Supporting Information

Fate and Uptake of Pharmaceuticals in Soil-Earthworm Systems

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Number of tables: 5

Number of figures: 2

Additional text: Sorption of study compounds to soil, *E. fetida* toxicity experiment, preparation of samples for analysis and detailed methods of metabolism study.

Table SI 1: Test soil characteristics († Analysis completed at The French National Institute for Agricultural Research (INRA) (Arras, France) *Analysis completed onsite at The Food and Environment Research Agency (FERA) (York, U.K.)).

Fine sand (50/200 µm) (g/kg) †	272
Coarse sand (200/2000 µm) (g/kg) †	136
Fine silt (2/20 µm) (g/kg) †	197
Coarse silt (20/50 µm) (g/kg) †	164
Clay (< 2 µm) (g/kg) †	231
pH (water) †*	6.31
Cation exchange capacity cmol +/kg †	10.3
Organic carbon (%) †	1.89
C/N †	11.2
Organic matter (%) †	3.27
Water holding capacity (%w/w) *	17.3

Table SI 2: LC-MS/MS parameters used for the analysis of the compounds

Compound	**Parent ion (*m/z*)**	**MRM product ion (*m/z*)**	**'Collision energy' setting**	**Collision cell exit potential setting**	**Retention time (min)**
Carbamazepine	237.3 ($M+H^+$)	194.3	13	15	1.8
Carbamazepine-D_{10}	247.5 ($M+H^+$)	204.2	13	15	1.8
Fluoxetine	310.3 ($M+H^+$)	148.3	25	12	1.6 – 1.9
Fluoxetine-D_5	315.2 ($M+H^+$)	153.2	25	12	1.6 – 1.9
Diclofenac	296.2 ($M-H^+$)	250.0	15	11	4.1
Diclofenac-D_4	298 ($M-H^+$)	254.1	15	11	4.1

Table SI 3: Analyte detection in earthworm samples from LC-MS/MS analysis. BSAF is the biota-soil accumulation factor.

Compound	**Soil spike (mg/kg)**	**BSAF**	**Expected (ng/g)**	**Average measured (ng/g) (± standard deviation)**
Carbamazepine	0.8	0.3	260	491.2 (± 18.5)
Diclofenac	0.8	0.6	456	< LOQ
Fluoxetine	1.6	0.3	466	803.0 (± 97.8)

The BSAF was estimated by dividing the maximum earthworm tissue concentration by the measured soil concentration in the radiolabelled studies. By dividing the BSAF by the nominal soil concentration in the un-labelled experiments we therefore calculated an expected earthworm tissue concentration which would allow for comparison to the measured earthworm concentration in the unlabelled experiments.

Table SI 4: FOCUS modelling results from dissipation of pharmaceuticals in soil

Pharmaceutical	**Kinetics**	**Chi (χ^2) (Tabulated χ^2)**	**DT_{50} (d)**	**DT90 (d)**
Carbamazepine	Single First order	2.0 (12.6)	68	226
Diclofenac	N/A	N/A	N/A	N/A
Fluoxetine	Single First order	5.1 (12.6)	66	220
Orlistat	Single First order	6.4 (12.6)	48	159

40 **Table SI 5**: Known diclofenac metabolites and transformation products detected in various

41 matrices for comparison against data obtained in this study using LC-FTMS analysis

Diclofenac metabolites / transformation products	**Matrice**	**Reference**
acyl glucuronide of diclofenac	Fish bile	Kallio et al., 2010
acyl glucuronide of 3′-hydroxydiclofenac	Fish bile	Kallio et al., 2010
acyl glucuronide of 4'-hydroxydiclofenac	Fish bile	Kallio et al., 2010
acyl glucuronide of 5-hydroxydiclofenac	Fish bile	Kallio et al., 2010
ether glucuronide of 4'-hydroxydiclofenac	Fish bile	Kallio et al., 2010
sulfate conjugate of 4'-hydroxydiclofenac	Fish bile	Kallio et al., 2010
sulfate conjugate of 5-hydroxydiclofenac	Fish bile	Kallio et al., 2010
sulfate conjugate of 4′,5-dihydroxydiclofenac	Fish bile	Kallio et al., 2010
monosulfate conjugate of dihydroxydiclofenac	Fish bile	Kallio et al., 2010
acyl-migrated isomers of acyl glucuronide of 3'-hydroxydiclofenac	Fish bile	Kallio et al., 2010
acyl-migrated isomers of acyl glucuronide of diclofenac	Fish bile	Kallio et al., 2010
5-hydroxydiclofenac	Fish bile/ Sewage effluent	Scheurell et al., 2009 Stülten et al., 2008 Kallio et al., 2010
8-Chlorocarbazole-1-yl-ethanoic acid	Effluent	Scheurell et al., 2009
3′-Hydroxydiclofenac	Effluent	Scheurell et al., 2009
1-(2,6-Dichlorophenyl)-1,3-dihydro-2*H*-indole-2-one	Effluent	Scheurell et al., 2009
1-β-*O*-acyl glucuronide of diclofenac	Rat liver	Lee et al., 2012
4'-hydroxydiclofenac	Fish bile/effluent/rat liver/plants	Kallio et al., 2010 Scheurell et al., 2009 Huber et al., 2012 Stülten et al., 2008

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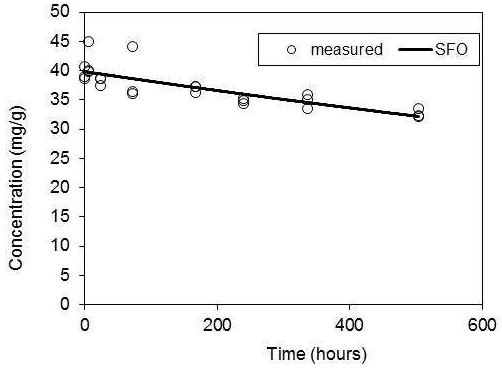
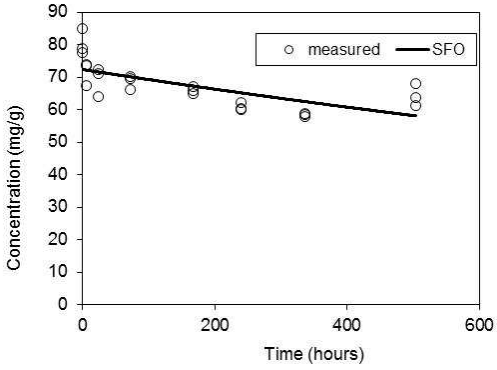
Figure SI 1: Measured soil concentration data from uptake phase for carbamazepine (A), fluoxetine (B) and orlistat (C) fitted with a single first order model. Diclofenac could not be modelled.

A)



B)



C)

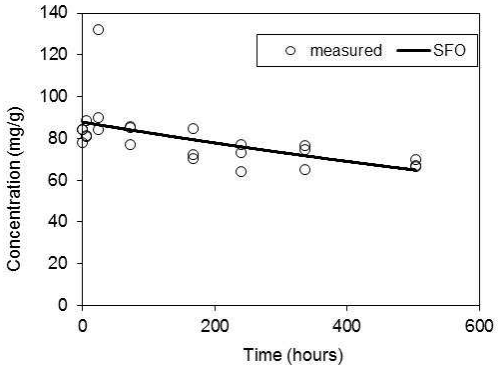
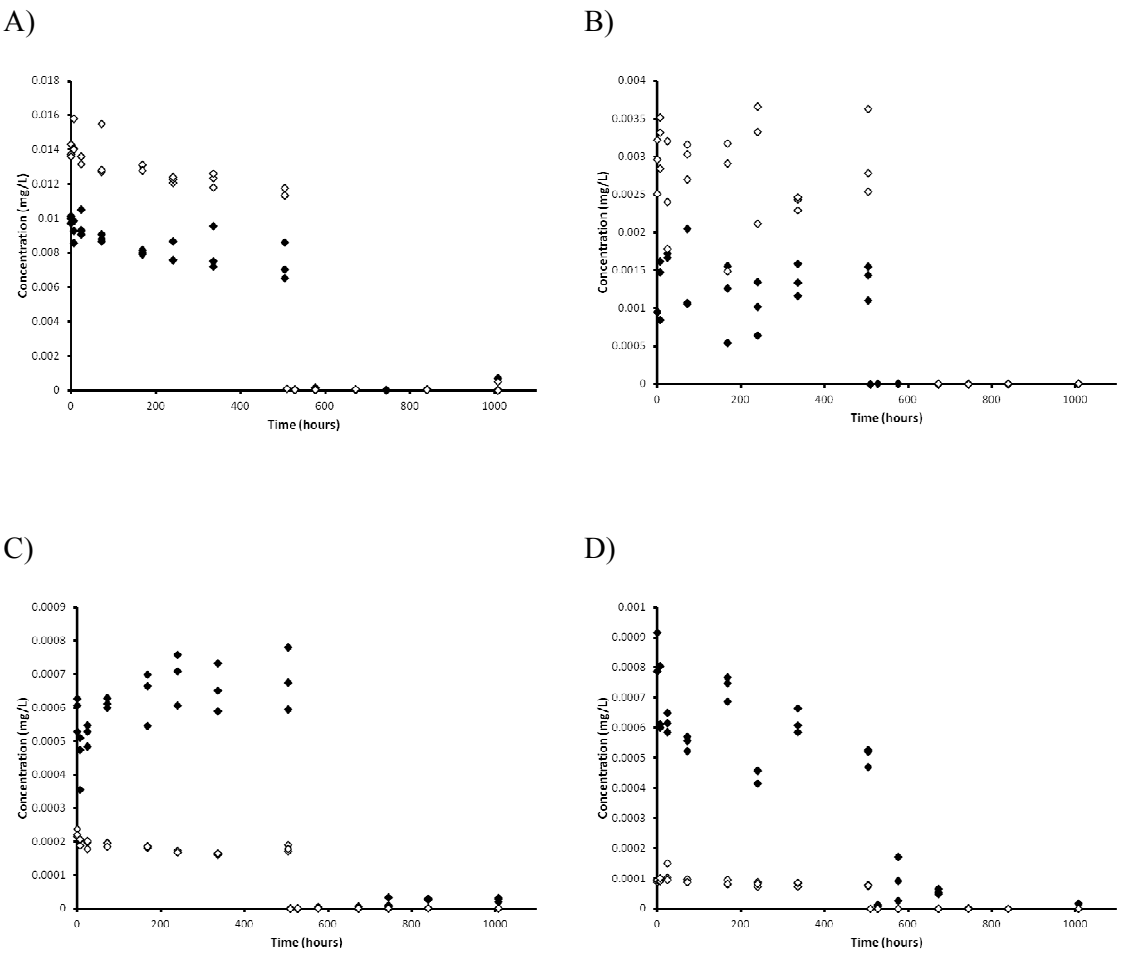


Figure SI 2: Comparison between measured pore water concentrations obtained from the uptake and depuration experiment and estimated pore water concentrations (PEC_{pw}) for each pharmaceutical for A) carbamazepine, B) diclofenac, C) fluoxetine, D) orlistat. The closed and open diamonds represent measured concentrations and estimated concentrations respectfully.

The predicted environmental concentration in pore water (PEC_{pw}) (Technical Guidance Document (TGD) on Risk Assessment Part II, 2003) was calculated based on measured soil data using the following equation: $PEC_{pw} = (C_{soil}*RHO_{soil})/(K_d*1000)$

Where C_{soil} is the measured concentration in the soil (this can be predicted soil concentration (PEC_{soil}) if measured data are unavailable – see TGD for equation), RHO_{soil} is bulk density of the soil (kg m^{-3}) K_d is the soil sorption distribution coefficient for each pharmaceutical in the test soil.



Sorption of study compounds to test soil.

The sorption behaviour of the study APIs in the test soil was assessed using a batch equilibrium method based on OECD guideline 106. Study pharmaceuticals were applied to a mixture of soil and a 0.1 M $CaCl_2$ solution contained in PTFE centrifuge tubes in triplicate. The soil solution ratios, selected based on preliminary investigations, were 1:5, 1:20, 1:30 and 1:30 for diclofenac, carbamazepine, fluoxetine and orlistat respectively. The resulting soil/solution mixtures were shaken in the dark (250 oscillations/min) at a temperature of 4 ^{o}C on a side-to-side shaker for 48 h, as preliminary studies showed that this was sufficient time for the test APIs to reach equilibrium between the soil and liquid phase. The samples were then removed and centrifuged at 3500 rpm for 10 minutes using a Heremle Z 513K Bench Top Centrifuge. A 1 mL aliquot of supernatant was then taken and mixed with 10 mL of Ecoscint A scintillation cocktail (National Diagnostics, Atlanta, Georgia) and the levels of radiation remaining in solution was determined by liquid scintillation counting (LSC) using a Beckman LS 6500 (Beckman Coulter Inc., Fullerton, USA). Soil sorption coefficients (K_d) values were then determined based on the amount of pharmaceutical applied and the amount remaining in the supernatant at equilibrium (Table 2).

Toxicity of study compounds to *Eisenia fetida*.

Methods:

Toxicity experiments were performed to ensure that the test concentrations used in the uptake studies were not toxic to the *E. fetida.* Earthworms were exposed in replicates of six to soil containing ten times and a hundred times the proposed test concentration for the main uptake study. The test vessel consisted of a 120 mL glass jar to which 50 ± 1 g of soil (dry weight) was added. One earthworm per vessel was added and beakers were incubated under similar conditions to the main experiment for the period of the exposure. Burrowing behaviour, potential weight change and mortality were compared to that observed in solvent controls and blank controls, to see if the pharmaceuticals had measurable effects on these variables.

Results and Discussion:

No mortality was observed in any of the toxicity experiments. There were no significant differences in the burrowing times of *E. fetida* after exposure to each of the pharmaceutical compounds (x 10 and x 100) in comparison to the blank and solvent controls ($F<0.709$, d.f. = 3, $p > 0.05$), with more than 90 % of earthworms burrowing beneath the soil within 10 minutes of being placed on the soil surface. Over the test period, the masses of *E. fetida* increased; however there was no significant difference in the growth rate of *E. fetida* exposed to pharmaceutical treated soils or to control soils (for carbamazepine and fluoxetine [$F<2.323$, d.f. = 3, $p>0.05$]) (for diclofenac and orlistat [$H<4.610$, d.f. = 3, $p>0.05$]). No unusual earthworm behaviour (e.g. coming to the soil surface, stiffening) or physiological differences (e.g. surface lesions) was noted for any of pharmaceutical-exposed worms. It was therefore concluded that as no visible effect on the earthworm behaviour was seen at 10 x and

100 x the proposed test concentrations for the main uptake and depuration experiments, uptake and depuration would unlikely be affected by pharmaceutical toxic effects.

In terms of pharmaceutical toxicity to earthworms, there is relatively little research. Previous studies have observed no *E. fetida* mortality after exposure to tetracyclines at environmentally relevant concentrations (Qu et al., 2005), similar to the results from this study. However, exposure to chlorotetracycline and tetracycline has induced changes in biochemical markers, including serious DNA damage to coelomocytes and enzyme activities in earthworms (Dong et al., 2012). As pharmaceutical toxicity was not evaluated on a biochemical scale in this study, further research is needed to establish if similar effects are observed with human pharmaceuticals comparable to what has been observed with tetracyclines.

Preparation of soil, pore water and earthworm samples for analysis.

To extract pore water, soil (25 ± 2 g) was placed in a disposable syringe with a layer of 3 cm of glass wool inserted into the bottom. The syringe was centrifuged for 40 minutes (2 x 20 minute runs) at 3000 RPM after which the pore water was collected from the bottom of the tube and transferred to a 2 mL plastic microfuge tube. The microfuge tubes containing the sampled pore water were then further centrifuged at 12000 RCF for 4 min to sediment loose particles. A 500 μL sample of pore water was then added to 10 mL of EcoScint A scintillation cocktail for analysis.

Soil samples were extracted by liquid extraction. For the carbamazepine study, 5 ± 0.5 g of soil was extracted twice for 45 min on a side to side shaker (250 oscillations min^{-1}) with 2 x 10 mL of methanol. A similar method was used in the fluoxetine and orlistat studies except that for fluoxetine a mixture of acetonitrile and water (7:3 v/v) was used as the solvent and for orlistat, acetonitrile was used. For the diclofenac study, 10 g samples of soil were extracted three times for 45 min each time with 20 mL ethyl acetate. Samples (1 mL) of extracts were then added to 10 mL of EcoScint A for analysis of the radioactivity present.

Even with the high extraction recoveries for diclofenac, after solvent extraction, the concentration at the start of the experiment was significantly lower than expected. A large amount of dissipation of radioactivity from the orlistat test beakers was also observed, which unlike the other test compounds, could not be explained by uptake into *E. fetida*. It was theorised that due to orlistat's particularly hydrophobic nature and high K_d value it would have a strong sorption capacity to the soil, to such an extent that a fraction of the compound may have become irreversibly bound to the soil. Combustion analysis of the diclofenac and orlistat soils was therefore performed to determine if there was radioactivity remaining in the soil which may account for the discrepancies. Combustion analysis was performed on a

139 Perkin Elmer 307 Sample Oxidiser. After solvent extraction to determine the total extractable
140 residues, the dried soils were homogenised into a fine powder. Each soil sample was prepared
141 in triplicate in combusto-cones in which 300 ± 25 mg of soil was mixed with equal amounts
142 of cellulose powder. After combustion consisting of a 1.5 min burn per sample, the ^{14}C
143 carbon dioxide was trapped by a vapour phase reaction with CarboSorb E forming carbamate
144 which was mixed with PermaFluor E + a scintialltion cocktail ready for counting the
145 radioactivity present on the Liquid Scintillation Counter (LSC). Regular checks were
146 performed throughout the analysis to ensure the recovery of the samples remained above 95
147 %.

148 *E. fetida* were extracted by liquid extraction using the same solvents as for the soil
149 extractions. Prior to extraction, *E. fetida* were defrosted, solvent (5 mL) was then added to the
150 defrosted samples and the worm/solvent mix was homogenised for 5 min using a LabGen
151 Series 7 homogeniser. The suspension was transferred from the beaker to a glass test tube and
152 the beaker was then rinsed with an additional 3 mL of solvent which was combined with the
153 suspension to give a total extract volume of 8 mL. The extracts were centrifuged at 415 g for
154 30 min (CHRIST Rotational Vacuum-Concentrator RVC 2-33 CD) and a 1 mL sample of the
155 resulting supernatant was then added to 10 mL of EcoScint A.

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157 **Potential metabolism in earthworms**

158 ***Methods:***

159 To ascertain whether the radioactivity we were measuring in our earthworm samples was that
160 of the parent compound or metabolite/transformation products, additional studies were
161 performed using unlabelled compounds. Studies were performed at 20 times the soil
162 concentration used in the radiolabelling studies to ensure that compounds were detectable in
163 the worm matrix.

164 *E. fetida* were exposed to unlabelled carbamazepine, diclofenac and fluoxetine for 21 days
165 (six replicates per compound) under similar conditions to the original studies, after which
166 they were allowed to purge their guts for 24 h and subsequently frozen (-20 ^{o}C) ready for
167 analysis. *E. fetida* were then injected with a a known amount of stable isotope-labelled
168 standard (carbamazepine d-10, diclofenac d-4 and fluoxetine d-5) and extracted using
169 methods outlined above. The supernatant from these extractions was taken to dryness under a
170 N_2 stream and reconstituted in 200 μL of methanol:water (50:50 v:v). This was further
171 centrifuged at 12000 RPM to sediment loose particles and the resulting extracts were
172 transferred to HPLC vials for analysis. Calibration (six concentrations, three replicates) and
173 quality control samples (three concentrations, six replicates at intermediary concentrations
174 across the calibration range) were also prepared in worm matrix for each of the respective
175 compounds. The calibration range was 0 - 1500 ng/mL; 0 - 800 ng/mL and 0 - 1375 ng/mL
176 for carbamazepine, diclofenac and fluoxetine respectively.

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180 ***Analytical methods:***

181 ***LC-MS/MS analysis***

182 Extracts were analysed for the pharmaceuticals by LC-MS/MS using a Dionex Ultimate 3000
183 and Applied Biosystems API 3000. HPLC separation was performed with a Symmetry C18
184 3.5 µm, 4.6 x 75 mm column and Symmetry C18 3.5 mm, 2.1 x 10 mm guard column
185 (Waters) with a mobile phase flow rate of 1 mL/min. The mobile phase composition was
186 aqueous 1 % formic acid (v:v) (mobile phase A) and 1 % formic acid (v:v) in acetonitrile
187 (mobile phase B) using a gradient program over 5 min for carbamazepine and fluoxetine and
188 7.5 min for diclofenac. For carbamazepine and fluoxetine the gradient was 0.0-2.5 min 43 %
189 B, 2.5-2.6 min 43-95 % B, 2.6-3.6 min 95 % B, 3.6-3.7 min 95-43 % B, 3.7-5.0 min 43 % B.
190 For diclofenac the relative flow of mobile phase B was 0.0-1.5 min 43 % B, 1.5-4.0 min 43-
191 80 % B, 4.0-4.2 min 80-95 % B, 4.2-5.5 min 95 % B, 5.5-5.7 min 95-43 % B, 5.7-7.5 min 43
192 % B. MS/MS analysis was undertaken using electrospray ionisation (ESI) in positive
193 ionisation mode, using the turbo ion-spray interface. Spray voltage was 5000 V, with the ESI
194 capillary line maintained at 550°C and collision gas (N_2) pressure set at 6. Fragmentation
195 parameters are detailed in Table SI 2.

196 Qualitative and quantitative analysis of compounds was based on multiple reaction
197 monitoring (MRM); measuring the relative peak areas of the product ion from analyte and
198 internal standard.

199 For the calibration range of 0 - 1500 ng/mL, 0 - 800 ng/mL and 0 - 1375 ng/mL the R^2 of
200 calibration fits are 0.92, 0.98, 0.99 for carbamazepine, diclofenac, fluoxetine respectively.
201 The mean accuracy for the QC's ranged between 88.6 – 108.5%, 95.8 – 97.6% and 82.1 –
202 96.3% and the mean standard deviation for QC's ranged between 5.3 – 9.1, 5.6 – 9.7 and 3.3

– 8.2 for carbamazepine, diclofenac and fluoxetine respectively. Lower limits of quantification (LLOQs) were 375 ng/mL, 12.5 ng/mL and 150 ng/mL for carbamazepine, diclofenac and fluoxetine respectively.

LC-FTMS

HPLC separation was performed with a Jupiter 4u Proteo 90A, 1.0 x 150 mm column with a mobile phase flow rate of 0.1 mL/min. The mobile phase composition was aqueous 1 % formic acid (v:v) (mobile phase A) and 1 % formic acid (v:v) in acetonitrile (mobile phase B) using a gradient program 0.0-20.0 min 10-90% B, 20.0-21.0 min 90% B, 21.0-21.5 min 90-10% B, 21.5-26.5 min 10% B. MS analysis was undertaken using positive mode electrospray ionisation (ESI). Spray voltage: 4500 V, 6 L/min dry gas (N_2) at 250 ^{o}C and 3 bar nebuliser gas. Acquisition range *m/z* 100-2000, with transient time 0.367 s giving an estimated resolution of 66000 at *m/z* 400.

Results – Determination of parent compound in earthworm tissue samples:

Based on previous results, a biota-soil accumulation factor (BSAF) was calculated. Using spiked soil concentrations, expected concentration of the pharmaceuticals in the worm tissue was estimated (see Table SI 3).

Both carbamazepine and fluoxetine were detected in the worm tissue at concentrations slightly greater than expected. Diclofenac was not detected. Diclofenac worm extracts were subsequently analysed using LC-FTMS to look for known diclofenac metabolites and transformation products using a Dionex Ultimate 3000 HPLC and solariX 9.4 T (Bruker) FT-ICR mass spectrometer (known metabolites are provided in Table SI 5).

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