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Chemicals with increasingly complex modes of action result in greater variation in sensitivity between earthworm species

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Capsule: Earthworm species sensitivity varies more widely for three specifically acting insecticides than for a non-specifically acting PAH.

Article type: Research article

Abstract

The scale of variation in species sensitivity to toxicants has been theoretically linked to mode of action. Specifically, it has been proposed there will be greater variations for chemicals with a putative specific biological target than for toxicants with a non-specific narcotic mechanism. Here we test the hypothesis that mode of action is related to variation in sensitivity in a specifically designed experiment for species from a single ecologically important terrestrial taxa, namely earthworms. Earthworm toxicity tests were conducted with five species for four chemicals, providing a series of increasingly complex modes of action: a putative narcotic polycyclic aromatic hydrocarbon (fluoranthene), and three insecticides (chlorpyrifos, cypermethrin, imidacloprid) with known neuronal receptor targets. Across all the chemicals, the standard epigeic test species *Eisenia fetida* and *Lumbricus rubellus*, were generally among the two least sensitive, while the endogenic *Aporrectodea caliginosa* and Megascolecidae *Amynthas gracilis* were generally more sensitive (never being among the two least sensitive species). This indicates a potential for bias in the earthworm ecotoxicology literature, which is dominated by studies in epigeic Lumbricidae, but contains few endogeic or Megascolecidae data. Results confirmed the lowest range of variation in sensitivities for effects on reproduction was for fluoranthene (2.5 fold). All insecticides showed greater variation for species sensitivity (cypermethrin: 7.5 fold, chlorpyrifos: 10.3 fold, imidacloprid: 31.5 fold) consistent with the specific mechanisms of the pesticides. Difference in toxicodynamics, based on mode of action specificity and receptor complexity was reflected in the magnitude of sensitivity variation. However, measurements of tissue concentrations also indicated the potential importance of toxicokinetics in explaining species sensitivity variations for chlorpyrifos and cypermethrin.

Keywords:

Species sensitivity, Earthworms, Toxicokinetics, Toxicodynamics, Pesticides

Introduction

Chemical risk assessment relies on using toxicity data from tests performed on a limited number of species to predict chemicals impacts for all organisms in an ecosystem. Since it is unfeasible to conduct toxicity tests for the myriad of ecological species, there is a need to extrapolate results from those few species that can be tested. In more data-rich cases, it may be possible to compile a statistical distribution of species sensitivity of, e.g. LCx, ECx, NOEC, values to support regulatory decisions (Posthuma et al., 2001; Posthuma et al., 2019). When data is more limited, an arbitrary 'safety factor' (usually division by 10, 100 or 1000 depending on the level of detail of the data) is applied to laboratory data during predicted no effect level (PNEC) derivation. This is done to account for differences in species sensitivity, as well as between laboratory and field conditions (Rico and Van den Brink, 2015; Van Leeuwen and Hermens, 1995). While both species sensitivity distributions (SSDs) and 'safety factors' represent pragmatic approaches to overcome the issue of missing data, they do not inform on the true nature and range of sensitivity of species, nor provide insights into how vulnerability may differ between chemicals.

The inherent sensitivity of species can be associated with differences in the toxicokinetic and toxicodynamic traits (Gergs et al., 2015; Rubach et al., 2012). In particular, the scale of variation in sensitivity has been theoretically linked to mode of action (Escher and Hermens, 2002). In a systematic study, a greater variation in sensitivities was found for chemicals with a specific biological target (e.g. neurotoxicity through acetylcholinesterase inhibition) than for non-specific chemical exhibiting non-polar narcosis (Vaal et al., 1997; Vaal et al., 2000). This conclusion was further supported in a study that also show lower LC_{50} variations for narcotics than for specifically acting chemicals (Hendriks et al., 2013).

The lower variation in species sensitivity to narcotics has been attributed to the conservation of their biological membrane targets (Vaal et al., 1997). This contrasts with the diverse nature of interactions that specifically acting chemicals may have with receptors, which may be conserved, diverse or missing between species (Escher and Hermens, 2002). Previous studies have associated receptor presence or absence with differential sensitivity (Fay et al., 2017; LaLone et al., 2016; Verbruggen

et al., 2018). The assumption being that when a receptor is present a given chemical can interact, leading to effects at lower concentrations than in species where the receptor is absent and for which only non-specific baseline toxicity effects (narcosis) may be relevant, as this is the minimal toxicity for any given chemical. This supposition underpins the targeting of pesticides and biocides to specific taxa. The phylogenetic conservation of receptors in species from bacteria to vertebrates has been investigated for human pharmaceuticals showing greater drug target orthologue presence in zebrafish (86%), than *Daphnia* (61%) or alga (35%)(Gunnarsson et al., 2008). Variations in orthologues presence was used to explain large difference seen in sensitivity for distantly related species (Gunnarsson et al., 2008; Rivetti et al., 2020).

When species come from a single taxon, there is a higher probability that a large portion of the target receptor complement is shared. Therefore, between such closely related species, more subtle differences such as receptor isoform number, protein sequences or expression may be critical to sensitivity. Given the potential complexity of these drivers of sensitivity, it is not yet established whether closely related species will show similar patterns of greater variations in sensitivity for specific versus non-specifically acting chemicals as found among distantly related species. To test this hypothesis, the aim of this study is to assess the comparative sensitivity of five earthworm species that were available in sufficient numbers for toxicity testing (four Lumbricidae, one Megascolecidae) to three neurotoxic chemicals and one polycyclic aromatic hydrocarbon, fluoranthene, selected as a representative narcotic (Stroomberg et al., 2004). The three neurotoxic insecticides represent chemicals which target increasingly complex receptors providing a set of progressively increasingly complexity, rather that being a simple binary comparison to the narcotic. The organophosphate (chlorpyrifos) targets the single sub-unit acetylcholinesterase; the pyrethroid (cypermethrin) the α subunit and small β sub-unit dimer voltage-gated sodium channel structure; and the neonicotinoid (imidacloprid) the pentameric nicotinic acetylcholine receptor. This study will therefore also allow us to assess whether chemicals with modes of action that target increasingly complex receptors show greater sensitivity differences in closely related species.

Material and Methods

Study species and genotyping

E. fetida were obtained from long term cultures kept at UK CEH. Other samples were field collected (*L. rubellus* from Dinays Powys, 51°26'35.6"N, 3°14'17.8"W, *D. octaheda* and *A. caliginosa* from the Whitley Wood, Hampshire, 50°50'58.3"N, 1°34'35.7"W and *A. gracilis* from Sao Miguel, 37°45'50.5"N+25°32'04.0"W). The genetic structure of the tested earthworm populations was analysed by CO1 genotyping representative individuals (15-20) from the tested cohorts. DNA was isolated from tail samples using the DNeasy Blood and Tissue Kit (Qiagen, Germany), and quantified (NanoDrop ND-1000). A region of the CO1 locus was amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994) by a PCR of 35 cycle with annealing at 48 °C and a 1 minute extension time in a 25 µl volume containing 40 ng of template, 1 U of Taq polymerase (Promega, UK), 2.0 mM MgCl2 and 10ug of BSA (NEB, UK). PCR products were visualised by electrophoresis and purified with the QIAquick-spin PCR purification kit (Qiagen, Germany) before products were sequenced (Eurofins MGW Operon, Germany). The resulting CO1 sequences were aligned and trimmed (Geneious v. 9.1.8) using reference CO1 sequences for *E. fetida*, *L. rubellus*, *A. caliginosa*, *D. octaedra* and *A. gracilis* available on NCBI (Table 1)

Chemical selection, test soil and spiking

Test concentration ranges were chosen using previous information on the toxicity of fluoranthene and chlorpyrifos to *L. rubellus* (Lister et al., 2011; Owen et al., 2008) and imidacloprid and cypermethrin to *E. fetida* (GomezEyles et al., 2009; Hartnik et al., 2008). The exposure ranges for each chemical are as follows (all in mg/kg dry weight soil): fluoranthene 0, 4.8, 14.4, 43.4, 130, 390, 1170; chlorpyrifos 0, 3, 9, 27.1, 81.3, 244, 732; cypermethrin 0, 6.2, 18.5, 55.6, 167, 500, 1500; imidacloprid 0, 0.041, 0.123, 0.37, 1.11, 3.33, 10, 30. All test chemicals were obtained as high-grade reagents or analytical standards of minimum 98% purity (Sigma-Aldrich, Poole, UK, Greyhound Chromatography, Birkenhead, UK). Four independent biological replicates were used for all test concentrations.

The soil medium used was a natural Kettering loam soil, with 24% sand, 35% silt and 41% clay, a pH 7.1 and 5% organic matter content (Broughton Loam, Kettering, UK), sieved to 2 mm and amended with 3% dry weight composted bark (LBS Horticultural, Colne, UK). The amount of soil and number of worms differed between species depending on the adult size. For *L. rubellus*, *A. caliginosa* and *A. gracilis*, five worms were added to 1400 g dry weight soil in a 2l container measuring 16.8 x 16.8 x 10cm; for *E. fetida*, 10 worms in 700 g soil in a container of 17.1 x 10.8 x 7cm, and for *D. octaedra* 10 worms in 350 g soil in a round pot 11.5 wide x 7.2cm. All containers were made of polypropylene. The density of earthworms chosen for the study was based on 1) their availability, particularly for those collected from the wild and 2) the density at which these organisms would exist normally and was reasonable for the volume of soil in the tests.

A stock solution of imidacloprid (0.84 mg/ml) was made in water and added to the soil to achieve the desired concentration. Further water was then added to reach 50% of soil water holding capacity. The spiked and wetted soils were mixed and left overnight before the earthworms were added. For the remaining three compounds, the soils were spiked with the chemical in an acetone-dissolved stock solution (fluoranthene 109.3 mg/ml, chlorpyrifos 170.8 mg/ml, cypermethrin 140 mg/ml). The spiked soil was left to evaporate until the soils were dry and odourless. Soils were then mixed, water added to 50% of water holding capacity, left to stabilise for one day and the earthworms then added. The controls for these three chemicals were spiked in the same manner, using acetone without chemical added, to ensure the acetone did not influence the earthworm responses.

A 28-day toxicity test was carried out using each species with survival assessed after 14 and 28 days (Organisation for Economic Co-operation and Development, 2004)and reproduction (number of cocoons produced) assessed after 28 days. Cocoon counting as a measure of reproduction was chosen instead of juvenile production rates because the hatching times from cocoon incubations differed between the different species, meaning cocoon production was a more reliable measure of reproduction. To provide food, horse manure from an animal grazing uncontaminated pasture not subject to recent medication was added to the soil surface. Added manure was dosed to the same concentration, and in the same manner, as the relevant test soil and rewetted to 80% moisture

content (Spurgeon et al., 2003a). Based on past work with these species, an amount of 6 g dry weight of manure was added for all species, except for 3 g for *D. octaedra* (Spurgeon et al., 2000). These amounts were selected to provide an excess of food to allow *ad libitum* feeding. All test containers were covered to prevent water loss and maintained at physiological optimum constant temperatures for each species (20˚C for *E. fetida* and *A. gracilis,* 13ºC for all remaining species) under a 16 : 8 hours light : dark regime for 28 days in order to carry out an earthworm reproduction assay. At day 14, any remaining manure was removed, the soils hand sorted and the number and weight of worms alive in each box recorded. Soil and earthworms were then returned to the containers and fresh food added. After 28 days, the soils were again hand sorted and the surviving earthworm counted and weighed. Soil samples (10 g) were collected to determine chemical degradation in sub-set (10%) of all samples. All remaining soil was wet sieved and the number of cocoons present counted to allow cocoon production rate (cocoon/worm/week) to be calculated. Surviving earthworms were counted and tail samples were collected from individuals (2 individuals per replicate) and snap frozen to preserve for analysis. For *L. rubellus*, tail samples from all exposure concentrations were analysed for tissue concentrations for each chemicals. In the case of the other three species, the tissue concentrations were measured at a single exposure concentration (130 mg/kg for fluoranthene, 81 mg/kg for chlorpyrifos, 55 mg/kg for cypermethrin, 0.37 mg/kg for imidacloprid), in order for comparisons between species to be carried out.

Soil and earthworm tissue chemistry

Fluoranthene: Earthworm tail and soil samples for fluoranthene analysis were homogenised in 25 ml dichloromethane and extracted by microwave extraction. Extracts were concentrated and lipids removed by size exclusion chromatography (Agilent, Stockport, UK) using two 19 mm Envirogel columns connected in series (Envirogel, London, UK). Analysis for fluoranthene was conducted by gas chromatography-mass spectrometry (GC-MS) using a 7890B GC fitted with a 5977B mass selective detector and a 7673 auto-sampler (all Agilent Technologies, Stockport, UK). The GC-MS was operated in selective ion mode with ionisation by electron impact. Compound identification was based on ion ratios (three per compound) and retention time. Residues were quantified using an internal standard (labelled fluoranthene) method and also calibration curves of the standard

fluoranthene and were recovery corrected. The mean recoveries were 95% (Range: 75-110.9%) and the LOD was 1.4 ng/g wet weight.

Chlorpyrifos: Worm tail and soil samples were homogenised in 25 ml dichloromethane and extracted by vortex for 30 min, centrifuged and the supernatant removed. This process was repeated twice. Extracts were concentrated and lipids removed by size exclusion chromatography (Agilent Technologies) using two 19 mm Envirogel columns connected in series (Envirogel). Analysis for chlorpyrifos was conducted by gas chromatography-mass spectrometry (GC-MS) using a 6890 GC fitted with a 5973 mass selective detector and a 7673 auto-sampler (all Agilent Technologies). The GC-MS was operated in selective ion mode with ionisation by electron impact. Compound identification was based on ion ratios (three per compound) and retention time. Residues were quantified using internal standard (labelled chlorpyrifos) method and also calibration curves of the standard chlorpyrifos and were recovery corrected. The mean recoveries were 102% (Range: 70.5-117%) and the LOD was 0.31 ng/g wet weight

Cypermethrin: Analysis of cypermetrin in earthworm tails and soils was performed after extraction and clean-up using liquid chromatography coupled to a triple quadrupole 'Xevo TQ-XS' mass spectrometer (Waters, Wilmslow, UK). Quantification was based on cypermethrin response factor to an internal standard (labelled cypermethrin) using the native standard calibration curve. Methods performance was assessed in terms of the limit of detection (LoD = 0.4 ng g^{-1}), limit of quantification (LOQ= 0.6 ng g^{-1}) and average recoveries of 81.1% (range 60.2-112.9). The LoD was derived as three times the signal to noise ratio and the LOQ as the LOD plus calculated expanded method uncertainty.

Imidacloprid: Concentrations in earthworm tail and soil samples were quantified based on Woodcock *et al*. (2017). Analysis was performed, after sample extraction using 50:50 methanol:water, and clean-up using Oasis HLB cartridges, using liquid chromatography coupled to a triple quadrupole 'Quantum Ultra TSQ' mass spectrometer (Thermo Fisher Scientific, UK). Quantification was based on imidacloprid response factor to an internal standard (labelled Imidacloprid) using the native

standard calibration curve (Magnusson et al., 2012. ; Woodcock et al., 2017). Methods performance was assessed in terms of the limit of detection (LoD = 0.4 ng g-1), limit of quantification (LOQ= 0.6 ng g^{-1}) and average recoveries (77-88% for 16 sample batches). The LoD was derived as three times the signal to noise ratio and the LOQ as the LOD plus calculated expanded method uncertainty.

Statistical Analyses

One-way analysis of variance was used to assess the effects of the fixed factor of soil concentration on survival (after square root transformation) and cocoon production rate. Probit analysis for the mortality data and a three parameter logistic regression conducted in the DRC package in R (Ritz and Streibig, 2005) were used to calculate species specific LC_{50} and cocoon production EC_{50} values (both with 95% confidence intervals) for each chemical. Tissue concentrations measured for *L. rubellus* across the concentration range were fitted with a linear model, although non-linearity was indicated in the data as this provided a clear and simple view of the pattern of accumulation with exposure level. Differences between species at a single concentration were visualised by simple comparison of mean values to identify difference in sensitivity as fold change differences based effect sizes.

3. Results

Soil concentrations.

Final measured concentrations (in 10% of soils) indicated concentrations at a mean of 45% of nominal values for fluoranthene, 77% for chlorpyrifos, 100% for cypermethrin and 75% for imidacloprid (see Supplementary Fig. 1). These remaining concentrations indicate chemical half-lives approximating to or longer than the 28 days exposure duration and evidence continuous exposure within 50% of nominal for fluoranthene and 25% of nominal for the three insecticides. As measured concentrations remained within an approximate factor of two of nominal and because exposure concentration would be changing over time, all effect concentrations were calculated from the nominal values that reflect starting exposure concentrations.

Earthworm genotypes

As some earthworm morphospecies used are known to comprise distinct genetic clades that may represent cryptic species, such as for *E. fetida* (Rombke et al., 2016), *L. rubellus* (Anderson et al., 2017; Andre et al., 2010), *A. caliginosa* (Shekhovtsov et al., 2016), *A. gracilis* (Novo et al., 2015a) and *D. octaedra* (Porco et al., 2013), mitochondrial cytochrome oxidase 1 (CO1) loci amplification and sequencing was conducted to better understand the genetic structure of the tested populations. Earthworm genotyping indicated that each of the species cohort used in the bioassays are dominated, to differing extents, by a single clade. CO1 sequences from the *A. caliginosa*, *D. octaedra* and *A. gracilis* populations suggest the presence of just one clade with relatively low genetic variability between individuals (Table 1). In contrast, the *L. rubellus and E. fetida* CO1 sequencing reveals evidence for two clades, indicating that the most common clade represents ~90% and ~65% of the respective populations. The dominant *L. rubellus* clade presents the largest intra-clade variability of all tested species (Table 1).

Fluoranthene

After 28 days of exposure, there was a 100% control survival for all species. Fluoranthene only affected survival for *A. gracilis*, with full mortality in the 390 and 1170 mg/kg treatments and a 28 day

LC_{50} of 189 (16.5 - 361) mg/kg (see Table 2, Supplementary Fig. 2). There was >50% survival in the top concentration of 1170 mg/kg for all other species (67% for *A. caliginosa*, >85% remaining species). All five species produced cocoons at sufficient rates in the control treatment (cocoons/worm/week) to allow the identification of reproductive effects (*E. fetida* = 0.72, *L. rubellus* = 1.11, *A. caliginosa* 0.47, *D. octaedra* = 0.46, *A. gracilis* = 0.11). Reproduction was a more sensitive endpoint (n.b. also the case for all chemicals in all species). Significant (ANOVA, Tukey' test, $p<0.05$) effects on cocoon production were found at >390 mg/kg for *E. fetida* and *L. rubellus* >130 mg/kg for *A. caliginosa*, *D. octaedra* and *A. gracilis*. Logistic fits gave a lowest EC_{50} values for *A. caliginosa,* 70.3 mg/kg (24.5 - 116) and a highest (2.5. fold higher) EC_{50} for *D. octaedra* of 178 mg/kg (116 – 240) (Table 2, Supplementary Fig. 2).

Tissue fluoranthene concentrations in *Lumbricus rubellus* were higher than the nominal soil concentration across the exposure range indicating biomagnification (Fig. 1). At 130 mg/kg, tissue concentrations were highest in *A. caliginosa*, the most sensitive species, and lowest in *A gracilis* and *E. fetida*, the latter being the least sensitive of the four species for which fluoranthene tissue concentrations data were available (fluoranthene was not measured in the most sensitive species *D.* octaedra because the tissue mass was too limited for the method). Tissue concentration ranking by species showed agreement with ranking for sensitivity (Table 3), suggesting a potential relationship between tissue fluoranthene concentrations and effect on reproduction. Further, the maximum variation in fluoranthene tissue concentrations between species of 2.35 fold approximately matches the 2.5 fold sensitivity variation for reproductive effects.

Chlorpyrifos

Control survival was ≥90% for all species. Chlorpyrifos exposure affected survival in all five species. The 28 day LC_{50} values ranged from 97.4 (77 - 124) mg/kg for *L. rubellus* to 505 (364 - 647) for *E. fetida*, (see Table 2, Supplementary Fig. 2). All five species produced sufficient cocoons (cocoons/worm/week) in the control treatment (*E. fetida* = 1.08, *L. rubellus* = 0.96, *A. caliginosa* 0.27, *D. octaedra* = 0.5, *A. gracilis* = 0.51) to allow assessment of reproductive effects. Reproduction was more sensitive than survival in all species. Significant effects on reproduction (ANOVA, Tukey's test,

296 *p<0.05*) were found at >3 mg/kg for *L. rubellus,* >9 mg/kg for *A. caliginosa*, *D. octaedra* and *A. gracilis*
297 and >81.3 mg/kg for *E. fetida*. Logistic fits indicated that *A. caliginosa* (most sensitive), *L. rubellus*
298 and *A. gracilis* had high sensitivity for chlorpyrifos (EC_{50} values from 5.86 – 7.64 mg/kg), compared
299 to *D. octaedra* (EC_{50} values 15.6 mg/kg) and especially *E. fetida* (EC_{50} 60.8 mg/kg) (see Table 2,
300 Supplementary Fig. 2).
301
302 Measurements of tissue chlorpyrifos in *L. rubellus* showed tissue concentrations consistently above
303 nominal soil concentrations, indicating biomagnification across the exposure range (Fig. 1). At 81
304 mg/kg, tissue concentrations were highest in *A. caliginosa*, the most sensitive species, and lowest
305 in *E. fetida*, the least sensitive species. Tissue concentration ranking by species showed good
306 agreement to ranking for sensitivity (Table 3), suggesting a direct relationship between tissue
307 chlorpyrifos levels and effect on reproduction across species.
308
309 *Cypermethrin*
310 Control survival was 100% for *A. caliginosa*, *E. fetida* and *A. gracilis* and >95% of the remaining
311 species. Cypermethrin had a <50% effect on survival at the highest tested concentration of 500
312 mg/kg for *L. rubellus* and 1500 mg/kg for *A. caliginosa*. For the remaining species, effects on survival
313 were seen. Calculated 28 day LC_{50} values ranged from 58.7 (30.8 – 86.5) mg/kg for *D. octaedra* to
314 731 (620 - 841) for *E. fetida* (see Table 2, Supplementary Fig. 2). Cocoon production rates
315 (cocoons/worm/week) in the control soils were sufficient to identify reproductive effects (*E. fetida* =
316 0.53, *L. rubellus* = 1.7, *A. caliginosa* 0.46, *D. octaedra* = 0.42, *A. gracilis* = 0.51 cocoons/worm/week).
317 Reproduction was more sensitive than survival. Negative effects on cocoon production rate (ANOVA,
318 Tukey's test, *p<0.05*) were found at >18.5 mg/kg for all species. Logistic models fits indicated that,
319 despite having low sensitivity for survival, *A. caliginosa* was most sensitive for effects on reproduction
320 (EC_{50} 3.5 mg/kg), with *L. rubellus* (EC_{50} 26.4 mg/kg) least sensitive (see Table 2, Supplementary
321 Fig. 2).
322
323 Tissue cypermethrin concentrations in *L. rubellus tissues* increased with exposure level (Fig. 1),
324 however, tissue values were always well below nominal (and final measured) soil concentrations,

reaching a maximum of 18.8 mg/kg tissue at 500 mg/kg. BCFs progressively reduced from a high of 0.2 at 6.2 mg/kg to a low of 0.037 at 500 mg/kg nominal soil concentration. At an exposure of 55 mg/kg, internal concentrations were lowest for *Eisenia fetida* at 2.03 mg/kg and highest for *A. gracilis* at 12.9 mg/kg. Cypermethrin tissue concentration rank showed partial agreement with ranking for sensitivity (Table 3). Thus the two most sensitive species *D. octaedra* and *A. gracilis* showed highest accumulation, and least sensitive species *E. fetida* the lowest. The position of *A. caliginosa* was more problematic as this species showed low sensitivity for survival, high sensitivity for reproduction and low (4th ranked) for tissue accumulation.

Imidacloprid

Survival in the control soils was 100% for all species except *D. octaedra* at 85%. Imidacloprid had no effect on survival up to 10 mg/kg for *L. rubellus* and *A. caliginosa*. The 28 day LC_{50} values for the remaining species indicated higher sensitivity for *A. gracilis* 0.79 (0.65 - 0.95) mg/kg than for *D. octaedra* of 1.93 (1.51 - 2.62) and especially for *E. fetida* of 5.58 (4.78 – 6.65) mg/kg (see Table 2, Supplementary Fig. 2). Cocoon production rates (cocoons/worm/week) in the control soils were sufficient (*E. fetida* = 0.39, *L. rubellus* = 1.21, *A. caliginosa* 0.91, *D. octaedra* = 0.57, *A. gracilis* = 0.6) to allow concentration response modelling. Effects on cocoon production rate (ANOVA, Tukey's test, $p<0.05$) were found at >3.3 mg/kg for *E. fetida* and *L. rubellus*, >1.1 mg/kg for *A. caliginosa* and >0.37 mg/kg for *D. octaedra* and *A. gracilis*. EC_{50} values were lowest for *A. gracilis* (EC_{50} 0.113 mg/kg) followed by *D. octaedra*, *A. caliginosa, E. fetida* and finally *L. rubellus* (EC_{50} 3.59 mg/kg) (see Table 2, Supplementary Fig. 2).

Tissue imidacloprid concentration increased in *L. rubellus* across the exposure range showing a non-linear relationship with soil concentration, being above nominal soil concentrations at 0.12 and 0.37 mg/kg, but lower than soil concentrations at higher levels, with a lowest BCF of 0.33 at 3 mg/kg (Fig. 1). Imidacloprid in tissue at 0.37 mg/kg varied approximately 3 fold between species from the lowest (*L. rubellus*) to highest (*D. octaedra*). There was poor agreement between species ranking by tissue concentration and ranking for sensitivity (Table 3). Thus, the most sensitive species, *A. gracilis,* showed the lowest tissue concentrations, whereas the second least sensitive species, *E. fetida* had

the second highest (Table 3). Tissue concentration ranking accorded with average body size (*D. octaedra* < *E. fetida* < *A. caliginosa* < *L. rubellus* < *A. gracilis*) from smallest to largest indicating uptake may be surface area : body size ratio limited. Any body size effect did not translate to a size-dependent pattern for sensitivity, since the largest species *A. gracilis* was also the most sensitive.

Comparison of sensitivity between species and for chemicals with different modes of action

The standard test species *E. fetida* generally showed low comparative sensitivity, being among the two least sensitive species for all chemicals tested. *L. rubellus* also showed low comparative sensitivity, being least sensitive for both cypermethrin and imidacloprid, although this species was most sensitive for chlorpyrifos (Table 3). Genotyping showed evidence for the presence of two clades in both *E. fetida* and *L. rubellus*, while the other three species, that generally showed higher sensitivities, were represented by a single clade (Table 1). To assess whether the presence of clades may result in greater variation in the responses of *E. fetida* and *L. rubellus* to exposure, we calculated the coefficient of variation (CV) of measured tissue concentrations for each species for all chemicals and an indication of individual variation in chemical handling. Between species, we found no evidence of greater variation (higher tissue concentration CVs) in those species with two genetic clade compared to those with a single clade (Fig. 1). Hence, a complex clade structure in a species did not appear to be associated with greater variation in chemical accumulation.

Variations in LC_{50} values could not be compared in relation to mode of action, because of the number of missing values (<50% effects on survival at the highest tested concentrations). For EC_{50} values, the lowest variation in sensitivity was found for the putative narcotic PAH fluoranthene, varying by 2.5 fold from the least sensitive species *D. octaedra* to most sensitive *A. caliginosa* (Fig. 2). Greater variation in sensitivity was observed for the three insecticides. For cypermethrin, the difference between the most sensitive species *A. caliginosa* and least sensitive *L. rubellus* was 7.5 fold; for chlorpyrifos the difference was 10.4 between *E. fetida* and *A. caliginosa* and for imidacloprid the differences in EC_{50} values from the most sensitive species *A. gracilis* and least sensitive *L. rubellus* was 31.5 fold (Fig. 2).

Discussion

In this study, we assessed the sensitivity of five earthworm species to chemicals with modes of action of different complexities. This allowed us to assess whether chemicals with modes of action that target increasingly complex receptors show greater sensitivity differences in closely related species. The consistent high survival and reproduction seen in the multiple tests conducted demonstrated the feasibility of reproductive toxicity testing using earthworm species not from the *E. fetida/E. andrei* complex. *E. fetida* is a compost dwelling earthworm species and is rarely found in natural soils. Hence, there have been frequent calls to include soil dwelling earthworms in regulatory testing, with *A. caliginosa* being a current focus (Bart et al., 2018; Lowe and Butt, 2007; Spurgeon et al., 2003b). The species used in this study included both endogenic (*A. caliginosa*) and epigeic species (*L. rubellus*, *D. octaedra*, *A. gracilis*); species from two genera, Lumbricidae (*A. caliginosa*, *L. rubellus*, *D. octaedra*) and Megascolecidae (*A. gracilis*) and species that are associated with more acidic (*D. octaedra*, *L. rubellus*) and neutral (*A. caliginosa*) soils. This repertoire of tested species, thus, provides a flexible set of options for testing of different chemicals (Bart et al., 2018). Further they include recognised invasive species, such as *L. rubellus* and *D. octaedra* in North America (Frelich et al., 2006) and *A. gracilis* in various regions (Novo et al., 2015a), that may be future targets for eradication.

Despite the persistent questions on its relevance, the *Eisenia fetida* complex is now fully established as the workhorse for terrestrial ecotoxicology testing. As this species is commonly used, it is important to know how the sensitivity of this species compares to other earthworms. Across the tested chemicals, *E. fetida* showed median or lower sensitivity in all cases, being least sensitive for chlorpyrifos, second least sensitive for cypermethrin and imidacloprid, and third least for fluoranthene. Following meta-analysis, Pelosi et al. (2013) showed that LC_{50} values reported for *E. fetida* were, on average, significantly higher than those for *L. terrestris* and *A. caliginosa* across a range of pesticides. Likewise for trace metals, *E. fetida* was least sensitive to zinc among four tested species (*L. terrestris*, *E. fetida*, *L. rubellus*, *A. caliginosa*) (Spurgeon et al., 2000) and for copper among three species (*L. rubellus, A. longa, E. fetida*) (Qiu et al., 2013). Hence, there is a consistent line of evidence to suggest that *E. fetida* frequently show low sensitivity to a range of chemical

411 classes and modes of action. Based on the dominance of the use of *E. fetida* for toxicity testing,
412 there is an indication of a significant bias in the earthworm ecotoxicology literature arising through
413 the standardised use of a relatively insensitive species. Efforts to integrate further endogenic
414 species, such as *L rubellus*, *A. caliginosa* or Megascolecidae species, such as *A. gracilis*, into testing
415 programs could provide a more representative set of sensitivities for different modes of action.
416
417 Regulatory requirements for earthworm testing according the OECD Guideline 222 (Organisation for
418 Economic Co-operation and Development, 2004) stipulate that tests should meet three quality
419 criteria for survival and cocoon production rates in control treatments. The performance of the tests
420 conducted with the other four earthworm species can be assessed against these criteria.
421
422 1) Adult control mortality over the test to be on average <10%. This criterion was met for all
423 species for all chemicals, except *D. octaedra* exposed for imidacloprid where control mortality
424 was 15%, although across all chemicals control mortality for this species was on average 7.5% .
425
426 2) Control replicates of 10 adults should produce on average > 30 juveniles. Performance for this
427 criteria is more difficult to judge as cocoon production rather than juvenile production was
428 measured, and other than for *E. fetida,* only 5 earthworms were used per replicate, rather than
429 10. However, based on 30 juveniles being equivalent to 15 *E. fetida* cocoons (i.e. two juveniles
430 hatching from each cocoon), this corresponds to a production rate of 0.375 cocoons/worm/week.
431 This rate is met in all species for all tests except two for *A. caliginosa* with fluoranthene and
432 chlorpyrifos and one with *A. gracilis* for fluoranthene. In these three cases concentration response
433 models could still be fitted.
434
435 3) Control coefficient of variation of reproduction should be < 30%. Of the three criteria, this one
436 was met in the fewest number of tests. Even so in three of the four species, at least three tests
437 showed acceptably low variation. The exception being *A. caliginosa* where only one test met this
438 criterion. Hence if this species is to be promoted as an additional soil dwelling standard test

species, further work is needed to optimise test conditions to ensure more consistent reproduction.

The sensitivities of the earthworm species to the four chemicals tested were largely consistent with those found previously. Cocoon production rate EC_{50} values for the PAH fluoranthene ranged from 70 - 178 mg/kg. These values are consistent with previous findings for PAH effects on earthworms. For example, Sverdrup et al. (2002) measured the reproductive toxicity of eight polycyclic aromatic compounds to *E. fetida*, finding EC_{50} values from 44 - 166 mg/kg. The EC_{50} for fluoranthene found here (158 mg/kg) is remarkably consistent with their value of 157 mg/kg. Similarly, the EC_{50} found here for *L. rubellus* of 137 mg/kg is consistent with that of Svendsen et al. (2008) of 182 mg/kg.

For chlorpyrifos, Ma and Bodt (1993) compared effects on survival in five earthworm species, three of which, *E*. fetida*, L. rubellus* and *A. caliginosa*, overlap with those used here. LC_{50} values found ranged from 129-1174 mg/kg, overlapping substantial with the range found in this study (97.4 - 505 mg/kg). For the three directly comparable species, the order of sensitivity for LC_{50} of *L. rubellus* > *A. caliginosa* > *E. fetida* was consistent in both studies. Further, the 8.3 fold sensitivity range found by Ma and Bodt (1993) between *E. fetida* and *L. rubellus* and 1.42 fold difference for *A. caliginosa* than *E. fetida* is also consistent with the 5.3 fold and 1.78 fold differences found here for these species pairs.

For cypermethrin, Hartnik et al (2008) found LC_{50} and reproduction EC_{50} values of 762 mg/kg and 31 mg/kg for *E. fetida*, consistent with those here (LC_{50} 731 mg/kg, reproduction EC_{50} 21.3 mg/kg). For imidacloprid, Kreutzweiser et al. (2008) found higher LC_{50} for imidacloprid (25 mg/kg for *E. fetida,* 5.7 mg/kg for *D. octaedra*) than those found here by a factor of 4.4 for *E. fetida* and 3 for *D. octaedra*. This difference might be explained by the use of forest litter as the test substrate by Kreutzweiser et al (2008) compared to the mineral soil used in this study. Kreutzweiser et al (2008) found *D. octaedra* to be 4.4 fold more sensitive than *E. fetida*, this order in sensitivity is conserved in this study, although the 8.3 fold difference is larger. The overall agreement between the current and past work indicates the technical validity of the experimental procedures used for generating toxicity data for research

on species to investigate the differences in response mechanisms between closely or distantly related species, or for chemicals with different modes of action and resulting effects.

Genotyping indicated the presence of two genetic clades in the tested *E. fetida* and *L. rubellus* cohorts consistent with previous findings for these species (Anderson et al., 2017; Andre et al., 2010; Rombke et al., 2016), but only a single clade for the remaining species. Previous studies have identified that differences within species earthworm clades may show different responses to chemical exposure and other environmental drivers (Anderson et al., 2017; Anderson et al., 2013; Andre et al., 2010; Novo et al., 2015a; Novo et al., 2015b; Spurgeon et al., 2016). Hence, there is the potential that species clade structure may influence individual responses to exposure for the tested chemicals. As both *E. fetida* and *L. rubellus* often showed comparatively low sensitivity (Fig. 2, Table 3), we conducted an initial assessment of variability in response to chemical at the individual level by assess variation in measured tissue concentrations in each species. No systematic evidence of increased variation in measured tissue concentrations was found for the two species that comprised of distinct genetic clades. Hence, there is no evidence from these initial results that the presence of genetic clade may increase variability in *E. fetida* and *L. rubellus* responses that could, for example, result in the presence of individuals in the population with relatively low sensitivity. As, however, the number of individuals measured per treatment was low (n=4), this issue requires further attention.

It has been proposed that the scale of species sensitivity variation varies depending on toxicant mode of action, with non-specifically acting chemicals (e.g. narcotics) showing lower variation than specifically acting chemicals (Escher and Hermens, 2002). This theory has been supported by meta-analyses (Hendriks et al., 2013; Vaal et al., 1997; Vaal et al., 2000). Such a pattern of variation has been attributed to the conserved nature of the biological membrane targets of narcotics, compared to the diverse nature of the receptor targets of specifically acting chemicals (Escher and Hermens, 2002). The chemicals tested here cover a range of complexities in modes of action. Fluoranthene, is recognised as a non-polar narcotic, while the three insecticides all have a putative specific mode of action. Chlorpyrifos targets acetylcholinesterase, a hydrolase enzyme receptor known to be active

497 in earthworms (Sanchez-Hernandez et al., 2018) consisting of a single protein sub-unit forming a
498 homo dimer or tetramer (Dvir et al., 2010); cypermethrin targets the voltage gated sodium channel
499 a neuronal surface receptor with a more complex structure consisting of a pore-forming α subunit
500 associated with ancillary β subunits (Catterall, 1984; Shen et al., 2017); imidacloprid interacts with
501 nicotinic acetylcholine receptor, a complex post-synaptic structure existing as a pentamer that can
502 comprise different subunits (Albuquerque et al., 2009). Thus, moving from the narcotic fluoranthene
503 through the simpler to complex receptors of chlorpyrifos, cypermethrin and imidacloprid, the
504 chemical targets present a range of conformations that support a robust assessment of the canonical
505 relationship between receptor complexity and species sensitivity variation.
506
507 In agreement with theory, the lowest variation in sensitivity for effects on reproduction (~2.2 fold)
508 was found for fluoranthene. This supports the hypothesis that, even among closely related species,
509 toxicity through non-polar narcosis is associated with low inter-species sensitivity differences,
510 although similar studies with further narcotic chemicals would be valuable to confirm this conclusion.
511 The three specifically acting insecticides all showed a greater range of interspecies variation than
512 fluoranthene. Variation was greatest (>30 fold) for imidacloprid, suggesting that the complex
513 structure of the nicotinic acetylcholinesterase target provides substantial variation in specific
514 structure or expression that results in differing strength of interaction and consequently different
515 levels of effect between species. Chlorpyrifos showed greater variation in sensitivity (10.4 fold) than
516 the narcotic fluoranthene but also cypermethrin, even though the acetylcholine receptor is
517 structurally simpler that the voltage gated sodium channel. It has been widely shown that chlorpyrifos
518 can interact with acetylcholinesterase in earthworms to cause inhibition (Collange et al., 2010;
519 Sanchez-Hernandez et al., 2014; Vejares et al., 2010) and that the extent of this inhibition can vary
520 for different tissues (Vejares et al., 2010). Such potential to change localised tissue activities of
521 acetylcholinesterases, as well as the precise nature of receptor-ligand interaction, may contribute to
522 the extent of variation in sensitivity seen for this organophosphate.
523
524 Variation in sensitivity among species to the insecticides was lowest for cypermethrin (7.5 fold).
525 While the two most sensitive species (*D. octahedra* and *A. gracilis* showed the highest accumulation

and the least sensitive (*E. fetida*) the least, the results from *A. caliginosa* imply there may be a more complex relationship between internal concentration and sensitivity compared to for example chlorpyrifos. This species showed low sensitivity for survival, high sensitivity for reproduction and low (4th ranked) for tissue. For earthworms, little is known about well characterised cypermethrin targets, namely the transmembrane voltage gated sodium channels (VGSCs). In *C. elegans*, VGSCs are not encoded in the genome, with the function replaced by calcium dependent channels (Yu et al., 2005), resulting in a low *C. elegans* sensitivity to pyrethroids (Svendsen et al., 2010). VGSC sub-units have been identified in earthworm (*L. rubellus*) genomes (unpublished data from our own work) and a function as ion channels is suggested by the fact that cypermethrin caused effects at lower concentrations than fluoranthene, even though they both have similar high lipopholicities (Kow: cypermethrin 6.6, fluoranthene 5.15). This indicates a probable specific mode of action for cypermethrin, acting through VGSCs and/or other targets that are currently uncharacterised. Whatever the mode or modes of action in earthworms, the relatively limited range of variation in species sensitivity suggests cypermethrin has similar mechanisms of effect that impacts on pathways and endpoints that are well conserved across the species tested.

In addition to toxicodynamic interactions, species variations could also arise from differences in toxicokinetics. To assess the contribution of toxicokinetics, tissue concentration in all species were measured at a single concentration found to cause effects on reproduction in the majority of species. For chlorpyrifos, good agreement was found between measured internal concentration and sensitivity, as species showing the highest internal concentrations also showed high sensitivity. This suggests that toxicokinetic, rather than toxicodynamic traits may be a key determinant of earthworm sensitivity for chlorpyrifos. In a study for 15 taxonomically diverse aquatic species, Rubach et al. (2010) found that sensitivity was correlated with high uptake rates and high elimination rates. Although less clear than for chlorpyrifos, a relationship, was also found between tissue accumulation and sensitivity for cypermethrin. This indicates again that xenobiotic metabolism may be a more important contributor to difference between species than, toxicodynamic traits. In contrast, for imidacloprid, ranking for tissue concentrations did not correspond with ranking for sensitivity, with the most sensitive species *A. gracilis* showing the lowest accumulation. This suggests that for this

555 insecticide, differences in the expression and specific residues at key places in ligand binding site of
556 the highly complex nicotinic acetylcholinesterase receptor and related nicotinic acetylcholinesterase
557 binding proteins play a primary role in determining species sensitivity (Short et al., in review).

Conclusions

Overall, the study conducted highlights that it is possible to produce toxicity data reliably for earthworm species besides *E. fetida*. In 3 of the 4 species it was possible to produce performance in controls that met existing guideline requirements in all chemicals and for 3 of 4 for *D. octaedra* and to assess effects on reproduction with increasing exposure that allow concentration response modelling for sensitivity assessment. Comparisons between the tested species, illustrated that earthworms can show large variation in sensitivity to chemicals ranging from 2.5 to >30 fold. Further, the standard test species *E. fetida* was always among the two least sensitive species, supporting previous findings that this species may not be a reliable surrogate for showing the potential chronic effects of chemical exposure on earthworms. This underpins the need to consider further earthworm species in soil ecotoxicological assessments. Our results support the hypothesis that species show lower variation in their sensitivities to chemicals with a narcotic mode of action than for specifically acting substances. This hypothesis has been supported previously through meta-analysis, but here, for what we believe is the first time, we find support of this hypothesis through a bespoke assessment conducted within a single taxon. In addition to differences in sensitivity ranges between narcotic and targeted compounds, our results suggest that chemicals targeting more complex receptors (e.g. imidacloprid) may be result in greater variations in sensitivity between species than those targeting structurally simpler receptors (e.g. chlorpyrifos). Ultimately understanding the mechanisms which underlie differences will mean that species sensitivity could be predicted based on the mode of action and target sites in the organism.

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Table 1. CO1 characterisation of earthworm populations used for chemical exposure experiments. Animals within a population are separated into distinct clades if individuals present ≥ 10% divergence across CO1 sequence.

Earthworm species	CO1 Sequences obtained	Clades present	Dominant clade %	Nucleotide variation between clades	Dominant intra-clade variation as % nucleotide change.	Accession top BLAST hit (dominant clade) against Genbank nucleotide collection nr/nt (%ID)
E. fetida	20	2	65% (13/20)	10.0-16.5%	0%	MF121780.1 (100%)
L. rubellus	20	2	90% (18/20)	12.9-14.1%	3.80%	FN658819.1 (100%)
A. caliginosa	17	1	n/a	n/a	1.1%	KY633766.1 (99.84%)
A. gracilis	18	1	n/a	n/a	0%	KP214557.1 (100%
D. octaedra	15	1	n/a	n/a	0.20%	MF121754.1 (99.0%)

761 **Table 2.** LC_{50} and EC_{50} for effects of cocoon production rate values, with 95% confidence interval
762 where available, for the effects of fluoranthene, chlorpyrifos, cypermethrin and imidacloprid on five
763 earthworm species exposed in a soil-based laboratory toxicity test system with seven treatments
764 and four independent replicate for 28 days.
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		Survival			Cocoon prouduction			
Earthworm species	Chemical	LC_{50} mg/kg	Low 95% CI	Upper 95% CI	LC_{50} mg/kg	Low 95% CI	Upper 95% CI	Ratio to lowest
E. fetida	**Fluoranthene**	**>1170**	-	-	**158**	0	464	2.2
L. rubellus	**Fluoranthene**	**>1170**	-	-	**137**	68.9	206	1.9
A. calaginosa	**Fluoranthene**	**>1170**	-	-	**70.3**	24.5	116	1
D. octahedra	**Fluoranthene**	**>1170**	-	-	**178**	116	240	2.5
A. gracilis	**Fluoranthene**	**189**	16.5	361	**110**	0	294	1.6
E. fetida	**Chlorpyrifos**	**505**	364	647	**60.8**	41.2	80.4	10.4
L. rubellus	**Chlorpyrifos**	**97.4**	77	124	**6.1**	2.87	9.33	1
A. calaginosa	**Chlorpyrifos**	**285**	137	432	**5.86**	2.62	9.1	1
D. octahedra	**Chlorpyrifos**	**117**	0	269	**15.6**	4.77	26.4	2.7
A. gracilis	**Chlorpyrifos**	**139**	137	142	**7.64**	0	15.5	1.3
E. fetida	**Cypermethrin**	**731**	620	841	**21.3**	16.9	25.6	6.1
L. rubellus	**Cypermethrin**	**>500**	-	-	**26.4**	21.6	31.2	7.5
A. calaginosa	**Cypermethrin**	**>1500**	-	-	**3.5**	0	8.74	1
D. octahedra	**Cypermethrin**	**58.7**	30.8	86.5	**11.6**	7.89	15.22	3.3
A. gracilis	**Cypermethrin**	**206**	153	258	**10.1**	7.51	12.7	2.9
E. fetida	**Imidacloprid**	**5.58**	4.78	6.65	**3.08**	1.07	5.1	27.0
L. rubellus	**Imidacloprid**	**>10**	-	-	**3.59**	2.64	4.53	31.5
A. calaginosa	**Imidacloprid**	**>10**	-	-	**1.13**	0.584	1.67	9.9
D. octahedra	**Imidacloprid**	**1.93**	1.51	2.62	**0.37**	0.28	0.461	3.2
A. gracilis	**Imidacloprid**	**0.79**	0.65	0.95	**0.114**	0.032	0.197	1

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769 **Table 3.** Ranking of species in relation to sensitivity for effects of each of the four tested chemicals
770 on LC_{50} and EC_{50} for cocoon production rate and internal concentration measured at the same
771 exposure concentration for all species
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Earthworm species	Chemical	Ranking LC_{50}	Ranking CPR EC_{50}	Ranking Tissue Conc.
E. fetida	Fluoranthene	=5	3	
L. rubellus	Fluoranthene	=5	2	
A. calaginosa	Fluoranthene	=5	1	
D. octahedra	Fluoranthene	=5	4	
A. gracilis	Fluoranthene	1	5	
E. fetida	Chlorpyrifos	5	5	5
L. rubellus	Chlorpyrifos	1	=1	2
A. calaginosa	Chlorpyrifos	4	=1	1
D. octahedra	Chlorpyrifos	2	4	4
A. gracilis	Chlorpyrifos	3	3	3
E. fetida	Cypermethrin	3	4	5
L. rubellus	Cypermethrin	=5	5	3
A. calaginosa	Cypermethrin	=5	1	4
D. octahedra	Cypermethrin	1	3	2
A. gracilis	Cypermethrin	2	2	1
E. fetida	Imidacloprid	3	4	1
L. rubellus	Imidacloprid	=5	5	4
A. calaginosa	Imidacloprid	=5	3	2
A. gracilis	Imidacloprid	1	1	2
D. octahedra	Imidacloprid	2	2	5

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Legends to Figures

Fig. 1. Left side plots: measured tissue concentrations of fluoranthene, chlorpyrifos, cypermethrin and imidacloprid in *L. rubellus* exposed across the exposure range, solid line indicates the linear regression fit of measured values, the dashed line the 1 : 1 ratio of measured to nominal soil concentrations; and Right side plots: tissue concentrations for the five earthworm species exposed at a single concentration of 130 mg/kg for fluoranthene, 81 mg/kg for chlorpyrifos, 55 mg/kg for cypermethrin, 0.37 mg/kg for imidacloprid.

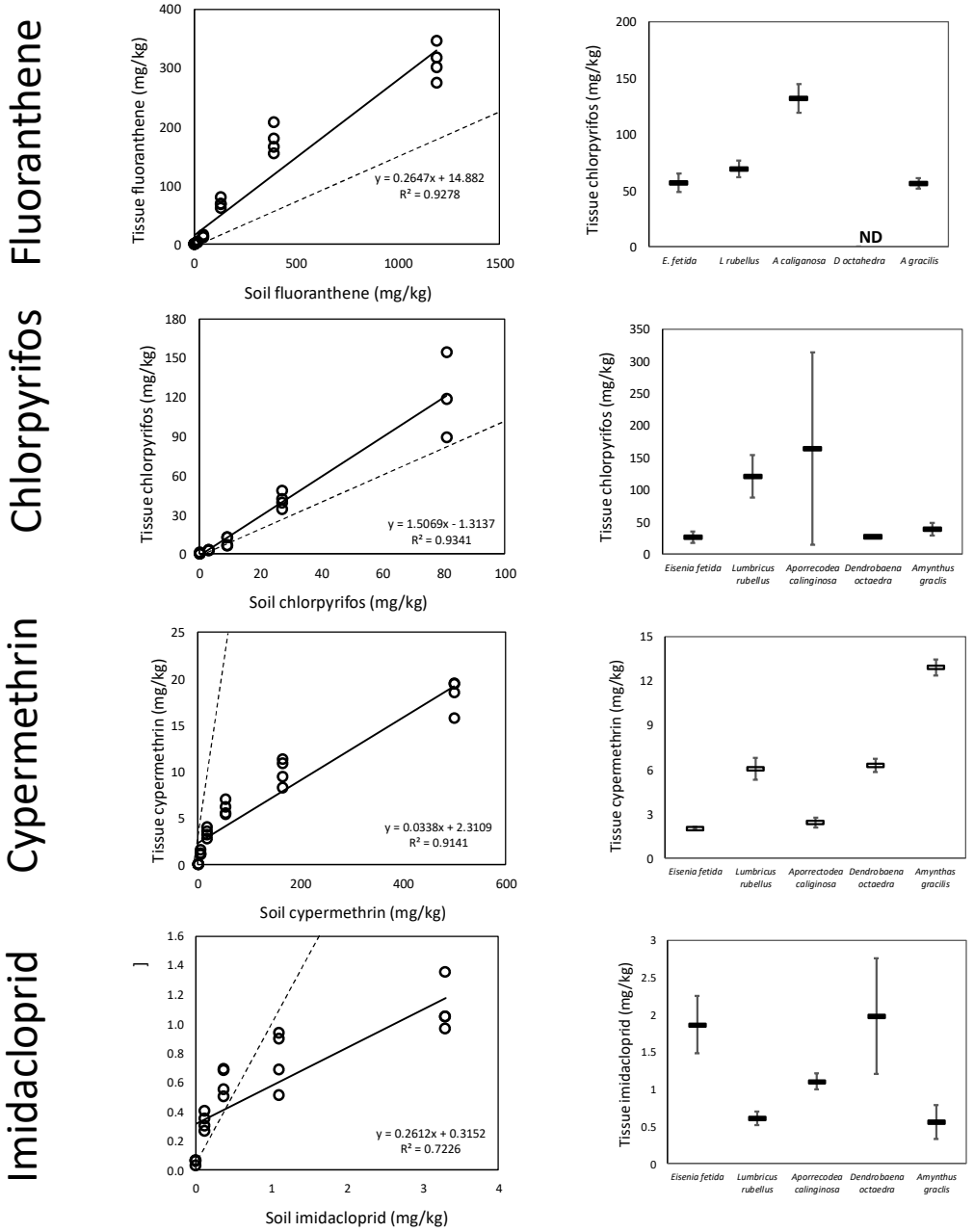
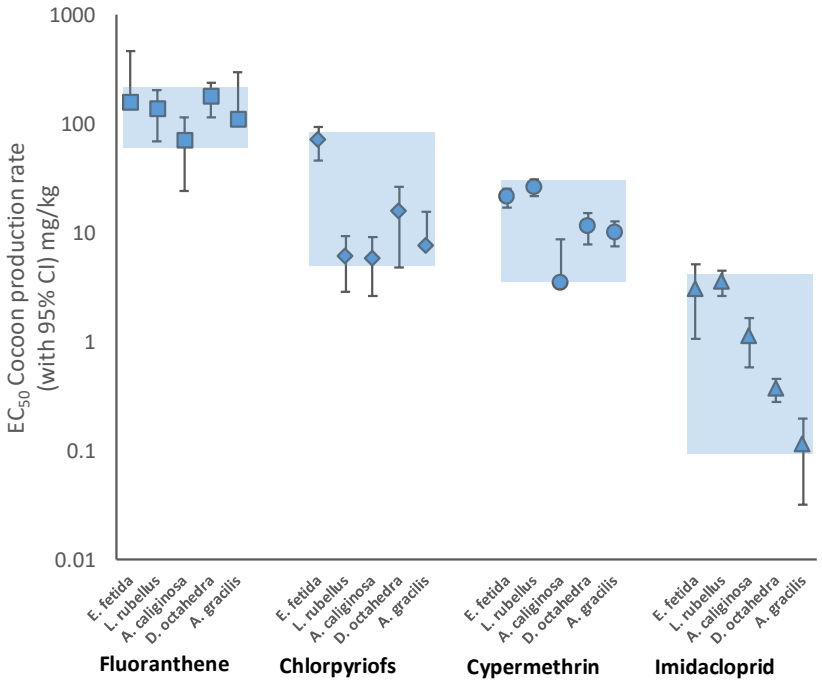


Fig. 2. Cocoon production rate EC_{50} values (with 95% Cis) for five earthworm species exposure to one narcotic, fluoranthene and three specifically acting chemicals, the blue box highlights the greater range of variation in values for the receptor targeting insecticides compared to the non-specific chemical.



796 **Legends to Supplementary Figures**

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798 **Supplementary Fig. 1.** Measured versus nominal concentrations of (A) fluoranthene, (B)
799 chlorpyrifos, (C) cypermethrin and (D) imidacloprid in soils collected at the end of the 28 day
800 exposure period.

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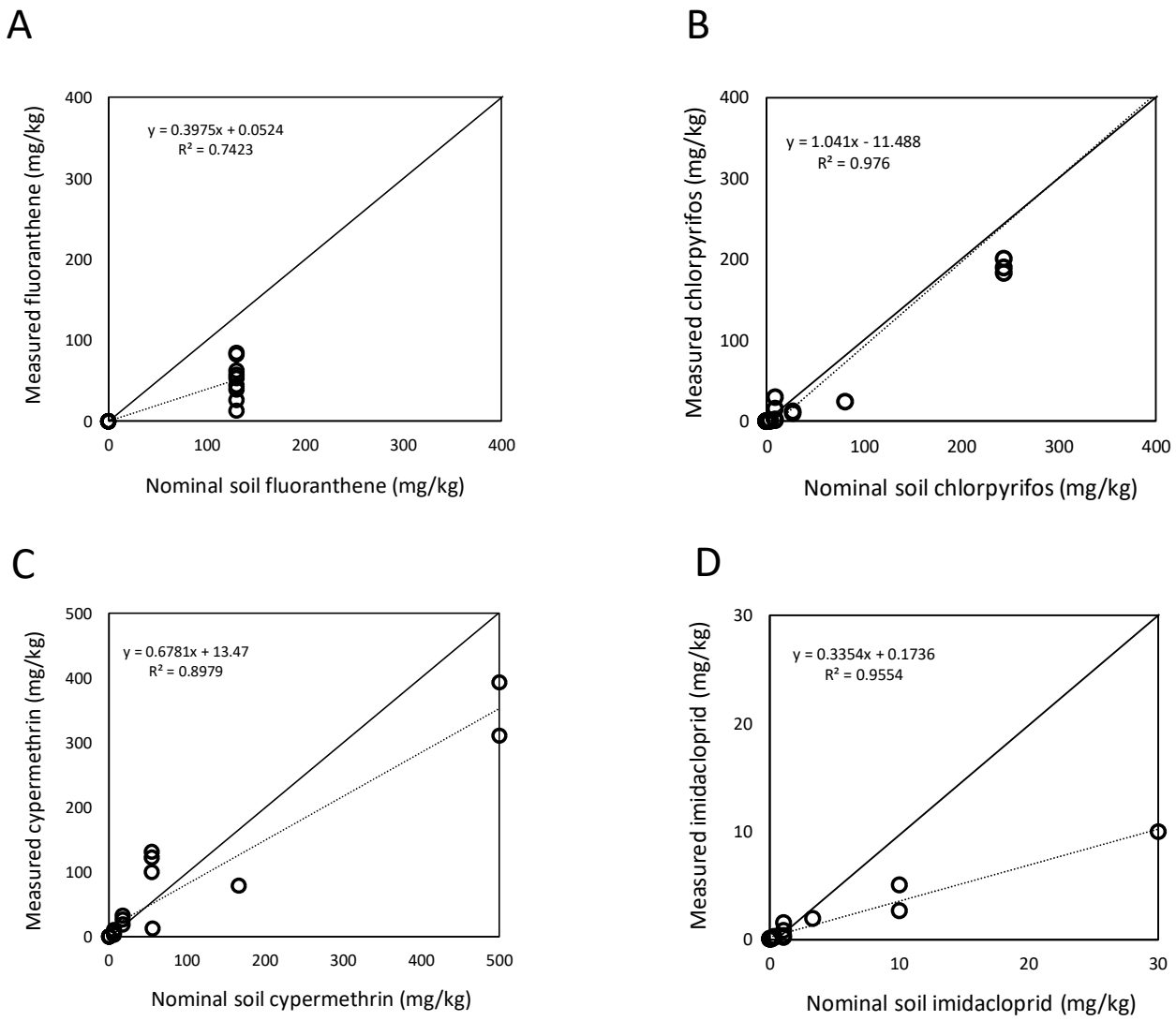

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Supplementary Fig. 2. Concentrations response relationships for fluoranthene, chlorpyrifos, cypermethrin and imidacloprid effects on cocoon production rate in five earthworm species (*E. fetida*, *L. rubellus*, *A. caliginosa*, *D. octaedra*. *A. gracilis* of versus nominal concentrations of fluoranthene, chlorpyrifos, cypermethrin and imidacloprid, individual point represents replicate values, the solid line in the fitted logistic regression model, the error bars indicate the 95% confidence interval of the cocoon production rate EC_{50}.