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Earthworm lipid content and size help account for differences in pesticide bioconcentration between species

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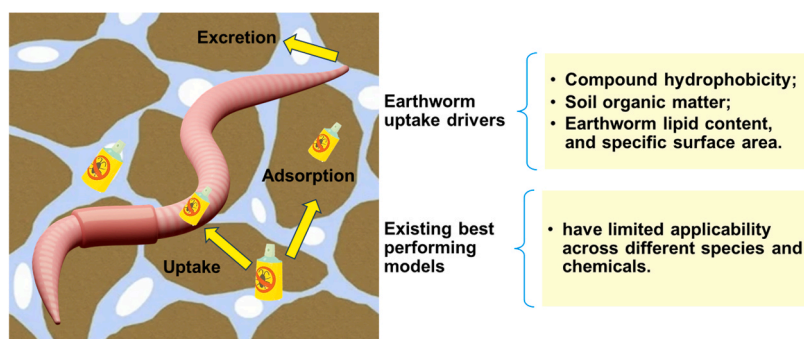
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HIGHLIGHTS

- Earthworm bioconcentration of pesticides increased with compound hydrophobicity.
- Earthworm bioconcentration of pesticides decreased with soil organic matter.
- Hydrophobic compound uptake varied with species lipid content and surface area.
- Existing bioconcentration models performed less well for larger earthworm species.
- Existing models performed less well for hydrophilic ($\log K_{ow} < 2$) compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

The uptake and elimination kinetics of pesticides from soil to earthworms are important in characterising the risk of pesticides to soil organisms and the risk from secondary poisoning. However, the understanding of the relative importance of chemical, soil, and species differences in determining pesticide bioconcentration into earthworms is limited. Furthermore, there is insufficient independent data in the literature to fully evaluate existing predictive bioconcentration models. We conducted kinetic uptake and elimination experiments for three contrasting earthworm species (*Lumbricus terrestris*, *Aporrectodea caliginosa*, *Eisenia fetida*) in five soils using a mixture of five pesticides ($\log K_{ow}$ 1.69 – 6.63). Bioconcentration increased with pesticide hydrophobicity and decreased with soil organic matter. Bioconcentration factors were comparable between earthworm species for hydrophilic pesticides due to the similar water content of earthworm species. Inter-species variations in bioconcentration of hydrophobic pesticides were primarily accounted for by earthworm lipid content and specific surface area (SSA). Existing bioconcentration models either failed to perform well across earthworm species and for more hydrophilic compounds ($\log K_{ow} < 2$) or were not parameterised for a wide range of compounds and earthworm species. Refined models should incorporate earthworm properties (lipid content and SSA) to account for inter-species differences in pesticide uptake from soil.

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1. Introduction

To achieve high levels of agricultural production, pesticides are used worldwide to protect crops from pest, weed and disease pressure [56,7]. Following application to crops or soil, some pesticides can accumulate in the soil which could pose risks to ecosystems [44,47,58]. Earthworms are recognised as important ecosystem engineers due to their ability to improve soil nutrient cycling, soil aeration, and soil structure [6,8]. Earthworms are known to accumulate a range of organic compounds in their tissues, e.g. [5,3,12,13,38,37,26]. As earthworms are consumed by various predators, including birds and mammals, biomagnification of pesticides through the terrestrial food web could be a cause for concern [20]. Therefore, earthworms are frequently included in the frameworks for risk assessment of pesticides in terrestrial environments [22,46] and for risk assessment from secondary poisoning via the terrestrial food chain [16,20].

Broadly, there are two modelling approaches for predicting chemical uptake in earthworms for risk assessment, empirical and kinetic models [17,20,21]. Empirical models predict earthworm body concentrations on the basis of statistical relationships derived from experimental data and primarily consider equilibrium partitioning. In these models, the steady-state earthworm body concentration is estimated assuming that chemicals partition between different phases (usually including earthworm lipids for organic compounds), e.g. [28,54]. In contrast, kinetic models predict body concentrations over time on the basis of uptake and excretion constants [17]. Both types of model can be used to characterise the risk of pesticides to soil organisms based on estimates of toxicity and the risk from secondary poisoning, based on estimates of body residues [17,20,30]. In order to support the development of kinetic models, research on the uptake and elimination kinetics of pesticides from soil to earthworms has steadily increased over the last two decades; these studies have revealed that bioconcentration differs between chemicals, soils and earthworm species [24,49,52,53,57,61,62].

Earthworms display a variety of ecological strategies, such as feeding habits and burrowing behaviours, resulting in varying capacities to access and digest soil organic matter, which may translate into varying exposure to soil-bound contaminants [19,32,45]. Goto and Sudo [24] found that soil concentration-based uptake and elimination rate constants for the herbicides trifluralin and pendimethalin varied between the earthworms *Eisenia fetida* (lipid content 1.3% wet weight) and *Pheretima spp* (lipid content 0.76% wet weight). The differences in rate constants between species were attributable in part to the lipid content of each species. Specifically, uptake rate constants in *E. fetida* were a factor of 5 larger than those in *Pheretima spp* while the elimination rate constants were factors of 2 to 4 smaller. Svobodová et al. [53] and Šmídová and Hofman [49] found that both the uptake and elimination rate constants for lindane ($\log K_{ow}$ of 3.72) were larger than those observed for more hydrophobic pesticides (e.g. p,p'-DDT, $\log K_{ow}$ of 6.63). Various studies have shown that uptake rate constants for pesticides such as lindane, p, p'-DDT, chlorpyrifos, and tebuconazole decrease with increasing soil organic carbon content (SOC), whereas elimination rate constants increase with increasing SOC content [52,53,57]. Several studies have shown that the kinetic bioconcentration potential for organic chemicals such as pharmaceuticals, polycyclic aromatic hydrocarbons and brominated flame retardants in earthworms may be influenced by factors such as the ionisation state of molecules at different soil pHs [12,26], the sorption affinity of chemicals in soil [13,19,40,39] and the surface area to volume ratio of the organism [11]. Additionally, the effects of species traits on bioconcentration also vary with chemical properties and soil properties [11,59]. Overall, existing bioconcentration studies for pesticides focus on a limited number of pesticides representing a narrow range of chemical properties (such as $\log K_{ow}$) and involve a small number of soil types and earthworm species; this severely hinders the identification of the mechanisms involved in uptake and elimination processes.

Li et al., (unpublished results, [34]) evaluated the performance of four empirical [14,5,28,54] and two kinetic [1,29] earthworm chemical

uptake models using independent datasets obtained from the literature. The models used measured soil concentration to derive a porewater concentration for the chemical of interest, the porewater concentration was then used to predict the earthworm body concentration. Of the models evaluated they found that the best performing empirical model was that of Belfroid et al. [5] which considered both equilibrium partitioning of compounds between soil porewater and earthworm tissues, but also dietary uptake calculated from feeding rate and uptake efficiency. The best performing kinetic model was that of Jager et al. [29]. However, they concluded that existing datasets lacked sufficient coverage of different earthworm species and more hydrophilic pesticides ($\log K_{ow} < 2$) to fully evaluate the models. Furthermore, the model of Jager et al. [29] considers two separate uptake routes, dermal and gut uptake, and kinetic datasets that are appropriate for assessing these are very scarce.

Therefore, this study aimed to generate a large independent dataset of earthworm kinetic bioconcentration data to aid the identification of factors determining pesticide bioconcentration and strengthen the evaluation of existing models. The specific objectives were to: 1) determine uptake and elimination kinetics of five pesticides for three contrasting earthworm species (*Lumbricus terrestris*, *Aporrectodea caliginosa*, *Eisenia fetida*) and five different soils; 2) use principal component analysis to identify properties of chemicals, soils and earthworms that influence earthworm bioconcentration; and 3) evaluate the predictive capacity of existing bioconcentration models using the resulting data.

2. Materials and methods

2.1. Study pesticides and reagents

Five pesticides (lenacil, flutriafof, dieldrin, hexachlorobenzene and p,p'-DDT) were selected for our study to represent pesticides that are persistent in soil and which have a wide range of hydrophobicity ($1.69 < \log K_{ow} < 6.63$). Detailed information on the physico-chemical properties of each compound is provided in Table S1. Pure forms ($\geq 95\%$) of the pesticides and two internal standards (4,4'-DDT-d₈, PCB 153) were purchased from Sigma-Aldrich (Gillingham, UK). Solvents including methanol, acetonitrile and acetone were LC-MS grade and were purchased from Fisher Scientific (Loughborough, UK). QuEChERS EN extraction kits containing buffered extraction salts (4 g magnesium sulphate, 1 g sodium chloride, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and dispersive solid phase extraction kits containing 400 mg primary secondary amine, 400 mg end-capped C-18 sorbent and 1200 mg magnesium sulphate were purchased from Agilent Technologies (CA, USA).

2.2. Test soils and organisms

Three surface soils were collected from Pollybell Farms (Doncaster, UK), Stockbridge Technology Centre (York, UK), and the University of York (York, UK). Two standard soils (LUFA 2.1 and 2.2) were obtained from LUFA Speyer (Speyer, Germany). The soils were air-dried, sieved through a 2-mm mesh to ensure homogeneity, and then stored in sampling bags at room temperature until further use. The soils were chosen to represent a broad range of soil properties that may influence the uptake of pesticides into earthworm tissues, including pH (5.23 - 7.06), organic matter content (0.972 - 39.9 wt%), cation exchange capacity ($1.41 - 88.8 \text{ cmol}^+ \text{ kg}^{-1}$), and clay content ($< 2 \mu\text{m}$, 4.02 - 50.0 wt%). Soil characteristics are provided in Table S2. Background concentrations of the pesticides in the soils used in our study were below detection (Table S3). Further information on the measurement procedures for soil characteristics are described in the Supporting Information (SI).

The earthworm species *L. terrestris*, *E. fetida*, and *A. caliginosa* were purchased from Worms Direct (Ipswich, UK), Blades Biological Ltd. (Kent, UK) and S.A.S PRODIGGA (Caumont-sur-Durance, France), respectively. Adult earthworms with a developed clitellum were kept in

a plastic box containing 10 kg of fresh University of York soil in a growth chamber under experimental conditions (see below) prior to use in the experiments. The three earthworm species display diverse biological and physiological characteristics. *L. terrestris* is an anecic earthworm that burrows deeply but feeds on soil surface litter during the night [48]. *E. fetida* is an epigeic earthworm which lives on the soil surface and feeds on leaves and manure [48]. *A. caliginosa* is an endogeic earthworm which lives in horizontal burrows in the upper soil layer and feeds on soil and organic matter [9]. In addition to their ecological traits, the *L. terrestris*, *E. fetida*, and *A. caliginosa* used in the experiments had significantly different (one way ANOVA-test, $p < 0.001$) lipid contents (9.21 ± 1.44 , 19.44 ± 1.44 , 13.64 ± 1.56 dry wt% respectively, mean values \pm standard deviation, $n = 6$), and specific surface areas (SSA, 0.70 ± 0.15 , 1.01 ± 0.10 and 1.45 ± 0.13 m² kg⁻¹), but similar water contents ($83.9 \pm 0.92\%$, $85.6 \pm 1.27\%$ and 85.1 ± 0.77 wet wt%). Background tissue concentrations of the pesticides used in our study in the earthworms were below detection (Table S3). Further information on the measurement procedures for earthworm characteristics are described in the SI.

2.3. Bioconcentration experimental design

A bioconcentration experiment was conducted with each earthworm species separately to investigate the uptake and elimination kinetics of test compounds from soil. The experimental design followed OECD Guideline 317 "Bioaccumulation in Terrestrial Oligochaetes." [41]. Exposure soils were prepared by adding a mixture of pesticides to dry soil using a carrier solvent (acetone) to achieve nominal soil concentrations of 7, 4, 4, 4, and 1.2 mg kg⁻¹ (dry weight) of lenacil, flutriafof, dieldrin, hexachlorobenzene and p,p'-DDT, respectively. In order to ensure that the mixture of pesticides had no adverse effect on the earthworms over the duration of the experiment, the exposure concentrations selected for the test compounds were at least 100-fold lower (lenacil, flutriafof, hexachlorobenzene) than the earthworm acute LC50 values reported in the Pesticide Properties Database (PPDB) or 8-fold lower (dieldrin, p,p'-DDT) than experimental concentrations reported in existing bioconcentration tests [31,53]. After the addition of the spiking solution to the soil and thorough mixing, the carrier solvents were allowed to evaporate for 72 h in a fume hood. The contaminated soil was then mixed with deionised water using a spatula to achieve a moisture content of 60% water holding capacity (WHC). Treated soil was kept in a growth chamber under experimental conditions (see below) for 24 h before adding the earthworms.

The experiments were conducted in the dark at temperatures appropriate for culturing the different species [35,41]: 20 ± 1 °C for *E. fetida* and 12 ± 1 °C for *L. terrestris* and *A. caliginosa*. For each compound, 45 plastic cups of treated soil (150 g of dry weight soil for *E. fetida*, and *A. caliginosa*; 280 g of dry weight soil for *L. terrestris*) were prepared for each soil type, along with solvent controls (no test substance) and negative controls (no solvent or test substance). At the start of the uptake phase, three mature adult, i.e. with a visible clitellum, *E. fetida* or *A. caliginosa* or one mature adult *L. terrestris* were added to each cup. More individuals of the lower weight *E. fetida* and *A. caliginosa* were used to ensure sufficient earthworm tissue for analysis. A previous study [51] found that varying the soil-to-earthworm ratio did not affect uptake rates. At each sampling point, all earthworms in one cup were pooled and constituted one replicate sample. The cups were then wrapped with garden fleece that was secured with an elastic band to prevent earthworm escape during the exposure. The uptake phase of the experiment lasted for 21 d, during which bulk soil, earthworm and soil porewater were sampled from three sacrificial replicate pots at 0 and 6 h and 1, 3, 7, 14 and 21 d. After 21 d, earthworms in the remaining cups were transferred to clean soil for another 21 d of elimination, with bulk soil, earthworm and soil porewater samples again being taken from 3 sacrificial replicates at 6 h and 1, 3, 7, 14, and 21 d after transfer. The weight and mortality of earthworms were recorded at each time point

throughout both phases. At each time point there was no mortality or weight change relative to initial weights within $\pm 20\%$ in any of the test systems, thus meeting the validity criteria established by the OECD 317 protocol. The soil moisture content in each cup was monitored via weighing during the exposure and maintained at 60% of the WHC by adding deionised water when necessary. After the exposure, earthworms from independent replicates were removed, rinsed with deionised water, gently dried with laboratory tissue, weighed and placed in a Petri dish with moistened filter paper for 48 h to allow them to purge their gut contents; the filter paper was replaced at the start and end of each day [2]. Earthworms were next dried with paper towel, reweighed, and the earthworms from each replicate were frozen together at -20 °C until analysis. Pot soil was homogenised and 50 g from each replicate was stored in a 50 mL centrifuge tube (Fisher Scientific, UK) at -20 °C for the determination of pesticide concentration in bulk soil and soil porewater.

2.4. Preparation of samples for analysis

Pesticides in earthworm samples were extracted and cleaned-up following the optimised QuEChERS procedure based on the methods given by Yu et al. [63] and Svobodová et al. [52]. Prior to extraction, all the frozen earthworms from a replicate were lyophilised using a freeze-dryer (Scanvac coolsafe, Labogene, Denmark). This material was reweighed, placed in a centrifuge tube (50 mL), and homogenised by shaking with two ceramic homogenisers. Forty μ L of 4,4'-DDT-d₈ (250 mg L⁻¹) as an internal standard was added into the tube to correct for variations caused by the subsequent extraction steps and the final GC-MS analysis. 10 mL of deionised water was added and allowed to stand for 60 min. Then, 10 mL acetonitrile was added, and the mixture was shaken for 2 min using a vortex device (Vortex Fischer Scientific FB15013 TopMix, UK). Next, the contents of the QuEChERS EN extraction kit were added and shaken vigorously and vortexed for 2 min, followed by centrifuging at 4000 rpm for 10 min (Rotanta 460 Centrifuge, Hettich, Germany). Eight mL of supernatant was transferred into a 15 mL centrifuge tube (Agilent Technologies, CA, USA) containing a QuEChERS dispersive solid phase extraction kit. The sample was shaken vigorously for 1 min with a vortex device, followed by centrifuging at 4000 rpm for 10 min. Then 4.5 mL of extractant was transferred into a glass tube and evaporated to near dryness under a gentle stream of nitrogen at 35 °C. The residue was reconstituted in 1 mL of acetone containing the second internal standard PCB-153 (5 mg L⁻¹) and filtered into amber glass vials for GC-MS analysis using 0.7 μ m glass fiber syringe filters (Agilent Technologies, CA, USA). PCB 153 was added to monitor the performance of the GC-MS.

Pesticides in soil samples were also extracted and cleaned-up using the QuEChERS method. This extraction captured both the pesticide in the soil porewater and that adsorbed to soil particles. The frozen soil samples were lyophilised using a freeze-dryer. A representative 5 g (dry weight) of the soil sample was weighed into a 50 mL centrifuge tube. Soil samples were then extracted using a similar procedure as for the earthworm sample extractions, but without the homogenisation step.

Soil porewater samples were separated from bulk soil samples using a centrifugation method described by Carter et al. [12]. Soil samples were defrosted at room temperature and then 30 g of soil was inserted into a 20 mL disposable syringe (Sigma-Aldrich, Gillingham, UK) with a layer of 3 cm of glass wool at the bottom. The syringe was placed in a 50 mL centrifuge tube and centrifuged at 3000 rpm for 30 min (2 \times 15 min runs, Hettich Rotanta 460). 1.5 mL of porewater was collected from the bottom of the centrifuge tube and transferred to a 2 mL microcentrifuge tube (Fisher Scientific, UK) for extraction of pesticide. This tube was centrifuged at 14000 rpm (Eppendorf Centrifuge 5424, Hamburg, Germany) for 5 min to sediment loose particles. One mL of the upper layer was pipetted into a new 2 mL microcentrifuge tube to which 20 μ L of the recovery internal standard (250 mg L⁻¹ 4,4'-DDT-d₈) was added. The solution was evaporated to near dryness using a vacuum sample concentrator (SP Scientific, Warminster, PA, USA). The residue was

reconstituted in 1 mL of acetone containing a second internal standard PCB-153 (5 mg L⁻¹) and filtered into amber glass vials for GC-MS analysis using glass fiber syringe filters.

2.5. Analytical method

Filtered samples were analysed using a Clarus 680/600 C GC-MS (PerkinElmer, UK) equipped with an Elite-5MS fused silica capillary column (L 30 m × 0.25 mm i.d. × 0.25 μm film thickness; PerkinElmer). The oven was programmed from an initial temperature of 70 °C held for 2 min and ramped at 30 °C/min to 170 °C without holding, then ramped at 15 °C/min to 310 °C without holding. One μL of sample was injected in split mode at 250 °C using helium as the carrier gas. The MS was operated in electron ionisation (EI) mode with an ionisation energy of 70 eV, source temperature of 180 °C and inlet line temperature of 240 °C. The quantification of pesticides was performed in selective ion monitoring (SIM) mode. The retention time and selected ions for each compound are provided in Table S3.

The performance of the analytical method including linearity, intra- and inter-day repeatability, limit of detection (LOD) and quantitation (LOQ) and extraction recovery as well as the details of method validation are provided in Tables S3-S5. Overall, the proposed analytical method achieved low LOQs (ranging from 0.06 to 0.10 mg L⁻¹) in acetone and good intra- and inter-day repeatability at the spiking levels of 1 mg L⁻¹ for all target compounds (relative standard deviation <5%). The average extraction recoveries for the pesticides at low (0.2 mg kg⁻¹ for soil and earthworm samples, 0.5 mg L⁻¹ for soil porewater samples) and high (2 mg kg⁻¹ for soil and earthworm samples, 5 mg L⁻¹ for soil porewater samples) spiking levels were 117.7 ± 11.3% and 94.7 ± 17.7% for soil samples (n = 25), 89.2 ± 25.4% and 82.6 ± 21.0% for earthworm samples (n = 15) and, 83.2 ± 24.5% and 88.5 ± 22.9% for soil porewater samples (n = 25).

2.6. Kinetic modelling

To determine whether bioconcentration was better related to bulk soil or soil porewater concentrations, uptake and elimination kinetic modelling was conducted for both concentrations. Rate constants were determined using a first-order kinetic model as described in Eqs. (1) and (2):

$$\frac{dC_{\text{earthworm}}}{dt} = k_{\text{in,soil}} \times C_{\text{soil}}(t) + k_{\text{out,soil}} \times C_{\text{earthworm}}(t) \quad (1)$$

$$\frac{dC_{\text{earthworm}}}{dt} = k_{\text{in,pw}} \times C_{\text{pw}}(t) + k_{\text{out,pw}} \times C_{\text{earthworm}}(t) \quad (2)$$

where t is time (d); $C_{\text{earthworm}}$ is the concentration of substance in the earthworm (mg kg⁻¹ wet weight); C_{soil} and C_{pw} are the concentrations of substance in the soil (mg kg⁻¹ dry weight) and porewater (mg L⁻¹), respectively; $k_{\text{in,soil}}$ and $k_{\text{in,pw}}$ are the uptake rate constants in tissue from soil (kg soil kg⁻¹ earthworm d⁻¹) and porewater (L porewater kg⁻¹ earthworm d⁻¹), respectively; $k_{\text{out,soil}}$ and $k_{\text{out,pw}}$ are the elimination rate constants (d⁻¹) calculated based on chemical concentrations in bulk soil (which includes porewater) and porewater, respectively.

The kinetic bioaccumulation factors based on soil and porewater concentrations ($BCF_{k,\text{soil}}$ and $BCF_{k,\text{pw}}$) were calculated from the ratio of the uptake rate constant in tissue, k_{in} and the elimination rate constant, k_{out} for soil or soil porewater (Eq. 3):

$$BCF_k = k_{\text{in}} / k_{\text{out}} \quad (3)$$

where $BCF_{k,\text{soil}}$ is soil-based bioconcentration factor (kg kg⁻¹), $BCF_{k,\text{pw}}$ is porewater-based bioconcentration factor (L kg⁻¹).

The model was implemented using the ODE solver in Matlab (R2021b) with the BYOM modelling platform (version 6.0) (<http://debttox.info/byom.html>). Statistical inference was based on likelihood-

ratio testing, and 95% confidence intervals on model parameters and model predictions were generated using likelihood profiling. Models were calibrated using the simplex algorithm to find the best fit parameter values.

2.7. Statistical analysis and evaluation of existing models

Principal component analysis (PCA) was conducted in SPSS (version 25.0) to identify which properties of chemicals, soil and earthworm species influence the bioconcentration of pesticides in earthworms. Separate PCAs were conducted for the BCF_k , k_{in} , and k_{out} values for both the bulk soil and soil porewater values. Chemical properties included in the PCA were log K_{om} (sorption coefficient calculated as the ratio of chemical concentration in soil to concentration in porewater and normalised to organic matter), log K_{ow} (octanol-water partition coefficient) and TPSA (fragment-based polar surface area from N, O, S, P polar co-efficients); soil properties were OM (organic matter content), Clay (clay content), CEC (cation exchange capacity) and pH; earthworm properties were Lipid (lipid content) and SSA. The water content of the earthworms was excluded due to the lack of significant variation between species (one way ANOVA-test, $p > 0.05$). The first three principal component axes were chosen to reduce the dimensionality of data according to the broken stick eigenvalue test [33]. Pearson statistical bivariate correlation analyses were applied between chemical, soil and earthworm properties using SPSS (version 25.0) to identify potential intercorrelation between them.

To further explore whether lipid content and SSA account for inter-species differences in pesticide uptake from soil, BCF_k values were normalised to earthworm lipid content and SSA. For each pesticide in each soil, the ratio of the maximum to minimum BCF values between the three species was calculated, giving 5 values per pesticide. A two-tailed t-test was performed in GraphPad Prism (version 9.0) to investigate significant differences between the maximum to minimum ratios before and after normalisation to determine whether normalisation significantly reduced variation in values between species.

The best-performing existing models (the empirical model of [5] and kinetic model of [29]) for estimating the bioconcentration of organic chemicals in earthworms as demonstrated by our previous study (Li et al., unpublished results, [34]) were evaluated using the uptake data obtained in this study. Model details are given in Table S6. The empirical model was evaluated using experimental data on the steady-state or maximum internal concentrations of earthworms exposed to all five different pesticides in five soil types. The kinetic model was calibrated previously for hexachlorobenzene to a single soil type and earthworm species [29]; therefore, only the data obtained for hexachlorobenzene in the present study were used to evaluate the performance of the kinetic model. The calibrated values reported by Jager et al. [29] for input parameters including the earthworm-soil organic matter partition coefficient (K_{ws}), rate constants for exchange across skin (k_s) and gut wall (k_g) and the degradation rate constant (k_d) as well as fixed values for the feeding process parameters were applied to predict the internal concentrations in earthworms over time. Predictions derived using those previously calibrated values were compared with predictions made using K_{ws} and k_d values that were measured in the present study. The applicability and accuracy of existing models were assessed by calculating Nash–Sutcliffe Efficiencies (NSE) and the percentage of predictions within a factor of 10 of the measured values. The calculation of NSE is described in the SI.

3. Results and discussion

Pesticide uptake and elimination kinetics were well described by the first-order kinetic model when using chemical concentrations in either bulk soil or soil porewater as input, with fitted R^2 values in the range 0.75 - 0.97 for soil concentration data (Table S7) and 0.71 - 0.97 for porewater concentration data (Table S8) ($p < 0.05$ for all values). The

soil-based BCF_k values varied by up to three orders of magnitude across pesticides, soils and earthworm species (Fig. 1A), whereas the porewater-based BCF_k varied by up to six orders of magnitude (Fig. S1A). $BCF_{k,pw}$ and $BCF_{k,soil}$ values were comparable for the most hydrophilic pesticide (lenacil), whereas $BCF_{k,pw}$ values were consistently higher than $BCF_{k,soil}$ values for the more hydrophobic pesticides (flutriafol, dieldrin, hexachlorobenzene and p,p'-DDT) because of their greater sorption affinities to the soil which resulted in lower porewater concentrations. Overall, values for BCF_k , k_{in} and k_{out} calculated from chemical concentrations in either bulk soil or soil porewater show similar trends, so the primary focus here is given to values derived from concentrations in bulk soil. Data derived from soil porewater concentrations are provided in the SI.

Bioconcentration of the pesticides we tested in earthworms has rarely been studied. Only a few experiments have investigated the uptake of p,p'-DDT by earthworms [49,50,53,57], and all of these studies were conducted only on the standard earthworm species (epigeic *E. fetida* or *E. andrei*) recommended in OECD Guidelines [41] for bioconcentration tests. The previous studies were performed in a range of soil types with SOC contents ranging from 0.47 to 20.19% and soil exposure concentrations ranging from 0.29 to 15.51 mg kg⁻¹ (dry weight). The steady-state or kinetic BCF values reported in the literature for p,p'-DDT ranged from 0.27 to 8.11 (kg kg⁻¹ wet weight), which is a similar range to the $BCF_{k,soil}$ values obtained in this study (0.48–11.03 kg kg⁻¹ wet weight for *E. fetida*). This observation suggests that our approach is robust and that the effect of experimental conditions on BCF values is negligible.

$BCF_{k,soil}$ values varied between pesticides by up to a factor of 736; values between soils varied by up to a factor of 114; and values between earthworm species varied by up to a factor of 6.3. Similarly, $BCF_{k,pw}$ values varied between pesticides by up to a factor of 1.05×10^6 , between soils by up to a factor of 17.4, and between earthworm species by up to a factor of 8.3. Generally, BCF_k increased with increasing hydrophobicity and with decreasing soil organic matter content. Variation in BCF_k values across earthworm species tended to increase with hydrophobicity of the pesticide. Values for *E. fetida* and *A. caliginosa* were more similar and higher than those for *L. terrestris*, particularly for the more hydrophobic compounds. The variation in BCF_k values across earthworm species tended to decrease with soil OM content. The k_{in} values for the five pesticides followed similar trends to the BCF_k values. These observations indicate that the variation in bioconcentration across earthworm species varies depending on the properties of the chemical and soil. Variation in the k_{out} values showed different trends to those of BCF_k and k_{in} . Values tended to decrease with increasing hydrophobicity of the pesticide and increase with increasing OM content. Variation between earthworm species was small with no evident trends between pesticides and soils.

3.1. PCA analysis

To further understand the factors driving variation in the BCF_k and k values, PCA analysis was carried out (Fig. 2 and Fig. S2). The PCA indicated that chemical properties explained the greatest variation in $BCF_{k,soil}$ values based on the strongest loading of log $BCF_{k,soil}$ on axis 1 (−0.86), followed by soil properties (loading of −0.32 on axis 2) and earthworm properties (loading of 0.15 on axis 3, Table S9). Similarly, chemical properties played a predominant role in explaining the variation in $BCF_{k,pw}$ values (loading of 0.97 on axis 1), whereas soil and earthworm properties played only minor roles (loading of 0.02 and 0.07 on axis 2 and 3, respectively, Table S10).

3.1.1. Chemical properties

The PCA indicated that log K_{om} and log K_{ow} had a strong positive effect on the uptake rate constants and a strong negative effect on the elimination rate constants resulting in a strong positive effect on $BCF_{k,soil}$ and $BCF_{k,pw}$. In contrast, the TPSA had a strong negative effect on uptake

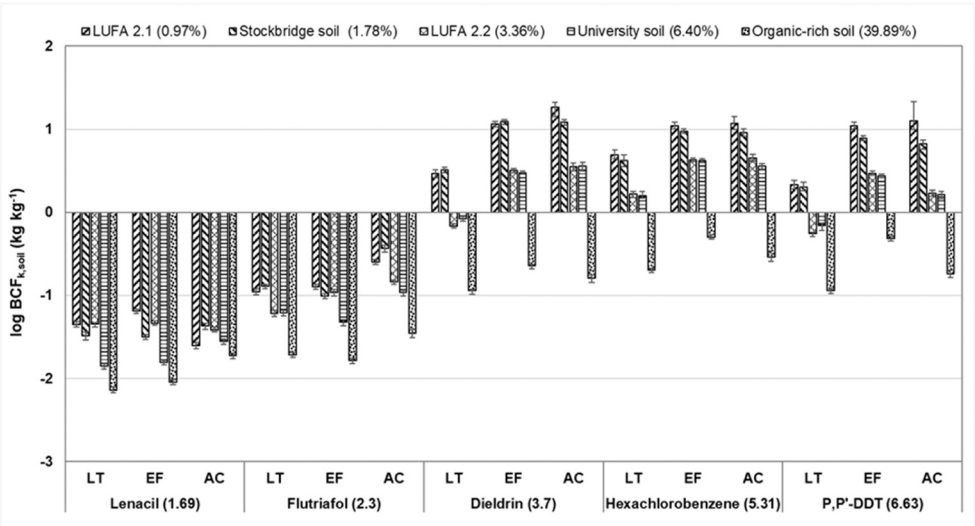
rate constants and a strong positive effect on elimination rate constants, resulting in a strong negative effect on kinetic bioaccumulation factors (Fig. 2 and Fig. S2). TPSA represents a fraction of the polarity of a molecule, accounting for the polar atoms on the surface of the molecule, such as oxygen, nitrogen, and their attached hydrogens [43]. The TPSA values in our study showed a strong negative correlation with log K_{ow} ($R = -0.93$, $p < 0.01$) and log K_{om} ($R = -0.87$, $p < 0.001$) indicating increased partitioning into the organic phase with decreased polarity most likely due to hydrophobic interactions [25,55]. The influence of hydrophobicity on pesticide bioconcentration in earthworms arises because more hydrophobic pesticides can diffuse more readily into the organism from the dissolved phase through the cell membranes of skin and intestinal walls, and have a greater tendency to accumulate in the lipid phase in earthworms, thus resulting in higher uptake rates [23]. The decrease in elimination with increasing hydrophobicity is consistent with results reported in previous studies [38,37,3]. Furthermore, the ingestion of soil may be an additional uptake pathway that results in a greater and more rapid uptake of hydrophobic chemicals; previous studies have observed increasing importance of the gut wall route of exposure with increasing log K_{ow} [5,29]. The negative effect of hydrophobicity on elimination may be attributable to the stronger partitioning of hydrophobic pesticides into lipids compared to their sorption affinity to soil, which may have retarded elimination.

3.1.2. Soil properties

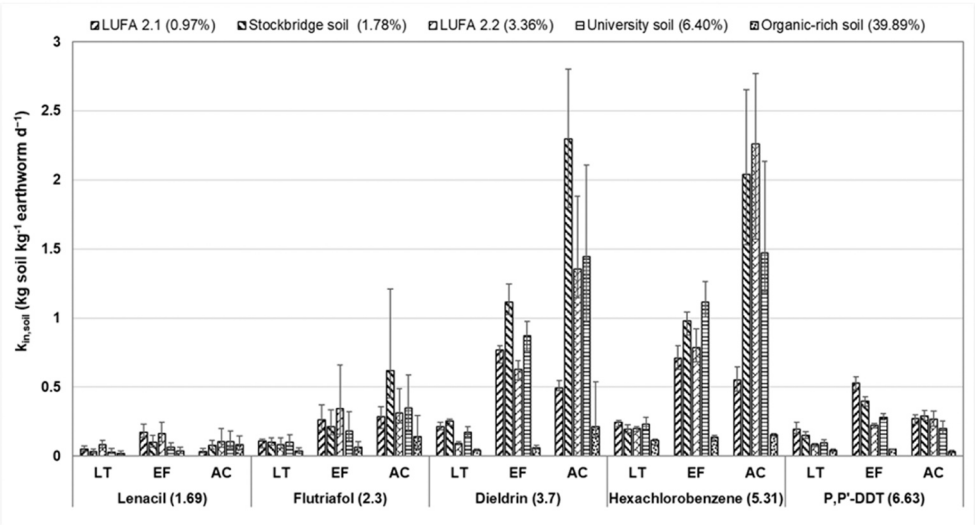
Soil properties including OM, Clay and CEC had a strong negative effect on $k_{in,soil}$ within the PCA (loading of −0.41 on axis 1, Table S9) and a positive effect on $k_{out,soil}$ (loading of 0.18 on axis 2, Table S9), which ultimately led to a strong negative effect on $BCF_{k,soil}$. In comparison, although these soil properties loaded positively and strongly on axis 2 (loading of > 0.97), log $k_{in,pw}$ loaded positively but weakly on axis 2 (loading of 0.09, Table S10), indicating a minor positive effect of soil properties on porewater-based k_{in} . Meanwhile, similar to $k_{out,soil}$, a weak positive effect of these soil properties on $k_{out,pw}$ was also observed, which overall resulted in a limited effect on $BCF_{k,pw}$.

The influence of soil OM on uptake and elimination kinetics has been well documented. Previous studies have shown that soil organic matter has a negative impact on soil-based uptake rates and a positive impact on elimination rates, which is in accordance with our PCA findings [49, 53]. This is probably due to the fact that the bioavailability of neutral pesticides for uptake by earthworms by dermal passive diffusion from porewater might be decreased by sorption of pesticides on soil organic matter, which in turn reduces the uptake rates [10,52]. Moreover, OM also displays a strong influence on the elimination rate of chemicals by earthworms. Belfroid and Sijm [4] demonstrated that the elimination process in earthworms is governed primarily by repartitioning from the gut wall to the soil particles in the gut. Consequently, the soil organic matter could compete with the organism for contaminants due to its strong binding affinity for neutral pesticides via sorption interactions, which facilitates their elimination from the gut [15,4]. Due to these dual effects, soil organic matter had a negative effect on earthworm $BCF_{k,soil}$.

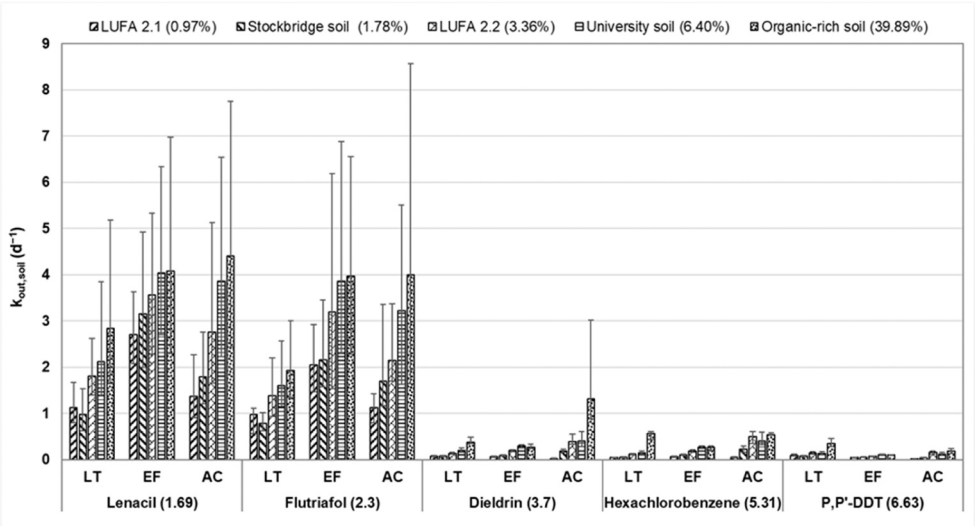
Compared to the extensive literature on soil OM, the influence of clay content and CEC on uptake and elimination kinetics has received comparatively less attention. However, soil properties including CEC, clay content, and pH have been reported as governing the fate of ionisable organic compounds in soil, influencing their bioavailability and hence their bioconcentration into earthworms [10,42,60,64]. Additionally, a few studies indicated that neutral organic compounds such as phenanthrene can sorb to clay minerals, especially in soils with low organic matter content, which may influence the bioconcentration of neutral pesticides in earthworms [40,36,39]. In our study OM, Clay, and CEC were found to be highly intercorrelated ($R = 0.99$, $p < 0.001$) which prevents our PCA from differentiating the relative importance of these parameters. However, based on literature studies, and given that our test compounds are neutral organic compounds, it seems likely that it is the OM rather than the CEC, clay content and pH that are governing uptake and elimination.



(A)



(B)



(C)

(caption on next page)

Fig. 1. Overview of the (A) $\log BCF_{k,soil}$, (B) $k_{in,soil}$ and (C) $k_{out,soil}$ values of five pesticides in three earthworm species in five soils. LT, EF, and AC represent the earthworm species *Lumbricus terrestris*, *Eisenia fetida*, and *Aporrectodea caliginosa*, respectively. The values in parentheses in the legend and on the X-axis are soil organic matter contents and $\log K_{ow}$ values of pesticides, respectively. Error bars represent the 95% confidence intervals of the values. Soil OM increased in the order of LUFA 2.1 < Stockbridge soil < LUFA 2.2 < University soil < Organic-rich soil. Equivalent figures for values calculated on the basis of pesticide concentrations in porewater are presented in [Figure S1](#).

3.1.3. Earthworm properties

Whilst soil and chemical properties have a greater impact on variation in the bioconcentration of pesticides, significant variation also exists between species. However, the effect of species traits on bioconcentration into earthworms varies with chemical and soil properties. PCA analysis of the entire dataset suggests that both Lipid and SSA had a strong positive effect on $k_{in,soil}$ (loading of 0.56 on axis 3, [Table S9](#)) but a negligible effect on $k_{out,soil}$ (loading of 0.08 on axis 3, [Table S9](#)), which ultimately led to a positive effect on $BCF_{k,soil}$. In comparison, Lipid and SSA had only a weak positive effect on $k_{in,pw}$ (loading of 0.11 on axis 3, [Table S10](#)) and a negligible effect on $k_{out,pw}$ (loading of 0.02 on axis 3, [Table S10](#)), which overall resulted in a minimal influence on $BCF_{k,pw}$.

To better understand the role of lipid content and SSA in the variation of pesticide uptake from the soil among different species, BCF_k values were normalised to the lipid content and SSA of earthworms. For the most hydrophobic pesticides (dieldrin, hexachlorobenzene, and p,p'-DDT, $\log K_{ow} = 3.7 - 6.63$) this significantly reduced the variation ($p < 0.05$) between the earthworm species ([Fig. 3](#), [Fig. S3](#)). However, the normalisation for flutriaol ($\log K_{ow} = 2.3$) which is less hydrophobic, did not result in a significant change to the variation in BCF_k values among the three earthworm species ($p > 0.05$). Finally normalising BCF_k values to lipid content and SSA for the least hydrophobic pesticide (lenacil, $\log K_{ow} = 1.69$) increased the variation between the three earthworm species significantly ($p < 0.05$). Previous studies have revealed that the lipid phase in earthworms is the dominant sorbing medium for hydrophobic compounds [28]. The higher BCF_k values for hydrophobic pesticides in *E. fetida* and *A. caliginosa* compared to *L. terrestris* may be primarily attributable to differences in lipid content between the three earthworm species. Moreover, for more hydrophobic compounds, previous studies have shown that uptake via the gut, which involves the consumption of contaminated soil, water, and food, is more significant than uptake via the skin [5,29]. Previous research has demonstrated that the internal surface area of the gut wall of isomorphic organisms is proportional to the outer skin surface area [27]. Therefore, a high SSA in earthworms may not only facilitate dermal uptake via the diffusion of chemicals through the skin, but it may also facilitate gut uptake, particularly for hydrophobic pesticides. This observation is consistent with the findings of Carter et al. [11], where the bioconcentration factor for orlistat ($\log K_{ow} = 8.95$) was 5.8 times larger for the small earthworm species *E. fetida* with a higher SSA than for the larger species *L. terrestris*. In comparison, BCF_k values here were comparable between the earthworm species for the more hydrophilic pesticide (lenacil), varying by an average factor of 1.9 for $BCF_{k,soil}$ and 2.1 for $BCF_{k,pw}$, which is probably attributable to the similar water content of the different earthworm species. Previous research revealed that the bioconcentration of hydrophilic compounds in earthworms is primarily determined by partitioning of the chemical to the water phase, rather than the lipid phase, within the organism [28].

The variation in $BCF_{k,soil}$ values for the five pesticides across the three earthworm species decreased with soil OM content, with the average ratio of the maximum to minimum $BCF_{k,soil}$ values ranging from 3.9 (LUFA 2.1) to 2.7 (organic-rich soil) ([Table S11](#)). The variation in $BCF_{k,soil}$ values between the earthworm species in the soils decreased after lipid and SSA normalisation, varying by an average factor between 1.9 and 2.5 ([Table S11](#)). This suggests that differences in lipid content and SSA of earthworm species remain key factors explaining inter-species variation in bioconcentration of pesticides in different soil types.

3.2. Evaluation of existing models against the experimental data

The empirical model developed by Belfroid et al. [5] to estimate the total internal concentration in earthworms from both dermal and gut uptake routes achieved reasonable predictions for the majority of pesticides, with 87% of predictions falling within a factor of 10 of the corresponding measured values and an overall NSE of 0.22 ([Fig. 4A](#)). However, this model consistently overestimated the steady-state internal concentrations for the more hydrophilic pesticide (lenacil) for all three earthworm species, by factors of up to 37.8 for *A. caliginosa*, 24.2 for *L. terrestris* and 23.9 for *E. fetida*. This is consistent with the results of our previous study, in which the model was evaluated using data extracted from the literature (Li et al., unpublished results, [34]). Although Belfroid et al. [5] suggest that their model is applicable over a $\log K_{ow}$ range of 2 – 7, the model estimates three key parameters - a bioconcentration factor, a sorption coefficient normalised to organic matter and an elimination rate constant - using data from chemicals with a more limited $\log K_{ow}$ range (4.2–5.7, 2.4–5.2 and 4.6–8.1 respectively). This could lead to substantial uncertainty in predictions for more hydrophilic compounds such as lenacil which has a $\log K_{ow}$ of 1.69 that lies outside both the stated applicability of the model and the data used to produce the relationships within the model. The performance of the Belfroid et al. model varied across species, with the model performing best for *A. caliginosa* and least well for *L. terrestris* according to NSE values ([Fig. 4A](#)). The model was developed based on *E. fetida* data, so it is perhaps surprising that it performs better (albeit only slightly) for *A. caliginosa*. The different performance between species suggests that some of the model parameters are not able to account for inter-species differences in pesticide uptake from soil. Porewater-based bioconcentration factors [11,18], uptake efficiency, and feeding rate [5] can vary between earthworm species. Our analysis above suggests that if lipid content and SSA were incorporated into empirical models this could improve predictions of pesticide bioconcentration in earthworms across species. Furthermore, lipid content and SSA are readily measured whereas factors like uptake efficiency and feeding rate are harder to quantify without the need for experiments or assumptions.

The kinetic model developed by Jager et al. [29] overestimated the internal concentrations of hexachlorobenzene for all three earthworm species when input parameter values were used that had previously been calibrated by Jager et al. [29]. However, over 93% of predictions were within a factor of 10 of the corresponding measured values (overall NSE 0.21). As with the Belfroid et al. [5] model, the model of Jager et al. performed better for *E. fetida* and *A. caliginosa* than it did for *L. terrestris* based on NSE values ([Fig. 4B](#)). However, the model provided accurate predictions for all three species when the model was implemented using the experimentally determined value of K_{ws} and k_d , obtained in this study, achieving an overall NSE of 0.81. There were improvements in NSE for each individual species with a particularly substantial improvement for *L. terrestris* from -1.19 to 0.560 ([Fig. S4](#)). Given that hexachlorobenzene was not readily degraded in soil, the K_{ws} , which is calculated as the ratio of the porewater-based bioconcentration factor to the sorption coefficient normalised to soil organic matter, is the primary factor in the model that influences model prediction and can thus explain variations in predicted bioconcentration between species. The remaining input parameters in the model were parameterised to *E. andrei* and include rate constants for exchange across skin (k_s) and gut wall (k_g) as well as fixed values for the feeding process parameters. These parameters appear to be applicable to other earthworm species if measured K_{ws} and k_d are used. In the absence of experimental K_{ws} data,

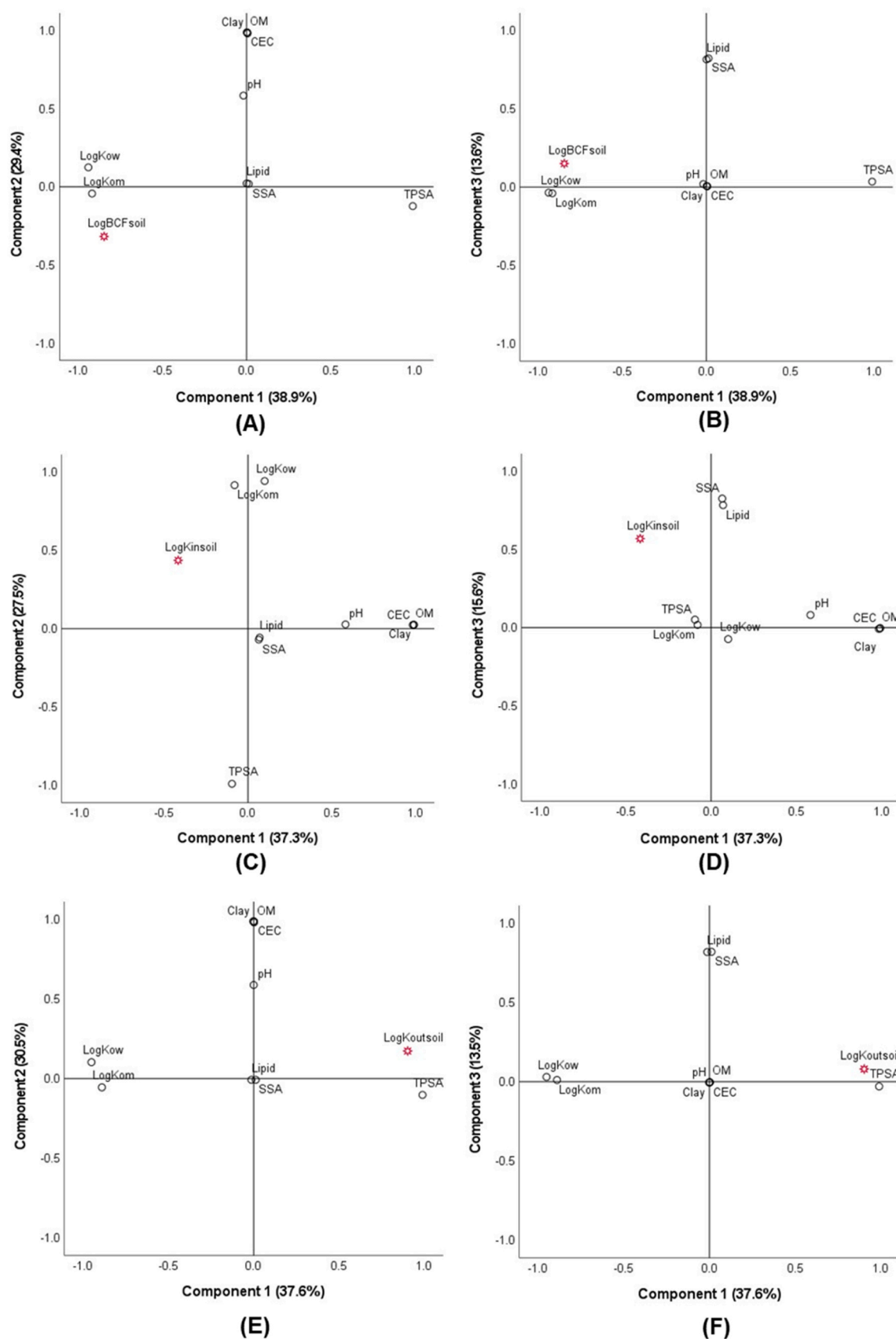


Fig. 2. Principal component analysis of chemical, soil, and earthworm properties and: $BCF_{k,soil}$ (A, B); $k_{in,soil}$ (C, D); and $k_{out,soil}$ (E, F). The values in parentheses on the axes represent the percentage of the variance that each principal component accounts for. Log BCFsoil, Log Kinsoil and Log Koutsoil represent the logarithmic transformations of $BCF_{k,soil}$, $k_{in,soil}$ and $k_{out,soil}$, respectively. Other abbreviations are provided in Section 2.7. Equivalent figures for values calculated on the basis of pesticide concentrations in porewater are presented in Fig. S2.

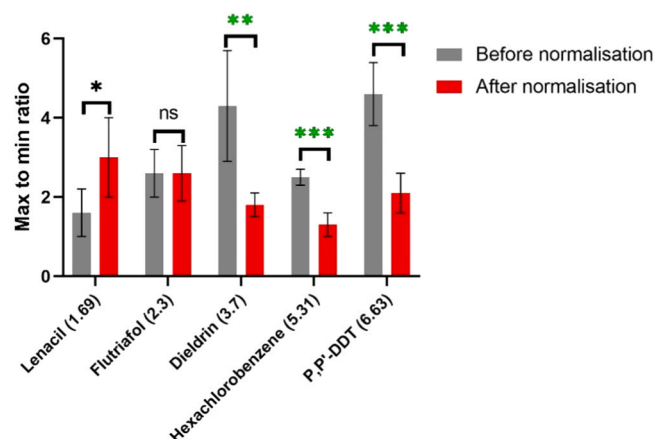


Fig. 3. Ratio between maximum and minimum $BCF_{k,soil}$ values between *L. terrestris*, *E. fetida*, and *A. caliginosa* earthworm species in five soils before and after lipid and SSA normalisation. For each pesticide in each soil, the ratio of the maximum to minimum BCF values between the three species was calculated, giving five values per pesticide which were then averaged; error bars represent the standard deviation around these averages. *, **, *** indicate significant differences at 0.05, 0.01, and 0.001 levels for each pesticide before and after normalisation. ns means not significant ($p > 0.05$). Green stars indicate that the variation decreased significantly after normalisation, while black stars indicate that the variation increased significantly after normalisation. Numbers in brackets are $\log K_{ow}$ values.

our PCA analysis suggests that this kinetic model coupled with a refined model for estimating K_{ws} that incorporates earthworm characteristics (such as lipid content and SSA) could better explain the variations in bioconcentration between earthworm species.

4. Conclusions

Our analysis demonstrates that the influence of earthworm characteristics on bioconcentration varies according to the properties of the chemical and soil. In soil environments with a homogeneous distribution of chemicals, inter-species variation in bioconcentration of hydrophobic pesticides is primarily attributable to differences in earthworm lipid content and specific surface area. In contrast, inter-species variation in bioconcentration of hydrophilic pesticides is relatively small, most likely because of the similar water content of the different species. Additionally, variation among species for all pesticides is influenced by soil organic matter; pesticide uptake, porewater concentrations and between species variation all decreased with increasing organic matter content. This suggests that uptake is dominated by uptake from the porewater, most likely via dermal diffusion. It seems likely that at lower porewater concentrations, this uptake is limited by the amount of chemical in solution such that variation between species is reduced. However, at higher porewater concentrations, surface available for uptake becomes more of a limiting factor, resulting in the species with higher SSA (*E. fetida* and *A. caliginosa*) showing more uptake than the species with lower SSA (*L. terrestris*) and consequently greater between-species variation. Therefore, from a risk assessment perspective, the smaller earthworm species such as *E. fetida* and *A. caliginosa*, which typically have higher lipid content and SSA, will take up more chemical relative to their body weight for hydrophobic compounds and are therefore more likely to give rise to biomagnification through the food chain than the larger species *L. terrestris*. Furthermore, it should be noted that, in soil environments with heterogeneous vertical distribution of chemicals or the specific contamination of food sources, the species habits such as feeding and burrowing behaviour may also influence earthworm bioconcentration. Thus, for risk assessments it becomes important to identify the range of earthworm species present rather than just earthworm numbers.

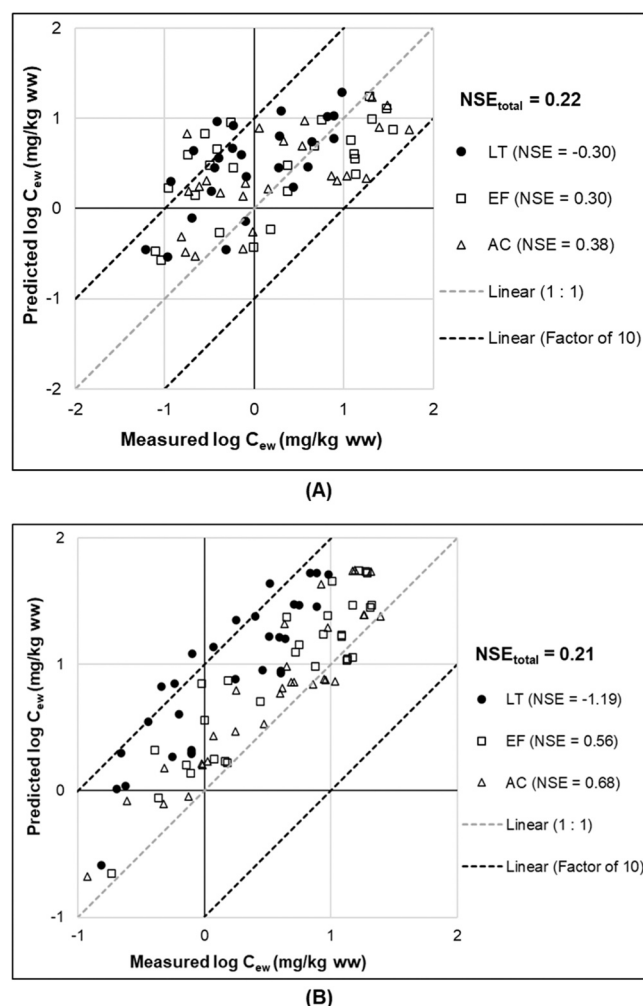


Fig. 4. Predictive performance of (A) the empirical model developed by Belfroid et al. [5] against the experimental data for five pesticides and (B) the kinetic model developed by Jager et al. [29] against the experimental data for hexachlorobenzene using the calibrated values reported by Jager et al. [29] for input parameters. NSE_{total} is the Nash–Sutcliffe Efficiencies of the model across all three earthworm species. The grey dashed line represents a perfect model fit (1:1 line), while the black dashed lines correspond to the predicted internal concentrations ± 1 unit against the measured values.

The empirical model developed by Belfroid et al. [5] provided a reasonable prediction of bioconcentration of pesticides in the $\log K_{ow}$ range of 2 to 7 for the smaller earthworm species. This model has the potential to be used instead of the two empirical models developed by Jager [28] and Connell and Markwell [14] that are recommended by the European Union [20,21] for assessing secondary poisoning in the terrestrial food chain. However, this model tends to overestimate pesticide bioconcentration for the larger species, which could result in an overestimation of the effect of pesticides on earthworm populations and the risk of secondary poisoning. Our results suggest that this could be addressed by incorporating normalisation to SSA and lipid content into the model. Similarly, to increase the applicability of Jager et al.'s. (2003a) kinetic model across species, rather than parameterising it to individual species a sub-model could be developed to better estimate K_{ws} by incorporating earthworm characteristics (such as lipid content and SSA). Based on the kinetic model's performance in predicting bioconcentration of hexachlorobenzene in our experiments, use of the model with either measured K_{ws} or a refined model for estimating K_{ws} holds promise for estimating internal concentrations over time series for various earthworm species and linking them to population effects to

refine current risk assessments for soil organisms. However, it is important to note that extensive calibration data are required for model parameterisation for some parameters (such as k_s and k_g) prior to making a prediction for a specific chemical. Such data sets do not currently exist except for tetrachlorobenzene, hexachlorobenzene or PCB 153, which were used in model development [29], so this potentially limits model use on a broad scale.

CRedit authorship contribution statement

Brown Colin D.: Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Conceptualization. **Bottoms Melanie J.:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Ashauer Roman:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Alvarez Tania:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Li Jun:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Hodson Mark E.:** Writing – review & editing, Visualization, Supervision, 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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133744.

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