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Confirmatory assays for transient changes of omics in soil invertebrates - copper materials in a multigenerational exposure

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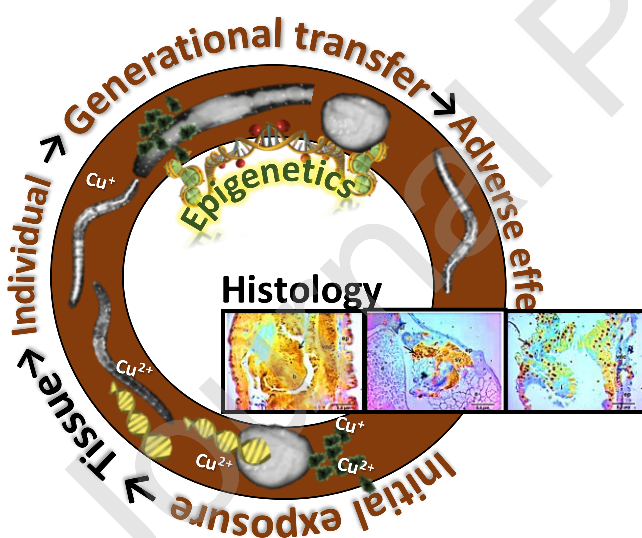

Highlights

- Organisms' tissues sampled along full life cycle and multigenerational exposure to Cu.
- Immunohistochemistry confirmed genetics and epigenetics effects.

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- Differentiated molecular initiating events between Cu NM and $CuCl_2$.
- Time series exposure allowed to collect time of events for building AOPs.

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, 46 and 224 days) 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;

1. 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

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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 three examples of draft AOPs for Cu NMs in soil (Bicho et al., 2017a; Gomes et al., 2018; Gomes et al., 2019) using *Enchytraeus crypticus* (Oligochaeta). Enchytraeids have a worldwide distribution (Pelosi and Römbke, 2018) being one of the most abundant taxa in several habitats (Hendrix et al., 1986; Pelosi and Römbke, 2016; Orgiazzi et al., 2015). These organisms are vital for soil ecosystems health, having an important role in soil nutrient cycling (Hendrix et al., 1986; van Vliet et al., 1995) and on the decomposition of organic matter (Didden, 1993; Briones and Ineson, 2002). For this species the traditional endpoints survival, reproduction and bioaccumulation are assessed within standardised guidelines (ISO, 2004; OECD, 2010, 2016). 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

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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. On the further relevancy of studying Cu NMs, these are widely used as wood-preservatives (Evans et al., 2008) and in many agricultural practices for example as fungicides (Gogos et al., 2012; Weitz et al., 2015), as fertilizers and additives for soil remediation, or growth regulators in plants (Zhu et al., 2012). Hence, the terrestrial compartment is a major sink, also for Cu NMs (Keller et al., 2013; Anjum et al., 2015).

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. We selected specific epigenetic and stress markers based on our previous study (Bicho et al., 2020): to target epigenetic changes, DNA methylation, histone modifications and miRNA immunostaining was performed for 5-methylcytosine, (5 mC), 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.

2. Material and Methods

2.1 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.

2.2 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.

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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.

2.3 *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).

2.4 Test procedures

2.4.1 *Apical endpoints*

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

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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). In short, 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 in order to correspond to the FLCt design. 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.

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

Exposure followed the standard guideline (OECD, 2016) 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).

2.4.3 *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 5 mC, 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 5 mC 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].

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2.5 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.

Adverse Outcome Pathway (AOP) was built based on the OECD guideline (OECD, 2018) including the provided templates format. Available AOPs were consulted (AOPWiki) to include established pathway key events when possible.

3. Results

All tests (FLCt and MGt) fulfilled the validity criteria described for the standard test (OECD, 2016), 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.

3.1 *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 $CuCl_2$

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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.

3.2 *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).

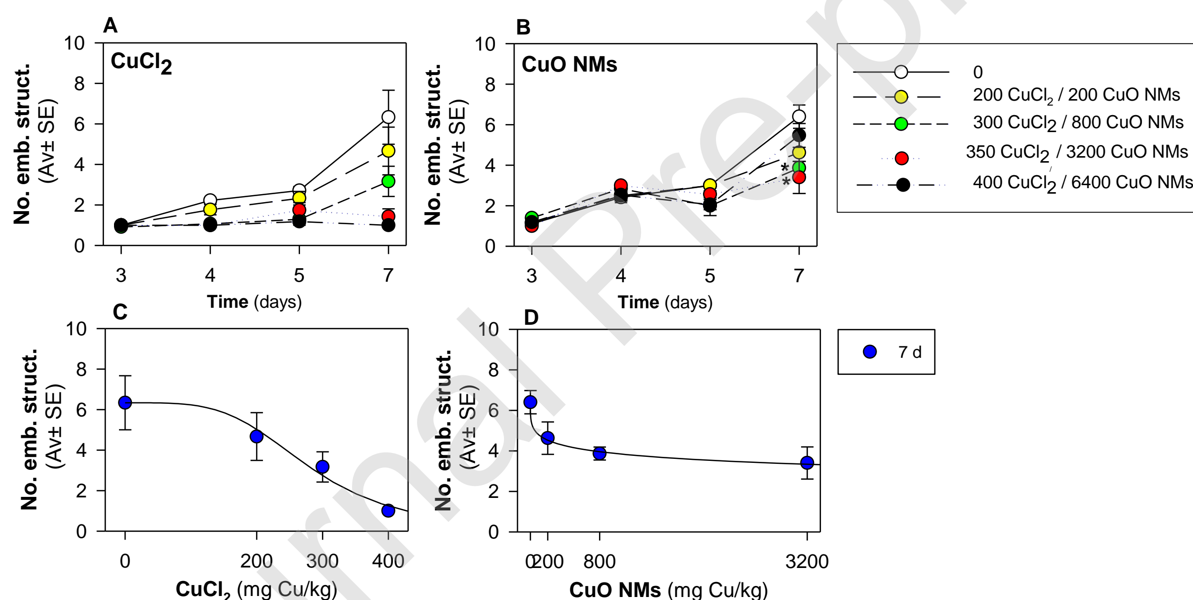

Figure 1: Number of embryonic structures of *Enchytraeus crypticus* in LUFA 2.2 soil during a full life cycle test (FLCt) after 3, 4, 5 and 7 days when exposed to A) $CuCl_2$ and B) CuO NMs (mg Cu/kg DW soil) and dose response results at day 7 (C and D). Line represents the model fit to the data. All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

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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.

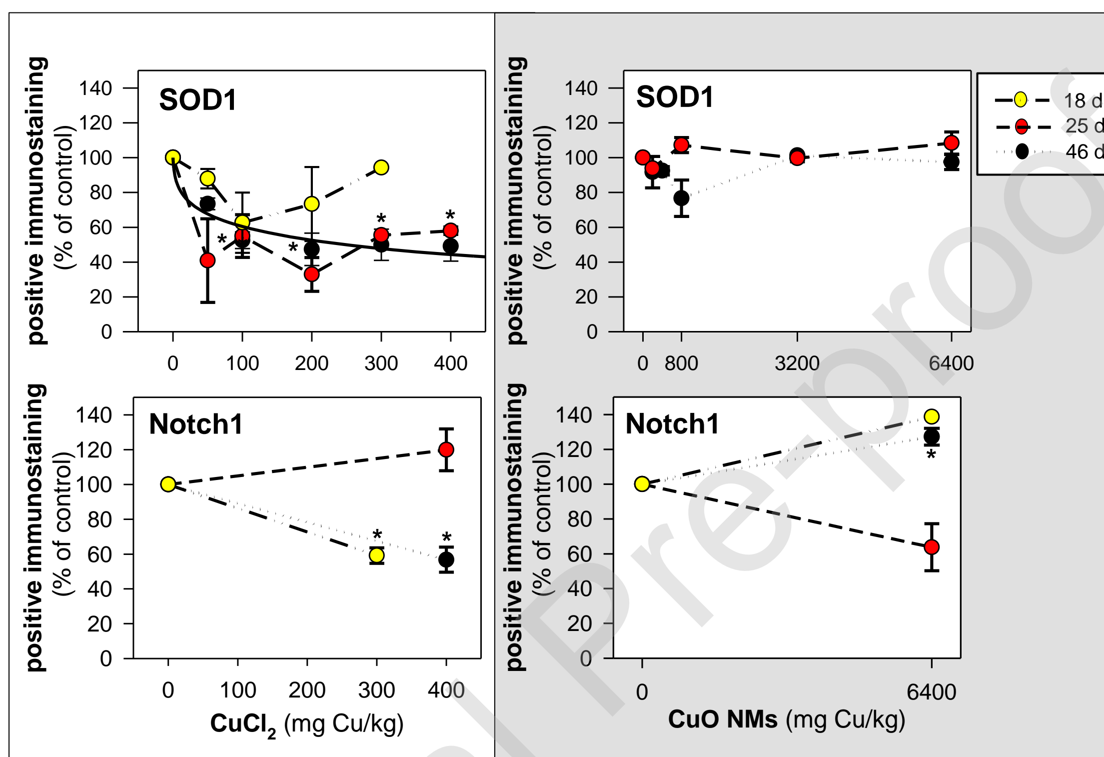

Figure 2: Immunohistochemistry quantification analysis of *Enchytraeus crypticus* when exposed to $CuCl_2$ and CuO NMs (mg Cu/kg DW soil) in LUFA 2.2 soil during the full life cycle test (FLCt). Organisms' covered ages: 18 (juveniles), 25 (mature) and 46 (adults post reproductive cycle) days. All values 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 5 mC was affected by both Cu forms, concentrations and across generations.

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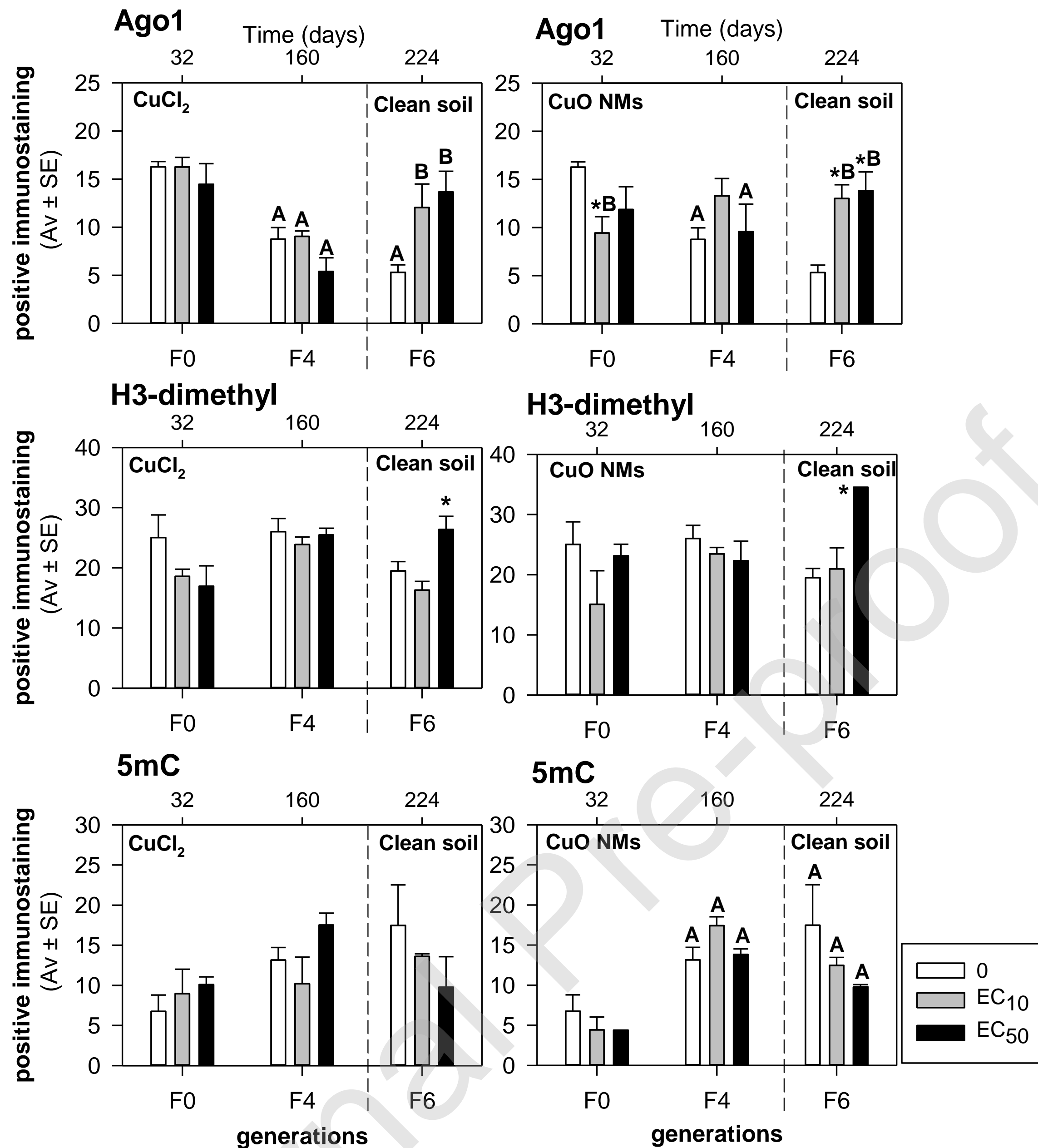

Figure 3: Immunohistochemistry quantification analysis for *Enchytraeus crypticus* when exposed to 0-20-180 mg Cu/kg soil for $CuCl_2$ and 0-500-1400 mg Cu/kg DW soil for CuO NMs, in LUFA 2.2 soil during a multigenerational test (MGt). Generations: F0, F4 and F6. * $p < 0.05$: one-way ANOVA, Dunnett's. [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

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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.

4. Discussion

4.1 *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 EC_{10} and EC_{50}. 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,

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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.

4.2 *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 (5 mC). 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 5 mC 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

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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 seem to be slower for CuO NMs than for $CuCl_2$. In the first generation (F0) these differences were most obvious, whereas in generations F4 and F5 were similar. This makes sense in light of the fast uptake for $CuCl_2$ that is known to occur for *E. crypticus*, reaching a maximum and equilibrium uptake after ca. 4 days, this can be slower or faster if at decreased or increased temperatures respectively, e.g. 11ºC and 25ºC (Cedergreen et al., 2013). 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).

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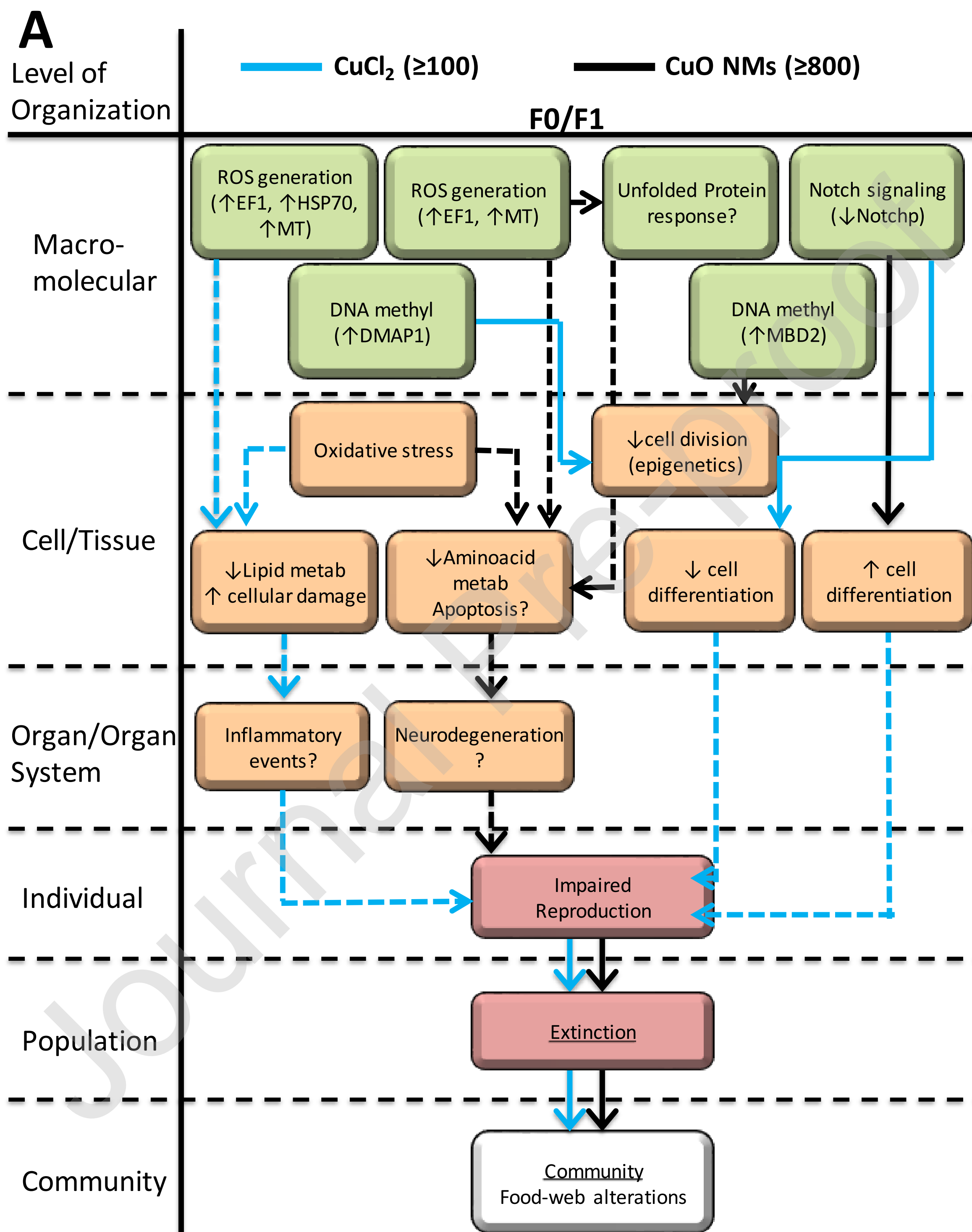
4.3 *AOPs*

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).



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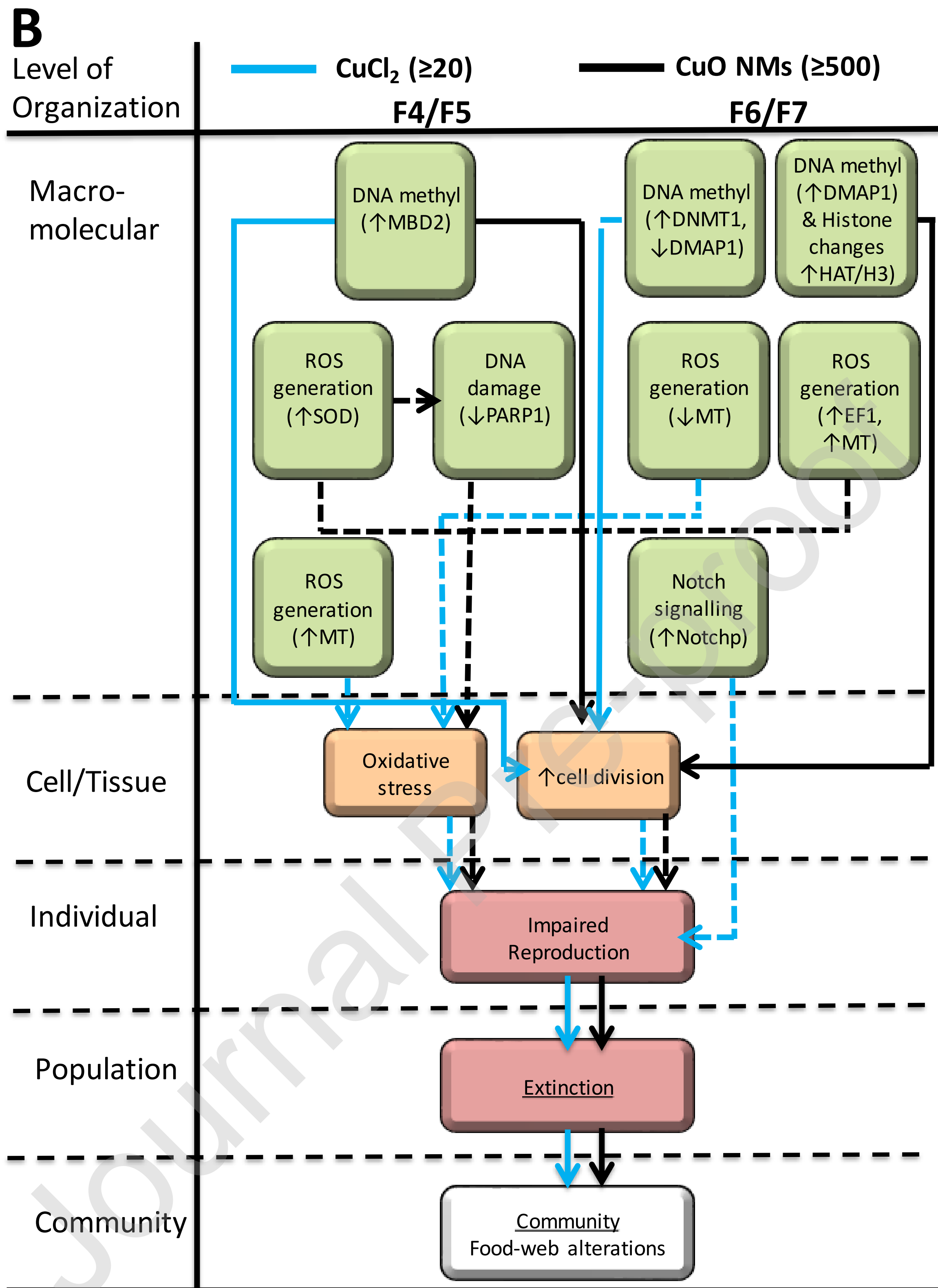

Figure 6: Adverse Outcome Pathway (AOP) for *Enchytraeus crypticus* from experimental results of A) full life cycle test (FLCt) (F0/F1) and B) multigenerational test (MGt) (F4/F5 and F6/F7) when exposed to $CuCl_2$ and CuO NMs in LUFA 2.2 soil. Continuous line: confirmed pathway; Dashed line: hypothesized pathway. Black: CuCl2; Blue: CuONMs

DNA methyl: DNA methylationDMAP1: 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; SOD: superoxide dismutase; NOTCHr: notch receptor; NOTCHp: notch protein.

The exposure to $CuCl_2$ and CuO NMs impact via differentiated pathways, as measured by initiating events of stress responses (ROS generation), notch signalling pathway and epigenetic mechanisms. The measured sequential key events, effects at the cell/tissue level, showed most differences between Cu materials at F0. $CuCl_2$ decreased lipid metabolism with subsequent cellular damage and reduction of cell division and differentiation (see also Sanchez-Alvarez et al., 2015), followed by inflammatory events (AOPwiki 282), impairing reproduction with negative outcome for individual/population and community. For CuO NMs exposure the Notch pathway is signalled, this together with protein folding malfunctions (AOPwiki 260), caused cellular stress responses reducing amino acid metabolism (see also Harding et al., 2003) with consequences at the cellular division and differentiation, and ultimately apoptosis and neurodegeneration processes can take place (AOPwiki 260). When exposed over multiple generations epigenetic mechanisms, via DNA methylation, were induced for both Cu materials. In the first generation DMAP1 expression increased for $CuCl_2$ and MBD2 expression increased for CuONMs, followed by a reduction in cell division for both Cu forms. Four generations later, at F4/F5, MBD2 expression increased for both Cu forms. Whereas $CuCl_2$ increased the Notchr expression and caused ROS generation, e.g. with an upregulation of MTs, CuONMs caused decreased PARP1 expression and DNA damage. At the cell/tissue level changes were identical for both Cu forms, showing an increase in cell division. When organisms were transferred to clean media in F6/F7, again both Cu forms caused changes in DNA methylation but $CuCl_2$ increased DNMT1 and decreased DMAP1 expression whereas CuONMs increased DMAP1. Both Cu materials caused ROS generation but triggering differentiated mechanisms: $CuCl_2$ decreased MT, CuONMs increased MT and EF1. The notch signalling pathway was affected in both but in opposite directions: $CuCl_2$ increase in Notchp and CuONMs decrease in Notchr. At the cell/tissue level changes were identical: increase in cell division. Adverse outcomes – impaired reproduction, population extinction and food-web alterations – were similar but the underlying pathways differed. The

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impact of CuONM was not predictable from $CuCl_2$ toxicity as suggested by (Gerloff et al., 2017) based on the ME or one generation results.

It is clear that the present AOP likely has further applicability domains in other materials, e.g. Ag and Ni. For silver materials (Ag NMs, $AgNO_3$) similar effects occurred in terms of cell division and apoptosis with consequent inflammatory responses and epigenetic mechanisms involved (Gomes et al., 2017). For nickel materials (NiNMs, $NiNO_3$), the pathway of protein damage induced by the ROS generation was also shown, with subsequent cellular damage, leading to apoptosis and inflammatory events (Gomes et al., 2019).

The development of AOPs will clearly contribute to the mechanistic understanding of toxicity and intelligent testing strategies, further supporting safer by design materials development. Although we are at its infancy, a scenario where a global overview of AOPs across materials, species, covering geographical boundaries, would be most interesting for the wide application onto risk assessment (understanding). Because mechanisms are conserved across species and especially for those living in similar environment – e.g. soil - and with similar exposure routes – e.g. oligochaetes - the AOPs presented here should be applicable across.

5. 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 epigenetic signals.

AUTHOR STATEMENT

RC Bicho: Methodology, Visualization, Investigation; first draft; **JJ Scott-Fordsmand**: Conceptualization, Data curation, Reviewing and Editing; **MJB Amorim**: Supervision, Conceptualization, Data curation, Reviewing and Editing.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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