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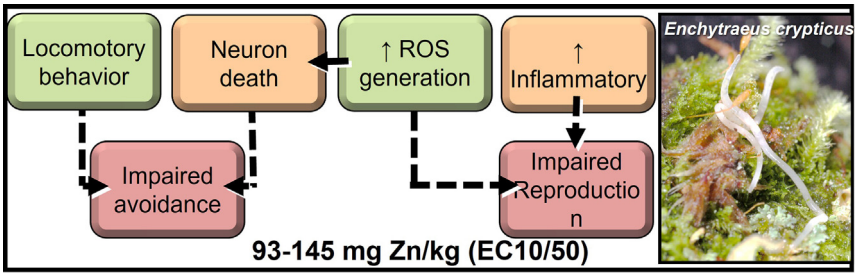
HIGHLIGHTS

- The initial (1–4 days) transcriptional response towards zinc exposure was investigated.
- A high-throughput (4x180K) microarray for *E. crypticus* was used.
- Key mechanisms included neuronal impacts, Zn trafficking and oxidative stress.
- Adverse outcome at organism level (EC50) could be predicted based on gene expression.
- Gene expression response was time-dependent and reflected the cascade of events.

GRAPHICAL ABSTRACT



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ABSTRACT

Zinc (Zn) is known to be relatively toxic to some soil-living invertebrates including the ecologically important enchytraeid worms. To reveal the molecular mechanisms of zinc toxicity we assessed the gene expression profile of *Enchytraeus crypticus* (Enchytraeidae), exposed to the reproduction effect concentrations EC_{10} and EC_{50}, over 4 consecutive days, using a high-throughput microarray (species customized). Three main mechanisms of toxicity to Zn were observed: 1) Zn trafficking (upregulation of zinc transporters, a defence response to regulate the cellular zinc level), 2) oxidative stress (variety of defence mechanisms, triggered by Reactive Oxygen Species (ROS)), and 3) effects on the nervous system (possibly the primary lesion explaining the avoidance behaviour and also why enchytraeids are relatively susceptible to Zn). The adverse outcome at the organism level (reproduction EC_{50}) could be predicted based on gene expression (male gonad development, oocyte maturation), with Zn at the EC_{50} affecting processes related to higher stress levels. The gene expression response was time-dependent and reflected the cascade of events taking place over-time. The 1 to 4 days of exposure design was a good strategy as it captured the time for sequence of events towards zinc adverse outcomes in *E. crypticus*.

1. Introduction

Zinc (Zn) is an essential trace element for animals and plants, involved in several biological processes such as structural, catalytic, intra- and intercellular signaling pathways. Zinc deficiency is recognized in humans and crops, and supplementation is recommended in those cases to improve soil productivity and the health of human populations (for further details see e.g. (Alloway, 2009; Vickram et al., 2021)). However, above certain levels Zn is toxic, e.g. the 50% lethal concentration in *Enchytraeus albidus* is 73 mg Zn/kg soil (Novais et al., 2011). This EC50 comes close to background Zn concentrations in many European soils. As enchytraeids

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dominate in acid soils (where they replace earthworms), bioavailability of metals is relatively high. Zinc is also present above threshold limits in 1% of agricultural soils sampled across Europe (Tóth et al., 2016). Enchytraeid worms are among the most sensitive components of a soil ecosystem. Hence, a better insight into the mechanism of zinc toxicity is needed to understand differential toxicity.

In ecotoxicology the hazard assessment of chemicals has been standardized for a number of biological species, having optimized test designs implemented, following guidelines, including a selected exposure time to assess e.g. survival and reproduction effects (ISO 16387, 2005; OECD 220, 2016). This is common and relatively well-established at the phenotypic level, although less so at the genotypic level, i.e. on adverse outcomes that are not easily observed, e.g., gene expression. Gene regulation is a transient process which may translate onto protein synthesis/inhibition, e.g. due to up- or down-regulation of genes. Such processes are known to commence within hours, while after reestablishment of homeostasis the signals are turned off (Inoue and Kaneko, 2013). Hence, to define a specific exposure time to assess gene expression would not make much sense, and this must be adjusted to the specific question, e.g. often the relationship with a specific observed adverse outcome. Gene regulation varies with time because the genes expressed reflect both the structural and functional capacities of the cell as well as the ability of the cell to respond to external stimuli (e.g., chemical exposure). Further, only a small fraction of the thousands of genes of an organism are expressed at a given time (Androulakis et al., 2007), therefore a gene expression profile provides a snapshot of the cellular response. Microarrays are excellent tools to monitor the expression variation of thousands of genes at a certain time. Previous microarray studies in enchytraeids, which are soil living invertebrates, and model species in ecotoxicology (ISO 16387, 2005; OECD 220, 2016), have shown that high transcriptional response occurs within few days of exposure (2 to 3) (e.g. in response to phenanthrene (Roelofs et al., 2016), silver (Gomes et al., 2017) or copper materials, including nano (Gomes et al., 2018)). However, the gene regulation peak, i.e. number of differentially expressed genes, will depend on various factors, namely concentration or chemical, e.g., for nickel, high transcriptional response was captured after 7 days of exposure (Gomes et al., 2019a) while for silver it was captured after 3 days of exposure (Gomes et al., 2017), reflecting the dynamics and specificity of gene expression responses. In any case, it is of interest to retain that even after 21 days of exposure, a case study with phenantrene (Roelofs et al., 2016) showed that although less, there were still significant gene responses.

In the present study we aimed at following the gene expression profiles of *Enchytraeus crypticus* (Oligochaeta) over 4 consecutive days (1, 2, 3 and 4) of Zn exposure, based on a high-throughput microarray developed for the species, a 4 × 180 K custom Agilent microarray (Castro-Ferreira et al., 2014). To be able to link to the adverse outcome and sub-lethal concentrations, the effects of Zn were also assessed at the survival and reproduction level. The gene expression investigation covered the concentrations known to cause adverse effects at organism level (10 and 50% reduction in reproduction), aiming to understand the dynamics of the mechanisms of Zn toxicity.

2. Materials and methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae, Oligochaeta), Westheide and Graefe (1992) was used as test species. The organisms were cultured on an agar substrate prepared with aqueous soil extract, fed ad libitum with ground oatmeal, at 16 °C with 75% relative humidity and 16/8 h light/dark photoperiod.

2.2. Test soil and spiking

Experiments were done on natural soil (LUFA 2.2, Speyer, Germany). The main characteristics of the soil (provided by the supplier) are pH (0.01 M $CaCl_2$) = 5.5; organic carbon = 1.61% of total dry weight, cation exchange capacity (CEC) = 10.0 $cmol_c$/kg, water holding capacity (WHC) = 43.3%, and a grain size distribution of 7.9% clay (<0.002 mm), 16.3% silt (0.002–0.05 mm), and 75.8% sand (0.05–2.0 mm).

Zinc chloride ($ZnCl_2$, Merk) was added to pre-moistened soil as aqueous stock solutions prepared with deionized water and serially diluted. Soil batches per concentration were homogeneously mixed and allowed to equilibrate for 3 days after which water was added up to 50% of the soil WHC.

For the enchytraeid reproduction test (ERT), organisms were exposed to $ZnCl_2$ at 0, 18.75, 37.5, 75, 150, 300, 600 mg Zn/kg soil (dry weight). For the microarray experiment, organisms were exposed to the reproduction effect concentrations EC10 (93 mg Zn/kg soil) and EC50 (145 mg Zn/kg soil), as determined based on the ERT results.

2.3. Survival and reproduction

2.3.1. Enchytraeid reproduction test (ERT)

Effects of Zn on *E. crypticus*' survival and reproduction were assessed following the standard guidelines (ISO 16387, 2005; OECD 220, 2016). In short, ten adults with well-developed clitellum and of similar size were introduced into each glass vial (100 mL) containing 30 g of moist soil (control or spiked). Oatmeal (20 mg) was supplied, and vessels were closed with perforated aluminium foil. The test ran for 21 days at 20 °C, 75% relative humidity and 16/8 h light/dark photoperiod. Five replicates were used per test concentration and control. Food (12 mg) and water were replenished every week, based on weight loss. After 3 weeks, all samples were fixed by adding 10 mL of 96% ethanol, transferred to a plastic container, and stained with Bengal rose solution (1% in ethanol). The containers were tightly closed, agitated vigorously for 10 s and incubated overnight at 4 °C to achieve optimal staining of the animals. Every sample was warily sieved over 160 μm to separate the enchytraeids from most of the soil. Subsequently, each sample was transferred into white trays (80 × 50 cm) and divided in fractions to optimise the counting of the stained enchytraeids under a magnifying glass, assessing the number of adults and juveniles per replicate.

2.3.2. Data analysis

To assess differences between treatments and control, one-way analysis of variance (ANOVA) was performed, followed by the post-hoc Dunnett's method for multiple comparisons at a significance level of 0.05 (SigmaPlot 11.0). Effect concentrations (ECx) were calculated modeling data to Threshold sigmoid or Logistic 2 parameters regression models, using the Toxicity Relationship Analysis Program (TRAP 1.30) software.

2.4. Gene expression

2.4.1. Exposure for microarray analysis

Ten adult enchytraeids with similar size were transferred into a glass vial (100 mL) with 30 g of moist test soil (control or spiked). Tests ran at 20 °C with 75% relative humidity and 16/8 h light/dark photoperiod. Exposure lasted a maximum of 4 days, with sampling at four time points: day 1, 2, 3 and 4. Four replicates were performed per test concentration and sampling time. Upon sampling, 5 to 7 enchytraeids were collected from the test soil by hand-sorting, washed in distilled water, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis.

2.4.2. RNA isolation and microarray hybridization

RNA was isolated using the SV total RNA isolation kit (Promega) according to the manufacturer's instructions. After isolation, RNA was precipitated with ethanol to remove any remaining isolation chemicals and to increase its concentration. RNA concentration and quality were determined using a Nanodrop ND1000 spectrophotometer (Thermo Scientific) and Bioanalyzer 2100 (Agilent Technologies). The RNA samples (input 500 ng per sample) were amplified and labelled using the Two-Color Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies), according to the manufacturer's instructions; complementary (c)RNA was purified using RNeasy (Qiagen). Labelled samples were hybridized to *E. crypticus* microarray (4 × 180 K

Agilent platform containing 86 K probes in duplicate) (Castro-Ferreira et al., 2014) using the Agilent Gene Expression Hybridization Kit, for 17 h at 65 °C and 10 rpm in the incubator. Two replicates per condition were labelled with the fluorescent dye Cy3 and the other two with Cy5 to include a dye-swap in the microarray design (loop design). After hybridization, the microarrays were washed using the Gene Expression Wash Buffer Kit (Agilent Technologies) and scanned on the SureScan Microarray Scanner (Agilent Technologies). Raw data of spot intensities were extracted using the Feature Extraction software (Ver 10.7.3; Agilent Technologies). Raw microarray data and Minimum Information About a Microarray Experiment (MIAME)-compliant metadata were deposited at the US National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under accession number GSE183377.

2.4.3. Microarray data analysis

Microarray raw intensities were LOESS normalized with a background subtraction according to the Edwards method which contained a minimal offset of 30. Significantly regulated transcripts were determined by directly contrasting the controls to the EC10 or EC50 exposures and fitting a linear model through the data, all using the Limma package for R/Bioconductor (Smyth, 2004) after which *P*-values were adjusted for multiple testing according to the Benjamini-Hochberg method (5% FDR, False Discovery Rate). Cluster analysis on differentially expressed genes was performed using the MultiExperiment Viewer (MeV, TIGR). A Gene Enrichment (GO) analysis was performed in Blast2GO using a Fisher's exact test with a *P*-value below 0.05.

3. Results

3.1. Survival and reproduction

The test fulfilled the validity criteria as described in OECD 220 (2016) with adult mortality (10%) below 20% and the number of juveniles (997 ± 53) higher than 50, with a coefficient of variation <50%, in controls. The results on survival and reproduction, as a function of zinc exposure, are shown in Fig. 1.

Exposure to Zn caused a significant reduction of *E. crypticus* survival at 600 mg Zn/kg, and of reproduction at 150 mg Zn/kg soil. The estimated effect concentrations (ECx) are shown in Table 1.

3.2. Gene expression

Exposure to Zn resulted in a total of 3820 significant differentially expressed genes (DEGs) (p adjusted <0.05), in at least one test condition, in comparison to the control (1, 2, 3 or 4 days). The total number of up- or down-regulated genes, affected by each Zn effect concentration (EC), is depicted in Fig. 2 and the list of DEGs is provided in Table S1.

Table 1
Summary of the effect concentrations (ECx with 95% confidence intervals – CI), expressed as mg Zn/kg soil, for the toxicity of $ZnCl_2$ to *Enchytraeus crypticus* in LUFA 2.2 soil. The dose-response models used are Threshold sigmoid 2 (Thres2P) or Logistic 2 parameters (Log2P). S: slope; y0: top point.

Endpoint	EC10 (95% CI)	EC50 (95% CI)	EC90 (95% CI)	Model & parameters
Survival	371 (216–526)	478 (393–564)	545 (456–634)	Thres2P (S: 5.16E-3; y0: 9; r^2: 0.932)
Reproduction	93 (68–117)	143 (135–151)	206 (180–233)	Log2P (S: 9.26E-3; y0: 1005; r^2: 0.967)

The overall transcriptional response increased over time, reaching the highest number of DEGs after 3 days of exposure, followed by a decrease at day 4, for both tested ECs. The EC50 affected more genes than the EC10, when compared over the identical exposure period. Overall, for the EC10 there were more down- than up-regulated transcripts, while the EC50 showed more up- than down-regulated transcripts.

The overall distinctive pattern of response between exposure times is shown by the cluster analysis of samples and gene results (Fig. 3A), with clear separation between days (days 2 and 4 clustered separate from days 1 and 3, with less than 50% similarity for the different time groups), but also between concentrations (there is no clustering of EC10 or EC50). The transcriptional response is also depicted in the Venn diagrams, both for time and concentration. It shows only few common DEGs across time (Fig. 3B) or concentrations (Fig. 3C). The exception is day 3, where the highest transcriptional response occurred (with a total of 1291 and 2374 DEGs at the EC10 and EC50, respectively), and where around 50% and 30% of the transcripts (657 in total) were shared between EC10 and EC50.

Gene ontology (GO) enrichment analysis results are presented in Table S2, with omission of the GO terms (Biological Processes (BPs)) supported by only one DEG. We only focused on GO terms related to BPs. For both EC10 and EC50, the number of GO terms regarding BP affected followed the same pattern as the number of DEGs in that most GO terms are affected at day 3. For instance, the EC10 affected 0, 8, 166, and 110 GO terms, and the EC50 affected 12, 18, 334, and 96 GO terms for 1, 2, 3, and 4 days of exposure, respectively.

In the case of EC10 exposure at 2 days, the affected GO terms were mainly related to translation, protein transport and modifications, and Reactive Oxygen Species (ROS) responses. At 3-day exposure, it included several GO terms related to regulation of transcription, regulation of translation, protein processing/modifications, neuron development, and locomotory behaviour. There were also 10 BPs related to transport and/or homeostasis of other ions (calcium, potassium, copper, sodium, magnesium, manganese). The processes inflammatory response and DNA damage response were also affected. After 4 days of exposure to the EC10, the

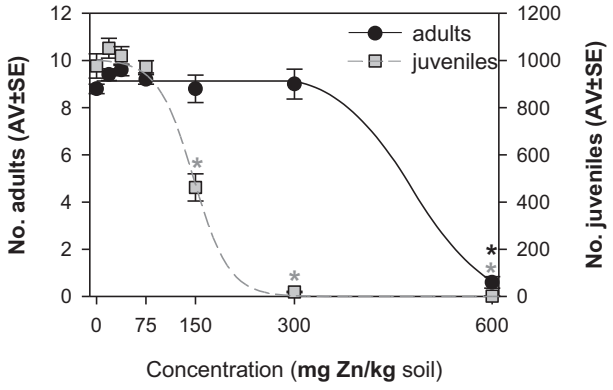

Fig. 1. Results of the standard Enchytraeid Reproduction test (ERT) for survival (no. adults) and reproduction (no. juveniles) of *Enchytraeus crypticus* exposed to $ZnCl_2$, in LUFA 2.2 soil. Results are expressed as average (AV) ± standard error (SE), $n = 5$. *$p < 0.05$ (Dunnett's method). Lines represents the models fitted to the data.

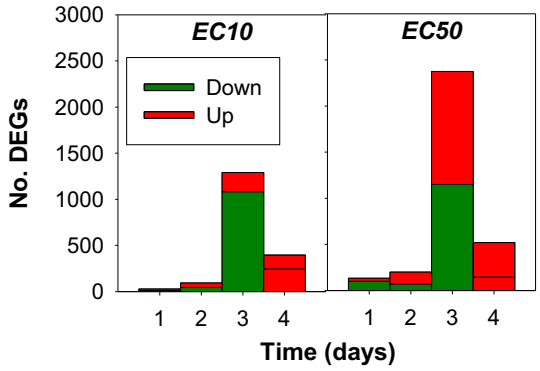

Fig. 2. Number of differentially expressed genes (DEGs) (adjusted $p < 0.05$) in *Enchytraeus crypticus* exposed to $ZnCl_2$ at reproduction effect concentrations EC10 and EC50, in LUFA 2.2 soil, for 1, 2, 3 and 4 days. Down: down-regulated, and Up: up-regulated in comparison to the control.

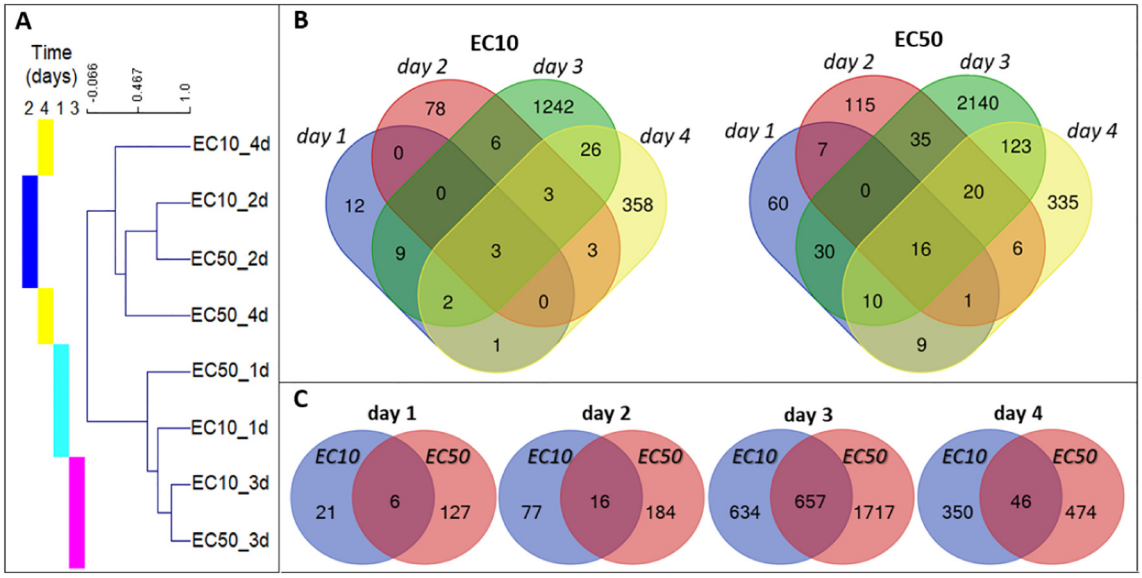

Fig. 3. Comparison of gene expression profiles in *Enchytraeus crypticus* exposed for different time intervals to $ZnCl_2$ at concentrations corresponding with the EC10 and EC50 for effects on reproduction. A) Dendrogram of samples, hierarchically clustered, based on the fold change of all differentially expressed genes (DEGs) [light blue: 1 day, dark blue: 2 days, pink: 3 days, yellow: 4 days], using Pearson uncentered correlation and average linkage (the heat map was truncated for improved visualization). B) Venn diagram representation of the DEGs, grouped by time of exposure, for the reproduction effect concentrations EC10 and EC50. B) Venn diagram representation of the DEGs, grouped by EC (10 or 50), for the different exposure periods.

effects on BPs related to other ions response/homeostasis persisted (at this time point, with the activation of the intrinsic apoptotic signaling pathway in response to osmotic stress), as well as some related to neuron development, but changing from positive regulation/development (day 3) to negative regulation of neuron death (day 4). Three processes related to osmotic stress were affected. There were more processes related to inflammatory response at day 4 (in comparison to days 1 and 3), and one process, related to DNA repair, persisted. Processes related to ROS generation (re)appeared. For an Adverse Outcome Pathway (AOP) draft overview see Fig. 4A.

Exposure to the EC50 for 1 day mainly affected locomotory behaviour. After 2 days, effects on the transport/homeostasis of other ions (calcium and sodium) emerged, as well as processes related to osmotic stress. These processes continued to be affected after 3 days of exposure, and to a higher extent (i.e., more processes). After 3 days of exposure, several processes related to locomotory behaviour and neuron development/neurotransmission, inflammatory/immune response, DNA damage and ROS response were also affected. Further, and detected for the first time, were processes related to reproduction (e.g., male gonad development, oocyte maturation). After 4 days, effects persisted on several biological processes such as those related to the transport/homeostasis of other ions, osmotic stress (including, as for EC10_4d, the activation of the intrinsic apoptotic signaling pathway in response to osmotic stress), neuron development/neurotransmission and ROS response (glutathione metabolic process appears affected for the first time). Detected for the first time at EC50_4days were processes related to energy metabolism (ATP synthesis and carbohydrate catabolism) (Fig. 4B).

4. Discussion

4.1. Survival and reproduction

Zinc exposure affected reproduction more severely than survival, which is commonly reported, e.g. for *E. albidus* (Novais et al., 2011). Impacts of Zn on enchytraeid reproduction were observed at concentrations that did not affect survival (150 and 300 mg Zn/kg), hence Zn must have a direct reproductive effect (this has been further supported by gene expression). The response to low Zn concentrations (< 75 mg Zn/kg) shows a small increase in reproduction performance and what seems a beneficial input (rather than *hormesis*).

Zinc was less toxic to *E. crypticus* than to *E. albidus*, both in terms of survival and reproduction, with LC50/EC50 of 478/145 mg Zn/kg for *E. crypticus* (current results) and 73/35 mg Zn/kg for *E. albidus* (Novais et al., 2011). This pattern has been found previously for certain chemicals, where *E. crypticus* was less sensitive than *E. albidus*, for instance to cadmium, carbendazim, phenanthrene (Castro-Ferreira et al., 2012) and atrazine (Gomes et al., 2019b). Results from studies in OECD artificial soil with *E. albidus* (Lock and Janssen, 2001) or *Eisenia fetida* (Spurgeon et al., 1997) showed Zn toxicity at slightly higher levels (reproduction EC50 of 267 and 308 mg Zn/kg for *E. albidus* and *E. fetida*, respectively). This is often the case when comparing toxicity results between soils with higher (OECD artificial soil) and lower organic matter contents (LUFA 2.2). Zn toxicity to *E. crypticus* occurred at comparable levels to copper, another essential and comparable trace element (LC50 = 303 mg Cu/kg, and EC50 = 179 mg Cu/kg soil in LUFA 2.2. soil (Bicho et al., 2017)).

4.2. Gene expression

Although previous studies with enchytraeids have considered the variation in gene expression responses at different exposure times (e.g. Gomes et al., 2019a, 2018, 2017, 2011; Novais et al., 2012a, 2012b; Roelofs et al., 2016), this is the first one to assess the transcriptomic responses over the initial four consecutive days of exposure (1 to 4 days), aiming to cover the early mechanisms of response towards Zn exposure. A previous study has shown that transcriptional response of *E. crypticus*, exposed to phenanthrene, significantly decreased from 2 to 21 days of exposure (Roelofs et al., 2016), indicating that after initial gene regulation other (cascade) mechanisms take place. Our results showed that there is a peak in gene expression at day-3 of exposure to Zn (for both EC10 and EC50), immediately followed by a steep decrease at day 4. Other studies, comparing exposure times of 3 and 7 days, also showed the gene expression response to be more pronounced after 3 days (e.g. for silver (Gomes et al., 2017) and copper nanomaterials (Gomes et al., 2018)). On the other hand, for nickel (Gomes et al., 2019a) and atrazine (Gomes et al., 2021), a higher transcriptional response was observed after 7 days compared to 3 days. Although the gene/molecular responses precede the effects at higher levels of biological organization, the temporal pattern

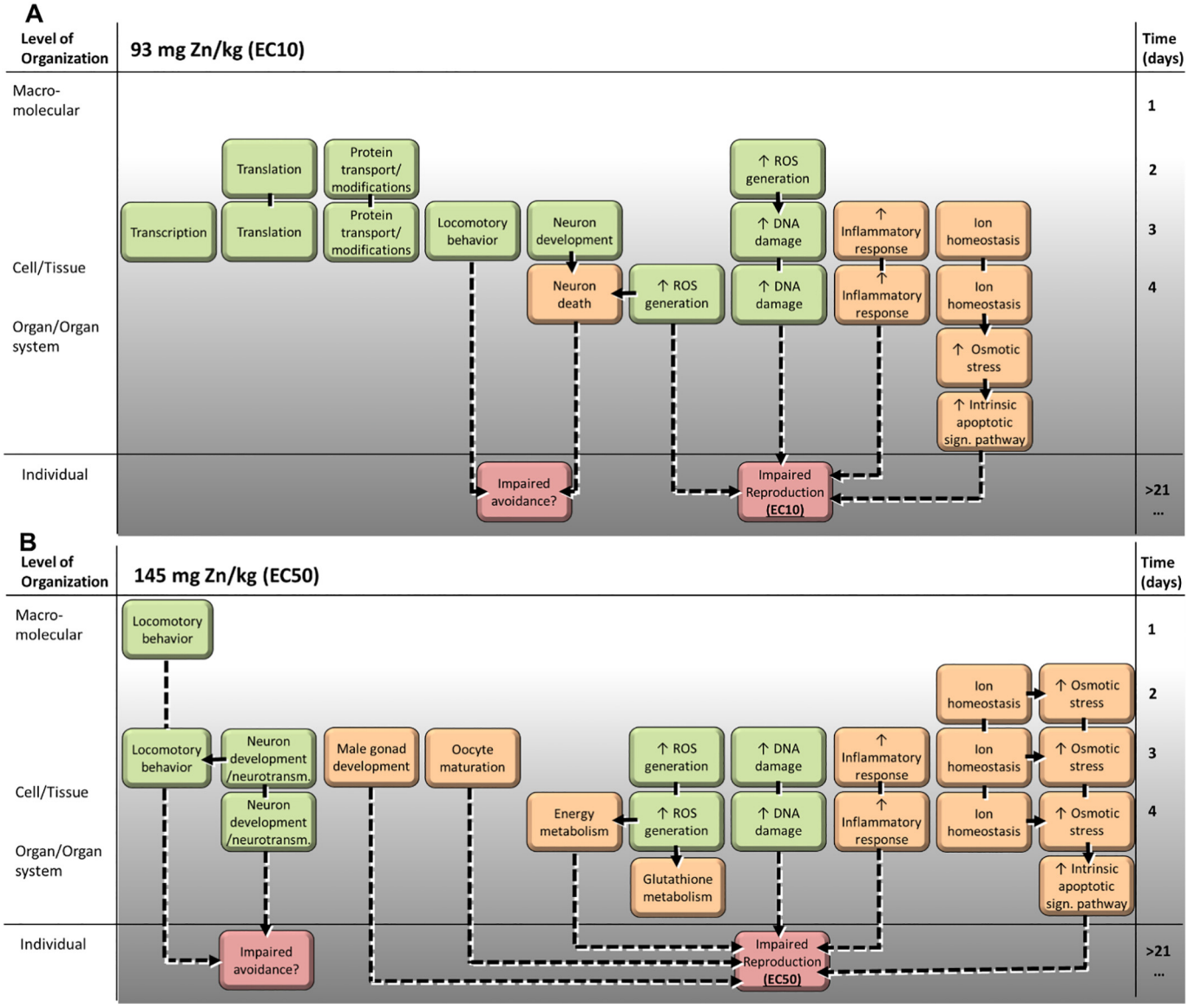

Fig. 4. Adverse Outcome Pathway (AOP) for the effects on *Enchytraeus crypticus* when exposed to $ZnCl_2$ at the EC10 and EC50 for effects on reproduction (93 and 145 mg Zn/kg, respectively) in LUFA 2.2 soil, from experimental results combining known gene expression and reproduction endpoints. Continuous line: confirmed pathway; Dashed line: hypothesized pathway. ROS: Reactive Oxygen Species. Box colours: Green: Macro-molecular; Orange: Cell/Tissue; Organ/Organ System; Pink: Individual/Population.

can be chemical specific, besides species specificity (Novais et al., 2012a). Also in *E. albidus*, while soil properties such as variations in clay and organic matter content and pH caused higher transcriptomic responses after 4 days compared to 2 or 21 days, the same pattern was not clear for exposures to phenmedipham or copper (Novais et al., 2012b). Although the gene expression response to Zn over time may seem to be very specific, as shown by the reduced number of DEGs shared between times (Fig. 2 B), in terms of the affected BPs there were several commonalities. The measured responses seem to indicate that a sequence of molecular events happens between 1 and 4 days, as discussed below.

Zinc exposure also caused an increase in the number of DEGs with increasing exposure concentration, i.e. with more DEGs at the EC50 than at the EC10. Such a response pattern has been also observed with other chemicals such as phenanthrene (Roelofs et al., 2016), nickel (Gomes et al., 2019a), and silver nanomaterials (Gomes et al., 2017), but not for all, e.g. copper nanomaterials (Gomes et al., 2018) or atrazine, including its nanoformulation (Gomes et al., 2021). Such a dose-dependent increase in the number of DEGs was not observed for *E. albidus* when exposure to Zn increased from EC50 to EC90 (Novais et al., 2012a).

Regarding the functional implications, being involved in a variety of BPs (e.g., as structural, catalytic, and intra- and intercellular signaling component), Zn homeostasis is tightly controlled, with zinc transporters being particularly important in these processes (Kambe et al., 2015). Our results showed that the transcript coding for the zinc transporter 1 (which plays a pivotal role exporting cytosolic Zn to the extracellular space (Nishito and Kambe, 2019)) was up-regulated in all the tested conditions, showing that the animals must be able to counteract Zn excess at the cellular level. Indeed, similar results were previously reported from the analysis of a sub-set of the data (Zn EC50 for 4 days) reported here (Castro-Ferreira et al., 2014). It has been suggested that ionic stabilizers have probably a key role in maintaining homeostasis in enchytraeids (Amorim et al., 2021). The plasticity of enchytraeids to survive in aquatic biotopes, such as marine interstitial environments, may be explained by dealing with the level of salts that is much higher than in terrestrial soil environments.

Affected for both ECx and across time points up to 3 days (i.e., EC10_3 days; EC50_1/2/3 days) were genes related to locomotory behaviour. This might be linked to the observed avoidance

behaviour, as measured after 2 days of exposure to Zn: an avoidance EC50 of 92 mg Zn/kg soil was determined for *E. albidus* (Amorim et al., 2008). Interestingly, although the same processes appeared affected by most of the treatments, it occurred via deregulation of different transcripts, which could reflect somehow a capture of a sequence of molecular events over time. Avoidance behaviour, or in fact the inability to do so, has been linked to affected neurotransmission. A previous study showed that *E. crypticus* (Bicho et al., 2015) was unable to avoid (attraction to) boric acid, which was linked to effects on gamma-aminobutyric acid (GABA) related neurotransmission mechanisms, and not to acetylcholinesterase (AChE). Our results showed that Zn exposure (at both EC10/50 and several time points) affected several GO terms related to neurotransmission and/or neuron development. Although, as discussed, *E. albidus* avoided Zn spiked soils (Amorim et al., 2008), the current results on neurotransmission indicate that avoidance behaviour might be compromised at higher concentrations and/or prolonged exposures. Further, and in line with our results, disturbances in Zn homeostasis have been associated with neuronal injury and death via effects on cellular energy production and mitochondrial ROS generation (Dineley et al., 2003). In fact, several of the test conditions (EC10_2/3/4 days, EC50_3/4 days) affected BPs related to ROS metabolism and/or biosynthesis, suggesting increase in ROS generation. However, GOs related to energy production such as ATP synthesis, Tricarboxylic acid (TCA) and carbohydrate metabolism were only affected at the EC50_3/4 days. These results indicate that, while ROS generation is common to both EC10/EC50, effects on energy production are related only to higher toxicity (EC50). Results in *E. albidus* (Novais et al., 2012a) were in agreement with *E. crypticus*, where exposure to Zn EC50 and EC90 affected several transcripts coding for mitochondrial proteins involved in the electron transport chain.

GO terms related to protein translation, transport and modifications were overrepresented across test conditions. Effects on genes involved in protein metabolism, have been reported before, in *E. albidus* (Novais et al., 2012a) and in *E. crypticus* (Castro-Ferreira et al., 2014). Since the effects were reported in all the test conditions with significantly enriched BPs/GO terms, this might be a transient mechanism from which organisms recover or, as suggested by Novais et al. (2012a), a mechanism to store energy for detoxification. In fact, despite the tendency to increase the energy consumption (estimated via electron transport system activity) in *E. albidus* exposed to Zn EC50 and EC90, the amount of energy available in the form of proteins was relatively constant, from 0 to 8 days (Novais et al., 2011).

Also common across test conditions (all except EC10_1 day without enriched GO terms, and EC10_2 days) was the clear interference of Zn with the transport and/or homeostasis of other ions (e.g. copper, iron, calcium, magnesium, sodium, potassium). Although the comparison between eukaryotic and prokaryotic organisms is not straightforward, it was previously shown that excess Zn disturbed Fe and Cu homeostasis, causing an increase in cellular Fe levels and a decrease in Cu levels in the bacteria *Escherichia coli* (Xu et al., 2019). Further, bacteria treated with excess Zn were more susceptible to Cu exposure (Xu et al., 2019). This can of course mean that also enchytraeids, when exposed to Zn, even at relatively low toxicity doses (EC10), can be more susceptible to co-exposure to other stressors. Zinc is also known to compete with Ca for its binding sites, causing a reduced absorption of this essential element, as well as posterior hypocalcaemia (McRae et al., 2016). The effects of Zn on Ca homeostasis were also shown at gene level in *E. albidus* (Novais et al., 2012a). The same competitive binding is reported for Na, which can impact cell membrane NaK pump, and consequently cellular ionic and acid–base homeostasis (Loro et al., 2014). The effects of Zn on *E. crypticus* cellular ionic homeostasis can also be related to the several enriched biological processes related to osmotic stress (e.g., response to salt stress, cellular hyperosmotic response, intrinsic apoptotic signaling pathway in response to osmotic stress), affected for both EC10 and 50 in several exposure periods (for the EC10_3/4 days; for the EC50_2/3/4 days). Interestingly, the evolution of the molecular response related to osmotic stress was captured within 1 to 4 days: after 3 days of exposure (to both EC10 and 50) the process cellular hyperosmotic response was enriched and after 4 days there was an activation of the intrinsic apoptotic signaling pathway in response to osmotic stress.

Processes related to inflammatory response were affected at the EC10 (for more than 3 days of exposure) and at the EC50 (for more than 2 days). Zn homeostasis is critical for proper immune cell function, and the mechanisms of its antioxidant and anti-inflammatory properties are well-known (Jarosz et al., 2017). However, both its deficiency and its excess affect immune cell function, often resulting in increased inflammation (Hall and Knoell, 2019). Our results point towards the increase in inflammatory response, which emerged associated to DNA damage mechanisms. The recently sequenced genome of *E. crypticus* (Amorim et al., 2021) shows that the innate immune system (and response to stress) is among the most expanded gene families.

Being detected only at the EC50, after 3 days of exposure, were processes directly related to reproduction (i.e., male gonad development, oocyte maturation). Although Zn is an essential element also for the proper functioning of all the reproductive processes (e.g.,(Vickram et al., 2021)) and, in many studies concerning mammals no negative effects were found, adverse effects are also reported on fertility and offspring viability (Khan et al., 2007). The fact that the gene expression results related to reproduction were only observed at the higher effect concentration (EC50) indicates that they must indeed be associated with reproductive toxicity.

Being detected uniquely at the EC50 after 4 days of exposure, were the effects on glutathione metabolism (as well as those related to energy metabolism, discussed above). Glutathione is an antioxidant present across the animal kingdom, the induction of its metabolism indicates that *E. crypticus* is facing oxidative stress. By measuring oxidative stress biomarkers at subcellular level (enzymes and substrates), Novais et al. (2011) showed that glutathione (in its oxidized form -GSSG) and dependent enzymes (glutathione peroxidase and reductase) play an important role in the antioxidant defence against Zn in *E. albidus*. The gene expression results corroborate the induction of higher levels of oxidative stress, at the higher effect concentration.

5. Conclusions

The gene expression response to Zn showed three main mechanisms: 1) Zn trafficking (upregulation of zinc transporters, an obvious defence response to regulate the cellular zinc level), 2) oxidative stress (variety of defence mechanisms, triggered by ROS generated by Zn, and affecting a variety of targets, hence also secondary effects of Zn toxicity), and 3) effects on the nervous system (possibly the primary lesion explaining the results of avoidance tests and also why enchytraeids are relatively susceptible to Zn). The specificity of gene expression over time (few commonly affected DEGs) reflected the cascade of molecular events taking place during Zn intoxication. The adverse outcome at the organism level (reproduction EC50) could be predicted based on gene expression responses, with the EC50 affecting processes related to higher stress levels. The use of a consecutive exposure sampling day approach, from 1 to 4 days in *E. crypticus*, showed to be a good design as it seemed to capture the time for sequence of events towards adverse outcomes.

CRediT authorship contribution statement

Conceptualization, CAMVG, NMVS, AMVMS, DR, MJBA; methodology, SILG, TEDB; formal analysis, SILG, TEDB; investigation, SILG; resources, MJBA; data curation, SILG, TEDB; writing—original draft preparation, SILG, MJBA; writing—review and editing, SILG, TEDB, CAMVG, NMVS, AMVMS, DR, MJBA; supervision, DR, MJBA; funding acquisition, MJBA. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153975.

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