UPTAKE OF PHARMACEUTICALS INTO TERRESTRIAL ORGANISMS

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Abstract

Over the past decade, there has been increasing scientific interest in the occurrence, fate and effects of pharmaceuticals in the environment. To date, the majority of this research has focussed on the aquatic environment whilst the terrestrial environment has remained relatively unexplored. Research carried out in the terrestrial environment has primarily focussed on the fate of pharmaceuticals in soils as well as the uptake of pharmaceuticals into plants. Less information is available on the uptake of pharmaceuticals into other soil dwelling species.

The studies presented in the thesis were therefore performed to investigate the uptake of pharmaceuticals into earthworm species (*Eisenia fetida* and *Lumbricus terrestris*) and plant species (radish and ryegrass). Experiments were designed to explore the effect of pharmaceutical physico-chemical properties, soil parameters and species traits on the uptake of pharmaceuticals from soils into terrestrial species. Understanding the factors and processes involved in the uptake of these compounds from soils, is vital to adequately assess the risks of pharmaceuticals in the environment.

Initial experimental studies evaluated the uptake of four pharmaceuticals, namely carbamazepine, diclofenac, fluoxetine and orlistat into the earthworm, *Eisenia fetida*. Pore water based bioconcentration factors (BCFs) increased in the order of carbamazepine < diclofenac < fluoxetine and orlistat.

As well as experimental research, a desk based investigation was perfomed to assess the applicability of a minimised design approach to estimate bioconcentration factors (BCFs) in terrestrial and aquatic species. A significant regression between $BCF_{minimised}$ and $BCF_{traditional}$ was found and this approach was therefore adopted to calculate earthworm BCFs in the soil parameters and species traits studies described below.

The uptake of the four study pharmaceuticals by *E. fetida* was therefore further evaluated in different soil types. The uptake and accumulation of pharmaceuticals

into *E. fetida* changed depending on soil type. Orlistat exhibited the highest pore water based bioconcentration factors (BCFs) and displayed the largest differences in uptake between soil types as BCFs ranged between 30.51 – 115.92. For carbamazepine, diclofenac and fluoxetine BCFs ranged between 1.05 – 1.61, 7.02 – 69.57 and 16.78 – 20.42 respectively.

Supplementary studies compared the uptake of the study pharmaceuticals in two earthworms (*Lumbricus terrestris* and *E. fetida*). All four pharmaceuticals were taken up by both *L. terrestris* and *E. fetida* tissue after 21 d exposure to spiked soil. Pore water based bioconcentration factors (BCFs) ranged between 6.69 and 83.79 for *L. terrestris* and 1.14 and 63.03 for *E. fetida*.

The effect of species type on the uptake of pharmaceuticals (carbamazepine, diclofenac, fluoxetine, propranolol, sulfamethazine) and a personal care product (triclosan) was also investigated in plant species (radish, *Raphanus sativus* and ryegrass, *Lolium perenne*). Five of the six chemicals were taken up into plant tissue, carbamazepine to the greatest extent in both the radish (52 µg/g) and ryegrass (33 µg/g) whereas sulfamethazine uptake was below the limit of quantitation (LOQ).

The results demonstrate the ability of plant species and earthworms to accumulate pharmaceuticals from soils with uptake apparently specific to both species, chemical and soil type. However the influence of these individual parameters does not affect BCFs to a significant amount. The research also highlights that a combination of factors and processes appear to be driving the uptake into soil dwelling species as further analysis was unable to find a single parameter to adequately explain pharmaceutical uptake into terrestrial species. For example, for plant uptake, results could only be partly explained by the hydrophobicity and extent of ionisation of each chemical in the soil.

Even though these chemicals are taken up by earthworms and plants, further analysis showed that the risk to predatory birds is minimal based on the current environmental scenarios as thousands of worms would have to be consumed by a bird to receive a single dose. Similarly, the potential risk to humans consuming crops contaminated with pharmaceutical residues is also minimal. However with

increasing loadings of pharmaceuticals to soils this may result in potential problems for human health and predatory birds in the future.

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And Rich, your endless love, encouragement and kindness has allowed me to finish this journey, and it is with great anticipation that I look forward to the next chapter in our lives. Love you always.

Authors Declaration

This thesis contains original work completed by myself as a PhD student at the University of York under the supervision of Professor Alistair Boxall (September 2009 – 2013).

This research was funded by a BBSRC PhD CASE award in collaboration with GlaxoSmithKline.

Research from **Chapter 2** was presented at the SETAC Europe meeting, 2012, Berlin, Germany and SETAC North America meeting, 2011, Boston, USA.

The data reported in **Chapter 4 and 5** was presented at the SETAC Europe meeting, Glasgow United Kingdom, 2013.

The results from **Chapter 6** were presented at The Food and Environment Research Agency (Fera) Student conference 2012

Research reported in **Chapter 2, 3, 4, 5 and 6** has been written as papers for international peer reviewed journals. These papers have been reworked, so they are presented in a consistent style format in this thesis. The current status of the papers presented in this thesis is presented in Table 0.1.

Table 0.1 Status of the papers presented in this thesis with respect to the publication process

Title	Journal	Status	Chapter
Uptake of pharmaceuticals into the earthworm, *Eisenia fetida*		Prepared for submission October 2013	2
Use and applicability of a minimised design to estimate bioconcentration factors		Prepared for submission October 2013	3
The effect of soil properties on the uptake of pharmaceuticals into earthworms, *E. fetida*		Prepared for submission	4
The effect of species type on the uptake of pharmaceuticals into earthworms, *E. fetida*		Prepared for submission	5
Which factors affect uptake of - pharmaceuticals into plants?	Journal of Agricultural and Food Chemistry	Submitted (September 2013)	6

With the exception of Chapter 6, the contents of this thesis have been produced solely by the candidate. Data for Chapter 6 was a result of a research visit to Commonwealth Scientific and Industrial Research Organisation (CSIRO), Adelaide, South Australia where plant uptake experiments were carried out. All experiments were designed and carried out by the candidate and samples were extracted then left ready for analysis on the LC-MS/MS before returning to the United Kingdom. Unfortunately before the HPLC vials were analysed they were spiked with cold stock solution instead of isotopically labelled internal standards and therefore the results were unable to be used. CSIRO therefore repeated the uptake experiments according to the original study design outlined by the candidate. CSIRO provided the repeated results to the candidate who then analysed and interpreted the data.

Chapter 1 Literature Review

1.1 Introduction

Pharmaceuticals are a group of chemicals used for diagnosis, treatment, alteration or prevention of diseases, health conditions or functions in the human body (Daughton and Ternes, 1999). Human medicines can be categorised according to their intended mode of action in the body. Pharmaceuticals have specialised properties and are designed to affect a specific target at a given concentration. Worldwide, pharmaceutical use has been on the increase for the past century (OECD, 2011). This trend is set to continue into the future with the development of new medicines to cure recently discovered diseases as well as previously untreatable problems and as a result of an ageing population (The Royal Commission on Environmental Pollution, 2011). Over the past fifteen years, there has been increasing interest in the potential effects of pharmaceutical in the natural environment. This Chapter reviews the current knowledge on the inputs, occurrence and fate of pharmaceuticals in the environment with primary focus on the terrestrial environment which is the subject of this PhD thesis.

1.1.1 Current situation in the environment

Although pharmaceuticals have been released into the environment for decades, researchers have only recently begun to quantify their levels and potential effects in the environment. Previously, studies into the uptake and effects of chemicals in the environment focussed on the presence of pesticides (e.g. Dieldrin) (De Silva and van Gestel, 2009; Lew- *et al*., 2009; Matsumoto *et al*., 2009) and metals (Mdegela *et al*., 2009; Nahmani *et al*., 2009) amongst other contaminants. A key example emerged during the period of 1932 – 1968 where it was established that a Japanese petrochemical plant was releasing methyl mercury into Minamata Bay. As a result, both total mercury and methyl mercury were seen to biomagnify through the Northwater Polynya food web, which caused many problems for wildlife especially for top predator mammals (Campbell *et al*., 2005).

However, pharmaceuticals are now a growing topic of scientific interest (Kummerer, 2001) and recently have been defined as emerging environmental contaminants (Beausse, 2004; Nikolaou *et al.*, 2007). Studies have already highlighted the effects of pharmaceutical residues on terrestrial wildlife after they are taken up by a variety of species. Experimental evidence demonstrates that diclofenac is strongly responsible for the rapid decline in vulture populations in Asia (Green *et al.*, 2007, 2006; Oaks *et al.*, 2004). Examination of the dead vultures revealed they had died from visceral gout, a condition caused by renal failure. It appeared that the vultures had scavenged on carcasses that had previously been given veterinary doses of diclofenac and that the uptake of diclofenac had caused kidney failure in several vulture species. The vulture populations are now classed as critically endangered after suffering a 97 % population decline, this may not be solely attributable to the presence of diclofenac residues in the environment but many scientists argue that it plays a substantial role.

Another important problem associated with the presence of pharmaceuticals in the environment is the selection of antibiotic resistance in bacterial populations (Levy, 2002). The emergence of resistant bacteria has led to widespread coverage in the scientific media. There is growing evidence that resistance to antibiotics (AR) is posing an emerging threat to both public and the future environmental health. Knapp *et al.*, (2010) were able to extract DNA from a series of archived historic soils from the Netherlands (1940 – 2008). Results showed that the AR gene from all classes of the 18 antibiotics tested had significantly increased since 1940. This was especially true of the group of antibiotics known as tetracyclines, where in some cases individual AR genes were more than 15 times more abundant now than in the 1970s.

Concern is now focussed, amongst scientists, environmental regulators and the pharmaceutical industry as to the risks and potential adverse effects of these agents on non-target organisms (GSK, 2011; Jorgensen and Halling-Sorensen, 2000; Pomati *et al.*, 2004; Stuer-Lauridsen *et al.*, 2000; Ternes, 1998). The detection of varying concentrations of numerous pharmaceuticals in all environmental compartments and incidents such as the vulture population decline as described above, has escalated this concern. As a result there has also been a noteworthy increase in the number of published studies on pharmaceuticals in the environment since 2001 (Figure 1.1). Although as Figure 1.1 also highlights the majority of studies have predominantly

focussed on the aquatic environment with the terrestrial environment being relatively unexplored in comparison.

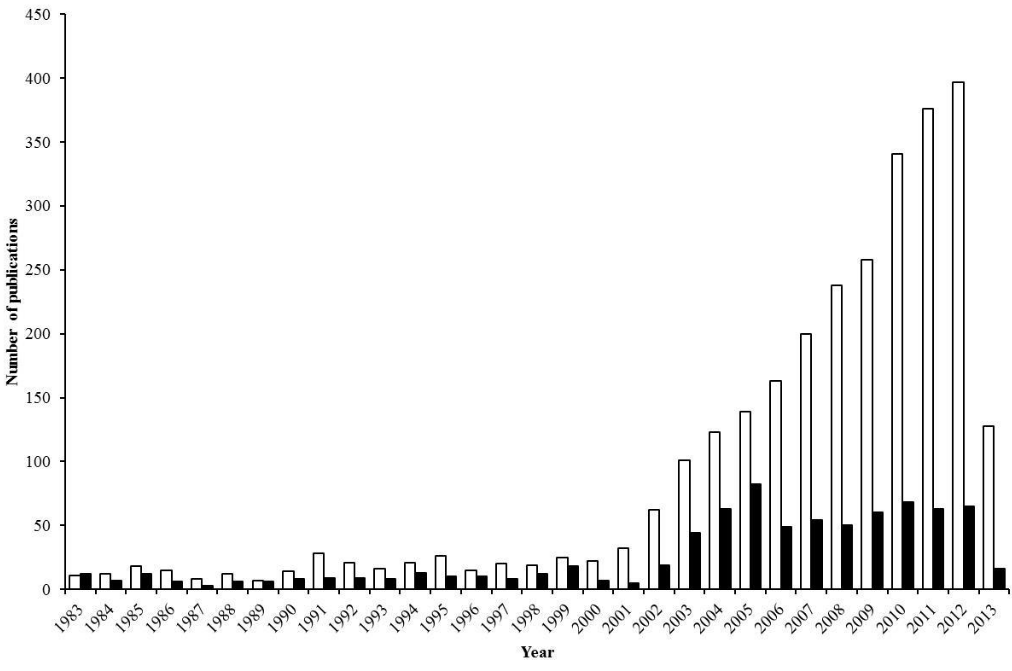


Figure 1.1 Number of publications regarding pharmaceuticals in both the aquatic (white) and terrestrial (black) environment. Graph obtained from web of knowledge citation report service on publications of environmental research, search terms were 'aquatic pharmaceutical' and 'terrestrial pharmaceutical.'

1.2 How do pharmaceuticals enter the terrestrial environment?

The various routes by which pharmaceuticals enter the soil compartment have been extensively documented (Diaz-Cruz *et al.*, 2003; Oppel *et al.*, 2004; Ruhoy and Daughton, 2008; Thiele-Bruhn, 2003). Land application of manure is the primary pathway for the release of veterinary pharmaceuticals into the terrestrial environment (Baguer *et al.*, 2000). The presence of veterinary pharmaceuticals in manure intended for land application has been demonstrated by Jacobsen and Halling-Sorensen (2006) with the detection of various tetracyclines (< 30 mg/kg dry weight) and sulphonamides (< 2 mg/kg dry weight). The two key pathways by which human pharmaceuticals enter the soil environment include *via* the use of reclaimed waste water (effluent) for irrigation and through the application of digested sewage sludge as a fertiliser onto agricultural fields (Diaz-Cruz *et al.*, 2003; Oppel *et al.*, 2004).

Sewage sludge and reclaimed wastewater are both by products of the sewage treatment plant (STP) process.

1.2.1 Sewage treatment

Because most pharmaceuticals are designed to not bioaccumulate and instead be rapidly eliminated from the body of the treated patient after administration, domestic raw sewage contains pharmaceuticals which have been excreted in urine and faeces to the wastewater system (Hirsch *et al*., 1999). Pharmaceuticals may also be metabolised in the patient so raw sewage will also contain metabolites of pharmaceuticals (Dollery, 1991; Huschek *et al*., 2004; Khan and Ongerth, 2002; Moffat, 2004; Ternes, 1998; Zuccato *et al*., 2005). Once released to the sewerage system, the pharmaceuticals and metabolites will typically be transported to a STP where the sewage will be treated (Lindqvist *et al*., 2005; Zorita *et al*., 2009).

Several in-depth studies have explored the fate of pharmaceuticals in STPs with different removal processes being investigated, including sorption to sludge (Kim *et al*., 2005), hydrolysis and aerobic and anaerobic biodegradation in sludge (Stumpf *et al*., 1999; Ternes, 1998; Wang *et al*., 1993; Xia *et al*., 2005). Different pharmaceuticals will behave differently in different treatment processes depending on their chemical functionality and physico-chemical properties (Castiglioni *et al*., 2006; Clara *et al*., 2005; Heberer, 2002; Stackelberg *et al*., 2004).

Whilst pharmaceuticals can be removed in STPs, as a result of complete mineralisation, on the whole, the elimination of pharmaceuticals is generally incomplete. Pharmaceuticals tend to not be removed from the raw sewage simply because the treatment processes are not designed to do this (Thomas and Hilton, 2004). Thus, the final effluent is likely to contain hydrophilic pharmaceuticals as these will remain in the aqueous phase whilst more hydrophobic pharmaceuticals may sorb to the sewage sludge.

1.2.2 Reclaimed wastewater effluent

Concentrations of pharmaceuticals in effluent are typically in the low µg/L range but have been recorded up to 200 µg/L for ketoprofen in Switzerland (Ollers *et al.*, 2001). The anti-epileptic drug carbamazepine is consistently detected in effluent samples with highest concentrations measured in Wales, United Kingdom < 4.59 µg/L (Kasprzyk-Hordern et al., 2009). A small number of studies have also investigated the detection of metabolites in sewage effluent with total mean concentrations of diclofenac metabolites ranging from 13 – 52 % of the measured parent compound concentrations. STP effluent is primarily emitted to water bodies such as rivers. However, recently effluent has been used to irrigate fields, a process which is sometimes described as 'reclaimed wastewater irrigation' or 'recycled wastewater irrigation.'

Irrigation comprises 65 % of all water use worldwide (Gielen *et al.*, 2009) and this demand is a growing year by year. Irrigation with effluent is a particularly attractive option to satisfy the demand for irrigation sources (Hamilton *et al.*, 2007). Many countries including the United States, Australia, Singapore, South Africa, Japan, China, Mexico and New Zealand have already adopted this strategy to cope with their water shortages (Levine and Asano, 2004). The Mezquital Valley in Mexico began using effluent for irrigation in 1912 (Siemens *et al.*, 2008). This is one of the oldest and largest examples of irrigation using municipal wastewater and is still in operation today, irrigating approximately 900 km^2 of land (Jimenez and Chavez, 2004).

With droughts and irrigation strains resulting from population pressures, the future use of recycled water is set to increase (Asano and Levine, 1996; Hamilton *et al.*, 2007). The Californian Government, for example, has set out aims to move towards a more sustainable management of water resources, including the increased use of recycled water over 2002 levels by a minimum of 1233 million cubic meters per year by 2020 and by at least 2467 million cubic meters per year by 2030 (California Water Resources Board, 2009). The increasing use of wastewater for irrigation will result in greater loads of pharmaceuticals reaching the soil.

1.2.3 Sewage sludge

Previously, sewage sludge was primarily disposed of at sea. However since 31 December 1998 when a ban was put in place prohibiting such actions in the European Union under the Urban Wastewater Directive (Section 1.4), the amount of sewage sludge applied to land has substantially increased (Andrews *et al.*, 1998) (e.g. Figure 1.2). Sewage sludge, produced from STPs, is now usually treated before it is used as a soil amendment (Jones-Lepp and Stevens, 2007). This treatment process typically involves digestion through biological, chemical and physical processes and then de-watering (Xia *et al.*, 2005). The sewage sludge can then be applied to fields either in a cake form, typically containing 30 % solids, or a slurry which comprises approximately 3 % solids (USEPA, 1999). In European countries, an average of 37 % of sewage sludge is land applied onto agricultural soils, equivalent to 2.39×10^6 dry tonnes per year (Chang *et al.*, 2002). Specifically, in the UK, over 60 % of the 1.4 million tonnes of sewage sludge produced annually is recycled to agricultural land (WATER UK, 2010). Recycling of sewage sludge occurs on a larger scale in the US with 60 % of the 6.2×10^9 kg of the sewage sludge generated in 1998 being land-applied (e.g. landfill cover, fertilizer, or soil amendments in land reclamation) and remaining disposed of *via* incineration for example (USEPA, 1999).

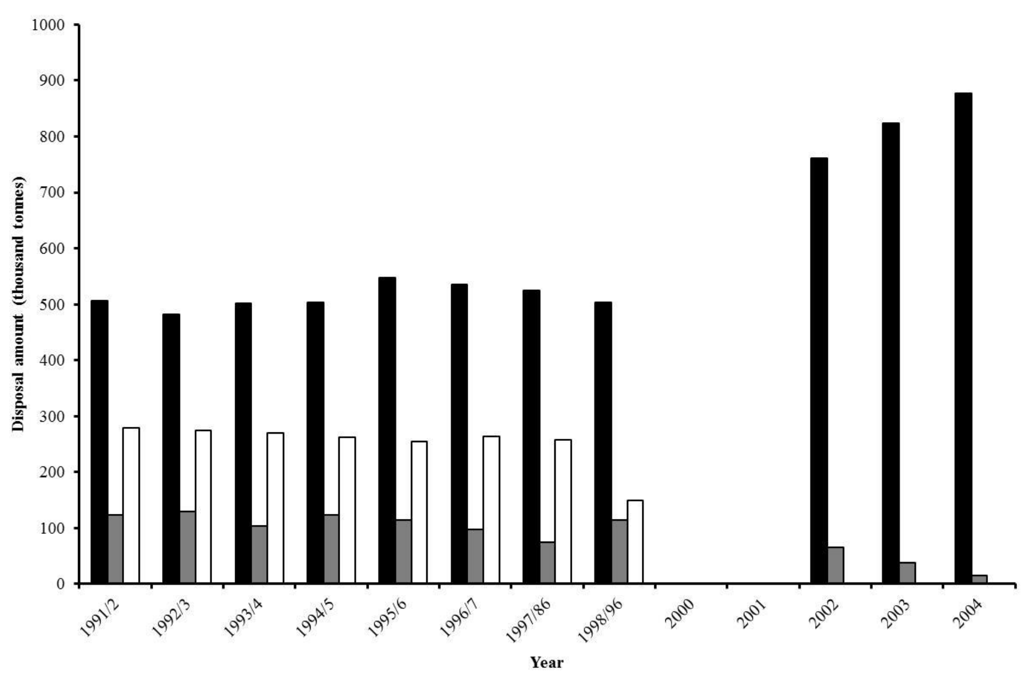


Figure 1.2 Primary disposal methods of sewage sludge in the United Kingdom between 1991 and 2004. Disposal methods include farmland (black) landfill (grey) and sea (white). Graph constructed with data obtained from e-Digest Statistics about Waste and Recycling from the defra website. Data was unable to be obtained for the period 2000 / 2001. Date accessed 30.09.12.

Sewage sludge is applied to agricultural fields for a number of reasons. From an agricultural perspective it is an inexpensive source of nitrogen, phosphorus, organic matter and other nutrients all of which can enhance soil physical properties and ultimately crop yield (Chitdeshwari and Savithri, 2007; Joshua *et al.*, 1998; Webber *et al.*, 1996). From a STP point of view land utilisation is desirable because it is an economic way of disposal. Land application of sewage sludge does however have its draw backs; it is also a source of many soil contaminants including organic compounds, pathogens and heavy metals (Rogers, 1996). Therefore through the use of the sewage sludge as a fertiliser there is the potential for pharmaceuticals to be added to soils. Recently studies have detected pharmaceutical compounds in sewage sludge destined for land application (Kinney *et al.*, 2006; Metcalfe *et al.*, 2003) as well in sewage sludge amended soils (Golet *et al.*, 2002; Kinney *et al.*, 2008, 2006) (Table 1.1).

The most frequently detected pharmaceuticals in sewage sludge include carbamazepine, diclofenac, ibuprofen and trimethoprim which have been measured in μg – mg/kg range. Carbamazepine was detected in all of the nine sewage sludge

samples studied by Kinney *et al.,* (2006) along with fluoxetine and diphenydramine of which three of the sludge samples were due to be applied to agricultural land.

Acidic pharmaceuticals such as naproxen have been measured in a number of sludge samples however concentrations tend to be low and can often remain undetected. In contrast, triclocarban and triclosan sorb particularly well to sludge, in some cases it is retained completely, resulting in concentrations < 441 mg/kg (Barron *et al*., 2009; McAvoy *et al*., 2002; Thompson *et al*., 2005; USEPA, 2009; Ying and Kookana, 2007). Sulfonamide antibiotics are frequently detected in sewage sludge and recently sulphonamide metabolites have also been detected in sludge samples (< 9.81 ng/g; García-Galán *et al*. (2012).

1.3 Concentrations of Pharmaceuticals in Soil

Concentrations of pharmaceuticals tend to be lower in the soil environment than in sewage sludge and effluent. For example, ibuprofen has been detected in sewage sludge between 99.5 to 11 900 µg/kg dry weight and in soil at only 0.25 ± 0.04 ng/g (Durán-Alvarez *et al*., 2009). This is primarily because when sewage sludge and effluent are applied to fields they are diluted within the soil matrix. Other potential loss mechanisms include leaching into nearby groundwater, photolytic processes or degradation by soil microbes for example, all of which would result in a reduction of the compound. In comparison to concentrations of pharmaceuticals in the aquatic environment and in sewage sludge, there are relatively few published studies that have shown concentrations in the soil environment. A list of measured concentrations has been compiled in Table 1.1 and a few key examples will be discussed in more detail below.

Kinney and colleagues (2006) published one of the earliest studies that detailed the presence of pharmaceuticals in soil samples after irrigation with wastewater effluent from Colorado City's WWTP. Individual pharmaceuticals were detected in the soils with concentrations typically ranging between 0.02 and 15 µg/kg (dw). A number of pharmaceuticals appeared to be persistent (acetaminophen, caffeine, carbamazepine, erythromycin, sulfamethazole, 1, 7-dimethylaxanthine and dehydronifedipine) and acetaminophen, fluoxetine, caffeine, erythromycin and carbamazepine were found to

accumulate in the soil in mass amounts consistently higher than the estimated mass applied in any month.

Durán-Alvarez and colleagues (2009) were able to detect pharmaceuticals in field soil samples from Tula Valley, Mexico which had been irrigated with untreated wastewater for 90 years. Very low concentrations of ibuprofen and naproxen were found in both the soil types (< 1 ng/g). This low build-up of pharmaceutical compound is indicative of degradation in the soil environment and possibly the high temperatures of the area. Meanwhile carbamazepine showed evidence of persistence in both the Phaeozem (6.48 ng/g) and the Leptosol (5.14 ng/g) soil (Durán-Alvarez *et al*., 2009). Similar to the results from Duran-Alvarez *et al*., (2009), carbamazepine was also detected in field soil samples obtained from marsh lands in Spain (Vazquez-Roig *et al*., 2010). In fact carbamazepine was the only compound detected in both soil samples with concentrations in the range of 1.43 - 5.77 ng/g.

Even though the concentrations of pharmaceuticals in sewage sludge and effluent are often at low levels, pharmaceuticals can build up in the soil compartment through long term, repeated, application (Dalkmann *et al*., 2012; Kinney *et al*., 2006; Redshaw *et al*., 2008a; Xu *et al*., 2009b). This is especially true of persistent pharmaceuticals that are known to not easily degrade in the environment, such as fluoxetine and carbamazepine (Clara *et al*., 2004; Durán-Alvarez *et al*., 2009; Monteiro and Boxall, 2009).

Table 1.1 Measured concentrations of pharmaceuticals in various soil matrices, ± standard deviations where reported in literature.

Pharmaceutical	Concentration (µg/kg)	Soil type	Reference
Acetaminophen	< 1.8	Pego-Oliva	(1)
Benzafibrate	0.67	Mezquital Valley	(2)
Bisphenol A	14.8 ± 3.2	Leptosol	(3)
	5.3 ± 0.9	Fangcun 0-10cm	(4)
	3.6 ± 2.2	Fangcun10-20 cm	(4)
Carbamazepine	< 6.96 ± 1.80	Mezquital Valley	(2)
	< 1.5	Pego-Oliva	(1)
	6.48 ± 0.59	Phaeozem	(3)
	5.14 ± 0.48	Leptosol	(3)
	5.77	Prat soil	(5)
Ciprofloxacin	< 2.82 ± 0.82	Mezquital Valley	(2)
	< 4.6	Pego-Oliva	(1)
Clarithromycin	< 3.89 ± 2.19	Mezquital Valley	(2)
Clofibric acid	0.49 ± 0.3	Fangcun20-30 cm	(4)
Codeine	< 1.7	Pego-Oliva	(1)
Diazepam	4.65	Prat soil	(5)
	< 0.3	Pego-Oliva	(1)
Diclofenac	0.40	Mezquital Valley	(2)
Enrofloxacin	< 0.64 ± 0.54	Mezquital Valley	(2)
Ibuprofen	0.25 ± 0.04	Phaeozem	(3)
Metoprolol	< 0.3	Pego-Oliva	(1)
Naproxen	2.86	Mezquital Valley	(2)
	0.55 ± 0.01	Phaeozem	(3)
	0.73 ± 0.2	Leptosol	(3)
Norfloxacin	< 8.4	Pego-Oliva	(1)
Ofloxacin	< 3.3	Pego-Oliva	(1)
Propranolol	< 0.4	Pego-Oliva	(1)
Sulfamethazine	11	-	(6)
Sulfamethoxazole	< 5.31 ± 0.22	Mezquital Valley	(2)
Triclosan	4.4 ± 0.1	Phaeozem	(3)
	18.6 ± 1.2	Leptosol	(3)
	3.2 ± 7.1	Fangcun10-20 cm	(4)
Trimethoprim	< 2.39 ± 0.29	Mezquital Valley	(2)
	< 0.2	Pego-Oliva	(1)

References: **(1)** (Vazquez-Roig *et al.*, 2012)**;** **(2)** (Dalkmann *et al.*, 2012)**;** **(3)** (Durán-Alvarez *et al.*, 2009)**;** **(4)** (Chen *et al.*, 2010)**;** **(5)** (Vazquez-Roig *et al.*, 2010)**;** **(6)** (Hoper *et al.*, 2002)**.**

1.4 Fate of pharmaceuticals in the soil environment

Several reviews have highlighted the current scientific knowledge on the fate of pharmaceutical compounds in the environment (Halling-Sørensen *et al.*, 1998; Heberer, 2002; Kummerer, 2004, 2001; Monteiro and Boxall, 2010; Thiele-Bruhn, 2003). The fate of pharmaceuticals in the terrestrial environment depends heavily on the physico-chemical properties of the pharmaceutical as well as the properties of the surrounding soil matrices.

The loss of pharmaceuticals from the soil can be broadly attributable to a number of processes, namely degradation, leaching and uptake by soil dwelling organisms. In general, the major processes that govern the fate of these compounds in the soil environment are sorption and degradation (Pignatello and Xing, 1996). These processes are important because they determine the overall persistence of pharmaceuticals which can therefore influence the potential for uptake of these compounds by terrestrial organisms.

1.4.1 Persistence

The chemical properties of human pharmaceuticals, such as high water solubility, high polarity and low volatility are largely responsible for their environmental persistence and thus their occurrence in terrestrial environments (Kummerer, 2008). Persistence in the environment is an inevitable result of the way most current medicines work. Pharmaceuticals need to be able to resist rapid metabolism in the body to ensure an adequate pharmacological effect (Richman and Castensson, 2008) and be stable enough to have a useful shelf life. Pharmaceuticals are developed with the intention of performing a biological effect; therefore they must be lipophillic to pass membranes whilst also being persistent to avoid the substance becoming inactivated (Halling-Sørensen *et al.*, 1998). It is these specific properties which mean

that pharmaceuticals which are present in the terrestrial environment could potentially bio-accumulate in organisms.

Persistence of a pharmaceutical in soil mostly depends on the photo-stability of the medicine, its binding and adsorption capability, its biodegradation rate and potential to leach into nearby water bodies (Diaz-Cruz *et al*., 2003). Studies have shown that pharmaceuticals can accumulate and persist in soil after application of sewage sludge or manure. For example, Hamscher *et al*. (2002) demonstrated that tetracyclines occur in soil which had been repeatedly fertilised with liquid manure. The persistence of each pharmaceutical is specific to the chemical and soil properties and thus persistence can vary across soil types. For example the persistence of the same antibiotic in the soil environment may range from less than one day to several months (Gavalchin and Katz, 1994).

1.4.2 Degradation

A number of studies have explored the degradation of human pharmaceuticals in the terrestrial environment (Monteiro and Boxall, 2009; Topp *et al*., 2008a, 2006; Xu *et al*., 2009a).

Xu *et al*., (2009a) established that the degradation of pharmaceuticals differs between soil types and the pharmaceutical compound in agreement to the findings by Monterio and Boxall (2009). Whilst ibuprofen had a half-life of 6.09 days in a silt clay soil it also had a considerably shorter half-life in loamy sand soil at 0.91 days. Only triclosan was observed to exhibit similar degradation patterns in all of the agricultural soils investigated. Regression data from Xu *et al*., (2009a) demonstrates that degradation rate constants were negatively correlated with soil clay content, with the exception of clofibric acid (r^2 between 0.42 – 0.56).

The degradation of organic chemicals can also be affected by organic carbon (OC) content of the soil, soil microbial activity as well as physico-chemical properties of the chemical (Monteiro, 2009; Topp *et al*., 2008b; Xu *et al*., 2009c). Specifically, a soil with a high organic matter content may inhibit organic compound (pharmaceutical) degradation due to increased adsorption of the chemical and thus reduced bioavailability (Johnson and Sims, 1993; Xu *et al*., 2009). Alvey and

Crowley (1995) have also postulated that soil organic matter may also serve as an alternative nutritional source for microorganisms involved in degradation.

The presence of sewage sludge can also affect the degradation of organic chemicals in soils. Sewage sludge alters the soil pH whilst also increasing the input of organic carbon (OC) (dissolved and solid) and microbial activity in the soil (Furczak and Joniec, 2007; Tsadilas *et al*., 1995). Specifically, pesticide degradation has been shown to be influenced by OC input and microbial activity (Kah *et al*., 2007). Monteiro and Boxall (2009) hypothesised that the presence of biosolids may result in the formation of bound residues between pharmaceuticals and the biosolid, thus decreasing its mobility and its potential for degradation. However conflicting results were observed in other studies where the degradation of pharmaceuticals increased with the introduction of biosolids (Topp *et al*., 2008a, 2006). In the 2006 paper by Topp *et al*., caffeine degradation was shown to be influenced by temperature, moisture, addition of liquid, the presence of caffeine degrading bacteria and soil type.

Whilst it is clear pharmaceuticals can be degraded in the soil environment, the continual input of these pharmaceuticals ensures that some chemicals are persistent and thus may present a threat to soil inhabiting organisms.

1.4.3 Sorption

The sorption of organic compounds such as pharmaceuticals is the partitioning of the chemical between the water and solid phase, i.e. soil (Schwarzenbach *et al*., 2003). Sorption can be characterised by a soil–water partition coefficient (K_d) which is defined as the ratio of the concentration in the solid phase to the concentration in the solution phase at equilibrium.

In terms of the environmental implications, the sorption behaviour of pharmaceuticals is critical because this regulates the transfer and distribution of compounds between phases which ultimately determines the mobility, bioavailability and availability of the compound for degradation (Thiele-Bruhn *et al*., 2003). It is the bioavailable fractions which have the potential for uptake into terrestrial organisms. A high K_d would suggest strong retardation in soils (Table 1.2; e.g. triclosan and

bisphenol A), whilst smaller K_d's (Table 1.2; e.g. ibuprofen) would infer that these pharmaceuticals could move downwards with percolating water, leaching possibly into groundwater or nearby streams thus posing less of a risk to terrestrial organisms (Chefetz *et al*., 2008; Oppel *et al*., 2004; Topp *et al*., 2008).

Physico-chemical properties specific to the pharmaceutical, including for example the octanol-water partition coefficient (K_{ow}), the degree of ionisation, the charge of molecule and its hydrophobicity are important in determining the adsorption of the chemical to soil (Diaz-Cruz *et al*., 2003). Specifically, K_{ow} indicates the tendency of a chemical to partition between the organic phase and the aqueous phase. Therefore compounds with a high K_{ow} are hydrophobic with low water solubility and should sorb, to a greater extent to the soil, thus generating larger K_d values than compounds with a low K_{ow}. For example triclosan has an extremely high K_d of 127 L/kg (Barron *et al*., 2009) and also a high log K_{ow} at 4.8 (Aranami and Readman, 2007).

This simple relationship between the K_d and K_{ow} does however not always hold as there are a number of different mechanisms which are fundamental to the way in which pharmaceuticals sorb to soils. Sorption can occur through the direct and induced ion-dipole and dipole-dipole interactions. These interactions arise from the reciprocal attraction of two permanent dipoles or an induced dipole and can be responsible for the sorption of polar and ionic compounds (Von Oepen *et al*., 1991). Other mechanisms of sorption can include chemisorption, hydrogen bonding, ion exchange (including cation exchange), cation bridging and formation of complexes with ions such as Ca^{2+}, Mg^{2+}, Fe^{2+} or Al^{3+}, (Diaz-Cruz and Barcelo, 2004; Tolls, 2001; Xia *et al*., 2005). For example the group of antibiotics, tetracyclines, have particularly low K_{ow}'s (e.g. Chlortetracyline log K_{ow} -0.62) yet bind strongly to soil because they form complexes with the metal ions present in the soil (Ca^{2+}/ Mg^{2+}) (Avisar *et al*., 2010; Bui and Choi, 2010).

Table 1.2 Reported sorption coefficients (K_d) in published literature for pharmaceuticals in various soil matrices, ± standard deviations where reported in literature.

Pharmaceutical	K_d soil (L/kg)	Soil type	Reference
Atenolol	15	Sandy mud	(1)
Benzafibrate	14	Sandy mud	(1)
Bisphenol A	17.00 ± 3.05	Loamy sand	(2)
	27.89 ± 3.93	Sandy loam	(2)
	42.22 ± 15.92	Silt loam	(2)
	15.93 ± 4.77	Silt clay	(2)
	20	Average of soils	(3)
Caffeine	25	Sandy mud	(1)
Carbamazepine	37 ± 1.6	High OC[a]	(4)
	0.49 ± 0.01	Low OC[a]	(4)
	13	Sandy mud	(1)
Chloramphenicol	42	Sandy mud	(1)
Cimetidine	11	Sandy mud	(1)
Ciprofloxacin	427	Centric Flurisol	(5)
Citalopram	250	Sandy mud	(1)
	42579	Loamy sand	(6)
	20691	Sandy loam	(6)
	17540	Loamy sand	(6)
Clofibric acid	9	Sandy mud	(1)
	5.36 ± 1.49	Loamy sand	(2)
	4.54 ± 1.68	Sandy loam	(2)
	3.36 ± 1.55	Silt loam	(2)
	200.73 ± 59.93	Silt clay	(2)
	5.38 ± 0.17	High OC[a]	(4)
Clotrimazole	1029	Sandy mud	(1)
Diazepam	30	Sandy mud	(1)
Diclofenac	164.5 ± 6.6	High OC[a]	(4)
	0.45 ± 0.03	Low OC[a]	(4)
	1.21 ± 0.36	Loamy sand	(2)
	3.47 ± 0.73	Sandy loam	(2)
	17.72 ± 7.45	Silt loam	(2)
	2.83 ± 1.05	Silt clay	(2)
Enrofloxacin	3037	Rhodic Ferralsol	(5)
	5612	Glegic Cambisol	(5)
	1230	Haplic Podsol	(5)
	260	Rendzic Leptosol	(5)
	496	Centric Flurisol	(5)

Pharmaceutical	K_d soil (L/kg)	Soil type	Reference
Erythromycin	68	Sandy mud	(1)
Flurbiprofen	11	Sandy mud	(1)
Fluoxetine	12546	Loamy sand	(6)
	2602	Sandy loam	(6)
	992	Loamy sand	(6)
Ibuprofen	0.56 ± 0.22	Loamy sand	(2)
	0.56 ± 0.17	Sandy loam	(2)
	3.71 ± 0.46	Silt loam	(2)
	1.24 ± 0.26	Silt clay	(2)
Indomethacin	32	Sandy mud	(1)
Ketoprofen	9	Sandy mud	(1)
Methadone	82	Sandy mud	(1)
Metoprolol	20	Sandy mud	(1)
Metronidazole	0.67±0.10 (mL/g)	Sandy loam	(7)
	0.54±0.02 (mL/g)	Sand soil	(7)
	0.62±0.02 (mL/g)	Sandy loam	(7)
	0.57±0.05 (mL/g)	Loamy sand	(7)
Naproxen	11	Sandy mud	(1)
	1.24 ± 0.31	Loamy sand	(2)
	1.65 ± 0.52	Sandy loam	(2)
	16.49 ± 5.17	Silt loam	(2)
	6.99 ± 2.33	Silt clay	(2)
Ofloxacin	309	Centric Flurisol	(5)
	3554 ± 194	High OC[a]	(4)
	1192 ± 122	Low OC[a]	(4)
Oxytetracycline	680 ± 69 (mL/g)	Sandy loam	(7)
	670 ± 149 (mL/g)	Sand soil	(7)
	1026 ± 374 (mL/g)	Sandy loam	(7)
	417 ± 97 (mL/g)	Loamy sand	(7)
Paracetamol	32	Sandy mud	(1)
Paroxetine	6386	Loamy sand	(6)
	886	Sandy loam	(6)
	355	Loamy sand	(6)
Propranolol	199 ± 9.6	High OC[a]	(4)
	16.3	Low OC[a]	(4)
Salbutamol	26	Sandy mud	(1)
Salicyclic acid	82	Sandy mud	(1)

Pharmaceutical	K_d soil (L/kg)	Soil type	Reference
Sertraline	787	Loamy sand	(6)
	270	Sandy loam	(6)
	149	Loamy sand	(6)
Sulfadiazine	2	Silt loam	(8)
Sulfamethazine	9	Sandy mud	(1)
Sulfamethoxazole	8	Sandy mud	(1)
	37.6 ± 1.2	High OC[a]	(4)
	0.23 ± 0.08	Low OC[a]	(4)
Tamoxifen	1626	Sandy mud	(1)
Triclocarban	438	Sandy mud	(1)
Triclosan	127	Sandy mud	(1)
	9.72 ± 3.82	Loamy sand	(2)
Triclosan contd.	132.83 ± 30.01	Sandy loam	(2)
	273.22 ± 78.43	Silt loam	(2)
	51.67 ± 24.37	Silt clay	(2)
Trimethoprim	26	Sandy mud	(1)

[a] Organic carbon is described as OC

References: (1) (Barron *et al*., 2009)**; (2)** (Xu *et al*., 2009b)**; (3)** (Ying and Kookana, 2005)**; (4)** (Drillia *et al*., 2005)**; (5)** (Nowara *et al*., 1997)**; (6)** (Kwon and Armbrust, 2008)**; (7)** (Rabolle and Spliid, 2000)**; (8)** (Thiele-Bruhn and Aust, 2004).

Soil components are important in the sorption of organic compounds, (Kah and Brown, 2006) as well as soil pore water distribution (Tsai *et al*., 2006) and soil particle size (Casey *et al*., 2003). For example clofibric acid in a silt clay soil has a K_d of 200.73 ± 59.93 (L/kg) which is larger than the K_d of 3.36 ± 1.55 (L/kg) obtained for the same compound in a silt loam soil (Xu *et al*., 2009) (Table 1.1). Therefore in addition to pharmaceutical physico-chemical properties, the extent to which a pharmaceutical sorbs to soils is determined by soil parameters such as the cation exchange capacity (CEC), solution chemistry (pH) and type of mineral and organic sorbents, (Boxall *et al*., 2002; Drillia *et al*., 2005; Tolls, 2001; Williams *et al*., 2006). Key soil parameters affecting sorption will be discussed further in sections 1.4.3.1 - 1.4.3.4.

1.4.3.1 pH

Ionisable chemcials posess either weak acidic or basic functional groups and their behaviour depends on paramters such as surrounding medium pH and chemical pKa, where pKa is the negative log of the acid dissociation constant (pH at which 50 % of the chemical is dissociated). As a result of their potential to protonate or deprotonate they will either be positively or negatively charged molecules. The pH of the soil is therefore important in determining the sorption of pharmaceuticals because most pharmaceuticals are ionisable.

For example, the sorption of acidic pharmaceuticals (e.g. naproxen) is pH dependant and it follows such that at typical soil pH most of these compounds are in their anionic state hence why their sorption to soils is particularly low. Conversely, at low pH, strong adsorption of basic chemicals is observed because the neutral species are present in high amounts (Monteiro, 2009). The pH of the soil solution has also been shown to affect the sorption of triclosan. An increase in pH (4 – 8) caused a decrease in the sorption of triclosan (Wu *et al*., 2009). Increasing pH decreased the amount of triclosan existing in the neutral from 100 to 39 % and the anionic form of triclosan was less attracted to the negative soil particles. Additional studies have also found that the sorption of sulphonamides is affected by pH (Boxall *et al*., 2002; Thiele-Bruhn *et al*., 2004).

1.4.3.2 Soil organic matter

Soil organic matter (SOM) consists of varying proportions of raw plant residues and microorganisms, an active organic fraction and humus (stable organic matter) (Lickacz and Penny, 2001). The concentration and type of SOM is a very important factor in determining sorption of a pharmaceutical compound and thus its fate in the terrestrial environment. A positive correlation between SOM and sorption of pharmaceuticals to soil has been found on several occasions (Chefetz *et al*., 2008; Williams *et al*., 2006; Xu *et al*., 2009a). When a substance is neutral and in its un-dissociated form, for example carbamazepine, it is quite likely to interact with SOM and it follows that with higher SOM there will be higher pharmaceutical retardation (Williams *et al*., 2006; Chefetz *et al*., 2008). However, if carbamazepine was in a poor SOM environment it would be significantly less retained by the soil. For neutral

organic compounds soil organic carbon has been postulated as the most important property to describe sorption in soils (Monteiro, 2009).

In comparison, the retardation of a charged compound, for example naproxen (negatively charged because the carboxyl functional group is deprotonated), will be less influenced by SOM and thus the compound will be highly mobile (Chefetz *et al.*, 2008). However, there are exceptions, diclofenac is a negatively charged pharmaceutical but it is slightly retarded in soils (Scheytt *et al.*, 2006). This can be attributable it's more hydrophobic nature meaning that it interacts more with SOM.

1.4.3.3 Clay content

The adsorption of some pharmaceuticals has been previously shown to be dependent on the clay content of the soil (Chen *et al.*, 2006; Rai *et al.*, 2000; Zheng and Cooper, 1996). Wu *et al.*, (2009) postulated that high clay content might hinder the interaction between the chemical and SOM. In one particular soil, characterised by high clay content, the average K_d for triclosan was 178 L/kg whilst the remaining three soils, with lower clay contents, all had K_d values over 200 L/kg. Contrastingly, the clay content of soils has been shown to be positively correlated with the sorption of clofibric acid (Wu *et al.*, 2009). This can be explained by the low pKa of clofibric acid (as previously discussed) which means that there are more hydrogen ions available for interaction with the negative clay particles.

1.4.3.4 Dissolved organic matter

Treated wastewater contains high levels of dissolved organic matter (DOM) (Fine *et al.*, 2002). When this wastewater is used to irrigate fields then correspondingly the DOM will be transferred to the soil compartment. If the pharmaceutical compound forms a complex with DOM or the organic and inorganic suspended materials present in treated wastewater, this can enhance the mobility of pharmaceutical compounds in soils as DOM behaves as a water-soluble carrier. Interactions between DOM and pharmaceuticals have, in the past, been also shown to affect transport and sorption and thus mobility of pharmaceuticals (Chefetz *et al.*, 2008; Nelson *et al.*, 1998; Totsche and Kogel-Knabner, 2004).

The presence of DOM can reduce the sorption of pharmaceuticals by competing for sorption sites in the soil (Celis *et al.*, 1998; Flores-Cespedes *et al.*, 2006, 2002; Nelson *et al.*, 2000). The presence of DOM can also decrease the mobility of chemicals due to cumulative sorption and co-sorption where two chemicals with different preferences on surface sorbent both interact with DOM (Flores-Cespedes *et al.*, 2006; Chefetz *et al.*, 2008).

1.5 Uptake of pharmaceuticals in the terrestrial environment

The bioavailable fraction of the pharmaceutical is the portion of pharmaceutical that is available for uptake, specifically in the terrestrial environment this would be uptake by soil dwelling organisms such as plants and invertebrates. Bioavailability is influenced by the fate of pharmaceuticals in soils, for example by sorption and mobility. If a chemical is degraded this can also influence its bioavailability (Jjemba, 2006); a more persistent pharmaceutical will remain in the environment for longer, and therefore have the potential to accumulate to higher concentrations and thus have a greater potential to be taken up by an organism. As discussed in section 1.4 the fate of pharmaceuticals in the terrestrial environment is extremely complex and depends on numerous soil properties; and thus it follows that the bioavailability and uptake of pharmaceuticals in the soil environment is also multifaceted.

Previous research has postulated that chemicals have to be in a dissolved state to be bioavailable to earthworms (Belfroid *et al.*, 1993; Oste *et al.*, 2001; Peijnenburg *et al.*, 1999; Saxe *et al.*, 2001; Vijver *et al.*, 2003) and additional studies has concluded that plant uptake of chemicals from soil are mediated by uptake from soil pore water during transpiration (Schrèoder and Collins, 2011). Therefore how the chemical behaves in the soil environment is important in determining the ultimate fate and uptake into terrestrial species.

1.5.1 Previous research on the uptake of pharmaceuticals in terrestrial organisms

Studies have explored the uptake of pharmaceuticals into plants from hydroponic culture mediums (Herklotz *et al.*, 2010; Kong *et al.*, 2007; Redshaw *et al.*, 2008b;

Shenker *et al*., 2011) as well as from soils (Boxall *et al*., 2006; Dolliver *et al*., 2007; Holling *et al*., 2012; Shenker *et al*., 2011; Wu *et al*., 2012, 2010). A number of pharmaceuticals with different physico-chemical properties and a range of therapeutic uses are taken up by a variety of crop species including soybean, carrot, lettuce and potato. Studies have also revealed variations in plant uptake between different species exposed to pharmaceuticals (Boxall *et al*., 2006; Herklotz *et al*., 2010; Wu *et al*., 2012). Recent studies have also investigated uptake into plants with the addition of sewage sludge to test systems and uptake following the application of reclaimed waste water effluent to soils to simulate realistic environmental exposures (Holling *et al*., 2012; Shenker *et al*., 2011; Wu *et al*., 2012, 2010). Results indicate that plant uptake is higher in the biosolid amended soils, probably a result of higher exposure concentrations, however, pharmaceuticals introduced by irrigation water appear to be more available for translocation (Wu *et al*., 2010).

Very little research has demonstrated the uptake of pharmaceuticals into terrestrial invertebrates. One published study assessed the bioaccumulation of anthropogenic waste indicators (including the pharmaceuticals: trimethoprim, caffeine, carbamazepine, thiabendazole and diphenhydramine) into earthworms from agricultural soil amended with biosolid or swine manure (Kinney *et al*., 2008). In this study, trimethoprim was the only pharmaceutical detected in the earthworms at concentrations of 127 $\mu g\ kg^{-1}$ (dw) in a biosolid amended field and 61 $\mu g\ kg^{-1}$ (dw) in the manure amended field. More recently, Kinney *et al*., (2012) continued their research with earthworms and investigated the effect of biosolids containing pharmaceuticals on earthworm reproduction and survival. Observations included biosolid toxicity to earthworm increased with biosolid aging and both survival and reproduction were sensitive at agronomic rates.

1.6 Aims and objectives of research

It is crucial that we understand how pharmaceutical physico-chemical properties, species traits and the distribution of pharmaceuticals between the soil and pore water and relate to the degree of uptake and depuration (including how metabolism can affect uptake) in the terrestrial environment (Figure 1.3).

The main aim of the PhD research was therefore to explore the factors and processes which affect the uptake of pharmaceuticals in the terrestrial environment. The specific objectives were to:

1) Explore the effects of chemical properties (e.g. pKa, log K_{ow}) on the uptake and depuration behaviour of pharmaceuticals in earthworms, *Eisenia fetida* (Chapter 2)
2) Explore the use of a minimised design approach to estimate bioconcentration factors in terrestrial and aquatic invertebrates, using reduced sampling points and kinetic definitions (Chapter 3)
3) Investigate the effects of soil properties on the fate of pharmaceuticals in the soil and pore water and the subsequent uptake and depuration of pharmaceuticals in earthworms, *Eisenia fetida* (Chapter 4).
4) Compare the uptake and depuration of pharmaceuticals between *Eisenia fetida* and *Lumbricus terrestris* earthworm species in order to establish the importance of species traits (Chapter 5).
5) Determine the impacts physico-chemical properties and plant type on the uptake of pharmaceuticals into plant species (Chapter 6).

Data from such investigations presented in the following chapters will be useful for risk assessment purposes as it will allow for the accurate prediction of uptake of chemicals into terrestrial species and mean that risks can be better characterised.

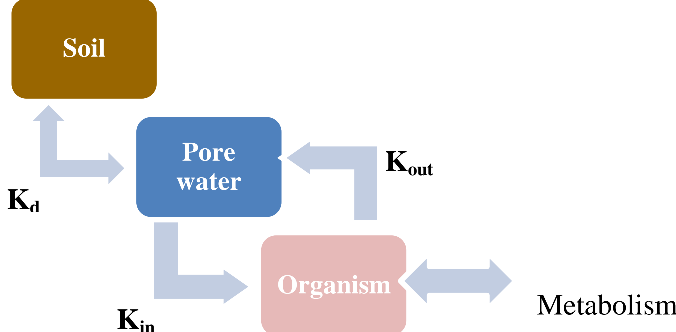


Figure 1.3 A conceptual model of uptake into terrestrial organisms from pore water exposure, where K_{in} and K_{out} are the uptake and depuration rates respectively.

1.7 Study compounds

The study compounds were chosen specifically to provide a range of pharmaceutical physico-chemical properties (log K_{ow}, pKa) and different therapeutic uses. Chemicals used in the earthworm uptake and depuration experiments (Chapter 2, Chapter 4 and Chapter 5) were ^{14}C radiolabelled (carbamazepine, diclofenac, fluoxetine and orlistat) (Table 1.4). Radiolabelled pharmaceuticals were used to allow for lower limits of detection and thus environmentally relevant concentrations could be used in the experiments. Additional studies, using cold compounds, were performed to determine any potential metabolism in the earthworm studies. Plant studies were carried out using unlabelled pharmaceuticals (carbamazepine, diclofenac, fluoxetine, propranolol, sulfamethazine and triclosan) (Chapter 6).

Carbamazepine is in a class of medications called anticonvulsants. It can be used to control certain types of epileptic seizures in patients, as well as in the treatment of trigeminal neuralgia (a condition that causes facial nerve pain) (Dale and Rang, 2011). Carbamazepine is the most widely used anti-epileptic drug (Dale and Rang, 2011). In England, 2256 x 10^3 carbamazepine prescription items were dispensed in 2000 resulting in an annual use of over 40 thousand kg of this drug (Jones *et al.*, 2002). Due to its wide use and thus potential to transfer to the environment, carbamazepine has been detected in numerous soil profiles throughout the world, in concentrations ranging up to 6.69 ± 1.80 μg/kg (Dalkmann *et al.*, 2012; Durán-Alvarez *et al.*, 2009; Vazquez-Roig *et al.*, 2012, 2010). With regards to the terrestrial environment, studies have explored the uptake of carbamazepine into cucumber and soybean plants (Shenker *et al.*, 2011; Wu *et al.*, 2010). Carbamazepine however has remained undetected in research into the bioaccumulation of anthropogenic waste indicators in earthworms after it was detected in soils receiving biosolid amendment (Kinney *et al.*, 2012).

Diclofenac is a non-steroidal anti-inflammatory (NSAID) taken to ease pain and reduce inflammation in patients suffering with, amongst other problems, rheumatoid arthritis, migraines and musculoskeletal injuries and pain for example (Dale and Rang, 2011). NSAIDs are drugs that suppress prostanoid synthesis in the inflammatory cells by the inhibition of the cyclo-oxygenase (COX)-2 isoform of the

arachidonic acid COX enzyme and in doing so provides three major therapeutic actions; anti-inflammatory action, analgesic effects and antipyretic effects (Dale and Rang, 2011). Diclofenac is a widely used pharmaceutical and statistics in 2000 indicated that 26 121 kg was consumed in England on an annual basis (Jones *et al.*, 2002). Diclofenac has been detected in soils in the Mezquital Valley, Mexico at low concentrations (0.4 µg/kg) after irrigation using wastewater (Dalkmann *et al.*, 2012). However research on the uptake of this pharmaceutical is limited; one recent study was unable to detect diclofenac in either wheat or soybean crops after 110 days grown in soil treated with sewage sludge containing NSAIDs. In terms of the wider environmental impacts of diclofenac, experimental evidence indicates that the casual factor in the decline of vulture populations in the Indian sub-continent is from these birds scavenging on cattle carcasses containing high levels of diclofenac (Green *et al.*, 2007, 2006; Oaks *et al.*, 2004). Therefore further research is needed to explore the uptake and effects of this pharmaceutical in the environment.

Fluoxetine (also known by the trade name Prozac) is used in the treatment of depression. It is a selective serotonin (5-HT) reuptake inhibitor (SSRI). Statistics from Spain indicate that it is a highly used drug as over three thousand kilograms of fluoxetine were consumed in 2003(Carballa et al., 2008). Fluoxetine has been detected in soil samples across the U.S. which were previously irrigated with reclaimed wastewater irrigation (Kinney *et al.*, 2006). The uptake of fluoxetine has been comprehensively explored in the aquatic environment (Brooks *et al.*, 2005; Meredith-Williams *et al.*, 2012) and the compound has been demonstrated to be taken up by plants from a hydroponic culture medium (Redshaw *et al.*, 2008b) as well as from soil (Wu *et al.*, 2010).

Propranolol belongs to a group of pharmaceuticals called beta blockers that primarily work on the heart and blood vessels. It can be used to treat a number of symptoms such as high blood pressure, irregular heartbeats, angina and anxiety. Propranolol is a non-selective β antagonist; it targets the beta receptor which can usually be found on cells of heart muscles, arteries and other tissues of the sympathetic nervous system. When stimulated by adrenaline (epinephrine) these receptors can lead to stress responses. Beta blockers interfere with the binding between epinephrine and other stress hormones to the receptor and thus weaken the effects of stress hormones

(Dale and Rang, 2011). Even though propranolol has been detected in soils at concentrations < 0.4 µg/kg (Vazquez-Roig *et al*., 2012) very little research has investigated the uptake of this pharmaceutical into terrestrial species. In a large scale experiment looking at the potential uptake of 118 pharmaceuticals into four crop species after biosolid amendment of soil resulted in no detection of propranolol residues in any of the plant material (Sabourin *et al*., 2012).

Sulfamethazine has antibacterial properties and comes from a well-known group of pharmaceuticals called sulfonamides. Recently, sulfamethazine is more commonly used for veterinary purposes however in the past it has been prescribed to treat a variety of bacterial diseases in humans. This has resulted in the detection of sulfamethazine in concentrations up to 11 µg/kg (Hoper *et al*., 2002) in soils. Some of the first plant uptake research with regards to pharmaceutical exposure demonstrated that sulfamethazine could be taken up into plant material and accumulate to concentrations in the range of 0.1 % of the amount applied to the soil in manure (Dolliver *et al*., 2007).

Triclosan is a synthetic anti-microbial agent that is commonly used in consumer products such as soaps, deodorants and toothpastes. It should be noted that triclosan is not strictly a pharmaceutical and is more often referred to as a personal care product. In a U.S. Geological Survey study of 95 different organic wastewater contaminants in U.S. streams, triclosan was one of the most frequently detected compounds, with some of the highest concentrations (Kolpin *et al*., 2002). Since its detection in soil (Chen *et al*., 2010; Durán-Alvarez *et al*., 2009) work has been undertaken to explore the uptake of triclosan into various plant species (Karnjanapiboonwong *et al*., 2011; Wu *et al*., 2010) with bioconcentration factors up to 12 reported in plant roots.

Orlistat is the only drug currently licensed in the UK for the treatment of obesity. (2010) (Dale and Rang, 2011). It works in interfering with the way that fat is digested and absorbed in the body. Specifically, the presence of orlistat in the intestine prevents the breakdown of dietary fat to fatty acids and glycerols by reacting with the serine residues at the active sites of gastric and pancreatic lipases and in doing so irreversibly inhibiting these enzymes (Dale and Rang, 2011). Whilst it has been available on the market as a prescription drug since 2001, under the sale

name of Xenical, it has been difficult to obtain usage and consumption statistics on this particular pharmaceutical. Orlistat has yet to be detected in soils. However its high log K_{ow} suggests a large affinity for sorption to soil and potential bioaccumulation into terrestrial species if it makes its way into the soil environment and thus further research on this pharmaceutical is important.

Table 1.3 Physico-chemical properties for selected study compounds

Test chemical	Therapeutic use	Chemical formula	Molecular weight (g/mol)	pK_a [a]	Log K_{ow} [b]	Soil-water distribution coefficient (K_d) (L/kg) [c]	Structure
Carbamazepine	Anticonvulsant	$C_{15}H_{12}N_2O$	236.27	N/A	2.25	4.83	
Diclofenac	Anti-inflammatory	$C_{14}H_{11}Cl_2NO_2$	318.13	4.12	4.02	28.65	
Fluoxetine	Anti-depressant	$C_{17}H_{18}F_3NO$	345.79	9.53	4.65	608.42	
Propranolol	Beta-blocker	$C_{16}H_{21}NO_2$	259.34	8.99	3.5	-	

Table 1.3 Continued

Test chemical	Therapeutic use	Chemical formula	Molecular weight (g/mol)	pK_a [a]	Log K_{ow} [b]	Soil-water distribution coefficient (K_d) (L/kg) [c]	Structure
Sulfamethazine	Antibacterial	$C_{12}H_{14}N_4O_2S$	278.32	6.0,1.55	0.9	-	
Triclosan	Antimicrobial	$C_{12}H_7Cl_3O_2$	289.54	8.1	4.8	-	
Orlistat	Weight loss aid	$C_{29}H_{53}NO_5$	497.74	N/A	8.19	1493.98	

[a] pKa values were predicted using the University of Georgia SPARC database v. 4.2 (http://ibmlc2.chem.uga.edu/sparc) Accessed: 25/05/2012
[b] Log K_{ow} values obtained from KOWWIN v. 1.68 database, USEPA EPI suite 4.1 programme
[c] Soil water distribution coefficients (K_d) for selected pharmaceuticals were determined experimentally following OECD 106 for study soil 280 used in earthworm uptake studies in Chapter 2 (2.3.1) (unpublished data).

Table 1.4 Radiolabelled chemical properties of selected study compounds

Test chemical name	Specific Activity (GBq/mmol)	Structure (including position of radiolabel)
Carbamazepine [carbonyl-^{14}C]	0.74	
Diclofenac sodium, [phenylacetic acid ring – ^{14}C (U)]-	2.294	
Fluoxetine [N-methyl-^{14}C] hydrochloride	2.035	
Orlistat [tridecanyl-2-^{14}C]	2.051	

1.8 Study species

1.8.1 Earthworms

Earthworms can be split into three categories; endogeic, anecic and epigeic species (Edwards, 1996). The descriptions are largely based on their habitats in the soil. Endogeic species rarely come to the surface of the soil preferring to reside in a series of complex lateral burrow systems through the soil layers. Meanwhile anecic worms, such as *Lumbircus terrestris*, also form deep burrows however these tend to be vertical from the soil surface and go deep into the mineral soil layers. In comparison, to endogeic species which eat soil, anecic species feed primarily on decaying surface litter and therefore come to the soil surface more regularly. Worms which are typically used in vermicomposting, including *E. fetida*, tend to be epigeic species. Epigeic worms inhabit the top soil layers or can be found on the soil surface residing in loose organic litter and debris (Bouche, 1992; Edwards, 2004; Edwards, 1996).

Earthworms can comprise of 60 – 90 % of total soil biomass in a selection of locations (Bouche, 1992; Lee, 1985). Earthworms are key organisms in the terrestrial environment; their presence is central to a healthy and sustainable soil environment, for example earthworms help to establish and maintain the structure and fertility of the soil (Edwards, 2004; Killham, 1994). The physical motion of earthworm burrowing can bury plants deep in the soil which is crucial for the recycling of nutrients whilst the structure of the burrows is important in draining and aerating the soil (Edwards and Bohlen, 1992; Edwards and Lofty, 1972; Edwards, 2004). Earthworms being at the base of a food chain hold an integral position. Uptake and accumulation of contaminants into earthworms not only poses a risk to the earthworm directly, but may also result in far wider reaching ecosystem effects due to bioaccumulation and contaminant transfer through the food chain to top predators such as birds where there is the potential for secondary poisoning (Romijn et al., 1994; Spurgeon and Hopkin, 1996).

Earthworms are known to bio-magnify inorganic and organic soil contaminants, including polycyclic aromatic hydrocarbons (PAHs), brominated flame retardants, pesticides and metals (Giovanetti *et al*., 2010; Grumiaux *et al*., 2010; Harris *et al*.,

2000; Heikens *et al*., 2001; Hinton and Veiga, 2008; Jager *et al*., 2005; Janssen *et al*., 1997; Ma *et al*., 1998, 1995; Matscheko *et al*., 2002; Qi and Chen, 2010; Sellstrom *et al*., 2005; Van Gestel and Ma, 1988). Earthworms take up contaminants in a number of ways: through living in the soil environment, earthworms are in direct contact with the soil and therefore the ingestion of soil particles may lead to chemicals passing across the gut wall and into the earthworm tissue. Uptake via diffusion across the earthworm skin from chemicals in the pore water is also possible.

1.8.1.1 Earthworm biological properties

The uptake and accumulation of chemicals in the soil environment can vary between species as a result of species specific traits, a number of studies have highlighted these differences. Work by Kelsey *et al*., (1997) demonstrated the bioavailability of atrazine and phenanthrene to bacteria was far greater than that to earthworms. In an early study; the earthworm *Lumbricus rubellus* accumulated more calcium and zinc but less lead and cadmium than the earthworm *Dendrobaena rubida* living in the same soil environment (Morgan and Morris, 1982). Further work regarding earthworms established that *E. fetida*, had BCFs that were approximately ten-fold higher than those for the other species in the study including the anecic species *Lumbricus terrestris*, and the endogeic species *Aporrectodea caliginosa* after exposure to 2,2-bis (p-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) (Kelsey and White, 2005). Differences in their processing of soil organic matter, ecological strategy, and lipid content are potential explanations as to the observed variation among earthworm BCFs (Kelsey *et al*., 2005).

1.8.1.2 Biotransformation

As well bioaccumulation of chemicals in earthworms, biotransformation of chemicals can also occur. Biotransformation is the chemical modification made by an organism on a chemical compound. Biotransformation together with bioaccumulation are key toxicokinetic processes that can modify the toxicity of chemicals and sensitivity of organisms (Ashauer *et al.,* 2012). Metabolism of chemicals, such as pharmaceuticals, is an example of biotransformation. The

metabolites may reach higher concentrations in the organism that that of the parent compounds.

The biotransformation of soil contaminants, such as polychlorobiphenyls, polycyclic aromatic hydrocarbons, pesticides and metals in a number of earthworm species has been extensively studied (Button *et al.,* 2009; Lydy and Linck, 2003; Belfroid *et al.,* 1995; Stroomberg *et al.,* 2004). Pharmaceuticals can remain unchanged during urinary excretion and leave the human body as parent compounds (Bound and Voulvoulis, 2005). However, it is well known that pharmaceuticals can undergo metabolism in the human body, including the study compounds selected in this thesis (Pearson and Wiehkers, 2008). For example, fluoxetine is extensively metabolized in the liver. The only identified active metabolite, norfluoxetine, is formed by demethylation of fluoxetine (Dale and Rang, 2011). Biotransformaton of diclofenac has also been demonstrated after uptake into fish (Lahti *et al.,* 2011). There is therefore the potential for pharmaceuticals to undergo biotransformation after being taken up by earthworms.

Understanding the uptake of chemicals into earthworms is a prerequisite to understanding the risks chemicals pose to earthworm populations, as well as the potential effects of secondary poisoning to predators like birds. Earthworms are at the base of many food chains and thus if chemicals are taken up into the earthworms they can facilitate the movement of chemicals into the food web via bioaccumulation and bio-magnification processes. The two earthworm study species are discussed in detail below.

1.8.1.3 Eisenia fetida

The typical species of earthworm outlined in standard test guidelines and used for terrestrial bioaccumulation and toxicity testing is *Eisenia fetida* (International Standard Organisation, 1998, Organisation for Economic Cooperation and

Development, (OECD) guidelines 207, 222, 317 (OECD, 2010, 2004, 1984). This primarily reflects its ease of laboratory culture as it is a robust species, has a fast reproduction time (8 weeks) and a shorter generation time than many other earthworm species.

E. fetida belongs to the Lumbricidae family of earthworms, characterised by their segmented body. There are typically 80 -120 segments and each *E. fetida* is approximately 60 – 120 mm in length (Sims and Gerard, 1985). *E. fetida* are known under various common names including redworm, red wiggler and brandling worm. Interestingly the name 'fetida' originates from the Latin word meaning 'fetid' which refers to a foul smelling odour. When they are roughly handled or in fear of predation *E. fetida* release a pungent liquid from their body. It is the release of this coelomic fluid from which the name originates.

E. fetida is a particularly robust species of earthworm, they are found in a number of soil environments throughout the world. It is therefore a suitable species to work with in laboratory experiments as they can tolerate a wide range of environmental conditions such as soil pH, moisture, temperature and soil types (Edwards, 1996). For example Edwards (1988) reported that *E. fetida* can tolerate a pH range from 4.0 -7.0. *E. fetida* typically thrive in organic rich habitats such as rotting vegetation, compost and manure piles.

1.8.1.4 Lumbricus terrestris

Similar to *E. fetida, Lumbricus terrestris* belongs to the Lumbricidae family of earthworms however it is a larger species. Compared to other earthworms in its genus, *L. terrestris* is the longest having approximately 140 - 155 segments and reaching a maximum length of 160 mm (Sims and Gerard, 1985). It is commonly known as the night crawler as this species tends to crawl to the soil surface through permanent constructed burrows during the night to feed.

Whilst *E. fetida* is a species of earthworm commonly used in terrestrial uptake and toxicity testing it has been suggested that *Lumbricus terrestris* is a more ecologically relevant and sensitive species (Dean-Ross, 1983). One of the key issues is that *E. fetida* is a manure compost worm and does not naturally inhabit the soil environment. Additionally some authors observed differences in the sensitivity of *Eisenia* species with *E. fetida* being less sensitive to contaminants than other earthworm species (Edwards and Bohlen, 1992; Edwards and Coulson, 1992; Langdon *et al*., 2005; Spurgeon and Weeks, 1998). Yet in a more recent study, after exposure to imidacloprid, *E. fetida* were more sensitive concerning cellular alterations and mortality than both *Aporrectodea caliginosa* and *L. terrestris* (Dittbrenner, 2012). *L. terrestris* which are commonly found in the soil environment may therefore be a more useful species in terms of the risk assessment of chemicals.

However *L. terrestris* worms have a long generation time, do not do well in high density cultures and require a stable burrow environment in order to thrive. Cultures must also allow sufficient burrowing depth for the worms, and this is difficult to achieve with burrow depths for *L. terrestris* reported to easily exceed 40 cm below the soil surface (Shipitalo and Butt, 1999). Without access to this burrow, anecic worms will encounter difficulties in both breeding and growing which are necessary for a successful standardised laboratory experiment.

In terms of the thesis, uptake studies began with *E. fetida* as optimised cultures were easier to obtain. Later research then explored, in a small number of studies, the uptake of pharmaceuticals into *L. terrestris.*

1.8.2 Plants

Plants were specifically chosen to provide enough biomass for extraction and analysis at time of harvest. Radish also provided the opportunity to differentiate between above and below ground crop concentrations by separating the leaf from the root material.

1.8.2.1 Radish (Raphanus sativus)

Radish (*Raphanus sativus*, Cherry belle variety) is an edible root vegetable of the Brassicaceae family. Specifically cherry belle is a bright red-skinned ('cherry' colour) round variety of radish with a firm white interior. Cherry belle germinate in 4 - 6 days and are fully grown in approximately 21 days where they typically reach a diameter of 2 cm and the leafy green tops can reach up to 15 cm. Previous research has predominantly explored the uptake of metals, polychlorinated biphenyls and pesticides in radish plants (Davies, 1992; Mikes *et al*., 2009; Zhou *et al*., 2005). Apart from research investigating the phytotoxicity to and uptake of the fluoroquinolone antibiotic, enrofloxacin in a variety of vegetable crops, including radish, the uptake of pharmaceuticals into radish species has been relatively unexplored.

1.8.2.2 Ryegrass (Lolium perenne)

Ryegrass (*Lolium perenne,* Guard variety) is a commonly used plant species in toxicity and uptake testing in the terrestrial environment (Gu *et al*., 2013; Li *et al*., 2002; Winker *et al*., 2010). It is a cool-season perennial grass native to Europe, temperate Asia, and North Africa and widely distributed throughout the world. Perennial ryegrass is important for forage and livestock farming in temperate regions of the world. It has a shallow root system which is highly branched and can be characterised by its fast growing and rapid establishment properties making it a high yielding species. Previous work with pharmaceuticals has demonstrated that carbamazepine can be taken up into ryegrass aerial plants and roots however ibuprofen remained undetected in any plant material (Winker *et al*., 2010).

Chapter 2 Uptake and Depuration of Pharmaceuticals in the Earthworm, *Eisenia fetida*

2.1 Introduction

A number of studies have explored the uptake of pharmaceuticals into aquatic invertebrates and fish (Brooks *et al*., 2005; Karlsson, 2013; Meredith-Williams *et al*., 2012; Paterson and Metcalfe, 2008; Schultz *et al*., 2010). However, much less work has been done to assess uptake of pharmaceuticals in the terrestrial environment; work that has been done has focused on the uptake of human and veterinary pharmaceuticals into plants (Boxall *et al*., 2006; Shenker *et al*., 2011; Winker *et al*., 2010; Wu *et al*., 2010) with very few studies looking at uptake into terrestrial invertebrates such as earthworms.

Earthworms are known to bio-magnify inorganic and organic soil contaminants, including polycyclic aromatic hydrocarbons (PAHs), brominated flame retardants, pesticides and metals (Giovanetti *et al*., 2010; Grumiaux *et al*., 2010; Heikens *et al*., 2001; Hinton and Veiga, 2008; Jager *et al*., 2005; Janssen *et al*., 1997; Ma *et al*., 1998, 1995; Matscheko *et al*., 2002; Sellstrom *et al*., 2005; Van Gestel and Ma, 1988). Through living in the soil environment, earthworms are in direct contact with the soil and soil pore water and therefore uptake of chemicals from these two media is possible. The ingestion of soil particles may also lead to chemicals passing across the gut wall and into the tissue.

Only one published study so far has explored the uptake of pharmaceuticals into earthworms (as previously discussed in section 1.5) (Kinney *et al*., 2008). Additional research is required to fully characterise the potential for pharmaceutical uptake into terrestrial invertebrates as this is something which we currently know little about. Pharmaceuticals are emerging contaminants and there have already been a number of notable effects on non-target organisms as a result of their presence in the environment (see section 1.1.1).

Earthworms are key organisms in the terrestrial environment (section 1.8.1). Earthworms, being at the base of a food chain, hold an integral position. Uptake and accumulation of contaminants into earthworms not only poses a risk to the

earthworm directly, but bioaccumulation and contaminant transfer through the food chain to top predators such as birds has the potential to cause secondary poisoning (Romijn et al., 1994; Spurgeon and Hopkin, 1996).

To assess the potential for chemicals to be taken up by earthworms, a number of quantitative structure-activity relationships (QSARs) allow for the prediction earthworm pore water based bioconcentration BCFs. QSARs can be used for initial screening purposes or to avoid lengthy experiments whilst still providing a measure of potential chemical uptake and thus wider environmental implications. Belfroid and colleagues (Belfroid *et al.*, 1993) were one of the first to develop a QSAR based on water exposure of chlorobenzenes and work by Jager (1998) followed, which is included in the Technical Guidance Document (TGD) on Risk Assessment Part 2. Both of these QSARs utilise log K_{ow} as the primary descriptor in BCF determination, mimicking the partitioning between the aqueous and lipid phases. The applicability of these relationships for pharmaceuticals is currently unknown.

The aim of this study therefore was to investigate the uptake and depuration of a range of pharmaceuticals, including carbamazepine, diclofenac, fluoxetine and orlistat (Table 1.3) into the earthworm *Eisenia fetida.* The results would then be used to evaluate existing predictive models, such as QSARs for estimating uptake of pharmaceuticals into earthworms.

2.2 Experimental materials

2.2.1 Pharmaceutical compounds and reagents

^{14}C labelled compounds were used in the uptake studies. Labelled fluoxetine [methyl-^{14}C] and carbamazepine [carbonyl-^{14}C] were obtained from American Radiolabelled Chemicals (*Missouri, USA*), diclofenac [U – ^{14}C] was obtained from Perkin Elmer (Boston, USA) and orlistat [tridecanyl-2-^{14}C] was provided by GlaxoSmithKline (GSK, UK). Unlabelled fluoxetine, carbamazepine and diclofenac were obtained from Sigma Aldrich (UK) and unlabelled orlistat was provided by GSK. Acetonitrile (99.9 %), methanol (99.9 %) and ethyl acetate (99.9 %) were obtained from Fisher Scientific (Loughborough, UK).

2.2.2 Test soil

The study soil was a clay loam soil obtained from LandLook (Midlands, U.K.). Prior to use in experimental studies, the soil was air dried for 48 hours then sieved to 2 mm to ensure homogeneity within the soil matrix. Detailed characteristics of the study soil are given in Table 2.1.

Table 2.1 Test soil characteristics provided ± standard deviation where provided (*n* = 6). († Analysis completed at INRA [Arras, France] *Analysis completed at 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 ± 0.15
Cation exchange capacity cmol +/kg †	10.3
Organic carbon (%) †	1.89
C/N †	11.2
Organic matter (%) †	3.27
Water holding capacity (%w/w) *	17.25 ± 2.52

2.2.3 Test organism

E. fetida were obtained from Blades Biological Ltd (Kent, UK) and cultured in a medium of peat and cow manure (50:50), kept moist with deionised water at room temperature (20 ± 3 ^{o}C). The animals were fed twice weekly with homogenised mashed potato powder which was added to the surface of the culture. The *E. fetida* were obtained from a single species culture and cultures were maintained for at least four generations before being used in the studies. The lipid content of *E. fetida,* determined using the method of Folch *et al*., (1957), was 5.11 ± 0.29 % (wet weight ± standard deviation).

2.3 Experimental methods

2.3.1 Sorption of study compounds to test soil

The sorption behaviour of the study pharmaceuticals 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 pharmaceuticals to reach equilibrium between the soil and liquid phase. The samples were then removed and centrifuged at 3500 rpm for 10 minutes (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 radioactivity remaining in solution was determined as per section 2.3.3.2. Soil sorption coefficients (K_d) values were then determined based on the amount of radioactive pharmaceutical applied and the amount remaining in the supernatant at equilibrium.

2.3.2 Toxicity of study compounds to *Eisenia fetida*

Toxicity experiments were performed to ensure that the test concentrations used in the uptake studies were not toxic to the *E. fetida.* Earthworms were individually exposed to soil containing ten times and a hundred times the proposed test concentration for the main uptake study. The experimental set up was similar to the main uptake and depuration studies as described in section 2.3.3. Burrowing behaviour, potential weight change and mortality were compared to that observed in solvent controls and blank controls to see if the pharmaceuticals had any effect on the earthworms.

2.3.3 Uptake and Depuration study

The uptake and depuration experiments followed OECD Guideline 317 'Bioaccumulation in Terrestrial Oligochaetes'. Tests were performed in glass jars containing 50 ± 1 g of test soil as this was an adequate amount to allow sufficient burrowing depth (approximately 4 -5 cm) for *E. fetida*. All exposures were performed in a growth chamber at 20 ± 2 ^{o}C, using a 16:8 light/dark cycle, and at 60 % humidity. Prior to exposure to test chemicals, worms were acclimated to the experimental conditions in the growth chamber for 48 hours using a non-treated test soil.

Exposure soils were prepared by adding the labelled pharmaceuticals to the soil using 100 – 200 µl of a carrier solvent to give concentrations of 39, 49, 80 and 65 µg kg^{-1} of carbamazepine, diclofenac, fluoxetine and orlistat respectively (wet weight). For carbamazepine and fluoxetine, ethanol was used as the carrier; for diclofenac, methanol was used and orlistat was applied in acetonitrile. After spiking, each test beaker was left for 2 hours and then mixed to allow for even distribution of the pharmaceutical within the soil sample. Following spiking and mixing, the carrier solvents were allowed to evaporate off for a period of 48 hours. For each study, blank study soils and test soils spiked with carrier solvent only were prepared as controls. Following preparation, the moisture content of the exposure and control soils was adjusted to between 40 – 60 % of the MWHC by addition of deionised water.

For each compound, 45 beakers of spiked soil were prepared along with solvent and non-solvent controls (Figure 2.1). At the start of the exposure one mature adult *E. fetida* (200 – 500 mg), with a visible *clitellum,* was added to each test beaker and the burrowing time of each of the worms was recorded. Beakers were then covered with garden fleece, attached with an elastic band to prevent earthworms from escaping while allowing sufficient air supply to be maintained. The uptake phase of the experiment lasted for 21 days with samples taken at 0 and 6 hour and 1, 3, 7, 10, 14, 21 day. *E. fetida* in the remaining beakers were then transferred to clean soil for a 21 day depuration phase with samples taken at 6 hour and 1, 3, 7, 10, 14, 21 day after transfer. Soil moisture content of the soil in each of the test beakers was monitored

throughout both phases, and adjusted, where necessary, by adding deionised water to ensure that it remained between 40 – 60 % of the MWHC. The pH of the soils was measured at the beginning and end of the uptake phase and at the end of the depuration phase. Worms were fed weekly with mashed potato powder (Norr and Riepert, 2007).

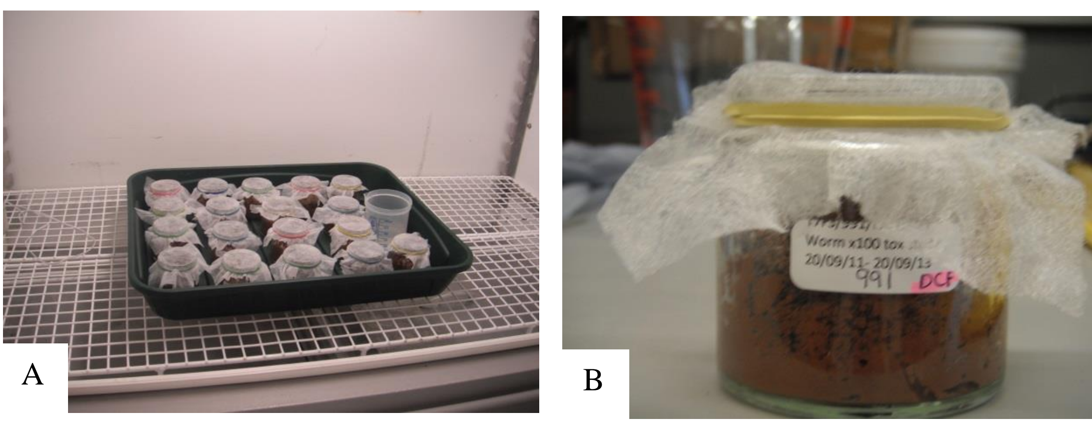


Figure 2.1 Test beakers for earthworm exposures in growth chamber (A) and single beaker containing earthworm before sampling (B).

At each sampling time point, three replicate beakers were taken of the pharmaceutical exposed worms. At the start of the uptake phase and end of both the uptake and depuration phases' four replicates were sampled from the solvent controls to obtain analytical background values. The earthworms were then removed, rinsed with deionised water, blot dried then weighed and placed on moist filter paper for 24 hours to allow the earthworm to void its gut contents (Dalby *et al*., 1996) (Figure 2.2). After 24 hours, earthworms were reweighed and then frozen (-20 ^{o}C) prior to analysis. A supplementary study indicated that maximum purging of gut contents occurred over 24 h with 77 % of the soil gut contents being eliminated so a correction had to be applied to the final worm concentration measurements (Appendix 1). Samples of soil were also taken for soil analysis and for immediate extraction of soil pore water.

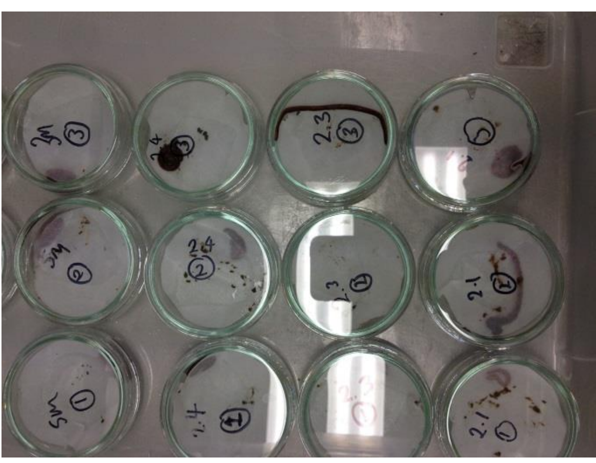


Figure 2.2 Earthworms purging their guts on moist filter paper after sampling.

Three replicates of soil spiked with radiolabelled pharmaceutical but containing no earthworm were also prepared and sampled at the end of the uptake phase to check for changes in concentrations of the pharmaceuticals in soil and pore water in the absence of the test organism.

2.3.3.1 Preparation of samples for analysis

To extract pore water, test 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 Eppendorf tube. The Eppendorf tubes containing the sampled pore water were then further centrifuged at 12000 rcf for four minutes to sediment any loose particles. A 500 µL sample of pore water was the-n 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 minutes 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 minutes with 3 x 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 in the test soils at the start of the experiment was significantly lower than what was expected. Combustion analysis of these soil samples was performed to determine i-f there was any radioactivity remaining in the soil which may account for the discrepancies. A large amount of dissipation of orlistat from the test beakers was seen 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 then 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 orlistat soils was also performed to check this. Combustion analysis was performed on a Perkin Elmer 307 Sample Oxidiser. After solvent extraction to determine the total extractable residues, the dried soils were homogenised into a fine powder. Each soil sample was prepared in triplicate in combusto-cones where 300 ± 25 mg of soil was mixed with equal amounts of cellulose powder. After combustion consisting of a 1.5 minute burn per sample, the ^{14}C carbon dioxide was trapped by a vapour phase reaction with CarboSorb E forming carbamate which was mixed with PermaFluor E + a scintialltion cocktail ready for counting the radioactivity present on the Liquid Scintillation Counter (LSC). Regular spec-checks were performed throughout the analysis to ensure the recovery of the samples remained above 95 %.

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

Method validation studies showed that average recoveries ranged from 82.8 (diclofenac) to 100.6 (carbamazepine) % for the soil methods and from 86.3 (fluoxetine) to 100.9 (carbamazepine and diclofenac) % for the earthworm extraction methods.

2.3.3.2 Liquid scintillation counting

Radioactivity in soil pore water and soil and worm extracts were determined using Liquid Scintillation Counting (LSC) using a Beckman LS 6500 LSC counter (Beckman Coulter Inc., Fullerton, USA). Samples were counted three times for 5 min. Counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method. Measured radioactivity of the pharmaceuticals in the earthworm extracts were corrected to account for soil-associated pharmaceuticals present in the gut.

2.3.4 Potential metabolism in *Eisenia fetida*

To ascertain whether the radioactivity measured in the earthworm samples was that of the parent compound or metabolite/transformation products additional studies were performed using cold compounds. Studies were performed at 20 times the soil concentration in the original studies to ensure that compounds were able to be detected in the worm matrices. Due to analytical limitations, studies to ascertain whether any metabolism had occurred in the earthworms were unable to be performed with orlistat.

E. fetida were exposed to unlabelled carbamazepine, diclofenac and fluoxetine for 21 days (six replicates per compound) under similar conditions to the main uptake studies (section 2.3.3), after which they were allowed to purge their guts for 24 hours and subsequently frozen (-20°C) ready for analysis. *E. fetida* were then injected with a stable isotope (carbamazepine d-10, diclofenac d-4 and fluoxetine d-5) and extracted using methods previously outlined in this study (section 2.3.3.1). The supernatant from these extractions was taken to dryness under a nitrogen stream and reconstituted in 200 µL of methanol:water (50:50 v:v). This was further centrifuged at 12000 RPM to sediment any loose particles. Resulting extracts were transferred to HPLC vials for analysis. Calibration (six concentrations, three replicates) and quality control (Q.C.) samples (three concentrations, six replicates at intermediary concentrations between the calibration range) were also prepared in worm matrix for each of the respective compounds (Appendix 4 and Appendix 5).

2.3.4.1 LC-MS/MS analysis

Extracts were analysed for the pharmaceuticals by LC-MS/MS using a Dionex Ultimate 3000 and Applied Biosystems API 3000. HPLC separation was performed with a Symmetry C18 3.5 µm, 4.6x75 mm column and Symmetry C18 3.5 mm, 2.1x10mm guard column (Waters) with a mobile phase flow rate of 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 over 5 min for carbamazepine and fluoxetine and 7.5 min for diclofenac. For carbamazepine and fluoxetine the gradient was 0.0-2.5 min 43 % 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. For diclofenac the relative flow of mobile phase B was 0.0-1.5 min 43 % B, 1.5-4.0 min 43-80 % 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 % B. MS/MS analysis was undertaken using atmospheric pressure electrospray ionisation (ESI) in positive ionisation modes, using the turbo ion-spray interface. Spray voltage was 5000 V and source collision induced dissociation was in positive ESI, with the ESI capillary line maintained at 550°C and collision gas (N_2) pressure set at 6 (additional information on LC-MS/MS methods can be found in Appendix 4).

Qualitative and quantitative analysis of compounds was based on retention time, multiple reaction monitoring (MRM) of two product ions and the ratios between the product ions. Limits of detection (LOD) were not assessed because sensitivity was not an issue with the amount of analyte. Lower limit of quantification (LLOQs) were 375 ng/mL, 12.5 ng/mL and 150 ng/mL for carbamazepine, diclofenac and fluoxetine respectively.

When LC-MS/MS analysis was unable to detect parent compound in the earthworm samples, extracts were subsequently analysed by LC-FTMS (solariX 9.4T, Bruker) to look for known metabolites and transformation products of the parent compound (Appendix 2).

2.3.5 Modelling

2.3.5.1 Earthworm kinetic modelling

A first order one-compartment model was used to estimate the uptake and depuration rates for each test compound from pore water. The toxicokinetic model, as described in Equation 1, was fitted to measured internal worm concentration data and based on principles outlined by Ashauer *et al*., (2010; 2006). The parameters were estimated using the software OpenModel (v 1.2; University of Nottingham, 2008; http://www.nottingham.ac.uk/environmental-modelling/OpenModel.htm). The model was parameterized using residual sum of squares with the Levenberg-Marquardt algorithm followed by Monte-Carlo Markov-Chain (MCMC) with the results from the Marquardt fit as input values. Confidence intervals were characterized by the 95 % percentile of the simulated variables. Pore water derived bioconcentration factors were calculated by setting the water concentration to 1 and by running the model until equilibrium was reached. Bioconcentration factors and their confidence intervals could then be read directly from the internal concentrations. The method is described in full in Ashauer *et al*., (2010; 2006).

$$dCorganism/dt = k_{in} * C_{pw}(t) - k_{out} * Corganism(t)$$ **Equation 1**

Where t is time (hours), k_{in} is the uptake rate constant (L/kg d^{-1}), C_{pw} is the concentration in the pore water (nmol/L), k_{out} is the depuration rate constant (d^{-1}) and $C_{organism}$ is the concentration in the organism (nmol/kg).

It is a valid assumption that a large percentage of the uptake into earthworms occurs dermally via the pore water based on previous work which has suggested this (Belfroid *et al*., 1995; Jager *et al*., 2003; Vijver *et al*., 2003) and hence omission of potential direct uptake from soil is justified.

2.3.5.2 Modelling dissipation of study compounds in soil

A simple first-order degradation kinetic model was fitted to the results of the soil analysis during the uptake phase. Model parameters were optimized according to recommendations by FOCUS (FOCUS, 2006) using the least squares method with Microsoft® Excel Add-Inn Solver. Half-lives (DT_{50}, the time for a 50 percent decline in the concentration of pharmaceutical) were then calculated using a true replicates FOCUS (FOCUS, 2006) spread sheet.

2.3.5.3 Comparison of data to predictive models

Models exist to predict environmental exposure scenarios such as those outlined in the Technical Guidance Document (TGD) on Risk Assessment (Part 2) (Equation 2). Pore water concentrations obtained in this study were compared to estimated concentrations (PEC_{pw}), calculated using sorption coefficients (K_d) for the selected pharmaceuticals (Table 2.2) based on equations outlined in the TGD (Equation 2). BCFs obtained in this study were compared to estimated BCFs using models outlined in Belfroid *et al*., (1993) and Jager, (1998) to evaluate the current models used in risk assessment.

$$PECpw = (PECsoil*RHOsoil)/(Kd*1000) \qquad \textbf{Equation 2}$$

Where PEC_{pw} (mg/L) is the predicted environmental concentration in the pore water, PEC_{soil} (mg/kg) is the concentration in the test soil, RHO_{soil} is the bulk density of the soil (kg/m^{-3}) and K_d is the soil sorption distribution coefficient for each pharmaceutical in the test soil (L/kg).

2.3.6 Statistical analysis

Statistical analysis of the data was performed using SigmaPlot (v. 12). For each compound, data on burrowing times and percentage weight gain from the toxicity study were first tested for normality using a Shapiro-Wilk test and then for equal variance. If these passed then a one-way ANOVA was performed to assess the differences in the values among the treatment groups. Where normality failed, analysis of variance was performed using a Kruskal Wallis analysis on ranks. Differences between the measured and predicted pore water concentrations were first tested for normality using a Shapiro–Wilk test, as normality failed for each pharmaceutical the difference between measured and predicted values was then evaluated by a Mann–Whitney U Rank test. The relative accuracy of the estimated results was estimated by calculating proportional deviation from the measured to the estimated value.

2.4 Results and discussion

2.4.1 Sorption of study pharmaceuticals to test soil

Sorption coefficients (K_d) for the study pharmaceuticals increased in the order of carbamazepine < diclofenac < fluoxetine < orlistat (Table 2.2). Whilst the sorption of a pharmaceutical can vary considerably depending upon the soil type (Tolls, 2001) the values for carbamazepine, diclofenac and fluoxetine all fall within the ranges previously reported in scientific literature (Barron *et al.*, 2009; Drillia *et al.*, 2005; Kwon and Armbrust, 2008; Xu *et al.*, 2009c). The results suggest that orlistat has a particularly strong sorption capacity to the soil. This may be due its particularly hydrophobic nature and the presence of a large clay fraction in the soil which has a high sorption capacity due to its small size and large surface area (McGechan and Lewis, 2002).

Table 2.2 Sorption of test pharmaceuticals to study soil (mean value provided ± standard deviation, *n* = 6).

Pharmaceutical	**Distribution coefficient (K_d) (L/kg)**[a]
Carbamazepine	4.83 ± 0.68
Diclofenac	28.65 ± 3.27
Fluoxetine	608.42 ± 87.57
Orlistat	1493.98 ± 92.01

[a] K_d values were determined experimentally following OECD 106.

2.4.2 Toxicity of study pharmaceuticals to *Eisenia fetida*

No mortality was observed in any of the toxicity experiments. There were no significant differences in the burrowing times of *E. fetida* for each of the pharmaceutical treatments (x 10 and x 100) in comparison to the blank and solvent controls ($F<0.709$, d.f. = 3, $p > 0.05$). More than 90 % of earthworms burrowed beneath the soil within 10 minutes of being placed on the 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 curling into a ball) or physiological differences (e.g. surface lesions) were noted for any of pharmaceutical exposed worms. It was therefore concluded that as no visible effect on the earthworm behaviour was seen at 100 x the proposed test concentrations, uptake and depuration would unlikely be affected by toxic effects of the study compounds.

There is relatively little research on pharmaceutical toxicity to earthworms, previous studies have observed no *E. fetida* mortality after exposure to tetracyclines at environmentally relevant concentrations (Qi *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 could

investigate if similar effects are observed with human pharmaceuticals comparable to what has been observed with tetracyclines.

2.4.3 Uptake and depuration of pharmaceuticals in *Eisenia fetida*

2.4.3.1 Main trends in soil and pore water data from uptake phase

For all of the pharmaceuticals, throughout the exposure phase, there was a decrease in radioactivity in the soil followed by an increase in uptake by *E. fetida*. Only small amounts of radioactivity were unable to be recovered from the test soil in the worm free exposure (> 94 % recovery) for carbamazepine, fluoxetine and orlistat. Thus *E. fetida* were consistently exposed to similar concentrations of the pharmaceuticals throughout the exposure phase. For diclofenac < 65 % of the radioactivity could be recovered using a combination of solvent extraction and soil combustion after 21 days (see discussion below).

The amount of radioactivity measured in the carbamazepine study decreased slightly in the pore water over the period of uptake phase which can probably be explained by the decrease in radioactivity extracted from the soil over 21 days (Figure 2.3). Only in the fluoxetine study was an increase in radioactivity measured in the pore water over the uptake phase (Figure 2.4). By the end of the uptake phase, in the pore water, only 50 % of the original radioactivity was measured in the orlistat study possibly due to the strong sorption of orlistat to the soil or uptake into *E. fetida.* In terms of concentration, carbamazepine had the highest concentration in the pore water which can explain the initial rapid uptake in the earthworms whilst the slow uptake of orlistat could potentially be explained by the lowest concentration in the pore water suggesting that this compound was not bioavailable.

2.4.3.2 Pharmaceutical degradation

Apart from in the diclofenac study, the dissipation of radioactivity from the test soil was modelled using single first order kinetics (Appendix 3). The soil data fit well according to single first order kinetics with Chi square values all below the accepted level (Table 2.3). The half-lives (DT_{50}) in the carbamazepine and fluoxetine studies would suggest these are the most stable compounds which is in agreement to

previous work where little to no degradation of carbamazepine and fluoxetine was observed with half-lives > 60 days (Monteiro and Boxall, 2010, 2009; Redshaw *et al*., 2008a). Previous research has shown that, depending on soil type, the half-life for diclofenac can range from 3.07 up to 20.44 days (Xu *et al*., 2009c). Regression has shown that diclofenac degradation rate constants are negatively correlated with soil clay content (Xu *et al*., 2009c). The test soil being of a particularly clayey nature may explain the larger DT_{50} values in this study (Table 2.3) due to less diclofenac degradation in comparison to previous work. Orlistat had a half-life of 48 days (11497 hours) which is the lowest of all the pharmaceuticals

2.4.3.3 Uptake and depuration of pharmaceuticals in *Eisenia fetida*

E. fetida were seen to take up all of the study compounds (Figure 2.3) but the degree and pattern of uptake into the worm was different for all of the compounds. Measured radioactivity in the carbamazepine study increased over the first 160 hours (~ 7 d) of exposure after which time it declined, possibly due to the observed dissipation of the radioactivity in the soil and soil pore water (Figure 2.3). Similarly, in the fluoxetine study measurements of radioactivity in the *E. fetida* tissue increased over the first 160 hours (~ 7 d) of the exposure phase and then appeared to reach a steady state (Figure 2.3). For diclofenac and orlistat, measurements of radioactivity continuously increased and did not appear to have reached a steady state by the end of the uptake phase (Figure 2.3).

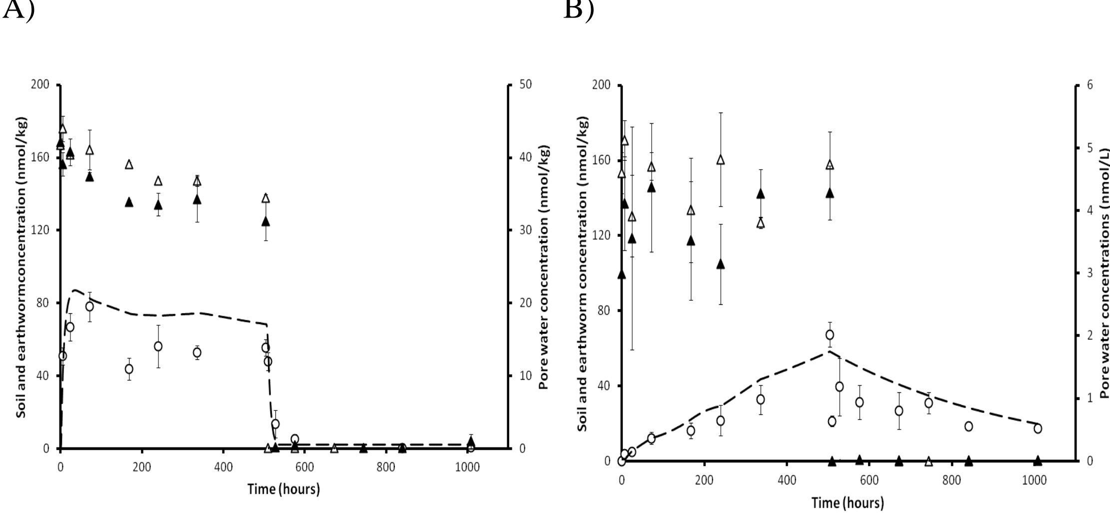
Table 2.3 Summary of key results from uptake and depuration experiment, including pH range of soil throughout each exposure (± standard deviation), the time taken for 50 and 90 % degradation of the pharmaceuticals in soil according to FOCUS modelling and the modelled *E. fetida* uptake and depuration rates including the pore water based BCF (BCF provided with 95 % confidence intervals in brackets, $n = 3$). Diclofenac soil concentrations could not be modelled.

Pharmaceutical	**pH**	**Kinetics**	**$\chi 2$**	**DT_{50} (d)**	**DT_{90} (d)**	**k_{in} (uptake rate) (L/kg d^{-1})**	**k_{out} (depuration rate) (d^{-1})**	**BCF (pore water)**
Carbamazepine	6.3 ± 0.2	First order	2.0	68	226	0.24	0.14	2.21 (1.3 – 3.5)
Diclofenac	6.2 ± 0.1	N/A	N/A	N/A	N/A	0.036	0.0021	21.5 (13.9 – 30.6)
Fluoxetine	6.3 ± 0.2	First order	5.1	66	220	1.11	0.047	30.8 (25.4 – 35.8)
Orlistat	6.2 ± 0.2	First order	6.4	48	159	0.071	0.0016	51.5 (40.0 – 65.3)

As soon as the depuration phase began, *E. fetida* immediately eliminated all of the pharmaceuticals from the earthworm tissue as measurement of radioactivity in the samples decreased. For carbamazepine and fluoxetine this was fairly rapid with complete elimination by day 3 and 7 of the depuration phase respectfully. Elimination in the diclofenac study was also fairly rapid at the start, it was not completely eliminated from the earthworm by 21 days with < 20 % of the radioactivity remaining in the tissue. Orlistat was eliminated the slowest from *E. fetida* with modelled depuration rates of 0.0016 d^{-1}. By the end of the depuration phase > 60 % of the radioactivity taken up in the orlistat study remained in the earthworm.

The first order, one compartment model, based on pore water measurements, was successfully fitted to the uptake and depuration data for all four study compounds (Figure 2.3 A-D). Uptake rates and depuration rates are provided in Table 2.3. The pore water-based BCFs obtained from the model increased in the order of carbamazepine < diclofenac < fluoxetine < orlistat. The relatively large BCF of 51.53 for orlistat can be attributed to the minimal elimination of this compound from the earthworm in the depuration phase whilst for carbamazepine the fast elimination of 0.14 d^{-1} is accountable for the smaller BCF of 2.21. The BCFs increase in a similar order to the increase in log K_{ow} values for the respective compounds perhaps inferring that the degree of hydrophobicity plays a key role in the uptake of pharmaceuticals from soils.

In comparison to aquatic BCFs for pharmaceuticals published in scientific literature, earthworms seem to have lower BCF values. For fluoxetine and diclofenac aquatic BCFs have been reported at values much larger than calculated for the earthworms (Brown *et al.*, 2007; Lahti *et al.*, 2011; Paterson and Metcalfe, 2008; Schwaiger *et al.*, 2004; Zhang *et al.*, 2010) with BCFs reported up to 185 900 for fluoxetine in the fresh water shrimp (*Gammarus pulex*) (Meredith-Williams *et al.*, 2012) which is over 6000 times greater than the BCF generated for earthworms. Aquatic BCFs for carbamazepine are similar to the BCF of 2.21 obtained in this study (Lahti *et al.*, 2011; Meredith-Williams *et al.*, 2012; Zhang *et al.*, 2010) with results from Vernouillet *et al.*, (2010) showing that algae (*Psuedokirchneriella subcapita*) has a BCF of 2.2 which is remarkably similar.



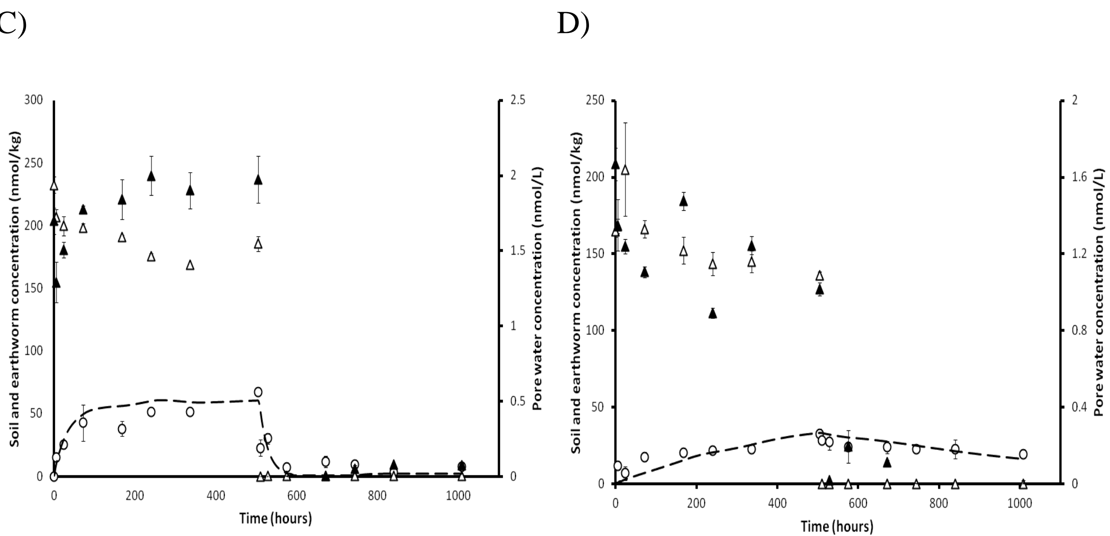


Figure 2.3 Uptake and depuration curves for *Eisenia fetida* exposed to A) carbamazepine, B) diclofenac, C) fluoxetine, and D) orlistat. Mean (n = 3 ± SE) measured concentrations in the worm are represented by the circles and the data lines represent the first order model fit. Mean Concentrations (n = 3 ± SE) in the soil and soil pore water are represented by the open and closed triangles respectively.

Biological attributes such as species size, number of segments, feeding habits and reproduction may play a key role in uptake and bioconcentration of pharmaceuticals. Previous work has suggested that an increase in organism size corresponds to a decrease in BCF (Hendriks *et al.*, 2001), and whilst this is true for p, p' – DDE as bioaccumulation in the smaller *E. fetida* was < 6 times higher than in *Lumbricus terrestris* (Peters *et al.*, 2007), pharmaceutical uptake into different earthworm species has not yet been evaluated to explore this concept further. In comparison to

fish species which are larger than *E. fetida* this relationship does not follow as has previously been shown fish BCFs for pharmaceuticals tend to be higher than BCFs observed in this study. Mercury accumulation in earthworms has demonstrated that species length and age is important in chemicals assimilating in tissues with decreased mercury contents following increased growth and development (Zhang *et al*., 2009). In the aquatic environment a positive relationship between lipid content and bioconcentration of chemicals has also been suggested (Barron, 1990; Hendriks *et al*., 2001; Schlechtriem *et al*., 2012). This would suggest differences in accumulation of pharmaceuticals in earthworms as lipid contents can range between 1 – 20 % (Dynes, 2003).

2.4.3.4 Mass balance results

A mass balance was performed to account for the radioactivity present in the experiment, using measurements in *E. fetida*, soil and pore water samples. At the end of the exposure phase > 89 % of the compound was recovered for carbamazepine, fluoxetine and orlistat. While soil data demonstrated dissipation of orlistat in the exposure phase, the soil combustion data confirms this is not due to mineralisation but instead due to the formation of bound residues. However for diclofenac, whilst combustion data showed some recovery of non extractable residues by the end of the uptake phase only 52 % of the initially applied compound could be recovered, suggesting perhaps a loss of ^{14}C - carbon dioxide released via mineralisation.

Formations of non-extractable residues in soils have been investigated since 1980’s (Calderbank, 1989) however very little work has explored pharmaceutical bound residues in soil (Kreuzig and Höltge, 2005; Kreuzig *et al*., 2003). Specifically, the persistent nature of ^{14}C sulfadiazine was shown in work by Kreuzig and Höltge (Kreuzig and Höltge, 2005) where only 1 % of the radiotracer was mineralized to ^{14}C-carbon dioxide and 82 % was transferred to non-extractable residues after 102 days. A pharmaceutical which may be irreversibly sorbed to soil may remain bio-available for uptake by soil organisms. Uptake of bound residues into earthworms was observed with pesticides however tissue to soil ratios were 2−10 times higher in soils with freshly spiked pesticides compared to soils containing previously non-extractable residues for the same compounds (Gevao *et al*., 2001).

2.4.4 Metabolism

A number of studies have reported the detection of metabolites and transformation products of the pharmaceutical parent compound in aquatic organisms (Lahti *et al.*, 2011; Paterson and Metcalfe, 2008). Little information is known about biotransformtion of pharmaceuticals in terrestrial organisms.

Both carbamazepine and fluoxetine were detected in the worm tissue at concentrations slightly greater than expected and thus we can assume that what was measured in the radiolabelled studies was the parent compound. Diclofenac was not detected (Table 2.4). A literature search was then performed to identify known diclofenac metabolites and transformation products (Appendix 2). Diclofenac worm extracts were subsequently analysed using LC-FTMS (solariX 9.4T, Bruker) to look for known diclofenac metabolites and transformation products collated from literature sources. However no valid matches were made. The measured radioactivity in the diclofenac study and subsequent BCFs therefore refer to diclofenac parent compound and any potential transformation products.

Table 2.4 Analyte detection in earthworm samples (n =6).

Compound	**Soil spike (mg/kg - nominal)**	**BSAF**	**Expected (ng/g)**	**Average measured (ng/g) (± standard deviation)**
Carbamazepine	0.78	0.33	260	491.16 (± 18.52)
Diclofenac	0.8	0.57	456	< LOQ
Fluoxetine	1.6	0.29	466	802.98 (± 97.77)

2.4.5 Evaluation of existing predictive models

2.4.5.1 Pore water concentrations

Pore water concentrations of pharmaceuticals throughout the uptake and depuration phase were estimated (Equation 2) and compared to the measured values obtained in

the study. For fluoxetine and orlistat, which had the highest K_d values, pore water concentrations were significantly under estimated ($U = 699$, $P = 0.012$; $U = 654$, $P = 0.004$ respectfully) in comparison to the measured data (Figure 2.4 C, D). For carbamazepine and diclofenac, which were less strongly sorbed to the soil, the estimated pore water concentrations were closer to the measured data but slightly overestimated with carbamazepine statistically different ($U = 761$, $P = 0.043$) whilst there was no significant difference for diclofenac ($U = 755$, $P = 0.076$). As there was a statistically significant difference between the measured and estimated data for a large proportion of the pharmaceuticals this infers that parameters other than the soil distribution coefficient (K_d) are important in estimating pore water concentrations. Alternatively, the results may also imply that batch sorption tests are not an appropriate way to calculate sorption coefficients (K_d) as it does not accurately stimulate the micro-environments of real soils. The inaccuracies of the K_d calculation may therefore be limiting the prediction of pore water concentrations. Nevertheless, the results show that the predicted environmental concentration in pore water for modelling purposes outlined the TGD may not be appropriate for pharmaceuticals.

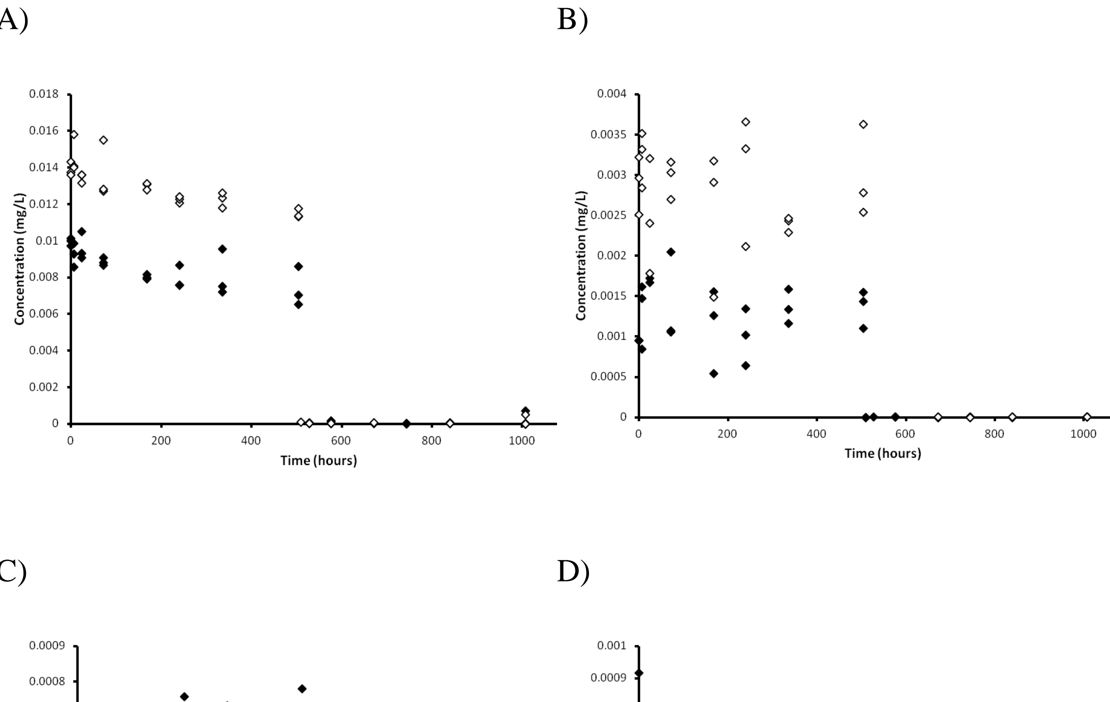


Figure 2.4 Comparison between measured pore water concentrations obtained from the uptake and depuration experiment and estimated pore water concentrations based on K_d values calculated for each pharmaceutical for A) carbamazepine, B) diclofenac, C) fluoxetine, D) orlistat. The closed and open diamonds represent measured concentrations and estimated concentrations respectfully.

2.4.5.2 Bioconcentration factors

The QSARs generally overestimated the pore water BCFs, particularly for orlistat where the measured BCF was up to 6000 times higher than the estimated value (Figure 2.5). There are a number of possible explanations for the lower than predicted BCF for orlistat. This may be because of the molecular weight cut off which is generally seen for compounds with a high log K_{ow}. According to REACH guidelines at log K_{ow} values between 4 and 5, log BCF increases linearly with log K_{ow}, however at very high log K_{ow} (< 6) a decreasing relationship between these two parameters is observed and reduced uptake due to molecular size may be

attributable. The QSARs do not represent true earthworm uptake, orlistat uptake into *E. fetida* may also be inhibited perhaps through irreversibly bound fractions and non-available residues in the pore water. Whilst previous work suggests that uptake across the gut wall is important for compounds with a log K_{ow} greater than 5 the particularly large log K_{ow} for orlistat may actually mean that the compound is so strongly bound to the soil that it is unable to desorb and enter the earthworm. Combustion results from this study and additional research (Ryan, 2013) has demonstrated appreciable degradation (DT_{50} 2.4 – 23.2 d), mineralisation and significant irreversibly bound residues (27.16 % [21 d]) of orlistat which supports the idea that the total applied orlistat concentration is not available for uptake. It should be noted however that both of the QSARs were not developed specifically to predict pharmaceutical uptake, and therefore may have limited use. The QSAR by Belfroid (1993) had a limited log K_{ow} window (4.2 – 5.7 which was later extrapolated to 2 -7) and was developed for specifically for neutral compounds.

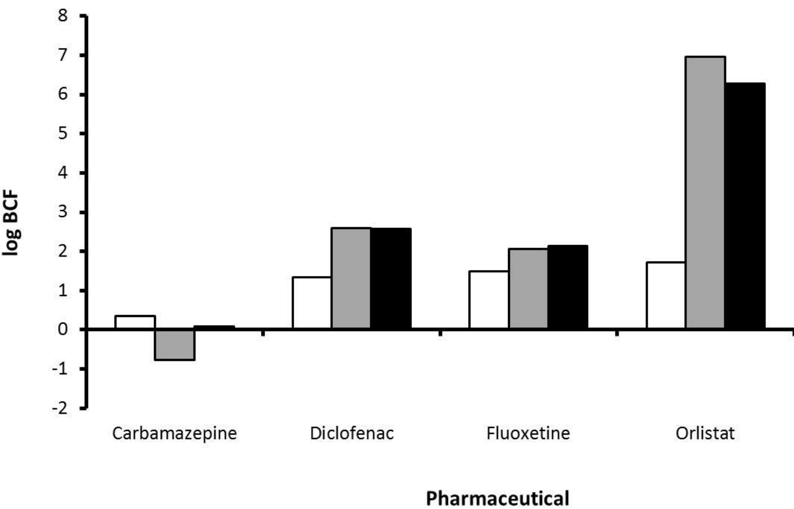


Figure 2.5 Comparison between earthworm BCFs obtained from the model in this study (white), predictions from the QSAR described in Belfroid *et al*., 1993 (grey) and predictions from the QSAR in the TGD based on Jager, 1998 (black) for carbamazepine (CBZ), diclofenac (DCF), fluoxetine (FLX) and orlistat (ORL).

These results suggest that there may be other descriptors or parameters which may be important in predicting the uptake of pharmaceuticals into earthworms, but most importantly the discrepancies between the estimated BCFs and those from the measured data highlight that the current QSARs are not applicable and new

estimation methods for predicting pharmaceutical uptake into earthworms are needed for future risk assessment. Approaches which consider the ionised state of the molecule are integral in obtaining realistic BCFs and might improve predictions of earthworm BCFs for pharmaceuticals. For chemicals in the neutral form, uptake is predicted to be higher while the bioavailability of ionised molecules will be dependent on the pH of the surrounding medium. Models which explore ionisation include the work by Lo and Hayton, (1981) and Erickson *et al.*, (2006) and the cell model (Trapp, 2004). Specifically the cell model uses Fick's first law of diffusion for the neutral molecules and Nernst-Planck equation for the ionisable fraction of molecules to predict the movement, by diffusion, of molecules in a living cell. This model includes principles of the ion trap effect and assumes only the freely dissolved molecules in the cell can undergo diffusion and when the end point of diffusion (net flux is zero) has been reached this is known as equilibrium. The equilibrium concentration ratio between the inside and outside of the cell can currently be used to predict fish BCFs and with further research could be adapted to predict accurate earthworm BCFs.

2.5 Conclusions

The work presented here demonstrates that pharmaceuticals present in soils at environmentally relevant concentrations can be taken up by the earthworm *Eisenia fetida.* A relatively simple one compartment first order model can fit the uptake into *E. fetida* based on the assumption that uptake into the worm occurs via the pore water. Carbamazepine and fluoxetine do not appear to be metabolised in the current studies and therefore for the remaining thesis, uptake of radioactivity of these compounds is assumed to be parent compound. For diclofenac metabolism does seem to occur, however this could not be characterised as a specific transformation product. Therefore the radioactivity measured in the diclofenac study will refer to that of the parent compound and potential transformation products. Current QSAR estimation techniques to predict bioconcentration factors in earthworms, for the large part, overestimate BCFs. The results suggest that the uptake of highly hydrophobic compounds such as orlistat does not scale according to log K_{ow}, implying a cut off point for a linear relationship between K_{ow} and BCF above which increasing log K_{ow} value does not appear to correlate with elevated bioconcentration. Even the higher

BCFs noted in this study are nonetheless quite low in absolute terms of plant and animal uptake.

Additional research is needed to establish the influence that soil parameters (e.g. pH, organic matter content) and species traits have on the uptake of pharmaceuticals into soil invertebrates. However studies described in this chapter are highly labour intensive. To explore the effects of environmental and species traits on uptake would be challenging using the methods. Therefore, in the next chapter, the use of a minimised approach is explored.

Chapter 3 Applicability of the Minimised Design Approach for Assessing Bioconcentration in Invertebrates

3.1 Introduction

Concern about bioconcentration, bioaccumulation and biomagnification of synthetic chemicals in biota has led to the establishment of bioconcentration tests, guidelines and assessment criteria (OECD 305 (OECD, 2012)). A bioconcentration factor (BCF) is a useful metric for the scientific evaluation of the risks of chemicals (pesticides, biocides, veterinary medicines, pharmaceuticals and industrial chemicals) in the environment (e.g. REACH). BCFs are typically compared to a threshold to determine whether there is a risk of bioaccumulation or not.

Bioconcentration studies generally consist of an uptake phase where test organisms are exposed to a chemical followed by a depuration (or elimination) phase where organisms are transferred to clean exposure medium free from chemical contamination, the concentration of the chemical in the organism at different time points in both phases is monitored (Figure 3.1). An example of such an approach can be found in Chapter 2.

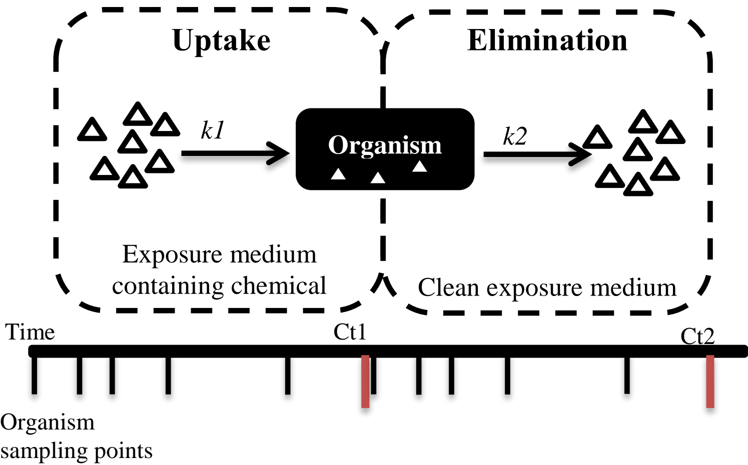


Figure 3.1 Schematic of an uptake and depuration experiment and comparison of sampling points between traditional designs (black lines) and minimised design (red line). Where *k1* and *k2* are the uptake and depuration rates respectively and Ct1 and Ct2 are the concentrations in organism measured at the end of uptake and end of depuration respectively.

Experimentally determined BCFs can be derived using a ratio of measured internal concentration and exposure medium concentration when steady state concentrations have been reached in the test organism. When steady state has not been achieved, measured data can be modelled to determine uptake and depuration rate constants and subsequently BCFs (sometimes termed kinetic BCFs) can be calculated from these. A full explanation of tokicokinetic modelling and BCF calculation can be found in Chapter 2 (section 2.3.5).

Generally, bioconcentration studies following OECD guidelines require a substantial amount of laboratory effort due to the degree of replication that is needed and the sampling frequency during the uptake and depuration phases. For example, terrestrial invertebrate bioconcentration guidelines, such as those used in Chapter 2 suggest that earthworms should be sampled in triplicate a minimum of six times during both the uptake and depuration phases (OECD, 2010). For aquatic invertebrates the total number of species used in published uptake and depuration study ranges between 33 - 98 (Ashauer et al., 2010; Karlsson, 2013; Meredith-Williams et al., 2012; Rubach et al., 2010) while original fish bioconcentration tests suggest a minimum of four fish to be sampled at least five times during uptake and four times during depuration (OECD, 1996). The rigour of the current guidelines means that large numbers of animals are required and that labour and analytical resources are costly. The magnitude of the tests may also be inhibiting our understanding of the factors and processes affecting uptake of chemicals in the environment as it almost physically impossible to perform large multi-factor uptake studies into chemical uptake using existing guidelines.

Recognising the labour intensity of BCF studies for fish, Springer and colleagues (2008) proposed a new minimised test design for the OECD 305 (OECD, 1996) and U.S. EPA (850.1730) test guidelines for fish. This design aimed to estimate BCF using the kinetic definition ($BCF_{minimised}$) (uptake rate constant/depuration rate constant) which meant that steady state tissue concentrations did not need to be achieved. However both uptake and depuration must follow first order kinetics. The proposed design requires that test organisms are collected and analysed only once at the end of the uptake phase/beginning of depuration (C_{t1}) and once at the end of the depuration period (C_{t2}). Water samples are also required on a regular basis

throughout the uptake period (C_w/C_{pw}) to calculate an average exposure medium concentration. Using simple algebraic expressions (Equation 3 and Equation 4) uptake and depuration rate constants and BCFs can then be estimated.

$$k_2 = (\ln C_{t_1} - \ln C_{t_2})/t_d$$ **Equation 3**

Where t_1 and t_2 are the beginning and end of the depuration period, respectively. The uptake rate constant (k_1) is then calculated based on the depuration rate constant (k_2) generated from Equation 3.

$$k_1 = k_2 * C_{t_1}/C_{w/pw}\ (1 - e^{-k_2 t_u})$$ **Equation 4**

Where k_2 is depuration rate constant, the mean concentration of the test substance in the medium during exposure phase is C_w or C_{pw} and t_u and t_d the length of uptake and depuration periods. Lastly a kinetic BCF from minimised design ($BCF_{minimised}$) can be calculated by dividing the uptake rate by the depuration rate (Equation 5). See Table 3.1 for a full explanation of parameters used.

$$BCF_{minimised} = k_1/k_2$$ **Equation 5**

Springer *et al*., (2008) showed that this design uses significantly fewer animals and resources, yet still provides useful BCF estimates. Since this publication a new approach has been adopted for the fish BCF test guideline (OECD 305,(2012)) which utilises fewer fish for both cost and animal welfare reasons. Some of the key changes include only using one test concentration (when the BCF is independent of the test concentration) and, if specific criteria are met then a minimised aqueous exposure test design could be used. This minimised aqueous exposure design is similar to that proposed by Springer *et al*., (2008) in that it allows for reduced fish sampling in the uptake and depuration phases and reduced exposure medium sampling. These changes in the guidelines indicate that regulatory agencies are recognising there is a need to change experimental designs to reduce organism usage.

Table 3.1 Parameters and definitions for minimised design equations.

Parameter	Definition	Units
k1	Uptake rate	L/kg d^{-1}
k2	Depuration rate	d^{-1}
C_{t1}	Concentration in organism at end of uptake	mg/kg
C_{t2}	Concentration in organism at end of depuration	mg/kg
C_w or C_{pw}	Mean concentration of exposure medium during uptake phase	mg/L
t_d	Length of depuration phase	d
t_u	Length of uptake phase	d
BCF	Bioconcentration factor	L/kg

While the minimised approach has been shown to be valid for fish, to date no-one has explored its wider applicability to other taxonomic groups. Therefore, this study was performed to assess the applicability of the minimised design for estimating BCFs in terrestrial and aquatic invertebrates. The study used existing datasets, published by a number of authors, on the uptake of a wide variety of pesticides and pharmaceutical compounds into different aquatic and terrestrial invertebrates to evaluate whether the minimised approach could generate reasonable estimates for rate constants and BCFs. The results were used to develop general guidance on the application of the approach.

3.2 Materials and Methods

3.2.1 Collation of uptake and depuration data

Datasets from a number of BCF studies were collated. The studies included different periods of uptake and depuration and different chemical classes. A summary of data collated is provided in Table 3.2. Studies were chosen specifically to provide a range of invertebrate species whilst also including a range of compounds with differing physico-chemical properties and modes of toxic action and different test matrices (Table 3.2, Appendix 6). For example, the log K_{ow} values of the chemicals in the

data set ranged from -0.81 to 8.19 and the dataset covered neutral compounds, weak acids and weak bases. Raw data from these previous studies was obtained, including measured internal concentrations and measured exposure medium concentrations for the duration of the experiments.

All of the studies used a one compartment first-order toxicokinetic model to simulate the internal concentrations in the organisms using the measured concentrations of the test chemicals in the exposure medium as the driving variable. The aquatic studies consisted of a water only exposure and therefore the exposure medium was C_w. For terrestrial species, uptake was assumed to come from the pore water (C_{pw}). First order toxicokinetic model equations are described further in Chapter 2 (2.3.5). The estimated $BCF_{traditional}$ (based on the full uptake and depuration studies) for the chemicals used in the studies ranged from 0.132 to 700 900 (Appendix 6).

3.2.2 Estimation of rate constants and BCFs using the minimised approach

Measured internal concentrations of chemicals in organisms from the last day of uptake and last day of depuration, for each study, were taken from the datasets along with measured data on concentrations of the study compound in the test media during the uptake phase (water or pore water). These data were then used in Equation 3 and Equation 4 to re-estimate the uptake and depuration constants and then $BCF_{minimised}$ values. $BCF_{minimised}$ values were subsequently compared to those previously published in the literature sources ($BCF_{traditional}$) to assess the applicability of the minimised design to estimate BCFs in a range of invertebrate species.

It should be noted in the Springer approach (2008), 28 d was used for t_u and t_d was 14 d. If the original study consisted of different time periods then measurements were rescaled and interpolated from reported measurement to provide the 28 d and 14 d measurements respectively. For the purposes of recalculating BCFs, in this study, the length of the uptake and depuration phases remained as they were in the original experiment (Table 3.2). This is an important difference, because it allowed us to test if the minimised design method is also applicable when much shorter experiments are used.

Table 3.2 Summary of data collated on published BCFs (more detaile d table can be found in Appendix 6).

Test species	Chemicals tested	Number of studies	log K_{ow} range[a]	Uptake period (t_u) (days)	Depuration period (t_d) (days)	BCF range
Gammarus pulex	Beta-blocker, anti-cancer, anti-epileptic, sedative, anti-depressant, insecticide, fungicide, herbicide, biocide, algaecide	25	(-0.81) - 5.31	< 2	< 6	1.64 - 185 900
Anax imperator	Insecticide	1	4.96	2	5	100
Asellus aquaticus	Insecticide	1	4.96	2	5	3242
Chaoborus obscuripes	Insecticide	1	4.96	2	5	2428
Cloeon dipterum	Insecticide	1	4.96	2	5	1782
Daphnia magna	Insecticide	1	4.96	2	5	541
Molanna angustata	Insecticide	1	4.96	2	5	5331
Neocaridina denticulata	Insecticide	1	4.96	2	5	1291
Notonecta maculata	Insecticide	1	4.96	2	5	407
paraponyx stratiotata	Insecticide	1	4.96	2	5	1601
Plea minutissima	Insecticide	1	4.96	2	5	654
Procambarus sp.	Insecticide	2	4.96	2	5	280 - 1295

Table 3.2 Continued

Test species	Chemicals tested	Number of studies	log K_{ow} range[a]	Uptake period (t_u) (days)	Depuration period (t_d) (days)	BCF range
Ranatra linearis	Insecticide	1	4.96	2	5	392
Culex pipens	Insecticide	1	4.96	2	5	13930
Sialis lutaria	Insecticide	1	4.96	2	5	9625
Planorbarius corneus	Beta-blocker	1	3.05	3	3	57.3
Notonecta glauca	Beta-blocker, anti-cancer, anti-epileptic, sedative, anti-depressant	6	(-0.81) - 4.65	2	2	0.13 - 1.60
Lumbriculus variegatus	Anti-epileptic, NSAID[b], anti-depressant, stimulant, antimicrobial, antibiotic	17	(-0.02) - 5.42	2	2	1 - 700 900
Eisenia fetida	Anti-epileptic, NSAID[b], anti-depressant, weight loss aid	4	2.25 - 8.19	21	21	1.14 – 63.03

[a] Log K_{ow} as reported in publications (specific log K_{ow} for chlorpyrifos not provided therefore Bowman and Sans (1983) reference used).

[b] NSAID – Non-steroidal anti-inflammatory drug.

3.2.3 Statistical analysis

The (log) $BCF_{traditional}$ and the (log) $BCF_{minimised}$ were plotted against each other in a correlation plot (Figure 3.2) and linear regression was used to test if the slope and intercept were significantly different from 0. As both X (log $BCF_{minimised}$) and Y (log $BCF_{traditional}$) were subject to error, linear regression was fit as a Deming (or Model II) regression. The null hypothesis (H_o) was that the slope is equal to zero whilst the alternative hypothesis (H_a) was that the slope is significantly different to zero. It also tested to see if the slope was significantly different from 1 (i.e. if confidence interval of slope includes 1), because a slope of 1 indicates perfect correlation between the two methods. Separate correlations were also made between the uptake ($k1_{traditional}$/ $k1_{minimised}$) and depuration rate ($k2_{traditional}$/ $k2_{minimised}$) constants as well as individual data sets used in the analysis using Deming regression.

3.3 Discussion and Results

3.3.1 Uptake and elimination rates

For both the uptake rate constants and the elimination rate constants, regressions between the minimised approach and original data do not correlate particularly well and deviated from the 1:1 line (Figure 3.2). In both the uptake and depuration rate correlations the regression line was significantly non zero ($p < 0.\ 0001$) however the slope was closer to 1 in the uptake rate figure (95 % confidence interval: 1.053 – 1.383; Figure 3.2) than the depuration rate figure (95 % confidence interval: 1.662 – 2.881;Figure 3.2). Therefore there was a better regression between uptake rate correlation (*k1*) than depuration rate correlation (*k2*).

Greater deviation around the regression and 1:1 line was most evident for the lower values of uptake and depuration rates in comparison to the larger values. There was less error around the larger rate constants. Interestingly the uptake rate data points for Karlsson, 2013 data set were always below the 1:1 line but in a linear fashion in comparison to the remaining data which were more scattered. The $k1_{traditional}$ appear

to be consistently underestimated by the constant amount however this does not occur in the *k2* figure.

In comparison, a greater proportion of the data points were above the 1:1 line for the Ashauer *et al*., data set (2007, 2006; 2010), this is particularly evident with the depuration rates. A lack of relationship between depuration rates is important as it can influence the time to reach steady state calculations. In this analysis, *G. pulex* exposure to 4-nitrobenzyl-chloride resulted in depuration rates of 3.16 d^{-1} and 0.0432 d^{-1} for the original and minimised calculations respectively which corresponds to either 3 or 0.41 days to reach steady state within the organism. The minimised approach may therefore not generate data applicable for use in toxicokinetic-toxicodynamic modelling.

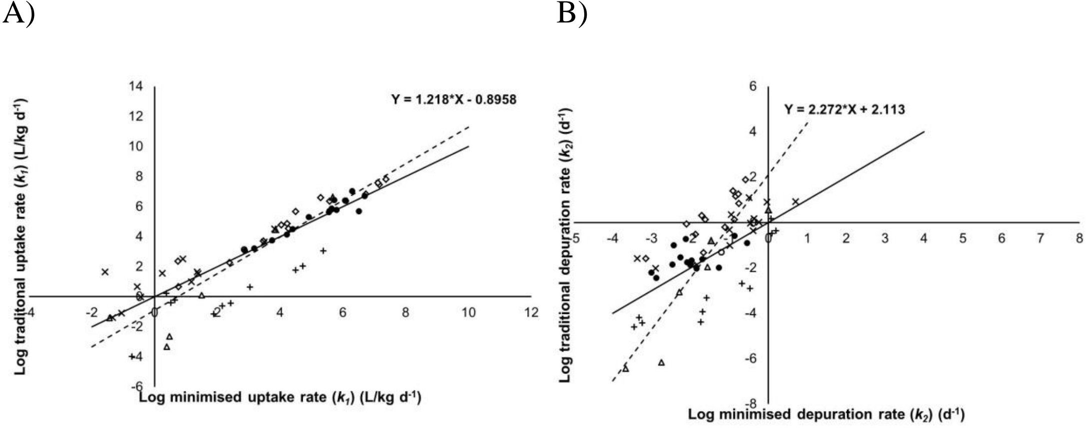
3.3.2 Bioconcentration factors

BCFs were unable to be estimated using the minimised approach when the concentration in the organism at the end of depuration phase was greater than internal concentration measured at the end of the uptake phase. This occurred in a few studies, particularly where the $BCF_{traditional}$ was very high e.g. triclosan (Table 3.2) (Appendix 7). In total 60 BCF values could be used from the $BCF_{traditional}$ and compared to $BCF_{minimised}$ estimates.

Deming regression analysis demonstrated a statistically significant correlation between BCF values obtained using the traditional and minimised approaches (Figure 3.2). The slope of the regression line was significantly non zero ($p = < 0.0001$) and the hypothesis that the slope is equal to 1 was not rejected (slope: 0.99, 95 % confidence interval: 0.91 – 1.07) therefore suggesting there is a significant linear relationship between the two variables (Figure 3.2). The intercept of the regression was also close to zero (intercept: -0.32, 95 % confidence interval: -0.77 – 0.13). Thus the $BCF_{minimised}$ estimates are in agreement with the $BCF_{traditional}$ values and there were no systematic and no proportional differences between the two methods. Specifically, 98 % (96 %; 65 %) of the minimised design BCF values fall within a factor of 10 (factor 5; factor 2) of the $BCF_{traditional}$ values (Figure 3.2). As

previously discussed, there was a weak correlation between the uptake and elimination rate constants generated by the minimised design and the original data. However this is interesting as it appears the respective inaccuracies of the $k1_{\text{minimised}}$ and $k2_{\text{minimised}}$ appear to compensate each other to calculate accurate $\text{BCF}_{\text{minimised}}$ values.

A) B)



C)

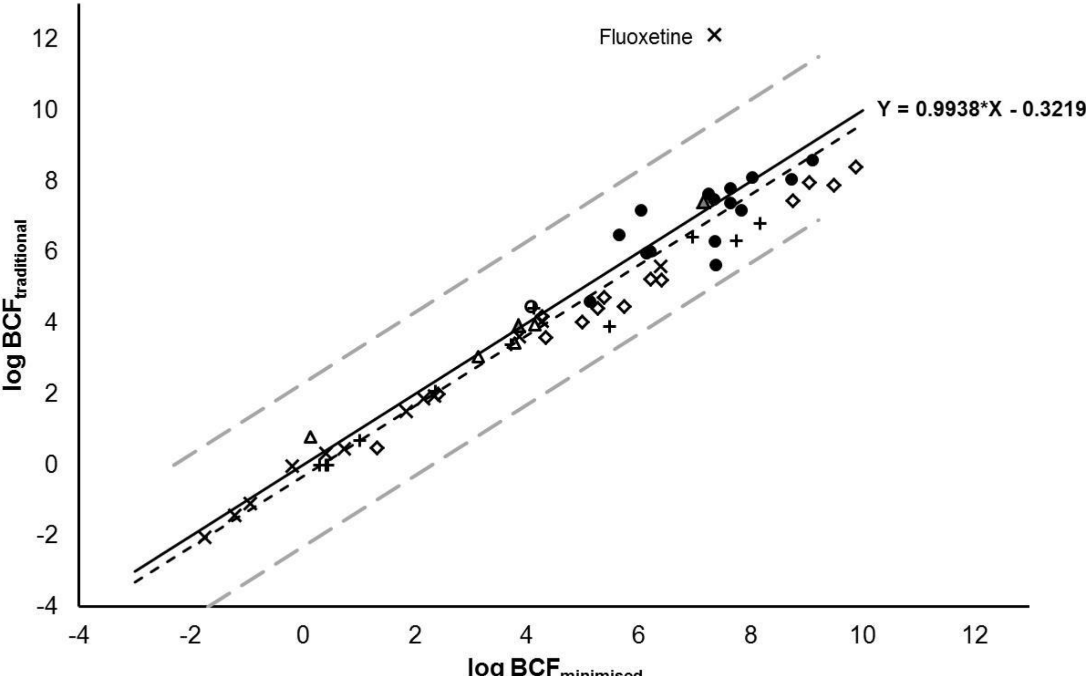


Figure 3.2 (A) Regression between uptake rate (*k1*) from minimised design and *k1* provided in literature. (B) Regression between depuration rate (*k2*) from minimised design and *k2* provided in literature. (C) Relationship between log $\text{BCF}_{\text{minimised}}$ estimates from the minimised design and log $\text{BCF}_{\text{traditional}}$ obtained from the literature. Data include Ashauer *et al*., 2006 (▲); Ashauer *et al*., 2007 (○); Ashauer *et al*., 2010 (◇); Rubach *et al*., 2010 (●); Meredith – Williams *et al*., 2012 (✖); Karlsson *et al*., 2013 (✚) and Chapter 2 (this thesis) (△). Deming regression line (black dash), with equation and 1:1 line (solid) with factor of 10 (grey dash) also provided.

Estimates of $BCF_{minimised}$ may not be accurate if the uptake and elimination kinetics differ greatly from first order. Some datasets used in this analysis exhibited small but systematic deviations from first order toxico-kinetics. However the significant correlation between the $BCF_{traditional}$ and $BCF_{minimised}$ suggests that the $BCF_{minimised}$ results are robust against slight deviations from first order toxicokinetics. Any deviation from the 1:1 line can probably be explained by the depuration rate constants from the minimised design, specifically the weak correlation between depuration rates ($k2_{minimised}$ and $k2_{traditional}$) as discussed previously (Figure 3.2).

Only 37 % of studies included in this analysis had reached steady state in the exposure phase duration. Apart from a few cases when the study length was much shorter than required to reach steady state (< 10 % steady state), there was no relationship between the ratio of rate constants ($k1_{minimised}/k1_{traditional}$) and the percentage of steady state reached in each phase (Figure 3.3). As the percent steady state reached increased, the ratio remained variable around 1 and when 100 % steady state had been achieved the greatest divergence around $k1_{minimised}/k1_{traditional}$ was noted. Thus the minimised design yields similar rate constants to the traditional design when the duration of the experiment allows for at least 10 % of steady state to be reached in the exposure and depuration phases.

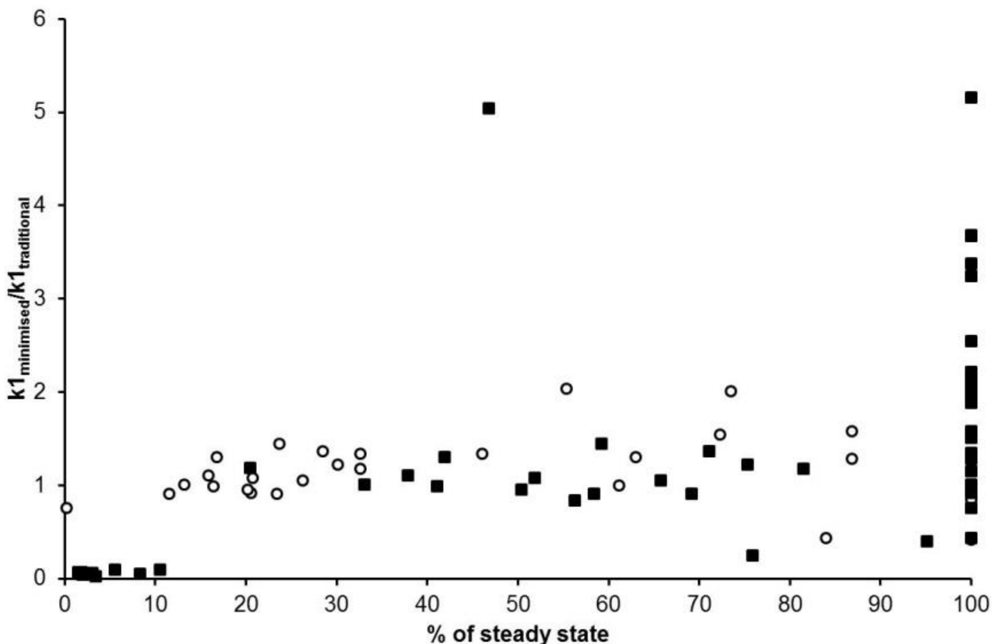


Figure 3.3 Relationship between uptake rate and percentage of steady state reached in uptake (○) and depuration phase (■) for each experiment.

In a number of experiments the concentration of the chemical in the exposure media decreased (< 72 % of initial concentration), a result perhaps of dissipation from the test beaker or uptake into the organism. Whilst in the depuration phase, the chemical sometimes reappeared in the exposure water. The minimised approach appears to be robust enough to deal with changes in exposure medium concentration as the degree to which the exposure medium concentration changes did not affect the BCF calculation. There were no significant relationships between correlations of $BCF_{minimised}/BCF_{traditional}$ and percent change in exposure medium concentration, with reported r^2 0.071 and r^2 0.276 for percent disappearing and reappearing respectively (Figure 3.4).

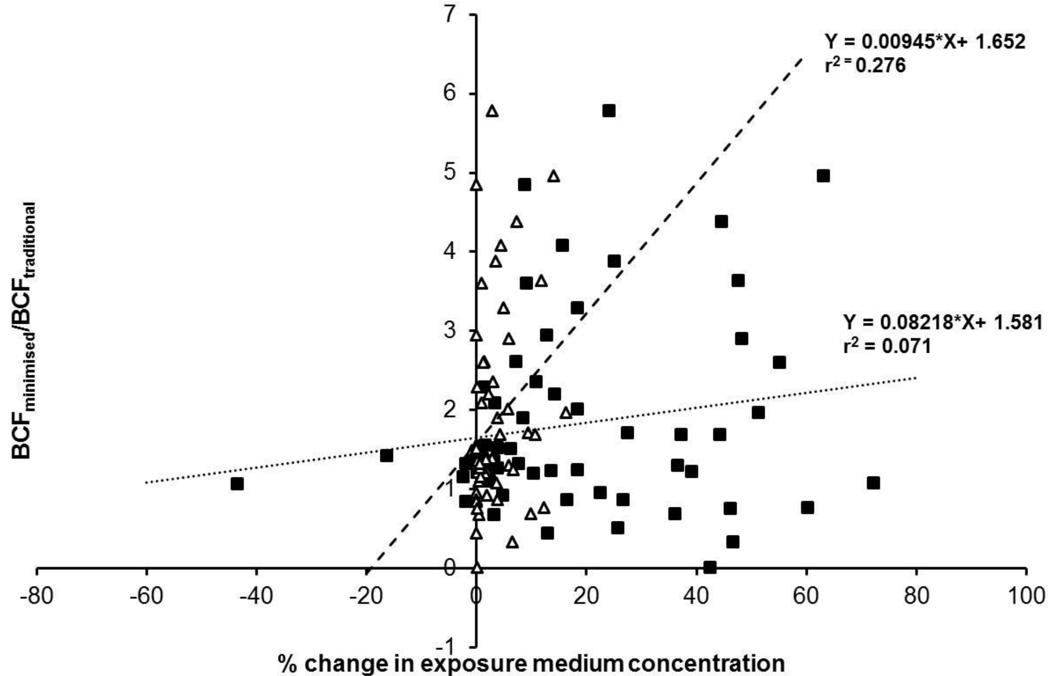


Figure 3.4 Regression between change in exposure medium concentration (% decline in exposure phase (■) and % reappearing (▲) in depuration phase) and BCF ratio. Regression lines provided by dashed lines for % decline (narrow dash) and % reappearing (wide dash).

Therefore it appears that there are no systematic errors in BCF calculation if changes in exposure medium concentration are observed, uptake is not entirely first order or if steady state has not been achieved in the test. This is important because it demonstrates the robustness of the design. When steady state does not need to be

reached this means that experiments can be shorter in terms of time scale which is advantageous with respect to time and effort costs.

3.3.2.1 Minimised further?

Further analysis explored whether the minimised design could be further reduced. Instead of taking an average of several measurements of exposure concentrations during the exposure phase, which can be up to eight sampling points for one study, an average was calculated by only using exposure medium concentrations from the start and end of the exposure phase. Using an average of these two sampling points yielded comparable $BCF_{minimised}$ values to those when a full average was used (Figure 3.5). Deming regression demonstrates that there were no systematic and no proportional differences between the two approaches (slope: confidence interval: 0.7986 – 1.036, Y-intercept confidence interval: -0.4562 – 0.8761). These results may offer an even smaller design to calculate accurate BCFs using considerably fewer materials.

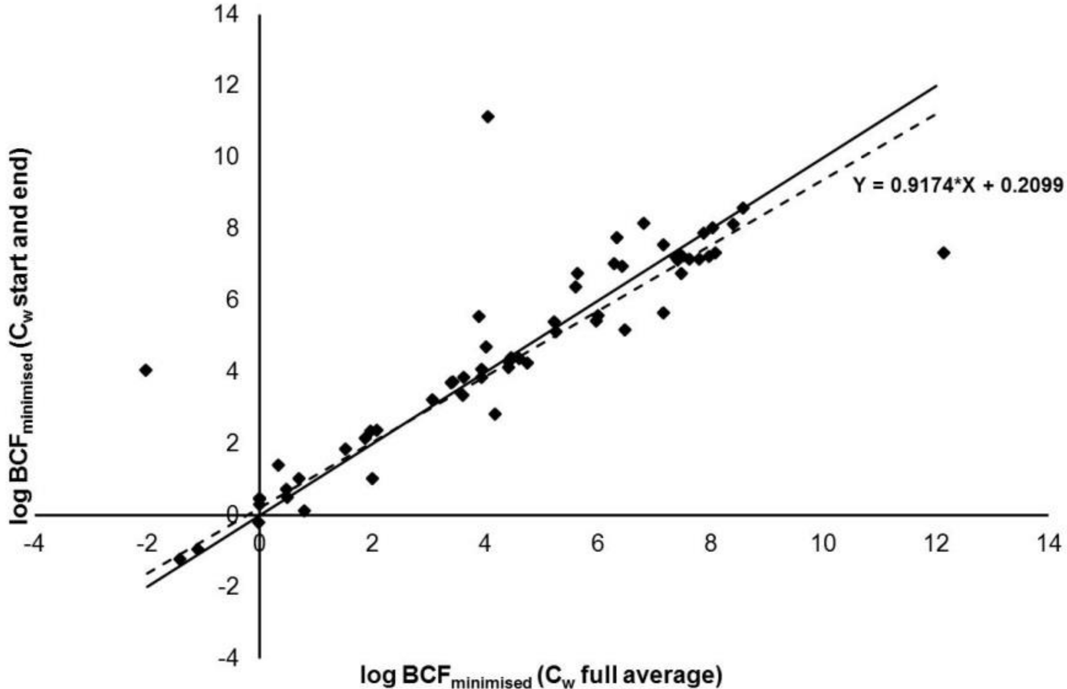


Figure 3.5 Regression between BCF values calculated using exposure medium concentration measured over a period of exposure and only the beginning and end of exposure. Regression (dash) and 1:1 (solid) lines also provided.

3.3.2.2 Study specific analysis

The $BCF_{traditional}$ were generally very small for pharmaceutical exposure in *Notonecta glauca* (0.13 – 1.60) (Meredith - Williams *et al*., 2012). The minimised design generated BCFs which were correspondingly very similar (0.17 - 2.11). However > 80 % of *N. glauca* BCFs were overestimated. This fits with the overall trend as the minimised design generally over estimates BCF values in comparison to the original published BCFs as more data points lie above the 1:1 line (Figure 3.2). At the other end of the scale, some of the largest published BCFs were obtained in the fluoxetine exposures with *Gammarus pulex* and *Lumbriculus variegatus* (< 218 500) (Table 3.2). Conversely, the corresponding $BCF_{minimised}$ for these were consistently under estimated by up to two orders of magnitude (Figure 3.2). In Figure 3.2, the data point for fluoxetine from the Meredith-Williams *et al*., (2012) study is a clear outlier. This is a result of very minimal fluoxetine elimination observed in *G. pulex* and resulted in a $BCF_{traditional}$ of 185 000 which is several orders of magnitude larger than those previously calculated in aquatic exposures for this compound (Nakamura *et al*., 2008). Following the minimised design, the corresponding BCF for this fluoxetine exposure was 1560.55.

Data from Ashauer *et al*., 2010 fitted very well to the 1:1 line, the slope of the regression line was also significantly different from zero ($p = < 0.0001$) and thus showed a significant relationship between the two methods for calculating BCF (Appendix 9). A wide range of chemicals were evaluated in this dataset with differing physico-chemical properties and BCF values which demonstrates that this is a fairly robust way to estimate BCFs with limited laboratory effort. When data for *G. pulex* from a number of publications were collected and analysed separately from the whole data set, Deming regression showed a significant relationship between the two methods with a slope of 1.082 (95 % confidence interval: 0.85 – 1.32), intercept of -0.88 (95 % confidence interval: -2.29 – 0.54) and slope significantly different from zero ($p = < 0.0001$) (Figure 3.6). A majority of data points lie below the 1:1 and whilst statistics show the minimised approach can accurately estimate BCFs this would infer that the approach generally overestimates BCFs for *G. pulex*.

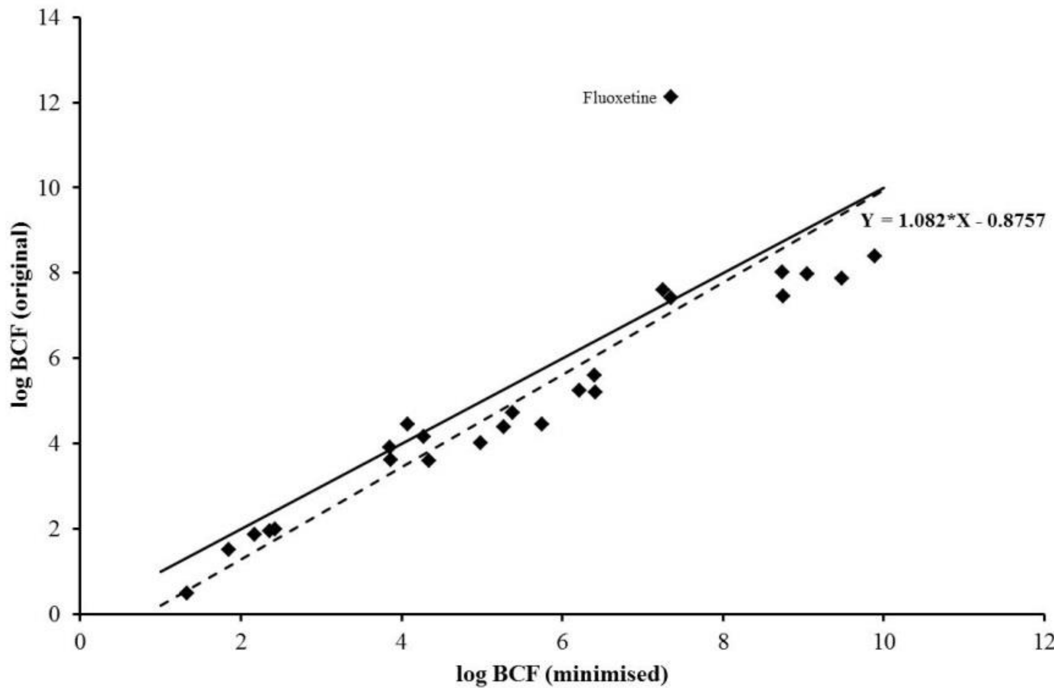


Figure 3.6 Relationship between log BCF (minimised) estimates from the minimised design and log BCF (original) obtained from the literature for *G. pulex* only. Deming regression line (dash), with equation and 1:1 line also provided (solid).

For earthworm data, correlations between BCFs presented in the original paper and those calculated by the minimised design results produced a slope of 0.77 (95 % confidence interval: 0.43 – 1.11) and intercept of 1.39 (95 % confidence interval: -12.23 – 15.01). Combined with a slope not significantly different to zero (p = 0.0105) there appears to be a good relationship between the two methods. This is interesting because the Springer approach was originally designed for aquatic BCF calculation but results presented here demonstrate that it is also probably suitable for terrestrial BCF calculations. Using the minimised design would reduce earthworm use by approximately 70 %; there would also be considerable savings in terms of time and cost of materials. There is a substantial lack of earthworm studies with regards to studying the uptake kinetics of organic chemicals, pharmaceuticals in particular and the minimised design may be an attractive option to resolve this.

Variation in data points around the 1:1 line and regression line for the Rubach *et al.*, (2010) data can be attributable to the fact that species differences are important in the uptake of chemicals (Rubach *et al.*, 2010). Specifically differences in BCFs amongst

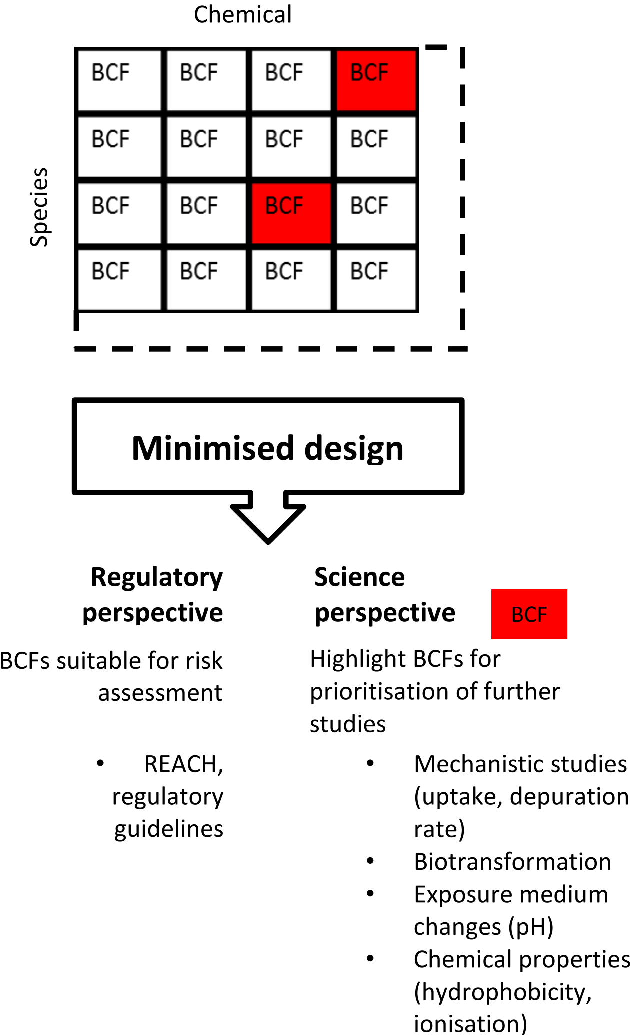
15 species of freshwater arthropods as well as between juvenile and adult species (*G. pulex* and *Procambarus* sp.) were observed in the original data set (Appendix 6). Even though all experiments consisted of a chlorpyrifos exposure only, this research demonstrates that various species at different life stages can accumulate and eliminate chemicals in differing amounts and thus generate a range of BCF values. Variation in BCF values for *L.variegatus* is also evident in the Karlsson *et al*., (2013) data set due to changing exposure medium pH affecting ionisable chemical uptake. The minimised approach seems to account well for both these factors affecting chemical uptake as the variation in the BCF values is within the general noise of the whole data set. In view of the complete data set it is evident that the variation around the 1:1 line increases as the BCF value increases (Figure 3.2). It appears that larger BCFs are subject to greater error.

Additional research has explored the use of the minimised design to estimate fish BCFs specifically for pharmaceutical exposures (Constantine, 2011). The results also yielded comparable, accurate BCFs with a reported r^2 of 0.99 and a slope of 1.02. This was shown to be a robust design as 55 pharmaceuticals were compared with BCFs ranging from 0.6 to 12 000.

3.3.3 Wider implications

As the minimised design yields very good proxies for BCFs (Figure 3.2), but poor estimates of the true uptake and elimination rate constants the minimised design may therefore offer an acceptable approach to calculate BCF values for regulatory purposes where risk assessments require BCFs to be reported within a range (Figure 3.7).

Figure 3.7 Schematic depicting potential applications of minimised design



For scientific purposes, as a result of reduced laboratory effort, the minimised design may allow for several studies to be carried out at once enabling many different parameters to be evaluated. Factors such as species differences (Meredith-Williams *et al.*, 2012; Rubach *et al.*, 2010) and effect of changing exposure medium properties (Díez-Ortiz *et al.*, 2010; Karlsson, 2013) published in recent research, require us to evaluate the uptake and bioconcentration of organic chemicals in greater depth. Combined with an increasing number of chemicals which are being discharged in the environment further research into the uptake of pharmaceuticals in particular into invertebrates are needed. As results would be cheaper and faster to generate the minimised design may reveal patterns amongst the numerous chemicals and the thousands of species exposed under a plethora of environmental conditions. Specific

exposure scenarios may therefore be highlighted which may need to be evaluated further, through toxicokinetic – toxicodynamic studies for example (Figure 3.7).

3.4 Conclusions

A comparison of BCFs generated from full study designs ($BCF_{traditional}$) and those estimated using the principles of the minimised design ($BCF_{minimised}$) would infer that the minimised design is a viable alternative approach to use. For a single experiment, test organism usage would be reduced by > 70 % as well as a reduction in experimental material and labour efforts required. Whilst the agreement between uptake and depuration rates generated by the two approaches is somewhat variable the minimised design is advantageous with respect to calculating overall BCFs. The approach is robust as steady state does not need to be achieved in the test system and BCFs are not affected by changes in exposure medium concentration. The approach therefore can provide a method to calculate reasonably good BCF estimates which may be used to determine if additional studies are required for example to explore if BCFs are concentration dependent. Care should however be taken when using the minimised design to calculate BCF values when compounds don't depurate from the organism and for rate constant determination, particularly when estimating depuration rates. It is important to note the lack of relationship between traditional and minimised rate constants. The use of toxicokinetic – toxicodynamic modelling may be more appropriate if rate constants are to be analysed as well as BCFs.

One of the most significant findings is that the minimised design appears to work well across a range of species (including both terrestrial and aquatic), chemicals and different exposure mediums offering a suitable alternative for BCF calculation in variety of environmental chemical exposure scenarios. Further analysis could explore the use of the minimised design concept for calculating additional BCFs, specifically for earthworm exposures as only a small number were collected and evaluated in the current work.

In the next Chapters, the minimised approach was therefore employed to explore the effects of environmental parameters and species type on the uptake of pharmaceuticals into earthworms.

Chapter 4 How Soil Properties Affect the Uptake of Pharmaceuticals into Earthworms

4.1 Introduction

Research presented in Chapter 2 and a small number of recent publications have demonstrated that pharmaceuticals can be taken up from soils into invertebrates such as earthworms (Kinney *et al.*, 2012, 2008). Specifically, Chapter 2 investigated the uptake and depuration kinetics of four pharmaceuticals into the earthworm *E. fetida*. Pore water based bioconcentration factors (BCFs) increased in the order of carbamazepine < diclofenac < fluoxetine < orlistat and ranged between 2.2 – 51.5. The results demonstrated that physico-chemical properties are important in the uptake of pharmaceuticals and that earthworm BCF's could not be predicted solely based on hydrophobicity of the chemical (log K_{ow}).

It is well known that the same pharmaceutical can behave very differently in different soil types (Drillia *et al.*, 2005; Monteiro and Boxall, 2009; Oppel *et al.*, 2004). For example, distribution coefficients for pharmaceuticals between soil particles and soil pore waters are known to vary by several orders of magnitude (Krogh *et al.*, 2008; Monteiro, 2009; Tolls, 2001). As diffusion across the skin from the pore water has been shown to be the primary exposure pathway for chemicals in the soil environment (Vijver *et al.*, 2003), it is therefore likely that uptake of pharmaceuticals could also vary significantly across soils.

As most pharmaceuticals are ionisable, the uptake of pharmaceuticals into organisms can also vary depending on the pH of the environment (Karlsson, 2013; Nakamura *et al.*, 2008). Different uptake from soils with different pH values could therefore be expected. Knowledge of the relationships between soil properties and pharmaceutical uptake is however very limited. There is therefore a real need to begin to generate data on the uptake of pharmaceuticals from soils with different characteristics in order to identify the key drivers affecting uptake and ultimately to develop uptake modelling approaches for use in environmental risk assessment.

The study described in this chapter was therefore peformed to explore the effects of soil properties on pharmaceutical uptake and depuration in the earthworm *Eisenia fetida*, and help elucidate the relationships between soil properties and uptake. The study focused on four pharmaceuticals, from a variety of therapeutic uses and covering a range of physico-chemical properties. To help explain any potential differences in uptake and depuration, parallel studies were performed to assess the fate and distribution of the study pharmaceuticals in test soils.

4.2 Materials and Methods

4.2.1 Pharmaceutical compounds and reagents

All studies were performed using ^{14}C labelled compounds. Labelled fluoxetine [methyl-^{14}C] and carbamazepine [carbonyl-^{14}C] were obtained from American Radiolabelled Chemicals (*Missouri, USA*), diclofenac [U – ^{14}C] was obtained from Perkin Elmer (Boston, USA) and orlistat [tridecanyl-2-^{14}C] was provided by GlaxoSmithKline (GSK, UK). Physico-chemical properties and specific activities for the pharmaceuticals can be found in Table 1.3. Acetonitrile (99.9 %), methanol (99.9 %) and ethyl acetate (99.9 %) were obtained from Fisher Scientific (Loughborough, UK).

4.2.2 Test soils

Five standard test soils were obtained from LUFA Speyer, Germany (Figure 4.1). The soils, 2.1, 2.3, 2.4, 5M and 6S, included clayey loam, silty sand and loamy sand varieties and were chosen to provide a range of soil characteristics including varying soil pH, organic carbon content, cation exchange capacity and particle size distributions (Table 4.1). Soils were air dried and sieved to 2 mm prior to testing.

Figure 4.1 Test beakers containing five different test soils (2.1, 2.3, 2.4, 5M and 6S) for earthworm exposures prior to addition of earthworms.

Table 4.1 Soil properties for the standard test LUFA Speyer soils. Mean values of different batch analyses are provided ± standard deviation (SD).

Standard soil type	2.1	2.3	2.4	5M	6S
Organic carbon in % C	0.65 ± 0.10	0.94 ± 0.10	2.26 ± 0.25	1.00 ± 0.2	1.64 ± 0.12
Nitrogen in % N	0.05 ± 0.01	0.08 ±0.02	0.2 ±0.04	0.11 ± 0.02	0.2 ± 0.02
pH value (0.01 M $CaCl_2$)	5.1 ±0.3	6.8 ±0.2	7.2 ± 0.2	7.3 ± 0.1	7.1 ± 0.1
Cation exchange capacity (meq/100g)	4.3 ± 0.5	10.9 ±1.1	31.4 ± 4.6	16.6 ± 2.8	27.2 ± 1.4
Soil type	Silty sand	Silty sand	Clayey loam	Loamy sand	Clayey loam
Water holding capacity (g/100g)	31.1 ± 2.1	37.3 ± 1.8	44.1 ± 1.2	39.5 ± 2.9	40.5 ± 2.1
Particle size (mm) distribution according to USDA (%)					
< 0.002	2.8 ± 1.1	8.5 ± 1.7	25.9 ± 2.1	11.1 ± 1.2	40.5 ± 2.1
0.002 – 0.05	10.2 ± 1.8	28.4 ± 4.5	40.5 ± 1.0	29.7 ± 2.8	35.0 ± 2.9
0.05 – 2.0	87.0 ± 1.5	63.1 ± 5.0	33.6 ± 1.8	59.2 ± 3.2	24.5 ± 3.5

4.2.3 Test organism

E. fetida were obtained from Blades Biological Ltd (Kent, UK) and cultured in a medium of peat and cow manure (50:50), kept moist with deionised water at room temperature (20 ± 3 ^{o}C). The organisms were fed twice weekly with homogenised mashed potato powder. *E. fetida* were obtained from a single species culture and cultures were maintained for at least four generations before being used in the uptake studies. The lipid content of *E. fetida,* determined using the method of Folch *et al*., (1957), was 5.11 ± 0.29 % (wet weight) (Chapter 2).

4.2.4 Fate studies

Triplicate beakers of each test soil (2.1, 2.3, 2.4, 5M and 6S) (35 ± 1 g) were prepared to sample at eight time points (0 and 6 h, 1, 3, 7, 10, 14 and 21 d) where pore water and soil samples would be analysed to allow for determination of the distribution of chemicals in the soil matrices over time. To each of the five soils, labelled pharmaceuticals were added using 125 – 165 µl of a carrier solvent to give concentrations of 26, 25, 28 and 44 µg kg^{-1} of carbamazepine, diclofenac, fluoxetine and orlistat respectively. For carbamazepine and fluoxetine, ethanol was used as the carrier solvent; for diclofenac, methanol was used and orlistat was applied in acetonitrile. After spiking, each test beaker was left for 2 h and then mixed to create an even distribution of the pharmaceutical within the sample. Following spiking and mixing, the carrier solvents were allowed to evaporate for 48 hours. Blank and solvent controls were also prepared free from test chemical. Following preparation, the moisture content of all soils was adjusted to 40 – 60 % of the MWHC by addition of deionised water. All beakers were incubated in a growth chamber at 20 ± 2 ^{o}C, using a 16:8 light/dark cycle [600 lx], and at 60 % humidity.

At each sampling point, soil was sampled for pH analysis and determination of pharmaceutical residues and pore water was extracted from the respective beakers using the method outlined in Chapter 2. Briefly, syringe’s containing 25 ± 5 g of test

soil were centrifuged for 40 minutes at 3000 rpm after which the pore water was collected from the bottom of the tube and transferred to a 2 mL Eppendorf tube. The Eppendorf tubes were then further centrifuged at 12000 rcf for four minutes to sediment any loose particles. A 1 mL sample of pore water was then added to 10 mL of EcoScint A scintillation cocktail for analysis. The pH of all soil and pore water (with the exception of carbamazepine) samples was measured using a Hanna pH electrode (HI-1093B) (Figure 4.2).

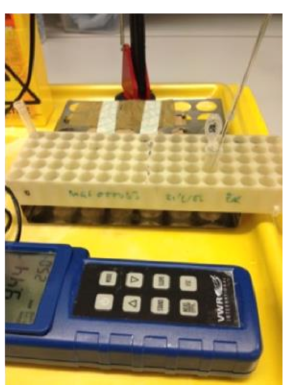


Figure 4.2 Pore water extractions of test soils after centrifugation (A) and pH analysis of pore water samples (B).

4.2.5 Uptake and depuration studies

The uptake and depuration studies followed the 'minimised' approach described in Chapter 3. Earthworms were exposed in glass jars containing 50 ± 1 g of each test soil. For each test, soil beakers were prepared and spiked with the four pharmaceuticals at similar concentrations and following similar methods to those in the fate studies. Adult *E. fetida* (200 - 500 mg) were then added to each test beaker after having been acclimatised under experimental conditions for 48 h in non-treated test soil. After addition, the time it took for each earthworm to completely burrow into the soil was noted. For each soil type, blank and solvent controls were prepared. Earthworm beakers were incubated in the growth chamber and moisture adjustments were performed as reported in the fate study. For each pharmaceutical treatment in each soil type, six replicates were sampled at the end of the uptake period (21 d) and six at the end of the depuration phase (42 d). *E. fetida* were then removed from the

vessels, and transferred to moist filter paper for 24 h to allow them to purge their guts. The worms were then frozen until analysis.

4.2.6 Preparation of samples for analysis

Soil and earthworms were extracted using methods similar to those outlined in Chapter 2. Briefly, soil samples were extracted by liquid extraction. For the carbamazepine study, 5 ± 0.5 g of soil was extracted twice for 45 minutes 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 a mixture of acetonitrile and water (7:3 v/v) and acetonitrile only were used as solvents, respectively. For the diclofenac study, 5 g samples of soil were extracted three times for 45 minutes with 3 x 10 mL ethyl acetate. Samples (1 mL) of extracts were then added to 10 mL of EcoScint A for analysis of the radioactivity present.

As previous work has shown that orlistat and diclofenac form irreversibly bound residues with soil (Chapter 2); combustion analysis of these soil samples was also performed using a Perkin Elmer 307 Sample Oxidiser according to similar methods outlined in 2.3.3.1.

E. fetida samples were defrosted and the internal pH of each worm was measured using a Thermo Scientific Orion pH microelectrode. Each worm was dissected across the segments in the direction from the anterior to the posterior. The pH probe was then inserted directly into the earthworm tissue taking care to avoid internal organs and the digestive tract. *E. fetida* were then extracted by liquid extraction using the same solvents as for the soil extractions. For each worm, 5 mL of solvent was added and the worm/solvent mix was homogenised for 5 minutes using a LabGen Series 7 homogeniser. The suspension was transferred to a glass test tube and the beaker was then rinsed with an additional 3 ml of solvent which was combined with the original suspension to give a total volume of 8 mL. This was centrifuged at 415 g for 30 minutes (CHRIST Rotational Vacuum-Concentrator RVC 2-33 CD) and a 1 mL sample of the supernatant was then added to 10 mL of EcoScint A for analysis of the radioactivity present.

Method validation studies showed that average recoveries ranged from 72.43 to 94.72 % for the pharmaceuticals in the five different soil types (detailed recovery information provided in Appendix 10). Recoveries ranged from 86.3 (fluoxetine) to 100.9 (carbamazepine and diclofenac) % for the earthworm extraction methods.

4.2.7 Liquid scintillation counting

Radioactivity in the soil pore water and soil and worm extracts were determined using Liquid Scintillation Counting (LSC) using a Beckman LS 6500 LSC counter (Beckman Coulter Inc., Fullerton, USA). Samples were counted three times for 5 min. Counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected for using the external standard ratio method.

4.2.8 Calculating BCF - kinetic modelling

Measured radioactivity of the corresponding pharmaceuticals in the earthworm extracts were corrected to account for soil-associated pharmaceuticals present in the gut after gut purging (see section 2.3.3 for more detail). Based on minimised design principles outlined in Chapter 3, earthworm tissue concentrations were then used to calculate uptake and depuration rates for the study compounds in each soil type using Equation 3 and Equation 4. The uptake and depuration rates were then used to estimate pore water based kinetic bioconcentration factors (BCFs) (Equation 5). For a full explanation of BCF calculations see section 3.2.2.

4.2.9 Calculating soil BSAF

Soil based bioaccumulation factors (BSAF) were estimated from the pore water based BCFs for all pharmaceuticals using soil water partition coefficients (K_d) calculated from fate studies (Equation 6). The K_d value used for each compound was an average K_d calculated across the different sampling points in the uptake phase.

$$BSAF\ soil\ = \frac{BCFpw}{Kd}$$ **Equation 6**

Regression analysis was then performed to compare BSAF values and soil properties and BCF values and pore water properties.

4.2.10 Statistical analysis

Statistical analysis of the data was performed on SigmaPlot (v .12). A two-way analysis of variance (ANOVA) with a significance level of 0.05 was performed, keeping study type (blank/treatment) as repeated and time as a variable factor. Endpoints tested included the differences in soil and pore water pH across time, and in comparison to control samples, after which additional pair-wise comparisons of the data were performed according to the Holm-Sidak method. Further two-way ANOVAs were performed to check differences in soil and pore water pH measurements made in the same soil type but under different pharmaceutical treatments over time. A one-way ANOVA was employed to assess differences in internal pH values of the worms in comparison to the controls and between uptake and depuration measurements. A three–way ANOVA was used to check for changes in internal pH for worms exposed in the same soil but under different pharmaceutical treatments at both the end of the uptake and depuration phases. Additionally, for each pharmaceutical, data on the burrowing times of *E. fetida* were tested against the control treatment burrowing times using a one-way ANOVA to assess the differences in the values among the treatment groups. Prior to all tests, normal distribution and equal variance were tested by performing a Shapiro–Wilk and Levene–Mediane test, respectively. If the normality test failed then the one-way ANOVA was instead performed on ranks.

4.3 Results

4.3.1 Fate studies

Measurements of radioactivity in the soil and pore water changed over time and these changes appear to be dependent on pharmaceutical compound and in a number of cases, on soil type (Figure 4.3). By 21 d radioactivity was detected in all treatment beakers in all soil types. In most soil types, measured radioactivity tended to decrease after 1d, however in soil 6S in the fluoxetine study, measured radioactivity increased from 0.017 to 0.021 mg/kg over the period of the uptake phase. Carbamazepine was fairly persistent in all soil types whilst initial results showed rapid dissipation of diclofenac and orlistat from the test beakers. However, combustion analysis confirmed the formation of nonextractable (bound) residues (NER's) in both the diclofenac and orlistat studies. NER fractions increased from 0.005 to 0.028 mg/kg and 0.012 to 0.032 mg/kg for orlistat and diclofenac respectively (Appendix 11).

Pore water concentrations of the pharmaceuticals differed to a greater extent, depending on soil type, in comparison to the soil concentrations (Figure 4.3). Soil 2.1 generally had the highest pore water concentrations for all pharmaceuticals while soil 2.4 generally had the lowest concentrations. From 10 d onwards pore water concentrations tended to decrease in all soil types especially for diclofenac, fluoxetine and orlistat. This was most evident in soil 2.1 for all pharmaceuticals.

The soil – water distribution (K_d) appears to be chemical specific and were affected by soil properties as there was a range of K_d values for each pharmaceutical in the five different soil types; namely carbamazepine (1.34 – 4.45 L/kg), diclofenac (5.63 – 18.37 L/kg), fluoxetine (55.48 – 71.44 L/kg) and orlistat (28.99 – 110.01 L/kg). Over the initial 10 d of the uptake phase, orlistat became less strongly bound to the soil as the amount recovered in the solvent extraction increased whilst the combustion analysis concentrations decreased. Interestingly, following this change, orlistat pore water concentrations began to decrease which coincided with a significant decrease in the pH of these pore water samples from all five soil types.

Additional changes in both soil and pore water pH over time, and in comparison to the controls were noted as a result of the presence of the pharmaceuticals (diclofenac, fluoxetine and orlistat) in the soil matrix (Figure 4.4). Whilst these changes appeared to be influenced by soil type it is important to note these changes were not statistically significant for all soil types and were not consistent over time.

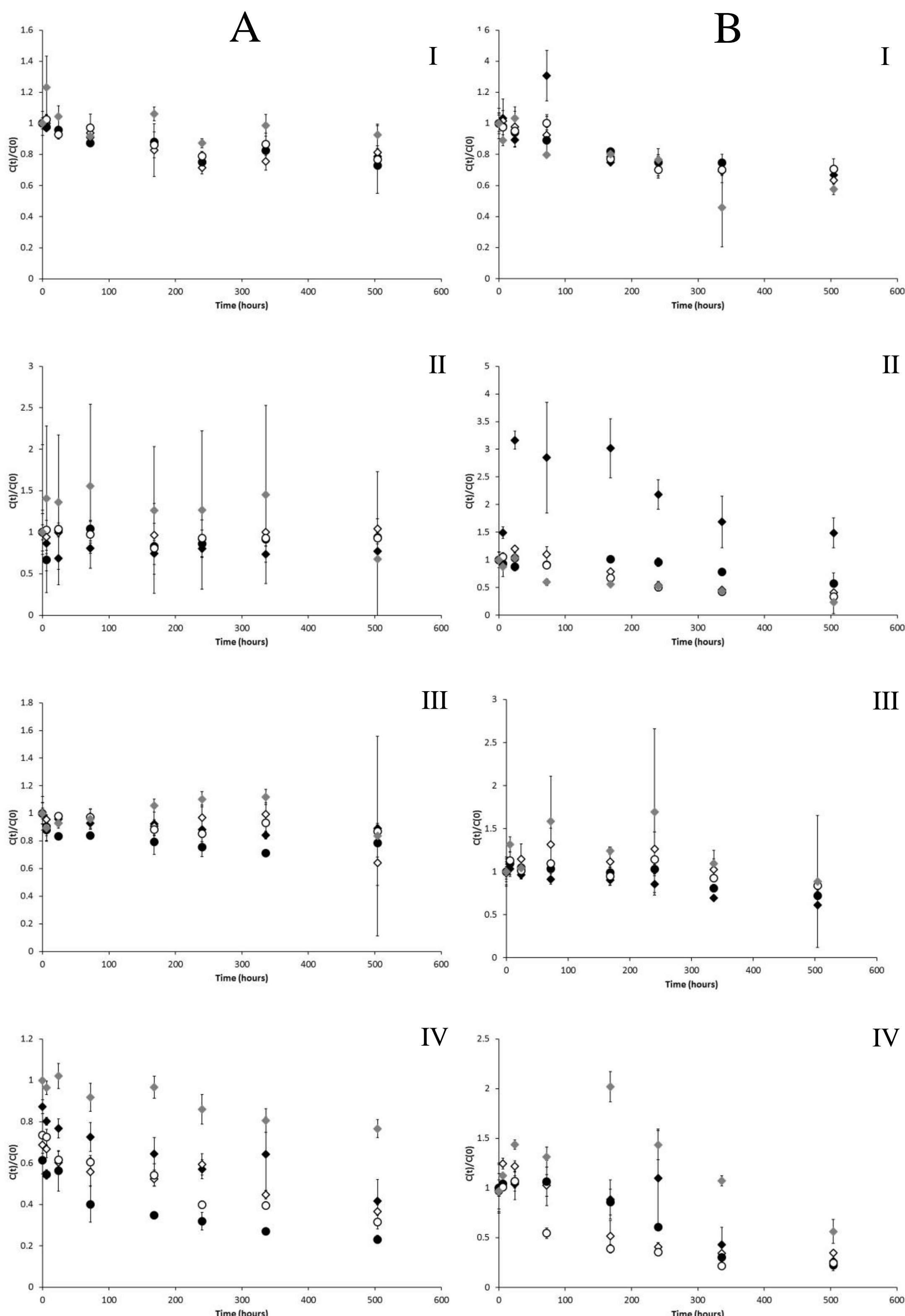


Figure 4.3 Dissipation of activity for soils treated withcarbamazepine (I), diclofenac (II), fluoxetine (III) and orlistat (IV) in soil (A) and pore water (B) throughout 21 day in five different soil types (2.1◆ , 2.3◇, 2.4 ●, 5M ○and 6S ◆. Average C(t)/C(0) ratio provided with ± standard deviation (*n* = 3), where C(t) is concentration at time of sampling and C(0) is concentration at 0 d.

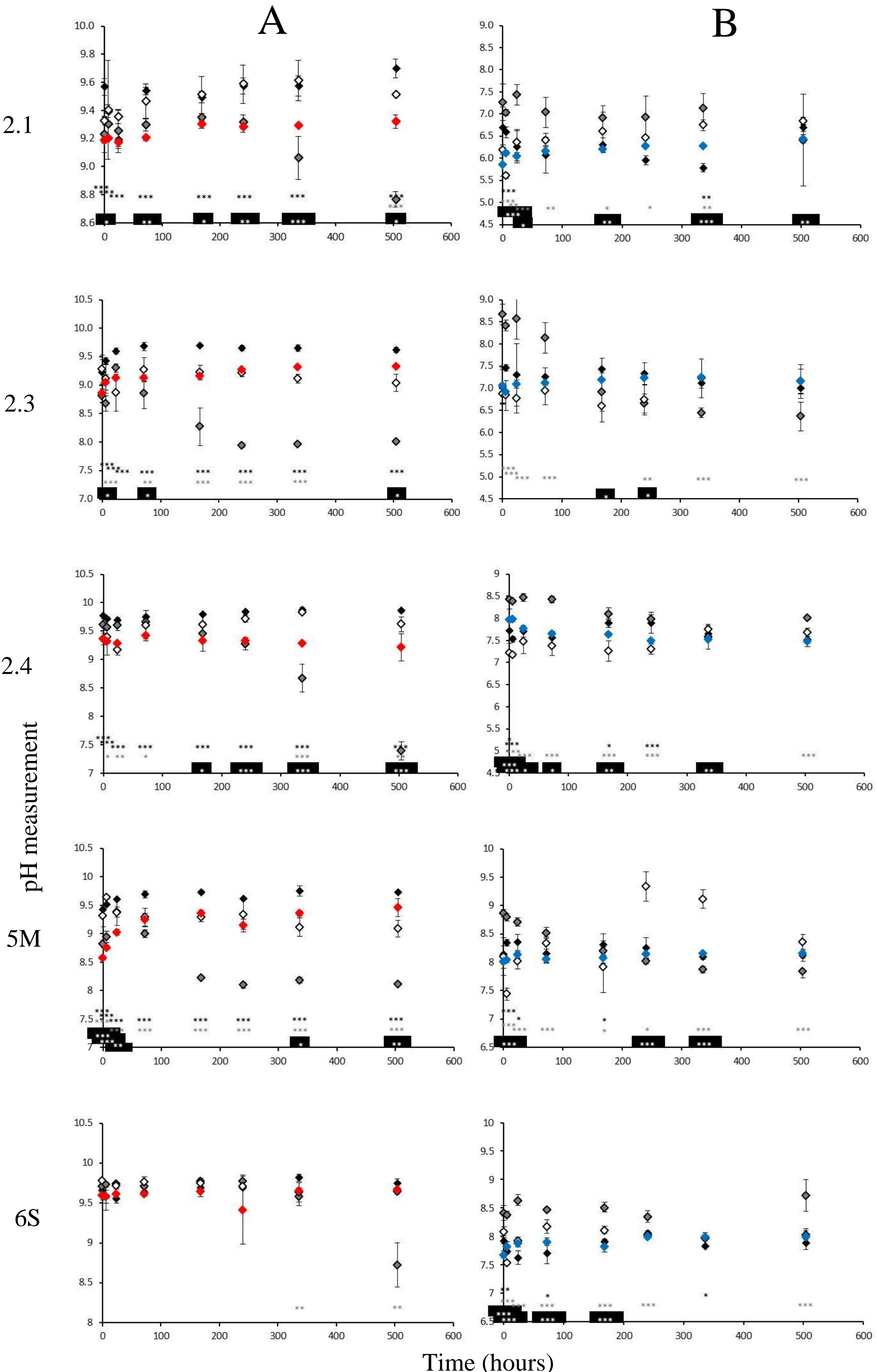


Figure 4.4 pH measurements made during the uptake phase in pore water (A) and soil samples (B) from the five soils (2.1, 2.3, 2.4, 5M and 6S) for the diclofenac (black diamonds), fluoxetine (white diamonds) and orlistat

(grey diamonds) studies. Mean pH values provided ± standard deviation (n = 3). Statistical analysis results from Holm-Sidak pair wise comparison provided (corresponding star colour to pharmaceutical treatment) ($p < 0.05$).

4.3.2 Earthworm uptake

Uptake into *E. fetida* from the four treatments, carbamazepine, diclofenac, fluoxetine and orlistat was seen from the five soils. Fluoxetine had the greatest uptake rate (*k1*) in all soils (1.138 – 2.351 L/kg d^{-1}) apart from soil 5M where orlistat had a higher uptake rate (1.477 L/kg d^{-1}), whilst carbamazepine had the fastest depuration rate (*k2*) in all five soils (0.16 – 0.243 d^{-1}) (Table 4.2). This is comparable to previous work in a single soil type (Chapter 2; 2.4.3.3) where fluoxetine had the fastest uptake rate and carbamazepine had the fastest depuration rate in *E. fetida.* Highest pore water-based BCFs were observed for orlistat (< 115.92) and the smallest BCFs for carbamazepine. Differences in BCFs were observed for the different soil types, especially in the diclofenac (7.02 – 69.57) and orlistat studies (30.51 – 115.92), whereas smaller variability of the BCFs was noted for fluoxetine (16.78 – 20.42) and carbamazepine (1.05 - 1.61) (Table 4.2).

Calculating soil pore water distribution coefficients (K_d) allowed the conversion of the pore water BCFs to soil based bioaccumulation factors (BSAFs) based on soil K_d values (Table 4.2). BSAFs were generally low (< 2), especially for carbamazepine and fluoxetine. Similarly to the BCF, the diclofenac exposure resulted in the largest range of BSAFs, up to 12.36 in soil 5M. Only very weak or no correlations we found between uptake and soil properties including properties such as organic carbon content, cation exchange capacity, soil concentrations and soil pH. Similarly individual pore water properties were unable to adequately explain uptake as weak relationships were observed between BCFs and pore water properties.

Table 4.2 Results from minimised design experiments in five soil types showing measured *E. fetida* concentration at the end of 21 d uptake phase (C_{t1}) and 21 d depuration phase (C_{t2}) and mean concentration of pharmaceutical in the pore water during the uptake phase (C_{pw}). Calculated uptake (*k1*) and depuration rates (*k2*) are presented along with pore water based BCF values derived using the minimised design approach. Soil/water adsorption coefficients (K_d) are also provided with soil BSAF estimates based on K_d values.

	C_{t1} mg/kg (internal)	C_{t2} mg/kg (internal)	Mean C_{pw} (mg/L) in uptake phase	k_2 (dep. rate) (d^{-1})	k_1 (uptake rate) (L/kg d^{-1})	Pore water BCF	Soil K_d (average 21 d)	Soil BSAF (based on K_d)
Carbamazepine								
LUFA 2.1	0.0243	0.0005	0.0191	0.187	0.243	1.30	1.34	0.97
LUFA 2.3	0.0089	0.0001	0.0059	0.243	0.372	1.53	3.87	0.40
LUFA 2.4	0.0082	0.0001	0.0052	0.215	0.345	1.61	4.45	0.36
LUFA 5M	0.0110	0.0002	0.0107	0.200	0.210	1.05	2.20	0.48
LUFA 6S	0.0112	0.0004	0.0075	0.160	0.249	1.56	3.44	0.45
Diclofenac								
LUFA 2.1	0.0567	0.0320	0.0046	0.027	0.777	28.56	6.88	4.15
LUFA 2.3	0.0047	0.0043	0.0043	0.004	0.054	15.04	7.25	2.07
LUFA 2.4	0.0043	0.0037	0.0013	0.008	0.175	21.50	18.37	1.01
LUFA 5M	0.0047	0.0047	0.0052	0.001	0.043	69.57	5.63	12.36
LUFA 6S	0.0093	0.0058	0.0035	0.023	0.159	7.02	6.37	1.10
Fluoxetine								
LUFA 2.1	0.0105	0.0009	0.0006	0.115	2.351	20.42	55.48	0.37
LUFA 2.3	0.0077	0.0013	0.0005	0.084	1.651	19.74	64.85	0.32
LUFA 2.4	0.0038	0.0009	0.0003	0.068	1.138	16.78	71.44	0.19
LUFA 5M	0.0059	0.0017	0.0004	0.059	1.128	19.18	64.06	0.29
LUFA 6S	0.0049	0.0005	0.0003	0.108	1.829	16.89	58.17	0.29

Table 4.2 continued

	C_{t1} mg/kg (internal)	C_{t2} mg/kg (internal)	Mean C_{pw} (mg/L) in uptake phase	k_2 (dep. rate) (d^{-1})	k_1 (uptake rate) (L/kg d^{-1})	Pore water BCF	Soil K_d (average 21 d)	Soil BSAF (based on K_d)
Orlistat								
LUFA 2.1	0.0284	0.0139	0.0018	0.034	1.039	30.51	28.99	1.05
LUFA 2.3	0.0138	0.0114	0.0007	0.009	1.051	115.92	75.10	1.54
LUFA 2.4	0.0086	0.0063	0.0004	0.015	1.092	74.40	110.01	0.74
LUFA 5M	0.0138	0.0065	0.0006	0.036	1.477	40.82	84.59	0.48
LUFA 6S	0.0092	0.0079	0.0010	0.007	0.485	68.36	51.30	1.33

There was a statistically significant difference in internal *E. fetida* pH after exposure to pharmaceuticals in comparison to control earthworms; however this was not true for all soil types except in the fluoxetine study (Figure 4.5). Significant differences were also observed between measurements made on the uptake and depuration samples. Interestingly, not only does the internal pH change between different soil types it was also significantly different between different pharmaceutical treatments in a single soil type at the end of the uptake phase (except soil 2.3 and 5M (p = < 0.001 – 0.003)) and the end of the depuration phase (except soil 2.1 and 2.3 (p = <0.001).

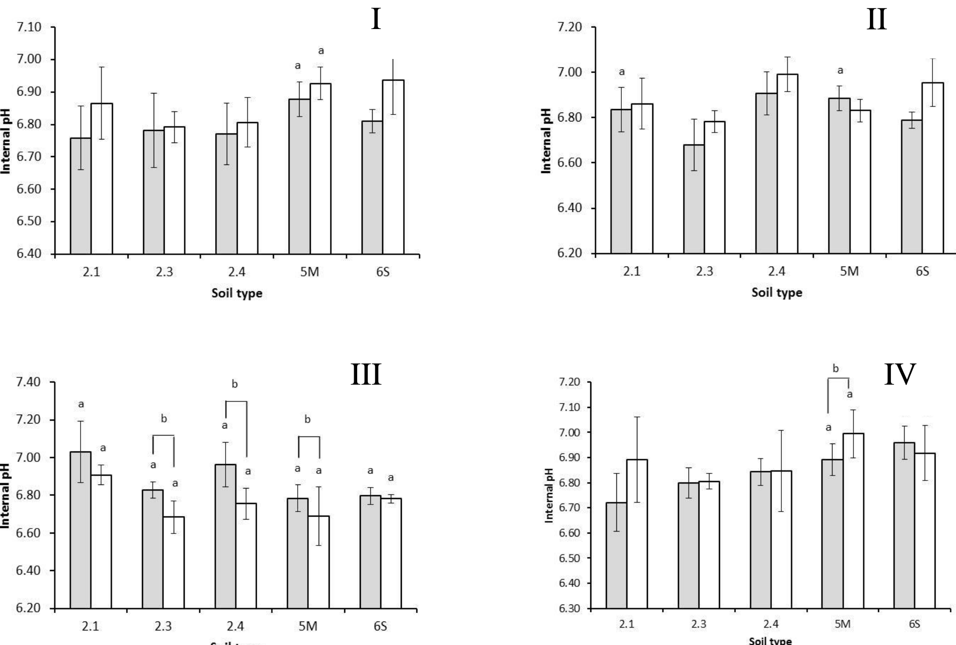


Figure 4.5 Average internal pH measurements (n = 6, ± standard deviation) of *E. fetida* exposed to pharmaceuticals (I = carbamazepine, II = diclofenac, III = fluoxetine, IIII = orlistat) in five different soil types. Grey bar shows measurement in uptake phase and white bar shows measurements made in depuration phase with error bars providing ± standard deviation. Where (a) there is statistically significant ($p < 0.05$) difference of in comparison to blank controls and (b) is a statistically significant ($p < 0.05$) difference between the uptake and depuration phases.

4.4 Discussion

4.4.1 Pharmaceutical fate in soils

In agreement with previous research and results from Chapter 2 carbamazepine was fairly persistent in all soil types (Kinney *et al*., 2006; Monteiro and Boxall, 2009; Williams *et al*., 2006). Conversely a decline in radioactivity was measured in the diclofenac study, reasons for this include volatilisation or a small proportion of the chemicals may have dissipated from the test system; a result perhaps of mineralisation (Figure 4.3). The K_d values reported in Table 4.2 fall within the ranges found in previous research for carbamazepine (0.49 – 37 L/kg,(Drillia *et al*., 2005)) and diclofenac (1.21 – 17.72 L/kg, (Xu *et al*., 2009c)) however lower than previously observed for fluoxetine (992 – 2546 L/kg, (Kwon and Armbrust, 2008)). For orlistat the K_d values are considerably lower than recorded in our own batch sorption experiments at 1494 L/kg (reported in Chapter 2).

Other than research primarily on veterinary antibiotics (Heise *et al*., 2006; Schmidt *et al*., 2008) this is some of the first work to demonstrate that human pharmaceuticals can form irreversibly bound residues with soil and the degree of NER can be influenced by soil type (Appendix 11). Previous work has shown non extractable pesticide residues remain bioavailable for uptake by earthworms and thus NERs may be contributing to some of the uptake observed in this study (Gevao *et al*., 2001). To the best of our knowledge, this is also some of the first research which demonstrates that soil and pore water pH can change after addition of chemicals to the soil environment. Both pharmaceutical physico-chemical properties and soil type appear to influence the degree of pH change, as changes in comparison to the controls and over time was not consistent across all five soil types (Figure 4.4). Further analysis should explore this with a wider range of chemicals and soil types.

Interestingly, the pore water pH measurements were not as you would expect from the corresponding soil pH for the range of soils evaluated. Pore water pH was consistently higher than soil pH and higher than pore water pH measurements made from floodplain sites contaminated with metals in a previous study which ranged between 7.51 – 7.88 (Vijver *et al*., 2007). In the fluoxetine study, changes in pore

water pH would result in a range between 36 to 64 % of the ionised fraction of fluoxetine. The observed changes in pH of soil and pore water samples would have minimal effect on the ionised percentage of diclofenac as it would remain extensively ionised (> 99 %) in the pH range of soils (Appendix 12). Similar results were observed in the fluoxetine study as soil pH changes would have had little change on the extent of ionisation of the parent compound (Appendix 13).

The environment comprises of a wide range of ionisable chemicals and different soil types and these initial results may have considerable impact on environmental modelling scenarios, which currently do not account for changes in pH. Changes in soil and pore water pH may have significant effects on the fate of chemicals in the terrestrial environment through processes such as sorption, leaching and degradation and should be considered in a modelling framework (Franco *et al*., 2009; Kah and Brown, 2006).

4.4.2 Relationships between soil and pore water properties with earthworm uptake

4.4.2.1 Soil based BSAF

Regression analysis between various soil properties and BSAF values failed to highlight key factors which may be responsible for pharmaceutical uptake into worm (Figure 4.6). Previously clay and organic matter content have been shown to influence bioavailability of organic pollutants in soils (Chung and Alexander, 1998; Weber and Weed, 1968; White *et al*., 1997; White, 1976). Research has shown greater earthworm uptake of phenanthrene in soils with higher clay content (White *et al*., 1997) however this was not observed with soil BSAF values calculated in this study.

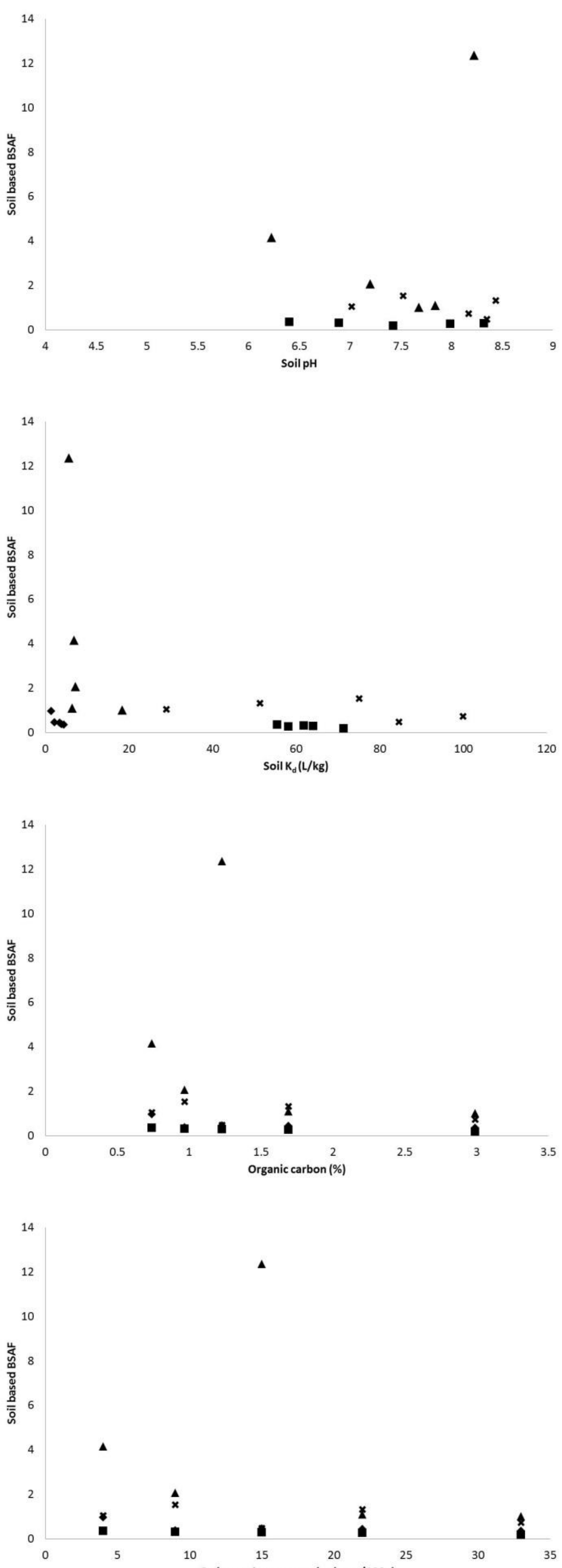


Figure 4.6 Regression plots between soil based BSAFs calculated in this study (carbamazepine – diamond, diclofenac – triangle, fluoxetine – square and orlistat – cross) and selected soil properties including soil pH, soil distribution coefficient (K_d), organic carbon content and cation exchange capacity.

This study used soils with an environmentally realistic pH range (6.6 - 8.2) (Figure 4.6). Therefore, this may account for the lack of clear effect of soil pH on the uptake of pharmaceuticals into earthworms as the pH range was fairly small. Where differences in $BSAF_{total}$ were observed in the diclofenac study (Table 4.2) and significant differences in soil pH between the five soil types were measured (Figure 4.4); diclofenac was always extensively ionised (> 99 %) and no relationship between $BSAF_{total}$ and soil pH were found. Additional studies could explore pharmaceutical exposure in soils with a wider pH range as research has shown *E.fetida* can survive in soils between pH 4.3 – 7.5 (Sims and Gerard, 1985).

As clear relationships with soil properties and earthworm BSAFs were unable to be found, it would suggest earthworm uptake is a complex interaction of a variety of factors and processes and does not exclusively rely on a single soil parameter. In addition, previous research has shown the ingestion of soil particles plays a minor role in the accumulation of chemicals (log $K_{ow} < 6$) into earthworm tissues (Jager *et al.*, 2003; Vijver *et al.*, 2003) and thus may contribute to the lack of clear relationships between soil based BSAFs and soil properties for carbamazepine, diclofenac and fluoxetine.

Instead, for a large proportion of chemicals uptake via diffusion across the earthworm skin dominates (Jager *et al.*, 2003; Vijver *et al.*, 2003). Therefore, understanding pore water properties may be a more appropriate approach to evaluate uptake. For this reason considering the sorption of pharmaceuticals is important as this will determine how much of the chemical is in the pore water and not sorbed to the soil surface.

4.4.2.2 Pore water based BCFs

Only weak relationships were found between pore water properties such as pH and pore water based BCFs. Unlike studies in the aquatic environment which found clear relationships between pH of the exposure medium and BCFs for ionisable chemicals (Karlsson, 2013; Nakamura *et al.*, 2008), the results presented in this chapter demonstrated that, like soil pH; pore water pH cannot account solely for the

differences in the accumulation of ionisable pharmaceuticals into earthworms (Figure 4.7).

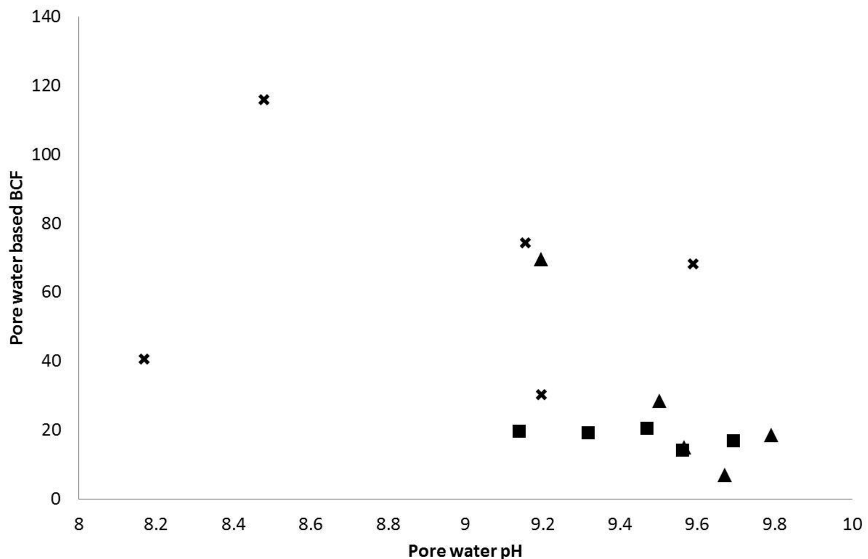


Figure 4.7 Regression plot between porewater pH and pore water based bioconcentration factors obtained in this study (diclofenac – triangle, fluoxetine – square and orlistat – cross).

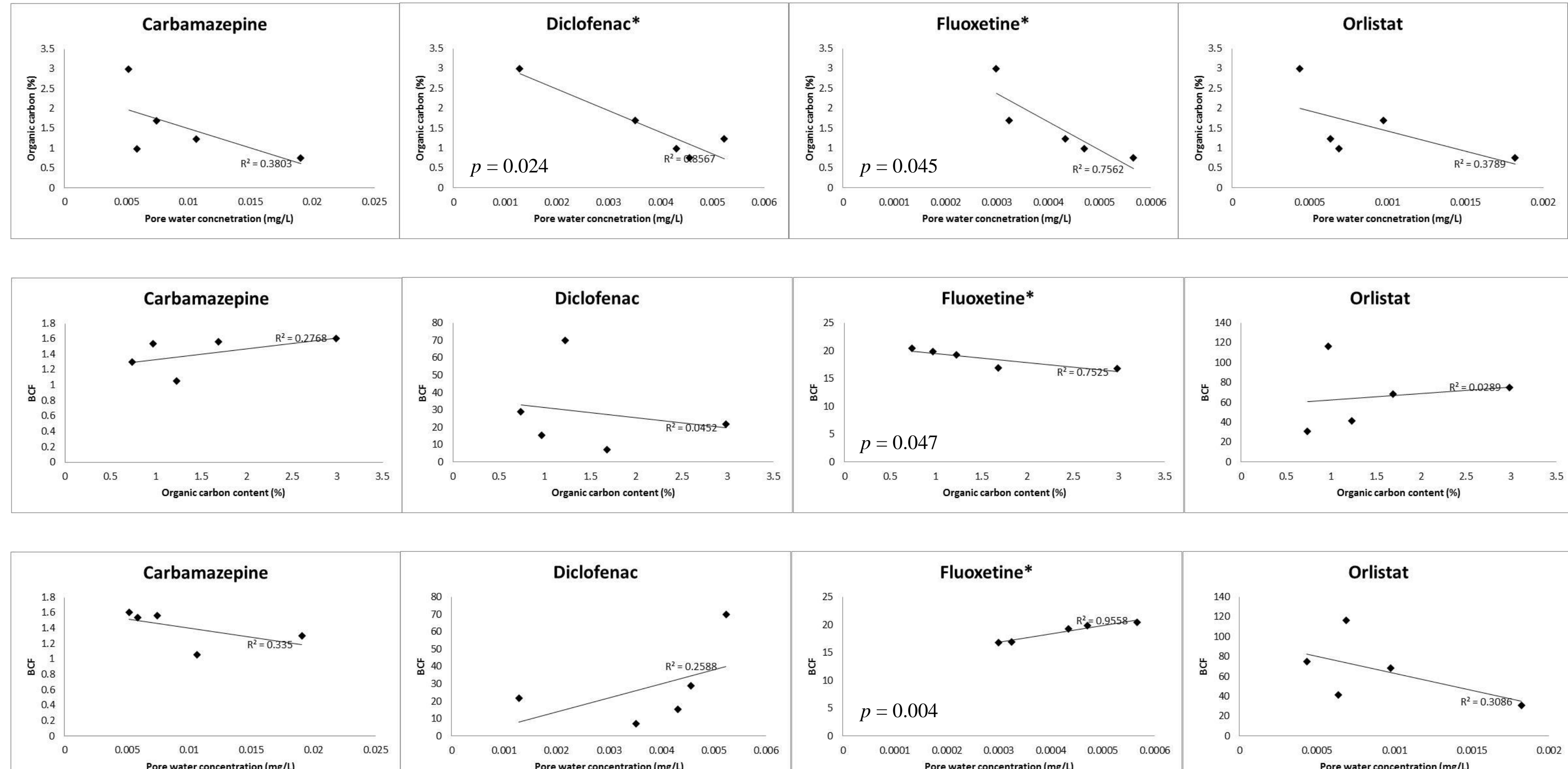
Regarding earthworm exposures specifically cadmium uptake from pore water was found to be pH dependent (Oste *et al*., 2001). In the current study, only a slight relationship was observed in the diclofenac exposure as the BCF increased with decreasing pore water pH. Even though the pore water pH was significantly different across soil types, diclofenac was always extensively ionised (>99 %) and therefore changes in pore water pH and the subsequent fraction of ionised:netural species is not expected to be solely responsible for controlling pharmaceutical uptake (Appendix 12, Appendix 13).

Highest internal concentrations were observed in exposures which had the highest pore water concentration of the respective pharmaceutical and therefore would suggest the bioavailability of chemicals in pore water is a limiting factor in earthworm uptake. For all pharmaceuticals, this was in soil 2.1, whilst soil 2.4 generally had the lowest pore water concentrations (Figure 4.3). However high internal concentrations at the end of the exposure does not necessarily translate into highest BCFs as other factors are at play such as depuration rates each of which are specific to an exposure scenario.

As pore water concentrations are clearly important, changing the soil moisture content has been suggested to enhance the sorption of some pesticides to soils, with a decreased moisture content potentially leading to partitioning of chemicals from bio-available fractions in the pore water to less accessible sites (Shelton and Parkin, 1991; Shelton *et al*., 1995). However as the moisture content was monitored daily and kept at around 60 % of the MWHC for each soil type this is an unlikely explanation as to the observed differences in uptake in this study.

Clearly many factors and processes in both the pore water and soil are governing the fate and subsequent uptake of pharmaceuticals into earthworms as current attempts to single out principal factors are yet to be successful. However considering uptake as a combination of both soil and pore water parameters may offer a better explanation. Different properties can interact to influence the bioavailable fraction such as explored in the work by Davis (1971) and White (1997). Regression analysis between BCF, organic carbon content and pore water concentration from the current study was performed to evaluate this concept further (Figure 4.8). Whilst it is important to note that not all regressions were significant, in general, fluoxetine and diclofenac results showed increased earthworm BCF in soils which had decreasing soil organic matter content (SOM). This could be explained by the presence of SOM decreasing the proportion of the chemical in pore water which in turn reduces potential for uptake. The results presented tend to agree that decreasing SOM leads to higher pore water concentrations of the pharmaceuticals (Figure 4.8). For fluoxetine a significant relationship in all regressions was noted, however for diclofenac this was only when a marked decrease in pore water concentration correlated to an increase in organic carbon content of the test soils. Relationships also showed an increase in organic carbon (OC) corresponded to a decrease in BCFs for the various soils and thus fits with previous research findings that the SOM is regulating the available fraction of pharmaceuticals in the pore water. This was most evident in the fluoxetine results and to a lesser extent in the diclofenac results, with weak correlations especially between BCF and OC (Figure 4.8).

Figure 4.8 Correlations between organic carbon content, pore water concentration and bioconcentration factor for carbamazepine, diclofenac, fluoxetine and orlistat, linear regression line provided with corresponding R^2 (* indicates significant relationship and p value provided for such regressions).



For the neutral pharmaceuticals, orlistat and carbamazepine, no significant correlations were observed, but in general, an increase in organic carbon content still follows a decrease in pore water concentration. However, in contrast a decrease in pore water concentration generally showed an increase in BCF (although not significant) (Figure 4.8). As numerous complex interactions exist between SOM, pore water concentrations and BCFs and no significant relationships were observed, apart from for fluoxetine, further experiments should be carried out using a wider variety of soil types to allow for appropriate exploration and conclusions to be drawn.

In conclusion as one single soil type did not generate the largest BCFs for all pharmaceuticals and this would suggest that earthworm uptake is both a factor of soil type (including soil and pore water parameters) and pharmaceutical physico-chemical properties. However, it is clear that for some pharmaceuticals the influence of soil type on the uptake and accumulation of pharmaceuticals is more significant (i.e. diclofenac) than for others (i.e. carbamazepine) with greater divergence in BCFs values reported. Exposure in the terrestrial system is a dynamic process and the availability of chemicals to organisms is highly changeable.

4.5 Conclusions

Earthworms differ in their ability to access chemicals in different soils types, whether they are sequestered or bio-available. The elimination of chemicals can also be influenced by differences in soil properties.

The complex nature of numerous interactions between pharmaceutical chemical properties and soil properties ensures that it is incredibly difficult to disseminate the key factors influencing pharmaceutical uptake in earthworms. Whilst different soil types may affect the uptake and accumulation of some chemicals, BCF and BSAF results presented in this study suggest that others are less influenced by soil chemistry. Further work could explore the influence of dissolved organic carbon (DOC) in the pore water which may increase the bioavailability of chemicals. Unfortunately this was not measured in the current study and additional experiments to explore this are necessary.

Information on how soil properties can affect chemical uptake are important in terms of both risk assessment and modelling. Currently used, generalised models are unlikely to accurately represent the potential uptake and risk associated with soil-borne contaminants and, as our research shows, numerous factors are involved in determining uptake. For modelling, a better understanding of biological factors influencing the uptake of chemicals residing in soils is important to accurately estimate the bioaccumulation potential. Additional work needs to explore the effect of changing pH in the earthworm tissue, soil and pore water samples on the uptake of ionisable chemicals and the subsequent implications of this for exposure modelling scenarios. Specifically, changes in earthworm tissue pH may result in wider implications such as the ion trap phenomena observed in plant cells (Trapp, 2004) being induced in earthworms, or negative effects on earthworm internal environments. However, as it is not clear which factors specifically lead to pH change further studies are needed to quantify and qualify these complicated processes.

This study represents the first attempt to evaluate the complex interplay between pharmaceutical chemical properties and soil chemical properties and how these govern potential exposure scenarios for a critical terrestrial organism. While there are many confounding complexities and unanswered questions this work represents a first important step in understand the terrestrial fate of pharmaceuticals, a critical component in understanding environmental risk.

Chapter 5 Does Uptake of Pharmaceuticals Vary Across Earthworm Species?

5.1 Introduction

In Chapter 2 and Chapter 4, the uptake of a range of pharmaceuticals, with different physico-chemical properties, into the epigenic earthworm, *Eisenia fetida* was explored. *E. fetida* is the preferred standard reference earthworm species in many international regulatory guidelines for risk assessment such as the Organisation of Economic and Cooperative Development acute earthworm toxicity test (OECD 207 (OECD, 1984)). Results showed that pharmaceuticals were accumulated by *E. fetida* and pore water based bioconcentration factors ranged from 2.25 (carbamazepine) to 51.53 (orlistat) (Chapter 2).

However, a number of different earthworm species co-exist within the soil environment. These species vary in their behaviour, physiological properties and in their preference of particular soil characteristics e.g. texture or pH (Edwards and Bohlen, 1996). Data for non-pharmaceutical contaminants (DDE and metals) indicates that chemical uptake and toxicity can vary across species (Kelsey and White, 2005; Langdon et al., 2005; Morgan and Morris, 1982; Spurgeon and Hopkin, 1996). These differences in uptake are thought to be due to differences in processing of soil organic matter, ecological strategy, and lipid content across the earthworm species studied (Kelsey *et al*., 2005). It is possible that the uptake of pharmaceuticals into other species could be very different from *E. fetida*. In order to fully understand the risks of pharmaceuticals in terrestrial systems it would be valuable to develop knowledge of the differences, if any, in uptake across different species.

This study therefore explored the uptake of four commonly used human pharmaceuticals into the earthworm, *Lumbricus terrestris* and compared the findings to previous results for the uptake of the chemicals into *E. fetida* from Chapter 2 in order to evaluate whether earthworm species traits are important in determining pharmaceutical uptake. Recently, *Lumbricus terrestris* have been suggested to be a more suitable earthworm test species for risk assessment as they reside in the soil environment unlike *E. fetida* which are more commonly found in manure/compost

matrices (Dean-Ross, 1983; Sims and Gerard, 1985). The test chemicals included the anti-epileptic drug carbamazepine, the anti-inflammatory diclofenac, the anti-depressant fluoxetine and orlistat which can be used as weight loss aid. Detailed physico-chemical properties of each pharmaceutical and study species can be found in Table 1.3 and Table 5.1 respectfully.

Table 5.1 Characteristics of *Eisenia fetida* and *Lumbricus terrestris.*

	Eisenia fetida	***Lumbricus terrestris***
Ecological grouping	Epigeic	Anecic
Time to maturity (days)	28 – 30[‡]	112 at 15 °C[^]
Colour	Brown and buff bands[‡]	Head darker, tail lighter[‡]
Optimal temperature (°C)	25 (0 – 35) [‡]	~ 10[a]
Length (mm)	60 – 120*	90 – 350*
Diameter (mm)	3 – 6*	6 – 10*
Number of segments (mm)	80 – 120*	140 – 155*
Mode of reproduction	Obligatory amphimictic	Obligatory amphimictic
Cocoon incubation time	18 - 26[‡]	90 at 15 °C[#]
Where in soil profile?	Leaf litter/surface*	Deep burrows*
Soil pH preference	4.3 – 7.5*	6.2 – 10.0*

(a) (Edwards and Lofty, 1972)

(*) (Reginald William Sims and Gerard, 1985)

(‡) (Edwards, 2004)

(^) (Svendsen *et al*., 2002)

(#) (Butt, 1991)

5.2 Materials and Methods

5.2.1 Pharmaceutical compounds and reagents

The test chemicals were ^{14}C labelled compounds to allow for lower limits of detection in the samples and thus the soil could be spiked with environmentally relevant concentrations in the uptake studies. Labelled fluoxetine and carbamazepine were obtained from American Radiolabelled Chemicals (*Missouri, USA*), diclofenac was obtained from Perkin Elmer (Boston, USA) and orlistat was kindly provided by GlaxoSmithKline (GSK, UK). Solvents including acetonitrile (99.9 %), methanol (99.9 %) and ethyl acetate (99.9 %) were HPLC grade and obtained from Fisher Scientific (Loughborough, UK).

5.2.2 Test soil

The test soil was a clay loam variety (soil 280) obtained from LandLook (Midlands, U.K.) and had been used in earlier earthworm uptake studies with *E. fetida* (Chapter 2; section 2.2.2). Prior to the uptake studies, the field fresh soil was air dried then sieved to 2 mm to ensure homogeneity within the soil matrix. Soil 280 had an organic matter content of 3 %, a pH of 6.3 and a total organic carbon concentration of 1.89 %.

5.2.3 Test organism

L. terrestris were obtained from Blades Biological Ltd (Kent, UK). *L. terrestris* were cultured in a plastic box containing 8 kg of soil 280 and kept in a growth chamber under experimental conditions (see below) prior to use in the uptake studies. They were fed twice weekly with birch leaves and pre-treated horse manure which was dried at 105°C and then rewetted, both of which were applied to the top of the culture medium. The mean lipid content of *L. terrestris* has been reported in literature as 1.23 ± 0.20 % based on fresh weight (Albro *et al*., 1992).

5.2.4 Experimental design

The *L. terrestris* uptake experiments followed the minimised design approach described in Chapter 3. Specifically; earthworms were exposed to each pharmaceutical, individually for a 21 d uptake phase. Exposures consisted of a 500 mL amber glass jar containing 350 ± 5 g test soil 280, and one *L. terrestris* earthworm (3 - 6 g). The soil had been previously spiked with one of the four chemicals (0.8 – 1.5 mL), mixed by placing on an end over end shaker for 24 h and then the lids were removed to allow solvent to evaporate off for 72 h. The resulting soil concentrations were 36, 9.9, 7.2 and 15.8 µg/kg for carbamazepine, diclofenac, fluoxetine and orlistat respectively. Earthworms were also added to blank control and solvent control beakers which were kept under test conditions.

The moisture content of the soils was monitored throughout the study and if necessary adjusted with deionised water to maintain the soil at 40 – 60 % of the maximum water holding capacity (MWHC). Earthworm beakers were incubated under controlled conditions to a constant dark cycle at 13 ± 2 ^{o}C and 60 % humidity and fed twice weekly (see culturing conditions). After 21 d, for each pharmaceutical treatment, six *L. terrestris* were removed from the spiked soil and left on moist filter paper for 30 h to purge their guts. The remaining earthworms were transferred to clean soil (350 ± 5 g) for a further 21 d for the depuration phase. After which the remaining six earthworms were removed from each treatment and allowed to void their gut contents (30 hours). All *L. terrestris* were then immediately frozen (-20°C) until analysis.

Soil samples were taken at the beginning and end of the uptake phase and frozen until analysis. Pore water was extracted from the soil in exposure beakers at the beginning and end of the uptake phase via centrifugation. Duplicate samples (25 g) of soil were taken from each beaker and the pore water extracted using the method outlined in section 2.3.3.1 then immediately analysed. Measurements of soil and pore water pH were also made on all samples using a Hanna pH electrode (HI-1093B) at time of sampling.

5.2.5 Sample analysis

Prior to worm analysis, each *L. terrestris* was defrosted and dissected along the earthworm through the cuticle and epidermis to reveal muscle tissue. A micro pH probe (Thermo Scientific Orion pH microelectrode) was inserted into the muscle tissue to record the internal pH. Worms were then extracted using an approach based on that used for *E. fetida* as described in section 2.3.3.1. The extraction solvents were methanol, ethyl acetate, acetonitrile:water (7:3) and acetonitrile for carbamazepine, diclofenac, fluoxetine and orlistat respectively and 25 mL of solvent was used per earthworm and each extraction took approximately 20 minutes. Suspended earthworm-solvent mixtures were centrifuged at 2000 rpm and a 1 mL sample of the resulting supernatant was taken and added to 10 mL EcoScint A scintillation cocktail ready for counting the radioactivity on a Liquid Scintillation Counter (LSC).

Soil was extracted using liquid extraction according to methods outlined in Chapter 2. Results from previous experiment confirmed the formation of irreversibly bound residues between diclofenac and soil 280 so combustion analysis of these soils was also performed to determine if there was radioactivity remaining in the soil according to methods reported in 2.3.3.1. Recoveries for all four pharmaceuticals in test soil 280 have been determined in previous validation studies (2.3.3.1). Briefly, method validation studies showed that average recoveries ranged from 82.8 (diclofenac) to 100.6 (carbamazepine) %.

5.2.5.1 Liquid scintillation counting

Measured radioactivity in pore water, soil and worm extracts were determined using LSC on a Beckman LS 6500 LSC counter (Beckman Coulter Inc., Fullerton, USA). Each sample was counted three times for 5 minutes. Counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method.

5.2.6 Kinetic model fitting

Measured radioactivity in the earthworm extracts allowed for calculation of *L. terrestris* tissue concentrations at the end of the uptake phase (C_{t1}) and end of depuration phase (C_{t2}). Along with measured pore water concentrations, tissue concentrations were input into Equation 3 and Equation 4 to calculate uptake (*k1*) and depuration rates (*k2*) for *L. terrestris* in each exposure (refer to Chapter 3 for a full explanation of minimised design calculations). The uptake and depuration rates were then used to estimate pore water based kinetic bioconcentration factors (BCFs) (Equation 5).

For comparison, data from the previous full uptake and depuration *E. fetida* experiments (Chapter 2) was resampled according to if the experiment had been carried out using the minimised design principles to generate equivalent minimised design pore water based BCFs to *L. terrestris*. Measured data used in the calculations was originally obtained from full uptake and depuration studies according to OECD 317 (OECD, 2010) outlined in Chapter 2.

5.2.7 Statistical Analysis

Statistical analysis of the data was performed on SigmaPlot (v .12). Prior to all tests, normal distribution and equal variance were tested by performing a Shapiro–Wilk and Levene–Mediane test, respectively. Firstly, measurements of soil and pore water pH made at 0 h and 21 d were compared to see if the average of each measurement was independent using a paired t-test. For the diclofenac exposure a one-way ANOVA was employed to assess differences in internal pH values of the worms in comparison to the control blanks and to see if there was a difference between measured pH values during the uptake period in comparison the depuration phase. For the remaining test chemicals as the normality test failed the one-way ANOVA was instead performed on ranks.

5.3 Results

For all pharmaceuticals there was a decrease in concentrations of radioactivity in the soil and soil pore which can be attributed to uptake into *L. terrestris*, formation of non-extractable residues (Chapter 2) or possibly small amounts of mineralisation (Al-Rajab *et al*., 2010). pH measurements indicated that over 21 d the presence of diclofenac increased the pore water pH (t (11) = -3.624, p =0.004) and decreased the soil pH (t (11) = 2.656, p = 0.022). The presence of orlistat decreased both the pore water (t (11) = 3.6534, p = 0.004) and soil pH (t (5) = 6.006, p = 0.002) over the uptake period. No pH differences were noted in the carbamazepine study, increases in soil pH from 0 h to 21 d (t (11) = -10.452, p = < 0.001) were found to be significant in the fluoxetine exposure (Figure 5.1, Figure 5.2).

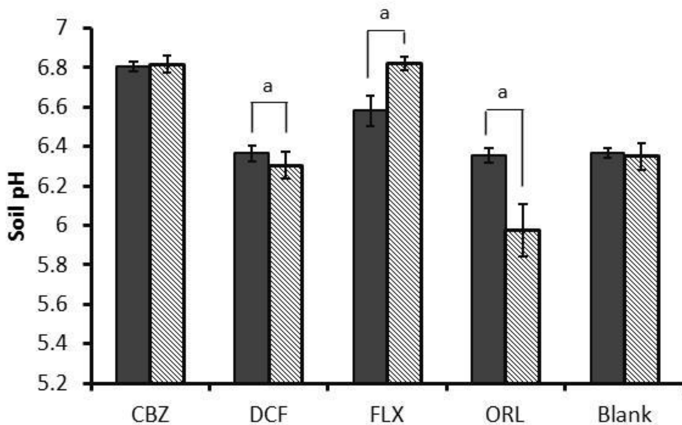


Figure 5.1 Measured soil pH in different study treatments during at start of study (grey bars) and end of uptake phase (21 d) (dashed bars). Average pH measurements provided with ± standard deviation (n = 6). Significant differences in measurements between 0 h and 21 d denoted by ‘a.’

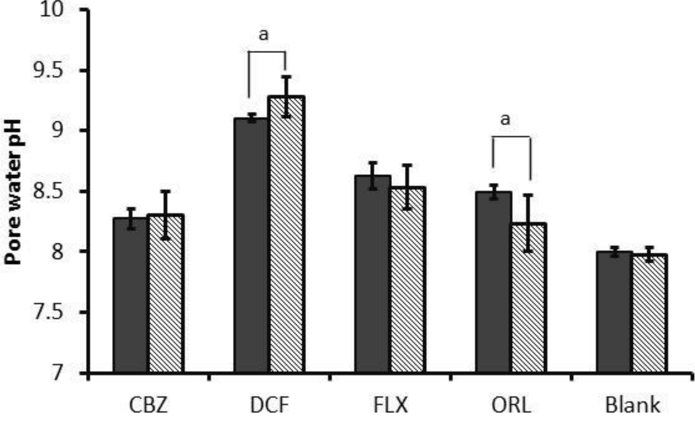


Figure 5.2 Measured pore water pH in different study treatments during at start of study (grey bars) and end of uptake phase (21 d) (dashed bars). Average pH measurements provided with ± standard deviation (n = 6). Significant differences in measurements between 0 h and 21 d denoted by ‘a.’

5.3.1 *Lumbricus terrestris* uptake

All four pharmaceuticals were taken up by *L. terrestris* over 21 d. After 21 d, 1.96 ± 0.65, 1.17 ± 0.57, 2.11 ± 1.04 and 0.72 ± 0.18 % of applied radioactivity was taken up by *L. terrestris* in the carbamazepine, diclofenac, fluoxetine and orlistat studies respectively. Similar average internal concentrations (C_{t1}) were observed after 21 d for diclofenac and orlistat and these two pharmaceuticals also had the greatest amount of chemical remaining within the tissue once the depuration phase had ended. The highest uptake rate (*k1*) was observed in the fluoxetine study with a mean of 11.685 L/kg d^{-1} whist the slowest accumulation was observed in the diclofenac exposure (0.468 L/kg d^{-1}). The diclofenac exposure also had, on average, the slowest depuration rate (*k2*) at 0.05 d^{-1} with approximately 90 % of the accumulated radioactivity remaining in the *L. terrestris* after the depuration period (0.00387 mg/kg). Comparatively, carbamazepine was eliminated fastest from the earthworm at 0.132 d^{-1} which resulted in a tissue concentration at the end of the depuration phase of 0.00162 mg/kg (Table 5.2).

Pore water based bioaccumulation factors (BCFs) increased in the order of diclofenac < fluoxetine < orlistat < carbamazepine for *L. terrestris* after exposure to the pharmaceuticals in soil 280 and ranged from 6.69 – 83.79 (Table 5.2). Internal pH differences were noted in the carbamazepine ($Q = 2.715$, $p = < 0.05$) and diclofenac ($t = 3.488$, $p = 0.007$) treatments at the end of the depuration phase in comparison to the controls and for fluoxetine at the end of the uptake phase ($Q = 2.788$ $p = < 0.05$). Meanwhile, orlistat was the only compound to have significant differences in *L. terrestris* internal pH measurements between the uptake and depuration phases ($Q = 3.327$, $p = < 0.05$) (Figure 5.3).

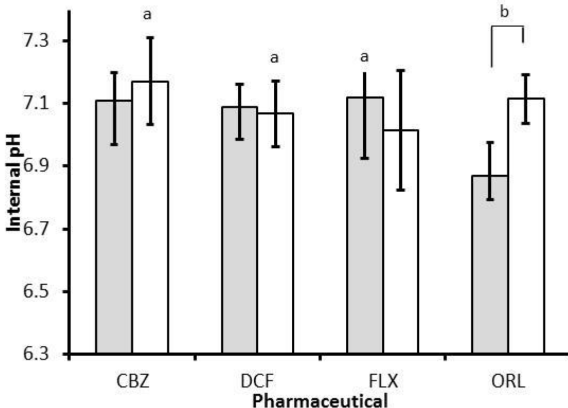


Figure 5.3 Internal pH measurements of *L .terrestris* exposed to four pharmaceuticals (carbamazepine, diclofenac, fluoxetine, orlistat) in test soil 280. Grey bar shows measurement in uptake phase and white bar shows measurements made in depuration phase with error bars providing ± SD (n = 6). Where (a) there is statistically significant ($p < 0.05$) difference of in comparison to blank controls and (b) is a statistically significant ($p < 0.05$) difference between the uptake and depuration phases.

Table 5.2 Results from *L. terrestris* study minimised design experiments together with previously calculated *E. fetida* BCFs showing average measured earthworm tissue concentrations (± standard deviation, $n = 6$) at the end of 21 d uptake phase (C_{t1}) and 21 d depuration phase (C_{t2}) and mean concentration of pharmaceutical in the pore water during the uptake phase (C_{pw}) (± standard deviation, $n = 6$). Calculated uptake ($k1$) and depuration rates ($k2$) are also presented along with BCF values derived using the minimised design approach.

	C_{t1} mg/kg (internal)	C_{t2} mg/kg (internal)	Mean C_{pw} (mg/L) in uptake phase	$k2$ (dep. rate) (d^{-1})	$k1$ (uptake rate) (L/kg d^{-1})	Minimised test design BCF
***Lumbricus terrestris* (this study)**						
Carbamazepine	0.0261 ± 0.0086	0.00162 ± 0.00005	0.00416 ± 0.0012	0.132	0.884	6.69
Diclofenac	0.0043 ± 0.0002	0.0038 ± 0.0011	0.00050 ± 0.00008	0.005	0.437	83.79
Fluoxetine	0.0059 ± 0.0009	0.0010 ± 0.00004	0.00011 ± 0.00007	0.086	5.744	66.90
Orlistat	0.0044 ± 0.0011	0.0022 ± 0.0006	0.00027 ± 0.00014	0.033	1.085	33.21
***Eisenia fetida* (Chapter 2)**						
Carbamazepine	0.0097 ± 0.0018	0.0001 ± 0.00001	0.00864 ± 0.001	0.209	0.238	1.14
Diclofenac	0.0214 ± 0.0036	0.0056 ± 0.0007	0.00126 ± 0.0004	0.064	1.476	23.03
Fluoxetine	0.0233 ± 0.0018	0.0028 ± 0.0021	0.00060 ± 0.0001	0.102	4.456	43.76
Orlistat	0.0162 ± 0.0026	0.0095 ± 0.0011	0.00062 ± 0.0001	0.025	1.601	63.03

5.3.2 Comparison between *Lumbricus terrestris* and *Eisenia fetida*

The uptake rates (*k1*) were considerably faster in the *L. terrestris* study in comparison to *E. fetida* for carbamazepine, diclofenac and fluoxetine however the depuration rates were comparable and fitted within the ranges found in previous *E. fetida* studies (Table 5.2). Nevertheless, in both earthworm species, fluoxetine was evidently taken up the fastest while carbamazepine was eliminated the quickest. The carbamazepine treatment had the highest average pore water concentration (0.0031 mg/L) and also the highest *L. terrestris* internal concentration (0.0261 mg/kg) after 21 days exposure which was also similar to the findings of the *E. fetida* study (Chapter 2).

For the smaller earthworm *E. fetida*, pore water based BCFs calculated based on the minimised design were 1.14 (carbamazepine), 23.03 (diclofenac), 43.76 (fluoxetine) and 63.03 (orlistat) (Table 5.2). In comparison, BCFs were larger in the *L. terrestris* studies for carbamazepine, diclofenac and fluoxetine. Specifically, the pore water based BCFs were 83, 73 and 35 % larger in *L. terrestris* than *E. fetida*. Conversely, for orlistat BCFs were almost two times larger in *E. fetida* (63.03) than *L. terrestris* (33.21) (Table 5.2, Figure 5.4).

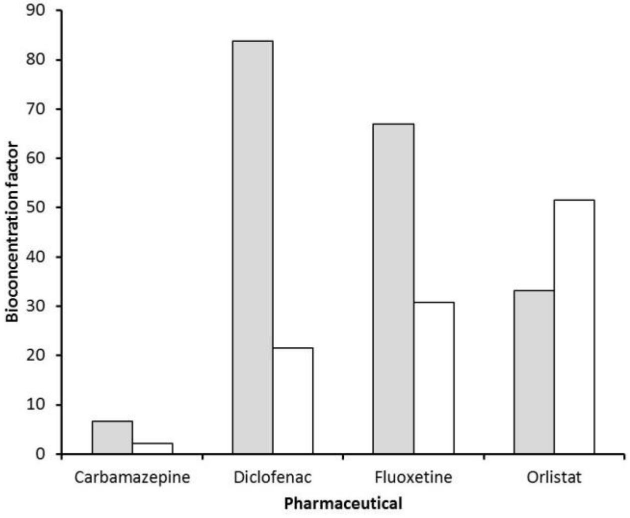


Figure 5.4 Comparison of *Lumbricus terrestris* (grey) and *Eisenia fetida* (white) bioconcentration factors (BCFs) and for the study compounds.

5.4 Discussion

There is increasing recognition of the importance of species traits in determining the uptake of chemicals, including pharmaceuticals, in the aquatic environment (Meredith-Williams *et al*., 2012; Rubach *et al*., 2012, 2010) traits such as respiration, locomotion and behaviour explaining observed differences in uptake. However, very little research has investigated the effect of species differences on the uptake of soil-borne organic contaminants. This study was therefore performed to explore the difference in uptake of pharmaceuticals from soil by two different earthworm species.

The larger BCFs in *L. terrestris* for three of the test compounds (carbamazepine, diclofenac and fluoxetine) in comparison to *E. fetida* present contradictory results to previous research findings which observed ten times higher BCFs for *E. fetida* than *L. terrestris* after exposure to the persistent DDT metabolite, *p,p'*-DDE (Kelsey *et al*., 2005). Comparatively, in agreement to the work by Kelsey *et al*., (2005), the BCF for orlistat was approximately 50 % smaller in the current *L. terrestris* study than the *E. fetida* pore water based BCF.

The differences in BCF's between *L. terrestris* and *E. fetida* may be due to differences in earthworm ecological strategy. *E. fetida* are epigeic species which primarily live at or near the soil surface and consume course particulate organic matter and surface litter whilst anecic species, such as *L. terrestris* live in deep burrows and come to surface to feed on surface litter (Bouché, 1983). Therefore as the soil in these experiments was mixed thoroughly, the deep burrowing action of *L. terrestris* would ensure they have the opportunity to explore the soil to a greater extent with more potential opportunities for chemical uptake in comparison to *E. fetida* which prefer to reside near the soil surface.

The uptake of chemicals has been postulated to be related to hydrophobicity and the lipid content of the organism. For carbamazepine, diclofenac and fluoxetine the results do not follow this trend as *L. terrestris* have a lower lipid content suggesting less accumulation which is reverse of what the results demonstrate. Differences in lipid content can however offer an explanation for the BCFs in the orlistat exposure because *E. fetida* have a higher lipid content (as reported in Chapter 2). Combined

with the fact orlistat is a particularly hydrophobic neutral compound with a large log K_{ow} value of 8.97 this would infer orlistat has a higher propensity for uptake into lipids and thus may account for the larger *E. fetida* BCF.

Furthermore, additional explanations for the larger *E. fetida* pore water based BCF in the orlistat study include size/volume ration principles. As the size of an object increases the surface area to volume ratio decreases therefore as *L. terrestris* are a larger species of earthworm this would infer that the smaller, *E. fetida*, have a greater potential for the diffusion of chemicals through their tissues.

Previous research elucidating the uptake of chemicals into earthworms demonstrated that a majority of uptake from the soil environment occurs via diffusion across the earthworm skin from pore water for a large proportion of chemicals (log $K_{ow} < 6$). However as the hydrophobicity of the chemicals increased uptake via the gut route became increasingly important (Jager *et al.*, 2003). A combination of large surface area to volume ratio ensuring minimal uptake via diffusion and the hydrophobic nature of orlistat restricting uptake to primarily across the gut wall may explain the smaller pore water based BCFs in *L. terrestris* in comparison to *E. fetida*.

Whilst this may explain the orlistat uptake, other mechanisms or processes must exist for carbamazepine, diclofenac and fluoxetine which ensure greater accumulation and higher BCFs in *L. terrestris*. These pharmaceuticals are also more hydrophilic than orlistat and therefore diffusion across the earthworm skin is the dominant route of exposure therefore the larger volume of tissue in the *L. terrestris* could facilitate a higher capacity for uptake, shown by faster uptake rates ($k1$), or greater storage ability of the chemicals in the tissue, as shown by the larger tissue concentrations (C_{t1}) at the end of the uptake phase (Table 5.2). Little is known about the metabolism of pharmaceuticals in earthworms but this may also be a factor influencing uptake in different earthworm species. Previous work presented in Chapter 2 demonstrates that diclofenac is metabolised in *E. fetida* studies however additional studies are required to see if similar transformation products are formed in *L. terrestris* exposures. Potential metabolism, together with gut load and retention time for ingested soil particles which may alter the bioavailability of the pharmaceuticals (Hartenstein *et al.*, 1981; Hartenstein and Amico, 1983).

These research findings demonstrate that species traits are important in determining uptake and BCF calculations. In terms of the wider environment, birds feeding on *L. terrestris* would generally be more at risk than if they were to ingest a similar number of the smaller, *E. fetida*, and thus the potential food chain effects as a result of bioaccumulation would also be greater. However for risk assessment purposes it may not be necessary to take into account species differences as all BCFs for the four pharmaceuticals were within an order of magnitude of each other. Nevertheless, it is important to recognise that only two species were evaluated in this study and therefore to draw more general conclusions it may be necessary to look at a wider range of earthworm species for example with differing burrowing habits, soil property preferences and sizes.

All test chemicals were thoroughly mixed in the soil to create an even distribution of the pharmaceuticals. This heterogeneity is not representative of the natural soil environment where pharmaceuticals will most likely be applied to the top layers of the soil profile after application of sludge and manure. Earthworms which prefer to reside at or near the soil surface would therefore have a greater exposure to chemicals than the deep burrowing species which come to the surface less often. Hydrophilic pharmaceuticals which have a greater potential for movement with percolating water flows may be more widely distributed in the soil profile than highly sorptive pharmaceuticals and therefore differences in pharmaceutical physico-chemical properties can also affect earthworm uptake in the natural environment. Recent research has demonstrated that the veterinary antibiotic, sulfadiazine accumulated in greater concentrations in the soil boundary layer between channel compartment and bulk soil. Over time, the shells of microaggregates also had a larger sulfadiazine concentration than the core (Reichel and Thiele-Bruhn, 2013). Pharmaceuticals accumulating in earthworm channels may present a greater risk to burrowing earthworm species where there is the potential for greater uptake of chemicals. Additional studies are required to determine whether this needs to be addressed with regards to risk assessment.

The observed changes in pore water, soil and internal pH (Figure 5.1, Figure 5.2, Figure 5.3) would also indicate that the presence of chemicals can alter the chemistry of the soil. This is important in terms of modelling exposure scenarios and risk

assessment exercises as soil properties such as pH are currently assumed to remain constant and changing these properties may alter chemical bioavailability.

5.5 Conclusions

After isolated exposure of test organisms (*Lumbricus terrestris*) to carbamazepine, diclofenac, fluoxetine and orlistat for 21 days uptake phase followed by 21 days depuration period the range of pore water based BCFs for these compounds were 6.69 to 83.79. These findings demonstrate bioaccumulation by *L. terrestris* appear to be highly compound specific. As a result of comparison to previous research on the uptake of pharmaceuticals into a smaller earthworm (*E. fetida*), the bioaccumulation of pharmaceuticals into earthworms also appear to be species specific, with the larger worm (*L. terrestris*) showing a greater capacity for the uptake of carbamazepine, diclofenac and fluoxetine and thus larger pore water based BCFs. In terms of risk assessment these results highlight that may be necessary to look at species differences when determining the effect of pharmaceutical residues in the soil environment. Further research is required to evaluate the effect of species traits on pharmaceutical uptake using a wider variety of test organisms before conclusions can be drawn as to whether a single species should not be used as a model to represent all organisms in the risk assessment of chemicals.

Chapter 6 Fate and Uptake of Pharmaceuticals in Soil – Plant Systems

6.1 Introduction

Due to the detection of pharmaceuticals in soils (Butler *et al*., 2012; Dalkmann *et al*., 2012; Durán-Alvarez *et al*., 2009; Golet *et al*., 2003; Kinney *et al*., 2006; Redshaw *et al*., 2008a; Vazquez-Roig *et al*., 2010), as described in Chapter 1, concerns have been raised over the potential for these substances to be taken up into human food items and to pose a risk to human health (Boxall *et al*., 2006; Wu *et al*., 2010). A number of studies have demonstrated the uptake of pharmaceuticals from both human and veterinary use, into plants (Boxall *et al*., 2006; Dolliver *et al*., 2007; Herklotz *et al*., 2010; Holling *et al*., 2012; Kong *et al*., 2007; Redshaw *et al*., 2008b; Shenker *et al*., 2011; Wu *et al*., 2010, 2012). Studies have explored the uptake and translocation of a variety of pharmaceuticals with a particular focus on the anti-depressant drug, fluoxetine and antibacterial chemicals including sulfamethazine, sulfamethoxazole and trimethoprim into numerous plant species including root and shoot crops such as soybean, lettuce and carrot.

A number of studies have explored plant uptake from a hydroponic culture medium such as the work by Herklotz *et al.,* (2010) and Redshaw *et al.,*(2008b). Specifically, uptake of fluoxetine was seen in the stems (5 % mean uptake of applied burden; 0.49 µg/g (wet weight)) and leaves (3 % mean uptake; 0.26 µg/g wet weight), however there was no evidence of uptake into the curd (Redshaw *et al.,* 2008b). Fluoxetine also remained undetected in plant roots whereas soil uptake studies have generally noted a concentration of the compound in the roots albeit smaller than the amount detected in the main plant (Winker *et al.,* 2010).

Studies have also revealed variations in plant uptake between different species exposed to pharmaceuticals (Boxall et al., 2006; Herklotz et al., 2010; Wu et al., 2012). Recent studies have also investigated the uptake into plants with the addition of sewage sludge to test systems and uptake via the application of reclaimed waste water effluent to simulate realistic environmental exposures in the field (Holling et al., 2012; Shenker et al., 2011; Wu et al., 2012, 2010). Results indicate that plant

uptake is higher in the biosolid amended soils, probably a result of higher exposure concentrations however pharmaceuticals introduced by irrigation water appear to be more available for translocation (Wu et al., 2010).

Many of the previous plant uptake studies have been done at unrealistic exposure concentrations. Studies typically have looked at uptake only with no attempt being made to understand the temporal fate of the pharmaceutical in soil matrices. Without understanding the dynamics of the distribution and fate of the pharmaceuticals in the soil, it is difficult to establish relationships between the properties of pharmaceuticals and uptake. This study was therefore initiated to explore the fate, distribution and uptake of a range of pharmaceuticals in soil-plant systems. The study was performed on two crop species with five pharmaceuticals and an antimicrobial personal care product covering a diverse range of physico-chemical properties (Table 6.1).

6.2 Materials and methods

Analytical grade carbamazepine (> 98 %), diclofenac (> 98 %), fluoxetine (> 98 %), propranolol (> 99 %), sulfamethazine (> 99 %), and triclosan (> 97 %) were obtained from Sigma-Aldrich (Sydney, Australia). Deuterated forms of selected study compounds (carbamazepine-D10 (99.8 %), diclofenac-D4 (98.5 %), fluoxetine-D5 (99.4 %), propranolol-D7 (99.6 %), and triclosan-D3 (98.6 %) were purchased from TLC Pharmachem (Canada) for use as internal standards in the the chemical analyses.

Tepko soil (obtained from near Tepko township in South Australia) was used for both the plant uptake and fate studies (pH 6.25, EC 0.09 dS/cm, OC 1 %, CEC 5.2 cmol(+)/kg, clay 8 %). Prior to testing, the soil was air dried then sieved to 2 mm to ensure homogeneity.

Ryegrass seeds (*Lolium perenne,* Guard variety) were obtained from Seed Services (SARDI, South Australian Research and Development Institute) and radish (*Raphanus sativus,* Cherry belle variety) from Mr Fothergills (Sydney, Australia).

Table 6.1 Selected properties of test chemicals (full description of physico-chemical properties are **Table 1.3**)

Test chemical	**p*K*$_a$**	**Log K_{ow}[a]**	**Log D_{ow}[b]**
Carbamazepine	N/A	2.5	N/A
Diclofenac	4.0	4.5	2.30
Fluoxetine	10.1	4.1	0.19
Propranolol	9.5	3.5	0.19
Sulfamethazine	7.4	0.9	0.87
Triclosan	8.1	4.8	4.80

[a] Unionised form of the drugs

[b] Log D_{ow} at pH 6.25

6.2.1 Fate study

Duplicate pots of soil were prepared (200 ± 5 g) and spiked with aliquots of 1 g/L (in acetone) solution of each study pharmaceutical to give a nominal concentration of 10 mg/kg. Following spiking, soil was mixed by hand to ensure a homogeneous distribution of the test chemicals; pots were then left for 2 h in a fume cupboard to evaporate off any solvent. Blank control pots were also prepared. Pots were then kept in controlled conditions (14 h light (23°C) 10 h dark (15°C)) until time of sampling. Moisture content adjustments were made on a daily basis, by addition of deionised water, to ensure levels remained at 60 % of the soil maximum water holding capacity (MWHC). Sampling points were 0 h, 1, 3, 7, 14, 40 d. At each sampling point, duplicate pots were removed and the soil pore water was extracted. Extractions were done by taking 2 x 25 g portions of soil from each pot and placing these on top of a glass wool insert in in 2 x 25 mL disposable plastic syringes. Syringes were placed in plastic centrifuge tubes and centrifuged at 3500 rpm for 45 minutes. The resulting pore water, collected in the centrifuge tubes for each single sample, was pooled and centrifuged again at 15000 rpm for an additional 30 minutes

and then transferred to vials ready for analysis. Samples of whole soils were also taken and stored at -20°C for later analysis.

6.2.2 Uptake of pharmaceuticals into plants

Plastic pots containing 500 ± 5 g and 200 ± 5 g were prepared for use in uptake studies with radish and ryegrass respectively. Pots were prepared in triplicate for each pharmaceutical and plant type and spiked as per the fate study to give a final soil concentration of 1 mg/kg. Solvent and blank controls were also prepared in triplicate. Soils were then left for 48 hours to equilibrate before a total of 6 and 16 seeds were initially added to each pot for radish and ryegrass respectively which were then lightly covered in test soil.

Plants were left to grow for 6 weeks in a growth chamber under the same conditions as the pots in the fate study (Figure 6.1). Pots were arranged in a randomised order (specific positions were determined based up on a random number generator in Microsoft Excel). A similar watering regime to that used in the fate study was adopted to maintain moisture levels at 60 % of the MWHC. As the experiment progressed, the growth of the plant was taken into account for the watering strategy. Germination counts were made at 11 days. After 12 days of growth, when approximately 80 – 90 % of plants had germinated the radish plants were thinned to leave behind three seedlings. This was to ensure maximum germination potential in order to gather enough biomass for the chemical analysis. After 50 % emergence, plants were fed Ruakura nutrient solution, where 5 mL was applied per 250 g soil twice weekly (for three weeks) instead of the DI water. After 3 weeks, addition of nutrient solution continued with one 5 mL application of nutrient solution per 250 g of soil per week (for nutrient solution preparation see Appendix 14).

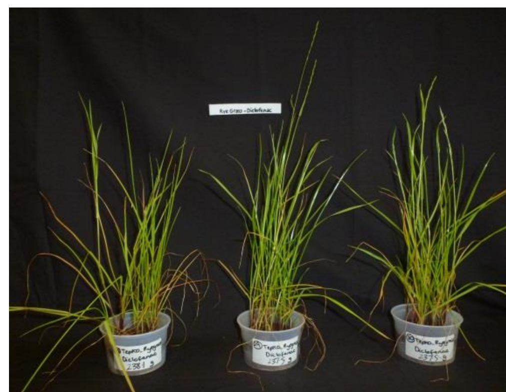


Figure 6.1 Radish and ryegrass plants before harvest.

At harvest, loose soil was removed from around the radish plant to allow for the intact removal of the whole radish (Figure 6.2). The radish plant was thoroughly rinsed in deionised water to remove any soil residues, patted dry with paper towel, weighed, divided up into root and above ground biomass and these were then re-weighed separately. For the ryegrass, after measuring the maximum height of the plants from each treatment, the above ground plant material was cut away, rinsed in DI water, patted dry and then weighed. All plant samples were cut into smaller pieces then freeze dried and stored at - 20°C until extraction for residue analysis. Soil was also taken from the plant pots, at the end of the uptake study, for analysis.

Figure 6.2 Radish plants after harvest.

6.2.3 Pharmaceutical analysis

6.2.3.1 Extraction from soil and plant material

Pharmaceutical compounds were extracted from soils and plants using validated methods chosen for their high percentage recoveries (Appendix 17). Prior to plant and soil extractions 1 mg/g of deuterated stable isotope standards were added to their respective samples (100 ug/L stock solution). Since stable isotopes were unavailable for sulfamethazine, control plants and control soil samples were spiked with a known amount of sulfamethazine to determine recoveries. For the soil extraction, 5 mL of methanol was added to 1g soil (wet weight) and 1 g of sand. After addition of the solvent the test tubes were vortexed for 1 minute and then ultrasonicated for 15 minutes. Lastly the tubes were centrifuged for 30 minutes at 1500 rpm and the supernatant was removed. The extraction process was repeated with a further addition of 5 mL methanol and then 5 mL acetone. The supernatants from the three extractions were combined and then evaporated to dryness before being reconstituted in 1mL methanol, sonicated for 5 minutes and then transferred into LC-MS/MS vials for analysis.

For the plant extractions, sand (1 g) was added to 1 ± 0.1 g of plant material for each of the samples and 5 mL of extraction solvent (70:30 acetonitrile:Milli-Q water solution) was then added to the test tube. After addition of the solvent the test tubes were vortexed for 1 minute and then ultrasonicated for 15 minutes. The samples were then centrifuged for 30 minutes at 1500 rpm and the supernatant was removed and the process repeated for two further extractions. The combined extracts (15 mL) were diluted with Milli-Q to make a maximum solvent concentration of 10 % and the extract was then applied to an Oasis HLB (Waters Corporation) 6 mL 200 mg solid phase extraction (SPE) cartridge that had been preconditioned with Milli-Q water and methanol. The cartridges were left to dry under vacuum, washed with 10 % methanol in Milli-Q water and eluted with 2 x methanol (3 mL) and 1 x dichloromethane (3 mL). The eluates were combined and evaporated to dryness under a nitrogen stream and reconstituted in 1 mL methanol. Lastly the test tubes were sonicated for 5 minutes ready for the extract to be transferred into LC-MS/MS vials.

6.2.3.2 LC-MS/MS analysis

Cleaned-up, extracts were analysed for the pharmaceuticals by LC-MS/MS using a ThermoFinnigan TSQ Quantum Discovery Max (Thermo Electron Corporation). HPLC separation was performed with a Kinetex C18 100 x 2.1 mm (2.6 µm particle size) column (Phenomenex, USA) with a mobile phase flow rate of 0.3 mL/min. The mobile phase composition was 0.1 % formic acid and acetonitrile using a gradient program over 12 min. The relative flow of 0.1 % formic acid was 95 % for 2 min, 20 % after 3 min, 2 % at 4 min and held for 3 min until 7 min before returning to 95 % by 9.5 min. MS/MS analysis was undertaken using atmospheric pressure electrospray ionisation (ESI) in both positive and negative ionisation modes. Spray voltage was 5000 V and source collision induced dissociation was -12 V in positive ESI and -4000 V and 10 V for negative ESI, with the ESI capillary line maintained at 350°C and collision gas (Ar) pressure set at 1.5 mTorr. Qualitative and quantitative analysis of compounds was based on retention time, multiple reaction monitoring (MRM) of two product ions and the ratios between the product ions (More details of the analytical method pertaining to each compound are in Appendix 15).

Lower limits of quantification (LOQs) were determined by repeat injections (n=6) of the lowest detectable concentrations of the compounds. The LOQ was defined as three multiplied by three times the standard deviation (3σ) of the responses. The LOQs relating to the soil and plant matrices were based on the respective recoveries within each matrix (Appendix 16).

6.2.4 Data analysis

6.2.4.1 Soil degradation

Concentrations of pharmaceuticals in soil and pore water were plotted against time of sampling. Where there was a significant difference in concentration to that measured at 0 d, three kinetic models were used to fit the data: a simple first order degradation kinetic (SFO; Equation 7) model, a first order multi-compartment model (FOMC; Equation 8) (Gustafson and Holden, 1990) and a bi-exponential first order

model (BFO; Equation 9) (FOCUS, 2006). Model parameters were optimized according to recommendations by FOCUS (FOCUS, 2006) using the least squares method with Microsoft® Excel Add-Inn Solver.

$C_t = C_0 * e^{-kt}$ **Equation 7**

$C_t = C_0\,(1+\beta t)^{-\alpha}$ **Equation 8**

$C_t = C_{t1}+C_{t2} = C_{01} * e^{(-k1t)} + C_{02}e^{*(-k_2t)}$ **Equation 9**

Where C_t is the concentration of pharmaceutical remaining in soil (µg/g) after t (days), C_0 is the initial concentration of pharmaceutical (µg/g), k the rate of degradation (day^{-1}), β is the location parameter, α is a shape parameter determined by coefficient of variation k values. For Equation 9, $C_{t1} + C_{t2}$ is the total amount of pharmaceutical applied at time, $t = 0$ (in two compartments), C_{01} and C_{02} are the amount of chemical applied to compartment 1 and 2 respectively and k_1 and k_2 are independent decay rate constants for compartments 1 and 2 respectively. Models used specific to each pharmaceutical, parameters and measurements to assess the goodness of fit for the optimised parameters are outlined in Appendix 18. For SFO and FOMC model fits the time it took for a 50 or 90 % decline in the concentration of the pharmaceutical (DT_{50}, D_{T90}) could then be calculated from the model fits (Appendix 18; Table 6.2). For BFO models no analytical solution exists to calculate degradation end points.

6.2.4.2 Uptake factors

Measured concentrations for each of the pharmaceuticals taken up by the radish and ryegrass were used to calculate soil and pore water-based uptake factors (UFs). UFs were derived using concentrations in the soil, pore water and plant material (Equation 10, Equation 11, Equation 12).

$$UF_{soil} = \frac{C_p}{C_s}$$ **Equation 10**

$$UF_{pore\ water} = \frac{UF_{soil}}{K_d}$$ **Equation 11**

$$K_d = \frac{C_s}{C_{pw}}$$ **Equation 12**

Where UF_{soil} is the soil-based UF, $UF_{pore\ water}$ is the soil pore water-based UF, C_p is the concentration in plant material, C_s is the concentration in soil, C_{pw} is the concentration in the pore water and K_d is the average soil sorption coefficient for each pharmaceutical calculated across seven sampling points in the fate study (Table 6.2).

6.2.5 Statistical analysis

Statistical analysis of the data was performed using SigmaPlot (v .11). A one-way ANOVA (significance level 0.05) was employed to assess differences in plant biomass (dry weight) between plants grown under treated soil and controls. Additionally, a one-way ANOVA was employed to assess any differences in concentration of the pharmaceuticals in the soil and pore water over 40 d exposures, with additional comparisons between sampling points assessed by Holm-Sidak pair-wise comparison. Prior to all tests, normal distribution and equal variance were tested by performing a Shapiro–Wilk and Levene–Mediane test, respectively.

6.3 Results and Discussion

6.3.1 Fate study

Over 40 d average K_d values ranged from 0.99 to 121.88 L/kg and increased in the order of sulfamethazine < carbamazepine < fluoxetine < diclofenac < propranolol < triclosan (Table 6.2). Some of the study compounds persisted in the soil throughout the 40 d uptake period whilst others were readily dissipated (Figure 6.3; Table 6.2). There was no significant difference between measured concentrations at 0 d and 40 d for carbamazepine (p = 0.026), fluoxetine (p = 0.162) and propranolol (p = 0.757). Triclosan dissipated from the soil after 14 d (p = 0.004).

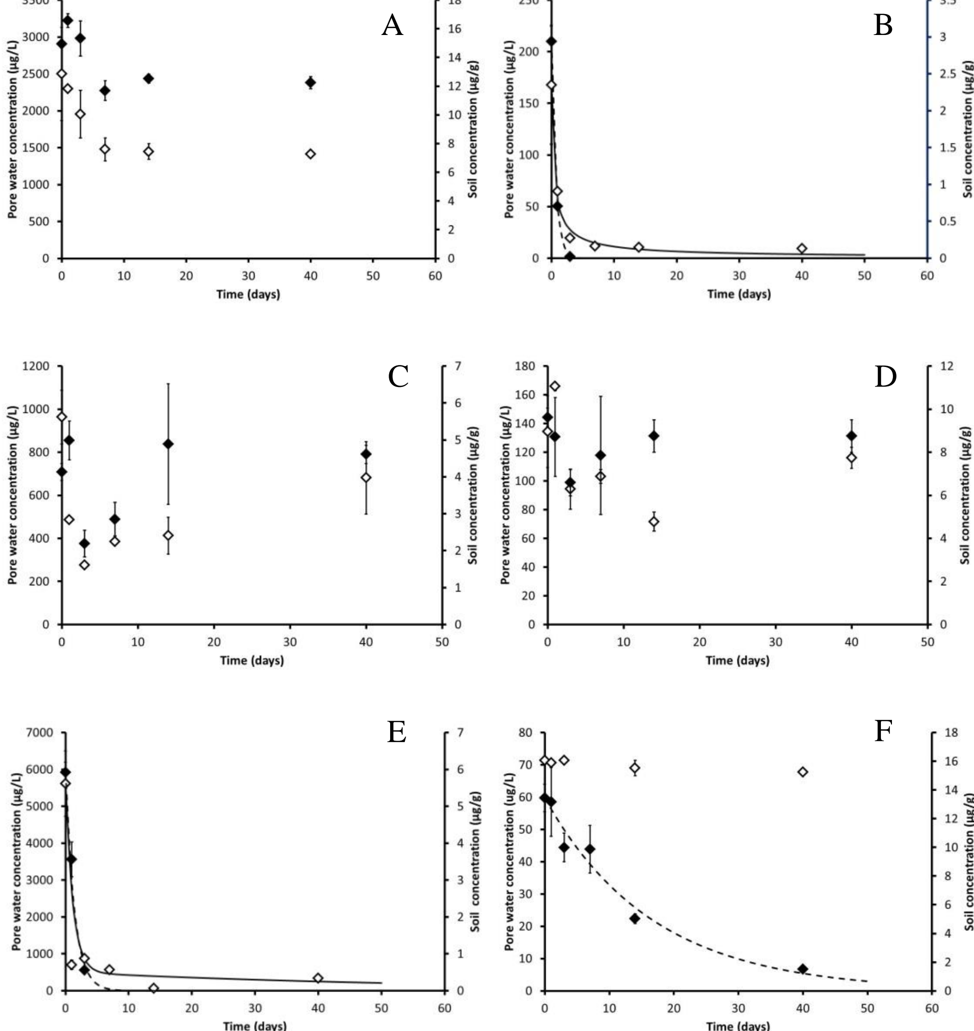


Figure 6.3 Average measured soil (closed points) and pore water (open points) concentrations during fate study (40 d) for test pharmaceuticals; carbamazepine (A), diclofenac (B), fluoxetine (C), propranolol (D), sulfamethazine (E) and triclosan (F). Best model fit provided by dashed line for soil and a solid line for pore water where necessary and error bars represent standard error of mean (n = 3).

Concentrations of diclofenac (p = 0.032) and sulfamethazine (p = 0.013) significantly decreased after 1 d and were undetectable after 3 d. The dissipation of these three compounds was fast (0.06 – 1.4 d^{-1}; Table 6.2); compounds followed single first order kinetics and corresponding DT_{50} values were 0.5 d, 0.99 d and 11.55 d for diclofenac, sulfamethazine and triclosan respectively. The persistent nature of carbamazepine is consistent with previous findings in this thesis (Chapter 2) and previous research (Kinney *et al*., 2006; Monteiro and Boxall, 2009; Williams *et al*., 2006). The observed degradation of triclosan is also consistent with previous research which has suggested a half-life of 18 days (Ying *et al*., 2007). Results

presented in this study show that in less than 40 days; only 10 % of the applied triclosan remained in the soil (Table 6.2) which has been previously reported to transform to methyl triclosan (Butler *et al*., 2012; Waria *et al*., 2011). Previous fate studies have also shown that diclofenac is not persistent and readily biodegradable from soils as a result of chemical mineralisation (Al-Rajab *et al*., 2010; Dalkmann *et al*., 2012) The half-life observed in this study (0.5 d) is therefore comparable to previous findings of < 5 d(Al-Rajab *et al*., 2010) and considerably faster than observations by Xu *et al*., (2009c) who reported DT_{50}'s ranging from 3.1 d (loamy sand) to 20.4 d (silty loam) (Table 6.2).

Even though diclofenac and sulfamethazine were not detectable in whole soil extracts after 3 d, detectable concentrations of these chemicals in the pore water were seen for the full duration of the fate study (Figure 6.3). By 40 d, concentrations of all test chemicals remaining in the pore water decreased in the order carbamazepine > fluoxetine > sulfamethazine > propranolol > triclosan > diclofenac (Figure 6.3).With the exception of sulfamethazine on 0 d, carbamazepine concentrations were consistently the highest in the pore water (1321– 3129 µg/L) over 40 d (Figure 6.3). Sulfamethazine concentrations were initially high (2932 – 6502 µg/L) however after 1 d, concentrations dropped to 832 – 2683 µg/L after which they decreased at a slower rate. Unlike soil dissipation, pore water dissipation did not follow first order kinetics. The models that described pore water dissipation better included first order multi-compartment model (FOMC; Equation 8) (Gustafson and Holden, 1990) and a bi-exponential first order model (BFO; Equation 9) (Table 6.2). Pore water concentrations decreased significantly in the diclofenac (p = 0.016) and sulfamethazine studies (p = < 0.001) resulting in DT_{50}'s < 20 d (Table 6.2) in comparison to DT_{50}'s for the remaining compounds of > 40 d. Whilst triclosan dissipated rapidly from the soil, pore water concentrations were not significantly different at any of the sampling points over 40 d (p = 0.266).

Table 6.2 Summary statistics from soil and pore water dissipation modelling (more detailed table including model fit provided in Appendix 18).

	Pore water					Soil				
Pharmaceutical	**Model**	**DT50 (d)**	**DT90 (d)**	**Rate constants**	**r^2**	**Model**	**DT50 (d)**	**DT90 (d)**	**Rate constants**	**r^2**
Carbamazepine	*	> 40	> 40			*	> 40	> 40		
Diclofenac	FOMC	19.65	2.57E+03	α = 0.79, β = 0.34	0.88	SFO	0.50	1.64	(1.4)	0.99
Fluoxetine	*	> 40	> 40			*	> 40	> 40		
Propranolol	*	> 40	> 40			*	> 40	> 40		
Sulfamethazine	BFO			C01 = 91, C02 = 9, k_1 =0.85, k_2 = 0.017	0.99	SFO	0.99	3.29	(0.7)	0.99
Triclosan	*	> 40	> 40			SFO	11.55	38.38	(0.06)	0.97

* No significant difference between 0 d and 40 d measured concentrations therefore data was not modelled to determine degradation rates.

6.3.2 Plant uptake

Plants contain ion channels and enzymes which could also be potentially targeted by pharmaceuticals and may initiate a response such as inhibition in the transport of essential elements required for plant growth for example (Williams and Cook, 2007). Previous research has highlighted the potential for pharmaceuticals to induce toxic effects on plants (Kong *et al.*, 2007). Dose response relationships with plants grown under triclosan treatment have been noted starting at 0.44 mg/L in hydroponic studies (Herklotz *et al.*, 2010) and low observed effect concentrations (LOECs) seen at 0.74 mg/kg after plant growth in quartz sand (Reiss *et al.*, 2009). In this study, however, no observed effect on plant growth was noted for any of the treatments in comparison to the controls (p = 0.08 – 0.966) for both radish and ryegrass, probably due to the more realistic exposure concentrations that were used (Figure 6.5). This is in support of previous research where concentrations of carbamazepine in root tissue ranging between 202 μg/kg - 426 μg/kg yielded no observed effect on ryegrass aerial plant growth (Winker *et al.*, 2010) (Figure 6.4).

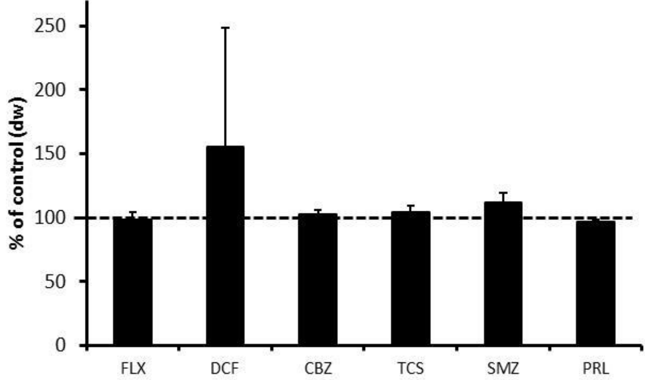
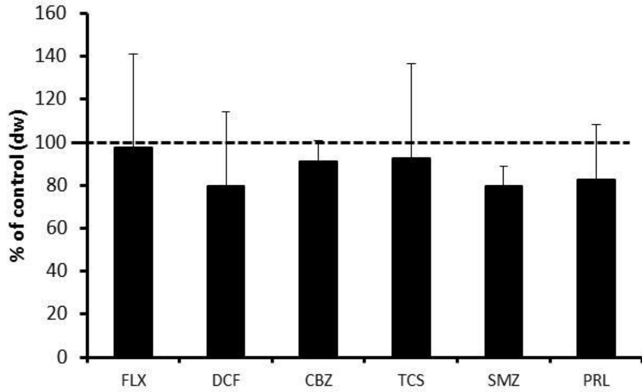


Figure 6.4 Percentage growth of control for ryegrass as a result of pharmaceutical treatment (fluoxetine [FLX], diclofenac [DCF], carbamazepine [CBZ], triclosan [TCS], sulfamethazine [SMZ], propranolol [PRL]). Average value provided with error bars representing standard deviation, based on dry weight of plant material (n = 6).



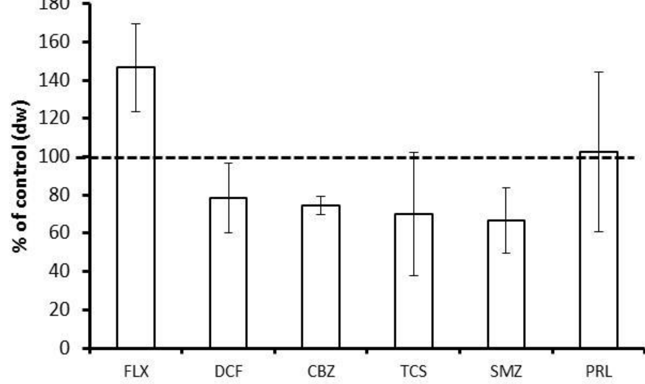


Figure 6.5 Percentage growth of control for radish leaf (A) and bulb (B) as a result of pharmaceutical treatment (fluoxetine [FLX], diclofenac [DCF], carbamazepine [CBZ], triclosan [TCS], sulfamethazine [SMZ], propranolol [PRL]). Average value provided with error bars representing standard deviation, based on dry weight of plant material (n = 6).

Five of the six test chemicals were taken up in detectable quantities into radish and ryegrass (Table 6.3; Figure 6.6). The degree of uptake varied across pharmaceuticals and plant species. With the exception of propranolol, greater uptake into radish was seen, after combining the concentrations in the bulb and leafy parts, compared to ryegrass. For both radish and ryegrass, carbamazepine was taken up the greatest extent with measured concentrations up to 52 μg/g in the radish leaf. Whilst sulfamethazine was taken up by both plants, concentrations were consistently below the LOQ. Therefore both radish leaf and radish bulb accumulated chemicals in the order of carbamazepine > triclosan > diclofenac > propranolol > fluoxetine > sulfamethazine whereas chemicals accumulated in the ryegrass in the order of carbamazepine > propranolol > triclosan > fluoxetine > diclofenac > sulfamethazine (Figure 6.6).

In the propranolol exposure, there was very high uptake into the ryegrass but this was not mirrored in the radish leaf where concentrations were some 16 times less (Figure 6.6). This was also true for triclosan, although to a lesser extent. For the remaining pharmaceuticals, concentrations in the radish leaf and ryegrass were generally similar.

Greater fluoxetine uptake into the roots was observed in this study (170 ng/g) in comparison to previous research where fluoxetine root concentrations were $< 22.2 \pm 5.3$ ng/g (Wu *et al*., 2010). The previous study involved fluoxetine application via biosolids and the effect of soil properties on uptake also must be considered. The amended soil concentration in the Wu *et al*., (2010) study was lower (0.07 mg/kg) than the current study whereas in an earlier study Redshaw and colleagues saw fluoxetine uptake by *Brassicaceae* tissue cultures from a hydroponic set-up comparable to the results from our study at 0.26 - 0.49 µg/g (Redshaw *et al*., 2008b).

Table 6.3 Average soil concentrations measured at the end of the experiment from soils collected from the plant pots, soil – water partition distribution coefficients (K_d) values calculated during fate study, measured plant concentrations (± standard deviation, *n* = 6), and calculated uptake factors (UF) for ryegrass, radish bulb and leaf.

	Radish soil (µg /g)	**Ryegrass soil (µg/g)**	**Soil K_d (average 21 d)**	**Ryegrass conc. (µg/g)**	**Radish leaf conc. (µg/g)**	**Radish bulb conc. (µg/g)**	**Ryegrass UF_{soil}**	**Radish leaf UF_{soil}**	**Radish bulb UF_{soil}**	**Ryegrass $UF_{pore\ water}$**	**Radish leaf $UF_{pore\ water}$**	**Radish bulb $UF_{pore\ water}$**
Carbamazepine	0.71 ± 0.1	0.46 ± 0.2	7.85 ± 1.5	30.23 ± 2.8	43.02 ± 9.3	5.88 ± 0.4	65.26	60.59	8.28	8.31	7.71	1.05
Diclofenac	0.07 ± 0.04	0.05 ± 0.02	12.40 ± 8.3	0.33 ± 0.1	0.79 ± 0.3	0.37 ± 0.02	6.82	11.53	5.39	0.55	0.93	0.43
Fluoxetine	0.47 ± 0.08	0.55 ± 0.03	8.39 ± 4.2	0.04 ± 0.01	0.04 ± 0.02	0.17 ± 0.15	0.08	0.10	0.36	0.01	0.011	0.043
Propranolol	0.16 ± 0.04	0.21 ± 0.04	79.44 ± 29.8	2.37 ± 0.7	0.14 ± 0.1	0.19 ± 0.1	11.04	0.91	1.20	0.14	0.011	0.015
Sulfamethazine	< LOQ	0.01 ± 0.001	0.99 ± 0.5	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Triclosan	9.31 ± 0.85	0.05 ± 0.01	121.88 ± 33.9	2.00 ± 0.5	0.91 ± 0.2	1.13 ± 0.64	37.59	0.10	0.12	0.31	0.0008	0.001

6.3.3 Uptake factors

The greatest UF_{soil} values for the ryegrass, radish leaf and radish bulb were obtained in the carbamazepine treatments, with values of 65.26, 60.59 and 8.28, respectively (Table 6.3). Relatively small UF_{soil} values were found for fluoxetine (0.08 – 0.36) which can probably be accounted for by the high soil concentration remaining at the end of the experiment and the observed low uptake (Figure 6.3). Work by Karnjanapiboonwong *et al*., (2011) found greater triclosan UFs between the soil and the root in the pinto bean (*Phaseolus vulgaris*), which ranged between 9 -12, in comparison to UF_{soil} (0.12) and $UF_{pore\ water}$ (0.001) values generated in this study for the radish root. However in the ryegrass exposure the triclosan UF_{soil} is considerably larger in the present study at 37.6.

Calculated $UF_{pore\ water}$ range between 0.01 – 8.31, 0.0008 – 7.71 and 0.001 – 1.05 for the ryegrass, radish leaf and radish bulb respectively (Table 6.3). Similar to UF_{soil} carbamazepine exposure resulted in the highest $UF_{pore\ water}$ in the ryegrass, radish leaf and radish bulb. Triclosan had the lowest $UF_{pore\ water}$ in the radish leaf (0.0008) and bulb (0.001) whereas fluoxetine had the lowest $UF_{pore\ water}$ in ryegrass (0.01).

6.3.4 Potential factors influencing the uptake of pharmaceuticals

Plant uptake is thought to be heavily dependent on the physico-chemical characteristics of the chemical, including the Henry's Law constant, water solubility and octanol-water partition coefficient (Bacci *et al*., 1990; Briggs *et al*., 1983; Duarte Davidson and Jones, 1996; Trapp *et al*., 1990). Physico-chemical properties are important because they can describe whether a chemical is neutral or ionisable at environmentally relevant pH values. A clear distinction has been made between the plant uptake of neutral chemicals and chemicals which are ionised (electrically charged) and separate models exist to predict uptake of chemicals in both these forms (Trapp, 2004). However it is important to note the total concentration in a plant cell comprises neutral, ionic and complexed forms of a compound (Trapp,

2004). In this study, carbamazepine was the only neutral chemical whereas the remaining pharmaceuticals were ionisable.

For neutral chemicals, hydrophobicity (usually expressed as log K_{ow}) has been postulated to be the most important property involved in the uptake of chemicals into a plant from the soil medium (Hellström, 2004) as the degree of uptake appears to be proportional to the octanol-water partition coefficient (Briggs *et al.*, 1982; Paterson *et al.*, 1994). Briggs *et al.*, proposed that plant uptake of neutral chemicals can be represented by a Gaussian curve distribution where maximum translocation of chemicals can be seen at a log K_{ow}, ~1.78 in comparison to particularly hydrophobic (high log K_{ow}) and hydrophilic (low log K_{ow}) chemicals which are taken up to a lesser extent.

The high pore water concentrations in the carbamazepine exposure may have played a crucial role in the large amount of uptake observed. However the consistently high carbamazepine uptake into leafy parts of the plants (< 52 µg/g) can more likely be attributable to the log K_{ow} of around 2 for this compound which is in the range of K_{ow} values for which maximum translocation of neutral compounds is expected (Briggs *et al.*, 1982). Greater carbamazepine concentrations were noted in the leaf material in comparison to the roots (Figure 6.6; Table 6.3) which is in agreement with previous research findings (Shenker *et al.*, 2011; Winker *et al.*, 2010; Wu *et al.*, 2010, 2012). It appears that the uptake of carbamazepine is passive and not restricted by root membranes. Carbamazepine has relatively low hydrophobicity and is mainly transported by mass flow from the roots and thus concentrates in the mature and older leaves (Shenker *et al.*, 2011).

Even though triclosan is slightly ionised at the test soil pH of 6.19 (1.2 %), most of the compound will be in the non-ionised form. The unionised molecule has a log K_{ow} of 4.80 so the low observed uptake for triclosan can be also explained by the Gaussian distribution relating uptake to hydrophobicity (Briggs *et al.*, 1982). Small radish $UF_{pore\ water}$ (0.0008 – 0.001) and UF_{soil} (0.10 – 0.12) values for triclosan uptake demonstrate that particularly hydrophobic chemicals are not taken up to a great extent in the plant material.

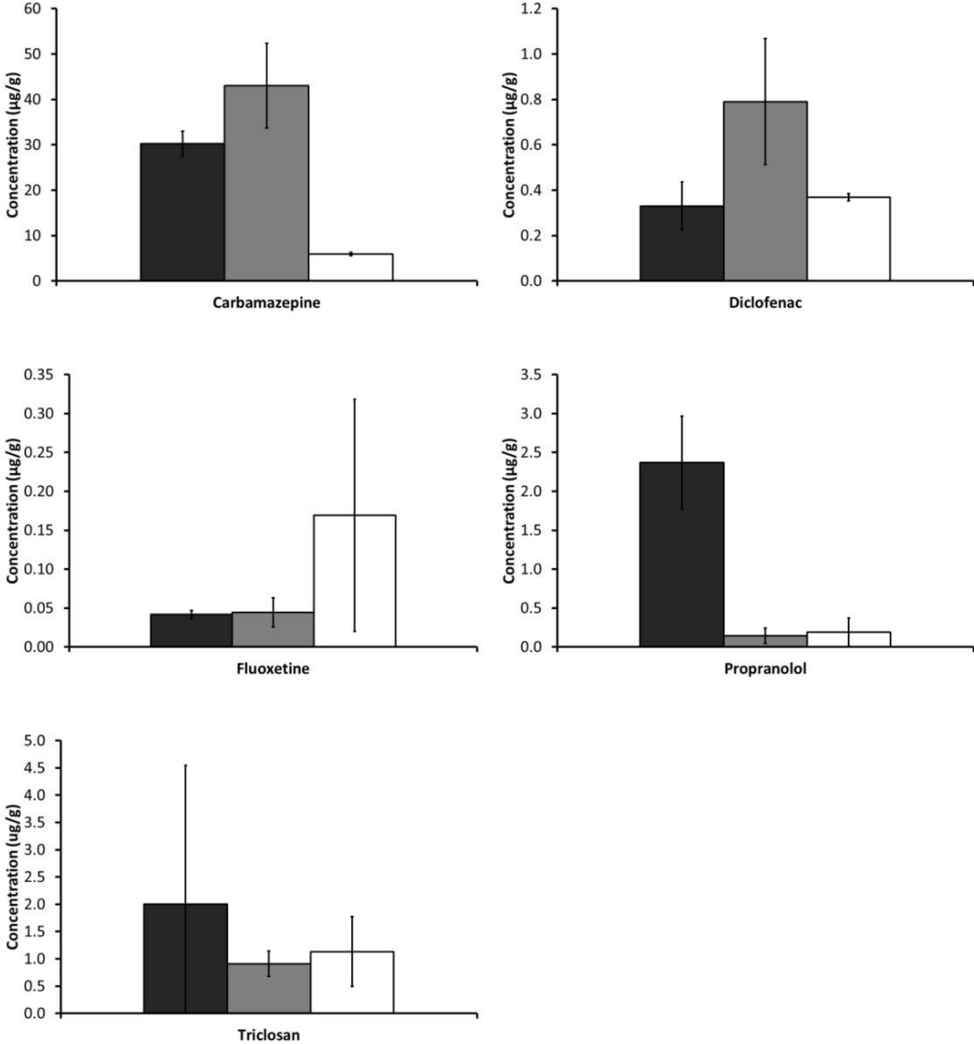


Figure 6.6 Total uptake of carbamazepine, diclofenac, fluoxetine, propranolol and triclosan into ryegrass (dark grey), radish leaf (grey) and radish bulb (white) after plants were grown from seed in pharmaceutically spiked soil for 40 days. Average concentrations provided with error bars representing the standard error (*n* = 6). Sulfamethazine uptake was below LOQ.

Similar to triclosan, only a small proportion of sulfamethazine (5.8 %) would be in the ionised form at the pH of the study soil. Based on the Gaussian distribution (Briggs *et al.*, 1982), the neutral form of sulfamethazine (log K_{ow} of 0.9) would not be expected to enter the root system - it is therefore not surprising that concentrations of sulfamethazine were below the LOQ and that UFs could not be calculated. The complete dissipation from the soil by 3 d may have also contributed to the unquantifiable sulfamethazine uptake (Figure 6.3).

Diclofenac, fluoxetine and propranolol are expected to be extensively ionised in the test soil (> 99 %). Previous research demonstrates that plant uptake of the

dissociated species of an ionisable compound is lower compared to the unionised species (Briggs, 1981; Trapp, 2000). As demonstrated in Figure 6.6 there were up to 600 times less uptake of diclofenac, fluoxetine and propranolol in the ryegrass in comparison to the neutral pharmaceutical, carbamazepine. Specifically for diclofenac, the large ionisation combined with the results from the fate study which show extensive dissipation from both the soil and pore water, and low measured concentrations can probably explain the minimal uptake of diclofenac into the radish and ryegrass.

Previous research findings demonstrate that some pharmaceuticals have a tendency to accumulate in the roots with the roots acting as a sink for hydrophobic neutral compounds (Herklotz *et al*., 2010; Wu *et al*., 2010). In general, organic chemicals with log $K_{ow} > 4$ are expected to have high potential for root retention and low translocation capacity (Duarte Davidson and Jones, 1996). Even though diclofenac and fluoxetine have log $K_{ow} > 4$ (Table 6.1), this is in their unionised form. As both diclofenac and fluoxetine are extensively ionised at test soil pH log K_{ow} is not applicable and thus cannot explain plant uptake. However log D_{ow} (pH corrected log K_{ow}) could be a better descriptor for ionised chemicals as our results show a general increase in log D_{ow} corresponds to an increase in $UF_{pore\ water}$ and UF_{soil}. For example fluoxetine and propranolol have low log D_{ow} values (Table 6.1) and smaller $UF_{pore\ water}$ (< 0.14) than diclofenac ($UF_{porewater}$ < 0.93) at log D_{ow} 2.3.

Similar to other studies, our results found differences in uptake between the two crop species (Boxall *et al*., 2006; Wu *et al*., 2012). Differences may be resultant of factors such as degree of root growth, transpiration rates and the size and shape of the leaf material. Differences in plant lipid contents may also be important as this can affect the affect the sorption of hydrophobic chemicals (Bromilow and Chamberlain, 1995; Orita, 2012). The reported lipid content of perennial ryegrass ranges between 2 – 4 % (Mir *et al*., 2006). Whereas radish bulbs only contain trace amounts of lipid which may explain the lower observed uptake of carbamazepine, diclofenac and propranolol in the radish (Figure 6.6).

Based on the results presented, the factors which affect the uptake of pharmaceuticals into plants include physico-chemical properties of the pharmaceuticals, species type including lipid content and distribution between above

and below ground plant. Additional research has also demonstrated that soil properties can also affect plant uptake (Chiou *et al*., 2001; Harris and Sans, 1967; Topp *et al*., 1986) and therefore to conclude, the uptake of chemicals into plants is a complex process governed by a combination of soil, plant and chemical factors. However on the whole, the uptake behaviour observed in this study makes sense based on the knowledge from previous research.

6.4 Conclusions

Radish and ryegrass can take up a variety of pharmaceuticals and personal care products from spiked soil. Using a combination of fate study and plant uptake data it is clear that relationships between plant uptake and the available fraction of the chemical are key. Whilst a chemical may have a log K_{ow} which fits within the Gaussian distribution correlating with a high propensity for uptake, this clearly is not the only property influencing uptake into plants. The ionisable state of the chemical together with its potential for degradation may result in diminishing concentrations in the soil matrix. The fraction available for uptake may therefore be very small and, correspondingly, the measured concentrations in the plant material will also be minimal. Interestingly, fate studies data show that whilst a chemical may dissipate from the soil it can still remain in the pore water. This may hold wider implications for risk assessment and screening techniques as chemicals present in the pore water may still be bioavaible for uptake into a organism.

The results presented in this Chapter demonstrate that, in some circumstances, uptake and distribution of pharmaceuticals in a plant can be related to hydrophobicity and ionisation of the molecule, (K_{ow}), and generally followed a Gaussian distribution, although this is not always the case. The results presented here would suggest that there are different drivers responsible for the uptake between different plant species. It is instructive to note that pharmaceuticals are predominantly ionisable organic chemicals, and in contrast to neutral organics, this is a characteristic that is likely to affect their partitioning behaviour in terms of bioavailability, plant uptake and molecular interaction with soil matrices of variable pH. It therefore may be important to question previous assumptions on plant uptake and specific models may be required to accurately predict plant uptake which

account for species differences, distribution of chemicals in the plant, chemical properties and the fate of the pharmaceutical in different soil matrices.

Chapter 7 Discussion

7.1 General discussion and recommendations

In recent decades a great deal of work has been undertaken concerning the fate and effects of pharmaceuticals in the environment. Whilst the aquatic environment has been comprehensively explored with regards to the presence, fate, uptake and effects of pharmaceuticals, the terrestrial environment has not yet been studied to the same extent. A large proportion of research in the soil compartment has focused on the development of extraction techniques combined with the improvement of appropriate analytical methods to determine the concentrations of pharmaceuticals in soil matrices. Several studies have also investigated the fate of pharmaceuticals in soils through sorption, leaching and degradation experiments. While research has also looked at the uptake pharmaceuticals, particularly of veterinary origin into crop species, fewer studies exist concerning the uptake of pharmaceuticals into soil dwelling organisms such as earthworms. As the detection of pharmaceuticals in the soil environment has been documented, the presence of pharmaceuticals may pose a risk to soil inhabiting species and thus studies exploring the factors and processes affecting uptake from soils is warrented.

Studying the uptake of pharmaceuticals in the soil environment is important because uptake into soil dwelling species particularly at the base of the food web has the potential for bioaccumulation through the food chain and far wider reaching effects to be seen. One such example of the wider implications of pharmaceutical residues in the environment was the rapid decline in vulture populations in the Indian sub-continent (Oaks *et al.*, 2004). Experimental evidence indicates that the casual factor of this decline is the consumption of meat by these scavenging birds from cattle carcasses containing high levels of the non-steroidal anti-inflammatory drug diclofenac (Green *et al.*, 2007, 2006; Oaks *et al.*, 2004). Uptake into vegetable crops also presents a potential human risk via the consumption of contaminated crops. The present studies were therefore initiated to explore the factors and processes which affect the uptake of pharmaceuticals into terrestrial species. The studies presented in this thesis primarily focussed on the uptake of pharmaceuticals into earthworms.

However additional studies were also performed to assess uptake into plants. Laboratory studies evaluated the relationship between uptake; and soil parameters (e.g. organic matter, soil pH); pharmaceutical physico-chemical properties (e.g. pKa, log K_{ow}); and species traits. A summary of data generated from the experiments detailed in this thesis can be found in Table 7.1.

Initial studies demonstrated that the pharmaceuticals, carbamazepine, diclofenac, fluoxetine and orlistat are taken up by earthworms, *Eisenia fetida* (Chapter 2). Pore water based bioconcentration factors ranged between 2.2 and 51.5 and increased in the order of carbamazepine < diclofenac < fluoxetine < orlistat. BCFs obtained in this study for earthworms were comparable to BCFs calculated in the aquatic environment for carbamazepine however considerably lower than previous research on aquatic invertebrates for fluoxetine (Meredith-Williams *et al*., 2012). Kinetic modelling demonstrated that pharmaceuticals were accumulated and eliminated to different extents in *E. fetida* depending on the pharmaceutical compound. Both carbamazepine and fluoxetine were taken up and eliminated the quickest and had reached near steady state in the organism after 21 days. Diclofenac and orlistat were accumulated at a much slower rate in the earthworm tissue and were not completely eliminated during the depuration phase.

The next steps in the research were to look at additional factors affecting the uptake of pharmaceuticals in the terrestrial environment. However the studies carried out in Chapter 2 were highly labour intensive and to explore many factors using these methods would be challenging. Therefore, the use of a minimised approach was explored in Chapter 3. Research successfully demonstrated that the minimised design approach, previously introduced by Springer *et al*., (2008), is viable alternative approach to calculate BCFs in aquatic and terrestrial invertebrates without having to carry out full length experiments such as OECD test guidelines. For a single experiment, test organism usage would be reduced by > 70 % as well as a reduction in experimental material and labour efforts required. The approach was robust as steady state does not need to be achieved in the test system and BCFs were not affected by changes in exposure medium concentration. One of the most significant findings is that the minimised design appears to work well across a range of species (including both terrestrial and aquatic), chemicals and different exposure

mediums offering a suitable alternative for BCF calculation in variety of environmental chemical exposure scenarios.

Using the minimised design, further research (Chapter 4) went on to establish that soil properties can also affect BCF calculations for pharmaceutical exposures in *E. fetida*. The largest differences in pore water based BCFs between the five soil types were observed in the diclofenac and orlistat studies with BCFs ranging between 7.02 - 69.57 and 30.51 - 115.92 respectively. Little deviation in BCF values was found between the soil types in the fluoxetine exposure (16.78 – 20.42) and the carbamazepine exposure (1.05 – 1.61). Largest BCFs obtained for each pharmaceutical were obtained in different soil types and no pattern of uptake corresponding to particular soil types was observed. Similarly, no relationships between soil pH, pore water pH, organic carbon content and pore water based BCFs were found, demonstrating that no single parameter can explain pharmaceutical uptake into earthworms. Numerous factors and processes appear to be governing the rate and amount of pharmaceutical uptake into earthworms.

Consistent with previous research findings which have demonstrated species traits are important in determining chemical uptake in organisms (Kelsey *et al*., 2005) differences in BCFs between *E. fetida* and the larger earthworm, *Lumbricus terrestris* were observed in Chapter 5. However contrary to previous research findings which observed larger BCFs for the smaller earthworm *E. fetida,* carbamazepine, diclofenac and fluoxetine accumulated to a greater extent in this experiment and resulted in larger *L. terrestris* BCFs. Conversely, in the orlistat study, BCFs were 50 % smaller in *L. terrestris* than in *E. fetida*. Disparities in uptake between the two species may be a result of varying lipid contents, differences in burrowing behaviour in the soil or species size.

Species differences governing the uptake of pharmaceuticals were also observed in plant experiments (Chapter 6). Certain pharmaceuticals accumulated to a greater extent in the radish roots (fluoxetine) in comparison to the leaf material (carbamazepine). Variations in uptake factors were also observed for the same chemical in the radish leaf and ryegrass. Differences in lipid content of the plant species or hydrophobicity (log K_{ow} /log D_{ow}) of the chemical are likely to be affecting the degree of uptake in the plant leaf and below ground root material.

The research presented in this thesis generated knowledge on the fate of pharmaceuticals in the soil environment, looking at the distribution of pharmaceuticals between the soil and pore water. It appears the fate of pharmaceuticals, similarly to the uptake of pharmaceuticals, is dependent both on pharmaceutical physico-chemical properties and soil parameters. Concentrations of pharmaceuticals in the soil and pore water changed over time. Larger decreases in soil concentration were observed for diclofenac in comparison to the more persistent pharmaceuticals such as carbamazepine in a range of soil types (Chapter 4). Similarly to soil, pharmaceutical pore water concentrations generally decreased over time, however interestingly in the initial earthworm studies (Chapter 2) fluoxetine increased in concentration. Through combustion of the exposure soils from the earthworm studies (Chapters 2 and 4) research established the formation of irreversibly bound residues of diclofenac and orlistat in a range of soil types. The degree of formation of non-extractable residues (NERs) was dependent on soil type and pharmaceutical compound (2.4.3.4).

The fate of a pharmaceutical in soil is important with regards to controlling any potential uptake into terrestrial species. The distribution of the pharmaceutical between the soil and pore water can regulate the bioavailable fraction for uptake. For example, in the plant study (Chapter 6) triclosan rapidly dissipated from the soil however remained fairly persistent in the pore water throughout the length of the study. Therefore if soil was sampled to check for pharmaceutical residues initial indications would suggest that there is no chemical present for uptake. However as the pore water fraction facilitates uptake into both into plants and earthworms pharmaceuticals may well accumulate in these organisms.

The experimental research presented in this thesis demonstrates that the uptake of pharmaceuticals into terrestrial species is a complex interaction of pharmaceutical physico-chemical properties, soil parameters and species traits. The fate and distribution of the pharmaceutical between the soil and pore water can also regulate the bioavailable fraction of the pharmaceuticals which has been shown to change between soil types.

Table 7.1 Summary of bioconcentration factor and fate data for test pharmaceuticals obtained in experimental chapters.

	Chapter	**Species**	**Fate in soil**	**Fate in pore water**	**Pore water based BCF / UF**	**Soil based BSAF / UF**
Carbamazepine	2	*E. fetida*	Persistent	Slight dissipation	2.21	Not calculated
	4	*E. fetida*	Persistent in all soil types	Slight dissipation all soil types (< 10 %)	1.05 - 1.61	0.36 - 0.97
	5	*L. terrestris*	Persistent	Slight dissipation	6.69	Not calculated
	6	Radish leaf	Persistent	Fairly persistent	7.71	60.59
	6	Radish bulb	Persistent	Fairly persistent	1.05	8.28
	6	Ryegrass	Persistent	Fairly persistent	8.31	65.26
Diclofenac	2	*E. fetida*	Dissipation and formation of NERs	Relatively constant	21.5	Not calculated
	4	*E. fetida*	Dissipation and formation of NERs	Fairly persistent, soil 2.1 dissipation	7.02 - 69.57	1.01 - 12.36
	5	*L. terrestris*	Dissipation and formation of NERs	Relatively constant	83.79	Not calculated
	6	Radish leaf	Complete dissipation (< 3d)	Rapid dissipation but traces remain at end	0.93	11.53
	6	Radish bulb	Complete dissipation (< 3d)	Rapid dissipation but traces remain at end	0.43	5.39
	6	Ryegrass	Complete dissipation (< 3d)	Rapid dissipation but traces remain at end	0.55	6.82
Fluoxetine	2	*E. fetida*	Persistent	Increase in concentration	30.8	Not calculated
	4	*E. fetida*	Persistent in all soil types	Fairly persistent	16.78 - 20.42	0.19 - 0.37
	5	*L. terrestris*	Fairly persistent	Relatively constant	66.9	Not calculated
	6	Radish leaf	Fairly persistent	Fairly persistent	0.011	0.1
	6	Radish bulb	Fairly persistent	Fairly persistent	0.043	0.36
	6	Ryegrass	Fairly persistent	Fairly persistent	0.01	0.08

Table 7.1 Summary of bioconcentration factor and fate data for test pharmaceuticals obtained in experimental chapters continued.

	Chapter	Species	Fate in soil	Fate in pore water	Pore water based BCF / UF	Soil based BSAF / UF
Propranolol	6	Radish leaf	Fairly persistent	Fairly persistent	0.011	0.91
	6	Radish bulb	Fairly persistent	Fairly persistent	0.015	1.2
	6	Ryegrass	Fairly persistent	Fairly persistent	0.14	11.04
Sulfamethazine	6	Radish leaf	Complete dissipation (< 3d)	Rapid dissipation but traces remain at end	< LOQ	< LOQ
	6	Radish bulb	Complete dissipation (< 3d)	Rapid dissipation but traces remain at end	< LOQ	< LOQ
	6	Ryegrass	Complete dissipation (< 3d)	Rapid dissipation but traces remain at end	< LOQ	< LOQ
Triclosan	6	Radish leaf	Rapid dissipation	Persistent	0.0008	0.1
	6	Radish bulb	Rapid dissipation	Persistent	0.001	0.12
	6	Ryegrass	Rapid dissipation	Persistent	0.31	37.59
Orlistat	2	*E. fetida*	Slight dissipation (< 20 %) and formation of NERs	Slight dissipation	51.5	Not calculated
	4	*E. fetida*	Slight dissipation (< 20 %)and formation of NERs	Some dissipation (< 30 %)	30.51 - 115.92	0.48 - 1.54
	5	*L. terrestris*	Slight dissipation and formation of NERs	Slight dissipation	33.21	Not calculated

7.1.1 Wider implications of research findings

The research presented in this thesis demonstrates that pharmaceuticals can be taken up by, and accumulate in, earthworms and plants from soil contaminated with pharmaceutical residues. The potential for this to cause secondary poisoning in higher tier predators or to present a risk to humans is evaluated in the sections below.

7.1.1.1 Risks of secondary poisoning

As pharmaceuticals can be taken up by earthworms this may present a risk to birds which feed on them. The predicted environmental concentration (PEC) in a worm was calculated using results obtained in Chapter 2 to estimate the potential for secondary poisoning. For this analysis a starling was used as a representative bird species which eats approximately 30 g of invertebrates per day (Markman *et al*., 2008). By extrapolating a human threshold value, calculated from the maximum therapeutic dosage for a human, a threshold value for the starling was computed. The daily dose is between 1.02 and 9.97 ng depending on the compound (Table 7.2). The results would infer the risk to be minimal for all of the pharmaceutical compounds as a starling would have to eat thousands of worms to reach the threshold dose, however these calculations should be used with caution as they involve a considerable amount of estimation and extrapolation. The compound with most risk is fluoxetine as 8000 worms would have to be eaten instead of over 250 000 to receive a greater than predicted threshold dose for carbamazepine.

Currently the risk of secondary poisoning to birds is minimal based on the BCF values obtained in this study, however further calculations involving less extrapolation and estimation would confirm these findings

Table 7.2 The risks of secondary poisoning to a starling, evaluated by consumption of worms containing predicted environmental concentrations of pharmaceuticals used in this study. The daily dose (DD) to a bird is compared to the threshold dose to provide the margins of exposure.

Pharmaceutical	PEC (mg/kg wwt worm^{-1}) [a]	Daily food consumption (g)[b]	DD to bird (mg/bird/ day)	Threshold dose for bird (mg/day)	Margin of exposure
Carbamazepine	0.0228	30	6.86 E-06	1.83	266 225
Diclofenac	0.0278	30	8.34 E-06	0.11	13 699
Fluoxetine	0.0339	30	1.02 E-05	0.09	8 979
Orlistat	0.0326	30	9.79 E-06	0.14	14 007

[a] PEC is the predicted environmental concentration in the worm and calculated from the TGD Part 2 where the total concentration in the worm = PECworm

[b] Markham *et al.,* 2008 – 30 g wet weight of invertebrates eaten per day for startlings (Markman *et al.,* 2008).

7.1.1.2 Human exposure

Uptake into plants, especially edible crops, may represent an important exposure pathway of these chemicals into the food chain and thus present a risk to humans and livestock which feed on them (Boxall *et al*., 2006; Sridhara Chary *et al*., 2008; Zhang *et al*., 2007; Zohair *et al*., 2006). An acceptable daily intake (ADI) value can be used calculate the amount of a substance, for example pharmaceuticals, which can be consumed by a human without resulting in appreciable risk to health. For a full explanation of calculated methods to determine the risk to humans from consuming contaminated crops from results in the present study see Appendix 19.

Results show that if all crops consumed were grown in soil containing the selected pharmaceuticals then humans would not consume levels greater than the ADI for any

of the pharmaceuticals in this study (Table 7.3). It should also be noted that all crops eaten must be grown in the contaminated soil as our analysis assumed ryegrass and radish bulb to be representative of all above and below ground crops consumed, which is not currently the case. A safety factor of 100 was also applied to the minimum therapeutic dose to calculate the ADI and for a large proportion of the population this is not needed which would make the actual ADI higher than the current threshold.

Table 7.3 Results from a comparison of acceptable daily intake (ADI) values for study chemicals and theoretical crop concentration (based on measured soil concentrations and UF_{soil} calculated in this study) shown as a percentage of ADI. With exception of sulfamethazine as plant concentrations were below LOQ. Ryegrass was used as a representative above ground crop species and radish as a representative below ground crop species.

		Ryegrass	**Radish**
	Soil[a] (mg/kg)	**% of ADI in 359.5 g crop**	**% of ADI in 159 g crop**
Carbamazepine	0.0065	3.81	0.21
Diclofenac	0.00054	0.18	0.06
Fluoxetine	0.0067	0.09	0.19
Propranolol	0.0004	0.20	0.01
Triclosan	0.019	83.80	0.12

[a] =Duran-Alvarez, 2009; Dalkmann *et al*., 2012; Vazquez -Roig *et al*., 2012

To date the health risks from pharmaceuticals in drinking water have been reviewed and several papers have also computed levels in crops fit for human consumption (Boxall *et al*., 2006; Bruce *et al*., 2010; Schwab *et al*., 2005). For fluoxetine, a comparison of measured concentrations in drinking water and predicted no effect concentrations in children yielded a ratio of 2.8×10^{-4} which would infer the risk to humans drinking water contaminated with fluoxetine would be minimal. Indeed, for all pharmaceuticals evaluated, approximate margins of safety for potential exposures ranged from 30 – 38 000 (Schwab *et al*., 2005). Presently, the risk to humans in terms of contaminated crops is therefore similar to drinking water exposures, in that it is very low.

However an important note for the future is with the growing demand for alternative irrigation resources in water stressed regions and projected increases in the application of sewage sludge on land, pharmaceutical loadings in soil will inevitably increase. The threat posed by pharmaceuticals taken up into crops may therefore be of more concern in the future than based on current exposure levels.

7.1.1.3 Risk assessment

Bioconcentration factors are important in the risk assessment of chemicals in the environment as they can be compared to threshold values to determine if there is a potential risk. The research presented in this thesis demonstrates that further refinement of previously accepted BCF estimation techniques, such as quantative structure relationships (QSARs), are needed for earthworm pharmaceutical exposure.

Work by Belfroid *et al*., (1993) and Jager (1998) both utilise log K_{ow} as the primary determinant in calculating BCFs however results show both estimation techniques consistently overestimated the pore water BCFs calculated in this study for diclofenac, fluoxetine and orlistat. For orlistat estimated BCFs were some 6000 times greater than obtained in this research (Chapter 2). This may be explained by the fact these QSAR's were not specifically designed for pharmaceutical exposures and highly lipophilic compounds; for example the work by Belfroid *et al*., (1993) has a limited log K_{ow} window of 4.2 – 5.7 (which was later extrapolated from 2 -7) and was based on a water only exposure scenario. The results suggest that the uptake of highly hydrophobic compounds such as orlistat do not scale according to log K_{ow}, implying a cut off point for a linear relationship between K_{ow} and BCF above which increasing log K_{ow} value does not appear to correlate with elevated bioconcentration.

There is therefore a need to improve the accuracy and applicability of currently available QSARs and models for the prediction of earthworm BCFs whereby parameters other than log K_{ow} are utilised to estimate pharmaceutical uptake. New models would need to account for physico-chemical properties (including potential ionisation), soil parameters (important in governing the fate of pharmaceuticals) and species traits, all of which have been shown to affect earthworm uptake. Similarly, results from Chapter 6 suggest that plant uptake models need to take into account the

ionisation state of the chemical and crop type, part of which has already been explored by Trapp (2000).

Refining models to calculate accurate BCFs may however not be necessary for general risk assessment. BCFs computed in this study were generally low and all BSAF values were < 1. Based on the current environmental scenario the measured internal concentrations of all four pharmaceuticals in *E. fetida* present little risk in terms of the wider threat of bioaccumulation and secondary poisoning (section 7.1.1.1). Similarly the results from the plant uptake study indicate that the present risk concerning of humans consuming food crops contaminated with pharmaceutical residues is very low (section 7.1.1.2). However with the projected increase in use and subsequent disposal of pharmaceuticals in the coming century this threat may develop into a more significant issue in the future and thus should be continually monitored and reassessed to account for the changing world we live in.

Interestingly through analysis of the fate study data we observed that the presence of pharmaceuticals in the soil matrices can affect soil chemistry. Changes in soil and pore water pH were noted which appeared to be dependent on soil type and pharmaceutical properties. Significant differences were also found between the internal pH of *E. fetida* exposed to pharmaceuticals and the internal pH of control earthworms. These differences were also dependent on soil type and in some circumstances after an initial increase during the exposure phase the internal pH decreased back to pH measurements similar to the controls after exposure in the clean soil. Significant differences in internal worm pH measurements were also recorded in the *L. terrestris* study. These results hold far wider reaching implications in terms of risk assessment and modelling as currently no attempt to account for changes in pH during exposure to chemicals is made. This may be important for pharmaceuticals, as many of which are ionisable chemicals, and changing pH may alter the bioavailability of the chemicals in the environment.

7.2 Conclusions

In conclusion, this thesis has demonstrated that that a combination of species traits, pharmaceutical physico-chemical properties and soil parameters are key to determining the uptake of pharmaceuticals from soil into terrestrial species. Currently used, generalised models for estimating earthworm BCFs are unable to adequately estimate the uptake of pharmaceuticals. Further refinement of these models is required, as it is necessary to account for these factors including species size, soil pH, log K_{ow} and pore water bioavailability.

It appears that pore water concentrations are important in regulating the amount of chemical available for uptake into earthworms. When comparing between the five soil types, greatest uptake into the earthworm, *E. fetida* occurred in the soil type which had the highest average pore water concentration throughout the uptake phase, and this was true for all four study chemicals. The closeness of the model fit to the measured earthworm data in Chapter 2 also supports the importance of pore water regulating the uptake of pharmaceuticals into earthworms, as the model was based on a pore water exposure only, and the contribution of uptake from ingestion of soil particles was ignored. Pore water concentrations have also previously been shown to be important for regulating the uptake of chemicals into plants.

Whilst pharmaceuticals can be taken up by earthworms and plants from residues in the soil matrices the potential for bioaccumulation through the food chain currently appears to be minimal.

7.3 Recommendations for further research specific to this thesis

The research presented in this thesis has generated novel information on the fate and uptake of pharmaceuticals in the terrestrial environment. However, it has also highlighted a number of additional research questions. In the future, work should consider the following aspects:

1. In the current studies (Chapters 2, 4, 5), for modelling purposes and bioconcentration factor calculations, uptake was based on the diffusion of chemicals across earthworm skin via pore water exposure as demonstrated in the Jager *et al*., (2003) study. However it would be useful to understand the percentage of uptake from the gut, specifically as a result of pharmaceutical exposure, to refine model fits and to provide better uptake and bioconcentration estimates. A series of experiments could elucidate the main uptake pathways of pharmaceuticals.
2. The research presented in this thesis has demonstrated different earthworm species (*Eisenia fetida* and *Lumbricus terrestris*) can affect uptake and bioconcentration factor estimates (Chapter 5). It would be useful to further understand the influence of species traits on the uptake of pharmaceuticals into soil organisms. A series of experiments could evaluate pharmaceutical uptake into additional species for example:
    - Organisms that primarily reside on the soil surface such as land snails (*Helix pomatia*) and slugs (*Limax maximus*), this would also provide an interesting comparison between species with and without a shell.
    - Soil dwelling organisms with an exoskeleton such as woodlice (*Oniscus asellcus*) to see if this restricts pharmaceutical uptake.
    - Nematode species to see if typically small and slender works (2.5 mm) can also take up pharmaceutical residues in soils.
3. Research from this thesis indicates some pharmaceuticals (diclofenac and orlistat) form irreversibly bound residues to the soil (Chapter 2). Experiments were unable to ascertain whether any uptake into *E. fetida* occurred from these non-extractable residues or if it was all from the soil and pore water. Additional research could explore this by exposing earthworms to soil

containing only non-extractable residues to investigate if these fractions can be taken up by earthworms.

4. The two main pathways by which pharmaceuticals enter, and become omnipresent in the soil environment, are via the application of sewage sludge and use of wastewater effluent as an irrigation tool. Rather than adding the compound directly to the soil via stock solution spiking (as described in Chapter 2, 4 and 5) it would be interesting to instead spike irrigation water and sewage sludge with the pharmaceuticals and apply this to the soil. Uptake into plants via this indirect exposure route has previously been investigated (Wu *et al*., 2010) however uptake into soil dwelling organisms such as earthworms is an area which still needs exploring. Changing the application method may change the bioavailability of pharmaceuticals, rate of uptake into earthworms and subsequent bioconcentration factor calculations. A series of experiments have already demonstrated that the presence of sewage sludge has been shown to affect pharmaceutical fate and behaviour in soils (e.g. Monteiro, 2009).
5. In terms of plant experiments, additional research could explore the risk to humans from pharmaceutical residues in vegetable crops in a stimulated gut bio-accessibility study. Evaluating this would provide better estimates as to the levels of pharmaceuticals that would be available and thus a risk to humans.
6. The results in Chapter 3 demonstrated that the minimised design, originally proposed by Springer *et al*., 2008, is a suitable alternative to full length uptake and depuration studies to estimate bioconcentration factors. Whilst the results demonstrated this approach works well for both aquatic and terrestrial species, data could only be obtained for four compounds in the terrestrial environment. Therefore the generation of new data (additional species and compounds) to test the comparison between $BCF_{minimised}$ and $BCF_{traditional}$ would be useful to fully validate this approach for use in terrestrial studies.

7.4 General recommendations for further research:

1. To date, a majority of reseach has focussed on parent compounds and more research is required into the occurrence, fate, uptake and effects of metabolites and transformation products. Parent compounds can be altered in their chemical structure at various stages; in the human body after administration to a patient; during the STP process or once released into the environment. Also, more research is needed to explore the fate and uptake of mixtures of chemicals. Pharmaceuticals do not occur by themselves in the environment, other pharmaceuticals and pesticides amongst other chemicals may also be present and the interaction between these different chemicals may alter their behaviour in the environment.
2. Human pharmaceuticals are prescribed and taken for a reason; they are designed to elicit an effect at a given concentration. In the terrestrial environment, little research has investigated the effects of pharmaceuticals on species. In comparison, several studies in the aquatic environment have observed effects in aquatic organisms (LOEC's) (Cleuvers, 2003; Fent *et al*., 2006; Ferrari *et al*., 2003; Skolness *et al*., 2012) such as reproductive failure in fish (Nash *et al*., 2004) and the feminisation of male fish (Jobling *et al*., 2006, 1998; Sumpter and Johnson, 2008). We now know that pharmaceuticals are taken up by soil dwelling species such as earthworms and plants so research must now progress to evaluate any potential toxic effects.
3. The use of reclaimed wastewater effluent for irrigation purposes and sewage sludge application to land is set to increase on a global scale and thus the loading of pharmaceuticals to the soil will subsequently increase. Projections for future concentrations of pharmaceuticals both in the soil and aquatic environment are needed to ascertain whether there may be problems as a result of this such as secondary poisoning, based on the results from current research.
4. Further research is required to establish the effect of chemical residues on soil and aquatic chemistry. Research presented in the previous chapters demonstrated that the presence of pharmaceuticals in the soil can affect pore

water and soil pH. Other soil or water quality parameters may also be altered as a result of the presence of pharmaceuticals in the environment. This may change the bioavailability or fate of chemicals for example and therefore needs investigating. Results from this may hold wider implications in modelling scenarios for example where changes in pH are currently not accounted for.

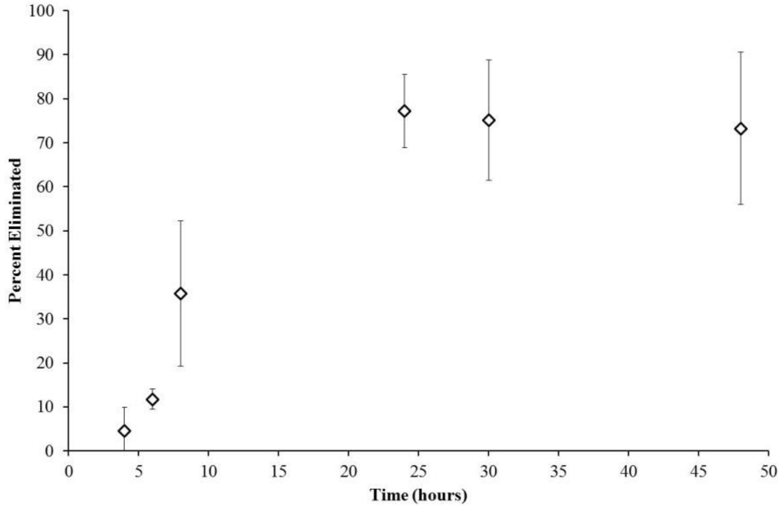
Appendix

Appendix 1 *Eisenia fetida* gut purge experiment

A preliminary experiment was performed to assess how long earthworms, *E. fetida,* required purging their gut contents on moist filter paper to ensure that a majority of soil is removed from the gut before analysis.

Methods:

Earthworms, *E. fetida*, were incubated under test conditions (see section 2.3.3) for 72 hours then removed from the soil, rinsed in deionised water and placed in individual petri dishes on moist filter paper. At various time sampling points (0, 4, 6, 8, 24, 30 and 48 hour) earthworms were removed (six replicates) from the filter paper, weighed to the nearest 0.0001 g then dried in an oven for 24 – 48 hour at 60°C until no further change in weight loss was recorded. Dried earthworms were then re-weighed before being placed in a muffle oven at 500°C for a minimum of four hours to burn off all the combustible parts of the earthworm (everything except the gut contents). The ash was then re-weighed and used to calculate the gut contents remaining in *E. fetida* at various stages of sampling. Below is is a figure of percentage of gut contents eliminated from *Eisenia fetida* after being removed from soil and placed on moist filter paper. Average value provided (± standard deviation).



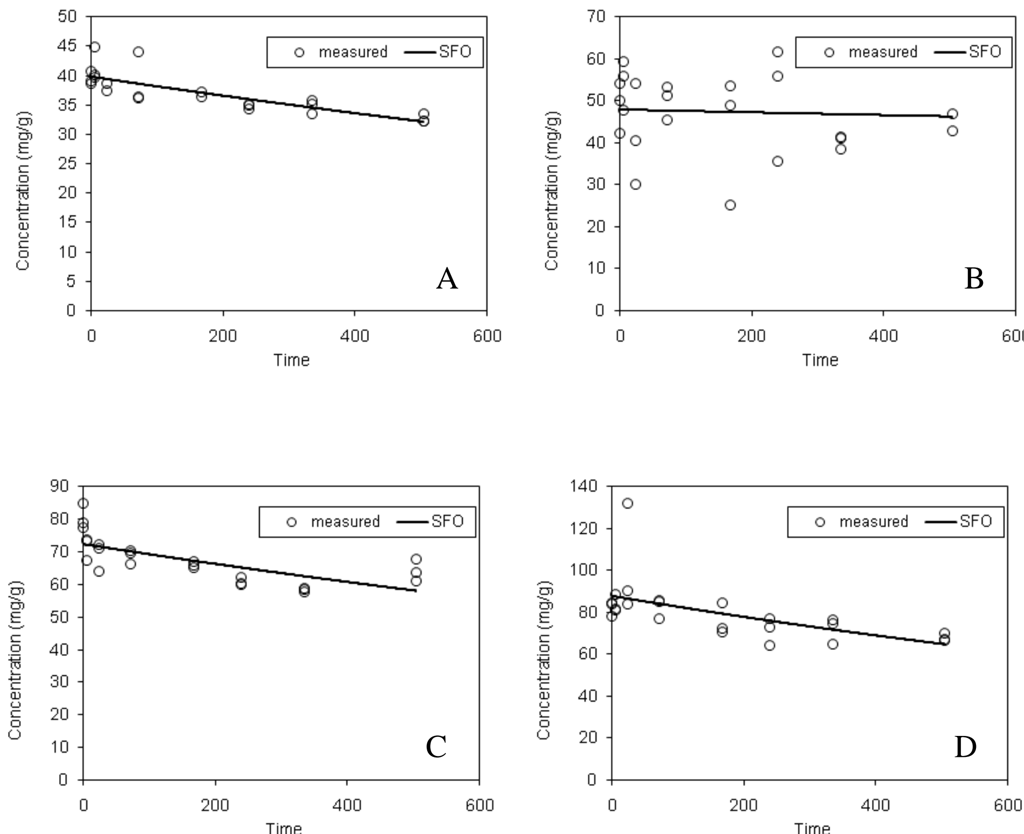
Results:

The amount of soil recovered in the earthworm samples over 24 hours decreased. After 24 hours of gut purge 77 % of the soil had been removed from the earthworm gut. At sampling 30 and 48 hours earthworms appeared to become distressed and started eating the filter paper. A gut purge of 24 hours was therefore chosen as an appropriate length of time, and measured concentrations pharmaceuticals in the earthworm samples were corrected for the 23 % of soil-associated pharmaceutical remaining in the gut in experiments detailed in Chapter 2 and Chapter 4.

Appendix 2 Literature search summary of known diclofenac metabolites and transformation products, including molecular weights provided (Huber *et al*., 2012; Kallio *et al*., 2010; Scheurell *et al*., 2009).

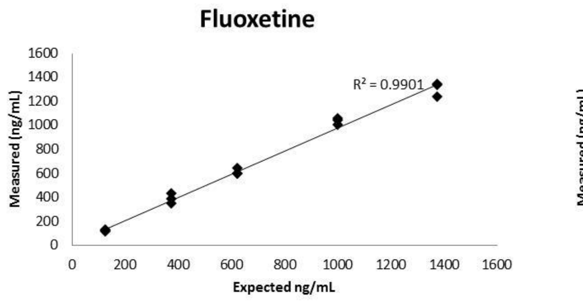
Diclofenac metabolites / transformation products	**Matrice**	**Molecular weight (g/mol)**	**Reference**
acyl glucuronide of diclofenac	Fish bile	472.275	Kallio *et al*., 2010
acyl glucuronide of 4'-hydroxydiclofenac	Fish bile	486.0364	Kallio *et al*., 2010
acyl glucuronide of 5-hydroxydiclofenac	Fish bile	486.0364	Kallio *et al*., 2010
ether glucuronide of 4'-hydroxydiclofenac	Fish bile	486.0364	Kallio *et al*., 2010
sulfate conjugate of 4'-hydroxydiclofenac	Fish bile	389.9611	Kallio *et al*., 2010
sulfate conjugate of 5-hydroxydiclofenac	Fish bile	389.9611	Kallio *et al*., 2010
monosulfate conjugate of dihydroxydiclofenac	Fish bile	405.9561	Kallio *et al*., 2010
4'-hydroxydiclofenac	Fish bile/effluent/rat liver/plants	312.15	Kallio *et al*., 2010; Scheurell *et al*., 2009; Huber *et al*., 2012; Stülten *et al*., 2008
acyl-migrated isomers of acyl glucuronide of 3'-hydroxydiclofenac	Fish bile	486.0364	Kallio *et al*., 2010
acyl-migrated isomers of acyl glucuronide of diclofenac	Fish bile	470.0415	Kallio *et al*., 2010
5-hydroxydiclofenac	Fish bile/ Sewage effluent	312.15	Scheurell *et al*., 2009; Kallio *et al*., 2010
3′-Hydroxydiclofenac	Effluent	312.148	Scheurell *et al*., 2009
1-(2,6-Dichlorophenyl)-1,3-dihydro-2*H*-indole-2-one	Effluent	278.13	Scheurell *et al*., 2009
1-β-*O*-acyl glucuronide of diclofenac	Rat liver	472.275	Lee *et al*., 2012

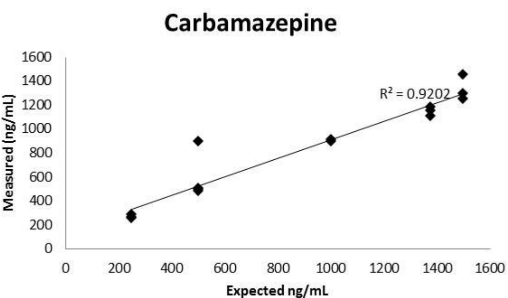
Appendix 3 Modelling the dissipation of test pharmaceuticals in exposure beakers from Chapter 2. Measured soil concentration data from uptake phase for carbamazepine (A), diclofenac (B), fluoxetine (C) and orlistat (D) fitted with a single first order (SFO) model.

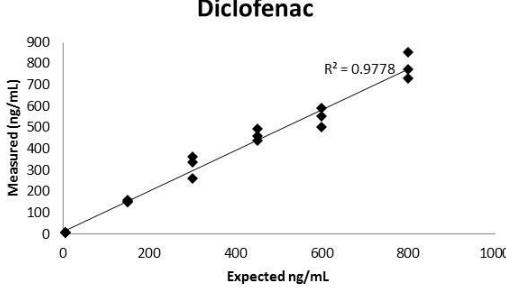


Appendix 4 LC-MS/MS parameters used for the analysis of the test pharmaceuticals in metabolism study.

Compound	**Parent ion (*m/z*)**	**MRM product ions (*m/z*)**	**Collision energy (V)**	**Collision cell exit potential**	**Retention time (min)**
Carbamazepine	237.3 ($M+H^+$)	194.3	13	15	1.83
Carbamazepine d10	247.5 ($M+H^+$)	204.2	13	15	1.83
Fluoxetine	310.3 ($M+H^+$)	148.3	25	12	1.4
Fluoxetine d5	315.2 ($M+H^+$)	153.2	25	12	1.4
Diclofenac	296.2 ($M-H^+$)	250.0	15	11	4.1
Diclofenac d4	298 ($M-H^+$)	254.1 15	11	4.1	







Appendix 5 Calibration plots for standards of carbamazepine, diclofenac and fluoxetine.

Appendix 6 Collation of bioconcentration factor data used in analysis of minimised design.Table of key points from studies used in this analysis including log K_{ow}, chemical use, test species, uptake and depuration period and BCF provided in the literature

Test compound	Log K_{ow} [a]	Pesticide (P) / Pharmaceutical (Ph)	Use	Test species	Uptake t_u (days)	Depuration t_d (days)	$BCF_{traditional}$	Author
4-Nitrobenzyl-chloride	2.61	P		Gammarus pulex	1	6	184.6	(1)
2,4-dichloroaniline	2.78	P	biodegradative intermediate of contact type herbicides	Gammarus pulex	1	6	55.73	(1)
2,4-dichlorophenol	3.28	P	Preparation of herbicide 2,4-D	Gammarus pulex	1	6	4466	(1)
4,6-Dinitro-o-cresol	1.96	P	Herbicide	Gammarus pulex	1	6	36.72	(1)
1,2,3-trichlorobenzene	4.05	P		Gammarus pulex	1	6	190.6	(1)
2,4,5-trichlorophenol	3.95	P	Fungicide, herbicide	Gammarus pulex	1	6	2635	(1)
Aldicarb	1.13	P	Insecticide	Gammarus pulex	1	6	1.64	(1)
Carbofuran		P	Insecticide	Gammarus pulex	1	6	65.14	(1)
Diazinon	3.81	P	Insecticide	Gammarus pulex	1	6	82	(1)
Ethylacrylate	1.32	P	Polymer production (resins, plastics, rubber, and denture material)	Gammarus pulex	1	6	86.97	(1)
Hexachlorobenzene	5.31	P	Fungicide	Gammarus pulex	1	6	2915	(1)
Imidacloprid	0.33	P	Insecticide	Gammarus pulex	1	6	7.35	(1)
Malathion	2.36	P	Insecticide	Gammarus pulex	1	6	114.3	(1)
Sea-nine	2.80	P	Biocide	Gammarus pulex	1	6	1732	(1)
Chlorpyrifos	4.70	P	Insecticide	Gammarus pulex	3	3	1660	(2)
Pentachlorophenol	2.75	P	Herbicide, insecticide, fungi cide, algaecide	Gammarus pulex	3	3	51	(2)
Carbaryl	1.85	P	Insecticide	Gammarus pulex	3	3	87	(3)

Test compound	**Log K_{ow} [a]**	**Pesticide (P) / Pharmaceutical (Ph)**	**Use**	**Test species**	**Uptake t_u (days)**	**Depuration t_d (days)**	**$BCF_{traditional}$**	**Author**
Chlorpyrifos	4.96[c]	P	Insecticide	Anax imperator	2	5	100	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Asellus aquaticus	2	5	3242	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Chaoborus obscuripes	2	5	2428	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Cloeon dipterum	2	5	1782	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Culex pipens	2	5	13930	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Daphnia magna	2	5	541	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Gammarus pulex juvenile	2	5	3083	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Gammarus pulex adult	2	5	2039	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Molanna angustata	2	5	5331	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Neocaridina denticulata	2	5	1291	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Notonecta maculata	2	5	407	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Parapoynx stratiotata	2	5	1601	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Plea minutissima	2	5	654	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	*Procambarus* sp. juvenile	2	5	280	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	*Procambarus* sp. adult	2	5	1295	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Ranatra linearis	2	5	392	(4)
Chlorpyrifos	4.96[c]	P	Insecticide	Sialis lutaria	2	5	9625	(4)
5-fluoruracil	-0.81	Ph	Anti-cancer	Notonecta glauca	2	2	0.132	(5)

Test compound	Log K_{ow} [a]	Pesticide (P) / Pharmaceutical (Ph)	Use	Test species	Uptake t_u (days)	Depuration t_d (days)	$BCF_{traditional}$	Author
Carbamazepine	2.25	Ph	Anti-epileptic	Notonecta glauca	2	2	0.244	(5)
Carvedilol	3.05	Ph	Beta-blocker	Notonecta glauca	2	2	1.596	(5)
Diazepam	2.70	Ph	Sedative	Notonecta glauca	2	2	0.98	(5)
Fluoxetine	4.65	Ph	Anti-depressant	Notonecta glauca	2	2	1.387	(5)
Moclobemide	1.16	Ph	Anti-depressant	Notonecta glauca	2	2	0.334	(5)
5-fluoruracil	-0.81	Ph	Anti-cancer	Gammarus pulex	2	2	6.48	(5)
Carbamazepine	2.25	Ph	Anti-epileptic	Gammarus pulex	2	2	7.094	(5)
Carvedilol	3.05	Ph	Beta-blocker	Gammarus pulex	2	2	270.8	(5)
Diazepam	2.70	Ph	Sedative	Gammarus pulex	2	2	37.47	(5)
Moclobemide	1.16	Ph	Anti-depressant	Gammarus pulex	2	2	4.55	(5)
Carvedilol	3.05	Ph	Beta-blocker	Planorbarius corneus	3	3	57.3	(5)
Fluoxetine	4.65	Ph	Anti-depressant	Gammarus pulex	3	3	185900	(5)
Chloramphenicol	-0.02	Ph	Antibiotic	Lumbriculus variegatus	2	2	2	(6)
Fluoxetine	4.16	Ph	Anti-depressant	Lumbriculus variegatus	2	2	911	(6)
Salicylic acid	2.30	Ph	NSAIDb /skin care product	Lumbriculus variegatus	2	2	82	(6)
Caffiene pH 5.5	1.03	Ph	Stimulant	Lumbriculus variegatus	2	2	1	(6)
Caffiene pH 7	1.03	Ph	Stimulant	Lumbriculus variegatus	2	2	1	(6)
Caffiene pH 8.5	1.03	Ph	Stimulant	Lumbriculus variegatus	2	2	1	(6)
Diclofenac pH 5.5	4.13	Ph	NSAID[b]	Lumbriculus variegatus	2	2	623	(6)
Diclofenac pH 7	4.13	Ph	NSAID[b]	Lumbriculus variegatus	2	2	30	(6)

Test compound	Log K_{ow} [a]	Pesticide (P) / Pharmaceutical (Ph)	Use	Test species	Uptake t_u (days)	Depuration t_d (days)	$BCF_{traditional}$	Author
Diclofenac pH 8.5	4.13	Ph	NSAID[b]	Lumbriculus variegatus	2	2	8	(6)
Fluoxetine pH 5.5	4.16	Ph	Anti-depressant	Lumbriculus variegatus	2	2	49	(6)
Fluoxetine pH 7	4.16	Ph	Anti-depressant	Lumbriculus variegatus	2	2	562	(6)
Fluoxetine pH 8.5	4.16	Ph	Anti-depressant	Lumbriculus variegatus	2	2	218500	(6)
Triclosan pH 5.5	5.42	Ph	Antimicrobial	Lumbriculus variegatus	2	2	568400	(6)
Triclosan pH 7	5.42	Ph	Antimicrobial	Lumbriculus variegatus	2	2	646400	(6)
Triclosan pH 8.5	5.42	Ph	Antimicrobial	Lumbriculus variegatus	2	2	559300	(6)
Triclosan	5.42	Ph	Antimicrobial	Lumbriculus variegatus	2	2	700900	(6)
Naproxen	3.36	Ph	NSAID[b]	Lumbriculus variegatus	2	2	72240	(6)
Carbamazepine	2.25	Ph	Anti-epileptic	Eisenia fetida	21	21	2.21	(7)
Diclofenac	4.02	Ph	NSAID[b]	Eisenia fetida	21	21	21.46	(7)
Fluoxetine	4.65	Ph	Anti-depressant	Eisenia fetida	21	21	30.8	(7)
Orlistat	8.19	Ph	Weight loss aid	Eisenia fetida	21	21	51.53	(7)

[a] log K_{ow} as reported in publications.
[b] NSAID – Non-steroidal anti-inflammatory drug
[c] Specific log Kow for chlorpyrifos not provided therefore Bowman and Sans (1983) reference used
(1) (Ashauer *et al*., 2010) (2) (Ashauer *et al*., 2006) (3) (Ashauer *et al*., 2007) (4) (Rubach *et al*., 2010) (5) (Meredith-Williams *et al*., 2012) (6) (Karlsson, 2013) (7) Chapter 2.

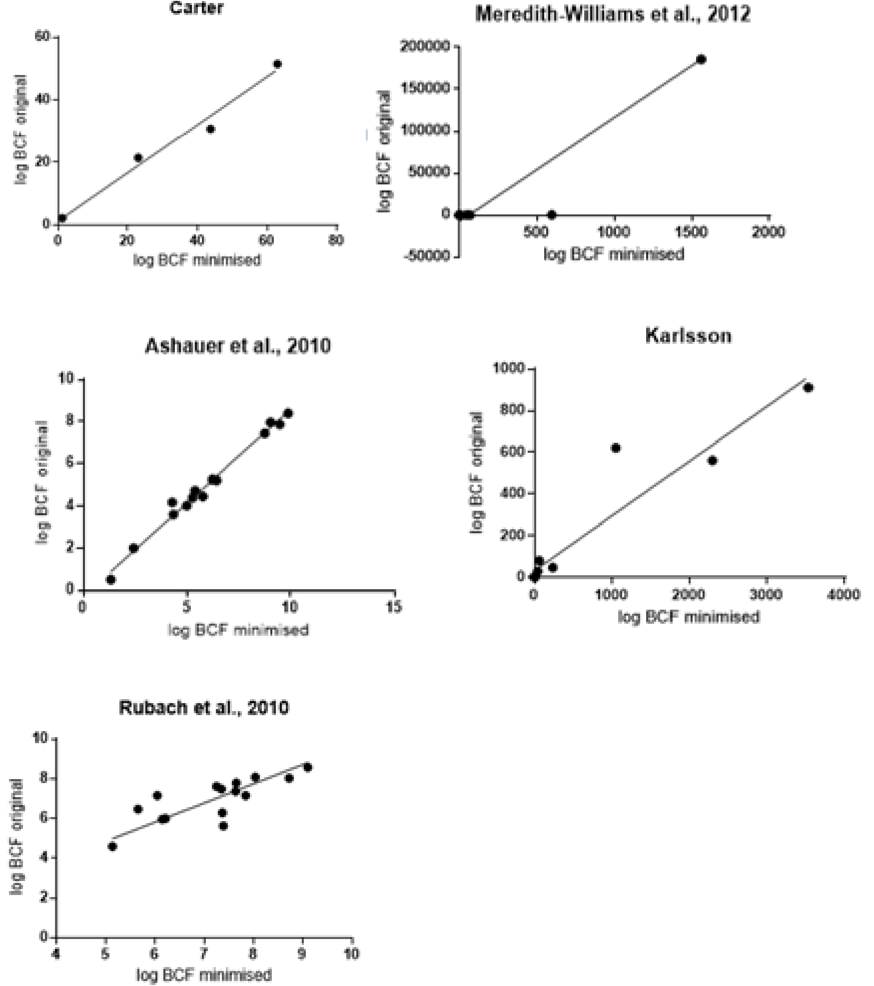
Appendix 7 Bioconcentration factors removed from minimised design analysis.

BCFs were unable to be estimated using the minimised approach when the concentration in the organism at the end of depuration phase was greater than internal concentration measured at the end of the uptake phase. For these situations the minimised design calculated a negative BCF value and thus were removed from the analysis.

Test compound	Pesticide (P) or Pharma-ceutical (Ph)	Use	Test species	Uptake t_u (days)	Depuration t_d (days)	Literature BCF	Author
Chlorpyrifos	P	Insecticide	Sialis lutaria	2	5	9625	Rubach *et al.*, 2010
Chlorpyrifos	P	Insecticide	Culex pipens	2	5	13930	Rubach *et al.*, 2010
Fluoxetine pH 8.5	Ph	Anti-depressant	Lumbriculus variegatus	2	2	218500	Karlsson, 2013
Triclosan pH 5.5	Ph	Anti-microbial	Lumbriculus variegatus	2	2	568400	Karlsson, 2013
Triclosan pH 7	Ph	Anti-microbial	Lumbriculus variegatus	2	2	646400	Karlsson, 2013
Triclosan pH 8.5	Ph	Anti-microbial	Lumbriculus variegatus	2	2	559300	Karlsson, 2013
Triclosan	Ph	Anti-microbial	Lumbriculus variegatus	2	2	700900	Karlsson, 2013

Appendix 8 Deming regression with associated regression line and additional details on slope and intercept for individual data sets analysed in Chapter 3.

Ashauer *et al*., 2010	
Equation	Y = 0.8885*X -0.2931
Slope	0.8885
95 % CI	0.8214 – 0.955
Intercept	-0.2931
95 % CI	-0.7265 – 0.1403
Slope sig. not zero?	$p = < 0.0001$
Rubach *et al*., 2010	
Equation	Y = 0.9630*X + 0.04606
Slope	0.9630
95 % CI	0.5111 – 1.415
Intercept	0.04606
95 % CI	-3.232 – 3.324
Slope sig. not zero?	$p = 0.0005$
Meredith - Williams *et al*., 2012	
Equation	Y = 124.0*X - 7673
Slope	124
95 % CI	91.91 – 156.0
Intercept	-7673
95 % CI	-22553 – 7208
Slope sig. not zero?	$p = < 0.0001$
Karlsson, 2013	
Equation	Y = 0.2623*X + 33.62
Slope	0.2623
95 % CI	0.1940 – 0.3307
Intercept	33.62
95 % CI	-56.08 – 123.3
Slope sig. not zero?	$p = <0.0001$
Chapter 2 (this thesis)	
Equation	Y = 0.7668*X + 1.394
Slope	0.7668
95 % CI	0.4268 – 1.107
Intercept	1.394
95 % CI	-12.23 – 15.01
Slope sig. not zero?	$p = < 0.0105$



Appendix 9 Results from Deming regression analysis on individual data sets comparing $BCF_{minimised}$ to $BCF_{traditional}$

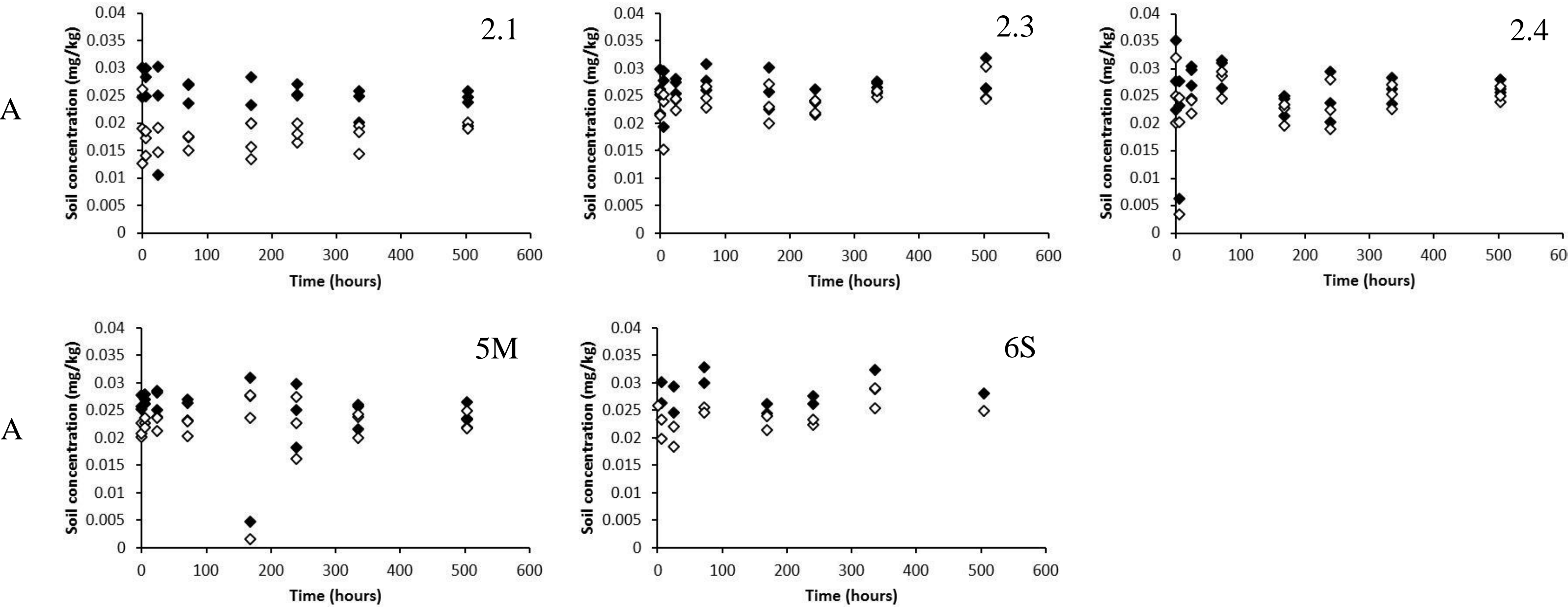
Appendix 10 Validation of extraction methods for pharmaceutical analysis in five soil types.

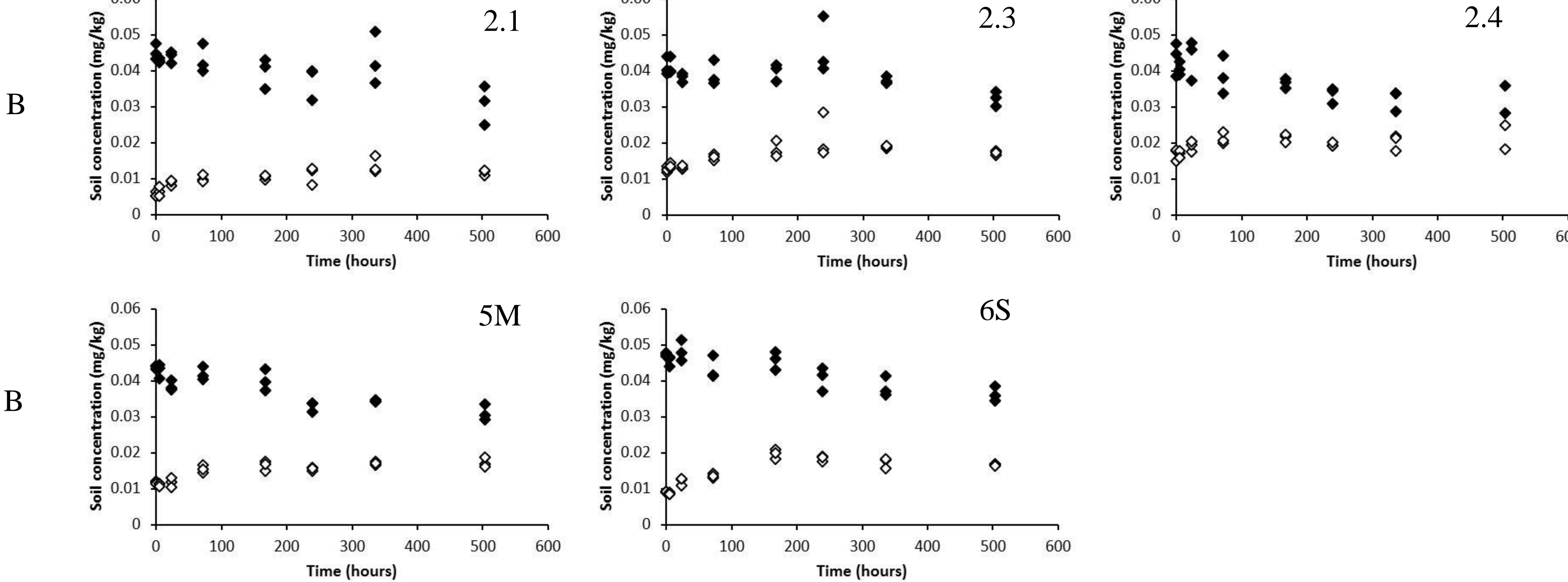
Experiments were carried out to determine the recovery of carbamazepine, diclofenac, fluoxetine and orlistat from five soil types using solvent extraction. For each soil type and each pharmaceutical 5 g of soil was prepared in triplicate and spiked with a known amount of pharmaceutical compound.

Average recoveries for each soil type and compound provided ± standard deviation provided in table below.

Pharmaceutical	Soil type	Solvent	1 x extraction (% recovery ± S.D.)	2 x extraction (% recovery ± S. D.)
Carbamazepine	2.1	Methanol	85.68 ± 10.12	93.65 ± 12.22
Carbamazepine	2.3	Methanol	79.25 ± 6.71	86.79 ± 5.00
Carbamazepine	2.4	Methanol	84.88 ± 1.94	92.61 ± 2.93
Carbamazepine	5M	Methanol	86.50 ± 2.84	93.00 ± 2.99
Carbamazepine	6S	Methanol	83.15 ± 2.81	94.72 ± 3.96
Diclofenac	2.1	Ethyl Acetate	93.05 ± 2.31	93.52 ± 2.00
Diclofenac	2.3	Ethyl Acetate	90.71 ± 1.79	94.67 ± 1.97
Diclofenac	2.4	Ethyl Acetate	90.11 ± 1.71	92.36 ± 0.70
Diclofenac	5M	Ethyl Acetate	77.04 ± 1.55	86.37 ± 4.31
Diclofenac	6S	Ethyl Acetate	83.72 ± 5.52	90.17 ± 5.89
Fluoxetine	2.1	Acetonitrile:Water (7:3)	73.96 ± 5.19	82.19 ± 5.07
Fluoxetine	2.3	Acetonitrile:Water (7:3)	66.16 ± 2.21	76.52 ± 1.48
Fluoxetine	2.4	Acetonitrile:Water (7:3)	61.33 ± 0.60	74.93 ± 1.31
Fluoxetine	5M	Acetonitrile:Water (7:3)	66.26 ± 0.80	78.06 ± 1.65
Fluoxetine	6S	Acetonitrile:Water (7:3)	36.78 ± 0.68	72.43 ± 1.61
Orlistat	2.1	Acetonitrile	84.36 ± 4.52	88.11 ± 2.19
Orlistat	2.3	Acetonitrile	82.00 ± 2.37	82.84 ± 1.94
Orlistat	2.4	Acetonitrile	79.07 ± 1.75	82.25 ± 2.04
Orlistat	5M	Acetonitrile	80.57 ± 2.59	82.28 ± 1.00
Orlistat	6S	Acetonitrile	81.83 ± 3.69	83.13 ± 3.62

Appendix 11 Combustion analysis results, the concentration of orlistat (A) and diclofenac (B) after solvent extraction are shown by the white diamonds and the combined residue concentration of combustion analysis and solvent extraction are shown by the black diamonds.




B
2.1
2.3
2.4
B
5M
6S
Soil concentration (mg/kg)
Time (hours)
0
0.01
0.02
0.03
0.04
0.05
0.06
100
200
300
400
500
600

Appendix 12 Percentage of ionised and neutral species in different soil types, pore water and worm samples for diclofenac exposure at 0H (start pH) and 21 d (end pH) (Chapter 5). Percentage ionisation calculated using Henderson-Hasselbalch equation: log Dow = log Kow - log $(1+10^{A(pH-pka)})$, where A is -1 for basic and 1 for acidic compounds.

Diclofenac

	pore water				pore water			
	start pH	**anion:neutral**	**% neutral**	**% ionic**	**end pH**	**anion:neutral**	**% neutral**	**% ionic**
Soil 2.1	9.57	279683.38	0.0004	100.00	9.70	377282.50	0.0003	100.00
Soil 2.3	9.22	126862.52	0.0008	100.00	9.62	313809.92	0.0003	100.00
Soil 2.4	9.78	453593.34	0.0002	100.00	9.86	549540.87	0.0002	100.00
Soil 5M	9.43	202612.70	0.0005	100.00	9.72	401174.51	0.0002	100.00
Soil 6S	9.66	346736.85	0.0003	100.00	9.74	420081.28	0.0002	100.00

	soil				soil			
	start pH	**anion:neutral**	**% neutral**	**% ionic**	**end pH**	**anion:neutral**	**% neutral**	**% ionic**
Soil 2.1	6.70	377.28	0.2644	99.74	6.69	368.69	0.2705	99.73
Soil 2.3	7.03	812.83	0.1229	99.88	7.00	758.58	0.1317	99.87
Soil 2.4	7.72	4011.75	0.0249	99.98	7.52	2531.24	0.0395	99.96
Soil 5M	8.14	10471.29	0.0095	99.99	8.12	10000.00	0.0100	99.99
Soil 6S	7.92	6358.19	0.0157	99.98	7.90	5979.52	0.0167	99.98

	worm				worm			
	end uptake pH	**anion:neutral**	**% neutral**	**% ionic**	**end dep pH**	**anion:neutral**	**% neutral**	**% ionic**
Soil 2.1	6.84	518.80	0.1924	99.81	6.86	551.65	0.1809	99.82
Soil 2.3	6.68	363.08	0.2747	99.73	6.78	459.20	0.2173	99.78
Soil 2.4	6.91	614.23	0.1625	99.84	6.99	744.16	0.1342	99.87
Soil 5M	6.89	582.10	0.1715	99.83	6.83	514.83	0.1939	99.81
Soil 6S	6.79	465.59	0.2143	99.79	6.96	683.91	0.1460	99.85

Appendix 13 Percentage of ionised and neutral species in different soil types, pore water and worm samples for fluoxetine exposure at 0H (start pH) and 21 d (end pH) (Chapter 5). Percentage ionisation calculated using Henderson-Hasselbalch equation: log Dow = log Kow - log $(1+10^{A(pH-pka)})$, where A is -1 for basic and 1 for acidic compounds.

Fluoxetine

	pore water				pore water			
	start pH	**anion:neutral**	**% neutral**	**% ionic**	**end pH**	**anion:neutral**	**% neutral**	**% ionic**
Soil 2.1	9.33	1.60	38.50	61.50	9.51	1.05	48.85	51.15
Soil 2.3	9.28	1.76	36.17	63.83	9.04	3.11	24.31	75.69
Soil 2.4	9.40	1.35	42.57	57.43	9.62	0.81	55.16	44.84
Soil 5M	9.31	1.66	37.60	62.40	9.09	2.78	26.49	73.51
Soil 6S	9.78	0.56	64.01	35.99	9.64	0.77	56.49	43.51

	soil				soil			
	start pH	**anion:neutral**	**% neutral**	**% ionic**	**end pH**	**anion:neutral**	**% neutral**	**% ionic**
Soil 2.1	6.19	2187.76	0.05	99.95	6.84	493.55	0.20	99.80
Soil 2.3	6.88	450.13	0.22	99.78	7.16	236.23	0.42	99.58
Soil 2.4	7.21	207.33	0.48	99.52	7.68	70.79	1.39	98.61
Soil 5M	8.10	26.92	3.58	96.42	8.35	15.14	6.20	93.80
Soil 6S	8.09	27.75	3.48	96.52	8.04	31.14	3.11	96.89

	worm				worm			
	end uptake pH	**anion:neutral**	**% neutral**	**% ionic**	**end dep pH**	**anion:neutral**	**% neutral**	**% ionic**
Soil 2.1	7.03	316.23	0.32	99.68	6.91	418.47	0.24	99.76
Soil 2.3	6.83	503.11	0.20	99.80	6.66	503.11	0.20	99.80
Soil 2.4	6.96	368.69	0.27	99.73	6.73	368.69	0.27	99.73
Soil 5M	6.81	530.88	0.19	99.81	6.66	530.88	0.19	99.81
Soil 6S	6.84	489.78	0.20	99.80	6.77	489.78	0.20	99.80

Appendix 14 Preparation of Ruakura nutrient solution for use during plant growth.

General method:

The solution is prepared by combining three prepared stock solutions (A, B, C) to 1.75 L of deionised water. Add 200 mL of stock B, then 200 mL of stock A and 100 mL of the micronutrient supplement, to make a final volume of 2.25 L.

5 mL of Ruakura solution per 250 g of soil (Smith *et al*., 1983) was applied twice weekly, (for three weeks), from the day that 50 % emergence is counted. After 3 weeks of additions, continued with 1 x 5 mL/250 g soil of nutrient solution per week.

Note: Pots were maintained to 60 % of the MWHC on a daily basis using deionised water (DI) or the nutrient solution on the prescribed one or two days per week.

Table below provided Nutrient stocks required for Ruakura solution.

Macronutrient Stock A (g/L)	
Chemical	Weight (g)
$Mg(NO_3)_2.6H_2O$	4.94
$Ca(NO_3)_2.4H_2O$	16.78
NH_4NO_3	8.48
KNO_3	2.28
Macronutrient Stock B (g/L)	
Chemical	Weight (g)
KH_2PO_4	2.67
K_2HPO_4	1.64 [or 2.149 g of $K_2HPO_4.3H_2O$]
K_2SO_4	6.62
Na_2SO4	0.60
NaCl	0.33
Micronutrient Supplement C (mg/L)	
Chemical	Weight (mg)
H_3BO_3	128.8
$CuCl_2.2H_2O$	4.84
$MnCl_2.4H_2O$	81.1
$(NH_4)6Mo_7O_{24}.4H_2O$	0.83

Appendix 15 LC-MS/MS parameters used for the analysis of compounds in plant study.

Compound	Parent ion	MRM product ions	Collision energy (V)	Retention time (min)
Propranolol	260 (M+H+)	116 183	25 25	5.25
Propranolol D7	267 (M+H+)	116 188	25 25	5.23
Sulfamethazine	279 (M+H+)	92 124	35 35	5.09
Carbamazepine	237 (M+H+)	193 194	40 30	5.41
Carbamazepine D10	247 (M+H+)	202 204	40 30	5.44
Fluoxetine	310 (M+H+)	44 148	20 20	5.33
Fluoxetine D5	315 (M+H+)	44 156	20 20	5.38
Diclofenac	294 (M-H+)	214 250	20 20	5.97
Diclofenac D4	298 (M-H+)	217 254	20 20	5.93
Triclosan	287 (M-H+) 289 (M-H+)a	287 289	2 2	6.09
Triclosan D3	290 (M-H+) 292 (M-H+)a	290 292	2 2	6.16

[a] ^{37}Cl isotope of TCS and TCS D3

Appendix 16 Analytical lower limits of quantification (LOQs) for the LC-MS/MS method used and within the plant and soil matrices in µg/L.

Compound	LC-MS/MS	Soil	Ryegrass leaf	Radish leaf	Radish bulb
Propranolol	1	2.6	5.3	4.4	5.9
Sulfamethazine	0.5	0.6	4.6	6.7	10
Carbamazepine	0.5	0.7	4.2	5	2.6
Fluoxetine	2.5	7.1	7.1	8.1	6.6
Diclofenac	2.5	2.7	2.1	3	4
Triclosan	5	17.9	11.1	7.3	6.3

Appendix 17 Validation of extraction methodologies for plant material.

Radish, ryegrass and soil were spiked with a known amount of each pharmaceutical and different extraction methods and clean up steps were followed to obtain the highest percentage recoveries. Plants were freeze dried prior to extraction (soil was not) and then either extracted with 2 x methanol, followed by 1 x acetone or three extractions of a 70:30 (v/v) acetonitrile and water solution. A comparison between using SPE and no SPE as a clean up step was also made. Results presented below indicate the methods which generated the highest recoveries for the different matrices and thus were adopted in the extraction techniques in this study.

		% Relative					
Matrix	Extraction	Carbamazepine	Diclofenac	Fluoxetine	Propranolol	Sulfamethazine	Triclosan
Leaf	ACN:H20 (SPE)	100.5 ± 4.7	118.7 ± 5.8	89.1 ± 4.3	98.8 ± 4.2	82.4 ± 4.9	117.2 ± 19
Root	ACN:H20 (SPE)	106.8 ± 4.1	139 ± 10	92.6 ± 5.2	103.9 ± 4.8	75.4 ± 1.7	181.8 ± 48
Soil	MeOH/Acetone	90.42 ± 7.12	85 ± 6.3	68.53 ± 10	109.40 ± 23	91.54 ± 7.54	98.57 ± 4.9

Results show that a ACN:H_20 extraction followed by SPE clean up yielded the best recoveries for radish and ryegrass (both leaf and root) for the range of pharmaceuticals and therefore this method was adopted to analyse plant samples. Best recoveries were obtained for the soil samples using a combination of methanol and acetone extractions.

Appendix 18 Soil and pore water dissipation: model parameters for plant study data.

Statistical indices for single first order (SFO), first order multi-compartment models (Gustafson and Holden, 1990) (FOMC) or bi-exponential models (BFO) using to model the degradation rates of the pharmaceuticals in the soil and pore water.

Soil:

Pharmaceutical	Model	DT50	DT90	SSRes	RMSE	χ^2 (tabulated χ^2)	Model error	Rate constant (k1)or (α/β)	r^2
Carbamazepine	*	> 40 d	> 40 d						
Diclofenac	SFO	0.50	1.64	0.51	13.68	0.084 (9.49)	29.59	(1.4)	0.99
Fluoxetine	*	> 40 d	> 40 d						
Propranolol	*	> 40 d	> 40 d						
Sulfamethazine	SFO	0.99	3.29	119.16	67.16	3.63 (9.48)	223.67	(0.7)	0.99
Triclosan	SFO	11.55	38.38	195.66	37.36	3.36 (9.49)	152.78	(0.06)	0.97

* No significant difference between 0 d and 40 d measured concentrations therefore data was not modelled to determine degradation rates

Pore water:

Pharmaceutical	Model	DT50	DT90	SSRes	RMSE	χ^2 (tabulated χ^2)	Model error	Rate constant (k_1/k_2,C01/C02,α/β)	r^2
Carbamazepine	*	> 40 d	> 40 d						
Diclofenac	FOMC	19.65	2.57E+03	61.47	8.75	7.58 (7.81)	40.81	(α = 0.79, β = 0.34)	0.88
Fluoxetine	*	> 40 d	> 40 d						
Propranolol	*	> 40 d	> 40 d						
Sulfamethazine	BFO	-	-	45.43	0.12	5.94 (5.99)	6.65	C01 = 91, C02 = 9, k_1 =0.85, k_2 = 0.0174)	0.99
Triclosan	*	> 40 d	> 40 d						

* No significant difference between 0 d and 40 d measured concentrations therefore data was not modelled

Equation for DT50/DT90:

For BFO models no solution exists.

Time for 50 % or 90 % decrease in chemical concentration can be modelled for the SFO using the rate constant (k):

DT50 = ln2/k and DT90 = ln10/k

For results using the FOMC model:

DT50 = β*(2(1/α)-1) and DT90 = β*(10(1/α)-1)

Appendix 19 Human exposure calculations.

The human risk of consumption from crops grown in pharmaceutically contaminated soil was calculated. Calculations were based on DEFRA statistics(Holmes *et al*., 2007) which estimate that in the United Kingdom an adult (70 kg) consumes 395.5 g of above ground crops and 159 g of below ground crops per day. Therefore ryegrass was assumed representative of an above ground crop species and radish bulb was representative of a below ground crop species. Acceptable daily intakes were based on the minimum therapeutic dose (mg/person/day) with a safety factor of 100 applied. Using calculated UF_{soil} and measured soil concentrations(Dalkmann *et al*., 2012; Durán-Alvarez *et al*., 2009; Vazquez-Roig *et al*., 2010) we could estimate realistic crop concentrations and thus how much would be in a human diet. A percentage of the ADI for each pharmaceutical was then calculated for each pharmaceutical. As sulfamethazine uptake was below LOQ this was removed from the analysis.

Ryegrass - assumed representative above ground crop

	Soil conc. (mg/kg)	**UF soil**	**Plant conc. (mg/kg)**	**Plant conc. (mg/g)**	**Consumption per person (g/day)**	**Conc. in 359.5 g of crop (mg/day)**	**Min. therapeutic dose (mg/person/day)**	**ADI (mg/person/day)**	**% of ADI in 359.5 g crop**
CBZ	0.0065	65.26	0.42	0.00042	359.5	0.1525	400	4	**3.81**
DCF	0.0005	6.82	0.0037	3.68E-06	359.5	0.0013	75	0.75	**0.18**
FLX	0.0067	0.076251	0.0005	5.11E-07	359.5	0.0002	20	0.2	**0.09**
PRL	0.0004	11.04	0.0044	4.42E-06	359.5	0.0016	80	0.8	**0.20**
TCS	0.0186	37.59	0.70	0.00070	359.5	0.2514	30	0.3	**83.8**

Radish - assumed representative of below ground crop

	Soil conc. (mg/kg)	UF soil	Plant conc. (mg/kg)	Plant conc. (mg/g)	Consumption per person (g/day)	Conc. In 159 g of crop (mg/day)	Min. therapeutic dose (mg/person/day)	ADI (mg/person/day)	% of ADI in 159 g crop
CBZ	0.0065	8.28	0.05	5.38E-05	159	0.00856	400	4	**0.21**
DCF	0.0005	5.39	0.00	2.91E-06	159	0.00046	75	0.75	**0.06**
FLX	0.0067	0.36	0.00	2.43E-06	159	0.00039	20	0.2	**0.19**
PRL	0.0004	1.20	0.00	4.79E-07	159	0.00008	80	0.8	**0.01**
TCS	0.019	0.12	0.00	2.26E-06	159	0.00036	30	0.3	**0.12**

List of Abbreviations

API	Active pharmaceutical ingredient
CBZ	Carbamazepine
DCF	Diclofenac
DOM	Dissolved organic matter
DW	Dry weight
FLX	Fluoxetine
K_d	Soil sorption distribution coefficient (L/kg)
LOEC	Low observed effect concentration
Log K_{ow}	Octanol-water partition coefficient (measure of hydrophobicity)
OM	Organic matter
ORL	Orlistat
pKa	Negative base-10 logarithm of the acid dissociation constant of a solution
PRL	Propranolol
SOM	Soil organic matter
SMZ	Sulfamethazine
STP	Sewage treatment plant
WW	Wet weight
WWTP	Wastewater treatment plant

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