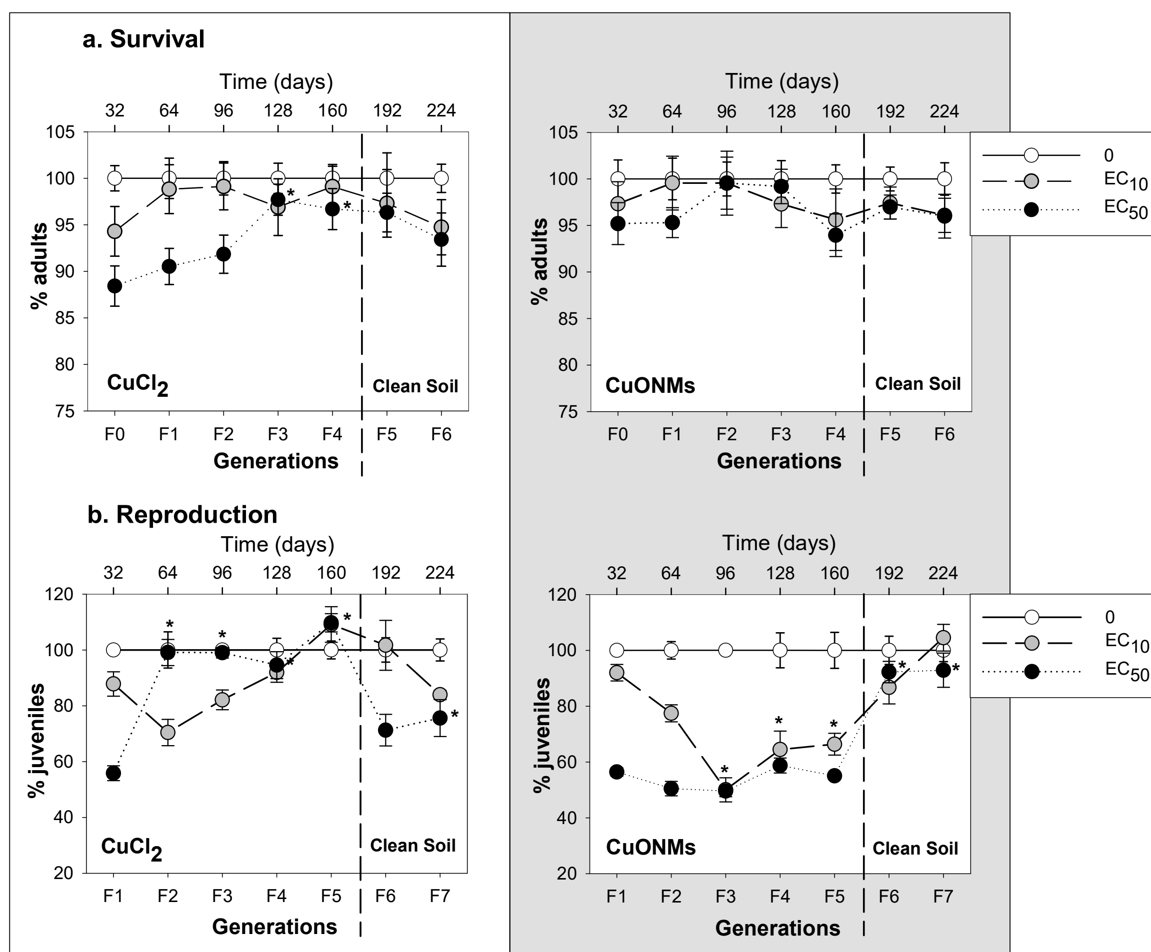

Figure 2. Results of the multigenerational test (MGt) for *Enchytraeus crypticus*. Exposure to the reproduction EC_{10} and EC_{50} of CuONMs and $CuCl_2$ (0-500-1400 mg Cu/kg and 0-20-180mg Cu/kg DW soil, respectively) in LUFA 2.2 soil in terms of survival (A) and reproduction (B). All values are expressed as % normalized to the control, average ± standard error (Av±SE). *p<0.05 (Dunnets' between parental generation (F0/F1) and Fx).

The effect depended on the Cu materials and the test concentration. Results for reproduction show that the selected EC_{10} and EC_{50} values were approximately confirmed in F1: this corresponded for CuONMs to 8 % (± 2.9) and 44 % (± 1.5) reduction, for $CuCl_2$ to 12 % (± 4.4) and 44 % (± 2.6) reduction respectively.

Results for the MG exposure with CuONMs in terms of survival showed that no significant effect occurred along generations for both exposure concentrations, which is not surprising given the sub-lethal concentrations. In terms of reproduction the EC_{10} exposure caused an increase in the toxicity followed by a levelling off while being exposed i.e. from F2→F5: F2

= 23 % decrease ($F_{2,21} = 77.21$, $p < 0.001$), F3 = 50 % decrease ($F_{2,21} = 99.12$, $p < 0.001$), F4 = 36 % decrease ($F_{2,21} = 20.57$, $p < 0.001$) and F5 = 34 % decrease ($F_{2,21} = 40.56$, $p < 0.001$) in the number of juveniles compared with control. For the EC_{50} exposure the effect was maintained in the same level as F1 in the subsequent generations from F2→F5. F2 = 50 % decrease ($F_{2,21} = 77.21$, $p < 0.001$), F3 = 50 % decrease ($F_{2,21} = 99.12$, $p < 0.001$), F4 = 41 % decrease ($F_{2,21} = 20.57$, $p < 0.001$) and F5 = 45 % decrease ($F_{2,21} = 40.56$, $p < 0.001$) in the number of juveniles compared with control. The transfer to clean soil (F5→F7) showed a recovery from the effect, i.e. by F6 the number of juveniles was similar to control levels.

For $CuCl_2$ the MG exposure in terms of survival showed that for EC_{10} there was a decrease in the effect from F1→F4, with the number of adults similar to control. EC_{50} exposure caused a similar effect with values being similar to control from F3→F4. After transfer to clean soil the organisms showed no effect, i.e. similar to control. In terms of reproduction, for EC_{10} the effect increased from F1→F2, i.e. there was a significant reduction in the number of juveniles (30 %) ($F_{2,21} = 8.92$, $p = 0.002$), after which there was a decrease from F3→F5, showing reproduction even higher than control at F5. The transfer to clean soil at EC_{10} showed for F6 values similar to control but in F7 there was an increase in the effect similar to the F1 (EC_{10}). For EC_{50}, transfer to clean soil showed an increase in the effect to values close to the F1 (EC_{50}), F6 = 30 % reduction ($F_{2,21} = 7.78$, $p = 0.003$) and F7 = 25 % reduction ($F_{2,21} = 4.78$, $p = 0.021$).

In situ characterization

Our measures show that the total Cu measured in the soil was ca. 100 % of the added total concentration for both CuONMs and $CuCl_2$. The total in soil solution was less than 0.07 % for the controls, less than 1 % of the total for CuONMs, and less than 3 % for $CuCl_2$. The free active Cu was less than 0.004 % in controls, and less than 0.001 % for both Cu forms exposure. As actual concentrations, the total Cu in soil solution for $CuCl_2$ was similar or up to 3 times higher than for CuONMs at total concentrations between 200-400 mg Cu/kg. It was not possible to identify whether the Cu in the soil solution was NM or free ions, due to the small size (approx. 10 nm, table 1) of our Cu particles.

Discussion

For both tested materials the MG effects were highly dependent on test concentration. For CuONM the EC_{10} exposure caused increased toxicity with a MG exposure, and by F3 the EC_{10} became similar to EC_{50} level, this being maintained in F4 and F5. On the other hand, for the EC_{50} MG exposure, the toxicity was similar from F1 to F5, hence no apparent increased tolerance or sensitivity occurred. For both ECx exposures transfer to clean soil induced full reset to no toxicity, i.e. values similar to control. The few literature results with MG with NMs show indications of either response. For example, a MG study in *D. magna* exposed to carbon NMs showed increased toxicity in generation F1 and its maintenance in F2[12]. In *C. elegans* exposed to AgNMs toxicity increased in F2 and this was maintained until F10[15]. For *C. elegans* transfer to clean media showed a similar toxicity as transferred, i.e. transgenerational effects, which could be related with epigenetic mechanisms[15] although not confirmed. Li et al.[4] also observed increased toxicity to mercury with MG exposures in *T. japonicus*, and then full recovery from effect when transferred to clean media, indicating only physiological effects rather than genetic or epigenetic changes. The same was observed to the same species on a MG exposure with Cu[24].

For $CuCl_2$ in terms of reproduction, the exposure to the EC_{10} caused an increase in toxicity for F2 and F3, and the opposite for F4 and F5, where an increased tolerance/resistance is observed after 2-3 generations. On the other hand, for the EC_{50}, there was an immediate decrease in toxicity after 1 generation, i.e. from F2, this being maintained until F5 (with an actual *hormesis* like effect in F5), again suggesting an increased tolerance/resistance after F1. Similar results were observed in a previous study with *E. crypticus* exposed to $CuCl_2$ for two generations[25]. In terms of survival, the effect also decreased after 1 exposure generation, although here to a lower extent. Such an increase in tolerance has been similarly observed in *D. magna*[1] exposed for six generations to $CuCl_2$ where the reproductive output increased with concentrations of 0.5 to 100 µg Cu/L, although the decreased toxicity did not reach values as similar to control as in our study. On the other hand, the opposite has also been shown by Yu et al.[9], where *C. elegans* was exposed for four generations to $CuCl_2$ and reproduction toxicity increased for both concentrations tested (0.1 mg Cu/L and 10 mg Cu/L) along generations. Multigenerational effects of Cu investigated in the marine invertebrate *Tigriopus japonica* [24] for two generations, (where for each generation the offspring was transferred to clean media to evaluate recovery effects), showed similarly a decrease in toxicity for reproductive output to both tested concentrations (10 and 100 µg Cu/L), this being higher for the highest

concentration (still below control); for the generations transferred to clean media, organisms presented a full recovery to same as control levels. So, not only MG effects seem dependent on the test concentration and materials, but are also species specific. Interestingly, in our MG exposed animals, the transfer to clean soil (F5→F7) there was an increase in toxicity, with the organisms changing from F5 no effect to similar F1 effect level, like a memory effect. Results may indicate that parent organisms which are exposed to $CuCl_2$ during one cycle produce offspring that developed defences or activated mechanisms to keep new homeostasis levels (Cu is an essential element); when the element is reduced in the transfer to clean media, it could be that there is now a deficit of Cu concentration for the new homeostasis levels, hence a shift in the stress type, from excess Cu to deficit. Hence, the mechanism does not necessarily seem to involve epigenetics, but rather a physiological adaptation, e.g. metallothyonein or Cu binding proteins activation. This is the first time that such a remarkable increased tolerance to Cu is shown (from EC_{50} to EC_0 in 4 generations) and then also regained after 2 generations in clean media. This could indicate a particular plasticity of *E. crypticus* to $CuCl_2$.

This experimental design (4+2) highlights that care should be taken regarding potential extrapolation of effects: one could assume that organisms exposed to Cu during 4 generations become more Cu-tolerant, although the transfer to clean soil shows that sensitivity was still embedded.

The differences observed between $CuCl_2$ and CuONMs indicate different mechanisms of toxicity or response. This is also confirmed via the full life cycle test performed with *E. crypticus*[26] where effects were life stage dependent and distinct for the two Cu forms.

An interesting observation was the increased toxicity for EC_{10} exposure for both Cu forms, which did not occur for the EC_{50} exposure. Similarly, Amorim et al.[27] in a MG study where *Folsomia candida* was exposed to cadmium EC_{10} and EC_{50} along more than one year, observed increased toxicity for EC_{10} exposure, leading to population extinction, although not for the EC_{50}. This highlights not only the need for MG studies but also that low concentrations can have a significant and higher impact.

A much discussed issue in relation to metallic NM exposure, is whether the organisms are exposed to the NM or a dissolved part of the metal, i.e. here Cu ions. As from soil measurements, the free active Cu was less than 0.001% for both Cu forms exposure, so this does not seem to be the source for differences. On the other hand, the total Cu in soil solution for $CuCl_2$ was 3 times higher than for CuONMs which could argue for a potential higher

bioavailable fraction and activate mechanisms differently, although this doesn't seem to account for all differences. Navratilova et al.[28] showed that it was possible to detect larger CuONMs by Single Particle ICP-MS (the theoretical detection limit being 15 nm), but due to the interaction with soil components it was not possible to separate Cu ions bound to small natural particles from CuONMs present in the sample. However, they showed that CuONMs persisted in the nanoform (even though in the form of agglomerates) and do not completely solubilize in the presence of soil components, i.e. organic matter. Another study showed that Cu was solubilized from CuONMs in a sand matrix and also that NM remained in the media: for 500 mg Cu/kg soluble Cu was 3 mg/L just after spiking, 2.5 mg/L after 1 day and 1 mg/L after 7 and 14 days[29]. This indicates the solubility of CuONMs tending to a stabilization/equilibrium point after some time. Nevertheless, a sand matrix is a fairly basic media compared to soil.

One should be reminded that in this MG test we are limited in terms of measured endpoints to survival and reproduction, hence a refined understanding is limited. Survival and reproduction are very important and will capture other effects, but as we learned from the full life cycle test[26, 30] including endpoints such as hatching and growth can help discriminate at what life stage the effects differs between nano and salt forms. Further studies, e.g. at the molecular level, should be performed to clarify the observed phenotypic effects, to measure the potential for epigenetics mechanisms is an obvious one.

Clearly CuONMs and $CuCl_2$ caused different mechanisms of toxicity or response in the long term, in this case MG, not predictable based on short term or one generation studies. The present results can contribute for the improvement of chemicals risk assessment, adding important information for the long term exposure and effects of Cu NMs.

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Supplementary information

Multigenerational effects of copper nanomaterials (CuONMs) are different of those of $CuCl_2$: exposure in the soil invertebrate *Enchytraeus crypticus*

Rita C. Bicho[a], Fátima C.F. Santos[a], Janeck J. Scott-Fordsmand[b], Mónica J.B. Amorim[a*]

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

*Corresponding author: mjamorim@ua.pt

Table S1: Summary overview of available literature data on multigenerational exposure of invertebrate species to nanomaterials. ↑: increase, ↓: decrease; ≈: similar toxicity. Ref: reference.

Species	NM	Media	Design	Endpoint	Effect	Ref
D. magna	MWCNTs	Water	Generations: F0-F2; Exposure: parent F0	survival	↑F1	12
				size (length)	↓F1-F2	
	C60-βCD	Water	Generations: F0-F2; Exposure: parent	survival	↑F1	
				reproduction	↓F1	
	C60-malonate	Water	Generations: F0-F2; Exposure: parent	survival	↑F1	
				reproduction	↑F1	
				size (length)	↑F1	
	SWCNT-CONH2	Water	Generations: F0-F2; Exposure: parent	reproduction	↑F1-F2	
				size (length)	↓F1	
D. magna	Ag	Water	Generations: F0-F4;	survival	↑F4	13

			Exposure: F0-F4 + clean F3-F4	reproduction	↓F1, ≈F2-F4, F3-F4 ≈ F2	
				population increase (*r*)	≈ F0-F4	
D. pulex	Ag	Water	Generations: F0-F4; Exposure: F0-F4	survival	↓F1,↑F2,↓F3-F4	
				reproduction	≈ F0-F4	
				population increase (*r*)	≈ F1 ↑F2 ↓F3-F4	
D. galeata	Ag	Water	Generations: F0-F4; Exposure: F0-F4	survival	↓F1,↑F2,↓F3-F4	
				reproduction	↑F1-F2;↓F3-F4;	
				population increase (*r*)	↓F1,↑F2,↓F3-F4	
C. elegans	Au	Agar	Generations: F0-F4; Exposure: parent F0	survival	none	14
				reproduction	↓F1,↑F2,↓F4	
C. elegans	Ag	SSPW agar	Generations: F0-F10; Exposure: F0-F10 + clean F6-F10	reproduction	↑F2-F10 F6-F10 ≈ F5	15

Chapter VI - Epigenetic effects of (nano)materials in environmental species – Cu case study in *Enchytraeus crypticus*

Rita C. Bicho[1], Dick Roelofs[2], Janine Mariën[2], Janeck J. Scott-Fordsmand[3], Mónica J.B. Amorim[1*]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[2]Department of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, The Netherlands
[3]Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark

*corresponding author: mjamorim@ua.pt

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Highlights

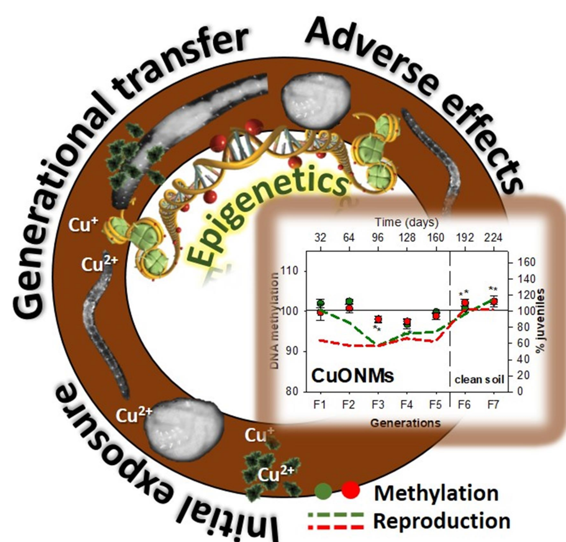
- First study on nanomaterials epigenetic effects in environmental species.
- Multigenerational exposure to nanomaterials induced epigenetic changes.
- Expression of epigenetic related genes was significantly regulated.
- The study links epigenetic and highly ecological relevant endpoints.
- Global DNA methylation showed a similar trend with reproduction.

Abstract

Chemical stressors can induce epigenomic changes, i.e., changes that are transferred to the next generation, even when the stressor is removed. Literature on chemical induced epigenetic effects in environmental species is scarce. We here provide the first results on epigenetic effects caused by nanomaterials with an environmental OECD standard soil model species *Enchytraeus crypticus* species. We assessed the epigenetic potential in terms of global DNA methylation, gene-specific methylation via bisulfite sequencing and MS-HRM (Methylation Sensitive - High Resolution Melting), and gene expression qPCR for genes involved in DNA methylation, histone modifications, non-coding RNA and stress response mechanisms).

We have exposed *E. crypticus* in a multigenerational (MG) test design to Cu (copper oxide nanomaterials (CuO NMs) and copper salt ($CuCl_2$)). To link possible epigenetic effects to population changes, we used exposure concentrations (ECx) that caused a 10 % and 50 % reduction in the reproductive output (10 and 50 % are the standards for regulatory Risk Assessment), the organisms were exposed for five consecutive generations (F1-F5) plus two generations after transferring to clean media (F5-F7), 7 generations in a total of 224 days. Results showed that MG exposure to Cu increased global DNA methylation and corresponded with phenotypic effects (reproduction). Gene expression analyses showed changes in the epigenetic, stress and detoxification gene targets, depending on the generation and Cu form, also occurring in post-exposure generations, hence indicative of transgenerational effects. There were in general clear differences between organisms exposed to different Cu-forms, hence indicate nanoparticulate-specific effects.

Graphical Abstract



Keywords: methylation; transgenerational; Methylation Sensitive High Resolution Melting

1. Introduction

It is well-known that both natural and anthropogenic environmental stressors can cause trans generational adverse effects via epigenetics mechanisms, even when the stress agent is removed.

One of the most well-known examples is famine, well documented episodes of famine include the 1836 famine that imprinted changes in genes of children born decades later (Epstein, 2013) and the Dutch Hunger Winter in 1944–45 which showed that 6 decades later there was less DNA methylation of the imprinted insulin-like growth factor II (IGF2) gene, compared with their unexposed same-sex siblings (Heijmans et al., 2008). Further examples show the impact of maternal nutrition in new born children (Geraghty et al., 2015), e.g. high fat diet can cause epigenetic imprinting of diabetes or obesity later in offspring´s life (Williams et al., 2014).

Epigenetic changes as induced by chemical stressors have been a topic of increasing concern for human toxicology (Bahadori et al., 2016; Smolkova et al., 2015; Stoccoro et al., 2013), but much less studied in ecotoxicology (Ray et al., 2014; Vandegehuchte and Janssen, 2014, 2011). Nevertheless, the number of studies with environmental species has been increasing recently. For example, studies with invertebrates have shown epigenetic changes caused by exposure to metals and metalloids (Kille et al., 2013; Santoyo et al., 2011; Šrut et al., 2017; Sussarellu et al., 2018; Vandegehuchte et al., 2009) and endocrine disruptors (Lee et al., 2018; Novo et al., 2018). Further, studies with fish showed effects with endocrine disruptors (González-Rojo et al., 2019; Olsvik et al., 2014) and pesticides (Bachère et al., 2017). Among the major concerns is the transgenerational epigenetic inheritance (e.g. Seong et al 2011, Xia et al. 2015), where the associated changes in the phenotype persist after the chemical is removed (Vandegehuchte and Janssen, 2014). Only two of the mentioned studies covered this aspect (Bachère et al., 2017; Vandegehuchte et al., 2009). These changes can result in stress adaptation (e.g. tolerance) or adverse effect (e.g. increased sensitivity) with consequences for population dynamics (Bahadori et al., 2016; Vandegehuchte and Janssen, 2014). Although well-known in humans, epigenetic effects have been far less studied in environmental species.

Epigenetic changes can occur via different mechanisms: DNA methylation (methylation, demethylation), histone modifications (methylation, phosphorylation, acetylation) and changes in non-coding RNA (e.g. microRNA (miRNA)) expression (Bahadori et al., 2016; Ray et al., 2014; Smolkova et al., 2015; Stoccoro et al., 2013; Vandegehuchte and Janssen, 2014). DNA methylation is the most studied mechanism, when focusing on induction by

environmental chemicals (Baccarelli and Bollati, 2009) and is a key epigenetic mechanism for gene regulation (Moore et al., 2013; Stoccoro et al., 2013). Usually, methylation of cytosines in the dinucleotide sequence CpG and methylation in the promoter regions supresses transcription in vertebrates (Smolkova et al., 2015; Stoccoro et al., 2013). Further, DNA methylation is also present in the gene body itself in vertebrates (Smolkova et al., 2015; Stoccoro et al., 2013) and is the most observed in invertebrates (Bonasio et al., 2012; Cramer et al., 2017; Wang et al., 2013). The exact function of gene body methylation in invertebrates is not always clear (Bonasio et al., 2012; Cramer et al., 2017; Wang et al., 2013). Studies in honey bees and ants suggest that gene body methylation may modulate alternative splicing (Bonasio et al., 2012; Lyko et al., 2010), function also suggested for humans (Smolkova et al., 2015). Such mechanisms of DNA methylation are maintained through the action of important enzymes, DNA methyltransferases (DNMTs) and proteins like methyl-cytosine binding domain proteins (MBD) which functions seem to have been maintained throughout evolution (Albalat et al., 2012; Cramer et al., 2017; Dabe et al., 2015; Suzuki et al., 2007; Wang et al., 2014). These mechanisms can be quantified on the global scale (whole genome scale) or the local scale (specific gene methylation).

Several classes of chemicals can induce epigenetic changes, e.g. metals (Baccarelli and Bollati, 2009; Kim et al., 2012; Vandegehuchte and Janssen, 2011), persistent organic pollutants (Baccarelli and Bollati, 2009; Vandegehuchte and Janssen, 2011), and nanomaterials (NMs) (Kim et al., 2012; Stoccoro et al., 2013). Given the potential long-term slow release and sub-lethal effects of NMs, these are of special interest in relation to epigenetic effects. Human health *in vitro* studies show that NMs can change the DNA methylation pattern, e.g. silica dioxide (SiO_2) (Gong et al., 2010), titanium dioxide (TiO_2) and zinc oxide (ZnO) (Patil et al., 2016). Changes can also occur via other epigenetics mechanisms, histone modifications, as shown when exposing human cells to cadmium telluride quantum dots (CdTeQDs) (Choi et al., 2008). Further, altered miRNA expression was observed following gold (Au) NMs exposure (Ng et al., 2011) in human cells, and multi-walled carbon nanotubes (MWCNTs) (Li et al., 2011a) and CdTeQDs (Li et al., 2011b) in mouse cells. Less is known from *in vivo* studies, but there are examples where miRNA expression changed in mice exposed to TiO_2 NMs (Halappanavar et al., 2011), SiO_2 NMs (Nagano et al., 2013) and Au NMs (Balansky et al., 2013). Similarly, the DNA methylation changed when mice were exposed to Ag NMs (Zhang et al., 2015), MWCNTs (Brown et al., 2016) and copper oxide (CuO) NMs (Lu et al., 2015).

Studies on epigenetics effects of chemicals with environmental relevant species are few, and relating to the exposure to nanomaterials are even less. The examples include the plant *Nicotiana tabacum*, where changes in miRNA expression were observed after exposure to aluminium oxide (Al_2O_3) NMs (Stoccoro et al., 2013). For zebrafish (*Danio rerio*), changes in DNA methylation were shown after exposure to single-walled carbon nanotubes (SWCNT) and MWCNTs (Gorrochategui et al., 2017) and silver (Ag) NMs (Xu et al., 2018). For nematodes, MWCNTs have been shown to cause changes in miRNA expression in *C. elegans* (Zhao et al., 2014).

Multigenerational (MG) studies are scarce, the few available studies include MG effects of Ag NMs in *C. elegans* with transgenerational transfer of sensitivity, in this study epigenetic effects were hypothesised but no epigenetic markers were evaluated (Wamucho et al., 2019)). A MG study with *Daphnia magna* initially exposed to functionalized SWCNT-$CONH_2$ showed that the biological effects remained even two generations after transfer or organisms to clean media, again epigenetic mechanisms were hypothesized but not measured (Arndt et al., 2014). Recent progress with environmental species revealed the total level of DNA methylation for the first time for two important soil model invertebrate species: *Folsomia candida* and *Enchytraeus crypticus* (Noordhoek et al., 2018), showing approximately 1.4% global DNA methylation for *E. crypticus* and none for *F. candida*.

In the present study we assess the epigenetic effect of CuO NMs in the species for which we previously showed global methylation i.e. *E. crypticus* (ISO, 2004; OECD, 2004a). The study hypothesis is that Cu nano and salt stress cause epigenetic responses in *E. crypticus.* Cu NMs are already extensively used as wood-preservatives e.g. estimated as 50% of the Cu containing wood-preservatives on the North American market (79.000 tonnes valued at $4.9 billion, see (Evans et al., 2008). CuO NMs are of particular importance also for this organism, since they are used in many applications, e.g. fungicide, (Gogos et al., 2012; Weitz et al., 2015) fertilizer, additive for soil remediation, and as growth regulators in plants (Zhu et al., 2012). Organisms were exposed in a MG design to CuO NMs (and $CuCl_2$) in soil (Bicho et al., 2017). Epigenetic and phenotypic endpoints were evaluated along five generations of continuously exposed organisms plus 2 generations in clean media. Hence, the transgenerational effect was also evaluated. The epigenetic endpoints were 1) global DNA methylation, 2) gene-specific methylation and 3) changes in gene expression of target genes. A key important aspect for the species is whether the exposure induced physiological change affected their fitness. For this reason, the epigenetic measurements were assessed at exposure concentration where population fitness (phenotypic, reproduction) were known to be

impaired. We use the exposure Effect Concentration (ECx) at the 10 % and 50 % level, since these are the most used in risk assessment and they are the statistically most reliable estimates.

2. Experimental section

2.1 Test organisms

A laboratory culture of *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) is kept in agar media plates prepared with a salt solution of $CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$, fed *ad libitum* with oatmeal and maintained in laboratory under controlled conditions at 18 ºC and a photoperiod of 16:8 (light: dark). Synchronized cultures were prepared as described in (Bicho et al., 2015), using juveniles with 17-18 days after cocoon laying.

2.2 Test soil

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

2.3 Test materials and spiking

Copper (II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$ > 99.9 % purity, Sigma-Aldrich, CAS number 10125-13-0) and copper oxide nanomaterials, CuO NMs (PlasmaChem GmbH) were used. For details see table 1. The tested concentrations were 0-20-180 mg Cu/kg soil (DW) for $CuCl_2$, and 0-500-1400 mg Cu/kg soil (DW) for CuO NMs, selected to be in the sub-lethal range and correspond to the reproduction effect concentrations EC_{10} and EC_{50} (Bicho et al., 2017).

For CuO NMs spiking followed the recommendations for nanomaterials (OECD, 2012). In short, 5 g of soil per replicate were mixed with the corresponding amount of the test materials (as dry powders) to obtain the final concentration range. The spiked soil was added to the remaining soil (35 g) and homogeneously mixed, per individual replicate, to ensure total raw amounts. Soil was equilibrated for 1-day prior test start. Soil moisture was adjusted to 50 % of the WHC_{max}. For $CuCl_2$ a stock aqueous solution was prepared and serially diluted; spiking was done onto the pre-moistened soil batches per concentration, this being homogeneously mixed and split onto replicates. Soil was freshly spiked 1-day prior each and every generation exposure.

Table 1: Characteristics of the tested CuO NMs (Source: FP7-SUN project).

Characteristics	CuO NMs
Manufacturer	Plasma Chem
CAS number	1317-38-0
Primary size distribution (average)	3-35 (12)
Mode (1st quartile - 3rd quartile) [nm]	10 (9.2-14)
Shape	Semi-spherical
Average crystallite size [nm]	9.3
Crystallite phases (%)	Tenorite 100 %
Dispersability in water: D50 [nm]	139.5 ± 4.6;
average agglomeration number (AAN)	346
Dispersability in modified MEM: D50 [nm]	85.2 ± 2.7;
average agglomeration number (AAN)	77
Z-potential in UP water [mV]	+ 28.1 ± 0.6
Isoelectric point [pH]	10.3
Photocatalysis: photon efficiency [unitless]	1.5 x 10-4
Specific Surface Area [m2 g-1]	47.0 ± 1.7
Pore sizes [nm]	13.5 ± 1.6 (BJH)
	23.0 ± 0.9 (AVG)
Surface chemistry [atomic fraction]	Cu = 0.46 ± 0.05; O = 0.47 ± 0.05; C = 0.07 ± 0.01

2.4 In situ characterisation

The amount of Cu was measured in the test soil and in soil solution (for method details see (Gomes et al., 2015)) in a concurrent experiment over the test duration. In the soil the total Cu was measured (by Graphite Furnace Atomic Absorption Spectroscopy: AAS-GF) and in soil solution both the total Cu (AAS-GF) and free active form (ion-selective electrode) were measured. The CuO present as nanomaterials was not determined in the soil, due to the technical difficulties, e.g. the particle size is below the theoretical detection limit of 15 nm (Navratilova et al., 2015).

2.5 Multigenerational test

Exposure followed the standard guideline (OECD, 2004a) with adaptations as follows. Forty (40) juveniles (17-18 days' age) per replicate were used. Organisms were collected and introduced in test vessels, containing 40 g of moist soil and food supply. For each generation tests ran during a period of 32 days at 20 ºC and 16:8 h photoperiod. The total test duration was 224 days. Test design involved 4 + 2 parental generations, 4 in spiked soil (F0-F4), 2 in clean soil (F5-F6). All biological material was collected from offspring generation (juveniles) for epigenetic analysis, i.e., F1-F5 in spiked soil and F6-F7 in clean soil. Food and water was replenished weekly. For control and the EC_{10} six replicates per treatment were used, for the EC_{50} ten replicates were used to ensure enough number of organisms for next generations and analysis. At the end of each generation, deionized water was added to each replicate and soil was left to deposit for 20 min, upon which organisms (adults and juveniles) were carefully transferred to freshly made reconstituted ISO water (OECD, 2004b). From each replicate, 400 juveniles of larger and medium size (n=100 and n=300 for DNA and RNA extractions respectively) were sampled, snap-frozen and kept at -80 ºC for further analysis; for DNA extraction organisms were freeze-dried. The soil and remaining organisms were counted, results are described in (Bicho et al., 2017).

Juveniles of medium size (n = 40) were collected and transferred to freshly spiked test soil for another generation. These juveniles of medium size (ca. 2 mm±0.2) correspond to 14 (±1) days of development after cocoon laying as described for *E. crypticus* (Bicho et al., 2015). At this stage, juveniles do not have differentiated reproductive tissue. This ensures that when first parental generation in clean soil (F5) develops germ cells there is no chemical exposure. Hence the measured effects in the two generations in clean soil (F6 and F7) allow to evaluate transgenerational effects.

The test design was developed to ensure that it is "truly" transgenerational effects in this study, i.e. to ensure that the observed effect will not be just a result of that egg cells of F2 may already be developing in the F1 embryo during F0 generation – which then would not be a transgenerational effect but just a very early "life-stage" stage exposure. Hence, the design included transfer of 12-14 days' juveniles from the last generation exposed to the next generation. At this stage, juveniles do not have reproductive tissue differentiated. Subsequently organisms only become sexually matured and develop first clitellum to lay the cocoon when they are already in next generation exposure. Hence, the respective descendants are not exposed even in the germ lines.

2.6 Epigenetic (methylation) analyses

2.6.1 DNA extraction

Genomic DNA was extracted, each replicate containing 100 pooled juveniles. Three biological replicates per treatment were used. The Wizard® Genomic DNA Purification Kit (Promega) was used following manufacture´s protocol. An extra purification step was performed using phenol chloroform isoamyl alcohol extraction, followed by sodium acetate and ethanol precipitation. The quantity and purity of isolated DNA was measured with NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific).

2.6.2 Global DNA methylation

Liquid chromatography-mass spectrometry (LC-MS) was used to measure global DNA methylation. Genomic DNA samples were digested to deoxyribonucleosides as previously described (Tian et al., 2012). Digestion solution (10 µL) was added to DNA samples (200 ng) and left to incubate at 37 ºC for 6 h. The 20 µL digested product was diluted with 80 µL of ultrapure water and 100 µL of internal standard solution. After this, samples were transferred to HPLC vials and were injected in an Agilent 1200 µHPLC system. Detection of the samples was performed on an Agilent 6460 QQQ mass spectrometer. Single deoxynucleosides were separated with an Agilent ZORBAX Eclipse Plus C18 column (2.1 x 100 mm, 1.8 µM). The mobile phase contained 0.1 % (v/v) formic acid in water (A) and in methanol (B). The gradient started at 5 % B, increasing to 15 % B in 3 min, kept at this composition for 1 min and returned to 5 % B in 10 min. The source conditions were applied during LC/-MS run: nebulizer gas pressure was set to 40 psi, drying gas (nitrogen) flow rate 10 L/min, and drying gas temperature of 350°C. Scanning of deoxynucleosides occurred with a dwell time of 50 ms per compound using multiple reaction monitoring. For method validation and DNA sample quantification compounds mass transitions were: 5mdC 242.1/126.1, D3mdC 245.1/129.1, dG 268.1/152.2, C10N5dG 243.3/162.2. A collision energy of 8 V was used for 5mdC and D3mdC, whereas for dG and C10N5dG a collision energy of 4 V was used. A standard curve with increasing amounts of 5mdC (0-2.07 µM, 0 % - 3 %) was included against a fixed amount of G (345 nM). After the LC/MS run, data analysis was performed with Agilent QQQ quantitative software.

2.6.3 Gene-specific DNA methylation

Gene-specific determination of gene body DNA methylation level was performed for 1 gene target (elongation factor-1 alpha (EF1)) given the known DNA sequence (exons) (Noordhoek

et al., 2018), for all generations and both Cu forms, using methylation sensitive high resolution melting curve analysis (MS-HRM) trough quantitative real-time PCR (qPCR) following (Newman et al., 2012). Melting profiles of unknown samples are compared with standards of known methylation percentage, after bisulfite conversion. Additionally, to confirm the results provided by the MS-HRM analysis, EF1 methylation level was also evaluated through bisulfite genomic sequencing analysis, in generation F5 for both Cu forms.

Primers were designed using the Bisulfite Primer Seeker program (Zymo Research). One CpG site was allowed for each primer pair. Primer pairs were selected to obtain highest content of CpG sites and smallest amplicon size of the amplification product.

Unmethylated DNA was obtained from genomic DNA (50 ng) from culture organisms using the Repli-g Mini Kit (Qiagen, 150023) according to manufacture´s protocol. Fully methylated DNA was obtained from genomic DNA (2000 ng) from culture organisms using the CpG methyltransferase (M.SssI, NEB, M0226S). After both unmethylated and methylated DNA were ready, an extra purification step was done using phenol chloroform isoamyl alcohol extraction (as described).

Genomic DNA (500 ng for all experimental samples, including both unmethylated and fully methylated standard DNA) was bisulfite modified using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Catalog No. D5005 & D5006) (according to manufacturer's protocol). The quantity and purity of bisulfite converted DNA was measured with the NanoDrop spectrophotometer, after all samples and both standards were diluted with nuclease-free water to 20 ng/µl. For the standard curve, bisulfite converted DNA standards were mixed to obtain new standards with different percentages of methylated DNA: 0 % (unmethylated DNA), 20 %, 40 %, 60 %, 80 % and 100 % (methylated DNA).

Real Time-PCR cycling and MS-HRM (Methylation Sensitive - High Resolution Melting) were performed in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Real Time-PCR was performed in a 20 µL reaction mix containing: 1 µL sample (equivalent to 20 ng of bisulfite converted DNA), 1 µL of each forward and reverse primer, 10 µL of SensiMix™ SYBR No-ROX mastermix (Bioline) and 8 µL of water (Sigma-Aldrich). Cycling conditions consisted of a pre-heating start at 95 ºC for 10 min, followed by 45 cycles with one denaturation step at 95 ºC for 30 secs, one annealing step at 61 ºC for 30 secs and one extension step at 63 ºC for 1 min, after a final extension step at 63 ºC for 3 min. Melting curve analysis occurred from 60 to 90 ºC raising 0.1 ºC every 30 sec. All amplification reactions were performed in duplicate. Calculations were performed as described in (Newman et al., 2012). Melting curves were normalized in between two normalization

regions (at temperatures before and after melting peak of PCR product). Differences in DNA methylation were assessed using the average difference between the melting curves of samples and standards and the fully methylated standard. This difference is named the Net Temperature Shift (NTS). The NTS was plotted with the standards of known methylation percentage to obtain standard curve and methylation level of experimental samples was extrapolated.

2.6.4 Bisulfite genomic sequencing

This procedure followed a series of steps as explained in (Li and Tollefsbol, 2011), consisting of PCR amplification, cloning and sequencing. To have good confidence in the methylation level, recommendations are that minimum 10 clones should be sequenced (Li and Tollefsbol, 2011; Wang et al., 2016). From each sample (16 in total), 10 clones were selected and sent for sequencing (Eurofins Genomics, Germany).

EF1 primers used for PCR were the same bisulfite primers used for MS-HRM PCR. PCR cycling was performed in T100™ Thermal Cycler (Bio-Rad). The reaction mix was in a total volume of 25 µL containing: 1 µL sample (bisulfite converted DNA) and 24 µL master mix (GoTaq® Hot Start Polymerase, Promega) containing 1 µL of each forward and reverse primer. Cycling conditions consisted of a pre-heating start at 95 ºC for 5 min, followed by 40 cycles with one denaturation step at 95 ºC for 15 secs, one annealing step at 61 ºC for 30 secs and one extension step at 72 ºC for 1 min, after a final extension step at 72 ºC for 5 min. PCR products were confirmed in agarose gel (1.5 %). Further these were purified using the PCR Clean-up Kit (Macherey-Nagel).

Ligation of purified PCR products to vector was performed using pGEM®-T Easy Vector Systems Cloning Kit (Promega) during overnight at 4 ºC. After transformation of JM109 Competent cells (Promega) performed according manufacture recommendations. Competent cells were left to grow overnight at 37 ºC in agar plates with ampicillin/X-gal/IPTG. Colonies with vectors inserted with target PCR product (white colonies) were selected from colonies with empty vector (blue colonies). After colonies colour selection, white colonies were picked and a new PCR was performed with exact same conditions as described in previous section, to confirm the PCR product insert. Selected white colonies were left to grow in LB medium during 16 h at 37 ºC and shaking at 400 rpm. Further plasmid extraction was performed with Wizard® Plus SV Minipreps DNA Purification Systems Kit (Promega) according manufacture recommendations. New DNA samples were sent for sequencing. Sequence alignments were performed using Vector NTI software (Thermofisher).

2.7 Gene expression analysis

2.7.1 RNA extraction

RNA was extracted from a pool of 300 organisms per replicate. Three biological replicates per treatment were done. Generations F1, F5 and F7 for both Cu forms were selected to analyze. The SV Total RNA Isolation System (Promega) (according to manufacturer's protocol) was used. The quantity and purity of the isolated RNA were measured with nanodrop (NanoDrop ND-1000 Spectrophotometer), and its integrity was checked on a denaturing formaldehyde agarose gel electrophoresis.

2.7.2 Quantitative real-time PCR (qPCR)

Gene targets were selected to cover genes related to epigenetics mechanisms and stress response (for details see table 2): DNA (cytosine-5)-methyltransferase 1 (DNMT1); Methyl-CpG-binding domain protein 2 (MBD2); DNA methyltransferase 1-associated protein 1 (DMAP1); histone acetyltransferase (HAT); histone methyltransferase (HMT); histone 3 (H3); poly (ADP-ribose) polymerase 1 (PARP1); argonaute 1 (Argonaute); elongation factor-1 alpha (EF1); heat shock protein 70 (HSP70); metallothionein-like protein (MT); superoxide dismutase (SOD); NOTCH receptor (NOTCHr) and NOTCH protein (NOTCHp) as retrieved from the *E. crypticus* library (Castro-Ferreira et al., 2014).

Table 2: List of gene-protein related functions selected for the epigenetic related gene expression quantification (GO: Gene Ontology).

Gene code	GO - Biological Process	GOs
DNMT1	**DNA (cytosine-5)-methyltransferase 1** DNA methylation	P:transcription; P:regulation of gene expression, epigenetic;
MBD2	**Methyl-CpG-Binding Domain protein 2** methyl-CpG binding; negative regulation of gene expression, epigenetic	P:transcription
DMAP1	**DNA Methyltransferase 1-Associated Protein 1** DNA methylation; histone acetylation	P:transcription; P:regulation of gene expression, epigenetic;
HAT	**Histone AcetylTransferase** histone acetylation	F:transferase activity; F:protein binding

HMT	**Histone MethylTransferase** histone methylation	F:transferase activity; P:protein modification process; P:catabolic process; P:cellular amino acid and derivative metabolic process
H3	**Histone 3** positive regulation of gene expression, epigenetic; regulation of gene silencing by miRNA	C:chromosome; F:DNA binding; P:organelle organization; C:nucleus
PARP1	**Poly (ADP-Ribose) Polymerase 1**	F:nucleic acid binding; F:binding
Argonaute	**Argonaute 1** miRNA mediated inhibition of translation; miRNA metabolic process; production of miRNAs involved in gene silencing by miRNA	F:translation factor activity, nucleic acid binding; C:ribosome; P:regulation of biological process; P:translation; F:protein binding
EF1	**Elongation Factor 1 alpha** protein synthesis elongation	P:regulation of biological process; P:translation
HSP70	**Heat Shock Protein 70** stress response (general)	P:metabolic process; P:response to stress
MT	**Metallothionein-like protein** metal ion binding	
SOD	**Super Oxide Dismutase** positive regulation of oxidative stress	P:metabolic process;
NOTCHr	**NOTCH receptor** (highly conserved cell signaling system present in most animals)	P:cell differentiation; P:regulation of biological process; P:embryonic development; P:anatomical structure morphogenesis
NOTCHp	**NOTCH protein** cell differentiation; cell fate specification; embryonic morphogenesis; epidermis development; neural tube development	P:anatomical structure morphogenesis; P:multicellular organismal development; P:regulation of biological process; P:response to stress; P:growth;

Actin was selected as housekeeping gene being its suitability confirmed in all treatments (i.e. variation of less than 2 cycles in treated samples). Primers were designed with the software

Oligo ExplorerTM (version 1.1.2) (Tab. S1 for primer sequences). Efficiency was tested using a cDNA concentration range (1, 10, 100, 1000 ng/µl) and specificity of each primer was determined by observing the obtained standard and melting curves, respectively, for all primer sets. To perform qPCR, the total RNA (0.5 µg) from samples was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was diluted 4X and 2 µL were used in 20 µL PCR reaction volumes containing 2 µL of forward and reverse primers (2 µM), 10 mL of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 4 mL of nuclease free water. Each replicate was applied in triplicate. For each qPCR plate non-template control (NTC) was added in duplicate, were nuclease free water was used instead of cDNA. Amplification was performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad). Reaction conditions consisted of one initial cycle at 50 °C for 2 min, followed by 95ºC for 2 min and 40 cycles at: 95 ºC for 15 secs and at 60ºC for 30 sec.

2.8 Data analysis

For the global DNA methylation data, two-way analysis of variance (ANOVA) ($p \leq 0.05$) was used, the two independent variables being treatment (0-EC_{10}-EC_{50}) and generation time (F1-F7) to assess the significance of each of the variables and the interaction (SigmaPlot, 1997). For gene expression data, the mean normalized expression value was calculated from the obtained cycle threshold (Ct) values and statistical differences were assessed using the Relative Expression Software Tool (REST-MSC). Further, coefficient of correlation ($R^2 \geq 0.75$ was considered for acceptable fit) between gene expression and generation time was investigated using linear regression models (SigmaPlot, 1997). Gene expression data were explored by Principal Component Analysis and uni- and multivariate analysis of variance were all performed using SAS IML studio 14.2 (SAS 2013-2014).

3. Results

3.1 In situ characterization

The total Cu measured in the soil ranged between 96-106 % of the added total concentration for both CuO NMs and $CuCl_2$, no concentration-dependent pattern was observed. The total Cu in soil solution was less than 1 % of the total for CuO NMs and less than 3 % for $CuCl_2$. The free active Cu was less than 0.001 % for both Cu forms exposure. For controls the total Cu in soil solution was 0.07 % and the active Cu was 0.004 %.

3.2 Global DNA methylation

There were no significant differences between $CuCl_2$ and CuO when performing a 2-way ANOVA (Tab. S2). Results showed that there is a tendency to increase global DNA methylation (Fig. 1) in the $CuCl_2$ EC_{10} treatment up to F4.

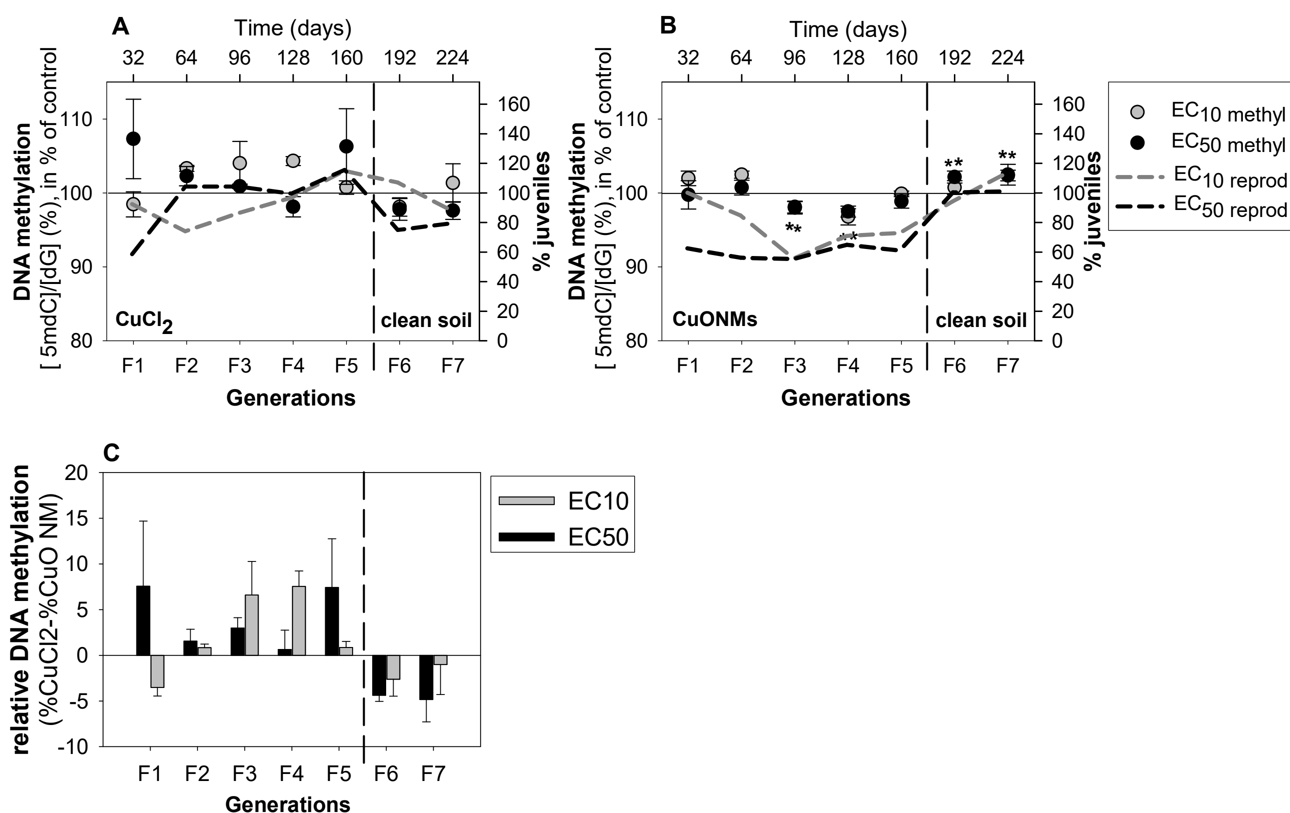


Figure 1: Global DNA methylation (LC-MS measurements) for *Enchytraeus crypticus* after a multigenerational exposure to the reproduction EC_{10} and EC_{50} (and control samples) of A) $CuCl_2$ (0-20-180mg Cu/kg DW soil) and B) CuO NMs (0-500-1400 mg Cu/kg DW soil) in LUFA 2.2 soil. C) Relative difference of DNA methylation between $CuCl_2$ and CuO NMs. All values are expressed as average ± standard error (Av±SE). *(2-way ANOVA, $p<0.05$): effect of time between F1 and Fx. The dashed lines represent the endpoint reproduction at the EC_{10} and EC_{50} MG (% of juveniles) for all generations [52].

For the CuO NMs, significant difference (up to 8 %) as explained by the impact of generation time was observed, with F3, F4, F6, and F7 significantly lower/higher from F1 ($p<0.001$) (see fig. 1 for details); the interaction between time and concentration was almost significant ($p<0.051$, 2-way ANOVA, Tab. S2). The pattern observed is that methylation decreases during prolonged NM exposure, but recovers during subsequent generations of culturing in

control conditions. The methylation showed a linear correlation with the corresponding reproduction (R^2=0.8) for CuO NM, but not for $CuCl_2$ (R^2=0.03).

3.3 Gene-specific DNA methylation

3.3.1 MS-HRM analysis (Methylation Sensitive - High Resolution Melting)

The EF1 gene body methylation level after MS-HRM analysis (Fig. 2) showed a melting profile similar to the 100 % methylated DNA standards, in all generations and in both Cu forms.

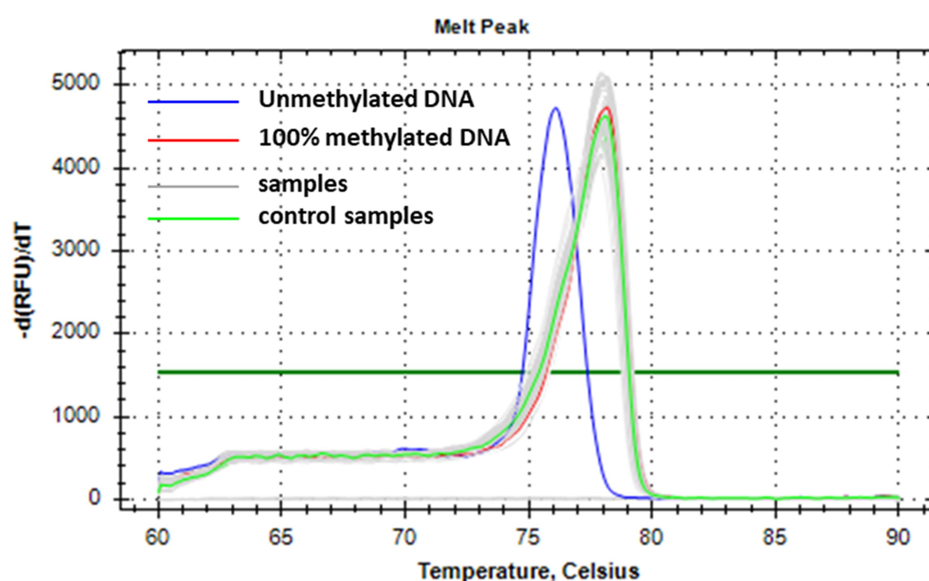


Figure 2: Melting profiles from Methylation Sensitive - High Resolution Melting of *Enchytraeus crypticus* EF1 gene after a multigenerational exposure to the reproduction EC_{10} and EC_{50} (and control samples) of $CuCl_2$ and CuO NMs (0-20-180mg Cu/kg DW soil and 0-500-1400 mg Cu/kg DW soil, respectively) in LUFA 2.2 soil. The presented melting profiles are for F1 and F5 generations for both $CuCl_2$ and CuO NMs as examples.

All melting curve profiles of the experimental samples showed that they match with the 100% methylated control EF1 curve with melting temperature of 78.5 °C (fig. 2). None of the experimental samples matched with the unmethylated EF1 control with melting temperature of 76.5 °C (fig. 2). Moreover, EF1 specific methylation patterns of both $CuCl_2$ and CuO NMs exposures did not deviate from the methylation pattern observed in control animals.

3.3.2 Bisulfite genomic sequencing analysis

We aimed at providing bisulfite sequencing data for 10 independent clones per samples. The cloning success of the total (16 samples) result was as follows: 10 clones for 9 samples, 9 clones for 3 samples, 8 clones for 2 samples, 7 clones for 1 sample, and 5 clones for 1 sample. The sequencing results could be compared (aligned) with the reference non-bisulfite converted and bisulfite converted sequences (Noordhoek et al., 2018) (where EF1 methylation level was 100 % methylated, i.e. all CpG sites were methylated) and the methylation level for each clone (from each replicate) was calculated (Fig. S1). Results display EF1 methylation levels for generation F5 and showed no differences between control and Cu tested materials, except for the small non-significant increase for CuO NMs EC_{10}. For $CuCl_2$ average methylation levels were: 93 % ± 1.5 (control), 93 % ± 1.2 (EC_{10}) and 93 % ± 1.3(EC_{50}). For CuO NMs average methylation levels were: 90 % ± 1.5 (control), 91 % ± 1.8 (EC_{10}) and 90 % ± 1.5 (EC_{50}) (average ± standard error). These data confirm the MS-HRM data showing almost complete methylation of the EF1 gene without significant deviations caused by toxicity exposure.

3.4 Gene expression results

Differences in gene expression (Fig. 3) showed significant up and down regulation depending on the Cu tested material, concentration (EC_{10}, EC_{50}) or generation. Across gene responses there were various levels of correlation between genes, in particular with a pronounced correlation between argonaute – HMT responses (R^2>0.9) and between HSP70 – MT responses (R^2~0.85/0.9), see Supplement Table S4).

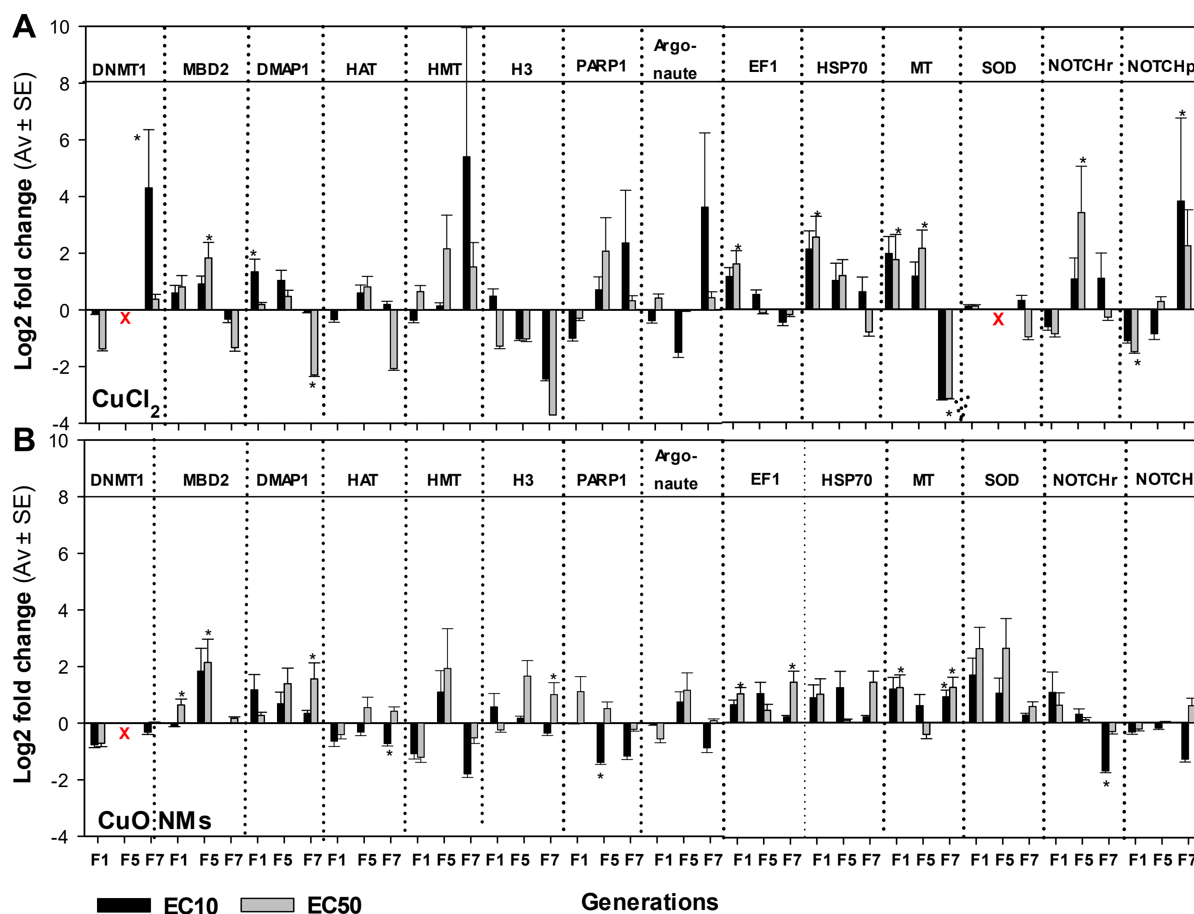


Figure 3: Results on quantitative gene expression using RT-qPCR for *Enchytraeus crypticus* after a multigenerational exposure (F1, F5, F7, i.e., after exposure during 1, 4 and 6 parental generations) to the reproduction EC_{10} and EC_{50} of A) $CuCl_2$ and B) CuO NMs, (0-20-180mg Cu/kg DW soil and 0-500-1400 mg Cu/kg DW soil, respectively) in LUFA 2.2 soil. All values are expressed as log2 ratio (fold change to control), average ± standard error (Av±SE). * ($p<0.05$). **X**: no expression. DNMT1: DNA (cytosine-5)-methyltransferase 1; MBD2: Methyl-CpG-binding domain protein 2; DMAP1: DNA methyltransferase 1-associated protein 1; HAT: histone acetyltransferase; HMT: histone methyltransferase; H3: histone 3; PARP1: poly (ADP-ribose) polymerase 1; Argonaute: argonaute 1; EF1: elongation factor-1 alpha; HSP70: heat shock protein 70; MT: metallothionein-like protein; SOD: superoxide dismutase; NOTCHr: NOTCH receptor; NOTCHp: NOTCH protein.

As can be observed, for certain genes there was a linear decrease in the gene expression with increase in the exposure generation (see Fig.3), while DMAP1 showed a linear decrease with increased generation time at CuO NMs EC_{10} level (R^2=0.99) (Table S3). The difference in expression was in some cases persistent in the absence of contaminant, indicating the

relevance of exposure history and persistence of epigenetic related changes. The higher correlation coefficients (Table S3) further support the transgenerational impact for both Cu forms.

An exploratory multivariate analysis (Principal Component Analysis) of the gene response data did not provide much explanatory power, with the first two components explaining approximately 60 % of the variation for both $CuCl_2$ and CuO NMs (Fig. 4). Including all generations, it was not possible to discriminate between $CuCl_2$ and CuO NMs exposure. When performing such analysis for the individual generations it was possible to separate the exposure type in F7 but not in F1 and F5 [see PCA figures in Figure S2].

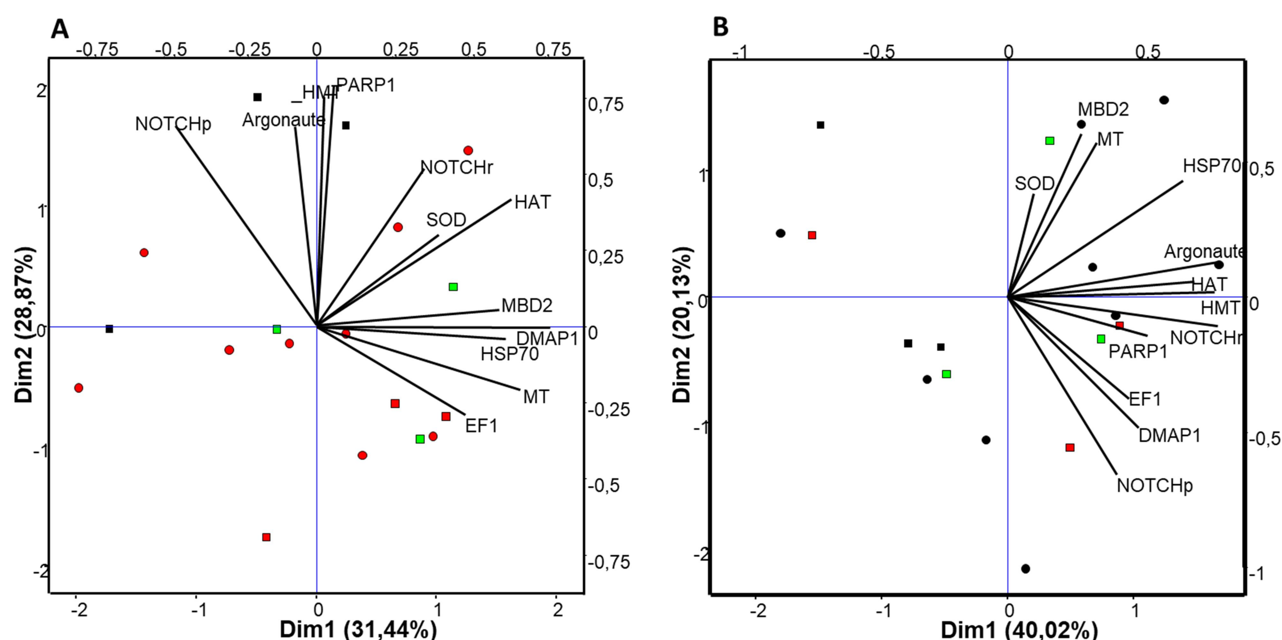


Figure 4: Principal component analysis (PCA) of qPCR data for the A) $CuCl_2$ and B) CuO NMs treatments, using covariance matrix with van der Waerden adjustment for skewness. Representation: Circle = EC_{50}, Squares = EC_{10}, Red = F1, Green = F5, Black = F7. DNMT1 and H3 were excluded due to missing values, but when included gave similar PCA plots.

4. Discussion

4.1 Materials in situ characterisation

Our soil measurements showed the free active Cu was less than 0.001 % for both Cu forms exposure, so this does not seem to be the source for observed differences. Navratilova and co-authors (Navratilova et al., 2015) showed that it was possible to detect larger CuO NMs by Single Particle ICP-MS, but due to the interaction with soil components it was not possible to separate Cu ions bound to small natural particles from CuO NMs present in the sample (the

CuO NMs used in the present study are below the 15 nm theoretical detection limit). As to the Cu characterisation in the soil media, it cannot be verified whether or to what extend the particles may have disintegrated. Our previous studies (e.g. (Gomes et al., 2015)) of the fate of Cu based NMs in soil indicate that the particles are not disintegrated (or at least only partially), which is probably due to a stabilisation by the organic carbon present in soil (Xiao et al., 2018). The particle-specific action, is also supported by the observation that biological responses are consistently different between $CuCl_2$ and CuO NMs exposure.

4.2 Biological characterisation

There has been only few (mainly aquatic) studies on methylation in environmental organisms, but much more on human related biological systems. Since the aquatic organisms (e.g. daphnia) are as different from Enchytraeids as are humans, the comparison throughout the discussion will be done with both other environmental organisms and human studies.

4.3 Global and specific gene methylation

Exposure to CuO NMs caused a significant change in methylation over the multigenerational exposure (F3, F4; F6, F7). Interestingly, phenotypic results in terms of reproduction (Bicho et al., 2017) showed a similar pattern: decrease in reproduction and decrease in global methylation, and vice-versa, increase-increase correspondence. In Figure 1C, it can be seen that for the EC_{10} exposed animals the methylation difference between the $CuCl_2$ and CuO NMs was 7 %, 9 % and 6 % for the F3, F4 and F5 respectively, with the $CuCl_2$ exposed organisms having the highest methylation level. When transferred to clean soil (the F6 and F7) the difference in methylation was 4 % and 5 %, but in this case the CuO NMs exposed organisms showed the highest values.

For $CuCl_2$ MG exposure, global methylation did not change significantly in a monotone manner. From a logic point of view this does not necessarily exclude that specific genes are methylated, as specific gene methylation can be "diluted" by non-methylated genes (depending on n) with a resulting non-significance on the global level. Previous results on global DNA methylation showed no change in methylation status in *E. crypticus* when exposed to CuSO4 (Noordhoek et al., 2018). However, in the present projects for $CuCl_2$ MG exposure, the reproduction variability pattern seemed to follow the global DNA methylation measurements, although less obvious than for CuO NMs.

The EF1 gene specific methylation analysis (and the MS-HRM), did not show a change in methylation status for $CuCl_2$ exposure nor for CuO NMs, the gene remained fully methylated

throughout all exposure treatments over all generations. EF1 gene expression changed significantly across generations (discussed ahead in detail). This obviously shows that other mechanisms rather than DNA methylation regulated the expression of the EF1 gene. This is in agreement with previous observations that have shown that for Wasps and Ants genes important in basic metabolism had extensive gene body methylation but is not correlated with correlated with gene expression (Bonasio et al., 2012; Wang et al., 2013). Another study with the marine mollusc *Crassostrea gigas* showed gene body methylation positively correlated with gene expression (Sussarellu et al., 2018). So it seems that the role of gene body methylation can differ among invertebrates. In fact also in humans the relationship between gene body methylation and gene expression levels can vary (Jjingo et al., 2012). Depending on the type of tissue this relationship can be either monotonic or non-monotonic (Jjingo et al., 2012). Clearly is a fascinating topic and needs further investigation. Again the lack of change in EF1 methylation status during exposure does not preclude the occurrence of other gene specific methylation. The study with *C. gigas* embryos exposed to Cu showed no changes in global DNA methylation but presented changes in the methylation status of specific genes (Sussarellu et al., 2018). A gene-by-gene qPCR for the whole genome would obviously be impossible, other approaches with high-content e.g. whole-genome bisulfite sequencing, will be more appropriate but was not performed here.

4.4 qPCR gene expression of selected targets

4.4.1 Methylation

The gene involved in the methylation during DNA replication (Cramer et al., 2017), DNMT1, was down-regulated in F1 and silenced in F5 for both Cu forms, being then significantly up-regulated in F7 (clean media) for $CuCl_2$ (especially EC_{10}) but not for the CuO NMs. Gene silencing at transcriptional level, has been described to occur mediated by genes like DNMT1 (Devailly et al., 2015; Stoccoro et al., 2013), although it is unclear why DNMT1 was silenced in F5. Since the overall level of global DNA methylation was maintained, there obviously must be another mechanism for DNA methylation during DNA replication. Moreover, up-regulation of DNMT1 (only in $CuCl_2$ exposed organisms), as observed in F7, was recorded in rat cells exposed to CuSO4 (Sun et al., 2014). A similar pattern was observed in human lung cells exposed to carbon-based NMs where there was an increase in global DNA methylation and a decrease in the expression of DNMTs (Li et al., 2016). Similarly, an *in vivo* study with mice exposed to CuO NMs, showed a stable/increase (not significant) in global DNA methylation while there was significantly reduced DNMT1 gene expression (Lu et al., 2015).

Hence, there must be additional players in the regulation of this methylation mechanism, although we are not aware of which. This topic needs further investigation. Moreover, up-regulation of DNMT1 (only in $CuCl_2$ exposed organisms), as observed in F7, was recorded in rat cells exposed to $CuSO_4$ (Sun et al., 2014).

The MBD2 gene (which causes transcriptional repression in methylated DNA) was up-regulated after MG exposure to both Cu forms, showing an increase of ca. 1 to 3 fold from F1 to F5. The expression levels of DNMTs and MBD2 has often been reported correlated for sick patients (Sheng et al., 2013), e.g. in human tumoral tissues showed a significantly higher expression of DNMTs and MBD2 compared to control tissue (He et al., 2013); human cells exposed to SiO_2 NMs showed a dose-dependent decrease in gene and protein expression levels of DNMTs and MBD2 (Gong et al., 2010). This correlation was not the case here for Cu exposed enchytraeids (see Table S4 and S5), hence these two DNA methylations did not seem to act together. The potential DNA methylation through the up-regulation of MBD2 was clearer for CuO NMs exposure, for which the transfer of organisms to clean soil (F7) changed the regulation back to control levels. For $CuCl_2$, both DNMT1 and MBD2 were regulated in opposite directions in F7, clearly different from what happened in the CuO NMs exposed organisms.

Additionally, DNA methyltransferase associated protein 1 (DMAP1) gene expression changed significantly: for $CuCl_2$ an up-regulation in F1/F5 and down-regulation in F7, for CuO NMs a continuous up-regulation for EC_{50}, including transgenerational F7. This could be indicative of transgenerational inheritance of CuO NMs. DMAP1 is known to have a critical role in stimulating DNA methylation, interacting with DNMT1 (Lee et al., 2010). This again supports evidence that CuO NMs MG exposure caused changes in DNA methylation mechanisms.

4.4.2 Histone modifications

DMAP1 can also interact with histone deacetylase (Kang et al., 2007) and may indicate histone modifications. Histone tail modifications, like acetylation or methylation, alters chromatin structure making it looser or tighter, i.e. facilitating or blocking the access to DNA, and consequently the transcriptional regions which ultimately regulate transcription (Kang et al., 2007; Stoccoro et al., 2013; Verdone, 2006). Our results showed no changes for histone acetyltransferase (HAT) or histone methyltransferase (HMT) gene expression in Cu MG exposed, but when transferred to clean media (F7), there was a down-regulation of HAT, most pronounced for $CuCl_2_EC_{50}$ and CuO NMs_EC_{10}, and an up-regulation of HMT for

$CuCl_2$. Changes in expression levels of HATs and HMTs have been reported from exposure to NMs, e.g. to Ag NMs in *in vitro* mouse cells showing a decrease in HMTs (Qian et al., 2015), to CdTeQDs in human breast cancer cells showing global hypoacetylation (Choi et al., 2008).

On the other hand, histones function like building blocks to which DNA cloaks around to form the nucleosome, the basic unit of chromatin (Dong and Weng, 2013). Hence a disproportion of histone levels can affect gene expression, also making chromatin more loose or tight (Rattray and Müller, 2012). Exposure of *E. crypticus* to $CuCl_2$ showed a down-regulation of H3 in F1 and F5, this being even more pronounced when transferred to clean soil (F7). CuO NMs caused the opposite change, an up-regulation of H3 in F5 and F7. This would be indicating opposite regulation of certain genes depending on exposure to Cu salt and Cu nano.

4.4.3 PARPs mechanisms

Poly(ADP-ribose) polymerases (PARPs) is a group of enzymes that mediate the post-translational modification of proteins called Poly(ADP-ribosyl)ation (Quénet et al., 2009). Our results showed that for $CuCl_2$ PARP1 was down-regulated in F1 and up-regulated in F5 and F7. For CuO NMs was up-regulated in F1 and down-regulated in F5 and F7. Hence, also here we observed the opposite responses between the two Cu forms.

PARPs have a key role in mechanisms like DNA repair and genomic stability (Bürkle, 2001) and it is shown that PARPs inactivation induces cellular apoptosis (Gangopadhyay et al., 2011; Shall and de Murcia, 2000). Moreover, PARPs are associated with several epigenetic mechanisms, playing a major role in regulating the composition and structure of chromatin (Quénet et al., 2009). PARP1 is the main member of the PARP family, it binds to histones and there is an interplay with Poly(ADP-ribosyl)ation and histone modifications (Quénet et al., 2009; Shall and de Murcia, 2000). It has also a role in DNA methylation: DNMT1 binds to PARP1, with the consequent Poly(ADP-ribosyl)ation which inhibits DNMT1 activity (Quénet et al., 2009; Reale et al., 2005). For instance the exposure of human cells to SiO_2 NMs showed a dose dependent down-regulation of PARP1, associated with up-regulation of DNMT1 (Gong et al., 2012). No correlation was observed between DNMT1 and PARP1 gene expression for *E. crypticus* exposed to Cu. Actually, in F5, where a significant down-regulation was observed for PARP1 with CuO NMs, there was no expression of DNMT1, which could indicate that there is no interplay between DNMT1 and PARP1 in enchytraeids. Moreover a study with human cells exposed to $CuSO_4$ showed a dose-dependent decrease in

Poly(ADP-ribosyl)ation and PARP1 activity with a consequent dose-dependent increase in oxidative DNA damage (Schwerdtle et al., 2007). A recent study, also with human cells exposed to $CuCl_2$, CuO NMs and microscale CuO particles (CuOMPs) showed a reduction of Poly(ADP-ribosyl)ation catalysed by PARP1 (Semisch et al., 2014). This reduction was more severe for CuO NMs exposure. This is in agreement with the phenotype level (reproduction) results (Bicho et al., 2017): the opposite response to $CuCl_2$ and CuO NMs can partly explain the loss of toxicity for $CuCl_2$ exposed generations and maintenance of toxicity in CuO NMs exposed generations.

4.4.4 Non-coding RNA

Micro RNA (miRNA) can bind to specific regions of the messenger RNA (mRNA) regulating transcription either through degradation or translational inhibition (Stoccoro et al., 2013), acting via double stranded RNA degradation, binding antisense RNA molecules like miRNAs. Argonaute proteins bind to miRNA and are important components of this complex system where miRNAs mediate transcription (Okamura, 2004). Changes in gene or protein expression of argonaute proteins can elucidate on the epigenetic potential. Our study showed changes in argonaute gene expression: for $CuCl_2$ a down-regulation in F5, and up-regulation in F7, for CuO NMs the opposite trend, with up-regulation in F5. Interestingly, Argonaute expression in F7 is anti-correlated with HSP70 and MT, both in $CuCl_2$ and in CuO NMs, which suggests that stress response genes may be regulated via RNAi over prolonged exposure.

There was a clear linear relationship between argonaute and HMT (R^2>0.9), which could indicate that the RNA induced transcriptomal silencing complex is positioned at a specific part of the genome so it attracts HMTs. No correlation with DMNT1 was observed.

4.4.5 Non-epigenetics stress related responses

EF1 has a key role during protein synthesis in the elongation step (Bebianno et al., 2015; Talapatra et al., 2002). There was a significant up-regulation of EF1 in F1 for both Cu forms indicating what could be a compensatory mechanism, the overexpression of EF1 as a compensation of apoptosis has been shown in human cells (Talapatra et al., 2002). The MG exposure to Cu (F5) and transfer to clean soil (F7) show a divergent pattern: regulation towards no change and up-regulation, for $CuCl_2$ and CuO NMs respectively. Again, this could be linked to the cascade of events that resulted in recovery of toxicity to $CuCl_2$ (organisms were adapted to Cu and no longer needed the compensatory mechanisms) and

toxicity to CuO NMs continued (EC_{50}) or increased (EC_{10}) (organisms have compensatory mechanisms activated for the adverse effect). In particular, in F7, for $CuCl_2$ there was a down-regulation (decrease of proteins synthesis when reproduction was affected), for CuO NMs the up-regulation was maintained, and the reproduction results showed a recovery from toxicity. The regulation (down) of EF1 has been previously reported for *Enchytraeus albidus* exposed to $CuCl_2$ and CuNMs (Gomes et al., 2012).

The HSP70 protein, known as a general stress response (Homa et al., 2007, 2005), showed upregulation in $CuCl_2$_F1, similar to the EF1 profile, except that in F5 HSP70 continued up-regulated. There was a general clear relationship between the HSP70 and the MST response, with R^2 larger than 0.8 ($CuCl_2$) and 0.9 (CuO NMs). On the other hand, the regulation of the well-known antioxidant enzyme SOD showed no changes in gene expression, except from a down-regulation in F7. To note that all gene expression measurements are a time snap-shot which means priori and posteriori regulations can occur and are not captured. The co-occurrence of changes in gene expression of HSP70 and antioxidant enzymes, namely SOD, is commonly triggered by numerous chemicals, including metals (Brulle et al., 2010; Homa et al., 2007; Shi et al., 2015; Wang et al., 2015; Xiong et al., 2014). For $CuCl_2$ MT was significantly up-regulated during F1 and F5, and down-regulated in F7, typical on-off Cu binding in the cell to detoxify. MT binds metals, including Cu, and has an important role in detoxification, tolerance and the right balance of essential metals like Cu, protecting against oxidative stress (Janssens et al., 2009; Xiong et al., 2014). There are many examples of significant up-regulation of MT during Cu exposure in soil invertebrates, e.g. in earthworm species (Mustonen et al., 2014; Spurgeon et al., 2004), or collembolans (*Folsomia candida*), e.g. at the protein level (Maria et al., 2014). Similarly, in the absence of Cu exposure, the MT gene expression was down regulated, and not just turned off. This seems to indicate that it may take several more generations in clean media to re-establish the original homeostasis level. That would explain the measured increased toxicity as a consequent Cu deficiency (Bicho et al., 2017).

For CuO NMs MG exposure, the gene expression profile was different from $CuCl_2$. HSP70 was up-regulated after F1 and continued in F5 and F7; SOD was upregulated in F1 and F5 but decreased in F7; MT was upregulated and continued activated after transfer to clean media (F7). This could be due to differences in CuO NMs kinetics, i.e. uptake and elimination rates may be slower than for $CuCl_2$. Additionally, the organisms may uptake CuO NMs and accumulate the particles in cells, with a potential long-term release of Cu ions, including longer after transfer to clean media. Measurements of Cu body burdens in *Folsomia*

candida showed that $CuCl_2$ was comparatively faster uptake than CuO NMs, but that CuO NMs could be uptake to high internal levels as time progressed, this for a similar concentration of Cu on a mass basis (Mendes et al., 2018). The upregulation of MT, HSP70 and SOD, 2 generations after transfer to clean soil (F7), clearly shows that the defence mechanisms are still activated. In contrast, Argonaute is down regulated in CuO NMs in generation 7. We speculate that the upregulation of stress response genes in recovery F7 maybe epigenetically regulated by RNAi. This could also be an indication of transgenerational effects caused by NMs.

NOTCH signalling mechanism had been previously reported affected by both Cu salt and Cu NMs on the gene expression profile of *E. crypticus* (Gomes et al., 2018). Hence we here analysed it in the current MG exposure study. The NOTCH signalling is an important pathway for cell-cell communication and during the development of both vertebrate and invertebrate species (Baron, 2003). There were significant changes for both NOTCHr and NOTCHp for $CuCl_2$ exposure, during both the exposed (F1 and F5) and in the unexposed (F7) generations, again a possible evidence of transgenerational effects. For CuO NMs the change only occurred in F7, with a significant down-regulation of NOTCHr. This again shows that, although a recovery at the phenotypic level (reproduction) was visible, there are underlying active mechanisms that compensate ongoing imbalances.

5. Conclusions

Multigenerational long-term exposure to Cu, both as salt and nano form, clearly induced changes in epigenetic markers, including transgenerational, i.e., when the chemical stressor was removed. However, global DNA methylation or gene specific methylation did not confirm the epigenetic effect. Specific gene expression of epigenetic related genes showed a clear regulation, e.g. DNMT1, MBD2, DMAP1, PARP1, H3, etc. Moreover, also the general stress related genes, like HSP70, SOD, MT, were regulated probably to promote cell detoxification. This, in association with Argonaute down-regulation in recovery may indicate RNAi, and not methylation, as potential epigenetic mechanism affecting MG exposure of metal stress. Hence, the present study shed further light on the mechanisms of toxicity as caused by $CuCl_2$ and CuO NMs. For $CuCl_2$ MG there was increased tolerance that could be explained by the activation of general stress response mechanisms, i.e. MT, HSP and EF; the transgenerational increased toxicity seems to be the result of a higher Cu homeostasis level, and hence a deficiency when transferred to Cu absence. For CuO NMs MG, toxicity is maintained (EC_{50}) or increased (EC_{10}->EC_{50}), but eliminated (or recovered) when transferred

to clean media, while organisms still have activated compensatory mechanisms of protein synthesis and detoxification, and impaired developmental mechanisms. Hence, the apparent good fitness of the population hides active stress mechanisms at gene level. Further confirmation of the response patterns could be attempted via gene knock-out studies.

This study highlights the importance of studying long-term effects of environmental chemicals, and how to link to include epigenetic markers and phenotypic responses in a format useful for risk assessment.

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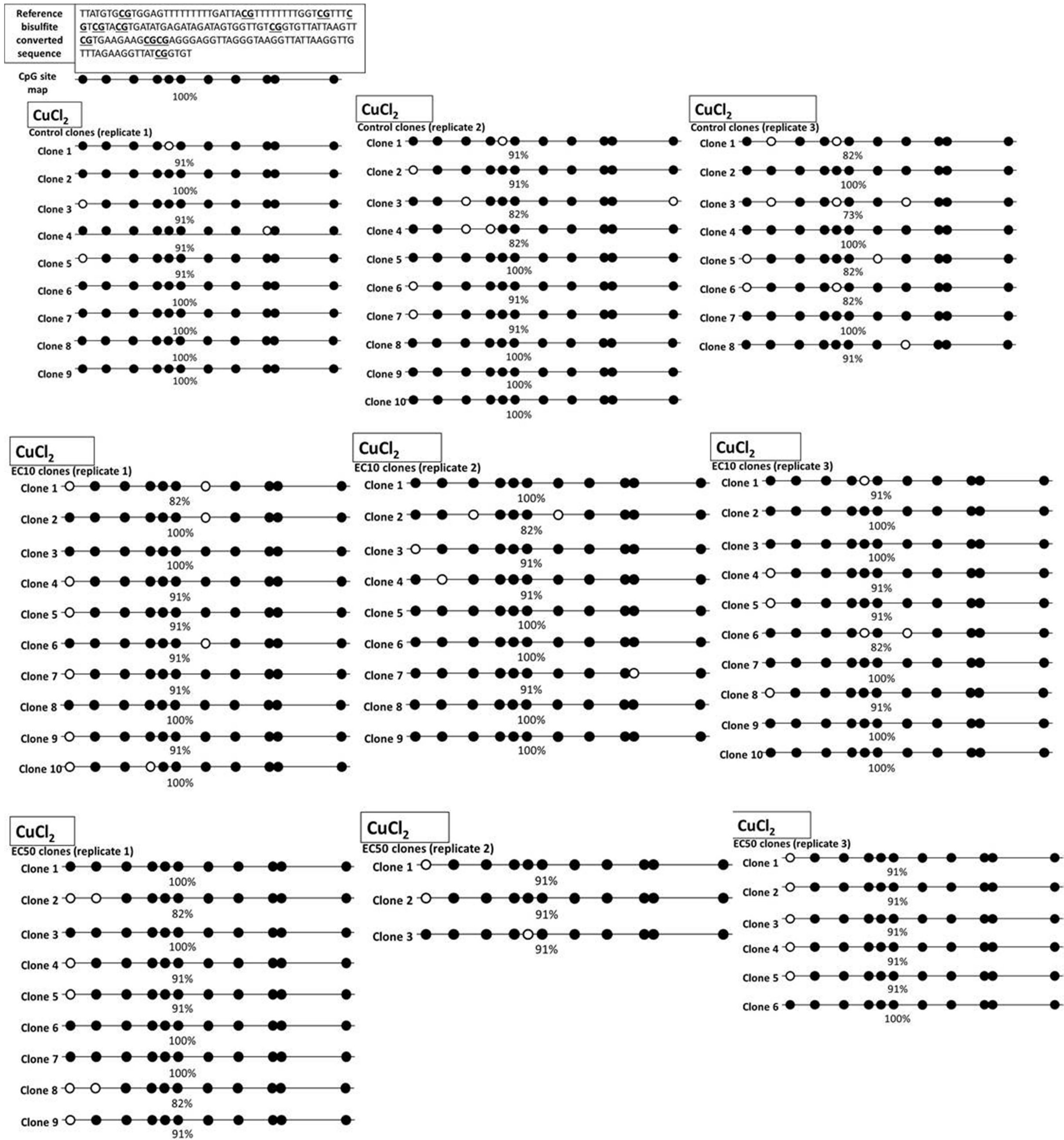
Supplementary information

Epigenetic effects of (nano)materials in environmental species – Cu case study in *Enchytraeus crypticus*

Rita C. Bicho[1], Dick Roelofs[2], Janine Mariën[2], Janeck J. Scott-Fordsmand[3], Mónica J.B. Amorim[1*]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[2]Department of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, The Netherlands
[3]Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark

*corresponding author



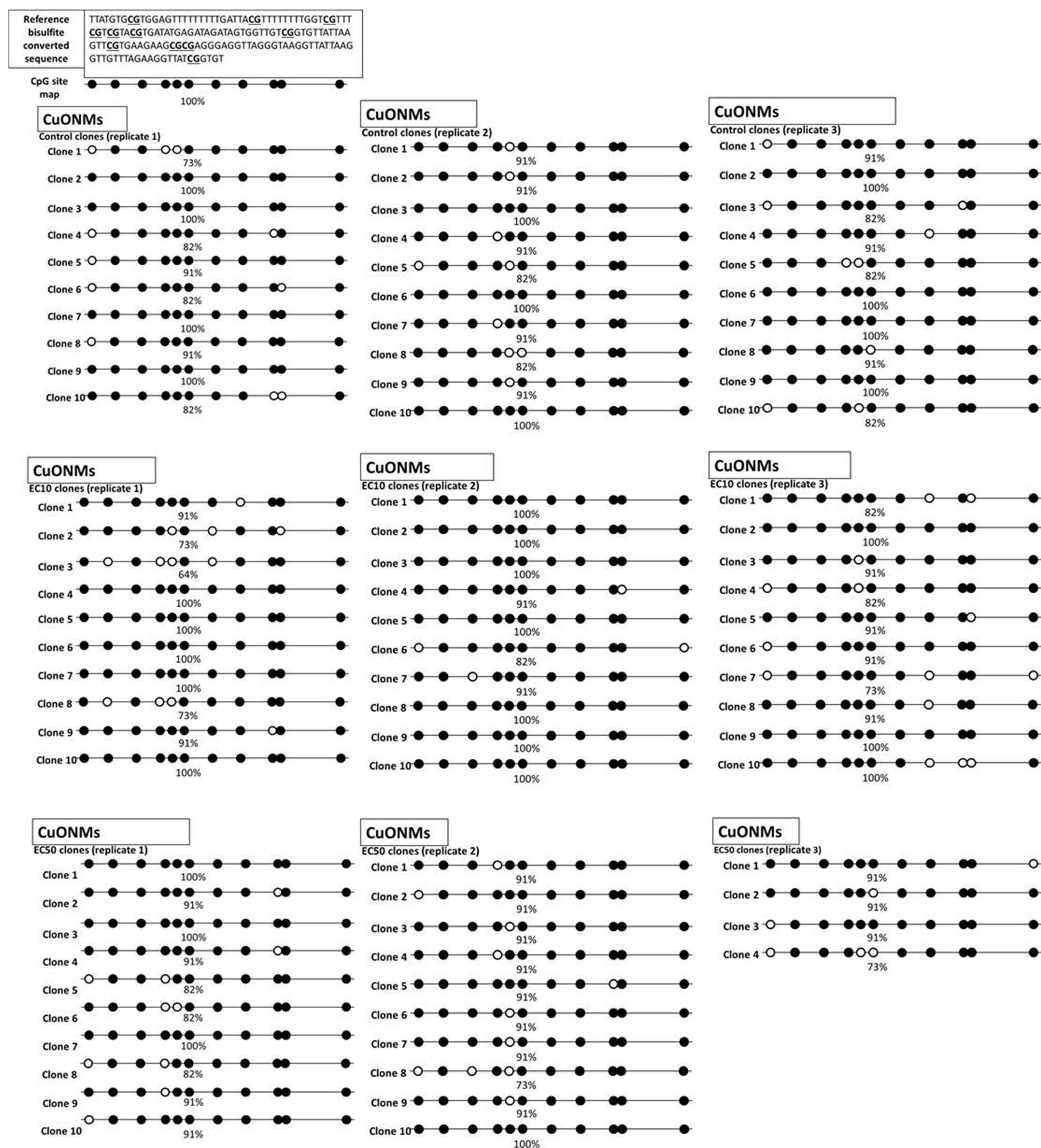


Figure S1: DNA CpG methylation level of EF1-α gene for *Enchytraeus crypticus* after a multigenerational exposure to the reproduction EC_{10} and EC_{50} (and control samples) of $CuCl_2$ (0-20-180mg Cu/kg DW soil) and CuO NMs (0-500-1400 mg Cu/kg DW soil) in LUFA 2.2 soil. Results are relative to generation F5. A reference sequence bisulfite converted with the location of CpG sites and respective percentage of methylation is shown. Further is shown the percentage of methylation for each replicate and respective clones for both Cu tested materials. Each circle represents one CpG site. black circle: methylated CpG site; open circle: unmethylated CpG site.

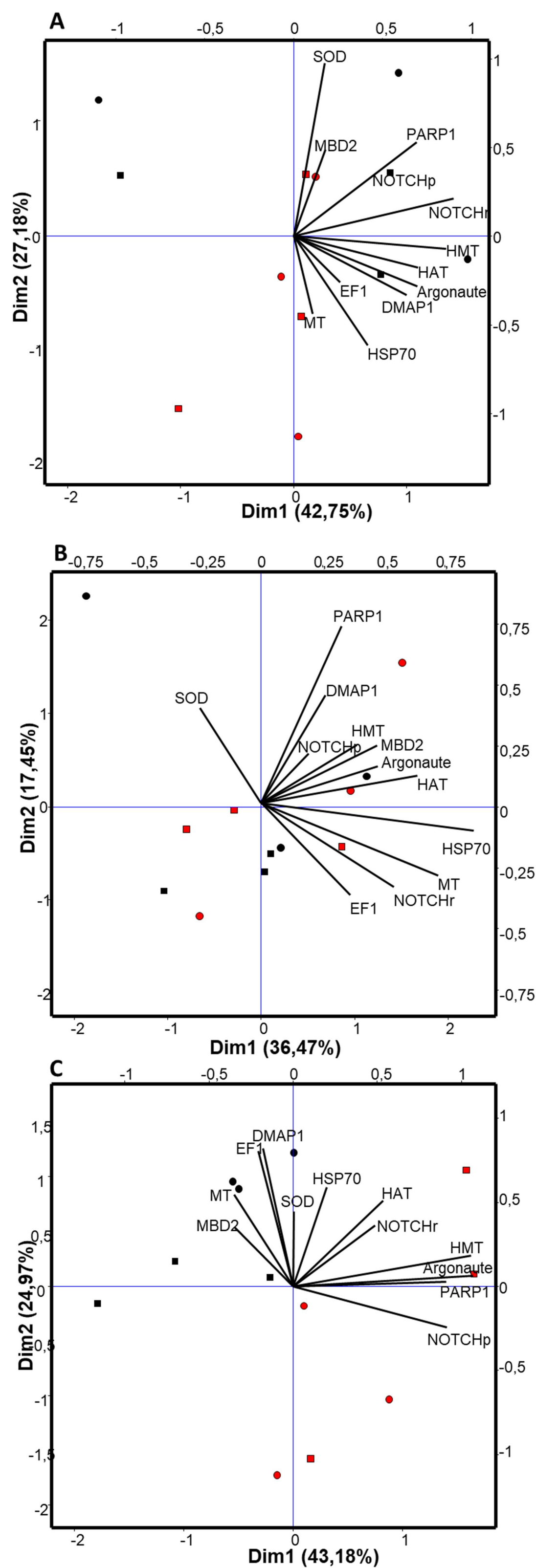


Figure S2: Principal component analysis for the $CuCl_2$ and CuONM treatments for each of the generations (A) F1, B) F5 and C) F7), using covariance matrix with van der Waerden

adjustment for skewness. Representation: Circle = $CuCl_2$ exposure, Squares = CuONM, Red=EC10m, Black=EC50.

Table S1: Primer sequences used for qPCR gene expression and efficiency values.

Gene code	Forward primer	Reverse primer	Efficiency
DNMT1	5´- TATTTCACGTCCTATCTCC - 3´	5´- ATCACTACCGTTTCTATGG -3´	1.68
MBD2	5´- CTCATCGTTTCACCACTC - 3´	5´- TCATCCAGACAGCAAGAC - 3´	1.86
DMAP1	5´- GCCTGACACTGACCTTTG - 3´	5´- TGAGAGAGCGTTTGACTAG -3´	2.12
HAT	5´- GCCAAACAGATAGCACAC -3´	5´- GTTTCCGTACCACAAGGG - 3	1.82
HMT	5´- GAGCCTAAAGAATCTGTGC - 3´	5´- CGTTGTTGTTGTTGTTGG - 3´	2.02
H3	5´- AGACTGGTGAGAGAGATTG - 3´	5´- AGAGATAGACGAGAAACGC - 3´	1.93
PARP1	5´-TTAATCCTCTCTGTCTACCAC -3´	5´- GCTCAATCTCCATCTACTTC - 3´	1.86
Argonaute	5´- AGGTATCTGAACATTGGC - 3´	5´- GCTGCAGCGTATCTCTAAC - 3´	1.99
EF1	5´-CCATGTGCGTGGAGTCCTTCTCTG -3´	5´- GCACCGGTGGCCTTCTGGGCAGCC - 3´	1.84
HSP70	5´- GCCGCCAAGAACCAGGTGGCCATG - 3´	5´- ATCACCTTAAAGGGCCAGTACTTC -3´	1.96
MT	5´- ATAGCTGCCTGTACTGTTC - 3´	5´- TGGTGATGGTTATGGTTC - 3´	1.87
SOD	5´- CTTGGTGATGCCGATAAC - 3´	5´- GAAGAACGATGGTGATTC - 3´	1.44
NOTCHr	5´- CGTCAATCCAATCCTATG - 3´	5´- TCAACCTCGCTTCGTATC - 3´	1.72
NOTCHp	5´- GTGCTGAAAATCCTTGTG - 3´	5´- CCGTGAATCCTTCTCTAC - 3´	1.85

Table S2: Results of the two-way ANOVA analysis (and *Holm-Sidak pos-hoc when significant), with two independent variables, i.e. 1) treatment (0-EC_{10}-EC_{50}) for both $CuCl_2$ and CuONMs and 2) generation time (F1-F7).

Source of Variation	**DF**	**SS**	**MS**	**F**	**p**
$CuCl_2$ ($EC_{10/50}$)	2	0.00364	0.00182	1.093	0.345
Generation time (F1-F7)	6	0.0206	0.00343	2.061	0.080
Interaction ($CuCl_2$ x generation time)	12	0.0387	0.00323	1.938	0.059
Residual	40	0.0666	0.00167		
Total	60	0.129	0.00215		
	DF				
CuO NMs	2	0.000122	0.0000608	0.0870	0.917
Generation time (F1-F7)	6	0.0269	0.00448	6.399	* <0.001: F1vs F3 F1 vs F4 F1vs F6 F1 vs F7
Interaction (CuONMs x generation time)	12	0.0168	0.00140	1.998	0.051
Residual	40	0.0280	0.000700		
Total	60	0.0729	0.00121		

Table S3: Coefficient of correlation ($R^2 \geq 0.75$ considered) between gene expression treatment (obtained for EC10, EC50) and generation time (32, 160 and 224 days). Linear regression equations information is included.

Gene	$CuCl_2$		CuO NMs	
	EC_{10}	EC_{50}	EC_{10}	EC_{50}
DMAP1	R^2= 0.764 (1.697-0.0066x)	-	R^2= 0.992 1.325-0.00426x	R^2= 0.955 0.101+0.00702x
HAT	-	-	-	R^2= 0.801 -0.466+0.00473x
H3	R^2= 0.97 1.052-0.0147x	-	R^2= 0.927 0.772-0.000458x	-
PARP1	R^2= 0968 -1.650+0.0169x	-	R^2= 0.784 0.0666-0.00664x	R^2= 0.938 1.386-0.00660x
EF1	R^2= 0.905 1.508-0.00792x	R^2= 0.909 1.811-0.00994x	-	-
HSP70	R^2= 0.994 2.361-0.00797x	R^2= 0.913 3.274-0.0165x	-	-
NOTCHr	R^2= 0.899 -0.804+0.00955x	-	R^2= 0.817 1.723-0.0132x	R^2= 0.983 0.798-0.00476x
NOTCHp	-	R^2= 0.953 -2.253+0.0187x	-	R^2= 0.842 -0.417+0.00398x

Chapter VI - Epigenetic effects of (nano)materials in environmental species – Cu case study in *Enchytraeus crypticus*

Table S4: Correlation matrix among variables. The darker the background grey the higher the R2. Five grey/white levels used between 0 and 1.

CuCl2 - Correlation Matrix

	Argonaute	DMAP1	DNMT1	EF1	H3	HAT	HMT	HSP70	MBD2	MT	NOTCHp	NOTCHr	PARP1	SOD
Argonaute		0,1173	0,2445	-0,0716	-0,1504	0,3364	0,9148	0,2538	0,0561	0,062	0,3126	0,219	0,4202	-
DMAP1	0,1173		-0,3214	0,6209	-0,258	0,5235	0,1793	0,7025	0,3679	0,7163	-0,5238	0,3281	0,1378	0,2584
DNMT1	0,2445	-0,3214		-0,6864	0,5475	-0,1651	-0,0406	-0,6437	-0,2262	-0,6873	0,5749	-0,2266	0,0853	-
EF1	-0,0716	0,6209	-0,6864		-0,3606	0,0475	0,0757	0,7081	0,031	0,5962	-0,6174	-0,0202	-0,1799	0,1288
H3	-0,1504	-0,258	0,5475	-0,3606		-0,5035	-0,3652	-0,2146	-0,5643	-0,3704	0,2197	-0,6296	-0,5516	-
HAT	0,3364	0,5235	-0,1651	0,0475	-0,5035		0,3891	0,3668	0,7543	0,3028	-0,1048	0,513	0,7611	0,4571
HMT	0,9148	0,1793	-0,0406	0,0757	-0,3652	0,3891		0,3304	0,1692	0,227	0,2564	0,4473	0,4888	-0,057
HSP70	0,2538	0,7025	-0,6437	0,7081	-0,2146	0,3668	0,3304		0,1375	0,8454	-0,668	0,0876	-0,1289	0,1917
MBD2	0,0561	0,3679	-0,2262	0,031	-0,5643	0,7543	0,1692	0,1375		0,2208	-0,0278	0,5889	0,754	0,6887
MT	0,062	0,7163	-0,6873	0,5962	-0,3704	0,3028	0,227	0,8454	0,2208		-0,6822	0,4319	-0,1712	0,2033
NOTCHp	0,3126	-0,5238	0,5749	-0,6174	0,2197	-0,1048	0,2564	-0,668	-0,0278	-0,6822		0,0748	0,4043	-
NOTCHr	0,219	0,3281	-0,2266	-0,0202	-0,6296	0,513	0,4473	0,0876	0,5889	0,4319	0,0748		0,5189	0,3841
PARP1	0,4202	0,1378	0,0853	-0,1799	-0,5516	0,7611	0,4888	-0,1289	0,754	-0,1712	0,4043	0,5189		0,3362
SOD	-0,2577	0,2584	-0,3523	0,1288	-0,1915	0,4571	-0,057	0,1917	0,6887	0,2033	-0,1405	0,3841	0,3362	

CuONM Correlation Matrix

	Argonaute	DMAP1	DNMT1	EF1	H3	HAT	HMT	HSP70	MBD2	MT	NOTCHp	NOTCHr	PARP1	SOD
Argonaute		-0,0631	-0,0379	0,1709	0,0148	0,6274	0,9164	0,3503	0,2426	0,1151	0,2475	0,5003	0,4338	0,3381
DMAP1	-0,0631		-0,2242	0,498	-0,0114	0,2738	0,03	-0,0402	-0,3532	-0,0928	0,5047	0,247	0,3256	-0,1195
DNMT1	-0,0379	-0,2242		-0,2315	-0,2622	-0,2503	-0,0025	0,1847	0,5965	0,1072	-0,215	0,013	0,1806	0,14
EF1	0,1709	0,498	-0,2315		-0,2946	0,4984	0,1258	0,4216	-0,1239	0,4688	0,4273	0,5036	-0,0274	-0,4942
H3	0,0148	-0,0114	-0,2622	-0,2946		-0,0151	-0,0273	-0,6388	0,0319	-0,5651	0,1779	-0,4784	-0,045	0,3268
HAT	0,6274	0,2738	-0,2503	0,4984	-0,0151		0,5562	0,4766	0,0286	0,4387	0,2396	0,4355	0,2249	0,1288
HMT	0,9164	0,03	-0,0025	0,1258	-0,0273	0,5562		0,2733	0,2543	0,0719	0,334	0,6074	0,6411	0,4759
HSP70	0,3503	-0,0402	0,1847	0,4216	-0,6388	0,4766	0,2733		0,2961	0,9158	-0,2642	0,5598	0,0483	-0,1564
MBD2	0,2426	-0,3532	0,5965	-0,1239	0,0319	0,0286	0,2543	0,2961		0,19	-0,3798	-0,0135	0,1455	0,476
MT	0,1151	-0,0928	0,1072	0,4688	-0,5651	0,4387	0,0719	0,9158	0,19		-0,2814	0,5024	-0,1676	-0,3368
NOTCHp	0,2475	0,5047	-0,215	0,4273	0,1779	0,2396	0,334	-0,2642	-0,3798	-0,2814		0,4336	0,311	-0,0656
NOTCHr	0,5003	0,247	0,013	0,5036	-0,4784	0,4355	0,6074	0,5598	-0,0135	0,5024	0,4336		0,361	-0,1297
PARP1	0,4338	0,3256	0,1806	-0,0274	-0,045	0,2249	0,6411	0,0483	0,1455	-0,1676	0,311	0,361		0,5421
SOD	0,3381	-0,1195	0,14	-0,4942	0,3268	0,1288	0,4759	-0,1564	0,476	-0,3368	-0,0656	-0,1297	0,5421	

Chapter VII - Impact of copper materials (Cu NMs and $CuCl_2$) exposure on soil invertebrates –histology and immunohistochemistry – confirmatory assays for transient changes in *Enchytraeus crypticus*

Rita C. Bicho[1], Faustino A.M.R.[2], Rêma A.[2], Janeck J. Scott-Fordsmand[3], Mónica J.B. Amorim[1*]

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

[2] Department of Pathology and Molecular Immunology, Biomedical Sciences Institute of Abel Salazar, University of Porto, 4050-313 Porto, Portugal

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

*corresponding author: mjamorim@ua.pt

Manuscript submitted

Abstract

Environmental risk assessment (ERA) based on effects caused by chronic and longer term exposure is highly relevant. Further, if mechanistic based approaches (e.g. omics) can be included, beyond apical endpoints (e.g. reproduction), the prediction of effects increases. For Cu NMs (and $CuCl_2$) this has been studied in detail, covering multi-omics and apical effects using the soil standard species *Enchytraeus crypticus*. The intermediate level effects like tissue/organ alterations represent a missing link. In the present study we aimed to: 1) perform long term exposure to Cu materials (full life cycle and multigeneration) to collect samples; 2) perform histology and immunohistochemistry on collected samples at 12 time points and 17 treatments; 3) integrate all levels of biological organization onto an adverse outcome pathway (AOP) framework. CuO NMs and $CuCl_2$ caused both similar and different stress response, either at molecular initiating events (MIE) or key events (KEs) of higher level of biological organization. Tissue/organ level, post-transcriptional and transcriptional mechanisms, through histone modifications and microRNA related protein, were similarly affected. While both Cu forms affected the Notch signalling pathway, $CuCl_2$ also caused oxidative stress. Different mechanisms of DNA methylation (epigenetics) were activated by CuO NMs and $CuCl_2$ at the MIE.

Keywords: Nanospecific effect; Adverse outcome pathway (AOP); Nanoecotoxicology; Oligochaeta; Long term; Epigenetics;

Introduction

Environmental risk assessment (ERA) based on effects caused by chronic and longer term exposure gains on relevancy and is less uncertain (Dekkers et al., 2016; Gedda et al., 2019; Oomen et al., 2018; Scott-fordsmand et al., 2018). Further, ERA can gain substantial explanatory power if mechanistic based approaches (e.g. transcriptomics) are also used, i.e. explain toxicity mechanisms beyond information of apical endpoints (survival, reproduction) as usually covered in most standard guidelines (Allen et al., 2016; Leist et al., 2008). A continuous challenge is linking events (Key Events, KEs) across various levels of biological organization (from molecular to organism) as in the principle of the Adverse Outcome Pathway (AOP) framework (Ankley et al., 2010). For AOPs it is important to identify the molecular initiating event(s) (MIE) for the pathway leading to the related adverse outcome at a higher level of biological organization. Stressors, such as (nano)materials and chemicals, can initiate a number of (maybe similar) KEs along the various levels of biological organization, the main difference may be the initial KE at the molecular level, i.e. the MIE (Gerloff et al., 2017). Hence, mechanistic based knowledge is essential to enlighten potential initiating differences and to study whether these lead to different longer term adverse consequences. Further, based on such understanding it may be possible to show that (dis)similar structures of nanomaterials (NMs) can be grouped or categorised into a certain KE and AOP pattern facilitating risk assessment procedures. This is similar to evaluation of various chemicals where well know molecular structures are known to relate to certain modes of action (e.g. insecticides, herbicides). In line with this, *in silico* QSAR (Quantitative Structure-Activity Relationship) based grouping and read-across models should be validated (Dekkers et al., 2016; Oomen et al., 2018; Scott-fordsmand et al., 2018) and here AOPs will be a particularly interesting tool to identify MIEs and related KEs (Allen et al., 2016; Mansouri and Judson, 2016).

AOPs for NMs in environmental species are virtually absent in the literature, especially for the terrestrial compartment. There are two examples of draft AOPs for Cu NMs in soil (Bicho et al., 2017a; Gomes et al., 2018) using *Enchytraeus crypticus* (Oligochaeta). For this species the traditional endpoints survival, reproduction and bioaccumulation are assessed within standardised guidelines (ISO, 2004; OECD, 2010, 2004). However, there has been continuous progress towards increasing, refining and interrelating endpoints at: (A) sub-organism levels of biological organisation e.g. covering multi-omics and high-throughput transcriptomics (Castro-Ferreira et al., 2014; Gomes et al., 2018), metabolomics (Vera L Maria et al., 2018), proteomics (Vera L. Maria et al., 2018) and epigenetics (Bicho et al.,

2020; Noordhoek et al., 2018). (B) across organism level organisation, e.g. covering the full life cycle (Bicho et al., 2015) with hatching success, growth, maturity, the full life span test (Gonçalves et al., 2017), the effect over multi-generations (Bicho et al., 2017b), and the effects in multispecies systems (Mendes et al., 2018). For Cu NMs (and $CuCl_2$) all of these endpoints have been assessed making Cu probably one of the best case studies currently available. Since information on the effects on tissues and organs were absent we aimed to cover this gap. The test design included exposure of *E. crypticus* to CuO NMs and $CuCl_2$, and samplings from exposure via fully repeated 1) full life cycle and 2) mutigenerational (4 generations in spiked soil + 2 generations in clean soil) approach. For each of these A) histology and B) immunohistochemistry tools were used to assess epigenetic and general stress responses using specific targets. To target epigenetic changes, DNA methylation, histone modifications and miRNA immunostaining was performed for 5-methylcytosine, (5mC), Histone H3 dimethyl K9 (H3-dimethyl) and argonaute 1 (Ago1), respectively. Stress responses were investigated through immunostaining of superoxide dismutase 1 (SOD1) and Notch1. We hypothesize that integrating data of the various levels would allow us to identify KE and build onto the AOP.

Material and Methods

Test organisms

Enchytraeus crypticus (Oligochaeta: Enchytraeidae) were used. The culture is maintained at the University of Aveiro for many years. Synchronized cultures were prepared as described in (Bicho et al., 2015), using cocoons with 1-2 days and juveniles with 17-18 days after cocoon laying, according to the test setup, i.e. the full life cycle and the multigenerational test, respectively.

Test soil, test materials and spiking

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

Copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$ > 99.9 % purity, Sigma-Aldrich, CAS number 10125-13-0) and copper oxide nanomaterials, CuO NMs (PlasmaChem GmbH) were used, for details please see table S1.

For the FLCt, test concentrations were based on the previous study (Bicho et al., 2017a), for the multigenerational test, concentrations corresponded to the reproduction effect (EC_{10} and EC_{50}) (Bicho et al., 2017b) (table 1).

Table 1: Summary of the tested concentrations on the full life cycle test (FLCt) and multigenerational test (MGt) including the copper oxide nanomaterials (CuO NMs) and copper chloride ($CuCl_2$), as based on previous studies (as in references).

Test type	Test material	Concentrations (mg Cu/kg soil (DW))	Reference
FLCt	CuO NMs	0-200-400-800-3200-6400	(Bicho et al., 2017a)
FLCt	$CuCl_2$	0-50-100-200-300-400	(Bicho et al., 2017a)
MGt	CuO NMs	0-500-1400	(Bicho et al., 2017b)
MGt	$CuCl_2$	0-20-180	(Bicho et al., 2017b)

Spiking was performed as described previously (Bicho et al., 2017a, 2017b). In short, CuO NMs was spiked as dry powder to the dry soil, following the recommendations for non-dispersible nanomaterials (OECD, 2012), each replicate individually, after which moisture was adjusted to 50% of the WHC. For $CuCl_2$, an aqueous stock solution was prepared and serially diluted, added to each concentration batch of soil.

In situ characterisation

The amount of Cu was measured in the test soil and in soil solution (for method details see (Gomes et al., 2015)). The CuO present as nanomaterials was not determined in the soil, due to technical difficulties, namely the particle size was below the theoretical detection limit of 15 nm (Navratilova et al., 2015).

Test procedures

Apical endpoints

Full life cycle test (FLCt): [F0: hatching success, maturity, growth, survival & reproduction]

To assess embryo development, the test followed the detailed procedures as described in (Gonçalves et al., 2015). In short, synchronized (1-2 days old) cocoons (n = 10 per replicate) were used. Cocoons were introduced in 6-well plates with 5 g of soil in each well. Sampling

days included 3, 4, 5 and 7 days after cocoon laying. To assess the remaining endpoints: hatching success, growth, maturity, survival and reproduction test followed the procedures as described in (Bicho et al., 2017a). Cocoons (n = 10 per replicate) were introduced in each test container with 10 g of soil. Sampling points covered day 11, 14, 18, 25 and 46. Three replicates per treatment were used. For each sampling day 10 cocoons, 10 juveniles or 10 adults were fixated in 10% formaldehyde until further analysis (histology). The soil and remaining organisms were stored. Total number of organisms was counted.

Multigenerational test (MGt): [F0, F1, F2, F3, F4, F5, F6: survival & reproduction]

Exposure followed the standard guideline (OECD, 2004) with adaptations. Test design and performance as in (Bicho et al., 2017b), 4 + 2 generations, 4 in spiked soil (F0-F4), 2 in clean soil (F5-F6) [the offspring generations were respectively F1-F5 and F6-F7]. In short, twenty (20) juveniles (17-18 days' age) per replicate were used, placed in test vessels, containing 20 g of moist soil and food supply. Three replicates per treatment were used. For each generation tests ran during a period of 32 days and the total experiment duration was 224 days. At the end of each generation 10 adults and 10 juveniles from each replicate were fixated in 10% formaldehyde until further analysis (histology). For the next generation, 20 juveniles with medium size (ca. 2 mm±0.2) were selected. The soil and remaining organisms were stored in 96 % ethanol and organisms` counting was as described in (Bicho et al., 2015).

Organs and tissues – Histology and immunohistochemistry analysis

Sampled organisms from MGt (adults, n = 10) and from FLCt (adults, juveniles and cocoons, n = 10) were placed on the histology cassettes between two squares of filter paper and with a sponge inside. Cassettes were introduced in an automated tissue processor (Microm STP 120) for paraffin embedding and then transferred to embedding workstation to obtain paraffin blocks. Organisms' longitudinal sections (2 µm) were cut automated microtome (zmway). Sections were stained with Hematoxylin and Eosin (H&E) for light microscopic examination. Observations and registrations of organisms' tissues and photographs were made using a ZEISS AX10 microscope with an ZEISS (AxioCam ERc5s) camera attached.

Immunostaining of various targets was done using corresponding primary antibodies as summarised in table S2.

Juveniles collected with 11 and 14 days proved not feasible to perform histological cuts due to their small size/fragility and hence was not possible to proceed with the immunohistochemical analysis.

Sections were immersed in 10 mM sodium citrate (pH 6.0) buffer, and placed in water bath for 30 min at 100 ºC for antigen retrieval. Antigen visualization was done with the Novocastra Novolink Polymer Detection System (Leica Microsystems GmbH, Wetzlar, Germany) and involved the following steps: 5 min incubation with a protein blocking agent, overnight incubation at 4 ºC with the primary antibody diluted at 1:200 for 5mC, 1:500 for Ago1,1:250 for H3-dimethyl, 1:50 for Notch1 and 1:400 for SOD1 with BSA (5%). On the next day sections were washed in TBS-buffered saline solution with 1 % triton X-100 before incubation for 30 min with the secondary antibody system using diaminobenzidine (DAB) as a chromogen. Observations and photographs were made using a ZEISS AX10 microscope with a ZEISS (AxioCam ERc5s) camera attached. To quantify the expression of these proteins and molecule, whole tissues present in the entire section were observed and classified. For Ago1 and SOD1 positive expression is cytoplasmatic and for Notch1 positive expression is on the cell membrane. Therefore, for each tissue the semi quantitative scoring system used was: 0 = no positive cells in the tissue, 1 = positive cells in the tissue. For 5mC and H3 di-methyl positive expression is nuclear so for each tissue the semi quantitative scoring system used was 0 = no positive nucleus in the tissue, 1 = positive nucleus in ≤ 50% of the tissue, and 2 = positive nucleus in > 50% of the tissue. This semi quantitative scoring system was adapted from (Gonçalves et al., 2015). In total 19 categories for the whole tissues present in all samples were observed: brain, septal glands, epidermis (anterior part), epidermis (clitellum), epidermis (posterior part), ventral nerve cord, gut, intestine, spermathecal, chloragogen cells (anterior part), chloragogen cells (posterior part), developing oocytes, vitellogenic oocytes, eggs, seminal vesicle, sperm funnel, spermathozoa, penial bulb and coelomocytes. Because in each section not all tissues/categories of the organism were present, the quantification was corrected to the number of tissues present in each section. The following formula was applied: Quantification X total nº of tissues)/ nº. of tissues present. [For Notch1 due to technical issues, immunohistochemistry was done only for control and highest concentration (instead of all concentrations), for both Cu forms and at each sampling day].

Data analysis

One-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test ($p \leq 0.05$) was used to assess differences between treatments (SigmaPlot, 1997). Effect Concentrations (ECx) calculations were done modelling data to logistic and threshold sigmoid 2 or 3

parameters regression models, using the Toxicity Relationship Analysis Program (TRAP v1.3) software.

Two-way ANOVA ($p \leq 0.05$) was also performed, followed by Dunnett's the two independent variables being treatment (0-EC_{10}-EC_{50}) and generation time (F0-F6) to assess the interaction.

Quantification for antibodies positive expression from multigenerational test was also explored by Principal Component Analysis and uni- and multivariate analysis of variance were all performed using SAS IML studio 14.2 (SAS 2013-2014). These provide no explanatory power and hence are not reported.

Results

All tests (FLCt and MGt) fulfilled the validity criteria described for the standard test (OECD, 2004), i.e. in controls adults' mortality ≤20%, the number of juveniles ≥50 and the coefficient of variation <50%. The soil pH did not change significantly between concentrations and during all generations, for FLCt values for control, $CuCl_2$ and CuO NMs were: 6.27 ± 0.12, 6.52 ± 0.08 and 6.37 ± 0.03 (Av ± SE), respectively. For MGt values for control, $CuCl_2$ and CuO NMs were: 6.06 ± 0.1, 5.9 ± 0.12 and 6.15 ± 0.12 (Av ± SE), respectively.

Apical endpoints

FLCt [F0: hatching success, maturity, growth, survival & reproduction] and MGt [F0, F1, F2, F3, F4, F5, F6: survival & reproduction]

Overall, FLCt and MGt results confirm the previous experiments (Bicho et al., 2017a) (Bicho et al., 2017b). In the FLCt (Fig. S1) $CuCl_2$ and CuO NMs caused a decrease in a dose-response manner for hatching success, maturity status, survival and reproduction, confirming the lower toxicity of CuO NMs compared to $CuCl_2$. Similarly, in the MGt results (Fig. S3), the main pattern was confirmed, e.g. for CuO NMs, both the EC_{10} and EC_{50} increased toxicity in reproduction with generations, except F4, followed by a recovery in clean soil. For $CuCl_2$ EC_{10} there was a significant decrease in survival (F1 and F3) and no change in reproduction toxicity including when transferred to clean soil. For $CuCl_2$ EC_{50} reproduction toxicity increased with generations, except F4, followed by recovery in clean soil (F6) and relapse in F7.

Organs and tissues – Histology and immunohistochemistry analysis

Observations allowed to record and count the various embryonic structures of cocoons sections stained with H&E (9 in total), as described in Gonçalves et al (2015) during embryo development (fig. 1) of the FLC. Exposure to $CuCl_2$ caused a dose response decrease in the number of structures, this being more obvious at day 7 (Fig. 1 C, D). For concentrations ≥ 350 mg Cu/kg no differentiation occurred. Exposure to CuO NMs caused no effect on the number of embryonic structures up to day 5, with a clear dose response decrease at day 7, if excluding the effects at 6400 mg CuO NMs/kg. For effect concentrations see supplementary information (Table S3).

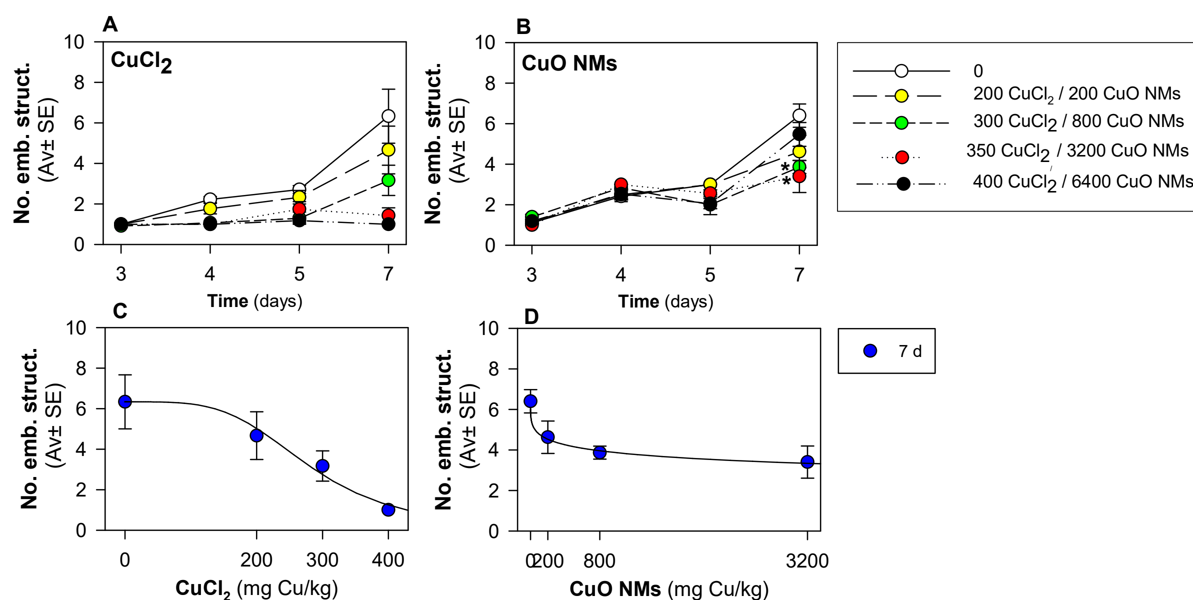


Figure 1: Number of embryonic structures of *Enchytraeus crypticus* in LUFA 2.2 soil during a full life cycle test (FLCt) after 3, 4, 5 and 7 days when exposed to A) $CuCl_2$ and B) CuO NMs (mg Cu/kg DW soil) and dose response results at day 7 (C and D). Line represents the model fit to the data. All values are expressed as average ± standard error (Av ± SE). *($p<0.05$: Dunnets').

Observations of sections from FLCt for juveniles and adults stained with H&E revealed no tissue alterations when exposed to both Cu forms and in all treatments (fig. S3, A, B, E). Results from immunohistochemistry quantification for FLCt (fig. 2 and S4) showed that the expression of SOD1 and Notch1 varied with the Cu form, concentration and age of the organisms. $CuCl_2$ caused a decrease of SOD1 expression in adults (46 days old) in a dose-response manner. The expression of Notch1 decreased ($p<0.05$) in both juveniles (18 and 25 days) and adults (46 days). For CuO NMs exposure, SOD1 expression in adults showed a

tendency to decrease up to 800mg/kg and Notch1 increased ($p<0.05$) at 6400 mg Cu/kg in adults.

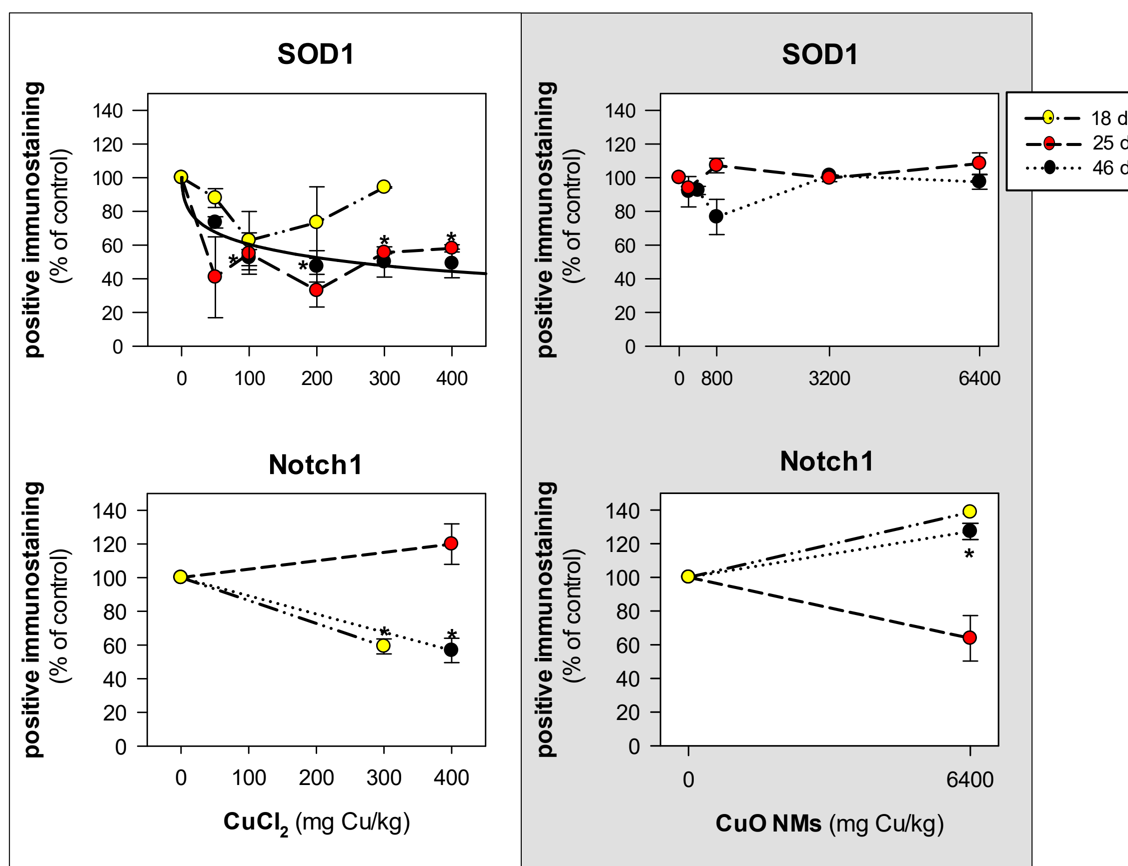


Figure 2: Immunohistochemistry quantification analysis of *Enchytraeus crypticus* when exposed to $CuCl_2$ and CuO NMs (mg Cu/kg DW soil) in LUFA 2.2 soil during the full life cycle test (FLCt). Organisms' covered ages: 18 (juveniles), 25 (mature) and 46 (adults post reproductive cycle) days. All values are expressed as average ± standard error (Av ± SE). The solid line represents the model fit to data. (*$p<0.05$: Dunnets').

Observations of H&E adults' sections from MGt exposure showed no tissue alteration in any of the treatments compared with control, including all generations (F0-F6) in CuO NMs and $CuCl_2$, EC_{10} and EC_{50} (fig. S3, C, D, F). Results from immunohistochemistry quantification (fig. 3) and representative images of positive immunostaining (fig. S5) show that the expression of Ago1, H3-dimethyl and 5mC was affected by both Cu forms, concentrations and across generations.

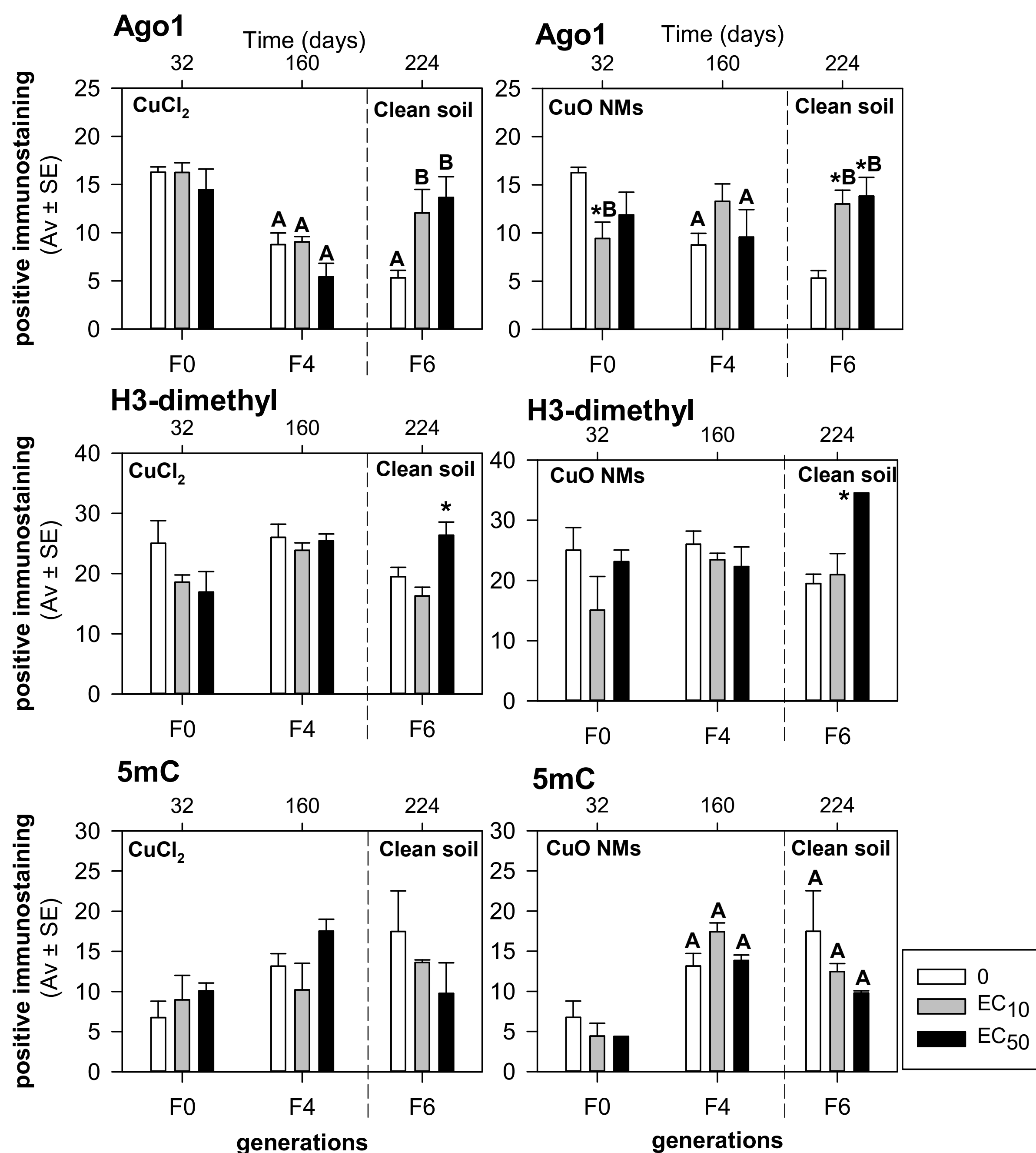


Figure 3: Immunohistochemistry quantification analysis for *Enchytraeus crypticus* when exposed to 0-20-180 mg Cu/kg soil for $CuCl_2$ and 0-500-1400 mg Cu/kg DW soil for CuO NMs, in LUFA 2.2 soil during a multigenerational test (MGt). Generations: F0, F4 and F6. * $p < 0.05$: one-way ANOVA, Dunnett's. ^{A} $p < 0.05$: two-way ANOVA Dunnett's, effect of time between F0 and Fx. ^{B} $p < 0.05$: two-way ANOVA, effect of treatment, within each generation (Fx).

Regarding $CuCl_2$ exposure, major differences occurred in F6 (clean soil), where an increase was observed for Ago1 expression for both EC_{10} ($p = 0.016$, Tab. S4) and EC_{50} ($p = 0.001$,

Tab. S4). The interaction between exposure time (generation) and concentration was significant (p=0.006, Tab. S4). Similarly, for H3-dimethyl, an increase in expression was observed for EC_{50} (p = 0.041, one-way ANOVA) in F6. For CuO NMs exposure, changes were observed in F0, with a decrease in Ago1 expression for EC_{10} (p = 0.019, Tab. S4). In F6 an increase for Ago1 expression for both EC_{10} (p = 0.028, Tab. S4) and or (p = 0.027, Tab. S4), and EC_{50} (p = 0.021, Tab. S4) and or (p = 0.013, one-way ANOVA) was observed. As well an increase for H3-dimethyl expression for EC_{50} (p = 0.001, one-way ANOVA). Again, there was a significant interaction between exposure time (generation) and concentration (p=0.004, Tab. S4). Overall significant differences were observed between F0 and FX (F4 or F6) for the three antibodies, depending on Cu form and generation, but these differences were also observed for control organisms.

Please note that to perform the organ and tissue histology and immunohistochemical analysis the long term exposures using the full life cycle (Bicho et al., 2017a) and the multigenerational (Bicho et al., 2017b) were repeated. Hence, we here have a repeated assessment of all the reported apical endpoints: hatching success, growth, maturity status, survival and reproduction plus the multigenerational exposure from F0 to F6 with survival and reproduction recorded along 224 days. Overall, previous results were confirmed, especially the longer exposure period showed high consistency and reproducibility of effects.

Discussion

Apical endpoints and histology

Results from full life cycle test (FLCt) apical endpoints combined with histological observations from embryo development, confirmed the evidence of life stage dependent effect depending on the Cu form (Bicho et al., 2017a). We here observed that $CuCl_2$ clearly caused a delay in embryo development whereas CuO NMs caused a post-hatching effect, i.e. in newly-hatched juvenile stage. Again, also the impact of CuO NMs on growth was confirmed.

The exposure for longer periods, from FLCt and MGt to both CuO NMs and $CuCl_2$, showed no tissue alterations in adult *E. crypticus*. It was expected to observe changes in the reproductive tissues, since organisms were exposed to the reproduction EC10 and EC50. This could be because effects occur in the earlier life stages as referred.

Contrasting with these results in *E. crypticus*, for example exposure of the earthworm *Eudrillus eugeniae* to Cu salt caused histopathological changes in the head, gut and intestine

(Sharma and Satyanarayan, 2011). Tissue alterations have also been seen in aquatic species, e.g. the snail *Pomacea canaliculata*, where the exposure to $CuSO_4$ caused several histopathological changes in the gill, digestive tract and gland (Dummee et al., 2015). Further examples in fish include underdeveloped liver in *Danio rerio* larvae exposed to CuO NMs (Sun et al., 2016), several histopathological effects in the gills, gut, liver, kidney, brain and skeletal muscle of *Oncorhynchus mykiss* exposed to Cu NMs and $CuSO_4$ (Al-bairuty et al., 2013), histopathological effects in the gills and liver of *Acipenser baerii* exposed to Cu NMs (Ostaszewska et al., 2016) and histopathological effects in the gills of *Cyprinus carpio* exposed to Cu NMs (Noureen et al., 2019). The "absence of evidence" in *E. crypticus* is not "evidence of absence". There is obviously the question of detection level, i.e. were we able to capture tissue alteration in sufficiently fine detail. Although we do not have the answer, the same methodology was used as in the other studies, so this is likely not the case.

Immunohistochemistry epigenetic markers

Regarding the stress markers in the FLCt exposure (observed mostly after 46d of exposure), tissue/organ stress responses were distinct: for $CuCl_2$ SOD1 and Notch1 protein expression decreased whereas for CuO NMs Notch1 increased. Copper is well-known for its essentiality to most life forms and both Cu deficiency and excess can cause oxidative stress disrupting the antioxidant system (Festa and Thiele, 2011; Uriu-adams and Keen, 2005). For soil invertebrates there are reports of metallothionein (MT) activation to Cu exposure, e.g. in earthworm (Mustonen et al., 2014; Spurgeon et al., 2004), or collembolan (*Folsomia candida*) species (Maria et al., 2014). Previous transcriptomic studies with *E. crypticus* showed that exposure to Cu salt ($CuNO_3$) caused oxidative stress and, exposure to Cu NMs, Cu-nanowires and aged Cu salt affected the Notch signalling pathway besides also causing oxidative stress (Gomes et al., 2018). The MG exposure to Cu materials induced differential expression of genes related with oxidative stress and the Notch signalling pathway (Bicho et al., 2020), this has also been reported after Cu exposure to *D. rerio*, at the gene level (Sonnack et al., 2017).

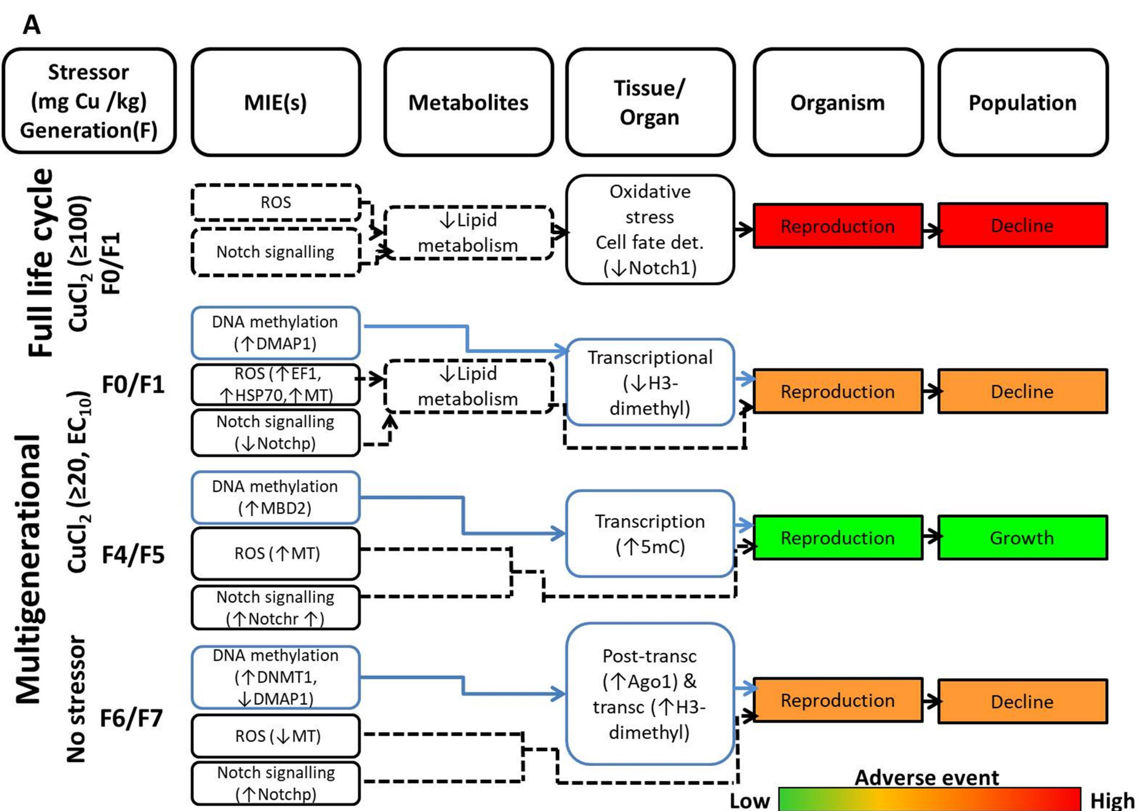
Results of immunohistochemistry from MGt confirmed at the tissue/organ level that MG exposure to Cu materials affected the measured epigenetic markers: non-coding RNA (Ago1); histone modification (H3-dimethyl) and DNA methylation (5mC). Previous results had confirmed that the genes were expressed differently (Bicho et al., 2020). For example, in the study by Bicho et al (2020) the 5mC molecule expression seemed to increase in F4 and decrease in F6 (clean media) - this same pattern was observed in terms of global DNA

methylation for CuO NMs (Bicho et al., 2020). Similarly, the pattern observed for Ago1 and H3-dimethyl gene expression across generations (Bicho et al., 2020) was confirmed at immunohistochemistry level.

Argonaute (like Ago1) proteins are known to bind to microRNA (miRNA), having an important role in post-transcriptional regulation (Okamura, 2004). Additionally histone modifications play a key role in changing DNA chromatin to regulate transcription (Stoccoro et al., 2013). Our findings showed that in *E. crypticus* both post-transcriptional and transcriptional regulation mechanisms were affected by $CuCl_2$ and CuO NMs. Post-transcriptional repression mediated by miRNA is known to be affected by the exposure to environmental chemicals like metals (Baccarelli and Bollati, 2009; Hou et al., 2013). For Cu these mechanisms are well known in plants (Gupta et al., 2014; Pilon, 2017). For example studies with *Oryza sativa* and *Brassica napus* showed that exposure to cadmium (Cd) affected the homeostasis of the complexes argonaute/miR168 and argonaute/miR403, respectively (Ding et al., 2011; Zhou et al., 2012). There is also a study reporting that Cu affected miRNA expression in *D. rerio* (Wang et al., 2013). So it seems that these mechanisms are conserved in *E. crypticus*. Moreover, regarding the transcriptional regulation by histone modifications, studies have shown that exposure to non-essential metals and NMs can alter these mechanisms: e.g. Cd alters the normal pattern of histone modifications to the metallothionein 3 (MT3) promoter in human cells (Somji et al., 2011); Ag NMs in mouse cells caused a decrease in histone methyltransferases (HMTs) (Qian et al., 2015), cadmium telluride quantum dots (CdTeQDs) lead to global hypoacetylation in human cells (Choi et al., 2008). The differences observed in tissue/organ responses could be due to differences in toxicokinetics between Cu forms, i.e. uptake and elimination rates may be slower for CuO NMs than for $CuCl_2$. The internalised CuO NMs have a potential long-term release of Cu ions due to acidic conditions in the lysosomes. Obviously there is also the issue of possible release of ions in the media and some uptake of Cu ions, however, as has been previously discussed, in complex media with organic matter and organisms present this is likely less of an issue (Arenas-Lago et al., 2019; Gomes et al., 2015; Xiao et al., 2018). In a previous study with *F. candida*, levels of Cu body burdens showed that uptake for $CuCl_2$ was comparatively faster than for CuO NMs but that with time, uptake increased to a similar internal concentration of Cu on a mass basis (Mendes et al., 2018).

AOPs

To overview the various known and measured events and integrate the time of event knowledge, a draft AOP for $CuCl_2$ and for CuO NMs were framed (Figure 6).



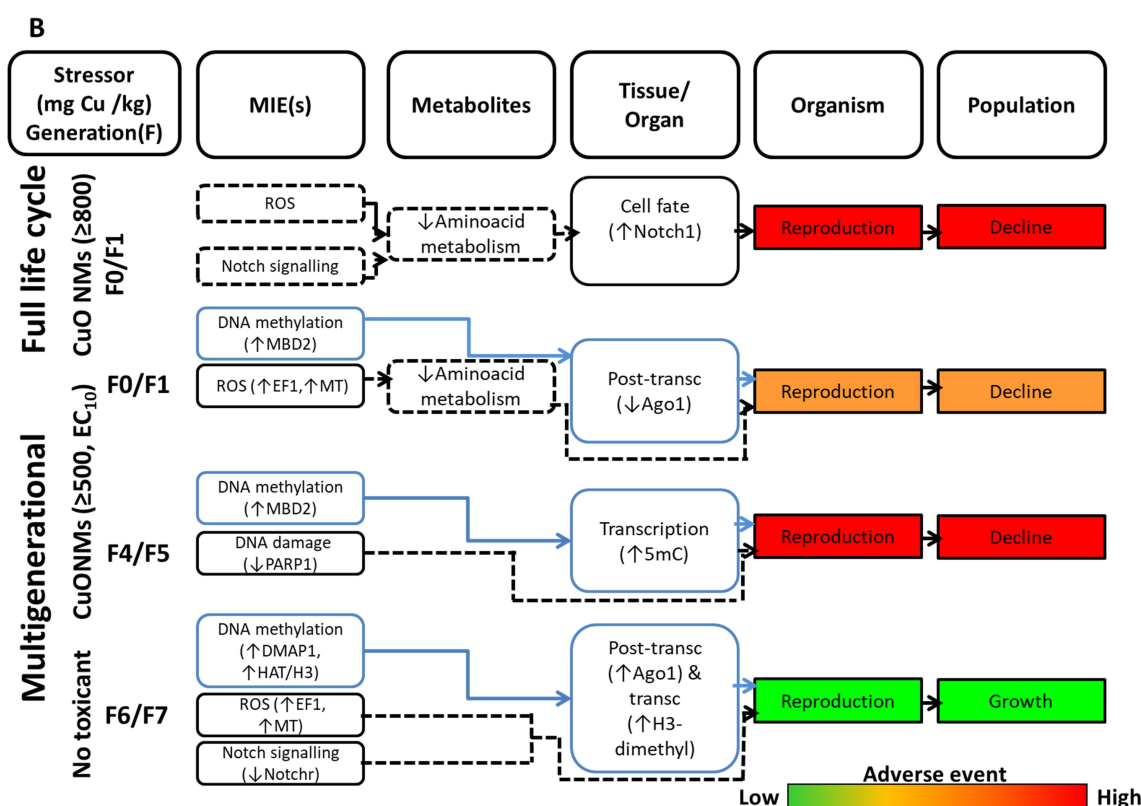


Figure 6: Adverse Outcome Pathway (AOP) for *Enchytraeus crypticus* from multigenerational test (MGt) and full life cycle test (FLCt) when exposed to (A) $CuCl_2$ and (B) CuO NMs in LUFA 2.2 soil. Red: adverse effect; Orange: semi-adverse effect; Green: no adverse effect; Blue arrows/lines: epigenetic responses; Black arrows/lines: stress responses. Dashed line: relationships hypothesized. Square boxes: final states for the organism. Rounded boxes: intermediate states. DMAP1: DNA methyltransferase 1-associated protein 1; MBD2: Methyl-CpG-binding domain protein 2; DNMT1: DNA (cytosine-5)-methyltransferase 1; H3: histone 3; HAT: histone acetyltransferase; PARP1: poly (ADP-ribose) polymerase 1; Argonaute: argonaute 1; EF1: elongation factor-1 alpha; HSP70: heat shock protein 70; MT: metallothionein-like protein; NOTCHr: notch receptor; NOTCHp: notch protein.

The $CuCl_2$ and CuO NMs cause impact via differentiated MIEs, as measured for epigenetic mechanisms. The measured sequential key events, effects at the tissue/organ level, showed no differences between Cu materials until exposed over multiple generations, showing differences (F4/F5 case) not found in the one generation study. Hence, the impact of CuO NMs was not predictable from $CuCl_2$ toxicity as suggested by (Gerloff et al., 2017) based on the MIE or one generation studies. The development of AOPs will clearly contribute to the

mechanistic understanding of toxicity and intelligent testing strategies, further supporting safer by design materials development.

Conclusions

Histological and immunohistochemistry analysis performed at the tissue/organ level proved to be important tools to test evidences of activated mechanisms, i.e. a good confirmatory assay for potentially transient responses like gene expression. CuO NMs affected the Notch signalling pathway, whereas $CuCl_2$ caused both oxidative stress and affected the Notch signalling pathway. Major differences occurred from multigenerational exposure, showing not only the impact of longer term exposure but also the transfer of effects from generation to generation and the importance of exploring epigenetics signals.

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Supplementary information

Impact of copper materials (Cu NMs and $CuCl_2$) exposure on soil invertebrates – histology and immunohistochemistry – confirmatory assays for transient changes in *Enchytraeus crypticus*

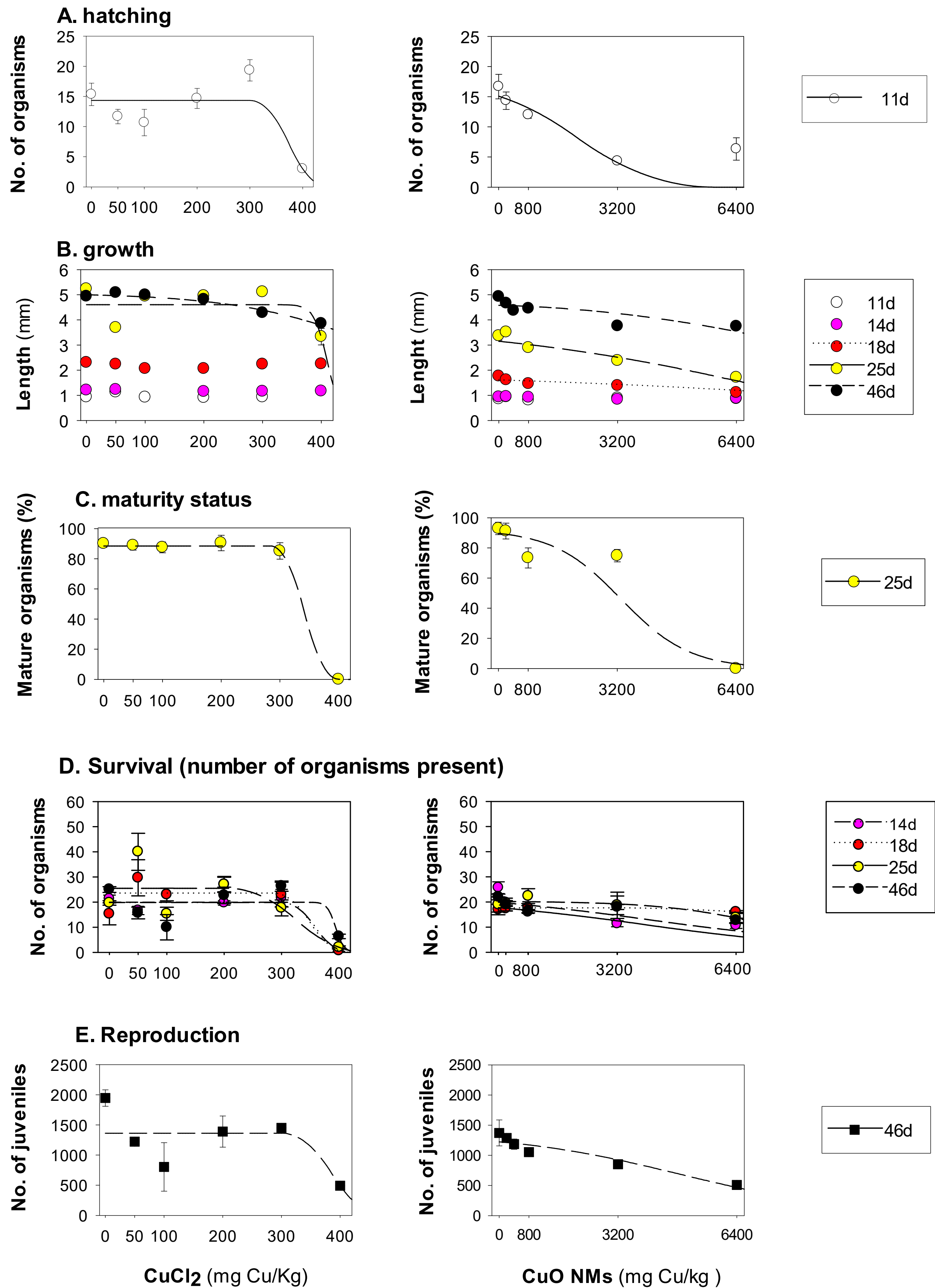
Rita C. Bicho[1], Faustino A.M.R.[2], Rêma A.[2], Janeck J. Scott-Fordsmand[3], Mónica J.B. Amorim[1*]

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

[2] Department of Pathology and Molecular Immunology, Biomedical Sciences Institute of Abel Salazar, University of Porto, 4050-313 Porto, Portugal

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

*corresponding author: mjamorim@ua.pt



A. hatching
No. of organisms
11d
B. growth
Length (mm)
Lenght (mm)
11d
14d
18d
25d
46d
C. maturity status
Mature organisms (%)
25d
D. Survival (number of organisms present)
No. of organisms
14d
18d
25d
46d
E. Reproduction
No. of juveniles
46d
CuCl2 (mg Cu/Kg)
CuO NMs (mg Cu/kg)

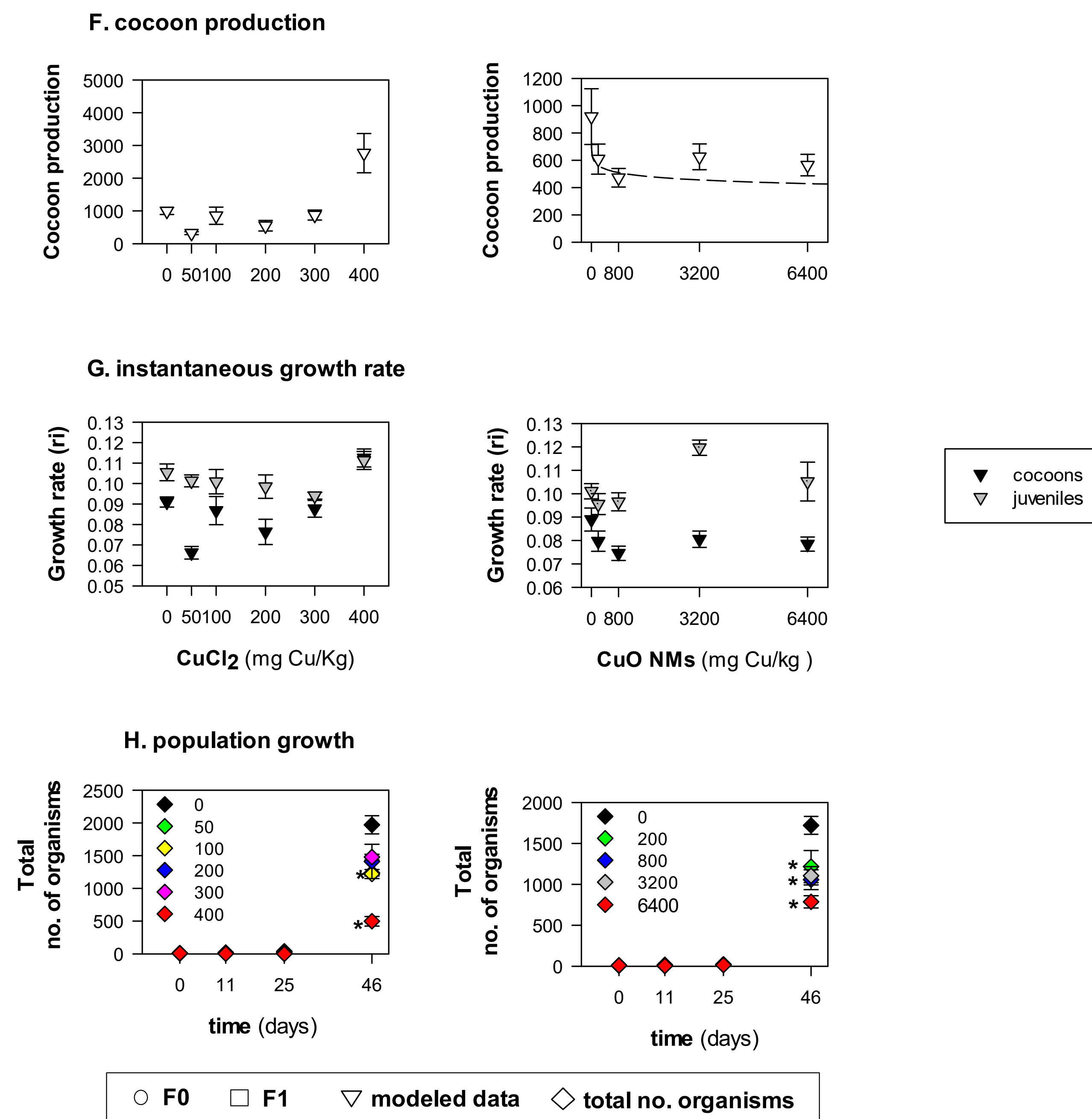


Figure S1: Results of the Full Life Cycle test (FLCt) for *Enchytraeus crypticus* when exposed to $CuCl_2$ and CuO NMs (mg Cu/kg DW soil) in LUFA 2.2 soil including the various endpoints: A: hatching; B: growth; C: maturity status; D: survival (number of organisms present); E: reproduction; F: cocoon production; G: instantaneous growth rate; H: population growth rate. All values are expressed as average ± standard error (Av±SE). The lines represent the model fit to data. d = days.

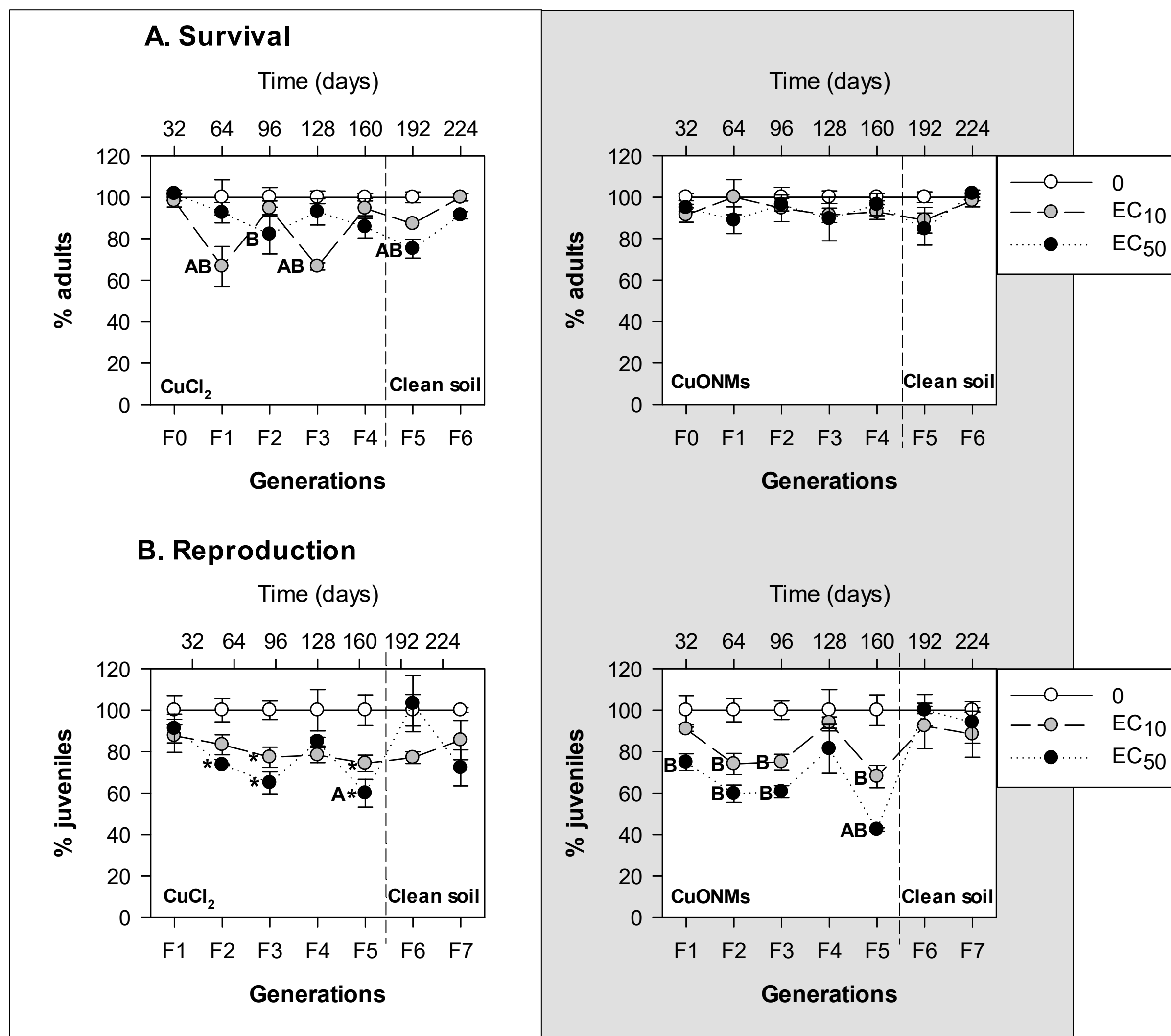


Figure S2: Results for survival (A) and reproduction (B) from the multigenerational test (MGt) with *Enchytraeus crypticus*. Exposure to the reproduction EC_{10} and EC_{50} of $CuCl_2$ and CuONMs (0-20-180 mg and 0-500-1400 mg Cu/kg Cu/kg DW soil, respectively) in LUFA 2.2 soil. All values are expressed as % normalized to the control, average ± standard error (Av ± SE). *p < 0.05 (Dunnets' between parental generation (F0/F1) and Fx).

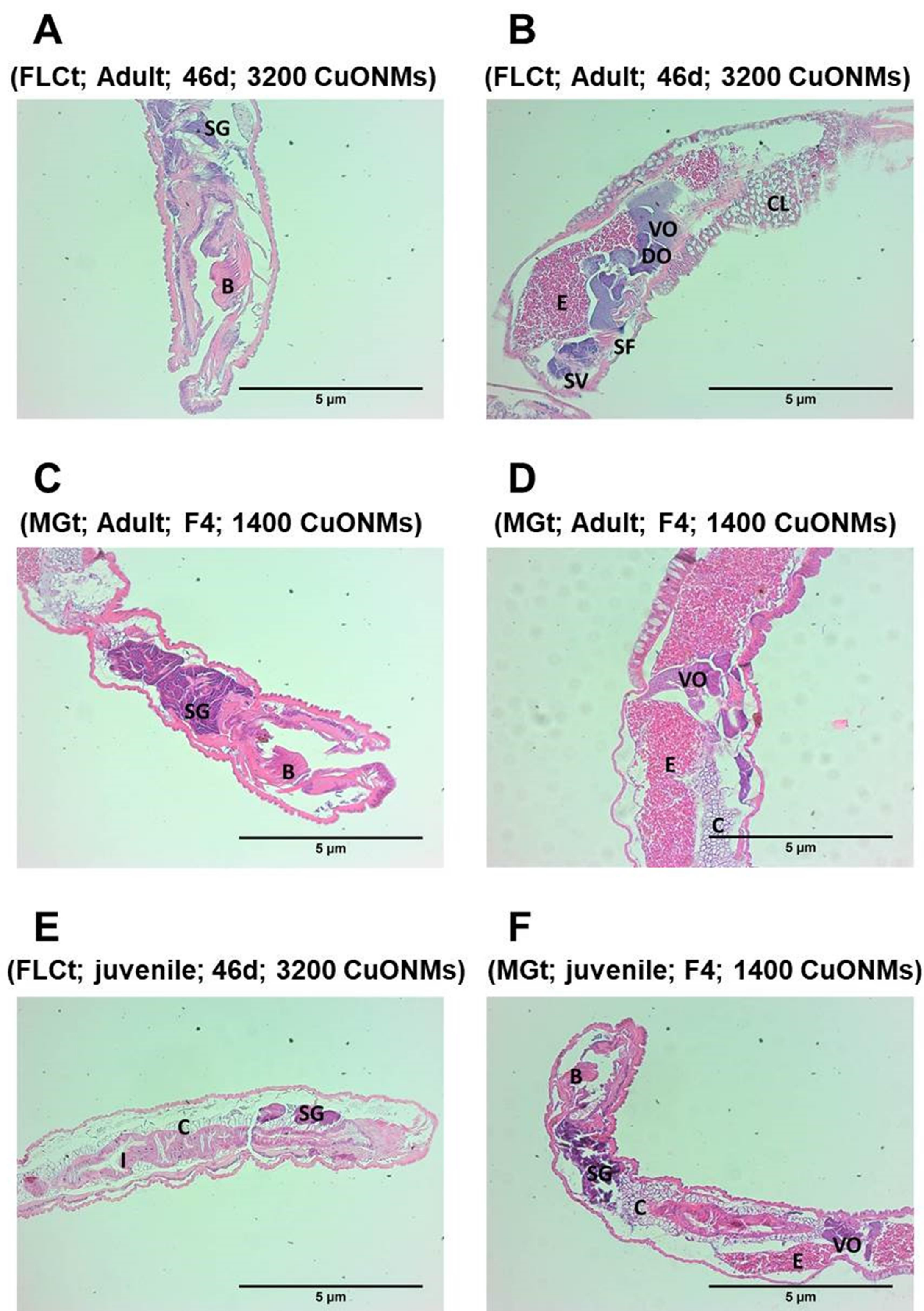


Figure S3: Representative H&E images of *Enchytraeus crypticus* tissues long term exposures to CuO NMs. Full life cycle test (FLCt): A, B (adults 46d) and E (juvenile 46d), 3200 CuO NMs (mg Cu/kg DW). Multigenerational test (MGt): C, D (adults, F4) and F (juvenile, F4),

1400 CuO NMs (mg Cu/kg DW). SG: septal glands; C: chloragogen tissue; I: intestine; B: brain; E: eggs; VO: vitellogenic oocyte; DO: developing oocyte; SF: sperm funnel; SV: seminal vesicle; CL: clitellum.

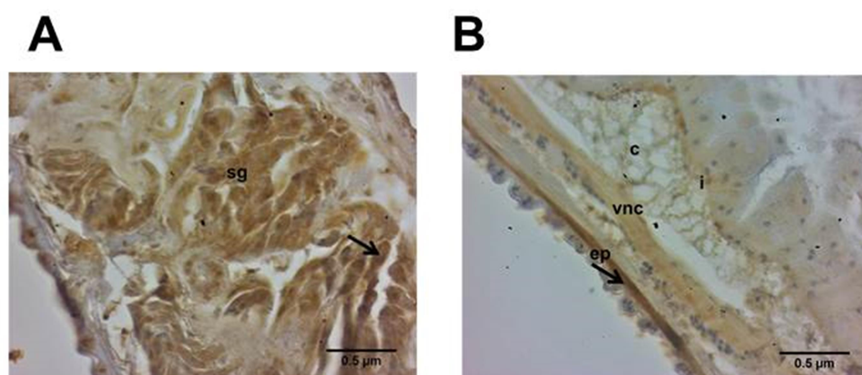


Figure S4: Representative images of the positive immunostaining of *Enchytraeus crypticus* tissues (adults, 46 d, control) in a full life cycle test (FLCt). A) SOD1, B) Notch1. sg: septal glands; vnc: ventral nerve cord; ep: epidermis; c: chloragogen tissue; i: intestine. Black arrow represents positive immunostaining (brown colour).

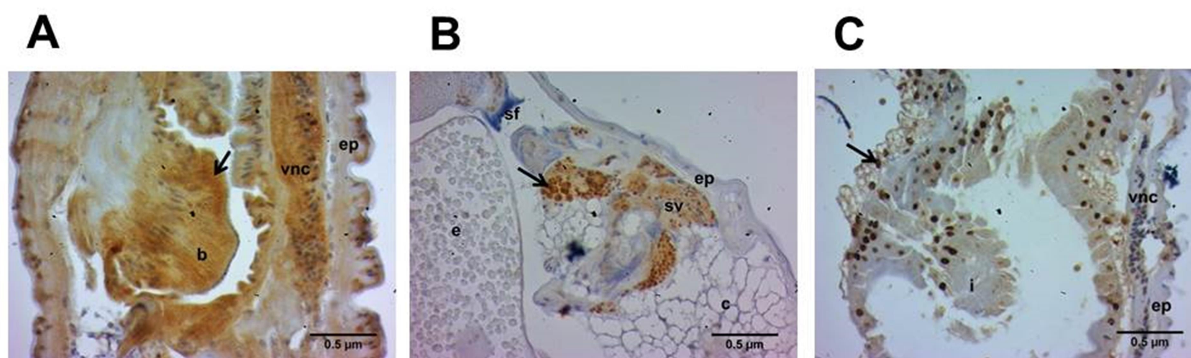


Figure S5: Representative images of the positive immunostaining of *Enchytraeus crypticus* (adults) tissues when exposed to $CuCl_2$ and CuO NMs in a multigenerational test (MGt). A) Ago1 (F4, EC_{50}, CuO NMs), B) H3-dimethyl (F0, EC_{50}, $CuCl_2$), C) 5mC (F0, control). b: brain; vnc: ventral nerve cord; ep: epidermis; sv: seminal vesicle; sf: sperm funnel; c: chloragogen tissue; i: intestine. Black arrow represents positive immunostaining (brown colour).

Table S1: Characteristics of the tested CuO NMs (Source: FP7-SUN project).

Characteristics	CuO NMs
Manufacturer	Plasma Chem
CAS number	1317-38-0
Primary size distribution (average)	3-35 (12)
Mode (1st quartile - 3rd quartile) [nm]	10 (9.2-14)
Shape	Semi-spherical
Average crystallite size [nm]	9.3
Crystallite phases (%)	Tenorite 100 %
Dispersability in water: D50 [nm]	139.5 ± 4.6;
average agglomeration number (AAN)	346
Dispersability in modified MEM: D50 [nm]	85.2 ± 2.7;
average agglomeration number (AAN)	77
Z-potential in UP water [mV]	+ 28.1 ± 0.6
Isoelectric point [pH]	10.3
Photocatalysis: photon efficiency [unitless]	1.5 x 10-4
Specific Surface Area [m2 g-1]	47.0 ± 1.7
Pore sizes [nm]	13.5 ± 1.6 (BJH)
	23.0 ± 0.9 (AVG)
Surface chemistry [atomic fraction]	Cu = 0.46 ± 0.05; O = 0.47 ± 0.05; C = 0.07 ± 0.01

Table S2: Summary of the list of targets for immunohistochemistry, including the used antibody, scoring system of positive expression, test type (full life cycle test (FLCt), multigenerational test (MGt)) and test concentrations for $CuCl_2$ and CuO NMs and life cycle stage (age) of the organisms used (adults or juveniles).

Target	Antibody	Positive Expression (% of present cells)	Test type/ generations/ treatment	Organism Sample
5-methyl-cytosine (5mC)	anti-5-mC (mouse monoclonal antibody [33D3] ab10805, Abcam, Cambridge, England)	Nuclear = 0%: 0 ≤ 50%: 1 > 50%: 2	MGt/ F0; F4; F6/ 0-500-1400 mg Cu/kg (CuO NMs) 0-20-180 mg Cu/kg ($CuCl_2$)	Adults (46 days)
argonaute 1 (Ago1)	anti-Ago1 (rabbit polyclonal antibody ab5070, Abcam, Cambridge, England)	Cytoplasmatic	MGt/ F0; F4; F6/ 0-500-1400 mg Cu/kg (CuO NMs) 0-20-180 mg Cu/kg ($CuCl_2$)	Adults (46 days)
Histone H3 dimethyl K9 (H3-dimethyl)	anti-H3-dimethyl K9 (mouse monoclonal antibody ab1220 (Abcam, Cambridge, England)	Nuclear = 0%: 0 ≤ 50%: 1 > 50%: 2	MG/ F0; F4; F6/ 0-500-1400 mg Cu/kg (CuO NMs) 0-20-180 mg Cu/kg ($CuCl_2$)	Adults (46 days)
Notch1	anti-Notch1 (rabbit monoclonal antibody [EP1238Y] ab52627, Abcam, Cambridge, England)	Cell membrane = 0%: 0 > 0%: 1	FLCt: 0-6400 mg Cu/kg (CuO NMs) 0-300-400 mg Cu/kg ($CuCl_2$)	Juveniles (18, 25 days)
superoxide dismutase 1 (SOD1)	anti-SOD1 (rabbit polyclonal antibody ab13498, Abcam, Cambridge, England)	Cytoplasmatic	FLCt: 0-6400 mg Cu/kg (CuO NMs) 0-300-400 mg Cu/kg ($CuCl_2$)	Juveniles (18, 25 days)

Table S3: Summary of the embryo development (emb dev) effect concentrations (ECx) for *Enchytraeus crypticus* when exposed to $CuCl_2$ and CuO NMs in LUFA 2.2 soil at day 7. Results show EC estimates (mg/kg) and the 95 % confidence intervals in brackets. S: slope; Y0: intercept; n.d.: not determined.

Cu form	EC_{10}	EC_{20}	EC_{50}	EC_{80}	Model & parameters
$CuCl_2$	163 (113-235)	198 (151-259)	274 (241-312)	381 (332-437)	Log 2 par (S: 2.43; Y0:6.3)
CuO NMS	2	33	4183	n.d.	Log 3 par (S: 0.16; Y0:6.4)

Table S4: Results of the two-way ANOVA analysis, with two independent variables, i.e. 1) treatment (0-EC_{10}-EC_{50}) for both $CuCl_2$ and CuONMs and 2) generation time (F0-F6) The statistical significant differences were tested with post-hoc test Holm-Sidak .

Source of Variation	DF	SS	MS	F	p	Post-hoc test	p
$CuCl_2$ ($EC_{10}/_{50}$) F6: EC_{10} EC_{50}	2	29.22	14.61	1.727	0.198	Holm-Sidak method	 0.009 0.001
Generation time (F0-F6) Ctr: F0 & F4 F0 & F6 EC_{10}: F0 & F4 EC_{50}: F0 & F4	2	366.331	183.166	21.654	<0.001		 <0.001 <0.001 0.006 <0.001
Interaction ($CuCl_2$ x generation time)	4	157.256	39.314	4.648	0.006		
Residual	26	219.93	8.459				
Total	34	791.592	23.282				
	DF						
CuO NMs	2	23.526	11.763	0.702	0.504		
Generation time (F0-F6) Ctr: F0 & F4	2	27.250	13.625	0.813	0.453	Holm-Sidak method	 0.008

F0 & F6							0.001
Interaction (CuONMs x generation time)	4	331.053	82.763	4.940	0.004		
Residual	30	502.626	16.754				
Total	38	875.834	23.048				

Chapter VIII - Multigenerational exposure to WCCo nanomaterials - epigenetics in the soil invertebrate *Enchytraeus crypticus*

Rita C. Bicho[1], Janeck J. Scott-Fordsmand[2], Mónica J.B. Amorim[1*]

[1]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

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

*corresponding author: mjamorim@ua.pt

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Abstract

It has become clear how important it is to assess longer term effects of (nano)materials in the environment given the present evidences showing how epigenetics drives the response mechanisms. We here studied global DNA methylation in standard soil invertebrate *Enchytraeus crypticus* over 224 days, when exposed in a multigenerational experiment to nanostructured Tungsten Carbide Cobalt (WCCo nanomaterials (NMs)) and to cobalt ($CoCl_2$). In order to assess transgenerational effect, we used a multigenerational (MG) test design consisting of 4 generations in spiked soil followed by 2 generations in clean soil. Results showed that MG exposure to WCCo NMs caused global DNA methylation to increase, which continued in unexposed generations and was associated with increase in reproduction (phenotypic effect). WCCo NMs caused in general more (and more consistent) methylation than $CoCl_2$.

Keywords: Global DNA methylation; environmental species; cobalt-based nanoparticles; carbon tungsten;

1. Introduction

Epigenetic mechanisms play a key role in regulating gene expression and consequently lead to phenotypic effects. To understand how environmental stressors can affect these

mechanisms will provide insight on organisms' response and its mechanisms linked to environmental changes. This can be of particular importance for phenomena like developmental reprogramming in early life exposures, with potential multigenerational (MG) and transgenerational effects [1]. There is an increasing amount of evidence showing that nanomaterials (NMs) can cause changes in epigenetic mechanisms, e.g. in humans [2]. In light of this evidences it has also become clear how important and urgent it is to address this topic for nanomaterial risk assessment [3]. However, epigenetic studies with environmental relevant species are very rare in the literature [4]. From the few studies reported with NMs, short term exposure has shown epigenetic effects in plants (*Nicotiana tabacum*) exposed to aluminum oxide (Al_2O_3) nanoparticles (NPs) [5], in fish (*Danio rerio*) exposed to single and multi-walled carbon nanotubes (SWCNT and MWCNTs) [6] and silver (Ag) NPs [7] and in nematodes (*C. elegans*) exposed to MWCNTs [8]. However, long term studies are among the top recommendations to assess effects of NMs [9-11], but long term exposures covering epigenetic effects of NMs in the environmental species are even more scarce. There are MG studies, one with *C. elegans* exposed to Ag NPs [12] and one with crustaceans (*Daphnia magna*) exposed to functionalized SWCNT-$CONH_2$ [13], and although epigenetic changes are suggested this was not assessed. It is clear that, especially for invertebrates, basic knowledge on epigenetic mechanisms is lacking. For *Enchytraeus crypticus*, a standard ecotoxicological soil species, a recent publication showed an approximately 1.4% DNA methylation [14], but no methylation was observed in Folsomia candida, another terrestrial model invertebrate. We recently observed that copper oxide (CuO) NMs caused changes in several epigenetic mechanisms like DNA methylation, histone modifications, and non-coding RNA in a MG study with *E. crypticus* [15]. Hence, epigenetic effects have been observed in E. crypticus following exposure to NMs, but no studies have dealt with nanostructured Tungsten Carbide Cobalt (WCCo NMs). Cobalt is an essential element for most biological species, being a component of vitamin B12 and essential to processes like DNA synthesis. Results supported that Co was also essential to E. crypticus. Tungsten Carbide Cobalt nanoparticles are used in many applications, e.g. tires or in the hard metal industry [16], giving the products increased wear resistance [17]. These materials are persistent and hence it is very important to assess the long term effects. Toxicity of WCCo particles is generally associated to an increase of activated oxygen species (AOS) given that cobalt (Co) oxidizes at the surface of tungsten carbide (WC) [18], causing proteins, lipids and DNA damage. Despite its essentiality Co can induce genotoxicity, inhibition of the DNA repair system and ultimately apoptosis.

We recently performed a WCCo and $CoCl_2$ MG study with *E. crypticus* which showed effects on survival and reproduction, besides an uptake of Cobalt (Co) [19]. We did not measure epigenetic effects at the time. Nevertheless, effects of Co on epigenetic mechanisms like DNA methylation were shown in other species such as plants [20,21] and humans [22], e.g. causing changes in histone modifications in human cells [23]. Further, it was also shown that cobalt-chrome NPs are genotoxicity in human cells [24-26] and that this genotoxicity may be caused through epigenetic mechanisms [27]. Other reports on WCCo NMs include observations of in vitro genotoxicity mostly attributed to oxidative stress [28-30]. Hence, there are ample indications that Co based NMs may cause epigenetic effects and maybe in E. crypticus.

The aim of this study was to evaluate global DNA methylation levels in *E. crypticus* when exposed to WCCo NMs (and $CoCl_2$) in a multigenerational design, including 4 generations exposed plus 2 after transfer to clean soil, hence including the transgenerational effect.

2. Materials and Methods

2.1 Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. Cultures are maintained in petri dishes with agar, please see Bicho et al [31] for further details. Synchronized cultures were prepared and juveniles (17-18 days) were used.

2.2 Test soil

LUFA 2.2 natural standard soil (Speyer, Germany) was used. This soil is very well characterized and has pH (0.01 M $CaCl_2$) of 5.5, 1.61 % organic matter, 10.0 meq/100 g CEC (cation exchange capacity), 43.3 % WHC (water holding capacity), grain size distribution of 7.9 % clay, 16.3 % silt, and 75.8 % sand as main properties.

2.3 Test materials, characterisation and spiking

Nanostructured Tungsten Carbide Cobalt powder (WCCo NM) and cobalt chloride ($CoCl_2.6H_2O$, 98% purity, Sigma-Aldrich) were used. Materials were fully characterized as synthesized (table 1) and also during exposure via a concurrent experiment. Spiking of the soil was done as reported in Ribeiro et al [19]. In short, concentrations used correspond to the approximate 10% and 50% effect (reproduction) concentrations EC_{10} and EC_{50}: 0–1200–1500 mg WCCo NMs/kg soil DW (0–100–120 mg Co/kg equivalent) and 0–110–180 mg Co/kg

soil DW for $CoCl_2$. WCCo NMs were spiked as dry powder following OECD [32] recommendations for dry powder non-dispersible NMs, done per individual replicate. Moisture was 50% of the maximum water holding capacity. $CoCl_2$ was prepared as concentrated aqueous solution, diluted to the required concentrations and spiked onto the pre-moistened soil batches per concentration and homogeneously mixed. Test started 1 day after spiking for both Co materials.

Table 1: Characterisation of WCCo NPs (FP7 SUN: Sustainable Nanotechnologies). TEM: Transmission Electron Microscope; XRD: X-Ray Diffraction; DLS: Dynamic Light Scattering; BET: Brunauer, Emmett and Teller; ELS: Electrophoretic Light Scattering; FTIR: Fourier-Transform Infrared Spectroscopy; XPS: X-ray Photoelectron Spectroscopy.

Characteristics	WCCo NP	Technique
Source	NBM Nanomaterialia, Italy	
Composition (%)	Tungsten carbide (WC<88% Wt., CAS 12070-12-1) Cobalt (Co=8.32% Wt., CAS 744-48-4)	ICP -MS
Primary Size distribution [nm] **Average (Min-Max)** **Mode [nm] (1st and 3rd quartile)**	170 (23-1446) 48 (69; 280)	TEM
Crystalline size (Average) [nm]	15.4	XRD
Iso Electric Point (pH)	<2	pH
Dispersability in water: D50 [nm]; Average Agglomeration Number (AAN)	182.8 ± 21.5; 31	DLS
Specific Surface Area [m2.g-1]	6.6±0.4	BET
Z-potential [mV]	7.1±0.5	ELS
Structure	O-W-O	FTIR and/or RAMAN
Pore size [nm]	Non-porous	BET
Surface Chemistry [atomic fraction]	Co=0.08±0.01 W=0.05±0.01 O=0.31±0.03 C=0.56±0.05	XPS

2.4 Exposure procedure and characterization

A multigenerational exposure test (MGt) was performed, for details see Ribeiro et al [19], and from which organisms were sampled to analyze here. For the exposure each replicate had 40 juveniles with 17–18 days of synchronized age. Organisms were placed in the test vessels

with 40 g of soil. Each generation was exposed for a period of 32 days. Design consisted of six generations for $CoCl_2$ and seven for WCCo NMs, during 224 and 256 days, respectively. The design included 4 + 2 ($CoCl_2$) and 5 + 2 (WCCo NMs) generations (spiked soil + clean soil). Each treatment had six replicates, except for the highest concentration, that had 10 replicates to compensate for mortality. At the end of each generation, organisms from offspring generation were used, collecting for each replicate juveniles of intermediate size (n = 40) and transferring these to the next generation. Further, for each replicate, adults (n = 20) and juveniles (n = 100) of large size were collected, snap-frozen in liquid nitrogen and stored at -80 ºC for DNA methylation analysis. At each generation adults and juveniles were counted to assess survival and reproduction.

Cobalt (Co) concentrations were measured in soil andsoil:water extracts [33] and also organisms' body, using Atomic Absorption Spectrometer (AAS, Perkin Elmer 4100, Ueberlingen, Germany). For soil samples, 1 g dry weight was digested using 65% HNO3 and heated to 120 ºC until all brown fumes disappear.

Co concentrations in soil:water extracts were measured at days 0, 0.13, 1, 3, 7, 14, 21 and 28 in a concurrent experiment as described in [33]. The supernatant of a soil:water solution (1:5) was mixed for 5 min at 250 rpm (lab shaker) and then settled for 2h. After 1 mL of the solution was digested using the same procedure as in soil. To measure Co concentrations in organisms these were dried at 80ºC and then weighted. The digestion was like previously described. Before the measurements, all samples were re-suspended in 2% HNO_3. Results are reported in Ribeiro et al [19].

2.5 Epigenetic analysis

2.5.1 DNA extraction

For each replicate genomic DNA was extracted from a pool of 100 juveniles. Three biological replicates were included. The procedures for the Wizard® Genomic DNA Purification Kit (Promega) were according with manufacture´s protocol. DNA quantity and purity was measured (NanoDrop ND-1000 Spectrophotometer).

2.5.2 Global DNA methylation

Global DNA methylation analysis was performed following the procedures as described in Bicho et al [15]. In short, measurements were done using Liquid Chromatography-Mass

Spectrometry (LC-MS). Genomic DNA samples were diluted (200 ng in 10 µL of nuclease free water) and the digestion of DNA samples into deoxyribonucleosides was performed with the addition of a digestion solution (10 µL). DNA samples were left to incubate at 37 ºC for 6 h. Further it was added to the digested product (20 µL) ultrapure water (80 µL) and the internal standard solution (100 µL). Samples were injected and detected in an Agilent 6420 Triple Quadrupole LC-MS system contained in HPLC vials. Single deoxynucleosides were divided in a column Agilent ZORBAX Eclipse Plus C18 (2.1 · 100 mm, 1.8 µM). The mobile phase had the following compounds: 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in methanol (B). The gradient for the mobile phase started at 5% B, was increased to 15% B in 3 min, maintained at this composition for 1 min and decreased to 5% B in 10 min. For the LC-MS run the following source conditions were applied: nebulizer gas pressure fixed to 40 psi; drying gas (nitrogen) flow rate at 10 L/min and drying gas temperature of 350 °C. Scanning of deoxynucleosides was done using a multiple reaction monitoring with a dwell time of 50 ms per compound. For the protocol validation and for the DNA sample quantification, the mass transitions of compounds were: 5mdC 242.1/126.1, D3mdC 245.1/129.1, dG 268.1/152.2, C10N5dG 282/162.2. The collision energy was of 8 V for 5mdC and D3mdC, whereas for dG and C10N5dG was of 4 V. The standard curve included increasing amounts of 5mdC (0-2.07 µM, 0 % - 3 %) to a fixed amount of G (345 nM). The software Agilent MassHunter Quantitative Data Analysis was used. 2.6 Data analysis

For the results on global DNA methylation, t-test and two-way analysis of variance (ANOVA) ($p \leq 0.05$) were performed, the two independent variables were the concentrations and exposure time (Fx generation) for the significance of variables and their interaction [34]. Linear regression analysis was used between methylation and reproduction data [34].

3. Results

Results from global DNA methylation across multiple generations (fig. 1) showed no significant differences between concentration, exposure time (generation) and the interaction between the two factors (two-way ANOVA). For WCCo NMs exposure there was an increase in DNA methylation from F1 to F3 [for the EC_{10} (p=0.04) and EC_{50} (p=0.006, here an outlier in F1 was removed], after which it remained generally elevated, including the after transfer to clean soil. For the F3 to F8 period there was a significant (P=0.03) linear regression between

DNA methylation and number of juveniles for EC_{10} (juveniles = -2.90 DNA-Methylation + 411; R^2=0.76). For $CoCl_2$ results presented higher variability and no clear pattern.

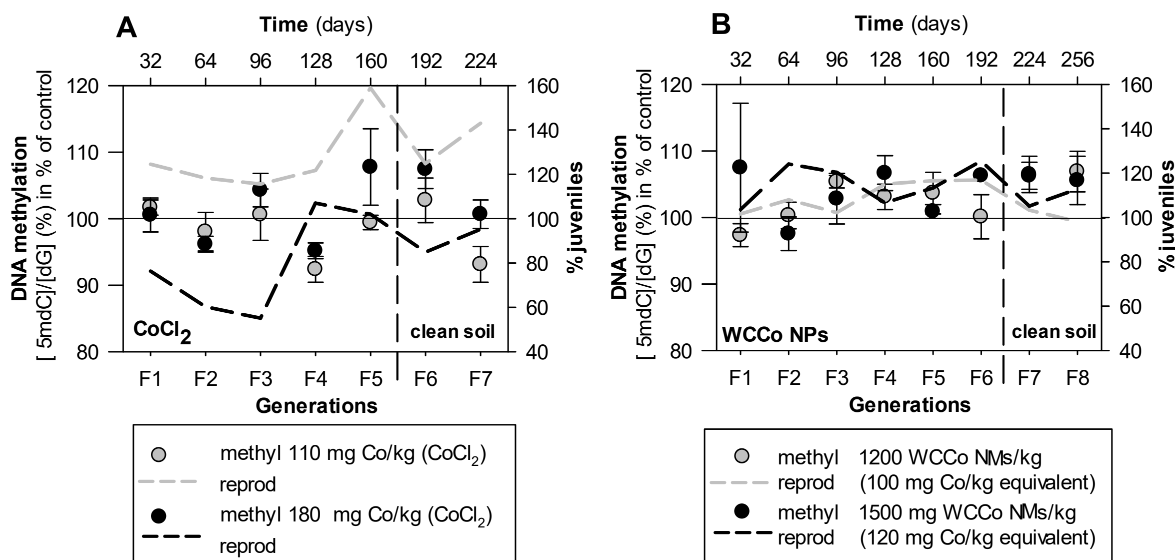

Figure 1. Global DNA methylation (LC-MS measurements) for *Enchytraeus crypticus* after multigenerational exposure to the reproduction EC_{10} and EC_{50} (and control samples) of A) $CoCl_2$ (0–110–180 mg Co/kg soil DW) and B) WCCo NMs (0–1200–1500 mg WCCo NMs/kg soil DW, 0–100–120 mg Co/kg equivalent) in LUFA 2.2 soil. All values are expressed as average ± standard error (Av±SE).

Results on the apical endpoints survival and reproduction (dashed plotted lines, Fig. 1), as well as measurements on internal Co body concentrations in E. crypticus were previously reported in Ribeiro et al [19].

4. Discussion

In summary, the effects at the organism level were observed on survival for $CoCl_2$ but not for WCCo. Cobalt caused a significant decrease on reproduction at 180mg Co/kg soil D.W. (ca. EC_{50}) [33], and this toxicity was fast increasing to lethal levels thereafter (LC50=260mg Co/kg soil D.W.). The analysis of $CoCl_2$ spiked soil showed relatively higher Co in solution in higher concentrations, i.e. more Co than the equivalent relative difference between concentrations [33] which explains the observed survival and reproduction effects. Cobalt is also well-known as an oxidative stress agent, hence, the effects (survival and reproduction) could indicate the irreversible impairment of antioxidant strategies, this for higher

concentrations. For WCCo, toxicity was shown to be due to a combined effect between WC and Co and the impact was clearly observed with increased exposure time, i.e. with twice the standard exposure period (56 days instead of 28). To assess the impact along generations the exposure was obviously at sub-lethal concentrations. Reproduction increased at lower concentrations of Co. Internal body measurements showed Co uptake from exposure to both CoC_{l2} and WCCo, but no toxicity occurred for WCCo; further Co from $CoCl_2$ exposure was apparently stored while for WCCo it was eliminated [19]. Results for the MG exposure to WCCo NMs showed an increase in reproduction along generations. A similar pattern was observed for $CoCl_2$ [19].

For WCCo NMs multigenerational exposure, the increased DNA methylation from F3 was inversely correlated with effects at reproduction level at the EC_{10} level. Since this increase methylation level continued in the clean media generations, this indicates a transgenerational effect. Although this is significant, this observation obviously calls for further studies, partly to wider confirm this and to identify the mechanism behind, e.g. what exact mechanism and specific methylation causes this change. We also assessed whether there was a better correlation between the DNA methylation of the Fn with the juvenile number of the Fn+1 generation, to see if methylation mainly caused an effect on the subsequent generation, but this was not the case. Bicho et al [15] who studied the MG effects of CuO NMs exposure also observed a pattern of increase for global DNA methylation levels, which corresponded with the phenotypic effects on reproduction, further indicating transgenerational effects. Epigenetic changes can lead to stress adaptation with consequences for population dynamics [35,36] causing a change in populations. We observed no similarity in the methylation pattern between the $CoCl_2$ exposure and the WCCo NMs, possibly due to difference in induction mechanisms by the two materials or because Co uptake patterns were different. Cobalt bioaccumulated in the $CoCl_2$ [33] exposure. Cobalt is an essential element and this could explain the increase in reproduction for the lowest $CoCl_2$ exposure concentration, hence its beneficial effects at lower concentrations [19]. For WCCo NMs this is much less clear. Uptake studies for CoCl2 indicated that it was bioaccumulated and apparently stored, as levels remain stable (ca. 120mg Co/kg body D.W.) and high even after transfer to clean soil [19]. For WCCo NMs exposure, Co accumulation increased along generations, up till F3, after which it started to decrease, similar to a detoxification activation mechanism process, with a complete elimination when transferred to clean soil. The observed decrease in the impact on reproduction could be associated with the observed epigenetic shift. Whether the

activated mechanisms were led by epigenetics remains uncertain and requires further evidences for CoCl2, but for WCCo NMs the epigenetic change remained in the absence of Co, hence was transferred between generations.

Hence, there is an indication that WCCo NMs in general cause more (and more consistent) methylation than $CoCl_2$. There was an increase after the F3 generation, although a linear correlation was observed at the EC_{10} level, there was not a clear 1:1 correlation between methylation and reproduction. Most studies done are in vertebrates, for instance, in humans, a correlation has been shown between reproductive history and DNA methylation [37]. However, for invertebrates, studies are very scarce, and to our knowledge the present study and the one performed with exposure to CuO NMs [15] are so far the only ones providing evidence of epigenetics (global DNA methylation) linked to impact on reproduction.

In summary, the conclusion from this study is that it supports the importance of assessing longer term effects of (nano)materials in the environment. We here studied global DNA methylation in standard soil invertebrate *Enchytraeus crypticus* exposed to WCCo nanomaterials (NMs)) and to cobalt ($CoCl_2$) over multiple generations showing that MG exposure to WCCo NMs caused global DNA methylation to increase, which continued in unexposed generations and was associated with increase in reproduction (phenotypic effect). WCCo NMs caused in general more (and more consistent) methylation than $CoCl_2$. The next interesting steps will obviously be to identify precisely where on the genome such methylation takes place and at which exact life cycle stage(s) this happens. Further, it seems obvious that such a methylation does not only comply to this species. Hence, studies with other species are also required, this especially because from a risk assessment perspective we wish to know whether this is a general phenomenon potentially affecting many invertebrate species.

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Chapter IX - Developing an epigenetics model species - from blastula to mature adult, life cycle methylation profile of *Enchytraeus crypticus* (Oligochaete)

Rita C. Bicho[1], Janeck J. Scott-Fordsmand[2], Mónica J.B. Amorim[1*]

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

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

*corresponding author: mjamorim@ua.pt

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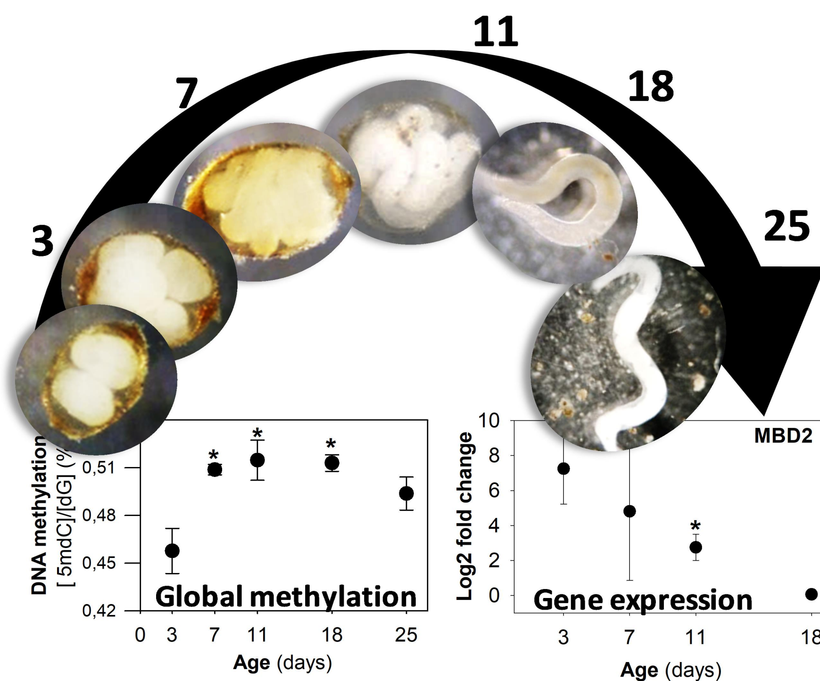
Abstract

DNA methylation is an epigenetic mechanism of particular importance in developmental biology, but methylation also varies along organisms' life cycle. Recent studies have deliberated copper (Cu) exposure induced epigenetic changes in *Enchytraeus crypticus*, a standard species belonging to one of the most common and important genera of soil invertebrates in many ecosystems. There is however no information on how DNA methylation levels change within the life cycle of this species. We here investigate the global DNA methylation profile along the life cycle of *E. crypticus* and compare this to the expression of target genes involved in methylation. Results showed that after the lowest DNA methylation level at day 3 (early embryonic stage, blastula) there was an increase by day 7 (organogenesis) after which levels were maintained at days 11, 18 and 25. DNA methyltransferase associated protein 1 (DMPA1) and Methyl Binding Domain 2 (MBD2) gene expression was highest during embryo stages (3 to 7 days), then decreasing (11, 18 days) and finally unregulated in adults (25 days). Hence, we here show that DNA methylation in *E. crypticus* changes among the different life stages, from cocoons to adults. Such

information is a key knowledge to use this endpoint and tool in an ecotoxicology context. This means that it is almost implicit that gene expression levels is age specific for a given stressor. It seems logic to recommend to always compare individuals with the same age between treatments, and to be careful when extrapolating results among life stages. Once, we understand more of these effects we may even be able to predict which life stage is more sensitive to specific stressors. An experimental design that aims to cover epigenetics of stressors in a multigenerational exposure, including transgenerational effects, should ensure the synchronous age of organisms for sampling analysis purposes.

Key words: Epigenetics; Life stages; Development; Risk assessment; Transgenerational effects;

TOC



Introduction

Epigenetic mechanisms are vital in most biological systems, playing a key role in gene regulation, not only transcriptional but also post-transcriptional level, being involved in

translational and post-translational changes (Moosavi and Ardekani, 2016). Epigenetic mechanisms and functions are well known in vertebrate developmental biology (Skinner, 2011), e.g. for tissue/organ differentiation (Sarmento et al., 2004; Skinner, 2011; Smolkova et al., 2015). It is also known, and gained particular attention, as a mechanism induced by environmental stressors (Halušková, 2010). There has been an increasing body of evidence showing that chemical stressors induce epigenetic changes, hence the relevancy of including this endpoint in environmental risk assessment (ERA) (Bahadori et al., 2016). Nevertheless, epigenetic studies with environmental relevant species are very scarce in the literature if compared to humans and other vertebrates (Chatterjee et al., 2018). So far, available studies show that environmental stressors can regulate epigenetic mechanisms causing phenotypic plasticity in organisms, both for vertebrates (Dolinoy, 2008) and invertebrates (Kucharski et al., 2008). For invertebrates, epigenetic changes have been observed from exposure to metals (Kille et al., 2013; Santoyo et al., 2011; Šrut et al., 2017; Sussarellu et al., 2018; Vandegehuchte et al., 2009), endocrine disruptors (Lee et al., 2018; Novo et al., 2018) and nanomaterials (Bicho et al 2020). For vertebrates like fish, examples include epigenetics due to exposure to endocrine disruptors (González-Rojo et al., 2019; Olsvik et al., 2014) and pesticides (Bachère et al., 2017).

The importance of epigenetic studies in the field of ecotoxicology relates to the identification of previous exposure to contaminants (Chatterjee et al., 2018; Mirbahai and Chipman, 2014) and the knowledge on organisms' adaptive responses to their environment over long periods of time (Chatterjee et al., 2018). Epigenetic changes are associated to long term exposure, through multigenerational, intergenerational and transgenerational effects (Barouki et al., 2018). For example, the occurrence of intergenerational inheritance of DNA methylation signatures is still unclear in humans (Barouki et al., 2018; Stenz et al., 2018) and other mammals (Lesch et al., 2019) and in molluscs (Fallet et al., 2020), although it was shown in

fish (*Oncorhynchus tshawytscha*) (Venney et al., 2020) and in corals (*Platygyra daedalea*) (Liew et al., 2020). Epigenetic changes can negatively affect populations, but can also lead to stress adaptation, causing an increase in populations or contributing for their evolution (Vandegehuchte and Janssen, 2011). Parental effects can affect offspring fitness and phenotype, which can cause changes at the population level (Venney et al., 2020).

DNA methylation has a particular role in development, namely via transposon silencing and gene repression (Bird, 2002; Hackett and Surani, 2012). For example, the DNA methylation pattern is believed to be `read' by a conserved family of proteins, the MBD (Methyl Binding Domain) family. Hence, these MBD proteins are important in connection with methylation. For example, MBD2 has a high affinity to bind to methylated DNA and regulates transcriptional repression (Fatemi and Wade, 2006). Other proteins like the DNA methyltransferase associated protein 1 (DMAP1) interacts with DNA methyltransferases (DNMTs) proteins which favours DNA methylation (Lee et al., 2010). For example, DNMT3 is responsible for de novo methylation, while DNMT1 maintains the DNA levels of methylation during DNA replication (Moore et al., 2013).

Further, DNA methylation levels and the associated gene expression levels are known to vary along organisms' life cycle. For vertebrates, in the case of humans' DNA, de-/methylation levels are dynamic during the first year of life (Wikenius et al., 2019), and later from 2 to 10 years of age these levels show a general increase (Gervin et al., 2016). Also in mouse models it is shown that DNA methylation levels are particularly dynamic during embryo development and directly after birth (Song et al., 2009). In fish (*Danio rerio*), it was shown that methylation present their lowest levels during early life stages and increase along with later developmental stages (Mhanni and McGowan, 2004). For invertebrates, examples include the case of the fly *Drosophila melanogaster*, where DNA methylation levels are much higher during embryonic stage than in adults' stage (Lyko, 2001). In the case of ants

(*Camponotus floridanus* and *Harpegnathos saltator*) levels were similar in all life stages (Bonasio et al., 2012). For the bivalve *Crassostrea gigas* it was shown that gene expression levels of target genes involved in DNA methylation are life stage related (Wang et al., 2014), being particularly high during embryo development, lowered during larval stage, and peaked again during juvenile and adult stage.

In the present study, the soil invertebrate standard model species *Enchytraeus crypticus* (ISO, 2014; OECD, 2004a) was used. Enchytraeids play a key role in maintaining soil ecosystems healthy, being involved in soil nutrient cycling (Hendrix et al., 1986; van Vliet et al., 1995) and food webs, besides their indirect action for the decomposition of organic matter (Didden, 1993; Briones and Ineson, 2002). These organisms have worldwide distribution (Pelosi and Römbke, 2018) being even among the most abundant in terms of biomass in many habitats (Hendrix et al., 1986; Pelosi and Römbke, 2016; Orgiazzi et al., 2015; Cragg, 1961). Recently we showed the existence of global DNA methylation in *E. crypticus* (Noordhoek et al., 2018). A follow up study on the multigenerational exposure showed that copper (Cu) materials induced epigenetic changes and indicate transgenerational effects (Bicho et al., 2020). Hence, with *E. crypticus* being a suitable candidate for epigenetic studies in this widely important group of annelids, especially in the context of environmental risk assessment, there is a need to gain further knowledge on the organism life cycle epigenetic dynamics. This information is also important to understand about epigenetic inheritance in enchytraeids.

This study aimed to investigate the global DNA methylation profile of *E. crypticus* within its life cycle (from early embryonic to adult stage). Further, we targeted the expression of specific genes (DMPA1 and MBD2) related to methylation.

2. Experimental

2.1 Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. Cultures are maintained for numerous years at the University of Aveiro, for more information see Bicho et al. (2015). Synchronized cultures were prepared as described (Bicho et al., 2015) and cocoons with 1-2 days after cocoon laying were used.

2.2 Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics are described as follows: pH (0.01 M $CaCl_2$) of 5.5, 1.61 % organic matter, 10.0 meq/100 g CEC (cation exchange capacity), 43.3 % WHC (water holding capacity), grain size distribution 7.9 % clay, 16.3 % silt, and 75.8 % sand.

2.3 Test procedures

The test procedures were performed based on the embryotoxicity test (Gonçalves et al., 2015), with due adaptations. In short, test started with synchronized (1-2 days old) cocoons. For the embryonic stage samplings (from blastula to organogenesis), cocoons were selected and introduced [this is done with the help of a paintbrush and cocoons are carefully covered by soil] in 6-well plates with 5 g of soil in each well. Cocoons (n = 300 per sampling day) were sampled after 3 and 7 days. For all other developmental stages, cocoons were introduced, with the help of a paintbrush and carefully covered by soil, in test vessels with 5 g of soil, to sample juveniles at days 11 and 18 (n = 300 start cocoons) and adults at day 25 (n = 20 start cocoons). Four replicates per sampling day were used. At each sampling day the organisms (cocoons, juveniles or adults) were carefully transferred from soil to a Petri dish with reconstituted water (OECD, 2004b) to remove the soil particles, snap-frozen in liquid nitrogen and kept at -80 ºC until further analysis. Due to the large amount of organisms to

synchronize (17000) and cocoons required (6800), the test had to be performed twice to have enough samples for both the global DNA methylation and gene expression analysis.

2.4 Global DNA methylation analysis

2.4.1 *DNA extraction*

Genomic DNA was extracted from each replicate with 100 pooled juveniles. Three biological replicates per treatment were used. The Wizard® Genomic DNA Purification Kit (Promega) was used according with manufacturer´s protocol. The quantity and purity of isolated DNA was measured with a NanoDrop ND-1000 Spectrophotometer.

2.4.2 *Global DNA methylation*

Global DNA methylation analysis was performed as described in (Bicho et al., 2020). In short, Liquid Chromatography-Mass Spectrometry (LC-MS) was used. Genomic DNA was digested to deoxyribonucleosides, adding digestion solution (10 µL) to the DNA samples (200 ng in 10 µL) and left to incubate at 37 ºC for 6 h. Ultrapure water (80 µL) plus the internal standard solution (100 µL) was added to the digested product (20 µL). Samples were transferred to HPLC vials and injected and detected in an Agilent 6420 Triple Quadrupole LC/MS system. Single deoxynucleosides were separated with an Agilent ZORBAX Eclipse Plus C18 column (2.1 · 100 mm, 1.8 µM). For method validation and DNA sample quantification compounds mass transitions were: 5mdC 242.1/126.1, D3mdC 245.1/129.1, dG 268.1/152.2, C10N5dG 282/162.2. A collision energy of 8 V was used for 5mdC and D3mdC, whereas for dG and C10N5dG a collision energy of 4 V was used. A standard curve with increasing amounts of 5mdC (0-2.07 µM, 0 % - 3 %) was included against a fixed amount of G (345 nM). Data analysis was performed with Agilent MassHunter Quantitative Data Analysis.

2.5 Gene expression analysis

2.5.1 *RNA extraction*

RNA was extracted from a pool of organisms in each replicate: 300 cocoons (embryonic development), 300 juveniles and 20 adults. Three biological replicates per treatment were done. The SV Total RNA Isolation System (Promega) (according to manufacturer's protocol) was used. The quantity and purity of the isolated RNA were measured with nanodrop (NanoDrop ND-1000 Spectrophotometer), and its integrity was checked on a denaturing formaldehyde agarose gel electrophoresis.

2.5.2 *Quantitative real-time PCR (qPCR)*

Gene targets were Methyl-CpG-binding domain protein 2 (MBD2) and DNA methyltransferase 1-associated protein 1 (DMAP1) as retrieved through homology from the *E. crypticus* library (Castro-Ferreira et al., 2014). Actin was selected as housekeeping gene being its suitability confirmed in all treatments (i.e. variation of less than 2 cycles in treated samples). For primers sequences and efficiency please see information in Bicho et al. (2020). To perform qPCR, the total RNA (0.5 µg) from samples was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was diluted 4X and 2 µL were used in 20 µL PCR reaction volumes containing 2 µL of forward and reverse primers (5 µM), 10 mL of SsoFast EvaGreen Supermix (Bio-Rad) and 4 mL of nuclease free water. Each replicate was applied in triplicate. For each qPCR plate non-template control (NTC) was added in duplicate, were nuclease free water was used instead of cDNA. Amplification was performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad). Reaction conditions consisted of one denaturation step at 95ºC for 30 sec, further 40 cycles at 95 ºC for 5 sec and 60 ºC for 10 sec.

2.6 Data analysis

For the global DNA methylation data, analysis of variance (ANOVA) was used followed by Dunnets' comparison post-hoc test ($p \leq 0.05$), where the independent variable was time (SigmaPlot, 1997). For gene expression data, the mean normalized expression value was calculated from the obtained cycle threshold (Ct) values. The 25 days adults were used as the control treatment , since this is the adult stage often used as a reference in experiments in gene expression studies. Statistical differences were assessed using the Relative Expression Software Tool (REST-MSC).

3. Results

3.1 Global DNA methylation

Global DNA methylation levels in *E. crypticus* during its life cycle (see figure 1) showed the lowest point at day 3, i.e. the very early embryonic stage (cocoons with an age of 3 days), after which it increases significantly at day 7 (still an embryo stage, before hatching), and then remaining similar at days 11 and 18 for juveniles, i.e. after hatching, with a slight decrease by day 25 (adult and mature stage).

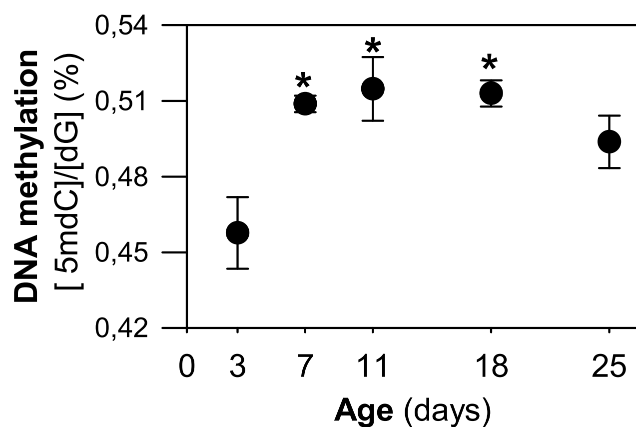


Figure 1: Results in terms of global DNA methylation (LC-MS measurements) in *Enchytraeus crypticus* life cycle when in LUFA 2.2 soil at various sampling days: 3, 7

(cocoons), 11, 18 (juveniles) and 25 (adults). All values are expressed as average ± standard error (Av±SE). *P<0.05 (Dunnets', compared to day 3).

3.2 Gene expression

Gene expression showed up regulation of target genes DMAP1 and MBD2 (figure 2), both with similar pattern, i.e. reduced upregulation with time increase. Embryonic stages (3 and 7 days) had significantly higher upregulation compared to adults (25 days).

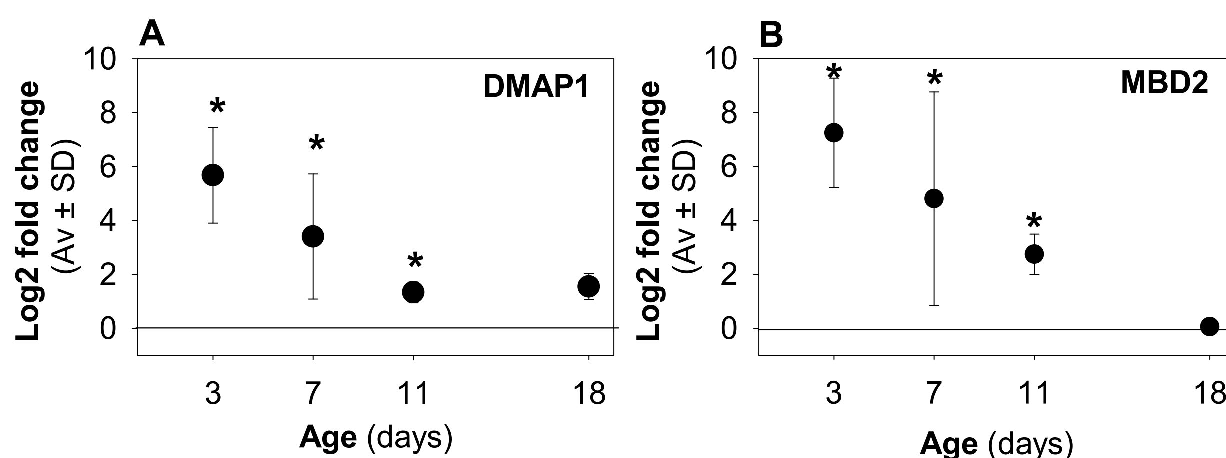


Figure 2: Quantitative gene expression (RT-qPCR) in *Enchytraeus crypticus* when in LUFA 2.2 soil during its life cycle at various sampling days: 3, 7 (cocoons), 11 and 18 (juveniles) [all compared to the control treatment at day 25 (adults)]. All values are expressed as $\log_2$ ratio [fold change relative to control (25 days' adults)], average ± standard error (Av±SE). * (p<0.05). A) DMAP1: DNA methyltransferase 1-associated protein 1 and B) MBD2: Methyl-CpG-binding domain protein 2.

4. Discussion

The assessment of stress related physiological impact cannot really be understood unless the mechanism behind is understood. The mechanism of the effect is initiated in the gene responses, which leads to subsequent protein induction, pathways, etc.. It is no secret that gene responses are tightly regulated by epigenetic mechanisms (Skinner, 2011), and it is

implicit that epigenetics response to stressors cannot be achieved without understanding its normal operating range within the life cycle stages. This is what we provide in this study. This represents one of the first steps in establishing a standard ecotoxicological organism with epigenetic tools, here covering the epigenetic profiling along the life cycle. This standard organism belongs to one of the most numerous and important invertebrate genera in many terrestrial ecosystems (Pelosi and Römbke, 2018), highlighting the ecological relevance for ecotoxicology and ERA.

Lowest levels of global DNA methylation were observed during the earliest life stage (3 days, blastula), which increased right after, during embryonic stage (7 days, organogenesis), and tended to stabilize from then onwards in juveniles and adults' stage. In a similar study, during the life cycle of the fly *D. melanogaster* (Lyko, 2001), the opposite was observed, i.e. highest levels in embryonic stage decreasing in the following developmental stages (from larva to adult). For example, for ants (*Camponotus floridanus* and *Harpegnathos saltator*) (Bonasio et al., 2012), DNA methylation levels were equivalent in all life stages. For *D. rerio* it is described that in early life stages there is a dramatically decrease in methylation (Mhanni and McGowan, 2004) and further de novo methylation occurs to restore methylation levels like in adults' stage (Mhanni and McGowan, 2004; MacKay et al., 2007). Hence, as observed so far, epigenetics mechanisms appear to differ substantially between species.

The relation between DNA methylation, effects on reproduction and its subsequent transfer to the next generation, as we studied for E. crypticus, is not well understood. A recent study with the fish species *O. tshawytscha* showed that despite the variability in methylation profiles, DNA methylation is one of the mechanisms responsible for the transmission of maternal effects to offspring generations (Venney et al., 2020). Hence, at least on a case by case, DNA methylation can hold a far large architecture of the effects that are observed in organisms and populations.

Few studies cover the full life cycle and focus is usually on DNA methylation levels for specific genes, showing large variability during embryo stage (e.g. for humans (Wikenius et al., 2019) or mouse (Song et al., 2009)). Interestingly, gene expression results for *E. crypticus* DNA methyltransferase associated protein 1 (DMPA1) and Methyl Binding Domain 2 (MBD2) showed the highest regulation at day 3, decreasing already at day 7 and then onwards till adult's stage (25 days), i.e. the opposite pattern of global DNA methylation. Given that both DMPA1 and MBD2 are involved in this epigenetic mechanism, it was expected a positive correlation with the global DNA methylation, as observed for *D. melanogaster* with DNA methyltransferase 2 (DNMT2) along the life cycle (Lyko, 2001). On the other hand, results in *E. crypticus* were in agreement with what was observed for the bivalve *C. gigas*, where the expression of MBD2 was higher during early embryonic development, and maintained lower during the remaining life stages (Wang et al., 2014).. The rational for these different observations is not clear at this stage, but could be because these genes are involved in specific mechanisms. In a multigenerational exposure to Cu materials (Bicho et al., 2020) the silencing of DNMT1 was observed in one of the generations while the level of global DNA methylation was maintained. Hence, DNMT1 can be involved in specific mechanisms not yet fully understood. Clearly, it is a fascinating topic that should be further investigated.

Enchytraeus crypticus DMPA1 and MBD2 gene regulation showed higher expression during embryo stages (3 to 7 days) decreasing after and reaching no variation level from juvenile to adult stage (18-25 days). Nevertheless, results here indicate that DNA methylation is very important during embryonic stages, but this mechanism is also maintained in the juvenile and adult stage. Hence, in the context of ecotoxicological testing and hazard assessment, one can opt for studying epigenetics in 1) one life stage, e.g. mature adults (25 days) as standard tests are performed with adults as a common reference, for comparison between treatments, or 2) a

time resolved setup, selecting a range of time points, e.g. 3, 7, 11, 18, 25 days of sampling, capturing variations that exist along the life cycle. Most of all it is important to clarify that DNA methylation in *E. crypticus* varies within its life stages, from cocoons to adults, hence comparisons should not be done between organisms of different age.

5. **Conclusions**

Enchytraeus crypticus DNA methylation varies within its life stages, being lowest in day 3 (cocoons) increasing at day 7 (organogenesis) and then stabilize from days 11-18 (juveniles) and 25 (adults). This knowledge is key for the future usage in ecotoxicology, adding epigenetics as an endpoint with an optimized tool. It seems logic to recommend to always compare individuals with the same age between treatments, and to be careful when extrapolating results among life stages. An experimental design that aims to cover epigenetics of stressors in a multigenerational exposure, including transgenerational effects, should ensure the synchronous age of organisms for sampling analysis purposes.

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Chapter X - General discussion

Life cycle tests have long been suggested as important ecotoxicity tests since they provide much more information than the usual standard tests, and allow to extrapolate organisms responses at the population level (Jager et al., 2004). However to today there is only one standardized life cycle test with the genus *Chironomus* (OECD, 2010). In chapter II a full life cycle test (FLCt) for the model soil invertebrate *Enchytraeus crypticus* was developed and optimized and this was a positive contribution for terrestrial ecotoxicology since it covers effects in all life stages of this organism. This test evaluates additional endpoints to the existing standard tests (ISO 16387, 2014; OECD, 2016a), so besides survival and reproduction, the FLCt evaluates: hatching success, growth and maturity status, population growth and allows data modelling for other population endpoints. To our knowledge, additionally to *E. crypticus* FLCt, with soil invertebrates, the literature shows only life cycle studies with the Collembola *Folsomia candida* (Guimarães et al., 2019; Jager et al., 2004). Another important addition to the enchytraeid standard test was the development of synchronized cultures. In the current standard test guideline it is suggested to start with adults from cultures. This brings much variability to the test, since like it was shown in Gonçalves et al (2017), along *E. crypticus* lifespan the reproductive output starts to decrease approximately after 180 days of age. This procedure of standardizing tests to start with organisms with synchronised age, is already common with other soil invertebrates like the *F. candida* (OECD, 2016b). The work developed in chapter II was indeed a stepping stone for this thesis. The use of synchronized cultures was an important improvement present in the remaining chapters, further the recurrence of the FLCt with other test substances like is depicted in chapters III, IV and VI only reinforces it´s reproducibility.

In chapters III and IV it was possible to confirm the utility of the FLCt for the evaluation of effects in long term exposures to nanomaterials (NMs). In both chapters it was possible to discriminate effects between life stages, and to observe different organisms' responses between nano and ionic form. For example in chapter III both exposure to silver (Ag) NMs and Ag ionic caused an effect during the embryonic stage, but effects were irreversible to Ag NMs (Bicho et al., 2016). In chapter IV the work done showed that between copper oxide (CuO) NMs and Cu ionic the effects are life stage dependent (Bicho et al., 2017a). Hence for CuO NMs the effects were mainly during juvenile growth and for Cu ionic effects were during embryonic stages. These studies contributed for mechanistic information that is much needed to better understand the effects of NMs, like suggested by (Dekkers et al., 2016;

Lamon et al., 2019; Scott-fordsmand et al., 2018). Further effects were not predictable by the performance of shorter assays like the standard enchytraeid test. This is in agreement with other authors that have also observed that long term exposures are needed to evaluate effects of NMs (Diez-Ortiz et al., 2015; Lead et al., 2018).

Numerous multigenerational (MG) and transgenerational effects are shown in the literature caused by several classes of compounds including endocrine disruptors (Massarin et al., 2010), non-essential metals (Anway and Skinner, 2006), pesticides (McCarrey et al., 2016; Paoloni-Giacobino, 2014) and NMs (Arndt et al., 2014; Kim et al., 2013; Schultz et al., 2016; Völker et al., 2013). All these evidences have made clear that short term assays are not enough to evaluate the effects of stressors. Hence for the improvement of hazard and environmental risk assessment (ERA) long term toxicity data is needed and MG studies are of particular importance. Particularly for the terrestrial compartment there is a big gap of such studies. There is however an interesting example with the soil invertebrate *F. candida*, which showed a scenario of increased sensitivity and tolerance to low and higher concentrations of cadmium (Cd), respectively, over several generations (more than 40) (Amorim et al., 2017). This motivated the work presented in chapter V, the development of a MG test (MGt) with *E. crypticus*. This was the first MG study with a soil invertebrate exposed to NMs, using soil as exposure media (Bicho et al., 2017b). The design of the test allowed for the evaluation of effects during subsequent exposed generations, followed by unexposed generations to evaluate transgenerational effects. Further the test design included the evaluation of apical endpoints (survival and reproduction), presented in chapter V, and evaluation of molecular endpoints, presented in chapter VI. At the phenotype level (reproduction), sequential exposure to Cu resulted in: increased tolerance (ionic form) and increased sensitivity (nano form). Obviously these observations could never be predicted from short term assays, further they allowed to distinguish organisms´ responses between Cu forms, thus improving on hazard and ERA of NMs.

Previous MG studies with *Daphnia magna* and *Caenorhabditis elegans* NMs (Arndt et al., 2014; Schultz et al., 2016) and more recent ones with *C. elegans* (Wamucho et al., 2019) have suggested that epigenetics could explain the MG and transgenerational transfer of reproductive toxicity. Further there is continuous evidence that NMs can change epigenetic mechanisms (Gedda et al., 2019; Smolkova et al., 2019), with no exception for CuO NMs (Lu et al., 2015). Like previous mentioned, in Bicho et al (2017b) the transference of effects was observed for Cu nano and ionic forms. Hence it was clear that epigenetic effects should be

investigated. This work is presented in chapter VI. This study, Bicho et al (2020), clearly showed that Cu multigenerational exposure, both in nano and ionic form, changed epigenetic mechanisms, showed by the changes in gene expression of target genes, including in non-exposed generations. Moreover, for CuO NMs exposure the MG profile of global DNA methylation corresponded with phenotypic effects (reproduction). This reinforces that for Cu, epigenetic mechanisms may be involved in the transference of reproductive toxicity, and there is evidence of transgenerational effects. This study is an add on to the idea that, epigenetics can be a potential tool to identify former exposures to contaminants (Mirbahai and Chipman, 2014). Further it attests that *E. crypticus* is suitable candidate for epigenetic studies, which can be an important contribution for identification of chemicals and non-chemicals hazard and for ERA of the terrestrial compartment.

Additionally, the combination of epigenetic effects with effects at the several levels of biological organization, as framed in the adverse outcome pathway (AOP), is suggested has an efficient approach for the inclusion of epigenetic studies on the improvement of hazard and ERA (Angrish et al., 2018; Chatterjee et al., 2018; Goodman, 2017; Vandegehuchte and Janssen, 2014). However the literature shows a major knowledge gap on effects, including epigenetic effects, at the different levels of biological organization to integrate or organize toxicoepigenetics data in the AOP framework (Chatterjee et al., 2018). This is no less true in the case of studies with NMs. Nevertheless for Cu NMs and Cu ionic, on *E. crypticus*, there was a major effort to obtain such data and, as mentioned, part of such work was performed in the context of this thesis. At lower levels several studies are published, e.g. covering multi-omics and high-throughput transcriptomics (Castro-Ferreira et al., 2014; Gomes et al., 2018), metabolomics (Vera L Maria et al., 2018), proteomics (Vera L. Maria et al., 2018) and epigenetics (Bicho et al., 2020). At higher levels, e.g. covering the full life cycle (Bicho et al., 2015), the full life span test (Gonçalves et al., 2017), the effect over multi-generations (Bicho et al., 2017b). However information at the tissue/organ level was a missing link. This motivated the work presented in chapter VII. Indeed in this study, Bicho et al (n.d.), previously known effects of Cu (nano and ionic form), plus the new investigated effects at tissue/organ were integrated in order to organise this (eco)toxicoepigenetics data into the AOP framework. From this organization, it was possible to observe that between Cu forms, in terms of epigenetics mechanisms, there were differences for the molecular initiating event (MIE), but similar effects at intermediate key events (KEs), tissue/organ level. Regarding the general stress pathways, there was a common pathway affected and another particularly

affected by Cu ionic. All effects combined resulted in different organisms' responses at individual and population level, being more evident from the MG exposure. These observations highlight once more the importance of long term exposure and the possibility of transference of effects from generation to generation, further the importance of including epigenetics markers in ecotoxicology data.

In chapter VIII, Bicho et al (n.d.), during another MG study, with another case study the Nanostructured Tungsten Carbide Cobalt (WCCo) nanoparticles (NMs), interestingly, like previously reported for CuO NMs in chapter VI, also MG profile of global DNA methylation was linked with phenotypic effects (reproduction). This study brings more evidence on the importance of epigenetic mechanisms for the transference of reproductive toxicity along subsequent exposed generations and transgenerational effects.

Further on the inclusion of epigenetics on ecotoxicology data, one important aspect is to understand the baseline of the epigenetic status of the organism studied, in particular, the dynamics of epigenetic markers during organisms' life cycle. It is reported in the literature, that for DNA methylation such profiles are specific for different taxa. Examples found are for humans (Wikenius et al., 2019), mouse (Song et al., 2009), fish (Macleod et al., 1999), flies (Lyko, 2001), ants (Bonasio et al., 2012) and bivalves (Wang et al., 2014). Important in the field of soil ecotoxicology and ERA for the terrestrial compartment, the model species used in this thesis *E. crypticus*, showed to be a potential good candidate for epigenetic studies as well, in chapters VI, VII and VIII. Therefore in chapter IX, Bicho et al (n.d.), global DNA methylation profile along *E. crypticus* life cycle was studied and revealed like reported in the literature, the importance of DNA methylation in development (Bird, 2002; Hackett and Surani, 2012).

Conclusions

The main novelty that resulted from this thesis was the development of ecotoxicity assays for the soil compartment, which allow for the evaluation of long term effects of compounds. This proved to be of particular importance to understand the hazard of nanomaterials (NMs). For example in the case of metallic NMs it allowed to distinguish organisms` responses between NMs and the ionic form, and evidences indicate nanospecific effects. Further both full life cycle (FLC) and multigenerational (MG) exposures made possible the observation of effects that were not predictable with the performance of shorter assays like the standard tests. In particular, for the NMs studied here, the MG exposure showed the transfer of effects,

including transgenerational. The work done on epigenetics was a plus on to the body of evidences that NMs cause epigenetic changes. Additionally evidences indicated that epigenetic mechanisms are involved in the transference of effects. This reinforced the idea that epigenetics should be included in the field of ecotoxicology and further in environmental risk assessment (ERA). Finally the integration of (eco)toxicoepigenetics data, at the several levels of biological organization, into the adverse outcome pathway (AOP) framework proved to be very useful to distinguish different molecular initiating and key events, between NMs and the ionic form.

In sum the work done brings a positive contribution for the improvement of NMs hazard and ERA.

Future recommendations

It is recommended to continue the investigation of epigenetic mechanisms in *Enchytraeus crypticus*. Namely, about the specific role of the different enzymes involved in DNA methylation used as epigenetic markers. Further to investigate more in deep about the other mechanisms of epigenetics. On the other hand, it would be important to better understand the link between global DNA methylation and reproductive toxicity and the mechanisms behind.

Additionally in order to increase more realism into the results, it could be interesting to adapt these long term exposures to NMs within a mesocosm system.

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