Enantioselective bioaccumulation and toxic effects of fipronil in the earthworm *Eisenia foetida* following soil exposure

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Abstract

BACKGROUND: Enantiomers of chiral pesticides often have different bioactivity, toxicity and environmental behaviours. Fipronil has been used in racemate for agricultural purposes against soil insects, leading to increased inputs into soil environments and complex biota exposures. To understand the potential risk associated with fipronil enantiomer exposure, subchronic toxicity and bioaccumulation tests with earthworms (*Eisenia foetida*) in fipronil-spiked soils were evaluated under laboratory conditions.

RESULTS: Enantioselective toxicity was measured in *E. foetida* biomass after 28 days of subchronic exposure, with increased toxicity from racemate and *S*-fipronil compared with *R*-fipronil. The bioaccumulation of fipronil in earthworm tissues was also enantioselective, with a preferential accumulation of *S*-fipronil, and the enantiomer fraction was approximately 0.56–0.60. During soil exposure, fipronil was transformed primarily into fipronil sulfide, sulfone and amide, and *E. foetida* rapidly accumulated fipronil and sulfone.

CONCLUSION: This work demonstrates the enantioselective subchronic toxicity and bioaccumulation of enantiomers of fipronil in *E. foetida*. The earthworm tissues exhibited a relative enrichment of fipronil and fipronil sulfone, and these compounds might biomagnify (with a biota-to-soil accumulation factor of $\geq 1.0 \text{ kg}_{\text{oc}} \text{ kg}_{\text{lip}}^{-1}$), allowing for the possible trophic transfer and/or bioaccumulation of all these chemicals if earthworms were consumed by predator organisms.

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Keywords: chiral pesticides; enantioselective; toxicity; bioaccumulation; soil; fipronil

1 INTRODUCTION

Fipronil – (*R,S*)-5-amino-1-(2,6-dichloro- α,α,α -trifluoro-*p*-tolyl)-4-trifluoromethyl-sulfinylpyrazole-3-carbonitrile (CAS number 120068-37-3) – is an important phenyl pyrazole insecticide used for insect control and plant protection. It is effective against a wide range of insect pests and has been used on a large number of crops.^{1,2} Fipronil contains one chiral centre and two mirror image forms or enantiomers called *R*- and *S*-fipronil.³ Fipronil was introduced into the market as a racemic formulation. Enantiomers of fipronil differ in their biological activity, toxicity, effects on beneficial and non-target organisms and environmental fate.^{4–8} These differences may lead to variations in microbial degradation rates and would indicate that one enantiomer is more persistent in the environment than the others. Many reports have documented enantioselective degradation or transformation in sediment,⁹ plants^{4,10} and animals¹¹ when *rac*-fipronil was applied.

In China, fipronil is primarily used as a seed-coating formulation for agricultural purposes against soil insects, such as wireworms. Raveton *et al.*¹² reported that only 7% of the fipronil had been absorbed by the sunflower plant from a seed-coating formulation, and most fipronil was found in the soil. Therefore, this application resulted in widespread soil contamination. Under field conditions, fipronil has a half-life of 5.6–135 days in soils, and its

average half-life in soils is approximately 65 days.^{13,14} Pesticide degradation is important because it permits a prediction of the potential risk associated with exposure.¹⁵ Fipronil reportedly underwent photolysis to fipronil desulfinyl,¹⁶ oxidation to fipronil sulfone,¹⁷ reduction to fipronil sulfide¹⁸ and hydrolysis to fipronil amide^{19,20} (Fig. 1). The rate and route of pesticide metabolism varied according to environmental factors.⁴

The earthworm (*Eisenia foetida*) is widely distributed and frequently dominant in soil communities. These worms have intimate contact with soils and have been designated as representative organisms for use in soil system bioassays. These organisms have played a major role in ecological assessments, particularly with reference to the bioaccumulation/bioavailability of chemicals and

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Figure 1. Metabolites or degradation products of fipronil.

heavy metals.^{21–23} Several papers have reported that *E. foetida* were used in both toxicity and bioaccumulation studies,^{24–26} but data about the enantioselective bioaccumulation of enantiomers of fipronil in earthworms have not been reported. Although fipronil had no acute toxicity in earthworms,²⁷ this compound did reduce the rate of reproduction ($EC_{20} = 23.16 \text{ mg kg}^{-1}$).²⁸ Fipronil and its soil metabolites may be accumulated by earthworms and then transferred and/or bioaccumulated into predator organisms that consume earthworms.²⁹

In the present study, the commonly used test species *E. foetida* was selected for subchronic toxicity and bioavailability tests under laboratory conditions. The objectives of this study were (i) to evaluate the subchronic toxicity of fipronil and its enantiomers to *E. foetida* by using fipronil-spiked soils, (ii) to investigate the enantioselective bioaccumulation of fipronil in *E. foetida* from soils and (iii) to quantify major fipronil metabolites in soils and *E. foetida* over the course of exposure.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Analytical standards for fipronil racemate (purity 99.0%, enantiomeric ratio $[R]/[S] = 0.99$) were obtained from the National Pesticide Quality Supervision and Inspection Centre (Beijing, China). Fipronil sulfide (MB45950, 99.5%) and fipronil sulfone (MB46136, 99.0%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Fipronil desulfinyl (MB46513, $100 \mu\text{g mL}^{-1}$) and fipronil amide (RPA200766, $100 \mu\text{g mL}^{-1}$) were obtained from the AccuStandard (New Haven, CT). *R*-Fipronil and *S*-fipronil were obtained by high-performance liquid chromatography on a Chiralcel OD-RH semi-preparation column ($20 \times 250 \text{ mm i.d.}$, particle size $5 \mu\text{m}$; Daicel, Shanghai, China) at 25°C with a mobile phase of 50/50 acetonitrile/water at 5.0 mL min^{-1} , and the UV detection wavelength was 280 nm . Both enantiomeric purities were 97.0% (supporting information Fig. S2 and S3). As in the published procedures,^{30–32} the *S*-fipronil in the acetonitrile/water mobile phase elutes first on a column cellulose tri-3,5-dimethylphenyl carbamate chiral stationary phase. The first eluted enantiomer was *S*-fipronil, and the second eluted enantiomer was *R*-fipronil. The water was purified with a Milli-Q system. Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ). Silica-based sorbents including C_{18} ($40 \mu\text{m}$ particle size) and primary secondary amine (PSA) ($\text{Si}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_2-\text{NH}_2$) ($40 \mu\text{m}$ particle size) were obtained from Agela Technologies (Tianjin, China). All other chemicals and solvents were of analytical grade and commercially available.

2.2 Eisenia foetida

Mature earthworms (*E. foetida*) were purchased from the northern suburban farm of Changping (Beijing, China), and they were maintained in a wooden breeding box ($50 \times 50 \times 20 \text{ cm}^3$) containing a mixture of soil and cattle manure. Active, sexually mature (with well-developed clitella) worms with body masses between 200 and 300 mg were used in the experiment.

2.3 Soil collection

The soil collection site was a farm in the Changping District of Beijing, China, which had not received any pesticide applications for at least 10 years. The topsoil (0–10 cm) was collected after removing vegetation. The soils were sieved (2 mm), air dried at room temperature and stored in a dark, dry place until a few days before they were used. The physicochemical properties of the soil were as follows: clay loam texture, organic matter $3.22 \pm 0.12\%$, clay $4.2 \pm 0.1\%$, silt $38.0 \pm 1.1\%$, sand $55.1 \pm 1.2\%$ and pH (soil:water ratio 1:2.5) 7.12 ± 0.20 .

2.4 Toxicity test

Toxicity tests were conducted by exposing the *E. foetida* to fipronil racemate, *R*-fipronil or *S*-fipronil for 28 days in the test soils. Based on a range-finding exposure test, a series of standard working solutions of fipronil racemate and each enantiomer were prepared in acetone. A vehicle control (acetone) was tested to evaluate the potential effects of acetone toxicity. Fipronil racemate and the individual enantiomers were each dissolved in 10 mL of acetone, and then the acetone solution was slowly added to dry soil (50 g) while mixing continued for approximately 5 min. The spiked soil was left in a fume cupboard overnight; after that time, the acetone had evaporated. The contaminated dry soil (50 g) was then mixed thoroughly with 200 g of uncontaminated medium, and then the contaminated soil (250 g) was transferred to circular glass containers with a diameter of 12.5 cm and a height of 9.5 cm. The final concentrations of fipronil racemate and each enantiomer in soil were 50, 100, 200, 400, 800 and 1000 mg kg^{-1} . Distilled water (90 g) was added to each jar to restore the content of water to 36%.

The *E. foetida* were acclimated to the test soils for 1 week prior to the start of the test. Use was made of three replicates (beakers) per concentration and ten vehicle control replicates. Before the start of the test, the worms were placed on moist filter paper to evacuate most of their gut contents for 3 h at 20°C ;²¹ the depurated worms were weighed individually, and ten individuals were then placed in each test container. This process, which eliminated the gut contents, had no effect on the growth or survival of *E. foetida* when comparing evacuated worms from untreated soils. Test vessels were randomly arranged in a cultivation cabinet and maintained at $20 \pm 2^\circ\text{C}$ with a photoperiod of 16 h light and 8 h dark (fluorescent, 400–800 lux). The loss of water by evaporation from inside the jars was compensated for by adding distilled water every 2 days according to the weight changes of the control jar. All worms were removed from their containers on the last day of the bioassay. The individuals that did not respond to mechanical stimulation of the anterior portion of their bodies were recorded as dead. No worm was found to be dead or missing in the treatment and control groups during the toxicity test. Live worms were washed, allowed to evacuate their gut contents, blotted dry and weighed (0.01 mg). Average weights were measured before and after the 28 day test. Soil samples were collected from test beakers (1, 2, 3, 7, 14, 21 and 28 days) and analysed separately to confirm the

concentrations of fipronil during the toxicity test. The growth (dry weight) of surviving *E. foetida* was regressed against exposure concentrations in Origin 8.0 (OriginLab Corp, Northampton, MA) to evaluate toxicity trends.

2.5 *Eisenia foetida* bioaccumulation and elimination

To ensure that 250 g of medium was spiked homogeneously with *rac*-fipronil, *R*-fipronil and *S*-fipronil, the procedure steps (dilution spike) were as follows. Firstly, 5.0 and 12.5 mg of *rac*-fipronil and 2.5 mg of *R*-fipronil and *S*-fipronil were dissolved in 10 mL of acetone, and the same process that was used for the toxicity test was used to prepare the spiked soils for the bioaccumulation test. The acetone solution was slowly added to dry soil (50 g). After the acetone had evaporated, the contaminated dry soil (50 g) was mixed thoroughly with 200 g of uncontaminated medium, and then the contaminated soil (250 g) was transferred to a 500 mL glass jar. The final concentrations of *rac*-fipronil in soil were 20 and 50 mg kg⁻¹. The concentrations of *R*-fipronil and *S*-fipronil in soil were 10 mg kg⁻¹ each. Distilled water (90 g) was added to each jar to restore the content of water to 36%.

The *E. foetida* used in the bioaccumulation test weighed between 200 and 300 mg and were acclimated to the test soils for 1 week prior to the start of the test. Use was made of three replicates per concentration and ten vehicle control replicates. The test vessels were randomly arranged in an incubator and maintained at 20 ± 2 °C with a photoperiod of 16 h light and 8 h dark (fluorescent, 400–800 lux). The *E. foetida* were exposed to the chemical when placed in each jar containing 340 g of contaminated soil after evacuating their gut contents on moist filter paper for 3 h at 20 °C. The jars containing contaminated soil and worms were weighed, and the loss of water by evaporation was compensated for by adding distilled water every 2 days.

For the uptake experiment, worms were collected after exposure periods (1, 3, 5, 7, 10, 14, 19, 25 and 32 days), rinsed with tap water and allowed to evacuate most of their gut contents on moist filter paper for 3 h. The water on the surface of the worms was dried carefully with absorbent paper, and the worms were then weighed and frozen at –20 °C (in 50 mL polypropylene tubes). Soil samples (6.8 g, wet weight) from each jar were also stored at –20 °C. All the incubations were carried out on three replicates at each sample point.

For the elimination experiment, the *E. foetida* were transferred to clean soil after 14 days of exposure to contaminated soil. During the elimination period, worms were re-collected after 0.04, 0.13, 0.25, 0.50, 1, 2 and 4 days. After evacuating their gut contents and having their external water removed, the worms were stored at –20 °C.

2.6 Sample extraction and purification procedure

All the samples were thawed for approximately 30 min at room temperature. Soil samples (6.8 g per sample) were mixed with anhydrous sodium sulfate (5.0 g) and ethyl acetate (25 mL) in a 50 mL polypropylene centrifuge tube. The tubes were capped, vortex mixed for 3 min, exposed to ultrasonic vibration (with a frequency of 40 kHz and an ultrasonic power of 500 W) for 20 min and then centrifuged at 3500 rpm (approximately 1150 × g) for 5 min. The extraction was repeated again by following the same step. The combined extracts were filtered through anhydrous sodium sulfate (5.0 g) and evaporated to dryness on a rotary

evaporator at 40 °C. The residue was reconstituted in acetonitrile (2.0 mL), and then PSA (40 mg) was added to the suspension for clean-up. After being shaken, the resulting solution was passed through a 0.22 µm filter. A 0.1 mL aliquot of filtrate was taken and diluted to 1.0 mL with acetonitrile, and then the solution was analysed by HPLC-MS/MS.

For the *E. foetida* analysis, the samples (2.0 g per sample) were blended with ethyl acetate (25 mL) in a 50 mL polypropylene centrifuge tube. The mixture was homogenised with an Ultra-Turrax T18 homogeniser for 30 s, vortex mixed for 3 min, exposed to ultrasonic vibration (with a frequency of 40 kHz and an ultrasonic power of 500 W) for 20 min and then separated by centrifugation at 3500 rpm (approximately 1150 × g) for 5 min. The upper organic phase was passed through a funnel (medium-speed qualitative filter paper) with anhydrous sodium sulfate (5.0 g). The extraction was repeated again by following the same step. The combined extracts were evaporated to dryness at 40 °C. Next, the residue was reconstituted in acetonitrile (5.0 mL), and *n*-hexane (3 × 5.0 mL) was then added for the liquid–liquid partition of the lipids. The upper layer of *n*-hexane was discarded, and the acetonitrile layer was evaporated to dryness by a rotary evaporator. The residue was diluted to 2.0 mL with acetonitrile. PSA (40 mg) and C₁₈ (40 mg) were added to the resulting solution. The mixture was shaken and then passed through a filter membrane (pore size 0.22 µm). A 0.1 mL portion of the filtrate was taken and diluted to 1.0 mL with acetonitrile, and then the solution was analysed by HPLC-MS/MS.

2.7 Chemical analysis

HPLC-MS/MS analyses were performed using a Thermo Fisher Scientific (Waltham, MA) instrument equipped with an Accela 600 pump, an Accela autosampler and a Quantum Access triple quadrupole mass spectrometer (TSQ Quantum Access MAX) equipped with a heated electrospray ionisation (HESI) source working in selective reaction monitoring (SRM) mode in negative ionisation.

A Chiralcel OD-3R (4.6 × 150 mm i.d.; Daicel, Shanghai, China) column with a 3 µm particle size was used at a flow rate of 400 µL min⁻¹ at 25 °C. Mobile phase A consisted of water/ammonium formate (10 mM), and mobile phase B (acetonitrile) was a mixed-gradient programme starting at 45% A/55% B and ending (32 min) at 30% A/70% B. The injection volume was 5 µL.

The mass spectrometer and HESI source were used in negative-ion mode to determine the concentrations of fipronil and its metabolites. The parameters for the MS detector were as follows: negative mode; spray voltage 2200 V; skimmer offset 0 V; sheath gas pressure 40 arb.; auxiliary gas pressure 5 arb.; vaporiser temperature 250 °C; capillary temperature 350 °C; ion sweep gas pressure 0 arb. Table 1 lists the selective reaction monitoring (SRM) transitions used to identify and quantify the target analytes. Racemic fipronil and its metabolites were ideally baseline separated, and no enantiomerisation was observed for fipronil under these analytical conditions.

The external calibration curves were evaluated for *S*-fipronil, *R*-fipronil and metabolites (sulfide, sulfone and desulfinyl) within the 1–1000 ng mL⁻¹ concentration range, and fipronil amide was evaluated in the 5–1000 ng mL⁻¹ concentration range. According to the procedure described above, a blank matrix was prepared, and also a series of matrix-matched calibration standards with the same concentrations. The absolute recovery of fipronil and its metabolites was determined in the soil and *E. foetida*. A series of blank samples were fortified with *rac*-fipronil and its metabolites at four different concentration levels (0.01, 0.1, 1.0 and 10 mg kg⁻¹,

Table 1. Characteristics of the HPLC-MS/MS method, LOD and LOQ employed for determining fipronil and metabolites

Pesticides	t_R (min)	Ionisation mode	Precursor ion (m/z)	Product ion (m/z) ^a	Collision energy (V)	Product ion (m/z) ^b	Collision energy (V)	LOD (ng g ⁻¹)	LOQ (ng g ⁻¹)
S-Fipronil	15.76	–	434.9	330.0	18	250.0	30	1.10	3.68
R-Fipronil	16.71	–	434.9	330.0	18	250.0	30	1.10	3.68
Fipronil amide	9.04	–	453.0	304.0	26	272.0	38	2.29	7.63
Fipronil sulfone	18.92	–	450.9	415.0	18	282.0	29	0.32	1.06
Fipronil desulfinyl	21.84	–	387.0	351.0	19	282.0	34	0.66	2.19
Fipronil sulfide	29.19	–	418.9	262.0	30	383.0	16	0.89	2.98

^a Used for quantitation.
^b Used for confirmation.

based on five replicates). The blank soil samples included 36% water, and the *E. foetida* individuals that had evacuated their gut contents were prepared the same way as the samples that were used in the *E. foetida* bioaccumulation experiment. The spiked blank samples were left for 1 h to ensure that the spiked pesticides were evenly distributed, and then extracted and determined as previously described. Recoveries were calculated by comparing the measured concentration with the fortified concentrations.

2.8 Equilibrium partitioning model and data analysis

Uptake of fipronil by *E. foetida* from the soils was described by the two-compartment model.^{33–35} The uptake kinetic parameters and the uptake curve were performed using the following model equations:³⁶

$$dC_{\text{worm}}/dt = k_1 C_{\text{soil}} - k_2 C_{\text{worm}} \quad (1)$$

$$C_{\text{worm}} = \frac{k_1 C_{\text{soil}}}{k_2} [1 - \exp(-k_2 t)] \quad (0 < t < t_c) \quad (2)$$

$$\text{BAF}_k = \frac{k_1}{k_2} \quad (3)$$

where C_{worm} is the concentration of fipronil in earthworm tissues (mg kg⁻¹), C_{soil} is the concentration of fipronil in the soil (mg kg⁻¹), k_1 denotes the uptake rate constant from the soil (day⁻¹), k_2 denotes the rate constant (day⁻¹) characterising the sum of the elimination, growth dilution and metabolic transformation of the two enantiomers in *E. foetida* and t_c is the time (days) at the end of the uptake phase. The kinetic bioaccumulation factor (BAF_k) is defined as the ratio of the soil uptake rate constant k_1 to the elimination rate constant k_2 .

When steady state is reached during the uptake phase (i.e. $t = \infty$),³⁶ equation (2) may be reduced to

$$C_{\text{worm}} = \frac{k_1}{k_2} C_{\text{soil}} \quad (4)$$

$$\text{BAF}_k = \frac{C_{\text{worm}}}{C_{\text{soil}}} = \frac{k_1}{k_2} \quad (5)$$

The $C_{\text{worm}}/C_{\text{soil}}$ ratio is also defined as the bioaccumulation factor (BAF) under steady-state conditions in equation (5).

According to equation (5), equation (2) is transformed into

$$C_{\text{worm}} = \text{BAF}_k \times C_{\text{soil}} \times [1 - \exp(-k_2 t)] \quad (0 < t < t_c) \quad (6)$$

The k_1 uptake rate constant is calculated from k_2 (deriving from the depuration phase) and BAF_k (deriving from the uptake curve):

$$k_1 = \text{BAF}_k \times k_2 \quad (7)$$

The bioaccumulation and biotransformation kinetics of the total fipronil (fipronil and its metabolites) in the soil and *E. foetida* were also fitted to the equilibrium partitioning model indicated above in equations (1) to (7). Here, C_{worm} and C_{soil} are the concentrations (mg kg⁻¹) of total fipronil (fipronil and its metabolites) in the *E. foetida* and the soil respectively.

The enantiomer fraction (EF) was used as a measure of the enantioselective behaviours of fipronil during the experiment. The EF values ranged from 0 to 1, with EF = 0.5 representing the racemic mixture. The EF was defined by the following equation:

$$\text{EF} = \frac{C_S}{C_S + C_R} \quad (8)$$

where C_S is the concentration of S-fipronil and C_R is the content of R-fipronil. A paired *t*-test was performed to compare the means of the EF values in the *E. foetida* and the soil samples with EF = 0.5.

3 RESULTS AND DISCUSSION

3.1 Calibration and method validation

Typical chromatograms of fipronil-fortified samples containing racemate and metabolite standard solutions are shown in supporting information Figure S1. Enantiomers of fipronil were baseline separated. There were no endogenous interference peaks at the same retention times as enantiomers of fipronil and metabolites. The matrix effect on the MS/MS (SRM) detector was investigated in the soil and *E. foetida* by comparing the standards in the solvent with the matrix-matched standards. A slight ion signal suppression for enantiomers of fipronil and metabolites was typically observed in this study, and the slope ratios (matrix-matched calibration/standard calibration in solvent) ranged from 0.842 to 0.967. Thus, the ion suppression caused by the matrix would not affect the analysis of fipronil and its metabolites in the soil or *E. foetida*.

Good linear calibration curves were obtained over a 1–1000 ng mL⁻¹ range for S-fipronil, R-fipronil, fipronil sulfide, fipronil sulfone and fipronil desulfinyl, and 5–1000 ng mL⁻¹ was used for fipronil amide. The average recoveries for enantiomers

of fipronil and metabolites at levels between 0.01 and 10 mg kg⁻¹ ranged between 89 and 99% in the soil and between 85 and 96% in earthworm tissues, with the relative standard deviations (RSDs) below 12% ($n = 5$ for each sample type). The limits of detection (LOD) and quantification (LOQ) for enantiomers of fipronil and metabolites were defined as the concentrations that gave signal-to-noise ratios of 3 and 10 (Table 1). The LOD and LOQ were estimated to be 1.10 and 3.68 ng g⁻¹ for enantiomers of fipronil, 0.89 and 2.98 ng g⁻¹ for fipronil sulfide, 0.32 and 1.06 ng g⁻¹ for fipronil sulfone, 0.66 and 2.19 ng g⁻¹ for fipronil desulfinyl and 2.29 and 7.63 ng g⁻¹ for fipronil amide.

3.2 Toxicity assay

The analyses of fipronil racemate and enantiomers in the soil tests showed that the average measured concentrations were within 20% of the target concentrations. Fipronil racemate and individual enantiomers were not lethal to *E. foetida* at concentrations of 50–1000 mg kg⁻¹ (soil). The surviving worms had reduced body weights, and their growth was inhibited. In some cases, these reductions could have been caused by changes in the physiological or biochemical processes of *E. foetida*, which resulted in reduced food intake and metabolism.³⁷ The weights of *E. foetida* that were exposed to racemic fipronil and both enantiomers decreased with increasing concentration. The weights of the worms decreased to 38–62% (*R*-fipronil), 23–53% (*S*-fipronil) and 30–57% (racemate) at concentrations of 1000–400 mg kg⁻¹ in soil. The weights of the worms in the control decreased to 70–80% of initial mass. The *E. foetida* in spiked soil treated with a lower concentration of fipronil lost less body weight than worms at higher concentrations in comparison with the *E. foetida* in the control. The slope intervals for racemate, *S*-enantiomer and *R*-enantiomer were -0.0531 ± 0.00585 , -0.0561 ± 0.00864 and -0.0398 ± 0.00399 respectively (Fig. 2). The *R*-fipronil slope was lower than the slopes of racemate and *S*-fipronil, implying lower toxicity of *R*-fipronil for earthworm growth. For subchronic exposures, the *R*-enantiomer had the lowest toxicity compared with racemate and the *S*-enantiomer. The toxicity of the racemate and *S*-enantiomer exhibited no differences. The same enantioselective subchronic toxicity was observed in the growth of juvenile fathead minnows (*Pimephales promelas*) that were exposed to fipronil-spiked sediment.³⁸

3.3 Transformation and persistence in the soil

Enantiomers of fipronil and metabolites in earthworm tissues and soils were determined (Figs 3 and 4). At the end of the exposure (32 days), the concentrations in soil decreased 10–30% for enantiomers of fipronil at different exposures (Fig. 3). The losses may be due to degradation, volatilisation or biotransformation, or to a combination of these processes. The residues were present primarily in the form of the parent compound and metabolites such as amide, sulfone and sulfide. Desulfinyl metabolite residues were not detected in the soil. The desulfinyl metabolite could have been produced by photolysis.^{16,19} Soil samples were not directly exposed to sunlight, which could be one reason why desulfinyl was not produced over the course of the experiment. The fipronil EF values in the soil samples remained largely unchanged (Fig. 5), and no significant difference was observed between the two enantiomers at different exposures (20 and 50 mg kg⁻¹).

3.4 Bioaccumulation and elimination in *Eisenia foetida*

The concentrations of enantiomers of fipronil in exposed *E. foetida* increased on day 1, as shown in Fig. 3. A higher rate of fipronil

Figure 2. Concentration–response relation for the growth of *Eisenia foetida* exposed to fipronil racemate, *S*-fipronil and *R*-fipronil. Error bars are the standard deviations of the means of toxicity tests on three replicates. The control was acetone, which was the solvent for the enantiomers of fipronil.

Figure 3. The concentrations of enantiomers of fipronil of 20 mg kg⁻¹ rac-fipronil treatments in the soil and *Eisenia foetida*. Error bars are the standard deviations of the means of bioaccumulation tests on three replicates.

application resulted in higher residues, and the concentrations of the two enantiomers reached their highest level on day 10. The concentrations exhibited a slight decline from day 14 to day 32, which may have been explained by the declining concentration in the soil. In addition, a concentration difference was observed between the two enantiomers in earthworm tissues at the same sample point, and *S*-fipronil had a higher concentration than *R*-fipronil as the duration of exposure increased. As shown in Fig. 5, the EF values in earthworm tissues deviated from 0.50 ($P < 0.001$) in the bioaccumulation experiment, and they were maintained at approximately 0.56–0.62. A paired *t*-test for the same time points between the EF values of spiked soil and the EF values of *E. foetida* yielded a *P*-value of < 0.001 . Therefore, enantioselectivity occurred when the two enantiomers of fipronil were bioaccumulated by *E. foetida*.

Metabolites of fipronil, such as sulfone and sulfide (Fig. 4), were detected in earthworm tissues, but the concentration of fipronil amide was low. Fipronil metabolites reached their highest level between day 7 and day 14, and the concentrations of fipronil

Figure 4. The concentrations of fipronil metabolites in the soil and *Eisenia foetida*: (a) 10 mg kg⁻¹ of *S*-fipronil; (b) 10 mg kg⁻¹ of *R*-fipronil. Error bars are the standard deviations of the means of bioaccumulation tests on three replicates.

Figure 5. Enantiomeric fraction (EF) of fipronil in the soil and *Eisenia foetida* by sampling days.

sulfone in worms were higher than the concentrations of fipronil sulfide (in the soil or *E. foetida*) and sulfone (in the soil) from day 7 to day 32 at *rac*-fipronil exposures of 20 and 50 mg kg⁻¹. In comparison with the soil, the concentrations of fipronil amide in earthworm tissues were lower throughout the exposure period. Based on the concentrations of the individual fipronil enantiomers, no enantiomerisation was observed for fipronil. The concentrations of *S*-fipronil were always higher than those of *R*-fipronil, and the residue of total fipronil metabolites in the *S*-enantiomer was less than that of the *R*-enantiomer in both the soil and *E. foetida* at the given exposure levels of the individual fipronil enantiomers. Therefore, *S*-fipronil was preferentially accumulated, and *R*-fipronil might have been previously metabolised into fipronil metabolites under laboratory conditions.

In this work, the biota-to-soil accumulation factor (BSAF) was used to express the bioaccumulation of fipronil and its metabolites in earthworm tissues. BSAF was a function of the relative sorptive capacities of the organism versus the surrounding environment, and the activities in this work were normalised to the lipid content of the earthworms and the organic carbon (OC) in the soil. A lipid content of 1%^{24,34} and a factor of 1.7 between the organic carbon

(OC) and organic matter (OM)³⁹ were assumed. The BSAF was defined as follows:

$$\text{BSAF (kg dry weight kg}^{-1} \text{ wet weight)} = \frac{C_{\text{worm}}}{C_{\text{soil}}} \quad (9)$$

$$\text{BSAF (kg}_{\text{OC}} \text{ kg}_{\text{lip}}^{-1}) = \frac{C_{\text{worm}} F_{\text{OM}}}{1.7 C_{\text{soil}} F_{\text{lip}}} \quad (10)$$

where C_{worm} and C_{soil} are the concentrations of enantiomers of fipronil or metabolites in *E. foetida* and the soil respectively, F_{OM} (soil) is the fraction of organic matter in the soil and F_{lip} (earthworm) is the fraction of lipids in the earthworms. The BSAF values of enantiomers of fipronil (Table 2) reached their highest level on day 10, and the BSAF values for the metabolites reached their highest level between day 7 and day 14. A paired *t*-test for the same time points between *S*-fipronil and *R*-fipronil yielded *P*-values of less than 0.05 at *rac*-fipronil exposures of 20 and 50 mg kg⁻¹. The paired *t*-test showed that there was a significant difference in the BSAFs between the two enantiomers of fipronil. The BSAF values for *S*-fipronil were larger than those for *R*-fipronil, indicating that *S*-fipronil was preferentially accumulated over *R*-fipronil in earthworm tissues. *E. foetida* take up organic chemicals through their skin in addition to their gut wall.⁴⁰ Thus, it can be assumed that enantiomers of fipronil in earthworm tissues accumulated via skin exposure and gut absorption were enantioselective. The same bioaccumulation phenomena for benalaxyl, metalaxyl, tebuconazole and alpha-cypermethrin were observed in spiked soil.^{24–26,40} A paired *t*-test for the same time points among the fipronil metabolites sulfide, sulfone and amide at different exposures yielded *P*-values that were all less than 0.05, except when they were between sulfide and amide ($P = 0.343$) at 10 mg kg⁻¹ (*S*-fipronil). The BSAF values revealed a significant difference among fipronil metabolites. Earthworm tissues exhibited a relative enrichment of fipronil sulfide and sulfone, and decreased weight gain in worms was linked to fipronil amide. This finding was also confirmed by the fact that much less lipophilic fipronil amide than sulfide and sulfone could be transferred and bioaccumulated in *E. foetida*.

Armitage and Gobas³⁵ tested the model for the soil–earthworm–shrew food chain and noted (i) that chemicals

Table 2. The calculated biota-to-soil accumulation factors [BSAF ($\text{kg}_{\text{OC}} \text{ kg}_{\text{lip}}^{-1}$)] for enantiomers of fipronil and metabolites by sampling days at different exposures^a

Exposure time (days)	Exposure level ($\text{kg}_{\text{OC}} \text{ kg}_{\text{lip}}^{-1}$)	S-Fipronil	R-Fipronil	Fipronil sulfone	Fipronil sulfide	Fipronil amide
1	<i>rac</i> -Fipronil (50)	0.577 ± 0.039	0.492 ± 0.133	2.640 ± 0.247	1.259 ± 0.180	ND
	<i>rac</i> -Fipronil (20)	0.490 ± 0.151	0.374 ± 0.125	3.880 ± 0.384	0.529 ± 0.121	ND
	S-Fipronil (10)	0.407 ± 0.123	ND	ND	ND	ND
	R-Fipronil (10)	ND	0.411 ± 0.086	3.830 ± 1.856	ND	ND
3	<i>rac</i> -Fipronil (50)	0.638 ± 0.119	0.496 ± 0.102	1.462 ± 0.194	1.024 ± 0.161	ND
	<i>rac</i> -Fipronil (20)	0.766 ± 0.258	0.556 ± 0.151	1.840 ± 0.233	1.052 ± 0.582	ND
	S-Fipronil (10)	0.464 ± 0.206	ND	1.785 ± 0.016	ND	ND
	R-Fipronil (10)	ND	0.430 ± 0.146	3.665 ± 0.683	ND	ND
5	<i>rac</i> -Fipronil (50)	1.023 ± 0.214	0.730 ± 0.001	1.925 ± 0.241	0.845 ± 0.146	0.020 ± 0.007
	<i>rac</i> -Fipronil (20)	0.842 ± 0.079	0.693 ± 0.250	1.912 ± 0.276	1.127 ± 0.537	ND
	S-Fipronil (10)	0.880 ± 0.165	ND	2.258 ± 0.698	ND	ND
	R-Fipronil (10)	ND	0.713 ± 0.164	1.920 ± 0.892	ND	ND
7	<i>rac</i> -Fipronil (50)	1.492 ± 0.267	1.127 ± 0.174	1.951 ± 0.432	0.850 ± 0.163	0.080 ± 0.049
	<i>rac</i> -Fipronil (20)	0.921 ± 0.301	0.722 ± 0.246	2.264 ± 0.471	1.170 ± 0.384	ND
	S-Fipronil (10)	0.960 ± 0.136	ND	4.232 ± 0.963	ND	ND
	R-Fipronil (10)	ND	0.766 ± 0.169	4.009 ± 1.437	ND	ND
10	<i>rac</i> -Fipronil (50)	1.759 ± 0.095	1.402 ± 0.276	4.856 ± 0.518	1.294 ± 0.111	0.209 ± 0.198
	<i>rac</i> -Fipronil (20)	1.391 ± 0.247	1.209 ± 0.386	2.341 ± 0.234	1.887 ± 0.491	ND
	S-Fipronil (10)	1.255 ± 0.230	ND	3.356 ± 2.271	ND	ND
	R-Fipronil (10)	ND	1.099 ± 0.282	2.471 ± 1.097	1.072 ± 0.145	ND
14	<i>rac</i> -Fipronil (50)	1.366 ± 0.149	1.166 ± 0.257	6.027 ± 0.718	1.710 ± 0.345	0.092 ± 0.069
	<i>rac</i> -Fipronil (20)	1.325 ± 0.493	1.013 ± 0.027	3.275 ± 0.534	2.103 ± 0.345	ND
	S-Fipronil (10)	1.216 ± 0.286	ND	1.503 ± 0.504	ND	ND
	R-Fipronil (10)	ND	1.149 ± 0.161	2.040 ± 0.282	1.028 ± 1.116	ND
19	<i>rac</i> -Fipronil (50)	1.365 ± 0.135	1.118 ± 0.171	4.558 ± 1.066	1.726 ± 0.400	0.134 ± 0.074
	<i>rac</i> -Fipronil (20)	1.212 ± 0.212	0.980 ± 0.291	2.682 ± 0.489	2.106 ± 0.767	ND
	S-Fipronil (10)	1.440 ± 0.504	ND	1.446 ± 0.731	ND	ND
	R-Fipronil (10)	ND	1.092 ± 0.174	1.948 ± 0.523	2.415 ± 0.655	ND
25	<i>rac</i> -Fipronil (50)	1.274 ± 0.327	1.103 ± 0.319	2.842 ± 0.775	1.602 ± 0.434	0.106 ± 0.076
	<i>rac</i> -Fipronil (20)	1.091 ± 0.195	0.881 ± 0.319	2.015 ± 0.246	1.320 ± 0.145	ND
	S-Fipronil (10)	1.171 ± 0.305	ND	1.221 ± 0.312	ND	ND
	R-Fipronil (10)	ND	1.088 ± 0.353	1.555 ± 0.241	0.617 ± 0.458	ND
32	<i>rac</i> -Fipronil (50)	1.350 ± 0.273	0.995 ± 0.392	3.142 ± 0.359	2.366 ± 0.461	0.068 ± 0.039
	<i>rac</i> -Fipronil (20)	0.990 ± 0.281	0.815 ± 0.021	2.320 ± 0.575	1.592 ± 0.705	ND
	S-Fipronil (10)	1.163 ± 0.504	ND	2.213 ± 0.823	ND	ND
	R-Fipronil (10)	ND	0.640 ± 0.246	1.314 ± 0.282	1.475 ± 0.532	ND

^a ND: <limit of detection; $\pm$ standard deviations of the BSAF values.

with an octanol–air partition coefficient (K_{OA}) of $<10^{5.25}$ did not biomagnify, and (ii) that chemicals with $K_{\text{OA}} \geq 10^{5.25}$ and an octanol–water partition coefficient (K_{OW}) between $10^{1.75}$ and 10^{12} had biomagnification potential unless they were metabolised at a sufficiently rapid rate. The K_{OW}^{13} for fipronil was 5.62×10^3 , and the K_{OA} was 3.51×10^{10} ($>10^5$), as calculated by the following equation:

$$K_{\text{OA}} = \frac{K_{\text{OW}}}{H} \quad (11)$$

where H is Henry's law constant at room temperature. Given the K_{OW} and the K_{OA} values, fipronil was expected to have biomagnification potential in the earthworm food chain according to this theory.

In the elimination experiments, *E. foetida* were transferred to fipronil-free soil without contacting and feeding on fipronil-spiked

soil. The quantity of fipronil and metabolite residues in the tissues decreased significantly within 4 days. The depuration of enantiomers of fipronil in *E. foetida* fitted first-order kinetics (Table 3). The half-life ($T_{1/2}$) of *R*-fipronil in *E. foetida* ranged from 1.2 to 2.0 days, and from 1.5 to 2.1 days for *S*-fipronil. This finding indicated that the degradation of fipronil in *E. foetida* was enantioselective, and *S*-fipronil was more persistent than *R*-fipronil.

3.5 Kinetic analysis

To evaluate the validity of the equilibrium partitioning model, tests were conducted to determine whether the temporal course of fipronil uptake into *E. foetida* that were exposed to soil could be described by equations (6) and (7). The average concentrations of each enantiomer and total fipronil (fipronil and metabolites) in *E. foetida* and the soil at four exposure levels for the corresponding time points were fitted to equation (6) by using the non-linear regression function in SPSS 16.0 (SPSS Inc., Chicago, IL). Non-linear dynamic fitting yielded an estimated value of BAF_k . The uptake

Table 3. The elimination equation and half-life ($T_{1/2}$) of enantiomers of fipronil in *Eisenia foetida*

Exposure group	Enantiomers	Elimination equation	$T_{1/2}$ (days)	Correlation coefficient
50 mg kg ⁻¹ of <i>rac</i> -fipronil	<i>S</i> -Fipronil	$C_{\text{worm}} = 7.969 e^{-0.325t}$	2.133	0.951
	<i>R</i> -Fipronil	$C_{\text{worm}} = 7.219 e^{-0.345t}$	2.009	0.930
	Total fipronil ^a	$C_{\text{worm}} = 16.26 e^{-0.352t}$	1.969	0.928
20 mg kg ⁻¹ of <i>rac</i> -fipronil	<i>S</i> -Fipronil	$C_{\text{worm}} = 4.905 e^{-0.401t}$	1.728	0.965
	<i>R</i> -Fipronil	$C_{\text{worm}} = 3.933 e^{-0.456t}$	1.520	0.989
	Total fipronil	$C_{\text{worm}} = 9.679 e^{-0.439t}$	1.579	0.984
10 mg kg ⁻¹ of <i>S</i> -fipronil	<i>S</i> -Fipronil	$C_{\text{worm}} = 3.757 e^{-0.466t}$	1.487	0.888
	Total fipronil	$C_{\text{worm}} = 3.800 e^{-0.470t}$	1.475	0.886
10 mg kg ⁻¹ of <i>R</i> -fipronil	<i>R</i> -Fipronil	$C_{\text{worm}} = 3.864 e^{-0.539t}$	1.286	0.988
	Total fipronil	$C_{\text{worm}} = 4.034 e^{-0.447t}$	1.551	0.961

^a Including enantiomers of fipronil and metabolites.**Table 4.** The uptake rate coefficient (k_1), elimination rate coefficient (k_2), kinetic bioaccumulation factor (BAF_k) and correlation coefficient (r) of enantiomers of fipronil and total fipronil at different exposures

Exposure group	Enantiomers	BAF_k	k_1	k_2	r
50 mg kg ⁻¹ of <i>rac</i> -fipronil	<i>S</i> -Fipronil	0.746	0.242	0.325	0.895
	<i>R</i> -Fipronil	0.581	0.200	0.345	0.871
	Total fipronil ^a	0.639	0.225	0.352	0.897
20 mg kg ⁻¹ of <i>rac</i> -fipronil	<i>S</i> -Fipronil	0.593	0.237	0.401	0.922
	<i>R</i> -Fipronil	0.465	0.212	0.456	0.896
	Total fipronil	0.545	0.239	0.439	0.914
10 mg kg ⁻¹ of <i>S</i> -fipronil	<i>S</i> -Fipronil	0.577	0.269	0.466	0.894
	Total fipronil	0.583	0.274	0.470	0.898
10 mg kg ⁻¹ of <i>R</i> -fipronil	<i>R</i> -Fipronil	0.467	0.251	0.539	0.812
	Total fipronil	0.492	0.220	0.447	0.851

^a Including enantiomers of fipronil and metabolites.

rate constant k_1 was calculated using equation (7). The regression results are shown in Table 4.

The model was suitable for describing the uptake kinetics of *S*-fipronil, *R*-fipronil and total fipronil at different exposures, as indicated by high coefficients of correlation ($r=0.894$ – 0.922 , $r=0.812$ – 0.896 and $r=0.851$ – 0.914 respectively). Uptake of enantiomers of fipronil in *E. foetida* fitted first-order kinetics and was accumulated from the soil in what was primarily an equilibrium partition process. The elimination rate coefficient (k_2) for the bioaccumulation model was accurately determined by the depuration processes and was 0.33–0.47 for *S*-fipronil and 0.35–0.54 for *R*-fipronil. The approximation uptake rate constant k_1 was 0.24–0.27 for *S*-fipronil and 0.20–0.25 for *R*-fipronil. The uptake rate coefficients (k_1) of *S*-fipronil were all higher than the rates for *R*-fipronil. In addition, the elimination rate coefficients (k_2) of *R*-fipronil were higher than those of *S*-fipronil. Rate constants BAF_k for *S*-fipronil and *R*-fipronil were less than 1.0, amounting to 0.58–0.75 and 0.47–0.58 respectively. This result indicated that the uptake rate that represented the motive power of accumulation in *E. foetida* was low when compared with the rate of elimination and metabolism. The accumulation, degradation and elimination of fipronil in earthworm tissues was enantioselective. *S*-Fipronil was preferentially accumulated, and *R*-fipronil might have been eliminated or metabolised.

4 CONCLUSION

In this study, the enantioselective subchronic toxicity and bioaccumulation of fipronil at four exposure levels (racemate 20 and 50 mg kg⁻¹, individual enantiomers 10 mg kg⁻¹) were assayed by soil contaminant. The results showed that *R*-fipronil was less toxic than racemate and *S*-fipronil to the non-target organism *E. foetida*, and there was a significant trend towards enantioselective bioaccumulation in *E. foetida*, with a preferential accumulation of *S*-fipronil (EF at 0.56–0.62, $P < 0.001$). The present study demonstrated the fate of fipronil and potential exposures in a simplified soil–earthworm system. The worms accumulated fipronil, fipronil sulfide and sulfone. In addition, the amount of fipronil amide was relatively low in earthworm tissues. Enantiomers of fipronil and metabolites (sulfone and sulfide) might be subject to a biomagnifying effect in the earthworm food chain. Although the present study demonstrated the variable fate of fipronil in the terrestrial environment, more research on the toxicity of fipronil sulfide and sulfone metabolites to non-target organisms should be investigated. Moreover, higher animals and variable levels of exposure should be employed for a more precise risk assessment in terrestrial food webs for future research.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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