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Carbamazepine-exposed earthworms are characterized by tissue-specific accumulation patterns and transcriptional profiles

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ABSTRACT

Pharmaceutically active compounds enter soils via wastewater reuse and biosolid application. A ubiquitous drug present in wastewater is carbamazepine, a frequently prescribed anti-convulsant. Its mode of action is not species-specific and affects the nervous system of non-target organisms, including most likely the soil dwelling earthworms, which in turn has the potential to negatively impact soil quality. In this project, soils were amended with carbamazepine to explore uptake dynamics and resultant changes in molecular and life cycle endpoints of earthworms (*Dendrobaena veneta*). Earthworms were maintained, under laboratory conditions, for 28 days in soil spiked with either a solvent control, 0.6 mg/kg carbamazepine (encountered in the terrestrial system) or 10 mg/kg carbamazepine (significantly above an environmental hotspot). Carbamazepine concentrations were quantified in soils and worms by liquid chromatography tandem mass spectrometry (LC-MS/MS) which revealed tissue, dose and time-dependent differences in accumulation. Carbamazepine also modulated the make-up of the microbiome in the soil as well as the earthworm's gut. *De novo* RNA sequencing identified novel transcripts and complex tissue-specific transcriptomic changes, where, for example, the expression of the *tubulin polymerisation promoting protein (tppp)* was inhibited (9-fold) in the gut but induced (11-fold) in the cerebral ganglion of exposed earthworms. However, the notable absence of a strong cytochrome P450 response across all conditions suggests that the terrestrial earthworm also relies on detoxification pathways that differ to those observed in well-studied aquatic models. The novel finding that carbamazepine exposure triggers tissue-specific impacts in non-target soil organisms highlights the value and need for a more comprehensive understanding of how contaminants of emerging concern behave within an ecotoxicological context. This, in turn, will lead to informed and reliable risk assessments defining the consequences of wastewater and biosolid amendment practices on soil ecology and ecosystem function.

1. Introduction

The escalating global demand for water and accompanying water restrictions pose significant challenges to sustainable agriculture (Zarei et al., 2020). Agricultural irrigation increasingly relies on the reuse of low-quality wastewater which, without adequate risk assessment and management, threatens both aquatic and terrestrial ecosystems, and potentially human health (Al-Hazmi et al., 2023). This is exacerbated by the notion that wastewater and agricultural run-off represent primary sources of contaminants of emerging concern (CECs) in the environment, including active pharmaceutical ingredients (APIs) and personal care products (PPCPs) (Ternes et al., 2004). Activated sludge processes in wastewater treatment are designed to remove pollutants, including many CECs (Rapp-Wright et al., 2023). However, the ban of their disposal at sea has resulted in an increased addition of deactivated and thermally treated sludge byproducts (i.e., biosolids) to farmlands which enhances soil nutrient levels and agricultural productivity. In most countries this procedure is tightly regulated, for example by the UK

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Water Services Regulation Authority (Ofwat, 2016), however existing regulations governing wastewater recycling practices have not been updated, and do not address the elevated concentration of emerging pollutants due to synthetic chemistry and pharmaceutical usage. Indeed, numerous PPCPs have been detected in wastewater outflows across the globe, suggesting that this route of contamination is the norm, rather than the exception (Ternes, 1998; Kolpin et al., 2002). A diverse range of compounds enter the environment, notably antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), and anticonvulsants such as carbamazepine (CBZ) (Kosma et al., 2014; Li, 2014). Given that they are frequently encountered in soil, raises concerns about their potential impact on primary consumers, but also the food chain. For example, carbamazepine and its metabolites were detected in the urine of humans following the consumption of fresh produce that was irrigated with treated wastewater (Paltiel et al. 2016).

Previous investigations suggested that concentrations of pharmaceuticals in the terrestrial domain remain below levels deemed harmful to humans and non-target organisms (Ohoro et al., 2019). However, given their 'pseudo'-persistence in wastewater (Rubirola et al., 2014), these compounds resemble a chronic threat to the receiving environment (Schnell et al., 2009). Psychiatric pharmaceuticals, with their intrinsic biological activity designed to target the nervous and endocrine systems, are of particular ecotoxicological interest (Argaluza et al., 2021) as anatomical structures and signalling pathways are phylogenetically conserved and present in many living organisms (Gunnarsson et al., 2008). The increased focus on chronic toxicity studies in non-target, albeit aquatic, organisms underscores the need to provide a comprehensive understanding of the long-term effects of pharmaceutical exposure (Radjenovic et al., 2007). CBZ is a commonly prescribed anti-convulsant, primarily used to treat epilepsy (Goodwin, 2003) and has recently been administered to treat post-COVID-19 olfactory dysfunction (Vasconcelos et al., 2022), thus presenting an example of an additional source of use and added exposure risk. CBZ exhibits slow absorption and undergoes autoinduction of metabolism, contributing to its persistence in the environment and is now a widely studied emerging pharmaceutical of concern (Puranik et al., 2013). This is, in part, due to the low removal efficiency of CBZ by wastewater treatment plants, which in some cases can even result in a negative removal efficiency (possibly due to the deconjugation), and this is notably not linked to a seasonal variation in concentration (Koba et al., 2016; Kråkström et al., 2020). Li et al. (2013) demonstrated that, in soil, CBZ was transformed into multiple intermediates, including 10,11-dihydro-10-hydroxycarbamazepine (DHC), whose high hydrophobicity (compared to CBZ) resulted in a reduced sorption and higher mobility in the terrestrial environment. This enables DHC to travel to deeper soil layers and thus poses a threat to the entire soil system, including groundwater (Malvar et al., 2020). Analogous epoxides have been observed when polycyclic aromatic hydrocarbons (PAHs) are metabolised through oxidation by cytochrome P450s (CYP450s). Other studies report additional intermediates (Koba et al., 2016; Paltiel et al., 2016; Thelusmond et al., 2016). These findings highlight the importance to elucidate the potential degradation pathway of pharmaceuticals in terrestrial compartments, as metabolites of CBZ might display higher mobility and toxicity than the parent compound.

CBZ has a high affinity to transfer from soil to plant tissues, not dissimilar to triclosan and triclocarban which are taken up by roots and then translocated to leaves and even fruits (Wu et al., 2012). For plants grown in commercial land, the potential human exposure to drugs via the daily consumption of root and leaf vegetables is estimated to be 0.01–0.21 % and 0.09–3.81 % of the acceptable daily intake (ADI), respectively (Carter et al., 2014). Exceptions are triclosan, lamotrigine and the metabolites of CBZ which can reach or even surpass the threshold of toxicological concern (TTC) (Malchi et al., 2014). Our limited knowledgebase concerning the human health risks derived from the plant-human food chain restricts our ability to predict the impact of the indirect ingestion of pharmaceuticals. However, Fremlin et al. (2020) assessed the trophic magnification of legacy persistent organic pollutants (POPs) in an urban food web encompassing some 13 species, including earthworms, and concluded that terrestrial food webs can suffer from higher biomagnification of certain organic compounds than aquatic environments, which might be due to the greater ability to absorb/digest their diet. However, within the terrestrial system, most work has focused on the transportation and transformation within the soil matrix and plant communities and only few have studied their uptake by soil invertebrates (Carter et al., 2014; Thelusmond et al., 2018; Dume et al., 2023). Earthworms play a crucial role in soil ecosystems by modulating physical characteristics and thus serve as key indicators of soil health (Stürzenbaum et al., 2009). Earthworms are omnipresent in temperate soils, contribute up to 90 % of soil fauna biomass, and the majority of soil will pass through the earthworm's gastrointestinal tract every 40 years (Ding et al., 2019). By occupying distinct ecological niches and pliant lifecycle strategies, earthworms showcase a spectrum of traits that contribute to their ability to adapt and succeed in diverse environments. Earthworms are not only a critical primary consumer but also a keystone organism for the assessment of the toxicity of chemicals, herbicides and pesticides. Recommended toxicity tests in respective OECD, EU and US guidelines have highlighted how earthworms can serve as an early warning system of deteriorating soil quality (European Commission, 2006; Peres, 2008; Cluzeau et al., 2012). The study of the toxicological responses of earthworms to environmental stressors provides crucial insights into the broader impact of these stressors on soil ecosystems and, by extension, on the health of the entire ecosystem (Asensio et al., 2013), which can be linked to the protection of human health. Earthworms are not merely simple soil-dwelling organisms but rather bridge the gap between soil ecology and genetic model organisms, offering valuable insights into the intricacies of the web of life. The applicability of using specific gene expression, e.g., metallothionein (*mt*) or glutathione (*gst*), as a biomarker of heavy metals and PAHs has been demonstrated (LaCourse et al., 2009; Dedeke et al., 2016).

This study utilized the earthworm *Dendrobaena veneta*, which is widely used in vermicomposting and has previously been used to study various biological and environmental applications (Natal-da-Luz et al., 2011; Molnar et al., 2015; Szederjesi et al., 2019). Herein, our objectives are: 1) to determine the correlation between soil carbamazepine levels and worm tissue concentrations, explore changes in the soil / worm microbiome and life cycle endpoints; and 2) to investigate the tissue-specific transcriptomic impact of exposing *D. veneta* to carbamazepine. The unravelling of how a widely consumed pharmaceutical such as carbamazepine affects non-target organisms will contribute to our understanding of earthworm physiology in a tissue-specific manner but also provide perspectives on potential environmental impacts and broader implications for soil health and ecosystem sustainability.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade and drugs (powder and 100 μg/mL in methanol) were purchased from Sigma-Aldrich (Gillingham, UK). Carbamazepine-10,11-epoxide and all other screened compounds were obtained from Sigma-Aldrich (Steinheim, Germany). Deuterated standards (100 μg/mL in methanol) were sourced from Merck (UK) and the other screened compounds were from QMX (Essex, UK). Ultra-pure (UP) water was obtained from a Millipore Milli-Q water purification system with a specific resistance of at least 18.3 M Ω cm (Millipore, Bedford, MA, USA). Stock solutions (10 mg mL^{-1}) were prepared in methanol and stored in vials (50 mL). Working solutions were prepared freshly in methanol, as required. All solutions were stored at 4 °C and in the dark for optimum stability.

2.2. Earthworms

Earthworms (*Dendrobaena veneta*) and soil were purchased from the Yorkshire Worm company (York, UK). Adult (clitellate) earthworms were acclimated in the dark at 15 °C in a constant temperature incubator (Sanyo MIR-154) for one week. The worm stock was cultured in topsoil (Yorkshire Worm Company, UK). Basic properties of the soil were: pH = 7.34, saturated water content 49 %, and 9.3 % organic matter. The soil was moistened with deionised (DI) water to reach 75 % of saturated water content and maintained at the 15 ± 1 °C. Worm stocks were fed with crushed oats monthly.

2.3. Experimental carbamazepine exposure

To prepare the incubation medium for each exposure group, 20 mL of methanol (solvent control) and diluted carbamazepine stock solution was spiked into the soil and the solvent residues allowed to evaporate for 48 h. Prior to exposure, adult worms were washed with DI water to remove attached soil particles and then weighed. In each bait box (1.9L), 10 acclimated *D. veneta* were randomly assigned to 200 g of soil. Containers with soil and earthworms were incubated at 15 ± 1 °C for 28 days. Each condition utilized five containers (replicates). By the end of the exposure duration, all surviving worms were cleaned with DI water, dried and weighted, then transferred for 48 h to a sterile petri dish containing moist filter paper (Whatman, UK) to purge the intestinal content.

2.4. Drug distribution in earthworms

Earthworm tissue samples were freeze-dried as described by Miller et al. (2019). The cuticle of earthworm could not be lysed by traditional methods, thus requiring the addition of a manual, pre-lysis grinding step under liquid nitrogen. For each sample, three replicates of 20 ± 1 mg were transferred to 2 mL of acetonitrile (MeCN): UP water + 0.1 % acetic acid (AcOH) (3:1) with 1 stainless steel bead and 50 μL deuterated standard (200 ng/mL). The lysis mixtures were simultaneously sonicated for 15 min and centrifuged at highest speed for 5 min at 0–5 °C. The supernatant was transferred to 100 mL 10 mM ammonium acetate (NH_4OAc) in UP water (pH 6.5). A Strata Alumina-N cartridge (6 mL, 1 g, Phenomenex Ltd., Cheshire, UK) coupled to an Oasis hydrophilic-lipophilic balanced (HLB) cartridge (6 mL, 200 mg, Waters Corp., Hertfordshire, UK) were used for tandem solid-phase extraction (SPE). The system was finally washed with 1 mL UP water and the HLB cartridges were dried on a manifold for roughly 30 min under vacuum, then eluted in 5 mL methanol. The extracts were reconstituted in 1 mL 0.1 % (v/v) formic acid in ultrapure water (mobile phase A), filtered with 750 μL nonsterile micro-centrifuge filters and immediately analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described in Miller et al. (2019).

2.5. Worm microsurgery

The earthworms were anaesthetised by submersion in cold (4–7 °C) carbonated water. The cerebral ganglion (CG) was identified, removed and immediately transferred into a sterile 1.5 mL microcentrifuge tube, then super-cooled in liquid nitrogen. The same earthworm was stretched, and its exposed segmental nerves were cut to release the intestine. The intestinal tract was removed and placed in a clean, super-cooled 1.5 mL tube and temporarily kept in a liquid nitrogen tank. Vertical incisions were made at the second and seventh posterior furrows to isolate the third to seventh most posterior tail segments, which was snap-frozen in liquid nitrogen. All samples were stored at −80 °C until further analysis. A further 3 anaesthetised worms per group were dissected under the microscopy, and the 5th to 20th post-/pre- clitellum segments were collected to represent the posterior/anterior tissues for chemical extraction.

2.6. Culturable bacteria in soil and earthworms

Microbiome cultures were isolated from soil and/or worms, then incubated in Luria broth (LB) overnight in a 37 °C shaking incubator at 150 rpm. The bacterial culture was then resuspended in 200 μL of water and transferred into a ZR BashingBead™ lysis tube for DNA extraction with the Quick-RNA™ Fungal/Bacterial Microprep Kit (Zymo research, Irvine, USA). Ultra-pure DNA was obtained by eluting with 50 μL DNA elution buffer into a clean 1.5 mL microcentrifuge tube. Bacterial DNA was directly used for 16S rRNA sequencing to genotypically identify species. Universal bacterial 16 s rRNA primers were utilized from which a 16S rRNA fragment corresponding to position 27 to 1392 was amplified with a series of selective polymerase chain reaction (PCR) runs. Products were gel purified, Sanger sequenced, and the resultant output blasted via NCBI for species identification.

2.7. Total RNA extraction from different sample types

TRIzol® Reagent (Invitrogen) was used to isolate total RNA from the posterior sections of the earthworms as well as the gut samples according to the manufacturer's specifications. The extraction of RNA from cerebral ganglion tissue samples by means of a standard TRIzol RNA extraction alone did not return satisfactory RNA yields in terms of quality nor quantity (the estimated concentration was below 50 ng/μL). By combining the TRIzol method with the Quick-RNA™ MiniPrep kit via an optimised extraction protocol facilitated superior sample concentrations and integrities which satisfied the requirements for downstream transcriptomic sequencing (Supplementary Fig. 1). In detail, each cerebral ganglion was mechanically homogenised in TRIzol® Reagent (Invitrogen) using an ultrasonic homogeniser (Model 120 Sonic Dismembrator, Fisher Scientific). The aqueous phase, after adding chloroform, was transferred to a new 1.5 mL microcentrifuge tube and 200 μL of 100 % ethanol (Merck) was added and mixed thoroughly. The content of the tube (~400 μL) was then transferred to a nuclease-free Zymo-Spin™ IC Column continuing as per the manufacturer's specification of the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, USA). To obtain sufficient RNA for reverse transcription, and subsequent qPCR, the cerebral ganglia from five individual worms were pooled. Samples prepared for RNAseq contained the RNA from an individual cerebral ganglion.

2.8. Characterisation of the Dendrobaena veneta population

The cytochrome *c* oxidase subunit II mitochondrial gene (COII) fragment was used to determine the genetical lineage of worms. All reactions were prepared in 0.2 mL tubes (ABGene) and the PCR reaction was performed in a thermal cycler (Applied Biosystems 2720). Additional details of all primers employed in this study are provided in Table S1.

The PCR products were purified via the Wizard® SV Gel and PCR Clean- Up System (Promega) and Sanger sequenced by Genewiz (UK). The sequencing results, together with COII sequences of five earthworm species (*Eisenia fetida*, *Lumbricus rubellus*, *Lumbricus terrestris*, *Dendrobaena octaedra* and *Dendrobaena veneta)* obtained either from NCBI or from previous projects were included to build a phylogenetic tree using the COII sequences on MegaX platform and annotated in iTOL.

2.9. De novo RNA sequencing of carbamazepine-exposed D. veneta

The transcriptomic profiling of carbamazepine-exposed earthworms was performed on earthworms inhabiting unexposed soil (control, CBZ_0) and compared to their counterparts exposed for 28 days to the same soil supplemented with a low dose of carbamazepine (0.6 mg/kg (CBZ_0.6)) or a high dose of carbamazepine (10 mg/kg (CBZ_10)). A bioanalyzer (2100, Agilent) was used to evaluate the quality and quantity of the RNA. Due to the possible divergence between

individuals, only samples that clustered in the same phylogenetic branch and with high-quality RNA samples were selected to construct the sequencing library.

Samples were sequenced on the Illumina platform (Illumina HiSeq X Ten). The *de novo* transcriptome, generated in Corset, was used as the reference in the alignment for gene expression analysis. Read count data was imported into the R platform and the normalisation validated prior to further analysis. The DESeq package was then used to identify differential gene expression between exposure groups. Subsequently, differentially expressed genes were queried against NR (NCBI non-redundant protein sequences), Nt (NCBI nucleotide sequences), Pfam (the prediction of protein structure domain), KOG (euKaryotic Orthologous Groups), COG (Cluster of Orthologous Groups of proteins), and KEGG (Kyoto Encyclopedia of Genes and Genome) with database-specific alignment thresholds. The successfully annotated genes were further grouped within the three gene ontology (GO) domains, namely the biological process (BP), the cellular component (CC), and the molecular function (MF). WGCNA34 (v1.67) was employed and a weighted signed network was built to conform to scale-free topology. The driver genes were defined by the weighted gene co-expression network analysis (WGCNA) connectivity algorithm. The dataset is available under gene expression omnibus (GEO) submission GSE268697.

2.10. Optimization of quantitative polymerase chain reaction (qPCR)

The expression level of target genes was assessed via qPCR using the QuantStudio™ 3 Real-Time PCR System in a MicroAmp Fast Optical 96-well plate format (Applied Biosystems) using a standard protocol. Each biological sample was run in quadruplicates, and for each condition a minimum of three biological replicate was quantified. To ensure the reliability of the qPCR reactions, a melting curve analysis was performed at the end of each qPCR. The choice of housekeeping gene was optimized by exploring the inter quartile range (IQR) of the variation in expression over the studied conditions. The expression of the reference genes *rla-1* and *gapdh* changed significantly across the experimental conditions ((IQR = 6.43 and IQR = 1.68, respectively) while *18S* varied least (IQR = 0.41). Thus, *18S* was chosen as the reference gene for the following analysis. Differences in gene expression levels were determined by means of the $2^{-\Delta\Delta Ct}$ Livak method. Primer sequences are provided in Table S1.

2.11. Statistical analysis

Statistical analysis was conducted with RStudio. One-way ANOVA and Student's t-tests assessed the significance between the groups with the statistical significance level threshold set to $p < 0.05$. Asterisks indicated significant differences in data (* = $p < 0.005$, ** = $p < 0.001$, *** = $p < 0.005$). All results were expressed as the mean ± SD.

3. Results

3.1. Acute toxicity test of carbamazepine exposed earthworms (Dendrobaena veneta)

To evaluate the toxic potential of carbamazepine exposure, a standard OECD acute mortality test was employed (Test No. 207: Earthworm, Acute Toxicity Tests, 1984), however substituting artificial soil for the topsoil inhabited by worms. Due to the poor solubility of carbamazepine an organic solvent with high volatility was required to achieve the desired soil concentration and minimize potential stress to earthworms from the carrier solution. Methanol was selected as the carrier solvent, suitable for the drug used in this study, and any residual solvent stress on worms was controlled by spiking the same amount of methanol into each soil sample before adjusting moisture levels to 75 % of the saturated water content. Exposure conditions and within-group variations were confirmed through LC-MS/MS analysis of the incubated soil. The presence of sharp peaks in matrix- matched standards facilitated the identification and quantification of carbamazepine in the samples (Fig. 1A).

In the methanol-only group (the control), carbamazepine was detectable at 0.47 ± 0.10 mg/kg, indicating the presence of low background concentrations of carbamazepine in the soil. The within-box variation was below 1 % in most groups, suggesting that the drug was evenly distributed in each exposure box; one exception was the 50 mg/kg condition where mean measured carbamazepine concentration in the soil was 56.28 ± 2.86 mg/kg (n = 5) (Fig. 1B). All worms (bar one, which died) moved actively and responded vigorously to touch during the 28-day test period, confirming that *D. veneta* tolerated the carbamazepine doses used (0 to 100 mg/kg dry soil weight, data not shown). As lethal concentration 50 (LC50) data could not be determined, an alternative "effect concentration" endpoint was chosen, namely percentage weight change. Carbamazepine exposure significantly impacted the body weight of *D. veneta* (Fig. 1C). Whilst the weight of earthworms in the control group presented no significant difference over the 28-day period, all carbamazepine-exposed worms gained weight, with the highest weight change ratio (16.83 ± 4.47 %) observed in worms exposed to 100 mg/kg carbamazepine (Fig. 1C). However, given the large data pool of 150 worms, no linear relationship between weight change ratio and environmental carbamazepine levels was observed.

3.2. Accumulation of carbamazepine in D. veneta

In *D. veneta* most organs are in the segments anterior to the clitellum, including reproductive (ovaries and seminal vesicles), neural (cerebral and suboesophageal ganglion), and circulatory organs (the heart-like aortic arches). In contrast, the post-clitellum segments mainly contain the intestinal tract. This study explored whether carbamazepine distribution within the earthworm body differed between these two distinct regions (Fig. 2A). The observed shift in retention times reflects the matrix effect between the (organ-rich) anterior and (mainly intestine containing) posterior segments (Fig. 2B). Therefore, all subsequent analyses were performed using two parallel calibrations established with *D. veneta* tissues from the anterior or the posterior segments, respectively. As with the control soil, carbamazepine could also be detected in earthworms from the negative control group (anterior segments: 8.9 ± 2.4 ng/g and posterior segments: 14.3 ± 1.1 ng/g). Upon exposing earthworms to soils spiked with carbamazepine (50 and 100 mg/kg), the accumulation pattern in the posterior segments started to plateau after one week but continued to increase in the anterior segments over time (Fig. 2C and D). Overall, the tissue concentrations of the anterior of the worm correlated with the soil dose, revealing a clear dose- and exposure time- responsive accumulation of carbamazepine ($R^2 > 0.90$). Posterior tissues exhibited a maximum accumulation of approximately 1500 ng/g, beyond which the drug either relocated to the anterior segments and/or was eliminated (excreted) (Fig. 2D). Carbamazepine-10,11-epoxide, the major carbamazepine metabolite, was not detected in worms (data not shown), suggesting that the metabolic pathway of carbamazepine in soil and its inhabiting earthworms may differ to higher animals.

3.3. Changes in microbiome population structure upon exposure to carbamazepine

It was deemed important to study the microbiome in the soil and earthworm upon exposure to 10 mg/kg carbamazepine. Focussing on the morphology of culturable bacterial populations suggested that the presence of rhizoid-type bacteria was generally reduced in carbamazepine-spiked soil, and irregularly shaped bacteria dominated the extracts derived from the guts of carbamazepine-exposed earthworms (Fig. 3, left panel). Individual bacterial colonies were subsequently submitted for 16S rRNA gene sequencing and 52 of 56 colonies were classified into nine bacterial families/genus. A shift in the relative abundance of bacteria was observed, with *Aeromonadaceae spp*

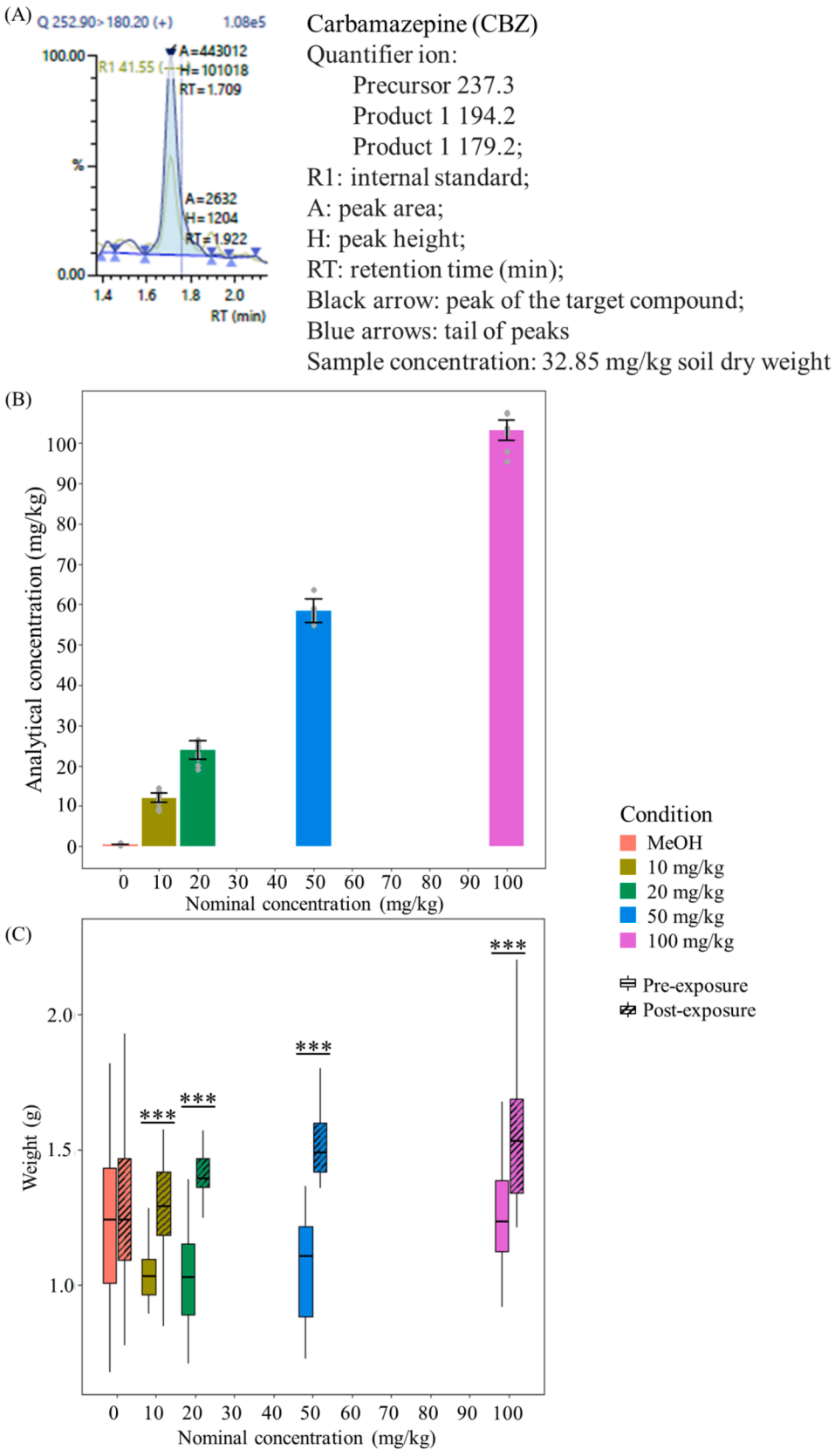

Fig. 1. Carbamazepine exposure-dependent changes in earthworm weight. (A) Optimized liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantitative detection of carbamazepine within the soil matrix. (B) The nominal doses of carbamazepine (0 to 100 mg/kg) added to soil in 1.9L boxes correlated with the analytical concentration quantified by LC-MS/MS (n = 5 per condition). (C) Changes in earthworm weights pre- and post-exposure to carbamazepine. Exposures were conducted by placing 10 randomly assigned earthworms (*Dendrobaena veneta*) into 200 g of soil (spiked with the appropriate carbamazepine concentration), then incubated at 15 ± 1 °C for 28 days. In total 50 worms (replicates) per condition were weighed pre-and post-exposure.

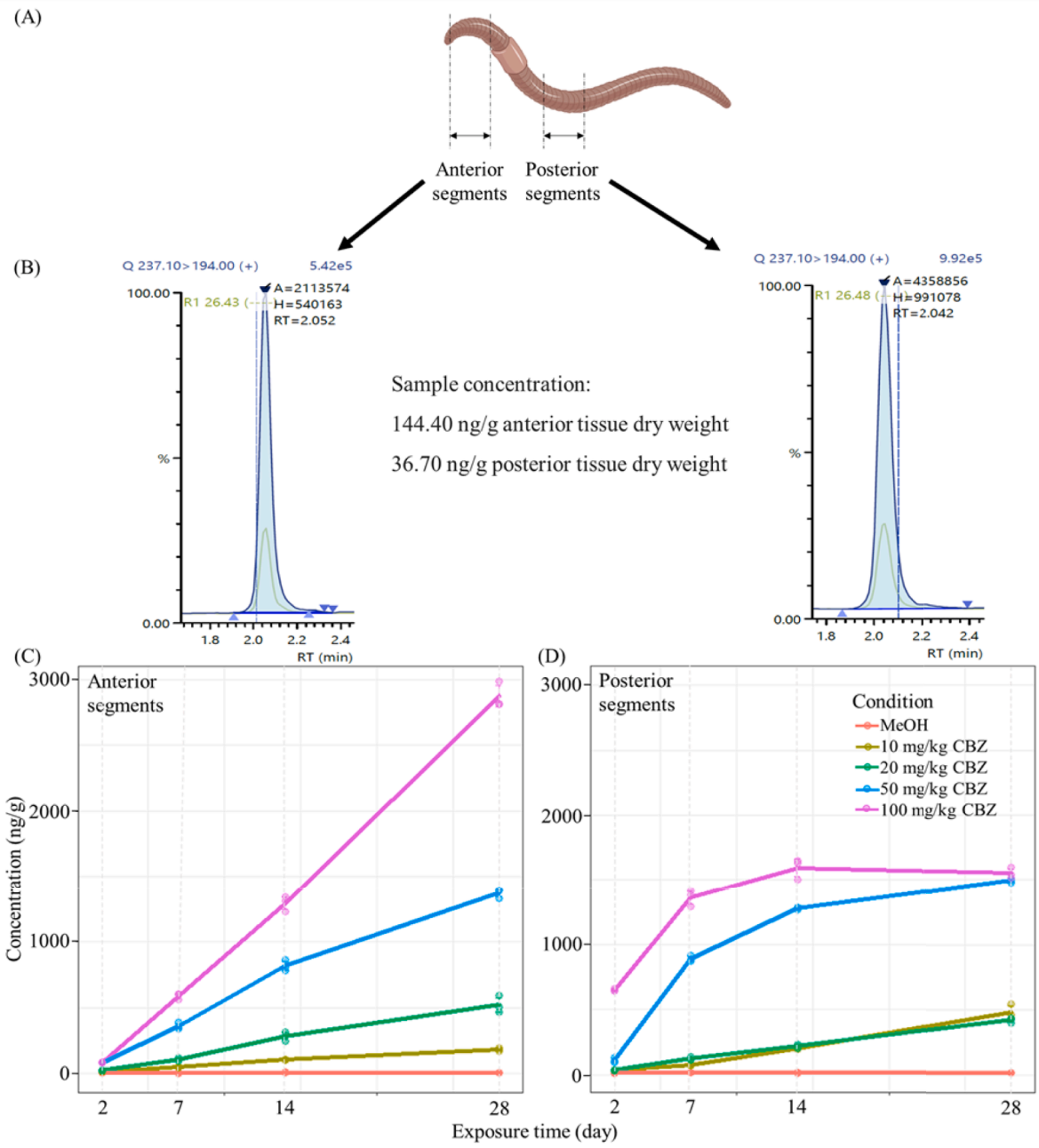

Fig. 2. Accumulation of carbamazepine in earthworm anterior and posterior segments. (A) Earthworm cartoon depicting the location of the anterior and posterior segments used for analysis. (B) Examples of carbamazepine LC-MS/MS chromatography signals captured for each tissue section. (C) Carbamazepine concentrations measured in the anterior (C) and posterior (D) tissues in earthworm exposed to carbamazepine (0 to 100 mg/kg).

significantly inhibited under carbamazepine exposure with a corresponding increase in *Enterobacteriaceae spp* (Fig. 3, right panel).

3.4. Tissue-specific adipogenic transcription responses of carbamazepine exposed earthworms

Carbamazepine primarily affects the late stages of adipocyte differentiation and enhances adipogenesis. Therefore, mRNA levels of adipogenic transcription factors (genes encoding sterol regulatory element binding proteins, *srebp* and the tubulin polymerisation promoting protein, *tppp*) were screened across a range of carbamazepine exposures (Fig. 4). Concentration gradients from 0.025 to 100 mg/kg were established, introducing additional doses to extend the detection range. In the cerebral ganglion (Fig. 4B), expressions of both transcripts were upregulated in all conditions, with the *tppp* presenting a concave dose response peaking at 0.6 mg/kg (19.2 ± 3.2 fold, $p < 0.05$). In the gut of the earthworm certain carbamazepine concentrations inhibited, whilst others significantly induced the transcription of the two genes (Fig. 4C). The expression of *srebp* was more induced in the gut tissue than in the cerebral ganglion, where relative fold changes remained below 1.5 across all conditions. Conversely, the expression of *tppp* was more dynamic in the cerebral ganglion than in the gut tissue, particularly in the exposure groups 10 and 20 mg/kg carbamazepine.

3.5. Transcriptomics of the gut and cerebral ganglion of carbamazepine exposed worms

Two carbamazepine exposure concentrations (0.6 and 10 mg/kg)

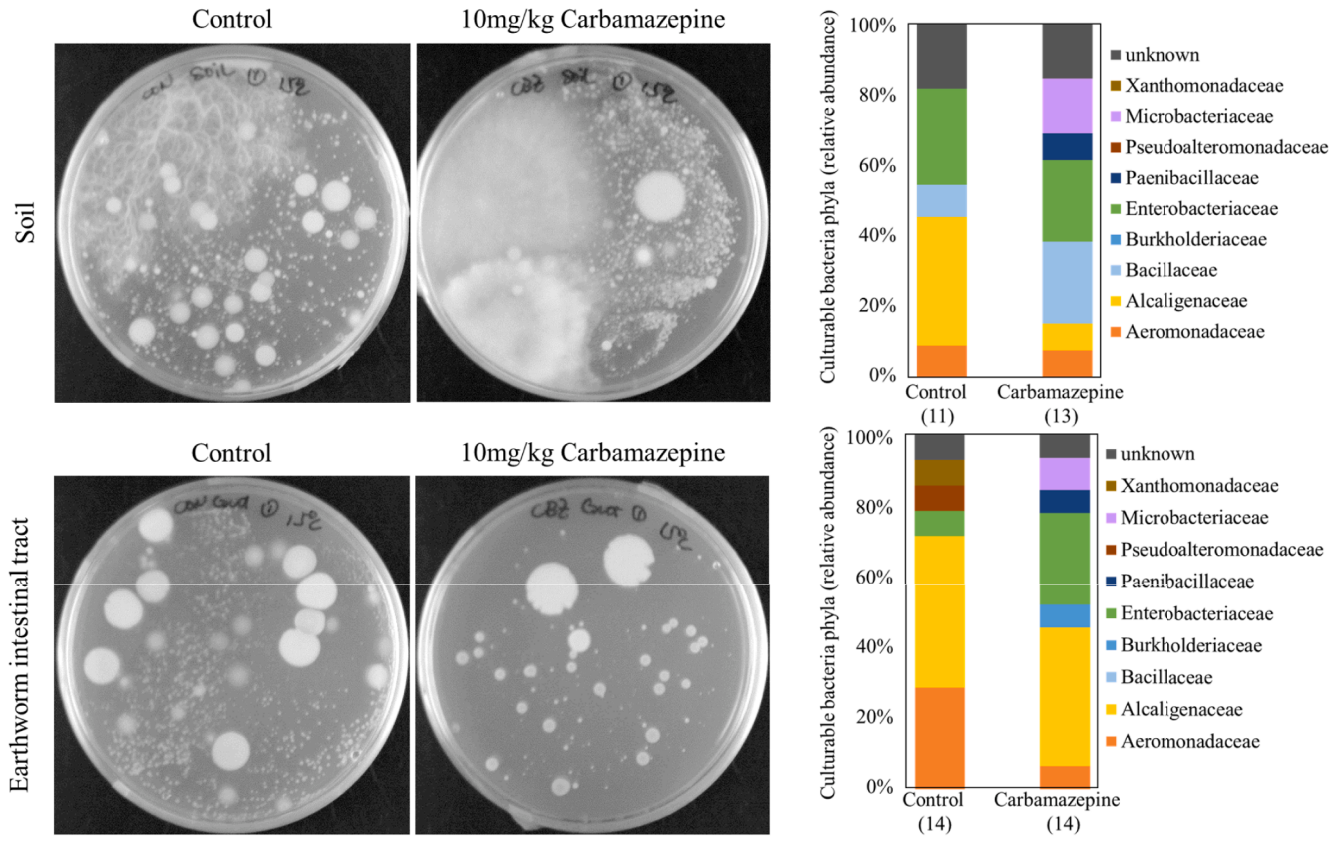

Fig. 3. Phenotypic appearance and identification of culturable bacterial colonies from soil and the intestinal tract of earthworms (in the presence or absence of 10 mg/kg carbamazepine). The bacterial cultures were grown on agar plates and the identity and relative abundance, was determined by 16S sequencing. The number of sequenced colonies is provided in brackets).

were selected for transcriptomic analysis by RNA sequencing. Given that the qPCR of individual genes identified intra-species variation in expression it was of paramount importance to identify and select closely related individuals. Therefore, 50 worms per exposure group were screened for phylogenetic relatedness by sequencing the COII gene. Overall, 5 distinct sub-populations of *D. veneta* were observed, marked as Group A-E (Fig. 5). Group C was the largest group with 6 % (18 base pair) within-group differences. The large group size provided sufficient statistical power to identify the genotypically most similar worms for downstream RNAseq.

The RNAseq analysis identified several significant differentially expressed (DE) contigs (Tables 1 and S2) which notably differed in the gut and cerebral ganglion samples, thus highlighting the differences in the dynamic nature of biochemical and metabolic/detoxification processes in these two organs. In another comparison, 7671 and 3573 DE transcripts were identified in worms exposed to 0.6 or 10 mg/kg carbamazepine revealing the presence of a dose-responsive change within a transient transcriptomic landscape.

The subsequent annotation of DE transcripts identified the most responsive transcripts, however at least 50 % did not have a reliable sequence annotation (Tables 1 and S3). This suggests that many DE transcripts are unique to earthworms or even to the species *D. veneta.* GO ontology enrichment analysis uncovered that the gut and the cerebral ganglion tissue responded uniquely to different carbamazepine exposure levels (Fig. 6) with only one shared molecular function identified in both tissues (proton transmembrane transporter activity, annotation rates $<$ 10 %, $p < 0.005$). In the dataset comparing the upregulated transcripts in CBZ_0_gut vs CBZ_0.6_gut, 8 transcripts were involved in the anion transport, five of which are members of the solute carrier family (*slc4a4*, *slc13a3*, *slc12a6*, *slc12a7*, and *slc5a5*) and the others relate to the chloride channel. In total some 750 significantly enriched BPs, 289 significantly enriched MFs, and 122 significantly enriched CCs were identified in the CBZ_0_cg vs CBZ_0.6_cg comparison, which demonstrates the dynamic biological activity in the cerebral ganglion. Common identifiers in the cerebral ganglion were typically related to energy processes, while the cerebral ganglion (10 mg/kg exposure) presented a neuron enriched GO term (Fig. 6). In all comparisons, more upregulated transcripts could be identified than downregulated transcripts. This was in particular the case in the CBZ_0_cg vs CBZ_0.6_cg comparison, which returned an 11-fold difference between upregulated and downregulated DE transcripts.

A shared response could be identified between two exposure concentrations within the same tissue type, and the KEGG pathway enrichment analysis with DE transcripts presented a more homogeneous pathway pool in the higher exposure group (Fig. 7). In the top 10 enriched pathways, 7 were related to metabolism. The cytochrome P450 pathway was only identified in the gut from the 0.6 mg/kg CBZ exposure group, and all enriched genes were annotated as UDP-glucuronosyltransferase (UDPGT) or glutathione-S-transferase (GST). Cerebral ganglion specific pathways mainly included signalling pathways, such as notch signalling, mechanistic target of rapamycin (mTOR) signalling and transforming growth factor-beta (TGF-beta) signalling, while in gut tissues the most enriched pathways were metabolism related (Fig. 7). Metabolic pathway was the only common down-regulated DE pathway.

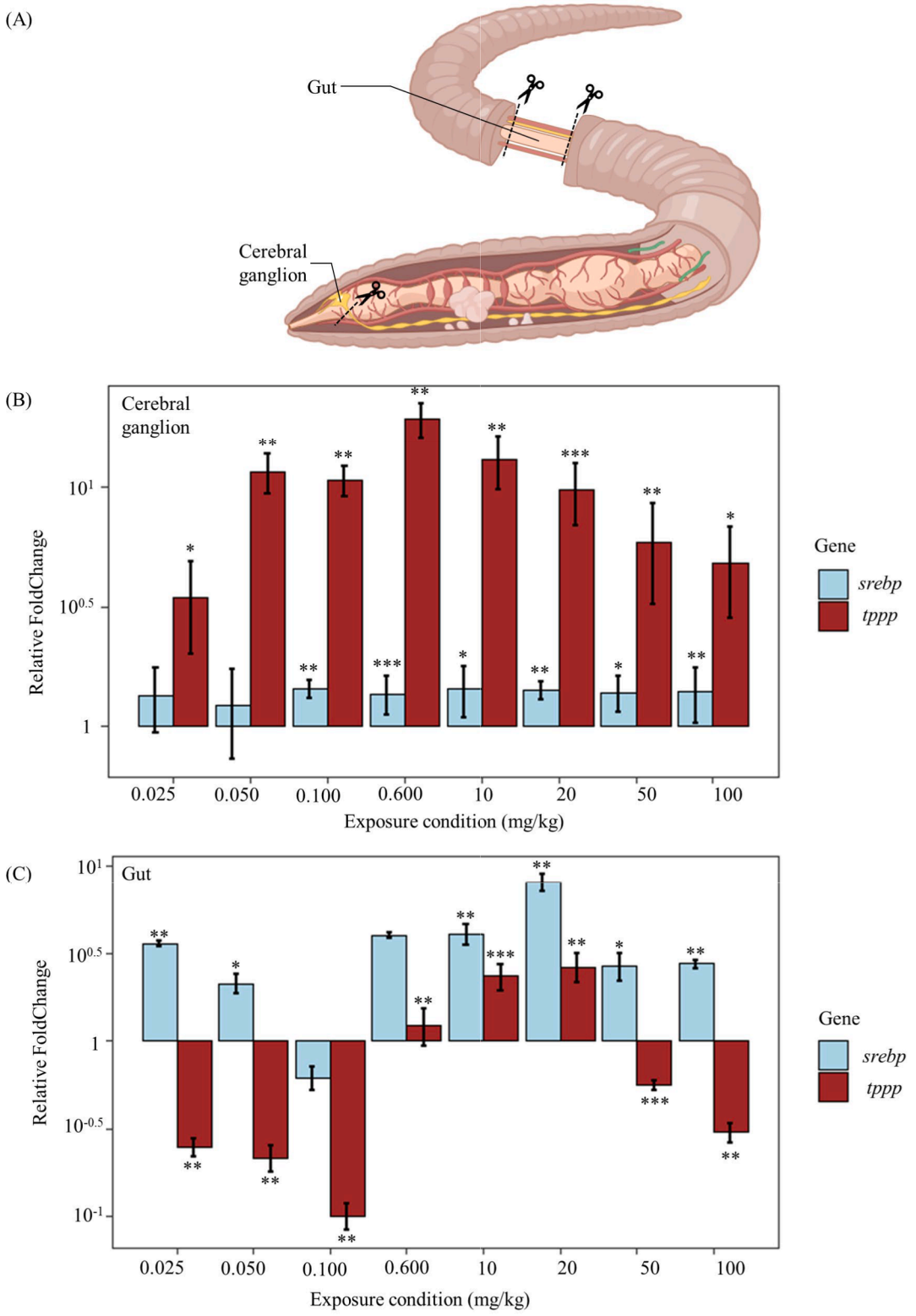

Fig. 4. The relative fold change of *srebp* and *tppp* expression in the cerebral ganglion and gut of carbamazepine exposed *D. veneta*. (A) Cartoon pinpointing the location of the cerebral ganglion (cg) and gut dissected from worms. Relative change in gene expression of *srebp* and *tppp* in the cerebral ganglion (B) and gut (C) of worms exposed for 28 days to dose range of carbamazepine (0—100 mg/kg). The expression was normalized to 18S. Each datapoint consists of quadruple technical repeats on three biological replicates. The statistical significance was labelled by stars, where * refers to $p < 0.01$, ** to $p < 0.005$ and *** to $p < 0.001$.

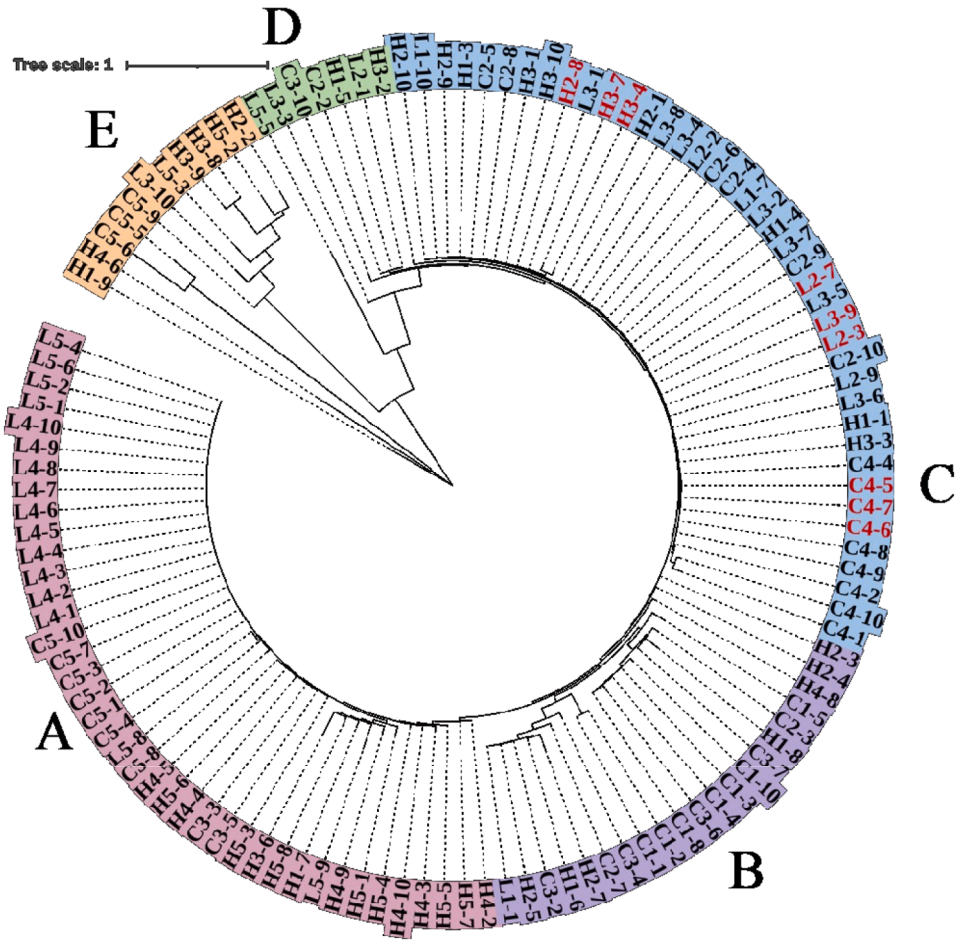

Fig. 5. Phylogenetic classification of earthworm genotypes. Total RNA was extracted from the posterior tissue of 50 worms in each of the three experimental conditions (control, C; low dose at 0.6 mg/kg, L; high dose at 10 mg/kg, H) and screened by Sanger sequencing based on their cytochrome *c* oxidase subunit 2 (COII) sequence. Distinct *D. veneta* sub-populations (A to E) were identified. The samples selected for RNAseq are highlighted in red font. The evolutionary relatedness was inferred via the Maximum Likelihood method and the Tamura-Nei model in MEGAX. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Number of differentially expressed transcripts in the cerebral ganglion and gut tissues of *Dendrobaena veneta* exposed to 0.6 or 10 mg/kg carbamazepine, compared to the matched tissue of unexposed worms. "CBZ_0" refers to the gut tissue extracted from worms in the unexposed control group, whereas "CBZ_0.6" and "CBZ_10" are samples derived from worms exposed to either 0.6 or 10 mg/kg carbamazepine, respectively. Two tissues were dissected, consisting of either the cerebral ganglion (cg) or the gut. Listed are the total number of differentially expressed genes and the subpopulation containing a functional annotation (for details of the identifiers of the most significantly changing transcripts see Tables S2 and S3).

Comparison	Number of Differentially Expressed Transcripts (padj < 0.05)	Number of Differentially Expressed Transcripts with annotations (padj < 0.05)
CBZ_0_gut vs. CBZ_0.6_gut	5216	2715
CBZ_0_gut vs. CBZ_10_gut	3052	1287
CBZ_0_cg vs. CBZ_0.6_cg	3523	1926
CBZ_0_cg vs. CBZ_10_cg	1296	522

3.6. Weighted gene co-expression analysis

One expression module had a strong positive correlation coefficient of 0.49 ($p < 0.001$) with earthworm weight change (Supplementary Fig. 2). The most enriched biological processes in this module were associated with the regulation of nerve system development as well as nucleobase-containing compounds, and metabolic process with the most enriched gene cluster related to the zinc finger family. Two other key modules identified in the cerebral ganglion were enriched in transcripts encoding neuropeptide and functioning in neuron cell adhesion, respectively. In contrast, the gut tissue module was dominated by activities in metabolic processes.

4. Discussion

In the past, the standard process to determine the species of an earthworm relied on phenotypic identifiers, however more recently the reliability and the resolution of the traditional method has been questioned (Pop et al., 2003; Dupont, 2009). In this study, most worms were characterised by red bands with yellow/white intersegmental furrows and three pairs of setae, thus classified as members of the *Dendrobaena* genus (Oligochaeta, Lumbricidae), however due to the cryptic features it was difficult to separate worms further, at least based on morphological approaches. The identification of an earthworm population to (sub)

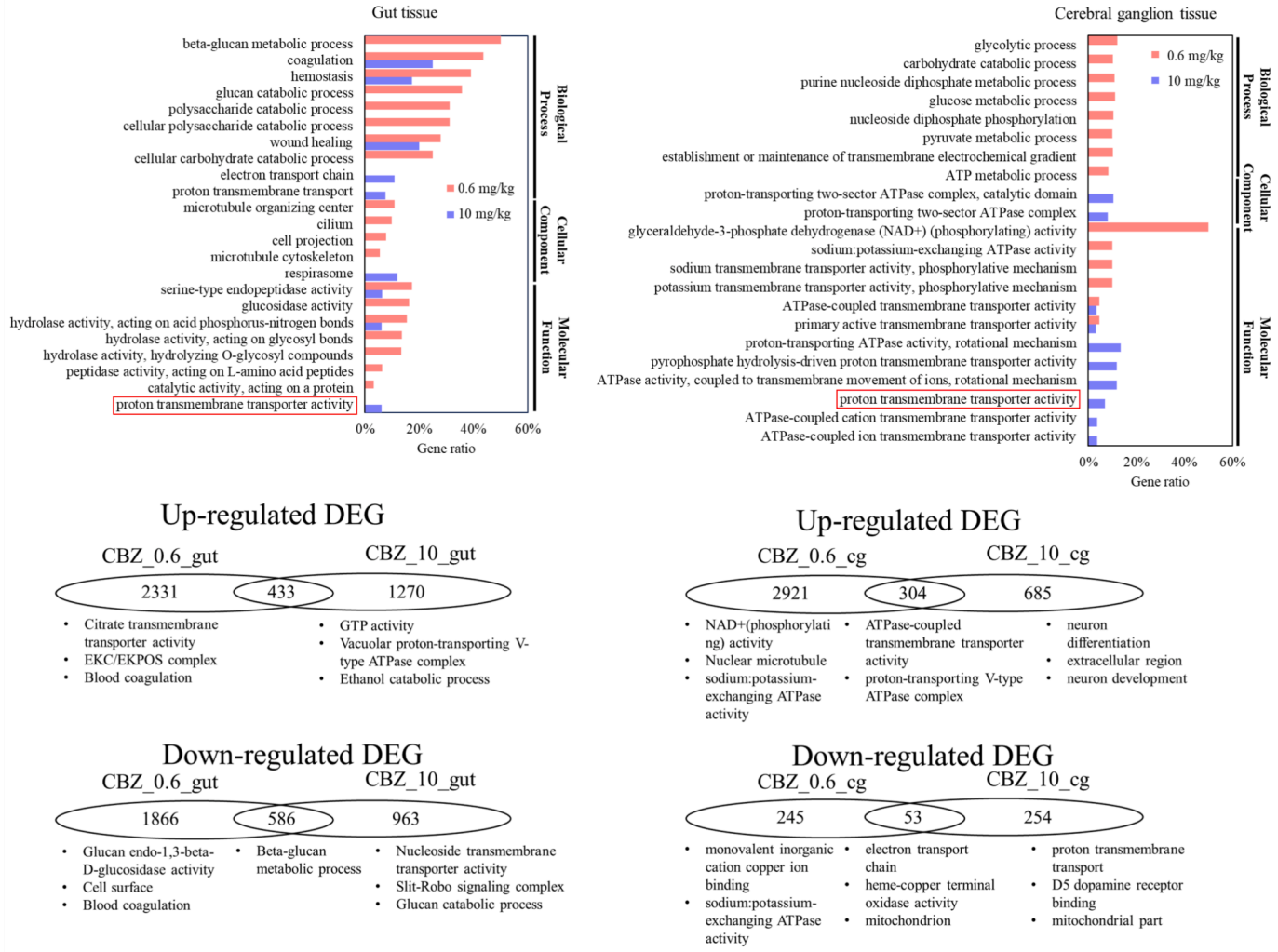

Fig. 6. Transcriptomic changes and GO enrichment analysis of differentially expressed genes (DEGs) in the gut and cerebral ganglion of worms exposed to 0.6 and 10 mg/kg carbamazepine. Top panel: Bar plot of the most enriched GO terms identified in gut (left) and cerebral ganglion (right) of carbamazepine exposed worms. Overrepresented biological processes (BP), cellular components (CC) and molecular functions (MF) from worms exposed to 0.6 mg/kg carbamazepine and 10 mg/kg are displayed in pink and purple, respectively; shared annotations are highlighted by a red box. Bottom panel: Venn diagrams comparing the overall number of significant GO annotations, in gut (left) and cerebral ganglion (right) tissues with a summary of highlighted terms listed below. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species level is challenging and error prone, a notion that was highlighted by studying the heterogeneity of earthworms provided by commercial suppliers in the UK (Katsiamides and Stürzenbaum, 2021). Similarly, a *Dendrobaena veneta* taxonomy study confirmed that the intra-clade genetic distance within the species is roughly 0.5 % (Szederjesi et al., 2019). However, the authors noticed that some Genebank entries generated a high intra-clade diversity exceeding 10 %, supporting the need to apply high-resolution techniques to confirm species identity. Indeed, only 60 % of 28 ecotoxicological test laboratories were able to apply phenotype-based taxonomic assignments to correctly identify earthworms to species level (Römbke et al., 2016). Misidentification of phenotypically similar species can have significant downstream ramifications on experimental design and most importantly on the interpretation of the results. For example, the mortality rate of *E. fetida* challenged with the phosphorothioate insecticide Fenitrothion was reported to be eight times higher than *D. octaedra* (Addison and Holmes, 1995). *D. veneta* was also described to be more susceptible to exposures of anaesthetics, glyphosate, and some pesticides (e.g., carbaryl, a cholinesterase inhibitor) than *E. fetida* (Stenersen et al., 1992). *D. veneta* has previously been described as an earthworm species which is less resilient to environmental toxic compounds (such as pesticides and microplastics) than *E. fetida* and *L. rubellus* (Stenersen et al., 1992; Suleiman et al., 2017; Short et al., 2021). This present project however demonstrated that even supratherapeutic concentrations of carbamazepine (10 to 100 mg/kg dry weight) was not lethal to *D. veneta*, possibly due to an efficient drug detoxification system which may guard the worm from carbamazepine induced (neuro)toxicity.

The biotransformation and biodegradation process of carbamazepine is dependent on bacteria strains (Gauthier et al., 2010; Sauvêtre et al., 2018) in the soil, but also the guts of its inhabiting earthworms. It is conceivable that the existence of carbamazepine in the soil can shift the abundance and activity soil and earthworm microbiome and the identification of *Pandoraea pnomenusa* and *Paenibacillus* sp. *RUD330* in the intestinal tract of exposed worms (but not in the unexposed earthworm) might contribute to the earthworm's response to carbamazepine exposure. CBZ has also been found to impact the gut microbiota of several other organisms. For example, CBZ altered the structure of the bacterial community in the gut of another soil model organism, namely the collembola *Folsomia candida*. The exposure to CBZ resulted in an enrichment of *Arthrobacter*, *Achromobacter*, *Gordonia*, and *Shinella* in their guts but also enhanced the abundance and diversity of antibiotic resistance genes (ARGs), including beta-lactams and multidrug resistance genes

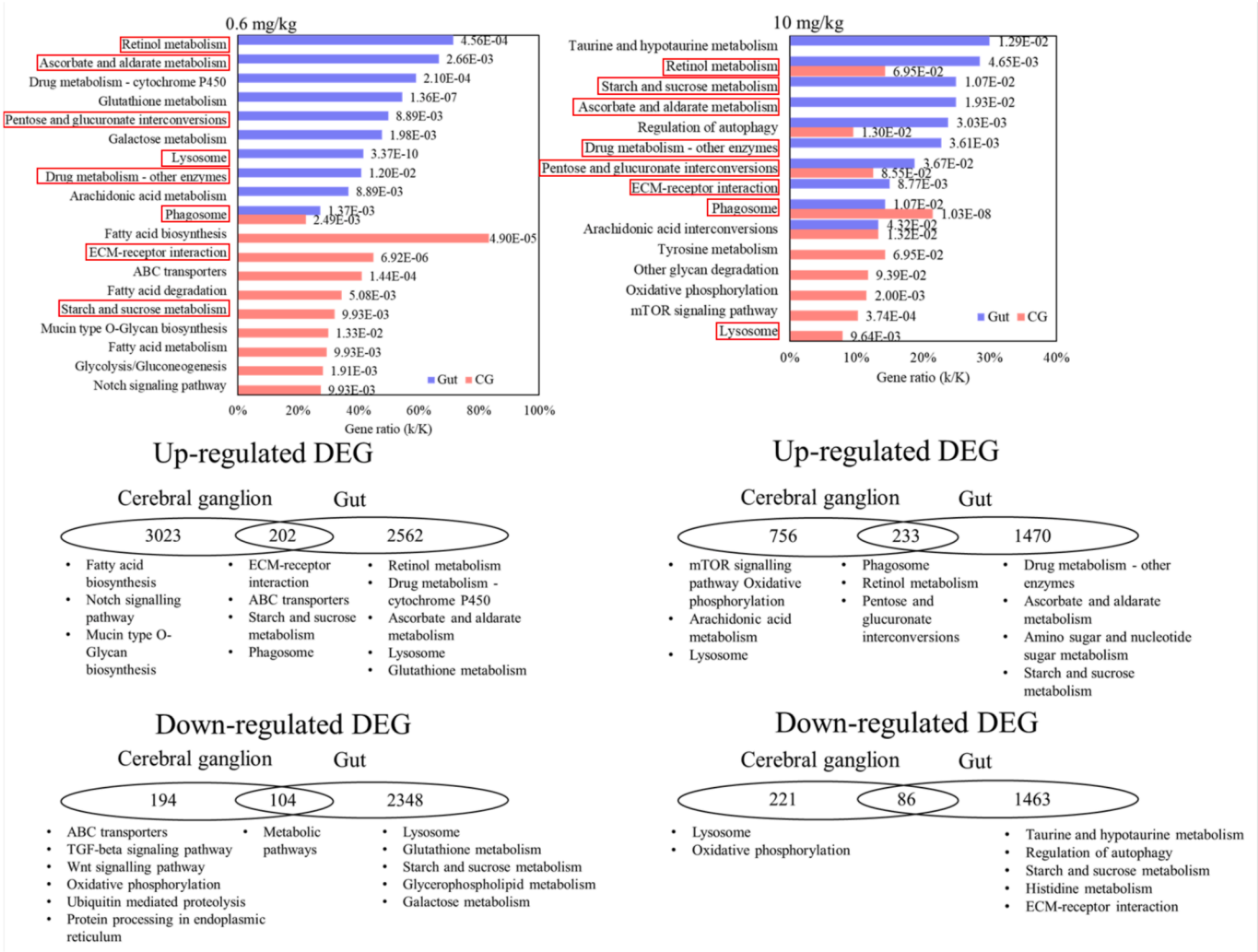

Fig. 7. KEGG pathway enrichment analysis of differentially expressed genes (DEGs) in the gut and cerebral ganglion of worms exposed to 0.6 and 10 mg/kg carbamazepine. Top panel: Bar plot of top 10 enriched KEGG pathways identified in the 0.6 (left) and 10 mg/kg (right) carbamazepine group. The data from gut and cerebral ganglion are highlighted in purple and pink, respectively; shared annotations are highlighted by a red box. Bottom panel: Venn diagrams comparing significant KEGG pathways identified using all DEGs in the 0.6 (left) and 10 mg/kg (right) carbamazepine exposure group with a summary of highlighted terms listed below. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Wang et al., 2020). In tadpoles, CBZ decreased the relative abundance of some bacterial genera (e.g., *Blautia*, *Prevotella*, *Bacillus Microbacterium*), while causing others (e.g., *Paucibacter*) to increase. Likewise, recent studies have found that CBZ impacts gut microbiota composition, such as ARG profiles and the expression of intestinal health related genes in a zebrafish model (Yang et al., 2023). In humans, exposure to CBZ reduced the growth of more than ten bacterial strains linked to the microbiota in young children with epilepsy (Ilhan et al., 2022). Overall, CBZ has anti-commensal activity across taxa, shaping the microbiota structure from diverse environments and host organisms. Whether changes in the structure and function of the microbiota induced by CBZ directly impacts host neural function and behaviour remains to be investigated. This is supported by further studies that show that the administration of atypical antipsychotics induce dysbiotic alterations to the gut microbiota composition and that antibiotic-induced dysbiosis has been linked to mental disorders such as anxiety and depression (Pryor et al., 2020). Shifts of both soil and gut microbiota might therefore result in the disruption of earthworm detoxification processes and immune responses (given that gut microbiota play a critical role in nutrient assimilation and pathogen defence), or the alteration of soil functionality by impairing nutrient cycling and organic matter decomposition. Although the amount of the carrier (methanol) used to spike the soil was small, its impact on the soil microbiome may pose a confounding factor. This was highlighted in previous laboratory experiments where only 67 % of CBZ could be accounted for by mass across the biosolid layer, soil and leachate fractions. This indicates the potential for microbial transformation, which may have been disrupted by the methanol solvent (Barron et al., 2010).

The observed weight gain in carbamazepine-exposed worms aligns with the findings derived from the diagnostic and statistical manual of mental disorders (DSM-III) in humans (Joffe et al., 1986). However, it is still debated whether weight gain is related to the pharmacokinetics of the drug or a consequence linked to the improvement of mental disorder status. Any correlation with earthworms made here are of course, at best, circumstantial but nevertheless intriguing. The overlap might suggest a potential conservation of mechanisms across taxa, a notion which warrants further investigation. The observed weight gain may originate from an enhanced adipogenesis or a reduction in metabolic rate, as suggested by the tissue-specific upregulation of adipogenic transcription factors such as *srebp* and *tppp*. The reduced difference in weight ratio between exposed and unexposed worms at the highest carbamazepine exposed group (Fig. 1) might be due to the higher starting weight in this experimental group, which could limit the relative growth potential compared to smaller (lighter) worms in other

groups. This is supported by the observed modulation of adipogenic transcription factors and suggests that baseline metabolic states or the microbiome composition influence the response to carbamazepine exposure. Indeed, a shift in microbiome population, such as the observed reduction in rhizoid bacteria, might impose changes linked to nutrient absorption and metabolic efficiency.

The tissue-specific accumulation of carbamazepine in *D. veneta* likely arises from differences in exposure, tissue function, and local metabolic activity. The anterior segments, housing organs like the cerebral ganglion and reproductive structures, displayed higher accumulation rates over time compared to the posterior segments, primarily composed of the intestinal tract. This pattern may reflect the differential vascularisation and permeability of tissues, with anterior tissues potentially being able to absorb more compounds. Another plausible mechanism involves the binding affinity of carbamazepine to tissue-specific proteins or lipids. The cerebral ganglion, rich in neural tissues, may selectively retain lipophilic compounds like carbamazepine, resulting in a prolonged exposure and neurotoxic effects (Banks, 2009; Starnes et al., 2022). Conversely, the gut is in direct contact with contaminants, therefore will drive initial uptake but also facilitate rapid excretion, accounting for the observed concentration plateau in the posterior tissue. The lack of detected carbamazepine metabolites, such as carbamazepine-10,11-epoxide, provides further evidence that the earthworm's metabolic pathways contain unique elements. Differences in enzyme expression profiles between tissues can explain the selective retention and processing of carbamazepine. Identifying these tissue-specific mechanisms is critical to provide a full understanding of how contaminants are distributed and metabolized in soil invertebrates, which in turn impacts the health status of the individual as well as the ecosphere.

This is the first study to specifically define and compare the transcriptomic profiles in neuronal and intestinal tissues of earthworms exposed to carbamazepine. Previous transcriptomic studies focussed on pharmaceutical and personal care product exposure, however they extracted RNA from the entire earthworm, rather than specific organs (Hattab et al., 2015). The methodology applied here ensured that the transcripts identified were differentially expressed in the tissue of initial contact (the intestine) or the target organ (the brain). Given that more differentially expressed genes (DEGs) were identified in the gut tissue, compared to the cerebral ganglion (CG), may suggest that transcriptomic activities are more dynamic in the intestine, the organ that is in direct contact with the drug, as opposed to the CG. This finding was supported by the chromatography-based results, where the accumulation of carbamazepine at the anterior segments (the area where the CG is positioned) had not reached a noticeable plateau by the time the experiment was terminated (as shown in Fig. 2). However, it has been argued that transcriptomics might not sufficiently serve as a reliable predictor of reaction rates, especially under environmental conditions where the combination of asynchronism, substrate dynamics, production and decay of transcripts/enzymes, and ambient conditions will contribute to a change in expression patterns (Störiko et al., 2021). When processing transcriptomic information from a mixed population of cells, certain responses might indeed be masked if only some cells are involved in the response to the stimulation. This phenomenon, known as bimodal gene expression, has been observed in numerous transcriptional experiments, especially studies exploring threshold stress levels (Paliwal et al., 2007; Pelet et al., 2011).

Transcriptomics focussed not only on the absolute number of transcripts expressed in each organ but also quantitative aspects of differential regulation. For example, the expression of a serine protease inhibitor was strongly inhibited in the gut but induced in the cerebral ganglion of earthworms exposed to 10 mg/kg carbamazepine, a concentration that is one magnitude above environmental hotspots. The expression of *wech*, a gene encoding a crucial component of the integrin-cytoskeleton during development (Löer et al., 2008), was strongly inhibited in the gut but induced in the cerebral ganglion of earthworms exposed to 10 mg/kg carbamazepine. Such activation in the cerebral ganglion of worms exposed to carbamazepine (0.6 mg/kg) may reflect the onset of tissue damage. Indeed, *wech* is thought to be a member of cytochrome b5 family and engages in the metabolism of xenobiotic compounds. The expression of *cyp2c8*, one of the major CYPs involved in carbamazepine metabolism activities in human liver microsomes (Zhang et al., 2015), was significantly modulated in the gut of earthworms, suggesting that it might play some role in drug metabolism.

The tubulin polymerisation promoting protein (*tppp*) was identified as a differentially expressed transcript in carbamazepine exposed *D. veneta*. In mammals, *tppp* promotes tubulin assembly and inhibits the formation of mitotic spindles and was shown to be highly expressed in colorectal, lung, and bladder cancer but supressed in liver cancer (Yamamoto et al., 2007; Inokawa et al., 2016a; Li et al., 2016; Ye et al., 2017). To date, no studies have identified a specific role of *tppp* in the gastrointestinal tract, but the GeneCards database indicates that TPPP is highly expressed in the colon and gastrointestinal tract (Safran et al., 2010). This concurs with our RNAseq analysis and RT-PCR data where *tppp* mRNA was down-regulated in the tissues of worms experiencing carbamazepine stress (Fig. 3). Others have stated that a decrease in *tppp* expression is an important feature in hepatocarcinogenesis, possibly due to promoter methylation (Inokawa et al., 2016). The observation that the expression patterns of target genes differed between *D. veneta* and humans highlights that the pharmacodynamic activities in the earthworm can differ to mammalian models and the resulting environmental risk assumptions cannot, or should not, be assumed to equal / superimposable.

Another notable observation was the absence of a strong cytochrome P450 response, known for their contribution to drug metabolism in many organisms, including mammals (e.g., *Homo sapiens* or *Rattus norvegicus*), fish (*Oncorhynchus mykiss*) and bacteria (Li et al., 2011, 2013; Burkina et al., 2018; Kato et al., 2019). CYP P450s are typically involved in the catalysis of drug metabolism, enhancing xenobiotic biotransformation and in humans, CYP3A4 is involved in the metabolism of carbamazepine (Pearce et al., 2002) but also about 50 % of pharmaceuticals on the market. Other phase I metabolising enzymes such as *cyp3a2*, *cyp3a5*, *cyp2c8*, *cyp11b*, *cyp17a* and *ephx1* (Kalgutkar *et al.*, 2005; Fraz et al., 2018; Ren et al., 2019; Liu et al., 2022) were shown to respond to carbamazepine exposure. The absence of a strong CYP response in earthworms exposed to carbamazepine, might be due to an overall lower abundance of the enzyme family as Milligan *et al.*, 1986 calculated a CYP content in *D. veneta* of 0.16–0.58 nmol/mg protein compared to 0.66–1.46 nmol/mg protein in rats and 1.22–3.63 nmol/mg protein in armyworm) corresponding to a reduced activity as determined by spectrophotometric methods. However, the observed absence of CYP family members might also be due to a yet to be identified earthworm specific CYPs (or alternative pathway). Putative candidates are perhaps any of the most-regulated sequence fragments with no functional annotation, and their characterisation may assist in the expansion of our knowledgebase of how earthworm detoxify carbamazepine and other contaminants of emerging concern (CECs).

Carbamazepine exposure is expected to also trigger phase II biotransformation activity which can be actioned by glutathione-S-transferase (GST) activity implicated in the conjugation of hydroxylated CBZ (Vernouillet et al., 2010). The activation of two isoforms of *udpgt* from higher carbamazepine exposure levels (0.6 and 10 mg/kg) but inhibition in lower levels might suggest that the UDP-glycosyltransferase phase II metabolism pathway is switched on at higher doses. The oxidative biotransformation of xenobiotics typically induces oxidative stress within cells (Laville et al., 2004), however glutathione-S-transferases (*gst*), quenchers of reactive oxidative stress, were not significantly induced in the RNASeq data and subsequent qPCR validations (data not shown).

The sensitivity and the intensity of transcriptomic responses are often regulated by signalling events. External signals, such as a pharmaceutical, typically activate transcription via signal transduction

pathways by directly binding to transcription factors (TFs). In doing so, the activated TF can acquire a function, for example nuclear retention enhancement, and/or stronger association with the cognate DNA motif, inhibitory factor attenuation, and chromatin-modifying coactivators and PolII complexes recruitment (Pascual-Ahuir et al., 2020). As the kinetics of gene induction, in response to a given stimulus, depends on how the TF reacts to the stimulation, and given that a single stressor can elicit different gene expression outcomes from activating a specific TF, explains why the observed gene expression differed in the different tissues of carbamazepine exposed earthworms.

5. Conclusions

Earthworms possess a remarkable capacity to shape the soil structure yet are sensitive to environmental change. This study aimed to study the earthworm's response to an exposure to carbamazepine (using concentrations typical of environmental hotspots and artificial laboratory exposures) within a simplified box exposure design. First, earthworm populations were screened (phenotypically and phylogenetically) to confirm, to species level, the identity of the test organism, an essential feature prior to any scientific experimentation. The gene list of differentially expressed genes that was forthcoming from the *de novo* RNASeq-based global transcriptomic experiment (and subsequently validated by qPCR) identified several transcripts that, to date, have never been associated with carbamazepine or other antidepressant exposure. The unique identification of these transcripts revealed the presence of specific detoxification response cascades in *D. veneta*, notably in the absence of members of the CYP family. Future work should evaluate how earthworms respond to carbamazepine exposure, as well as other pharmaceutical and personal care products which have been introduced continuously into terrestrial systems via anthropogenic activities. This should include different earthworm species as they inhabit unique ecological niches and are characterised by unique phylogeny, physiology and morphology profiles. With this information it will be possible to enhance the spatio-temporal modelling of terrestrial risk assessment, leading to informed decisions based on the transportation and transformation behaviours of substances in soil and biota.

CRediT authorship contribution statement

Chubin Zhang: Writing – original draft, Validation, Investigation, Funding acquisition, Formal analysis, Data curation. **Filipe Cabreiro:** Writing – review & editing, Methodology, Conceptualization. **Leon P. Barron:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Stephen R. Stürzenbaum:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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.envint.2025.109357.

Data availability

Data will be made available on request.

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