Uptake of pharmaceuticals into benthic invertebrates

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Environment and Geography

September 2019

Abstract

The occurrence, fate and, uptake of active pharmaceutical ingredients (APIs) in the environment has received increasing attention from the scientific community. Research investigating the uptake of APIs into invertebrates is still limited specifically for benthic invertebrates. Therefore, the research presented in this thesis, evaluated the fate of pharmaceuticals in sediment and their uptake into the sediment-dwelling worm *Lumbriculus variegatus.* The experiments in the different chapters were conducted in order to explore the influence of compound physicochemical properties and sediment characteristics on the uptake of ionisable pharmaceuticals into the lumbricids.

An initial evaluation of Quantitative Structure Actvity Relationships for bioconcentration was performed using existing representative fish and invertebrate bioconcentration data to elucidate whether these models were suitable for predicting the uptake of ionisable chemicals and what properties are essential in predicting the ionisable chemical uptake.

Laboratory experiments were performed to measure the uptake of four APIs into the worms *via* water-only exposure at different pH values. For the bases, the bioconcentration factor (BCF) increased with the increase of the water pH. The opposite was observed for the acidic diclofenac (higher uptake at lower pH).

The sorption behaviour of three APIs in four types of sediments was also evaluated. Sorption coefficients revealed that the sorption behaviour of the weak basic compounds was driven by the organic content, whereas, the sorption behaviour of diclofenac was driven by the pH.

The influence of sediment properties on the uptake of the ionisable APIs into sediment-dwelling lumbricids was investigated for three APIs on two sediment types. With these experiments, we demonstrated differences in bioaccumulation of the two basic APIs and one acid into *L. variegatus* and how uptake varies across two sediments possessing diverse properties.

In conclusion, our experimental findings produced knowledge on the fate and the uptake of ionisable pharmaceuticals in the water-sediment compartment.

List of Contents

List of Tables

List of Figures

Acknowledgements

I would like to thank Prof. Alistair Boxall, Dr. Roman Ashauer, and Dr. Laura Carter for their expertise and for giving me the chance to achieve this important step in my life.

I would like to thank all my colleagues at the Environment Department (in particular Verena Riedl) who have supported me throughout the Ph.D. project. They are not only colleagues with whom I may potentially work with in the future but they are also friends who constantly reassured me and were always willing to listen to my frustration.

I profoundly thank my parents, Caterina, and Bruno, and my brother Alberto for their long-distance support and my partner Silvio.
It has been a long journey, full of emotions and “ups and downs” but I do not think I could have made it without your encouragement.

Thanks also to all my friends around the world and those in Italy who were trying to understand the weird experiments I was conducting.

Ultimately, I thank the Environment Department, Prof. Alistair Boxall and the IMI (Innovative Medicines Initiative) and the iPiE project for funding.

Author's declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.
The presented work was supervised by Prof. Alistair Boxall, Dr. Roman Ashauer and, Dr. Laura Carter. I performed the work enrolled as a full-time PhD student at the Department of the Environment and Geography at the University of York, UK.

The chapters are presented as research articles for future publications in peer-review journals. The Supplemental Data for each chapter is presented in the Appendix section.

I played a leading role in the experimental design, data collection, analysis and authoring the chapters that I present in the thesis. The publication status of the presented work is given in Table 0.1.

Title	Status	Journal	Chapter
Evaluation of bioconcentration models for estimating the bioconcentration factor of ionisable compounds	/	To be decided	*Two*
Influence of pH on the uptake of ionisable active pharmaceutical ingredients (APIs) into a sediment-dwelling worm (*L. variegatus*)	Preparing for publication	To be decided	*Three*
Sorption of ionisable active pharmaceuticals in four different types of sediments	/	To be decided	*Four*
Influence of sediment properties on the uptake of ionisable APIs into a sediment-dwelling invertebrate (*L. variegatus*)	/	To be decided	*Five*

Table 0. 1. Status of the publications from the chapter presented in this thesis.

Chapter 1: Literature review

1.1 Introduction

Active pharmaceutical ingredients (APIs) are contained in medicinal products that we use every day and are manufactured to treat a disease or prevent symptoms. They are designed to have beneficial effects in humans and animals and their use is enormous and the release into the environment is ubiquitous (Boxall *et al.,* 2012). Therefore, in the ecotoxicology field, the interest in the environmental impacts of APIs has continuously increased since the early 1990s (Daughton, 2016). This increasing interest in the environmental impacts of APIs is partly due to the fact that it is not until recently that analytical methodologies have become available that are able to separate the substances with high efficiency in environmental matrices (Ternes, 2001). Researchers are also concerned that these molecules could elicit effects on organisms at low concentrations such as µg/L or ng/L as a result of their inherent biological activity (Daughton and Ternes, 1999).

To date, a large volume of literature has been published about the occurrence, fate and effects of many active pharmaceutical ingredients in different environmental compartments (including soil, fresh and marine surface water and sediments) and the organisms dwelling in these compartments across the globe. Therefore, in this chapter, our understanding of the environmental occurrence, fate, and effects of APIs is presented and the major gaps in our knowledge are identified. The Chapters focus more on the sediment compartment which is the main subject of this thesis.

1.2 Current situation of pharmaceuticals in the environment

1.2.1 Sources of pharmaceuticals entering in the environment

Pharmaceuticals enter into the natural environment through different pathways (Figure 1.1). In high-income countries, the main sources of APIs found in the surface waters are Wastewater Treatment Plants (WWTP). Generally, the medicine is taken by the patient and only a part of it is metabolized. The parent molecules are then released in the urine

and feces which are flushed down the toilet into the sewerage system. The sewage is then transported to a Wastewater Treatment Plant, where depending on the structure of the API and the nature of the WWTP, the API may be removed to some extent. Usually, the removal efficiency of APIs in WWTPs range between 60% and 90% (Carballa *et al.,* 2004). In Portugal, for example, the removal of 83 pharmaceuticals was investigated and their removal efficiency in WTP effluents was found to exceed 70% (Paíga *et al.,* 2019). In South Korea, the removal efficiency of 20 pharmaceuticals and personal care products in five Wastewater Treatment Plants averaged > 90% (Kumar *et al.,* 2011). The efficiency of removal of APIs depends mainly on the technologies available in the Plants, as well as the intrinsic physicochemical properties of the pharmaceuticals.

Another potential pathway of pharmaceuticals entering the environment is the use of veterinary pharmaceuticals for treating livestock and domestic animals. These medicines can be directly released into surface water *via* use in aquaculture practices. In addition, traces of APIs have been found in the soil compartment due to the release of slurry and manure from treated animals which are then used as fertilizers (Boxall, 2004; Kim *et al.,* 2011). Irrigation of soil with reclaimed wastewater has been demonstrated to be another source of contamination of APIs in some regions of the world (Wu *et al.,* 2014; Pan and Chu, 2017). A study by Biel-Maeso *et al.,* (2018) demonstrated the presence of several pharmaceuticals like diclofenac, acetaminophen and, caffeine in the soil irrigated by the water from a Wastewater Treatment Plant due to their scarce removal by the technologies present in the Plant. Recently, concerns have been raised over the contamination of soils with APIs because of the potential leaching of APIs to the groundwater (Oppel *et al.,* 2004).

Manufacturing facilities are another source of contamination of medicines entering into the environment (Larsson *et al.,* 2007; Larsson, 2014). For example, Larsson *et al.,* (2007) found an incredibly high amount of an antibiotic released from a manufacturing site into a wastewater treatment effluent. Incorrect disposal of medicines by the population (through the sink/toilet) could also lead to a discharge of APIs into the sewage system and then into the Wastewater Treatment Plants (Bound and Voulvoulis, 2005).

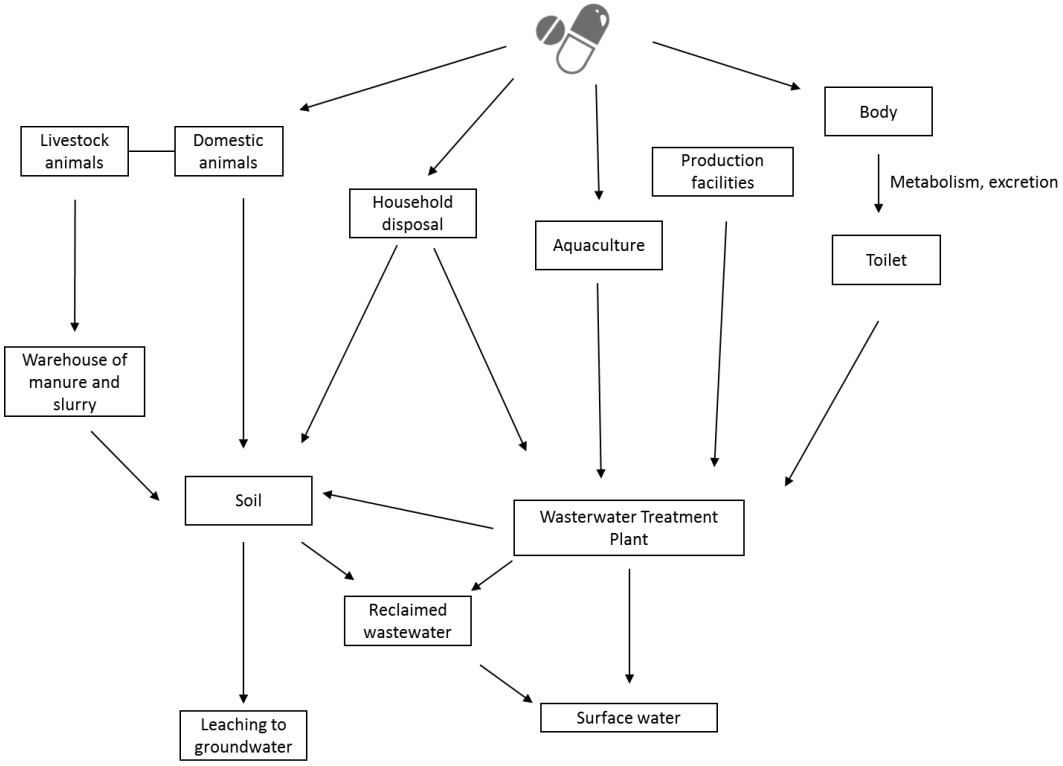


Figure 1. 1. The major pathways by which pharmaceuticals enter the environment.

1.2.2 Occurrence and effects of pharmaceuticals in the environment

Because of their continuous use and release, the environmental occurrence of active pharmaceutical ingredients and their metabolites has been investigated and they have been found to be omnipresent in natural environments around the world (Daughton & Ternes, 1999). Since the 1990s the literature has grown with monitoring campaigns being performed to assess the presence of APIs in different matrices such as surface water (Česen *et al.,* 2019; Sousa *et al.,* 2019; Mandaric *et al.,* 2019; Sharma *et al.,* 2019; Caldas *et al.,* 2018; Nkoom *et al.,* 2018; Praveena *et al.,* 2018; Moldovan *et al.,* 2018; Hossain *et al.,* 2018; Burns *et al.,* 2018); sediment (Battaglin *et al.,* 2018; Zhang *et al.,* 2018; Biel-Maeso *et al.,* 2017; Agunbiade and Moodley, 2016) and soil (Biel-Maeso *et al.,* 2018; Jaimes-Correa *et al.,* 2015; Aznar *et al.,* 2014) in many countries. In these studies, APIs are generally reported to be detected at low concentrations ranging from ng/L to µg/L in surface water and ng/g or µg/g in sediments and soils. The frequency of detection and concentrations of pharmaceuticals varies and depends on several factors

such as the sampling site or the proximity to the main source and the season. For instance, Kasprzyk-Hordern *et al.,* (2008) analyzed surface water samples from different sampling locations of two rivers in Wales. They found high concentrations of the APIs in the locations in proximity to the WWTPs. Further down the river, the concentrations decreased most likely due to dilution, biodegradation, sorption to sediments or photolysis. Weather conditions may influence the presence of pharmaceuticals in rivers too. For example, in a study conducted in the Alpine rivers, high detection was reported during low river flow (dry season) and the opposite during winter when higher water flows occur probably due to more frequent precipitations (Mandaric *et al.,* 2019).

Pharmaceuticals are designed to target a specific receptor or pathway in humans resulting in a beneficial effect but they may have undesired effects on non-target organisms possessing the same receptors (Gunnarsson *et al.,* 2008). An example is the positive correlation between the presence of endocrine disruptors (17β-estradiol and 17α-ethinylestradiol) and the feminization of male fishes observed in rivers of England and Wales (Gross-Sorokin *et al.,* 2006). In this example, scientists from the Environment Agency discovered that the direct exposure to endocrine disruptors induced vitellogenin in male fish. Vitellogenin is a female protein that results in an intersex exchange, particularly in fish living in treated sewage effluents (Gross-Sorokin *et al.,* 2006). Another study exposed fish species (*Betta splendens*) to three environmentally relevant concentrations of an anti-depressant, fluoxetine, to assess impacts on the aggressive behaviour of the organisms (Eisenreich and Szalda-Petree, 2015). The authors exposed the fish species to different concentrations of fluoxetine and found a reduction in the aggressive behaviour of the organisms overtime at higher concentrations of the API.

Studies have also evaluated the impact of APIs in terrestrial species. In 2004 a study conducted by Green *et al.,* (2004) showed a drastic drop in the population sizes of three species of vulture in India and Pakistan due to renal failure. They discovered that the constant feeding by the birds on livestock carcasses treated with diclofenac was the main cause of the decline of the vulture population. More recently, studies have demonstrated that environmentally realistic exposure concentrations of fluoxetine alter courtship behaviour of a songbird that is well known to feed on invertebrates that live nearby wastewater treatment plants (Whitlock *et al.,* 2018).

1.2.3 Previous studies on the uptake of pharmaceuticals in aquatic organisms

Along with toxicity studies, studies have explored the bioconcentration of APIs. Typically, these studies assess the uptake of APIs into non-target organisms under laboratory conditions. The exposure time used varies and for example for fish, chronic exposure of 28 days is recommended by the OECD guideline 305 (OECD, 2012). Understanding the uptake of pharmaceuticals is important because it gives an understanding of the uptake, depuration and the internal exposure of the APIs within the organism. From the 2000s, many studies have been published regarding the uptake of pharmaceuticals into fish species (Brooks *et al.,* 2005; Brown *et al.,* 2007; Paterson and Metcalfe, 2008; Schultz and Furlong, 2008; Mehinto *et al.,* 2010; Lahti *et al.,* 2011; Nallani *et al.,* 2011; Al-Ansari *et al.,* 2013; Steinbach *et al.,* 2016; Du *et al.,* 2016; Chen *et al.,* 2017). In these studies, the bioconcentration was evaluated by exposing the fish to environmentally relevant concentrations (ng/L and µg/L) over a certain period of time. Then, the bioconcentration was derived when an equilibrium between the organism and the water concentrations (steady-state) was reached or by deriving the kinetic bioconcentration factor. Different species are used such as fathead minnow, rainbow trout, *Danio rerio*, channel catfish, goldfish. Differences in the bioconcentration between the different studies were likely to be the time of exposure, the concentration and the experimental conditions. Over the same period of time, from 2000, the number of studies on the uptake of pharmaceuticals into invertebrates has been considerably lower than for fish species. For some years (2005, 2006, 2007 and 2010) no studies were found in the literature on uptake of APIs into invertebrates (Figure 1.2). Recently, research investigating the accumulation of APIs into invertebrates has increased in the freshwater and marine environment (Dussault *et al.,* 2009; Meredith-Williams *et al.,* 2012; Bossus *et al.,* 2014; Miller *et al.,* 2015; Ding *et al.,* 2016; Karlsson *et al.,* 2016; Miller *et al.,* 2017; Garcia-Galan *et al.,* 2017). Most of the studies use gammarids as non-target invertebrates for the experiments. Other organisms investigated include daphnids, lumbricids, insects and marine molluscs. Ding *et al.,* (2016) exposed groups of *Daphnia magna* to two APIs (roxithromycin and propranolol) *via* water-only exposure and the kinetic bioconcentration factor was derived. Another study by Miller *et al.,* (2017) sampled the organisms in different locations from a river in the outer area of south London and exposed the gammarids in the laboratory to eight APIs. They derived

different bioconcentration factors depending on the different compound and the physicochemical properties.

To date, the discrepancy between fish species bioaccumulation data and invertebrates is substantial. Also, research regarding the uptake into sediment-dwelling invertebrates is limited making it difficult to interpret the bioaccumulation results due to the diverse biological differences among the species. The species selected in the studied mentioned above have different species traits that are peculiar of the chosen organism such as habitat, feeding habit, respiration, therefore, the bioaccumulation has to be interpreted carefully taking into account these differences.

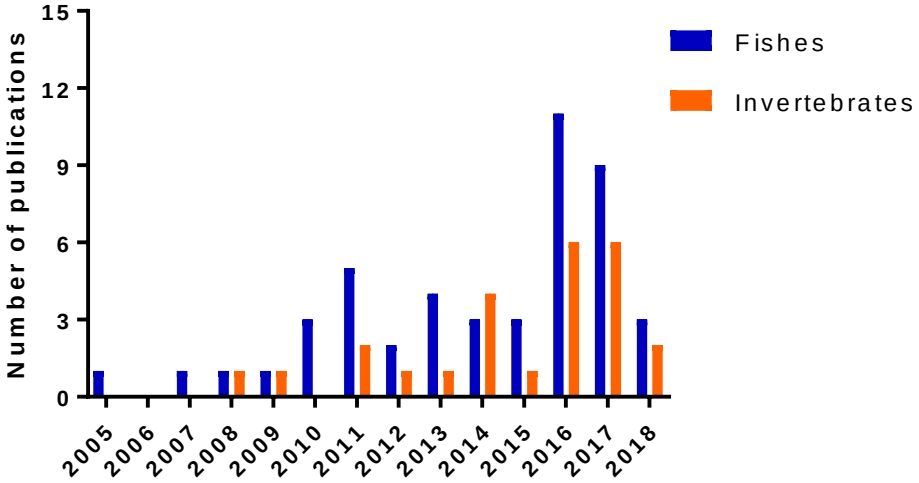


Figure 1. 2. The number of publications from 2005 of the uptake of APIs into invertebrates (orange columns) and fishes (blue columns). The research on the web was conducted using keywords such as “invertebrates”, “uptake”, “fish”, “bioconcentration”, “pharmaceuticals”.

1.3 Bioconcentration, bioaccumulation and biomagnification

Bioconcentration is a mechanism through which a chemical may be absorbed by non-target organisms passively *via* the skin. The degree of bioconcentration of a molecule is usually expressed as a bioconcentration factor (BCF), which is the ratio of the concentration of the xenobiotic between the body of the organism and the surrounding medium, for example, water or in the sediment (biota-sediment accumulation factor, BSAF) at the steady-state. The BCF has the unit of L/Kg (Equation 1).

$$\mathrm{BCF} = \frac{\mathrm{Corganism}}{\mathrm{Cwater}} \qquad \text{Equation 1}$$

Some guidelines have been developed on how to conduct bioconcentrations studies such as the OECD method 305 for fish species (OECD 2012) and method 315 for invertebrates (OECD, 2008).

When a steady-state is not reached, toxicokinetic models can be used to estimate the rates of uptake and depuration of the molecule. The bioconcentration factor is then calculated as the ratio of the uptake kinetic rate constant (K_{in}, L × kg^{-1} × d^{-1}), and the depuration rate constant (K_{out}, d^{-1}) into an organism over time (Equation 1).

$$\mathrm{BCF} = \frac{\mathrm{Kin}}{\mathrm{Kout}} \qquad \text{Equation 1}$$

Bioaccumulation is the process by which a substance is absorbed by an organism from both the surrounding environment and the dietary pathway. The degree of bioaccumulation of a molecule is usually expressed using the bioaccumulation factor (BAF).

Ultimately, biomagnification reported as the biomagnification factor (BMF) is the concentration of the substance between the prey and the predator. This factor measures the amount of chemical transferable from prey to predator, and thus provides an indication of the propensity for a molecule to move throughout the food chain.

1.4 Factors influencing the uptake and accumulation of ionisable APIs

Various factors influence the uptake of ionisable compounds into non-target organisms including the physicochemical properties of the compound, environmental conditions and biological traits of the study organism. Table 1.1 below gives an overall view of these factors that will be described in the following sections.

Factors influencing the bioconcentration of ionisable chemicals		
Chemical	**Environmental**	**Biological**
pK_a Log D_{ow} Volume distribution Water solubility	pH Carbon organic content Cation exchange capacity Texture (clay, silt, sand) Temperature	Size Feeding habits Life stage Active transport of APIs Mode of respiration Metabolism

Table 1 1. Main processes influencing the uptake of ionisable organic compounds in the sediment compartment.

Chemical characteristics

pK_a

The pK_a gives an indication of the amount of the ionic form of an ionisable chemical that will be present at a given pH value (Manallack, 2007). The majority of the APIs are ionisable possessing functional groups that accept or donate protons. These functional groups give an indication of whether the chemical acts as a base or an acid. Acidic compounds become increasingly undissociated at lower pH and the opposite occurs for bases (Rendal *et al.,* 2011b). The neutral and ionic form of the molecule posses diverse properties in regard to bioconcentration and toxicity. For instance, it is well documented that the ionized form of a molecule is less bioaccumulative than the neutral form due to different physicochemical properties and polarities (Rendal *et al.*, 2011b). Thus, the dissociation constant of a compound is a key chemical parameter that influences lipophilicity, solubility and permeability processes (Manallack, 2007).

Few experimental pK_a values have been measured but different software has been developed to predict the pK_a of a chemical such as ACD I-lab (ACD I lab, 2015) and chemicalize.org (Swain, 2012). For example, ACD I lab helps in understanding the charged and the uncharged part of a molecule that is more likely to be reactive with biological tissues at a specific pH.

Lipophilicity

Lipophilicity is a characteristic of a compound that tends to easily dissolve in fatty acids and phospholipids and it is described by the $logK_{ow}$ which is the logarithm of the n-octanol-water partition coefficient. Octanol is used as a surrogate to the lipid tissues of

an organism. Traditionally the $logK_{ow}$ is used to describe the hydrophobicity of a compound.For neutral chemicals, the $logK_{ow}$ has been used to predict the uptake into non-target organisms (Veith *et al.,* 1979; Mackay and Fraser, 2000).

The logarithmic octanol-water distribution coefficient ($logD_{ow}$) is a measure of the $logK_{ow}$ but corrected for the pH. For ionisable chemicals, it describes the partitioning of the dissociated and un-dissociated form of a chemical as a function of pH. The formula contains the dissociation constant (pK_a), the pH and the valency (A), therefore, $LogD_{ow}$ depends on the speciation of a chemical (Rendal *et al.,* 2011a). For ionisable chemicals, the $LogD_{ow}$ is a better descriptor of the distribution and partitioning of ionic compounds between the medium and the cell membrane of an organism (Kah and Brown, 2008), (Wen *et al.,* 2012) where pH along with the valency and pK_a are combined in a single formula shown in equation 2 below. A is -1 for acids and 1 for bases. It is generally assumed that the $LogK_{ow}$ of charged species is 3.5 units lower than the uncharged species (Fu *et al.,* 2009a).

$$\log D_{ow} = \log K_{ow} - \log (1 + 10)^{A\,(pH-pKa)} \qquad \text{Equation 2}$$

Volume of distribution

The volume of distribution is a pharmacological parameter that represents the amount of the medicine in the bloodstream of the human body and the fraction that is distributed in the tissues. It has been suggested as a potential predictor for estimating the sorption behavior of pharmaceuticals in natural soils and sediments and uptake into non-target organisms such as invertebrates (Williams *et al.,* 2009; Meredith-Williams *et al.,* 2012).

Water solubility

The water solubility of a chemical has been proposed as a molecular descriptor to predict the uptake of compounds into non-target organisms (Chiou *et al.,* 1977; Veith *et al.,* 1980). It has been argued that compounds not easily dissolved in water are more likely to sorb to hydrophobic surfaces and membranes (Piir *et al.,* 2010); thus, a

substance possessing high water solubility is less likely to partition into the lipids of an organism and will therefore have a low bioaccumulation factor (Pavan *et al.,* 2008).

Environmental characteristics

The bioavailability of organic chemicals is strongly affected by water and sediment characteristics such as the pH, carbon organic content, the cation exchange capacity (CEC), texture and temperature (Al-Khazrajy and Boxall, 2016).

pH

pH is a key environmental factor affecting the toxicity and uptake of ionizing compounds. Many studies have explored the pH-dependent uptake of organic contaminants in key non-target organisms from small invertebrates throughout the food-chain up to fish species.

For example, Nakamura *et al.,* (2008) explored the uptake of the antidepressant fluoxetine and its main metabolite norfluoxetine in a fish (*Oryzias latipes*) at three pH values (7, 8 and 9). They found a correlation between the accumulation of fluoxetine with pH where high bioaccumulation was seen at high pH. Similar findings were observed by Nichols *et al.,* (2015) analyzing the uptake of the weak base antihistamine diphenhydramine in fathed minnow at pH levels of 6.7, 7.7 and 8.7. Also, in this case, BCF increased with the increase of the water pH. The influence of pH on uptake into smaller organisms has also been investigated. For instance, the crustacean *Daphnia magna*, and a higher plant *Salix viminalis* were the organisms used to test the toxicity and uptake of the weak base chloroquine at pH values of 6, 7, 8 and 9 (Anskjaer *et al.,* 2013). Increasing toxicity was seen at higher pH. The same result was seen in the study of Neuwoehner and Escher, (2011) where they assessed the pH-dependent toxicity of five weak bases, fluoxetine, norfluoxetine, propranolol, lidocaine and trimipramine on the green algae *Scenedesmus vacuolatus*. An ion-trapping model was developed to describe the specific mode of toxic action of aliphatic amines inside and outside the green cell.

Carbon organic content

The organic carbon in waters and sediments is typically generated by decaying vegetation and microbial activity and it regulates the distribution and persistence tendency of chemicals in the water-sediment system (Rust *et al.,* 2004). Chemicals interact with the organic carbon content present in the water or sediment and adsorb to the organic carbon content being less free to be taken up by non-target organisms (Haitzer *et al.,* 1998). For instance, the toxicity and bioaccumulation of sediment-associated herbicides, ioxynil, pendimethalin and bentazone have been assessed in the sediment-dwelling organisms *Lumbriculus variegatus* and *Chironomus riparius*. The authors observed that the bioaccumulation and bioavailability of these organic pesticides were reduced by the increase of the sediment organic matter (Maënpaä *et al.,* 2003). Another study investigated the bioaccumulation of the antiparasite veterinary medicine ivermectin in *L. variegatus* in two sediments containing different organic carbon content. A decrease in the uptake of the ivermectin into the worms was observed in the sediment with the higher organic carbon content, probably due to the reduced availability of the compound bound to the organic matter (Slootweg *et al.,* 2010). Alsop & Wilson, (2019) studied the uptake of three pharmaceuticals into zebrafish and demonstrated how the dissolved organic carbon content reduced the bioavailability of the compounds and thus the uptake into the organisms.

Texture and cation exchange capacity (CEC)

Sediment texture (for e.g. clay, sand or silt) and CEC influence the sorption and mobility of ionisable chemicals. For example, some antidepressants and antibiotics are positively charged at pH around neutrality and they may be strongly linked to clay that is negatively charged (Kah and & Brown, 2007; Droge and Goss, 2013). In this way, pH which influences the speciation, along with texture and CEC, affects retardation and mobility of ionisable compounds (Schaffer *et al.,* 2012) in the natural sediment.

Temperature

Temperature is an environmental property that influences the bioaccumulation into non-target organism. For example, Muijs and Jonker, (2009) evaluated the influence of different temperature from 5°C to 24°C on the bioaccumulation of polycyclic aromatic

hydrocarbons into the worm *L. variegatus* and observed that the bioaccumulation decreased at lower temperatures probably due to changes in the lipid compositions of the worms.

Biological traits

The uptake of chemicals may vary among different or same taxonomic groups due to diverse feeding behaviour, size, life stage, diet composition, etc. (Rubach *et al.,* 2010; Rubach *et al.,* 2012).

Size

The size of an organism could explain the differences in bioconcentration of chemicals across species. Recently, several studies have demonstrated how biological traits like body-size could better explain the differences in toxicity and uptake among organisms belonging to the same taxonomic group or organisms belonging to different taxonomic groups. For example, Gergs, *et al.,* (2015) exposed three species that are taxonomically and physically different (*Daphnia magna*, *Chaoborus crystallinus* and *Mesocyclops leuckarti*) to the fungicide triphenyltin hydroxide and differences in sensitivity of toxicity were observed between small body-size and large body-size organisms. The authors calculated the LC_{50} after 48-h and 96-h exposure to the fungicide to the three species and lower LC_{50} values were found for smaller body size organisms than large body size. Another study conducted by Wang and Zauke, (2004) on *Gammarus zaddachi* found a negative correlation between the bioconcentration of metals and the body length of the organism.

Feeding habits

An additional biological factor that should be considered during the analysis of uptake of toxicants into organisms is their feeding habits and the diet route that may potentially influence the accumulation pattern. The feeding strategies of organisms have been shown to influence the accumulation of Polychlorinated biphenyls (PCBs) into four freshwater benthic invertebrates: *Chironomus riparius*, *Hyalella azteca*, *Lumbriculus*

variegatus, and *Sphearium corneum* (Sidney *et al.,* 2016). In this study, they demonstrated the difference in bioaccumulation of the four organisms having different feeding habits. For example, *H. Azteca*, *L. variegatus* and *C. riparius* are deposit feeders while the mollusca *L. corneum* is a suspension feeder. Furthermore, depending on the feeding habit and the contact with the sediment, some organisms may be partially exposed to contaminants attached to the sediment particles. For instance, *H. Azteca* swims mostly in the overlying water but this is not the case for the worm *L. variegatus* that tends to burrow in the sediment having the majority of the body submerged.

Life stage

The life stage of an organism has been recognized as an important biological trait to consider when assessing the bioconcentrations of toxicants. In this regard, differences in toxicity were observed for different life-stages (neonates and adults) of a cocepod *Mesocyclops leuckarti* exposed to th fungicide triphenyltin hydroxide (TPT) (Kulkarni *et al.,* 2013). Another study conducted on *Daphnia magna* showed that the bioconcentration of a nonylphenol isomer (p353-NP) between adults and neonates daphnids differed by a factor of 5.6 (Preuss *et al.,* 2008).

Active transport of APIs

Along with the passive diffusion transport, the carrier-mediated transport of ionisable APIs by specific proteins present on the cell membrane has been recognized as another uptake pathway of APIs through the cell membrane. Sugano *et al.,* (2010) explained that for pharmaceuticals with low passive permeability and high hydrophilicity, such as charged APIs, carrier-mediated transport is another possible uptake pathway of these medicines into the cell of the organism. In this case, the transporter needs to be present in the study species (Smith *et al.,* 2014).

Mode of respiration

Respiration type is a species trait that has been considered to indicate species sensitivity to different contaminants (Baird and Van den Brink, 2007; Rico and Van den Brink, 2015). For instance, Meredith-Williams *et al.,* (2012) exposed three invertebrates to several pharmaceuticals to evaluate the different uptake of the APIs in relation to the different organisms. The authors observed that the uptake in *Gammarus pulex* was greater than in the other organisms and explained the difference in the uptake by the different modes of respiration of the organisms. For their uptake experiments, they used the shrimp *G. pulex*, the aquatic insect *Notonecta glauca* and the aquatic snail *Planobarius corneus*. These organisms possess different modes of respiration: the shrimp uses the gills; the insect breathes mainly through the plastrons and the snail through the skin and the siphon (Meredith-Williams *et al.,* 2012). The authors argued that the shrimps accumulated the pharmaceuticals to a greater extent than the other species because they breaths through the gills which is the primary pathway of exposure of contaminants. Another study by Nyman *et al.,* (2014) exposed three pesticides to three freshwater species, two shrimps (*G. pulex* and *Gammarus fossarum*) and one snail (*Lymnaea stagnalis*), to assess the bioaccumulation. They found that the snail accumulated more of the compounds than the shrimps due to different species traits including the different respiration types.

Metabolism

The metabolic pathway is a species-specific trait that could explain the different bioaccumulative potential of different organisms to toxicants. For example, two fish species (*Gambusia affinis* and *Jenynsia multidentate*) were exposed to carbamazepine and different uptake was observed likely due to the differences in the metabolism of the compound between the two species (Valdés *et al.,* 2016).

1.5 Regulations

In Europe, the EMA (European Medicines Agency) is the body responsible for the evaluation and authorization of human and veterinary medicines. Generally, an environmental risk assessment is required for new human and veterinary medicinal

products before they can be marketed. The risk assessment consists of two phases: Phase I and Phase II. The Phase I is an initial screening of the active substance and its potential exposure in the environment based on the future use of the substance. The predicted environmental concentration (PEC) in the surface water has to be calculated and if it is ≥ 1 µg/L, then a Phase II has to be performed. The Phase II testing requires the estimation of the physicochemical properties of the substance along with ecotoxicity and fate studies following OECD guidelines. For the estimation of the physicochemical properties of the active ingredient, the water solubility, Octanol/Water partitioning and Dissociation in water are the required tests to perform. For fate, the adsorption/desorption studies and biodegradability tests are necessary and ultimately, aquatic toxicity studies such as assessment of effects on growth, mortality and reproduction with three species belonging to three different trophic levels (algae, crustacea and fish species) are needed. In this part, for compounds that accumulate throughout the food chain, secondary poisoning involving bioconcentration studies in fish species has to be determined.

The guideline also includes a hazard assessment for persistent, bioaccumulative and toxic properties of the active ingredients. Three essential criteria have to be assessed: persistence, bioaccumulation, and toxicity. At the end of the assessment, a substance may be classified as toxic, bioaccumulative and persistence (PTB) or very, bioaccumulative and very persistence (vPvB). For instance, the first assessment explored the persistence in different environmental compartments (for e.g. fresh-estuarine water, marine sediments, and soils) through degradation tests (OECD 301, 307, 308, 309). The second criterion is bioaccumulation (BAF) or bioconcentration (BCF). In this evaluation, if a substance possesses a $\log k_{ow} < 4.5$, further uptake studies are not required; on the other hand, a $\log k_{ow} > 4.5$ requires bioconcentration tests in aquatic, terrestrial and benthic organisms. Preferably the OECD guideline 305 for fish species is used. When studies on bioaccumulation in fish are lacking, then other species rather than fish may be considered. Toxicity is the final criterion and mortality endpoints such as EC_{10} or LC_{10} and NOEC are considered. It involves short and long-term toxicity data possibly on invertebrate species like daphnids, algae, and sediment-dwelling organisms as well as carcinogenic and mutagenic tests. For these latter tests, information from mammal experiments could be used.

At the end of the assessment, if the substance is identified of very high concern the emission and risk characterization are requested. The emission characterization consists of the analysis of all the potential routes of emission of the substance into the environment and consequently the risk that it may pose to ecosystems. If needed, risk mitigation approaches are proposed such as: proper labelling and risk mitigation through some actions such as the appropriate storage and responsible disposal, the releasing of SDSs and the training of personnel. Further, these substances should be replaced with alternatives when feasible and their use is granted only if no alternatives are found.

1.6 Bioconcentration from sediment

The presence of APIs in the sediment compartment has been widely investigated in different locations (Silva *et al.,* 2011; Camacho-Muñoz *et al.,* 2013; Radović *et al.,* 2015; Thiebault *et al.,* 2017). The classes of pharmaceuticals detected include anti-inflammatories, antidepressants, antibiotics, birth control substances, anti-histamines and many others. Concentrations detected in the environment are generally low (ng/g), yet, they may represent a potential risk to the organisms dwelling in the natural environment (Wilkinson *et al.,* 2017). For example, Liu *et al.,* (2018) detected 8 APIs in sediment samples at concentrations of ng/g and some of these APIs were also found to accumulate in different tissues of the common carp (*Cyprinus carpio*).

Benthic organisms are often exposed to pharmaceuticals associated with sediments. For example, worms such as *L. variegatus* are exposed to them *via* three pathways: the overlying water, the sediment and, the pore water present in between the sediment particles (Mäenpää *et al.,* 2008). Hydrophobic APIs entering the water ecosystem bind to the sediment being potentially toxic to the organisms dwelling in that compartment. Some studies have assessed the routes of uptake of sediment-bound chemicals into *L. variegatus*. Generally, the sediment is spiked with the toxicant which is left to equilibrate between the water and the sediment. Then, the organisms are introduced and the exposure is followed by a period of depuration where the organisms are introduced to the same sediment-water system without the chemical (Maënpaä *et al.,* 2003; Liebig *et al.,* 2005; Van Geest *et al.,* 2010).

It is important to estimate the exposure of the different routes that can reach the worms in order to understand which may pose a threat to a greater extent but little research regarding the uptake of sediment-associated chemicals in benthic invertebrates is known. Moreover, sediment characteristics play a key role in the uptake of APIs. Sediments possess heterogeneous properties (e.g. high/low organic content) and in combination with the contaminants' physicochemical properties (water solubility, $\log K_{ow}$), the APIs may be adsorbed to the sediment becoming less bioavailable to be accumulated by the organisms. In this way, an understanding of the sorption behaviour of the compound is essential to better evaluate the bioncentration of ionisable APIs for the overlying water, the sediment and the pore water exposure. The sorption behaviour of a pharmaceutical is generally described by the solid/water partition coefficient (K_d, L/Kg). This is the ratio of a chemical partitioned between the water and the soil or sediment phase (Equation 3). The standard method to measure this parameter is fully described by the OECD guideline 106 (OECD, 2000). For neutral chemicals, the organic carbon-normalized sorption coefficient (K_{oc}, L/Kg) is considered to be a better descriptor and, to derive this, information on the organic content of the sediment is required (Equation 4).

$$K_d = \frac{C_{sediment}}{C_{water}} \qquad \text{Equation 3}$$

$$K_{oc} = \frac{Kd \times 100}{OC} \qquad \text{Equation 4}$$

$C_{sediment}$ is the concentration of the compound in the sediment compartment (mg/g); C_{water} the concentration of the compound in the water phase (mg/ml) and OC is the organic carbon.

1.7 A theoretical model of possible pathways of uptake of ionisable APIs into sediment-dwelling invertebrates

The overlying water and sediment are linked. For instance, once a pharmaceutical ends up in the surface water, it can be adsorbed ny and be deposited onto the sediment. A

resuspension may occur when the flow rate of the river is high as for e.g. during perturbations of the system such as floods. Therefore, sorption and desorption processes are the main exchange routes between water and sediment, measured by the K_d (1). Thus, sediment-dwelling organisms, such as *L. variegatus,* are exposed to the APIs both through the sediment compartment and the overlying water and K_{in} and K_{out} can be estimated (2 and 3). Also, the pore water present among the sediment particles may be a potential route of exposure of the APIs to the worms (4). Because sediment may be a sink of contaminants through sorption processes but also a source though resuspension in the overlying water column, we must further investigate the extent of uptake from all these routes described above in order to better estimate the risk to the sediment-dwelling invertebrates. The conceptual scheme of different pathways of APIs in the water-sediment system is illustrated in the figure below. Parameterisation of the scheme would involve:

1) Adsorption/desorption studies being conducted to estimate the K_d value of the APIs sorbed to the sediment;
2) The estimation of the internal concentrations of the APIs into the worms from the overlying water consisting of the water-only exposure of the worms to the pharmaceutical followed by an elimination phase during which the internal concentration was measured. By using the first-order one-compartment toxicokinetic model, we derived the K_{in} and K_{out} (uptake and depuration rates) and calculated the bioconcentration factor (BCF);
3) The measurement of internal concentrations of the APIs fromthe sediment compartment can be derived similarly to the water-only exposure as described above with the only difference that the sediment concentrations are used to fit the one-compartment toxicokinetic model to derive the K_{in} and K_{out} and then estimate the BSAF (=biota-sediment accumulation factor).

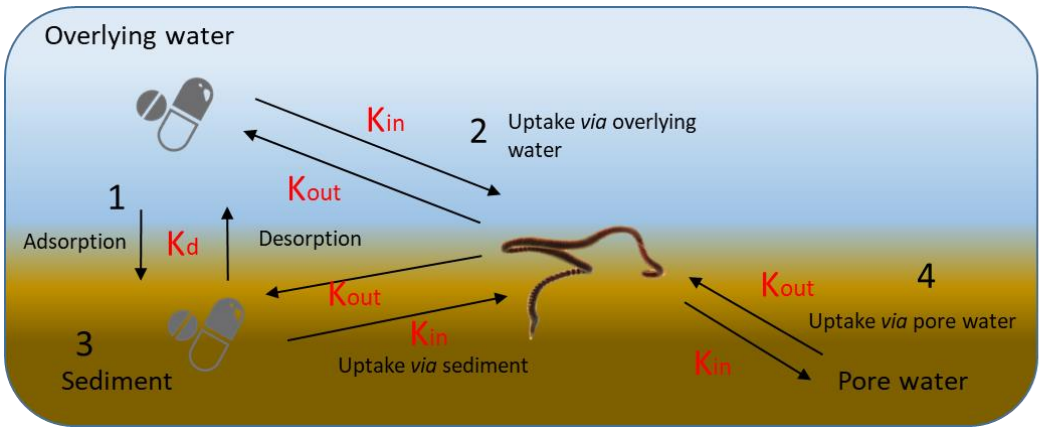


Figure 1. 3. Conceptual model of the uptake of APIs in the sediment-dwelling worms.

1.8 Aims and objectives

In the previous paragraphs, the limited knowledge of the sediment uptake of ionisable APIs into invertebrates has been highlighted. A better understanding of the uptake of ionisable chemicals into sediment-dwelling invertebrates is essential not only because there are few studies in the literature addressing this topic but also because it is important to comprehend the environmental factors such as pH of the sediments that could influence the uptake of these APIs.

Therefore, in this PhD research, the main aim was to further explore the influence of environmental conditions like pH and their interactions with the physicochemical properties of the APIs like pK_a on the uptake of ionisable APIs into a sediment-dwelling invertebrate *L. variegatus*. This was achieved through different objectives:

- To evaluate the applicability of eight existing representative fish and invertebrate bioconcentration models to elucidate whether these models are suitable for predicting the uptake of ionisable chemicals and what properties are essential in predicting the ionisable chemical uptake (Chapter 2);
- To assess the influence of different ranges of water pH on the uptake of ionisable APIs into benthic invertebrates (Chapter 3);
- To explore the sorption behaviour of ionisable APIs in different types of sediment (Chapter 4);

- To determine the impact that sediment properties could have on the uptake of ionisable APIs into *L. variegatus* (Chapter 5);
- To use the information generated from the above chapters in order to provide recommendations for future research and perspective of these chemicals and to better categorize the risk (Chapter 6).

1.9 Study compounds selected

The study compounds selected for the study belong to different therapeutic classes with different physicochemical properties. Their pK_a values range between 3.9 and 9.6 and $LogK_{ow}$ values from 4.06 to 4.92. They include acid and basic molecules and are available as 14-C radiolabelled pharmaceuticals. The use of radiolabelled compounds allowed for a lower detection limit meaning that lower exposure concentrations could be used in the experiments similar to the concentrations detected in the environment. (Table 1.2). A brief description and environmental issues of the tested APIs raised so far will be given below.

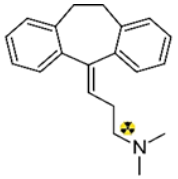
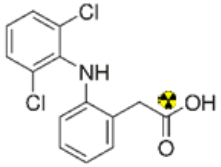
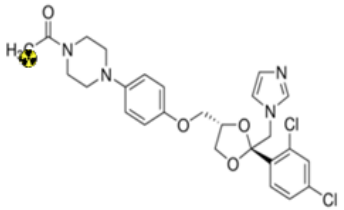
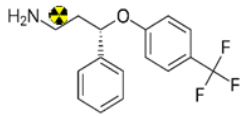
Test compound	CAS number	Acid/base	Therapeutic class	Molecular Weight (g/mol)	pK_a	$LogK_{ow}$	Chemical structure	(MBq)
Amitriptyline	549-18-8	Base	Antidepressant	279.19	9.4[c]	4.92[a]		6.00
Diclofenac	15307-86-5	Acid	Anti-inflammatory	296.15	3.9[b]	4.51[d]		1.85
Ketoconazole	65277-42-1	Base	Antimycotic	531.44	6.5[b]	3.78[b]		0.37
Norfluoxetine	57226-68-3	Base	Antidepressant	295.31	10.01[e]	4.16[f]		7.51

Table 1. 2. Structures and properties of the pharmaceuticals studied in the thesis.

[a] Predicted using Advanced Chemistry Development, Inc. (ACD/Labs), (https://ilab.acdlabs.com/iLab2/), accessed 09/01/2018.

[b] Experimental using iPie database, (http://i-pie.org/) accessed 09/01/2018.

[c] (Al-Khazrajy and Boxall, 2016).

[d] (Escher *et al.,* 2017)

[e] (Nakamura *et al.,* 2008a).

[f] (Karlsson *et al.,* 2016).

Ketoconazole- an azole fungicide

Azole fungicides are antifungal active ingredients used as pesticides to treat plant pathogenic fungi (e.g. propiconazole and tebuconazole) and as pharmaceuticals (e.g. fluconazole, clotrimazole, ketoconazole, miconazole). They are sold as topical creams or oral medications (Chen & Ying, 2015) and their use is large. For instance, in 2005, in Switzerland, they were sold with a total sale volume of 1 tonne (Kahle *et al.,* 2008). A part of them is flushed into WWTPs where they are not completely removed as a consequence, they reach rivers and lakes. Studies into their occurrence and fate in sewage waters and in rivers and lakes have been performed in Sweden (Lindberg *et al.,* 2009); in Belgium (Van De Steene *et al.,* 2010); in Ireland (Lacey *et al.,* 2012); in England (Thomas and Hilton,2004) and in China (Huang *et al.,* 2010; Peng *et al.,* 2012). In these studies the concentrations detected ranged between ng/L to µg/L and some of them, such as fluconazole and clotrimazole, were found to be persistent and not completely removed in the wastewater treatment plant and therefore present in the effluents (Lindberg *et al.,* 2009; Peng *et al.,* 2012).

Ketoconazole is an imidazole antifungal active compound. Commonly commercialized as an anti-dandruff product, it is administered on the scalp and, after application, it is flushed down the drain. It ends up in the receiving surface water through effluents of the WWTPs with a small contribution from hospital wastewaters. Ketoconazole is the most commonly detected antifungal in rivers, sediment, and soils with clotrimazole, miconazole, itraconazole and fluconazole (Liu *et al.,* 2016). The experimental pK_a is 6.5, the $LogK_{ow}$ is 3.78, and in the environment, it can be adsorbed to solid matrices such as sediments (Chen and Ying, 2015). In the past years, different papers have investigated the potential effect of this pharmaceutical on fish (Hasselberg *et al.,* 2008; Yan *et al.,* 2013). The results show that exposure, even at low concentrations of ketoconazole, compromises the expression of key enzymes present in the metabolic and steroid clearance. Studies into the chronic bioconcentration, distribution, metabolism and biomarker responses of this toxicant in common carp (Liu *et al.,* 2016) show that ketoconazole was highly concentrated in the liver and induces reactive oxygen species with increasing exposure time and concentrations.

Norfluoxetine and amitriptyline - antidepressants

Antidepressants are drugs used to treat depressive disorders. They act through different mechanisms of action including monoamine oxidase inhibitors, serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine inhibitors. A 2008 study showed that the SSRIs are the most prescribed antidepressants across Europe (Bauer *et al.,* 2008). Due to the large use of this group of medicines, antidepressants are still commonly detected in surface waters across the globe (Valcárcel *et al.,* 2011; Fedorova *et al.,* 2014; ter Laak, Kooij *et al.,* 2014; Gonzalez-Rey *et al.,* 2015a; Wu *et al.,* 2017). Therefore their potential risk to aquatic life is gaining more attention and many studies have investigated the adverse effects of different antidepressants on non-target aquatic species (Xie *et al.,* 2015; Estévez-Calvar *et al.,* 2017; Yang *et al.,* 2018). For example, Estévez-Calvar *et al.,* (2017) assessed the impact of the antidepressant sertraline on three different taxonomic marine species at their early development stage: the crustacean *Amphibalanus amphitrite*, the rotifer *Brachionus plicatilis* and the mollusk *Mytilus galloprovincialis*. Sertraline has been found to alter the swimming capacity of *A. amphitrite* and *B. plicatilis* and to cause the death and the immobilization of the organisms. In fish species, the same compound, sertraline, induced antioxidant responses when the organisms were exposed to the pharmaceutical and the authors also recorded an excessive increase of the swimming activity and a decrease of the shoaling activity of the fish (Xie *et al.,* 2015).

Norfluoxetine is the major metabolite of the SSRI fluoxetine. From the human body, fluoxetine is excreted principally in urine and 20% of the applied dose is excreted as norfluoxetine (Nałęcz-Jawecki, 2007). The detection of this drug in surface water started at the beginning of the 2000s (Kolpin *et al.,* 2002) and since then, numerous studies have detected fluoxetine and norfluoxetine ubiquitously (Metcalfe *et al.,* 2003; Kwon and Armbrust 2006a; Schultz and Furlong 2008; Calisto and Esteves, 2009). Although fluoxetine and norfluoxetine have been detected at low concentrations (ng/L to µg/L), in the environment, norfluoxetine has been found to be 50% more toxic than fluoxetine in 24 h lethal tests on a protozoan *Spirostomum ambiguum* and the crustacean *Thamnocephalus platyurus* (Nałęcz-Jawecki, 2007). Another study exposed fish species Japanese medaka at environmentally relevant concentrations (from 13 to 15 µg/L) at pH 7, 8 and 9 and the BCF for fluoxetine and norfluoxetine increased with an increase of the

pH of the medium in both the liver and the body (Nakamura *et al.,* 2008). The toxicity of fluoxetine and norfluoxetine was also assessed on other aquatic organisms such as algae (Neuwoehner and Escher, 2011). They exposed the green algae *Scenedesmus vacuolatus* at six different pHs (from 6.5 to 10) and the EC_{50} based on the concentrations of the cytoplasm was calculated. Here again, they observed an increase of the cytoplasmatic EC_{50} at higher pH. Regarding invertebrates, norfluoxetine was the most recurring antidepressant having the highest mean concentration in mussel tissues among the other antidepressants investigated by Silva *et al.,* (2015). Ultimately, norfluoxetine has been detected in the plasma of sharks at even higher concentrations than the parent compound fluoxetine. The possible explanation of this observation is due to the polarity of norfluoxetine compared to fluoxetine. In fact, once fluoxetine is metabolized to norfluoxetine, the compound becomes less polar and thus, it tends to bioaccumulate more than fluoxetine causing detrimental effects (Gelsleichter and Szabo, 2013).

Amitriptyline is a medicine used to cure symptoms of depression. It is a tricyclic antidepressant that inhibits the norepinephrine in the brain and it increases neurotransmitter concentrations (www.drugs.com). It has been detected at concentrations ranging from 2.2 to 11 ng/L in effluent samples (Togola and Budzinski, 2008; Kostich *et al.,* 2014). Few studies have investigated the toxicity of this drug in aquatic organisms such as freshwater invertebrate *Daphnia Magna* where an EC_{50} of 4.82 mg/L was calculated after 48 h of exposure (Minguez *et al.,* 2014). For fish species, bioconcentration assays have been conducted by Ziarrusta *et al.,* (2017): the organisms (*Sparus aurata*) were exposed to two environmentally relevant concentrations (0.2 and 10 ug/L) and they found a high accumulation of the drug in the brain and in the gills. The results were not surprising because amitriptyline is an antidepressant and the brain should be the main target organ. However, another study exposed a brook trout to several antidepressants including amitriptyline and, in this study, it was detected only in liver tissues (Lajeunesse *et al.,* 2011).

Diclofenac -an anti-inflammatory

Anti-inflammatory drugs are extensively used in medicine to treat general pain, minor injuries, and flu. They belong to the non-steroidal anti-inflammatory group (NSAIDs) and worldwide they do not require a prescription from a doctor but they are sold as over-

the-counter medicines. For example, it has been estimated that the annual global consumption of diclofenac was 1443 ± 48 tonnes from 2010 to 2013 (Acuña *et al.,* 2015). For this reason, anti-inflammatories are one of the most investigated groups of pharmaceuticals in the environment and they have become the subject of interest for researchers and regulators. Because of their large use, they are found ubiquitously in environmental compartments (rivers, lakes, and soil) with numerous studies investigating their presence in environmental matrices. To cite some studies: Camacho-Muñoz *et al.*, (2013) detected concentrations of diclofenac and naproxen in river sediments from Doñana National Park in Spain. Another monitoring study conducted by Rivera-Jaimes *et al.,* (2018) in a big city in SouthWest Mexico, Cuernavaca, detected ibuprofen, diclofenac, and naproxen as the most commonly present in the samples analyzed.

Diclofenac is one of the most popular analgesics used globally by humans and animals for veterinary purposes. Because it is an over-the-counter drug, it is very difficult to estimate its global consumption. It can be applied to the skin or orally administered. It is sold under different names such as Voltaren Emulgel in Canada and Europe, Vilini in India and Voltaren in USA (www.drugs.com). The first attention to this medicine as a potential anthropogenic pollutant started as a result of the sudden decrease (by 95%) in vulture populations in India observed during the early 2000s. In that study, the researchers discovered that the mortality of the vultures was due to feeding on the carcasses of cattle treated with diclofenac. Diclofenac was concentrated in the tissues causing renal failure in the vultures (Oaks *et al.,* 2004). From that episode, the number of studies regarding the toxicity of diclofenac in non-target organisms increased all over the world including studies with aquatic and terrestrial species. For example, a freshwater fish (*Rhamdia quelen*) exposed to environmentally relevant concentrations of diclofenac for 21 days showed biochemical reactions including reduction of the enzyme catalase, lipid peroxidation and inhibition of SODs in the brain (Guiloski *et al.,* 2017). Also, a study conducted on an earthworm *E. Fetida* exposed to short-term assays to 18 pharmaceuticals including diclofenac found out diclofenac as one of the most toxic NSAIDs in this species (Pino *et al.,* 2015).

To date, due to the numerous studies that have highlighted the potential negative effects of diclofenac in non-target organisms and its widespread detection in

environmental matrices, diclofenac has been inserted on the watch list of the European Water Framework Directive (2000/60/EC) as a priority substance to be monitored constantly in rivers and lakes.

1.10 Study organism

Lumbriculus variegatus is a freshwater oligochaete that inhabits sediments of ponds, lakes, or marshes of North America and Europe. It tends to burrow in the sediment by keeping the head submerged into the top layer of the sediment and the tail undulating in the overlying water for respiratory and sensory perception purposes. It feeds on decaying vegetation or microorganisms present in the sediment (EPA, Environmental Protection Agency, 2000). It possesses two reproduction strategies: sexual and asexual. The worm normally reproduces asexually under laboratory conditions. During the asexual reproduction, the worm fragments and the posterior part of the body then regenerates a new head and thus forms into a new organism (Martinez *et al.,* 2006).

L. variegatus has been used for decades as a key organism for sediment bioaccumulation and toxicity studies of contaminants. It is a species that is easy to cultivate in the laboratory, it can be supplied by different companies at a reasonable cost, it can be used for short and long-term experiments and it has a large tolerance of different types of sediments (Dermott and Munawar, 1992). Because *L. variegatus* feeds on suspended particulate matter in the sediment, it is an ideal organism to assess the dietary route of exposure of toxicants attached to the particles of the sediment and the biomagnification of these toxicants through the food chain. For example, Mount *et al.*, (2006) conducted long-term feeding experiments (21 and 30 days) where *L. variegatus* was used as a possible prey for carrying out dietary exposure experiments of toxicants to two species

of fish, fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*).

Therefore, nowadays, *L. variegatus* is a common species used for standardized bioaccumulation and toxicity tests (OECD, 2007), (OECD, 2008) and trophic-transfer assays (Ng and Wood, 2008; Dutton and Fisher, 2011).

In this PhD research, *L. variegatus* will be exposed to the APIs mentioned abover and the main findings will be presented in the following chapters.

Chapter 2. Evaluation of models for estimating the bioconcentration factor of ionisable compounds

2.1 Introduction

Man-made chemicals including pesticides, personal care products, and active pharmaceuticals ingredients (APIs) have been detected in various environmental compartments where they have the potential to accumulate in biota, (Pal *et al.,* 2010; (Huerta *et al.,* 2012; Gonzalez-Rey *et al.,* 2015b). The accumulation of these chemicals can cause physiological and behavioral alterations in non-target species exposed throughout their lifetime (Brodin *et al.,* 2014; Dzieweczynski *et al.,* 2016; Xie *et al.,* 2015; Richmond *et al.,* 2016; Ford and Fong, 2016). As a consequence, bioconcentration is a fundamental endpoint in Environmental Risk Assessment and regulatory agencies worldwide evaluate thousands of chemicals based on their bioconcentration potential during the chemical assessment process (Dimitrov *et al.,* 2005).

To assess the bioconcentration, a bioconcentration factor (BCF) can be measured based on a series of standard protocols for fish species (OECD, 2012) and for sediment-benthic oligochaetes (OECD, 2008). Considering the significant number of chemicals to be tested, the laboratory effort to determine BCFs (for example for fishes, up to 14 days of acclimatization phase is followed by generally the same time for the depuration phase) and the number of organisms required for each experiment, measuring BCFs for all chemicals of interest would require a massive laboratory effort (Carter *et al.,* 2014; Miller *et al.,* 2016). Therefore, several bioconcentration models have been developed in order to estimate BCFs from the chemical structure and thereby minimize the need for costly experiments. Such models include empirical correlation BCF models (Neely *et al.,* 1974; Veith *et al.,* 1979; Geyer *et al.,* 1991); and mechanistic models (Mackay and Fraser, 2000; Barber, 2003; Barber, 2008). These models have been proposed mainly for neutral compounds where the hydrophobicity ($LogK_{ow}$) is the main descriptor used to estimate the bioconcentration factor.

However, many chemicals are ionisable and become charged at environmentally relevant pH values, as monovalent, multivalent acids and bases and zwitterions (Karlsson *et al.,* 2016). For instance, Franco *et al.,* (2010) reported that 491 (33%) of a

random sample of 1510 chemicals registered in the European Union regulation Registration, Evaluation, Authorization and Restriction of Chemicals, (REACH), ionize at pH around neutrality. Therefore, in recent years, models to predict the BCF of ionisable chemicals have been proposed for selected fish species (Meylan *et al.,* 1999; Fu *et al.,* 2009b; Erickson *et al.,* 2006; Trapp *et al.,* 2010; Armitage *et al.,* 2013; Nichols *et al.,* 2015b). On the contrary, very few BCF models have been developed for invertebrates (Arnot and Gobas, 2003; Meredith-Williams *et al.,* 2012; Du *et al.,* 2015; Karlsson *et al.,* 2016). However, an extensive validation of these models has not yet been performed. Hence, the present study evaluates the applicability of eight existing bioconcentration models in order to elucidate whether these models are suitable for predicting the uptake of ionisable chemicals and what properties are essential in predicting the ionisable chemical uptake including organisms' traits and physicochemical parameters.

2.2 Materials and Methods

2.2.1 BCF Database

A total of 132 BCF measurements were collected from the literature. The dataset consisted of a total of 42 ionisable compounds including different classes of chemicals: pesticides, personal care products, and active pharmaceuticals ingredients.

Measured BCF data for APIs is limited so given the chemical similarity between pesticides and APIs this analysis was done on a large dataset encompassing pesticides, active pharmaceutical ingredients and personal care products. The APIs included in this study included 35 APIs, 5 pesticides and 2 personal care products. The entire dataset of both acids and bases is available in the Appendix A, Table A.1. They were collected from several sources, for example, the iPiE database (http://i-pie.org/) and the open literature. BCFs for pesticides were obtained from the Pesticide Properties Database (PPDB) (http://sitem.herts.ac.uk/aeru/ppdb/en/) (Lewis et al., 2016). Physicochemical properties such as $LogK_{ow}$ and pK_a were taken from the different papers and if the values were not available, they were estimated with predictive software such as Estimation Program Interface (v 4.11 EPI Suite™) (US EPA, 2019) and DrugBank (https://www.drugbank.ca/) (Wishart *et al.,* 2006). Different studies were selected to

provide BCF data from a range of fish species and invertebrates having a range of chemicals with different physicochemical properties. For instance, for the chemicals included in this study, the $logK_{ow}$ ranged between -0.09 and 6.15 and the pK_a values between 1.7 and 10.5. Experimental pK_a was preferred overpredicted, if available. Furthermore, the database included not only information about chemical properties (for e.g the acidic or basic nature of the chemicals, $LogK_{ow}$, molecular weight, water solubility, etc.) but, additional experimental details about the exposure or field conditions and organism species were also recorded. For water-only bioconcentration studies, different pH values and water properties such as dissolved oxygen and conductivity were included in the database, whilst, for sediment bioconcentration studies, cation exchange capacity and organic carbon content of the test sediment were added. Regarding the exposure conditions, details such as exposure duration, and experimental design (flow through, static or renewal) were reported. The organisms selected in the data collection were mainly fishes (n=82) and invertebrates (n=50).
Most of the BCF studies on fish species used a steady-state BCF test following the OECD 305 flow-through exposure (OECD, 2012) where the BCF is calculated as a ratio of total radioactivity measured in the fish and the water phase. For invertebrate studies, BCFs are generally kinetic BCFs based on the first-order one compartment toxicokinetic model (Ashauer *et al.,* 2006; Ashauer *et al.,* 2010; Grech *et al.,* 2017).

2.2.2 Models that were evaluated

An extensive literature review of bioconcentration models followed the database development. A total of eight BCF models were selected and evaluated to assess their suitability (Table 2.1) to predict the uptake of ionisable compounds by comparing the predicted BCFs to the measured BCFs in the database. The models included:

1. The Chiou *et al.,* (1977) model is a regression model that uses Log_S (logarithmic water solubility) as the only predictor of the equation. Log_S was retrieved from DrugBank (https://www.drugbank.ca/) (Wishart *et al.,* 2006) and when the data was not available, it was estimated from a Quantitative Structure-Property Relationship (QSPR) model (Shayanfar *et al.,* 2010) using the formula:

$$Log_S = 0.5 - 0.01 \times (mp-25) - LogP^{a} \quad \text{Equation 1}$$

Log_S (mg/L): it is the logarithmic of the water solubility of the medicine; mp (°C): it is the melting point of the compound;

[a] Log P= it is the logarithmic partition coefficient and it was recorded from Drugbank.

2. The TGD (1996) model is a linear model included in the Technical Guidance Document on Risk Assessment of the European Union based on a previous model proposed by Veith *et al.,* (1979) regarding the estimation of the BCFs for fish species.
3. The Meylan *et al.,* (1999) and the Arnot & Gobas, (2003) models are integrated into the predictive software EPI Suite and encoded in a computer program called BCFBAF, an extension of a previous program called BCFWIN (Ver v 4.11 EPI Suite™), (USEPA, 2012). The Meylan model consists of two input parameters: $LogK_{ow}$ and Σ_{fi} which is a summation of several correction factors to apply to the chemicals. Each chemical contains a specific functional group to which a specific correction factor is applied. For some chemicals, more than one correction factor may be applied.
4. The other model integrated into EPI Suite, is the model proposed by Arnot and Gobas, (2003) for lower, middle and upper trophic levels of fish species. The model consists of several uptake and elimination input parameters listed in Table 2.1.
5. The Meredith-Williams *et al.,* (2012) model has been proposed for two invertebrates: *Notonecta glauca* and *Gammarus pulex*. The only predictor is the pH-corrected liposome-water partition coefficient ($LogD_{lipw}$), calculated from the formula in Equation 2.
6. The Karlsson *et al.,* (2013) model is a specific model developed for ionisable chemicals uptake into a sediment-dwelling invertebrate, *Lumbriculus variegatus*. The input parameters are several: physicochemical parameters such as the pK_a (constant dissociation of a chemical), environmental parameters as pH internal of the species and external of the medium and biological traits like the water

content of the species F_w (the lipid content (f_{lip}) and the pH-corrected liposome-water partition coefficient (D_{lipw}). The latter parameter has been calculated based on a formula of Escher *et al.,* (2008):

$$LogD_{lipw} = 0.904 \times LogD_{ow} + 0.515 \qquad \text{Equation 2}$$

The $LogD_{ow}$ was obtained from the Henderson-Hasselbach equation, where the fraction of the ionized and neutral fraction of the substance at a specific pH was determined as shown in Equation 3 and 4.

$$\alpha_{ion} = \alpha_{neutral} \times 10^{i(pH-pKa)} \qquad \text{Equation 3}$$

$$D_{ow} = f_{ion} \times K_{ow\text{-}ion} + f_{neutral} \times K_{ow\text{-}neutral} \qquad \text{Equation 4}$$

α_{ion} is the activity of the ion at a particular pH; $\alpha_{neutral}$ is the activity of the neutral species; f_{ion} is the ionic fraction of the substance at the studied pH; $f_{neutral}$ is the neutral fraction of the substance at the studied pH; $K_{ow\text{-}ion}$ and $K_{owneutral}$ are the octanol-water partition coefficients for the neutral and ionic species respectively.
The partition coefficient between octanol and water of the ionic species is assumed to be 3.5 log units lower than the neutral species (Trapp and Horobin, 2005). All the biological traits (internal pH for both fishes and invertebrates; lipid content in % wet weight, the water content in % and organisms' weight in Kg) were searched in the open literature. A lack of data for these biological traits were found; in particular, the water content and the internal pH for invertebrates. Thus, specific biological traits of the species' order or class were used (Appendix A, Table A.2).

7. The Fu *et al.,* (2009) model in Table 1 is a linear regression model similar to the Technical Guidance Model on Risk Assessment 1996 where instead of the $Logk_{ow}$, the $LogD_{ow}$ (pH-corrected octanol-water partition coefficient) is replaced.
8. The last model of Table 1 is a model proposed by Dimitrov *et al.,* (2005). The model can be used to calculate the baseline BCF of a compound considering the K_{ow} and

the F_w (water content of the species); a and n are model parameters reported in the paper.

2.2.3 Statistical analysis

Predicted and measured BCFs were plotted as linear correlations using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The accuracy and applicability of the models were calculated based on percentages of BCF data that fit within a factor of 10. Also, the performance of the models was tested by performing the Nash-Sutcliffe efficacy test (NSE) with R in order to see how well the observed values versus predicted fit the 1:1 line. The Nash-Sutcliffe efficiency gives an indication of the goodness of fit of the data. When a value ≥ 0, a good match between the modeled and observed data can be concluded; if a value < 0, the observed data mean are more accurate than the modeled.

Model	Equation	Species coverage	Predictors of the model
(Chiou *et al.,* 1977)	Log BCF = 3.41 × Log S - 0.508	Fish (rainbow trout)	The model predicts the BCF for a wide range of chemicals such as hydrocarbons, aromatic acids, etc only with a physicochemical property such as **water solubility** of the chemicals.
Technical Guidance Document for Risk Assessment of the European Union, 1996	Log BCF = 0.85 × $\mathrm{LogK_{ow}}$ - 0.70	Fishes and specifically fathed minnow	The model calculates the BCF of organic chemicals, considering the $\mathbf{LogK_{ow}}$**,** a physicochemical property of the chemicals **only**.
(Meylan *et al.,* 1999)	Log BCF = - 1.37 × $\mathrm{LogK_{ow}}$+ 14.4 + $\sum_{\mathrm{fi}}$	Fishes and specifically fathed minnow	The hypothesis of the model is to provide a better estimation of BCF based on the physicochemical property of a chemical as $\mathbf{LogK_{ow}}$ and **different correction factors** to apply to each compound ($\sum_{\mathrm{fi}}$).

(Meredith-Williams *et al.,* 2012)	Log BCF = 0.71 × $\mathrm{LogD_{lipw}}$ - 0.23	Invertebrates and specifically *Gammarus pulex* and *Notonecta glauca*	The model uses the $\mathbf{LogD_{lipw}}$ which is assumed to be a better input physicochemical parameter for the prediction of uptake for ionizable compounds.
(Karlsson *et al.,* 2013)	$\mathrm{F_w} \times \frac{1+10^{(\mathrm{pHint}-\mathrm{pKa})}}{1+10^{(\mathrm{pHext}-\mathrm{pKa})}} + \mathrm{f_{lip}} \times \mathrm{D_{lipwater}}$	Invertebrates and specifically *Lumbriculus variegatus*	The model proves to better and deeply describe the uptake of ionizable compounds linking together **physicochemical properties** such as **pKa** (constant dissociation of a chemical); **biological traits** as $\mathbf{F_w}$, $\mathbf{f_{lip}}$ $\mathbf{and}$ $\mathbf{D_{lipw}}$ (the water content of the organism, the lipid content and the liposome water partition coefficient) and **environmental properties** such as **pH**.
(Arnot and Gobas, 2003)	$\mathrm{BAF} = \frac{C_b}{C_w} = (1 - L_b) + [(k_1 \times \phi + (k_d \times \beta \times \tau + \phi + L_d \times K_{ow})] / (k_2 + k_E + k_G + k_M)$	The model is applicable on three general trophic levels of fishes (lower, middle and upper)	The model assumes specific **fish traits**: the weight of the organism (**W**); the lipid content of the organism ($\mathbf{L_b}$); lipid content of the lowest trophic level ($\mathbf{L_d}$); **environmental conditions** such as **the** concentration of particulate organic carbon ($\boldsymbol{\chi_{POC}}$); concentration of dissolved organic carbon ($\boldsymbol{\chi_{DOC}}$) and **physicochemical properties** of the chemicals ($\mathbf{K_{ow}}$)

			in order to be more representative of natural uptake of chemicals for fish species in aquatic environments.
(Fu *et al.,* 2009a)	Log BCF = 0.85 × $\mathrm{LogD_{ow}}$ - 0.70	The model has been applied to fish species	The model proposes and assumes that the $\mathbf{LogD_{ow}}$, a **physicochemical property** (pH-corrected octanol-water partition coefficient) is a better input parameter to describe the uptake of ionizable compounds.
(Dimitrov *et al.,* 2005)	$\mathrm{Log\ BCF_{max}} = \log \frac{\mathrm{Kow^n}}{(\mathrm{aK_{ow}} + 1^{2n})} + \mathrm{F_w}$	Fish species and in particular for salmonids and cyprinids.	The model predicts base-line BCFs assuming several **mitigating factors** as **molecular descriptors**, the octanol-water partition coefficient ($\mathbf{K_{ow}}$) and **fish biological traits** such as **water content** ($\mathbf{F_w}$).

Table 2. 1. Summary table for selected BCF models including their predictors and applicability domain.

-$\mathrm{Log_S}$ (mg/L): water solubility of the chemical;
-$\mathrm{LogK_{ow}}$: octanol-water partition coefficient;
-Σ_{fi}: summation of correction factors to apply to the chemicals. Each chemical contains a specific functional group to which a specific correction factor is applied;
-$\mathrm{LogD_{lipw}}$: logarithmic of the liposome-water partition coefficient;
-$\mathrm{LogD_{ow}}$: logarithmic of the pH-corrected octanol-water partition coefficient;
-F_w (%): water content of an organism;
- $ʃ_{lip}$ (%, wet weight): lipid content of the organism;
-C_b (mg/Kg): concentration of a chemical in the upper trophic level;
-C_w (mg/L): is the concentration of the chemical in the unfiltered water;

-L_d (1 %): lipid content of the lowest trophic level organism;
-L_b (20 %): lipid content of the organism;
-W (Kg): the weight of the organism;
-χ_{POC} (5 X 10^{-7} g/mL): concentration of particulate organic carbon;
- χ_{DOC} (5 X 10^{-7} g/mL): concentration of dissolved organic carbon;
-T (˚C): mean water temperature;
-$k_1 \left(\frac{1}{[\left(0.01+\frac{1}{Kow}\right)\times W^{0.4}]} \right)$: uptake rate constant;
-$\phi \left(\frac{1}{(1+\chi_{POC}\times 0.35\times K_{ow}+\chi_{DOC}\times 0.1\times 0.35\times K_{ow}} \right)$: the fraction of the free bioavailable chemical to be taken up by the organisms in the water;
-$k_d \left(\frac{0.02\times W^{-0.15}\times e^{0.06\times T}}{5.1\times 10^{-8}\times K_{ow}+2} \right)$: is the rate of uptake of the chemical via the diet;
-β: biomagnification process; it is an empirical value to calibrate the model;
-τ: the maximum level of trophic dilution that occurs for substances that are metabolized at a significant rate in organisms of a food web and by default is 1;
-$k_2 \left(\frac{k1}{L_b\times K_{ow}} \right)$: is the elimination rate constant;
-k_E ($0.125\times k_d$): fecal egestion rate constant;
-k_G ($0.0005\times W^{-0.2}$): elimination rate constant through growth dilution;
- k_M (day^{-1}): metabolic transformation rate constant.

2.3 Results and discussion

Comparisons of experimental BCFs against BCF values predicted using the different models are shown in Figure 2.1. Specifically, for fish models, an over-prediction of BCFs was observed for the models proposed by Dimitrov *et al.,* (2005); TGD (1996) and Meylan *et al.,* (1999), while the Fu *et al.,* (2009) and Arnot and Gobas (2003) models show more scattered BCF data. In the Dimitrov *et al.,* (2005) model, BCFs typically fell below the 1:1 line, more precisely in the 1:0.1 range or even below the 0.1 line. The Chiou *et al.,* (1977) model is the only model where no BCFs were predicted within a factor of 10 of the experimental values .
Also, two models specifically developed for invertebrates have been evaluated: the Meredith-Williams *et al.,* (2012) and Karlsson *et al.,* (2013) models. They show two opposite trends: the Karlsson *et al.,* (2013) model over-estimated the BCFs, between 2 and 6 log-units, whereas, the Meredith-Williams *et al.,* (2012) model under-estimated BCFs. Most of the data are shifted in the 1:10 range or above resulting in a slight negative prediction.
Along with the correlations, a table showing the accuracy of the predicted BCF data that fall within a factor of 10 of the different models is given in Table 2.2. Correlations including the acids and zwitterions and the bases and zwitterions only have been tested too, assuming that some models may be more appropriate to predict the uptake of the acids rather than bases and vice-versa. The graphs of the models are provided in the Appendix Figures A.1 and A.2. The results of the Nash-Sutcliffe Efficacy test are shown in Table 2.3. All models showed poor performance with the NSE values below zero. The worst model performance was observed for the Chiou *et al.,* (1977) model with a NSE of -140.45 being obtained for acids and zwitterions; -92.37 for acids, bases and zwitterions and -93.8 for bases and zwitterions respectively. For the other fish models, the NSE ranged from -7.86 for the Dimitrov *et al.,* (2005) model for acids and zwitterions to -0.38 of Arnot and Gobas (2003) model for bases and zwitterions. The same negative results for the invertebrate models were seen with the Meredith-Williams *et al.,* (2012) model having slightly better performance than the Karlsson *et al.,* (2013) model.

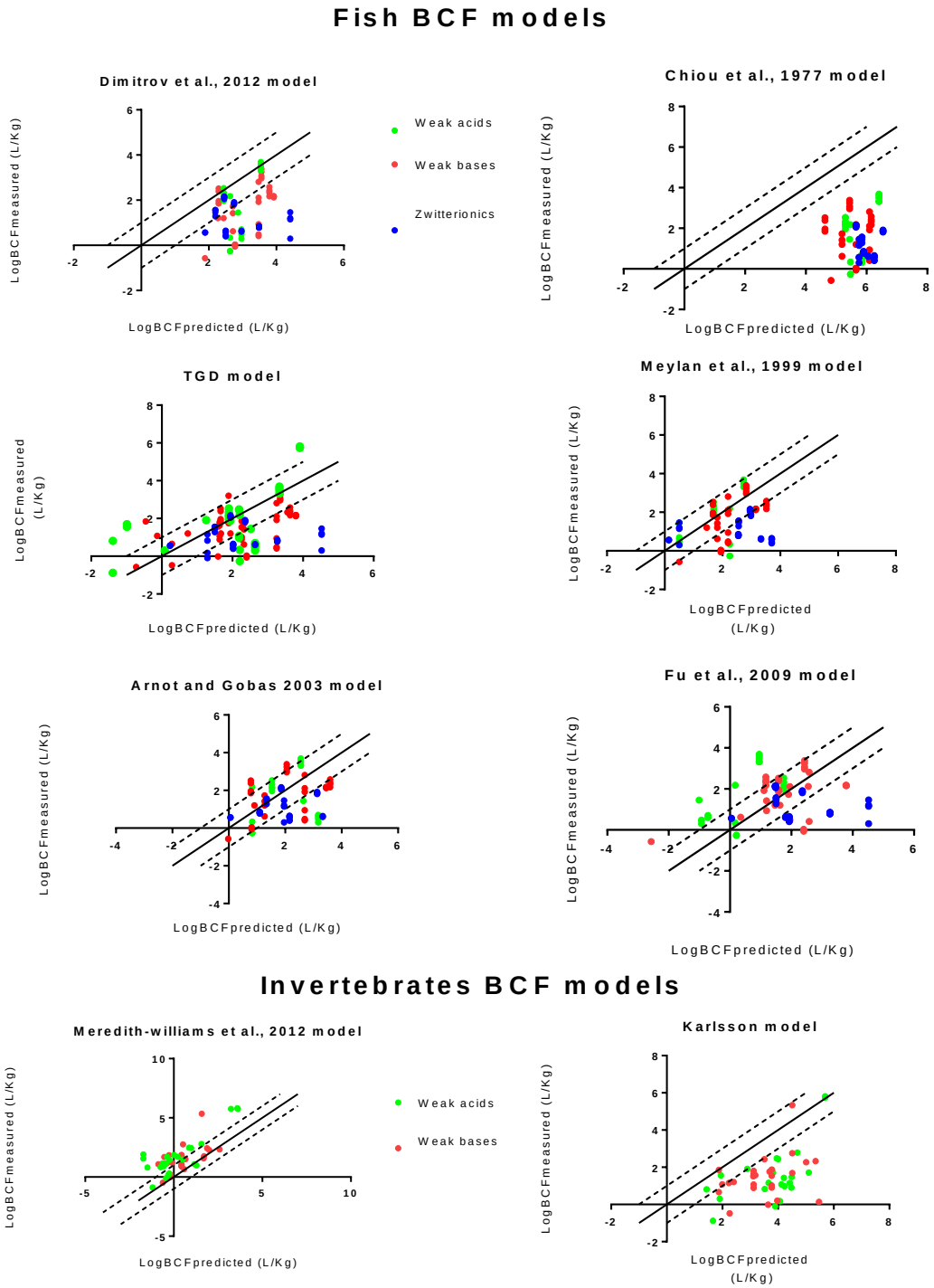


Figure 2. 1. Relationships of predicted and measured BCF data for the eight selected BCF models in fish and invertebrate species. Note the different scale of the axes. The fish BCF data include zwitterions obtained from the iPie database (http://i-pie.org/). In this database, BCF data were not available for invertebrates and zwitterions and therefore invertebrate data included acids and bases only.

Model	*Accuracy (%) for acids, bases and zwitterions*	*Accuracy (%) for acids and zwitterions*	*Accuracy (%) for bases and zwitterions*
		Fish BCF models	
Dimitrov et al., 2005	39	39.2	31.7
TGD, 1996	45.1	50.9	42.8
Meylan et al., 1999	46.3	50.9	33.3
Chiou et al., 1977	0	0	0
Fu et al., 2009	43.9	35.2	42.8
Arnot and Gobas 2003	46.3	47	49.2
		Invertebrate BCF models	
Karlsson et al., 2013	9.80	16.6	11.5
Meredith-Williams et al., 2012	35.2	20.8	38.4

Table 2. 2. Percentages of BCFs predicted within a factor of 10 for acids, bases, and zwitterions in fish and invertebrates species. Accuracy is determined as a percent of predicted values within a factor of 10 of the corresponding measured BCF.

Model	*NSE for acids, bases and zwitterions*	*NSE for acids and zwitterions*	*NSE for bases and zwitterions*
		Fish BCF models	
Dimitrov et al., 2005	-6.99	-7.86	-6.34
TGD, 1996	-0.49	-0.63	-0.82
Meylan et al., 1999	-1.14	-1.18	-1.52
Chiou et al., 1977	-92.37	-140.45	-93.8
Fu et al., 2009	-0.44	-0.56	-0.75
Arnot and Gobas 2003	-0.66	-1.27	-0.38
		Invertebrate BCF models	
Karlsson et al., 2013	-3.7	-3.32	-4.55
Meredith-Williams et al., 2012	-0.64	-0.43	-1.27

Table 2. 3. NSE calculated for acids, bases, and zwitterions of predicted and measured BCF data for the eight selected BCF models in fish and invertebrate species.

Fish BCF models

The chemicals selected in this study cover a range of physicochemical properties but also different exposure scenarios such as the presence or absence of food, the duration of the test exposure and the different exposure media. For example, water and sediment BCFs of diclofenac have both been recorded. Due to the complexity of the BCFs analyzed in the sediment compartment, where environmental parameters (such as the carbon organic content or the nature of the sediment) influence the uptake; a careful analysis and interpretation of the discrepancy of the predicted and measured BCFs is necessary.

Half of the fish BCF models clearly over-predict BCFs (Meylan, TGD, Dimitrov, Figure 1). A possible reason for the overestimation is due to the method used to measure bioconcentration. For example, many studies included in this work have analyzed the bioconcentration using radiolabelled chemicals measuring the total radioactivity. Generally, this method is very efficient in terms of laboratory effort but it is not suitable for distinguishing between the uptake of any metabolites and parent compounds. Therefore, the differences between the predicted and measured BCFs may be partially explained by the radioactivity measurements used to evaluate the BCF that do not distinguish the parent chemicals from their metabolites leading to a possible over-prediction scenario. An increase in BCF prediction model performance was observed for the Meylan *et al.* (1999) and TGD (1996) models when acids and zwitterions are considered on their own, with a maximum of 50.9% of the predicted BCFs being within a factor of 10 of measured data for both these models. They are the only two models that estimate a fairly satisfactory threshold (50% of the BCFs are predicted by the model), confirming the hypothesis that for some models a better prediction occurs when acids and bases are considered separately.

These two models use the parameter $LogK_{ow}$ as the sole descriptor in the simple linear regression equations proposed. Because of the simplicity of the two models, the difference in the prediction of BCFs is likely due to the different degree of hydrophobicity of the chemicals included in this study. While some compounds are highly hydrophobic, (for e.g. Eltrombopag, $LogK_{ow}$ 6.15), others are very hydrophilic such

as Atenolol ($LogK_{ow}$ of -0.03). However, this could just explain in part why some compounds are better predicted by the models than others.

The Arnot and Gobas (2003) model was able to predict 49.2% of bases and zwitterions BCFs to within a factor of 10 of experimental values. Whilst this is the best performing model for the bases and zwitterions class, it was unable to estimate at least 50% of the measured BCFs. The original model accounts for the uptake rate of the chemical *via* the gills and the elimination of the parent compounds through metabolic transformation. Hence, we believe that possible differences between the predicted and measured BCFs could be explained partially by different detoxification and biotransformation processes of the different fish species included in this work. Evidence suggests that different compounds are metabolized to a different extent; for example, fluoxetine is completely metabolized to norfluoxetine (Nakamura *et al.,* 2008a). Besides, it is well known that biotransformation influences the internal concentration of the parent compounds and metabolites, resulting in some cases of a higher internal concentration of the metabolites than their parent compounds (Ashauer *et al.,* 2012). Therefore, this parameter is the most uncertain because it is very dependent on the metabolic process of the species.

Moreover, previous studies have demonstrated that one of the major passive uptake routes of xenobiotics is at fish' gills (Erickson *et al.,* 2006). However, a new theory has been presented by some authors regarding the permeability of ionisable chemicals in biological membranes (Dobson and Kell, 2008). They account for the active transport of ionisable compounds at the fish' gills with either facilitated diffusion or carrier-mediated transport through the membranes. Thus, differences of BCFs could be partially explained by the fact that uptake is not only occurring through the passive adsorption of the neutral fraction of the compound, but, by the possibility that ionisable chemicals are transported across the bio-membrane by specific carriers (Sugano *et al.,* 2010; Miller *et al.,* 2015; Richmond *et al.,* 2016). For example, it has been shown that two members of a solute carrier family (SLC), isolated from the fish' gills of Rainbow trout, might act as principally responsible for the uptake of ionic and non-ionic drugs (Cooper *et al.,* 2007; Fardel *et al.,* 2012; Armitage *et al.,* 2017).

However, whilst this is a plausible explanation for the discrepancies in predicted and measured BCFs presented in this study, this is a new hypothesis that needs be further investigated considering a wider range of ionisable chemicals in order to be validated.

The Fu *et al.,* (2009) model is the only model that was able to better predict BCFs when all the chemicals are considered together (43.9%, Table 2.2), while the Dimitrov *et al.,* (2005) has a similar prediction for acids, bases and zwitterions and also for acids and zwitterions (39%, Table 2.2). The differences in the BCF prediction from the two models can be explained by exploring the input parameters, namely K_{ow} and the water content which are the main descriptors of the Dimitrov *et al.,* (2005) model and $LogD_{ow}$ (pH-corrected octanol-water partition coefficient) in the Fu *et al.,* (2009) model. The $LogD_{ow}$ is considered to be a better parameter for estimating the uptake of ionisable chemicals because it accounts for the pH, the dissociation constant (pK_a), the valency of a compound and the K_{ow} (Kah and Brown, 2008). For the Dimitrov *et al.,* (2005) model, a physicochemical parameter (K_{ow}) and a species-specific biological trait (water content) have been proposed in analyzing the bioconcentration factor. The Water Content is considered an important biological trait when assessing the bioconcentration (Rubach *et al.,* 2012) based on the theory that a chemical is partitioned between the lipid and the aqueous phase of an organism. However, there is a scarcity in data regarding the specific water content of the species investigated. In order to test the model, if such information for the specific fish species could not be found in the literature, the class or phylum's value of that species was recorded. This could be a critical point leading to the shift of the BCFs below the 0.1 line. In this case, this biological parameter is not so precise because it is not referred to the specific fish species. Besides, due to a difficulty to find this data, we suggest that more specific biological traits should be provided in the future, in particular when assessing the bioconcentration of xenobiotics organic chemicals across different species.

The final model evaluated is the Chiou *et al.,* (1977) model. The results show that BCFs are unable to be predicted within a factor of 10 of experimental values using this model and the general trend is an overprediction of the modeled BCFs (between 5 and 7 log units) compared to the experimental data.

Invertebrates models

The invertebrates models evaluated in this work include the Karlsson et al., (2013) model and the Meredith-Williams *et al.,* (2012) model. The former predicts lower than 20% of the modelled BCFs to within an order of magnitude of experimental values for both acids and bases. It considers biological traits, chemical, and environmental properties in order to describe the entire uptake process of ionisable compounds into a sediment-dwelling worm *Lumbriculus variegatus*. Due to the problem of data availability for biological traits, the same issue occurs with the Dimitrov *et al.,* (2005). Most of the biological traits were not provided and in order to test the model, we relied on the literature. Scarce data are available about the internal pH or the water content of invertebrates, thus, when no data were available, we had to rely on biological traits of the class or phylum of the considered species. In this way, the deviation from the best-fit line and factor 10 of the model may be in part due to the imprecision of the biological traits. On the other hand, the latter predicts more bases than acids; 38.4% to 20.8% respectively. It is the only model showing a clear underestimation of the BCFs, due in part to a negative estimation of the $LogD_{lipw}$ for some chemicals such as Atenolol or Sulfadiazine.

2.4 Conclusions

The present study has demonstrated that the uptake of ionisable compounds could not be reliably predicted by the current BCF models. The majority of the selected models under-predict BCFs while others over-predict BCF. Two models (Meylan *et al.,* 1999 and TGD 1996) estimate 50% of the BCF data to within an order of magnitude of experimental values, while the remaining models demonstrated a low accuracy in predicting BCFs. The poor predictive accuracy of the models was observed also by the performance of the Nash-Sutcliffe Efficacy test and all models showed negative values. The different performance between the eight bioconcentration models could be partially explained by the wide variety of physicochemical properties, test conditions, the method to measure the BCF and the different input descriptors not fully suitable for describing the mechanism of uptake of ionisable compounds. In addition, the models showed an improved prediction when the acids, bases, and zwitterions were considered

separately. Based on these findings, we suggest considering these charged species separately in developing and proposing a new uptake predictive model. Ultimately, of eight models, only two invertebrate models have been evaluated in this work and this clearly shows the gap of data and thus invertebrates' models available in the open literature compared to fish species. Further work should be addressed to develop more invertebrates BCF models, in particular for estimating the uptake of ionisable chemicals. It could be useful to focus on more than one biological trait, environmental and physicochemical parameters.

Therefore, the next chapter will focus on assessing the key role of an environmental factor, pH, on the uptake of APIs that possess diverse physicochemical characteristics into the sediment-dwelling worm *L. variegatus*.

Chapter 3. Influence of pH on the uptake of ionisable active pharmaceutical ingredients (APIs) into the sediment-dwelling worm (*L. variegatus*)

3.1 Introduction

Previous studies have explored the uptake and depuration of APIs into non-target organisms such as earthworms (Carter *et al.,* 2016), plants (Riemenschneider *et al.,* 2017; Santiago *et al.,* 2016; Carter *et al.,* 2015), fishes (Chen *et al.,* 2017; Zhao *et al.,* 2017; Valdés *et al.,* 2016; Du *et al.,* 2016) and invertebrates (Karlsson *et al.,* 2016; Miller *et al.,* 2015; Meredith-Williams *et al.,* 2012).

For example, Karlsson *et al.,* (2016) investigated the uptake of selected ionisable APIs into the benthic invertebrate *Lumbriculus variegatus.* Differences in uptake among the pharmaceuticals were observed which are believed to be due to differences in physicochemical properties of the study compounds and sediment properties. Differences in bioconcentration have also been assessed by Meredith-Williams *et al.,* (2012) in two non-target invertebrates: the freshwater shrimp *Gammarus pulex* and the aquatic insect *Notonecta Glauca*. They found that the different bioaccumulative degree of each API was explained by the influence of water chemistry (for example pH), physicochemical properties of the compound (for example pK_a) and different biological traits such as respiration and locomotion of the two organisms.

Mechanism of ionisation. Most of the APIs that are in use are ionisable at pH ranges found in natural aquatic systems (Franco *et al.,* 2010). Depending on the pH of the system, these compounds can be found in the neutral or ionized form as weak acids, bases or zwitterions. The fraction of the neutral and the ionized form of the chemical depends directly on the pK_a and the pH of the medium can be calculated using the Henderson-Hasselbalch (Henderson, 1908) equation:

$$\alpha_{ion} = \alpha_{neutral} \times 10^{i(pH-pKa)} \qquad \text{Equation 1}$$

where α_{ion} is the ionic fraction of the compound, $\alpha_{neutral}$ is the neutral fraction and I is 1 for acids and -1 for bases.

Generally, ionized APIs are believed to be less bioaccumulative compared to their neutral counterpart (Rendal *et al.,* 2011a) because they are more polar and they do not easily cross the plasma membrane which is comprised of fatty acids and phospholipids. Previous studies have analyzed the influence of pH on the toxicity and bioconcentration (BCF) of ionisable APIs. For example, a study by Nichols *et al.,* (2015) examined the accumulation of the weak base diphenhydramine in fathead minnows at pH values of 6.7, 7.7 and 8.7. They measured an increase in toxicity and bioconcentration with an increase in the water pH. Another study conducted by Anskjaer *et al.,* (2013) measured the toxicity and bioconcentration of the weak acid sulfadiazine in the invertebrate *Daphnia magna* at pH values of 6, 7.5 and 8.5 respectively. In this case, the lower pHs led to higher toxicity and bioconcentration due to the fact that the majority of the compound exists in its neutral state at the lower pH values. Opposite results were found by Ding *et al.*, (2016) who exposed *Daphnia magna* to two basic pharmaceuticals roxithromycin and propranolol at two different pH conditions (pH 8 and 9) and higher uptake was observed at higher pH exposure. Lastly, Rendal *et al.,* (2011b) found that *Daphnia magna* and the algae *Salix viminalis* showed higher sensitivity to and uptake of pharmaceuticals at higher pH values of 8 and 9 than pH 6 and 7. Even though an increasing number of studies are available in the literature regarding the uptake of ionisable compounds in non-target aquatic species such as fish, pH-related invertebrate uptake studies are very limited, in particular for sediment-dwelling organisms. Because the pH of rivers naturally may range between 3 to 10 (Bundschuh *et al.,* 2017), large differences may be expected in the ionization state of many APIs in natural waters which could have a large effect on the internal concentrations of these compounds in organisms. Recently, a model that accounts for the pH and the pK_a of the chemical has been proposed for assessing the uptake of ionisable APIs in the sediment-dwelling worm *L. variegatus* (Karlsson *et al,* 2017). In this study, supporting the development of the model, a 37-fold difference in uptake for fluoxetine and 47-fold for diclofenac was observed in a water pH ranging between 5.5 and 8.5. This study was the first attempt of its kind in understanding the role of physicochemical and environmental properties in measuring the uptake of ionisable APIs in sediment-dwelling invertebrates but the approach was limited to only three APIs. Additional data on the effect of pH on the uptake of ionisable APIs in sediment-dwelling organisms are therefore needed. Also,

studies with better characterisation of pH effects than the previous studies are essential for a more comprehensive environmental assessment of the risks posed by APIs in the environment.

Therefore, in this chapter, the uptake and depuration of four APIs were evaluated across pH values ranging from 5.5-9. Three of the APIs, amitriptyline, ketoconazole, norfluoxetine, are weak bases and the fourth API, diclofenac, is a weak acid. These pharmaceuticals were selected based on the literature and represent a diversity of physicochemical properties. The broad range of pHs was chosen in order to have each API fully and partially ionized. The aim of the study was to quantify the absorption and excretion of these emerging contaminants in sediment-dwelling invertebrates in order to gain more insight and understanding into how these APIs, which have different physicochemical properties, are taken up by the worms at different pH values.

3.2 Material and methods

3.2.1 Test chemicals

The antimycotic, ketoconazole (CAS 65277-42-1), and anti-inflammatory, diclofenac (CAS 15307-86-5), were purchased from American Radiolabeled Chemicals, Inc. (UK). The two antidepressants amitriptyline (CAS 64-17-5) and norfluoxetine (CAS 65277-42-1) were obtained from Merck & Co (New Jersey, USA) and from Sanofi (Paris, France). Test chemicals were all labeled with 14-C and they were selected to represent different physicochemical properties. Specific properties and activity data for each API are provided in Table (1.2) in Chapter 1. Stock solutions were prepared in ethanol and stored at -20 ˚C. The dosing stocks were prepared in either ethanol or artificial pond water (APW) (Naylor et al., 1989). Ethanol, acetate, 3-(N-morpholino)propane sulfonic acid (MOPS, CAS 1132-61-2), N-Cyclohexyl-2-aminoethanesulfonic acid (CHES, CAS 103-47-9), tris(hydroxymethyl)aminomethane (TRIS, CAS 77-86-1) buffers, hydrochloric acid and sodium hydroxide were obtained from Sigma Aldrich (https://www.sigmaaldrich.com), Soluene-350 and Hionic Fluor were purchased from PerkinElmer (http://www.perkinelmer.com) and Ecoscint A from National Diagnostics (https://www.nationaldiagnostics.com).

3.2.2 Lumbriculus variegatus cultivation

Lumbriculus variegatus were obtained from Blades Biological Ltd (Kent, UK) and reared in a 30 L glass aquarium. The aquarium contained artificial pond water with shredded unbleached tissue paper strips. The culture was maintained at a constant control temperature of 25 ± 2˚C with a light/dark cycle of 16h/8h. The organisms were fed with groundfish flakes (Tetramin, Tetra Werke) once a week.

3.2.3 Uptake and depuration studies at multiple pHs

The uptake and depuration experiments were carried out following the method of (Ashauer et al., 2010), consisting of a 24-hour uptake phase followed by 24-hour depuration phase. Worms were acclimatized to the pH test conditions for 24 hours prior to the beginning of the exposure. The APW was buffered at four pH values: 5.5, 7, 8 and 9 using 0.1 mol/L of acetate, MOPS, TRIS and CHES buffers. For the uptake phase, animals were exposed in groups of 3 worms in 20 mL of APW at concentrations of 10 µg/L (for the amitriptyline experiments 10 mL was used). The uptake phase included two sampling times (12 and 24 hours) and there were six replicates per sampling time point. An additional 12 groups of three animals were exposed for use in the depuration phase. After 24 hours of uptake, these worms were transferred to new beakers containing new APW without the test pharmaceutical with samples then being taken 12 and 24 h later. Six stability beakers (= APW only spiked with the APIs) and six control beakers (= beakers containing worms without the API) were also set up. To keep the pH stable additional uptake and depuration experiments were carried out increasing the amount of APW from 10 mL to 20 mL. Due to the high variability of pH throughout the experiments, an increase of the APW was necessary in order to keep the pH constant. The entire experiments were performed in the dark to avoid photodegradation of the APIs and exposure was performed at a constant temperature of 25 ± 2˚C. pH measurements were taken every 12 hours using a Mettler Toledo 51343104 InLab pH probe.

At each sampling time, 1 mL of test media was taken into a 20 ml scintillation vial to determine the remaining concentration of the API. 10 mL of Ecoscint A was added and the vials were ready to be analyzed. Likewise, worms were sampled, gently rinsed in deionized water, dried using filter paper and then transferred to a pre-weighed scintillation vial to measure the wet weight (g). Then, 2 mL of Soluene-350 was added and the vials were left overnight in order to allow the worms to be solubilized. Before analysis, 10 mL of Hionic Fluor was added. Internal concentrations and water concentrations of each API were analyzed using Liquid Scintillation Counting (HIDEX 300 SL). Samples were counted for 5 minutes three times and counts were corrected for background activity by using blank controls. Counting efficiency and color quenching were corrected using the external standard ratio method.

3.2.4 Estimation of uptake and depuration rate constant and bioconcentration factor

The model used to derive the uptake and depuration rate constants was a first-order one compartment toxicokinetic model (Equation 1), programmed in the software OpenModel, (University of Nottingham, http://openmodel.info/ downloaded 5th May 2017).

$$dC_{int}(t)/\,dt = k_{in} \times C_{water}(t) - k_{out} \times C_{int}(t) \qquad 1$$

Where C_{int} is the organism internal concentrations (pmol/g wet weight), C_{water} is the water concentration of the API (pmol/mL), k_{in} and k_{out} are the uptake (mL x g wet weight h^{-1}) and depuration rate constant (h^{-1}) and t is time (hours).

Measured internal and water concentrations were used as input data to derive the uptake and depuration constants k_{in} and k_{out}. A detailed description of the model code is provided in the Appendix B. First, least-squares optimization with the Levenberg-Marquardt algorithm was applied using the experimental data. Then, Monte-Carlo Markov-Chain (MCMC) with the Metropolis-Hastings algorithm was used to parameterize the model. 95 % confidence intervals were plotted along with the fit model curve. The bioconcentration factor (BCF) (Kg/L) was estimated by setting the water concentration (C_{water}) equal to 1 and by running the model until a steady state was

reached. The formula used for calculating the BCF (L/Kg) at steady state is given in Equation 2:

$$\mathrm{BCF} = \frac{\mathrm{Kin}}{\mathrm{Kout}} \qquad 2$$

3.2.5 Statistical analysis

One-way ANOVA test was performed with Graphpad Prism (www.graphpadprism.com) in the stability beakers. The analysis was done to verify whether the aqueous concentrations of the level of radioactivity changed overtime during the exposure. Prior to the ANOVA test, the Shapiro-Wilk test was performed to test for normality.

3.3 Results and discussion

3.3.1 Measured concentrations in the stability beakers

The analysis of the aqueous concentrations of the APIs in the stability beakers confirms that the level of radioactivity in solution was stable throughout the experiments for all the pH ranges meaning that there was no sorption of the test chemicals to the beakers (Figures 3.1, 3.2, 3.3, 3.4). Overall, the pH was stable for all the compounds remaining within the ± 0.3 units of the starting pH value.

At some pH ranges, (for e.g. ketoconazole pH 8 and 9 and norfluoxetine pH 5.5) a slight decrease in the concentrations of the compound was observed during the first 12 h from the beginning of the experiment ($p < 0.0001$). A possible explanation may be that a biotransformation process has occurred, however, the use of radiolabelled APIs does not distinguish between the parent compound and the transformation products, therefore, we assumed that the measured compound throughout the test was the parent compound. The same trend was observed for diclofenac at pH 9 ($p < 0.05$). Diclofenac has been reported to be photosensitive and degradable through photolysis and biotransformation (Koumaki *et al.,* 2015; Koumaki, *et al.,* 2017). For example, diclofenac has been exposed to natural sunlight and a half-life of 1.7 h was found (Poiger *et al.,* 2001). However, in this study, the experiments were conducted in the dark and

the photodegradation was therefore expected to be very limited. The concentration of the 14-C diclofenac in water was reported by Karlsson *et al.,* (2016) where the authors analyzed the API over 48-h exposure. They observed stable radioactivity in the beakers containing the API and water only. Once pharmaceuticals end up in the water, their dissipation could be explained by other processes such as changes in pH and biodegradation. A study conducted by Baena-Nogueras *et al.,* (2017) analyzed the fate of several PPCPs including diclofenac and amitriptyline and their removal from the water column. Photodegradation, hydrolysis and biodegradation were the processes that the authors investigated. Different results were found: diclofenac was highly degraded by the light but there were no significant changes in its removal with pH changes. This confirms the results of this study where the 14-C activity of diclofenac was stable at different pH ranges. Amitriptyline was not removed by any of the above mention processes (Baena-Nogueras *et al.,* 2017). Another study evaluated the photodegradation of amitriptyline and its major metabolite in water and no photodegradation occurred in deionized water but an increase of the photodegradation was observed at higher pH in deoxygenated solution (Chen *et al.,* 2017). However, in our experiments, the beakers were not deoxygenated and stable radioactivity of amitriptyline was observed.

Regarding norfluoxetine, previous fate studies have mainly been conducted on the parent compound fluoxetine. For instance, Yin *et al.,* (2017) evaluated the effects of hydrolysis and photodegradation on fluoxetine. They exposed fluoxetine to different pH values (from 2 to 10) over a year under dark and light conditions to assess the long-term effects of dissipation by the water chemistry and photodegradation. Fluoxetine was found to be persistent under both conditions (light and darkness), in particular, under dark conditions with a half-life of 858-13905 days at all the pH values. These half-life values show that the compound is hardly degradable under alkaline and acidic conditions and it is not photosensitive. Similar to the experiments of our study, the radioactivity of its primary metabolite norfluoxetine was stable at all pH values from 5.5 to 9 and it was exposed to very limited light. Therefore, we conclude that hydrolysis and photolysis are negligible and do not account for the removal of this compound in water. The radioactivity of ketoconazole was found to be stable for the whole duration of the exposure at the different pH ranges. Only a slight decrease of the radioactivity was

observed at higher pHs such as 8 and 9 within the first 12 h (Figure 3) ($p < 0.0001$) in which a potential transformation process may have occurred. Limited studies about the fate of ketoconazole in the water column were found, and to the best of our knowledge, this is the first attempt to measure the stability of this azole fungicide in the water under acidic, alkaline and dark conditions.

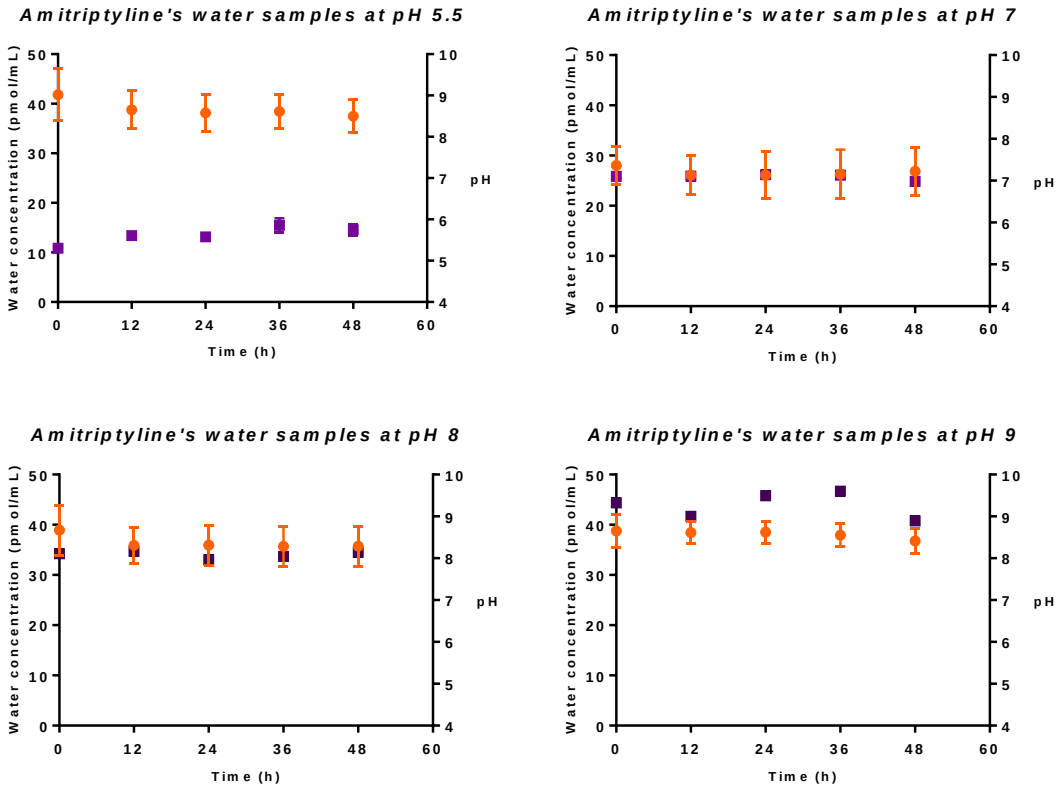


Figure 3. 1. Mean and standard deviations of concentrations in water (pmol/mL, orange points) and pH of the stability beakers (violet squares) for amitriptyline at pH 5.5, 7, 8 and 9 over 48 hours. Concentrations are based on measured activity and it is assumed this is the parent compound.

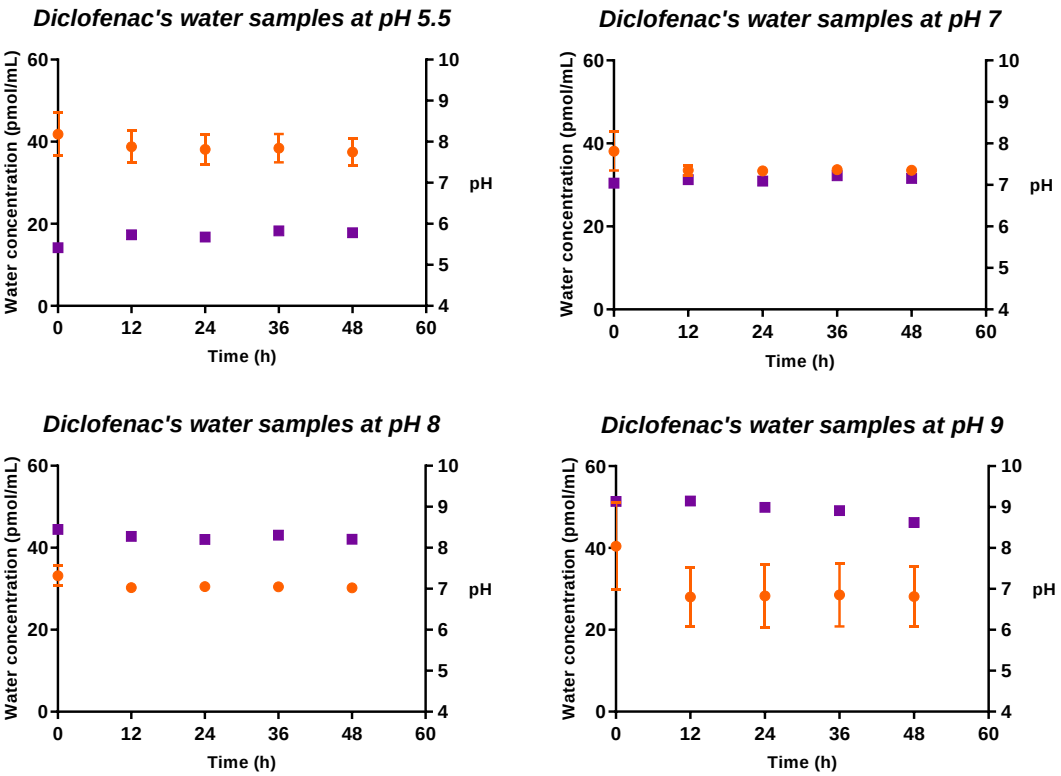


Figure 3. 2. Mean and standard deviations of concentrations in water (pmol/mL, orange points) and pH of the stability beakers (violet squares) for diclofenac at pH 5.5, 7, 8 and 9 over 48 hours. Concentrations are based on measured activity and it is assumed this is the parent compound.

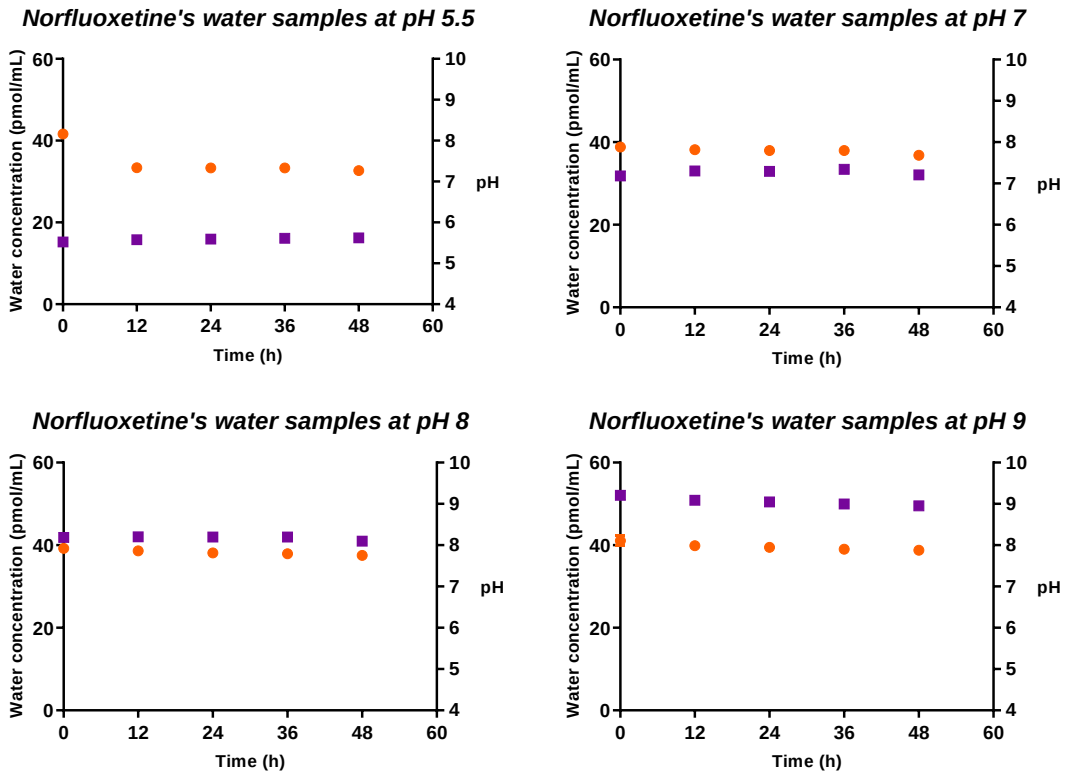


Figure 3. 3. Mean and standard deviations of concentrations in water (pmol/mL, orange points) and pH of the stability beakers (violet squares) for norfluoxetine at pH 5.5, 7, 8 and 9 over 48 hours. Concentrations are based on measured activity and it is assumed this is the parent compound.

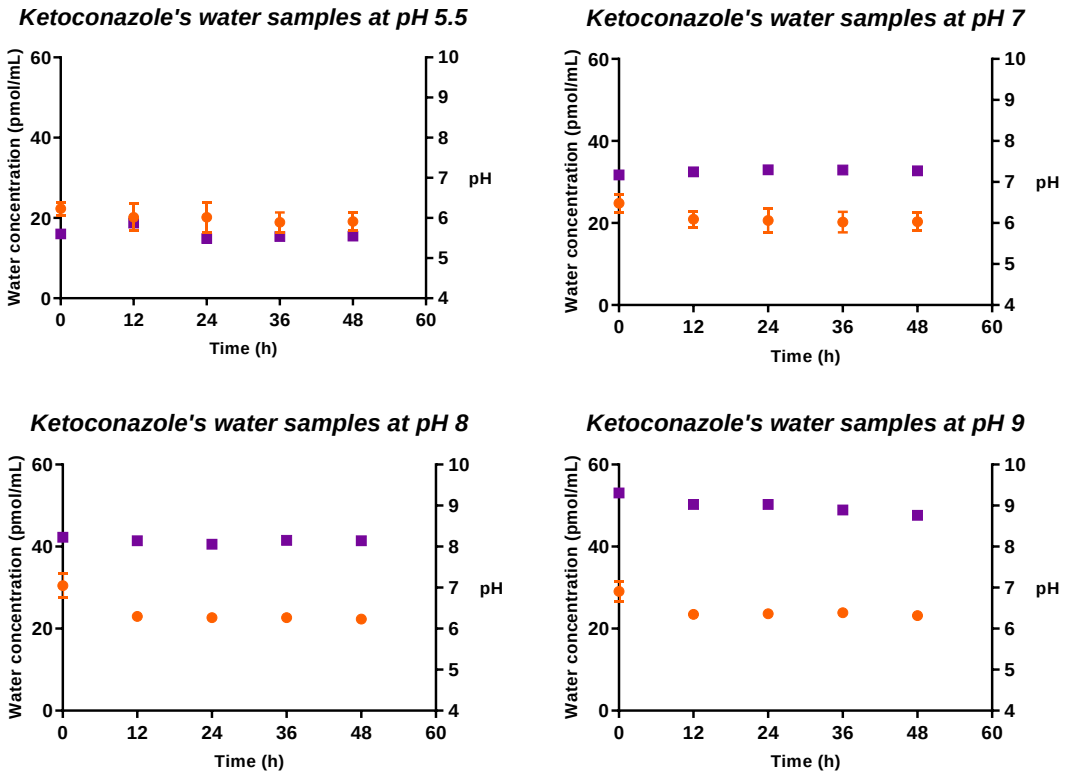


Figure 3. 4. Mean and standard deviations of concentrations in water (pmol/mL, orange points) and pH of the stability beakers (violet squares) for ketoconazole at pH 5.5, 7, 8 and 9 over 48 hours. Concentrations are based on measured activity and it is assumed this is the parent compound.

3.3.2 Uptake and depuration studies in *Lumbriculus variegatus* at multiple pHs

3.3.3 pH variability in uptake and depuration experiments.

The pH values of the exposure beakers were monitored throughout the experiments (Fig. 3.5). Different buffers were used to keep the pH constant for 48 hours as mentioned in the Material and Methods section. All the pH values remained within ± 0.3 pH units of the starting pH value per each time point except amitriptyline pH 5.5 treatment. In fact, during the exposure of amitriptyline, changes of pH by up to 0.5 log units were observed every 12 h (Figure 3.5, top left). Because the uptake of ionisable APIs is very sensitive to variations of pH, a drift of 0.5 log units could change the ionisation state of the molecule and thus influence the accumulation of the compound. Thus, to keep the pH stable, the addition of a few drops of 0.1 mol/L NaOH or HCl every 12 hours was necessary (Rendal *et al.,* 2012).

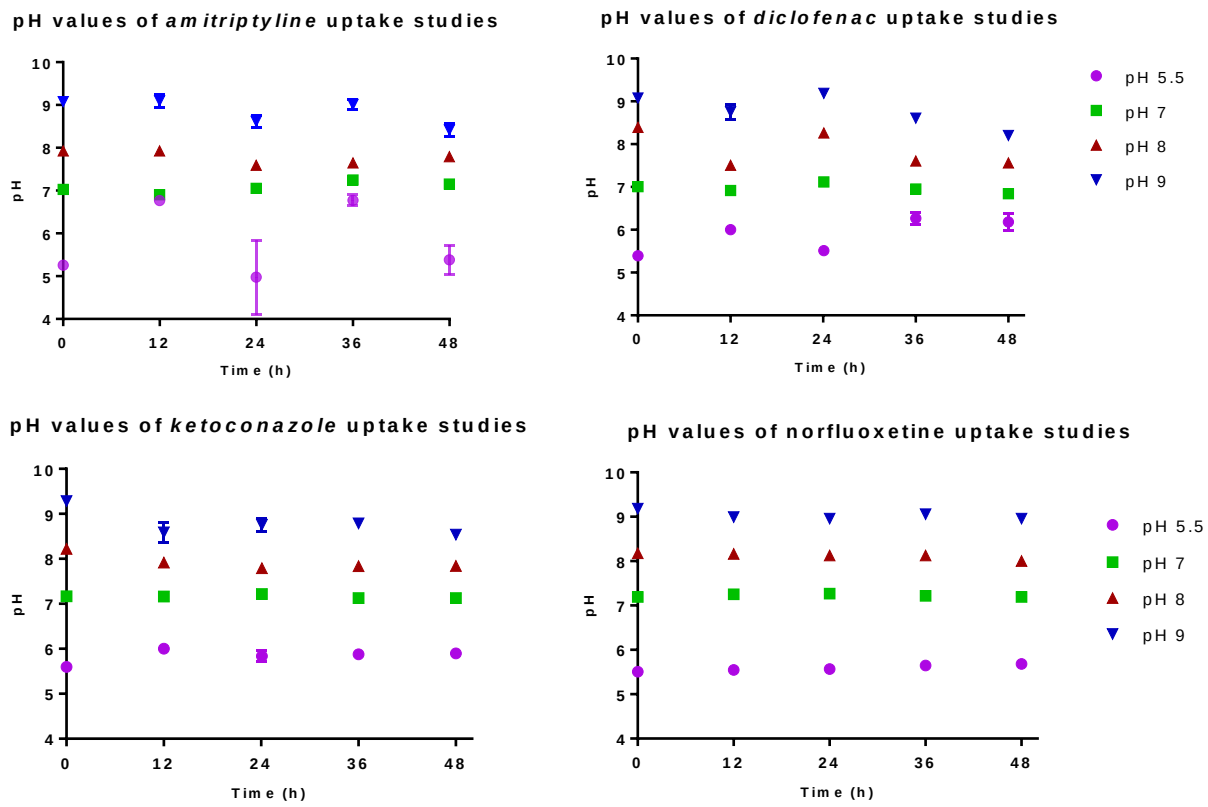


Figure 3. 5. pH measurements over 48-h of exposure and 95% confidence intervals of the APW buffered for the uptake and depuration experiments of amitriptyline, ketoconazole, diclofenac and norfluoxetine.

3.3.4 Uptake and depuration exposures.

No mortality of the organisms was observed in the treatment beakers and the growth dilution of the worms was minimized due to the short duration of the uptake and depuration experiments. The radioactive concentration of the compounds in the water was measured (Figures 3.7, 3.9, 3.11, 3.13). During the uptake phase, a decrease of the radioactivity in the exposure beakers was observed which is likely due to the uptake of the APIs by the worms. The opposite occurred during the depuration phase when the worms were placed in the new beaker without radioactivity.

The first order one-compartment toxicokinetic model was fitted to the measured internal concentration data for all the APIs (Figures 3.6, 3.8, 3.10, 3.12). Diverse results were observed depending on the compound and they will be discussed separately in the

following sections. The raw dataset of water concentrations, internal concentrations, and mass of the worms of each API are listed in Appendix B.

For amitriptyline, the model successfully fitted the uptake and depuration data for all the pH treatments with most of the data fitted within the 95% confidence intervals. Overall, a rapid uptake followed by a rapid depuration was observed, however, at higher pHs the worms did not depurate amitriptyline, particularly at pH 9 where a shallow depuration curve was seen (Figure 3.6).

Regarding ketoconazole, the model fitted the measured uptake and depuration data although, for the pH 7 experiment, the model slightly underestimated the 48-h depuration data (Figure 3.10). Also, a rapid uptake followed by a rapid depuration was seen.

The last base evaluated in this study was norfluoxetine. The model successfully fitted the uptake and depuration data and rapid uptake by the worms was observed for all the pH treatments (Figure 3.12). However, during the depuration phase, the worms slightly depurated the compound at pH 7 and 8 but they did not depurate norfluoxetine at the most extreme pHs of 5.5 and 9.

The only weak acid analyzed was diclofenac. The uptake of diclofenac showed a rapid increase of the 14-C activity of the target analyte for 24 hours, as well as a rapid depuration when the worms were transferred in the new medium (Figure 3.8). Generally, the model fitted the measured internal concentrations data but it slightly underestimated the 48-h depuration data of pH 8 and 9 experiments.

The results of the uptake, depuration rates and the BCFs are shown in Table 3.1. The K_{in} ranged from 0.365 ($mL \times g^{-1} \times h^{-1}$) of diclofenac pH 9 to 43.88 ($mL \times g^{-1} \times h^{-1}$) of norfluoxetine pH 9, while the K_{out} ranged from 1×10^{-7} (h^{-1}) of norfluoxetine pH 5.5 to 0.0014 (h^{-1}) of norfluoxetine pH 8. Also, at pH 5.5, the BCF values ranged from 93.04 (mL/g) to 1.06×10^{7} (mL/g) and increased in the order of ketoconazole < amitriptyline < diclofenac < norfluoxetine. For the pH 7 tests, the BCF values ranged form 81.42 to 870 (mL/g) and increased in the order of diclofenac < ketoconazole < amitriptyline < norfluoxetine. At pH 8, the BCF values ranged from 13.75 to 2902 (mL/g) and increased in the order of diclofenac < ketoconazole < amitriptyline < norfluoxetine. At pH 9, the

BCF values ranged from 3.78 to 1.11×10^4 (mL/g) and increased in the same order as pH 8.

Overall, the BCF increased with the increase of the water pH for the three bases and increased with a decrease of the water pH for diclofenac. In fact, it is worth noticing that a similar pattern of uptake among the three basic pharmaceuticals can be observed: higher uptake occurred at higher pH when the neutral fraction increased with an increase of the pH. For example, in a range of pH between 5.5 and 9, the neutral fraction of amitriptyline increased from 0 % to 30 %. For ketoconazole from 15 % to 99 % and for norfluoxetine from 0 % to 60 %. Likewise, the BCFs increased with an increase of pH (e.g. amitriptyline: 146 (pH 5.5) < 391 (pH 7) < 653 (pH 8) < 2284 (pH 9); ketoconazole: 93.04 (pH 5.5) < 100.9 (pH 7) < 101.1 (pH 8) < 211.4 (pH 9). This trend was not be observed for norfluoxetine because the largest BCFs were calculated for pH 5.5 and 9, respectively 1.06×10^7 and 1.11×10^4 mL/g.

On the other hand, for diclofenac, an opposite uptake trend was observed compared to the basic APIs: higher internal concentrations occurred at lower pHs and increased in the order of 3.78 (pH 9) < 13.75 (pH 8) < 81.42 (pH 7) < 353.4 (pH 5.5).

The results of this study demonstrate that *L. variegatus* can accumulate quantifiable concentrations of human active pharmaceutical ingredients and that the uptake of these APIs is pH-dependent. For example, for amitriptyline and ketoconazole, the BCFs calculated differed by approximately a factor of 15 and 2 between the highest and the lowest pH.

On the other hand, for the acidic diclofenac: the BCF values differed by approximately a factor of 93 between pH 5.5 and 9 with a change in the fraction of ionisation of the compound by only 2-3 %. Results from a previous study reported the BCF of diclofenac to be 623, 30 and 8 in *L. variegatus* at three different pHs (5.5, 7 and 8.5) (Karlsson *et al.,* unpublished) which are close to the values measured in this study. Also, the same uptake trend was seen: higher internal concentrations were observed at lower pHs when the compound increased its neutrality. Diclofenac has been inserted in the European watch list of priority substances as it has been demonstrated to alter fish feeding and behaviour (Nassef *et al.,* 2010) and affects fish kidney and gill integrity (Hoeger *et al.,* 2005). However, few studies have investigated the role of pH on

diclofenac uptake and effects in smaller organisms such as aquatic invertebrates. For instance, diclofenac was measured in two marine bivalves *Mytilus galloprovincialis* and *Ruditapes philippinarum* exposed to the API at low pH values. The reduction of pH affected biomarker responses of the organisms showing a significant decrease of COX activity which involves alterations in reproduction and osmoregulation (Munari *et al.,* 2018). Furthermore, the pH-dependent toxicity of diclofenac (EC_{50}) has been assessed on *Daphnia magna* at three pH ranges of 6, 7.5 and 9 and the results showed lower toxicity at higher pH (Boström and Berglund, 2015).

Regarding norfluoxetine, no studies on the mechanism of uptake of fluoxetine and its metabolite norfluoxetine into sediment-dwelling invertebrates have been studied so far. However, fluoxetine has been investigated at the sediment pH of 7.7 in lumbricids and a 48-h BASF of 0.33 L/Kg was found (Karlsson *et al.,* 2016). In our study, much higher BCFs were derived.

Norfluoxetine uptake has been also well documented in fish species (Brooks *et al.*, 2005; Chu and Metcalfe, 2007; Gelsleichter and Szabo, 2013). It has been detected in the different body parts of fish, including: liver, brain and muscle tissues where norfluoxetine has been detected at higher concentrations than the parent compound fluoxetine. Also, norfluoxetine has been found to be more hydrophobic tending to accumulate to a greater extent than fluoxetine eliciting the same effects (Gelsleichter and Szabo, 2013). Norfluoxetine has a pK_a value of 10.01 and the same trend of uptake (higher uptake at higher pH) as mentioned in the literature for basic compounds was seen. However, this trend was noticed for the experiments conducted at pH 7 and 8 while significantly high BCF values were derived at pH 5.5 and 9. These high values are most likely due to a non-existent depuration of the compound by the worms. An almost non-existent depuration by the worms was also reported by Karlsson *et al.,* (unpublished). Studies of the uptake of amitriptyline in non-target invertebrates are limited but, there has been a study that analyzed the bioaccumulation of amitriptyline into a freshwater mussel (de Solla *et al.,* 2016). The BAF value calculated was 6028 L/Kg which is much higher than the value derived in this study at the highest pH. The explanation could be that the authors calculated the BAF which consists of the analysis of the uptake of the chemical from both the surrounding medium and the diet; whereas in our study, the exposure from the surrounding medium only was considered. Thus, a

possible underestimation of the accumulation of this highly prescribed antidepressant in our study could have been reported. Another study investigated the bioconcentration of amitriptyline in a freshwater mussel *Lampsilis siliquoidea* and a BCF value of 253 L/Kg at pH 8.3 was found (Gilroy *et al.,* 2017). The value is the same order of magnitude with the value reported in our study, but, we derived a higher BCF of 653 L/Kg at the same pH range.

Generally, there is not much information available in the literature about the accumulation of amitriptyline in the invertebrates compared to fish species, particularly regarding sediment-dwelling invertebrates. Therefore, comparisons of our results with other studies are very difficult due to the fact the only studies available reported bioconcentration/bioaccumulation values at a single pH. In this study, we highlighted how pH influences the uptake confirming the general rule that the neutral fraction of a molecule is more bioaccumulative when compared to the ionic fraction. Therefore, we suggest further investigation of amitriptyline in other non-target invertebrate species, more specifically at different pH values. In this way, we will be able to effectively compare the data in order to have a more understandable picture of the environmental risk.

The last compound evaluated in this study was ketoconazole. Very little research has been conducted on the uptake in both fish species and invertebrates. Recently, a study investigated the toxicokinetic of some azole fungicides including ketoconazole in *Gammarus pulex* and the BAF calculated was 9.2 L/Kg at the exposure concentrations of 100 µg/L (Rösch *et al.,* 2016). The result is much lower compared to the BCF obtained in our study that ranged between 93.04 to 211.4 L/Kg at the exposure concentration of 10 µg/L. However, the study did not mention the tested pH which is very important to consider when evaluating the toxicokinetic of ionic compounds such as ketoconazole. Also, the difference in the BCF among the two species could be due to the different species traits that has been suggested by different studies in the literature (Meredith-Williams *et al.,* 2012; Rico and Van den Brink, 2015; Sidney *et al.,* 2016). For example, the two organisms possess different habitats and metabolism. To mention some, the shrimp inhabits the water column mainly, while the worm is normally submerged in the sediment and therefore subjected to exposure from the overlying water, the pore water between the sediment particles and the chemicals bounded to the sediment.

In addition, based on its pK_a (6.5), the neutrality of ketoconazole increased by 84 % between the lowest and the highest pH. Hence, this compound should show the most extreme changes in BCF compared to the other basic APIs. However, this does not seem the case because the BCF differed by only a factor of 2. For instance, in comparison, the BCF of amitriptyline differed by 15 times between pH 5.5 and 9 with an increase of the neutral fraction by only 30 %. This slight difference in BCF of ketoconazole could be explained by the hydrophobicity of the molecule compared to the other bases. The correlation between the bioconcentration and hydrophobicity has been well established in the literature (Mackay, 1982; Mackay and Fraser, 2000). Ketoconazole possesses a $logK_{ow}$ of 3.78 and therefore should not be expected to be as much bioaccumulative as norfluoxetine ($logK_{ow}$ = 4.16) and amitriptyline ($logK_{ow}$ = 4.92).

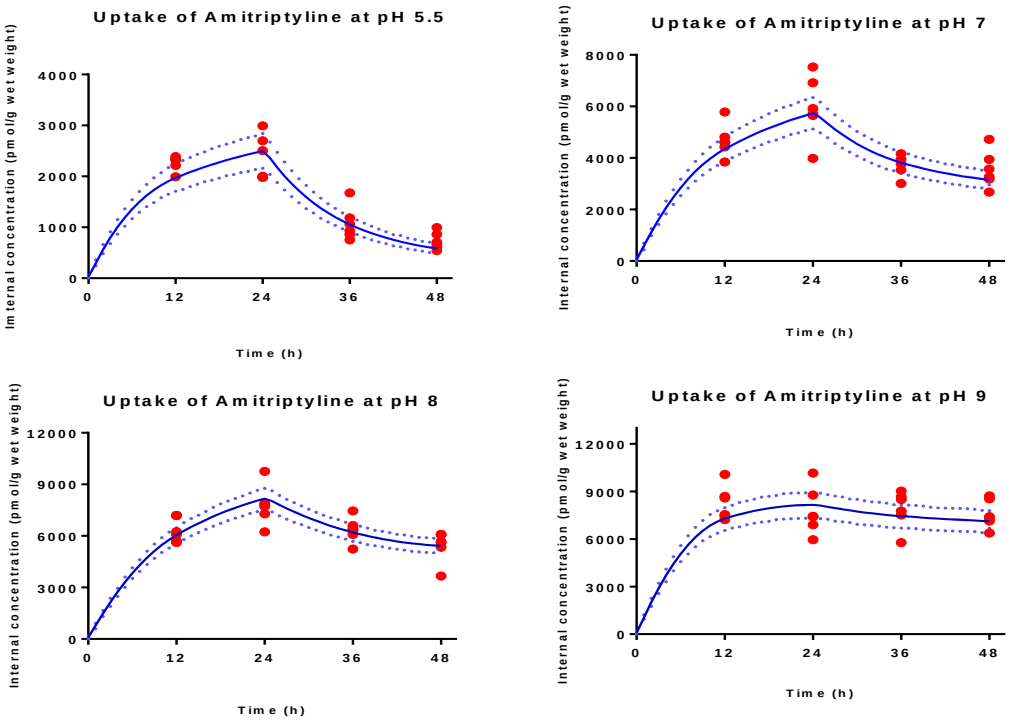


Figure 3. 6. Uptake (0-24-h) and depuration (24-48-h) graphs of amitriptyline at multiple pHs in *L. variegatus*. Red dots represent the internal measured concentrations (pmol/g wet weight). The thick line is the model fit of the toxikokinetic model and the dotted lines are 95% prediction intervals of the model. The concentrations are based on measured activity and it is assumed it is the parent compound.

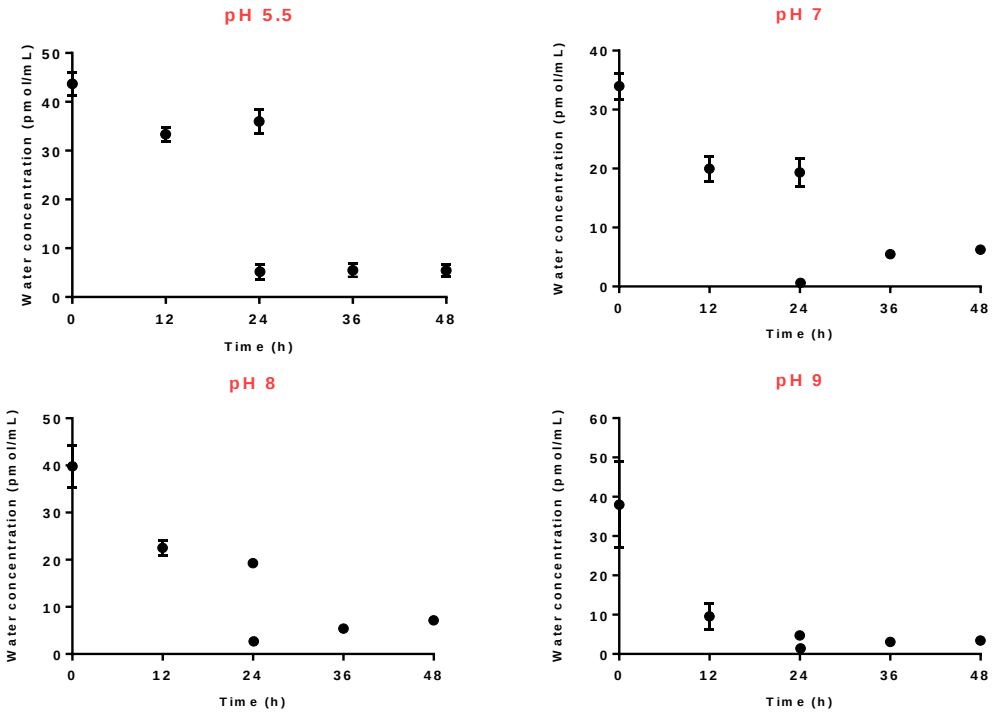


Figure 3. 7. Exposure water concentrations (pmol/mL) and 95% confidence intervals of amitriptyline at multiple pHs over 48 hours in *L. variegatus*. The concentrations are based on measured activity and it is assumed it is the parent compound.

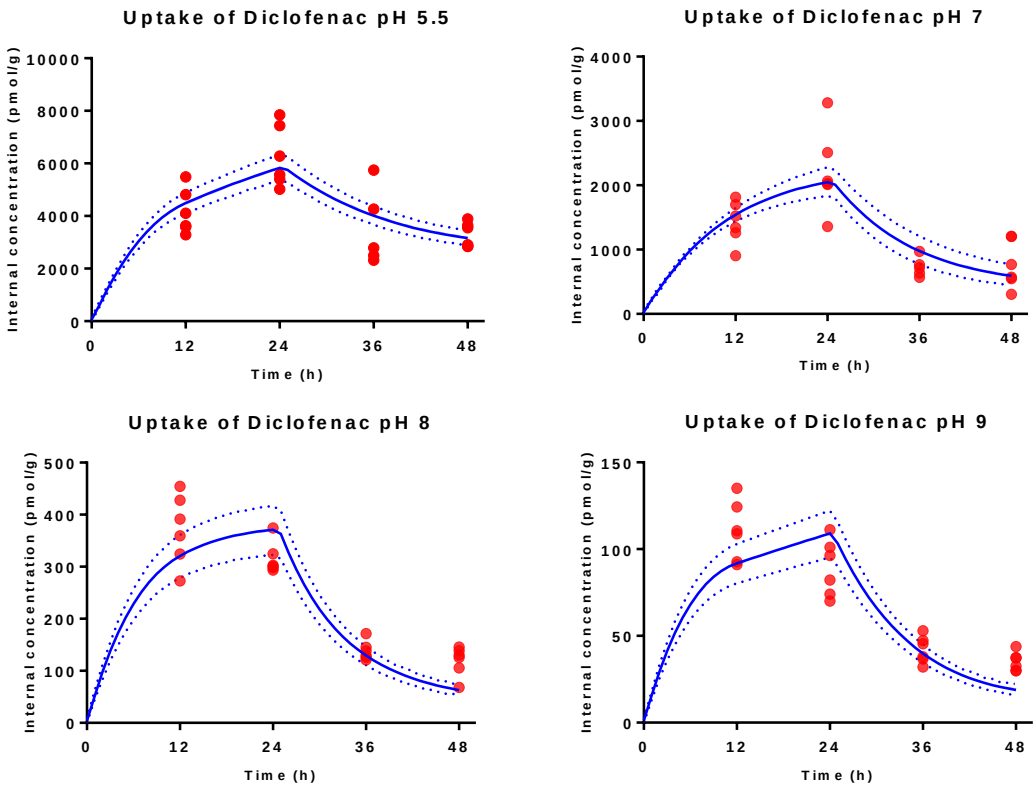


Figure 3. 8. Uptake (0-24-h) and depuration (24-48-h) graphs of diclofenac at multiple pHs in *L. variegatus*. Red dots represent the internal measured concentrations (pmol/g wet weight). The thick line is the model fit of the toxicokinetic model and the dotted lines are 95% prediction intervals of the model. The concentrations are based on measured activity and it is assumed it is the parent compound.

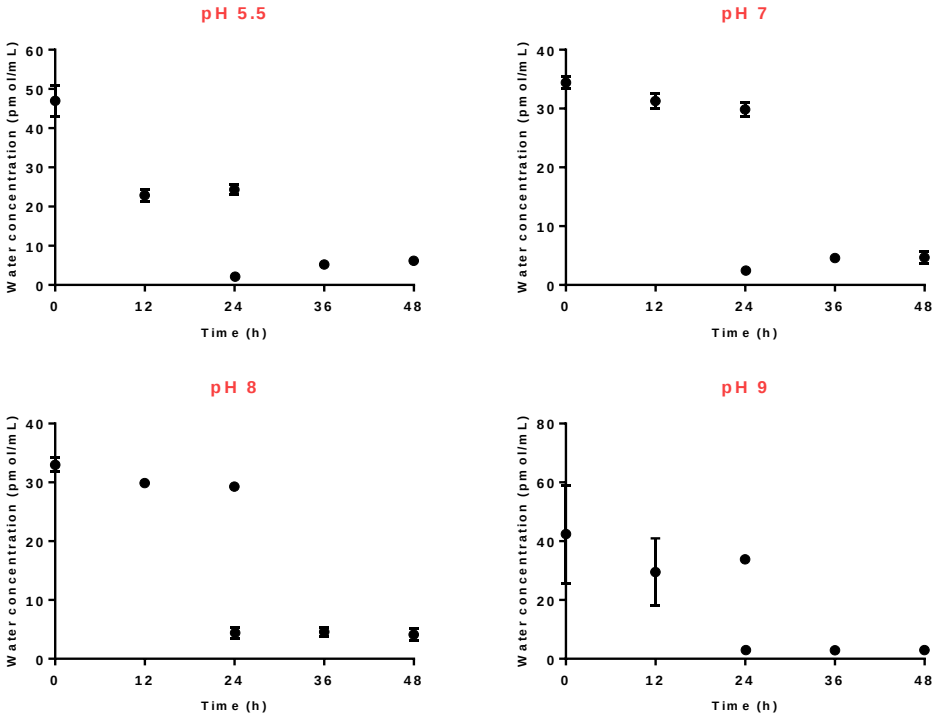


Figure 3. 9. Exposure water concentrations (pmol/mL) and 95% confidence intervals of diclofenac at multiple pHs over 48 hours. The concentrations are based on measured activity and it is assumed it is the parent compound.

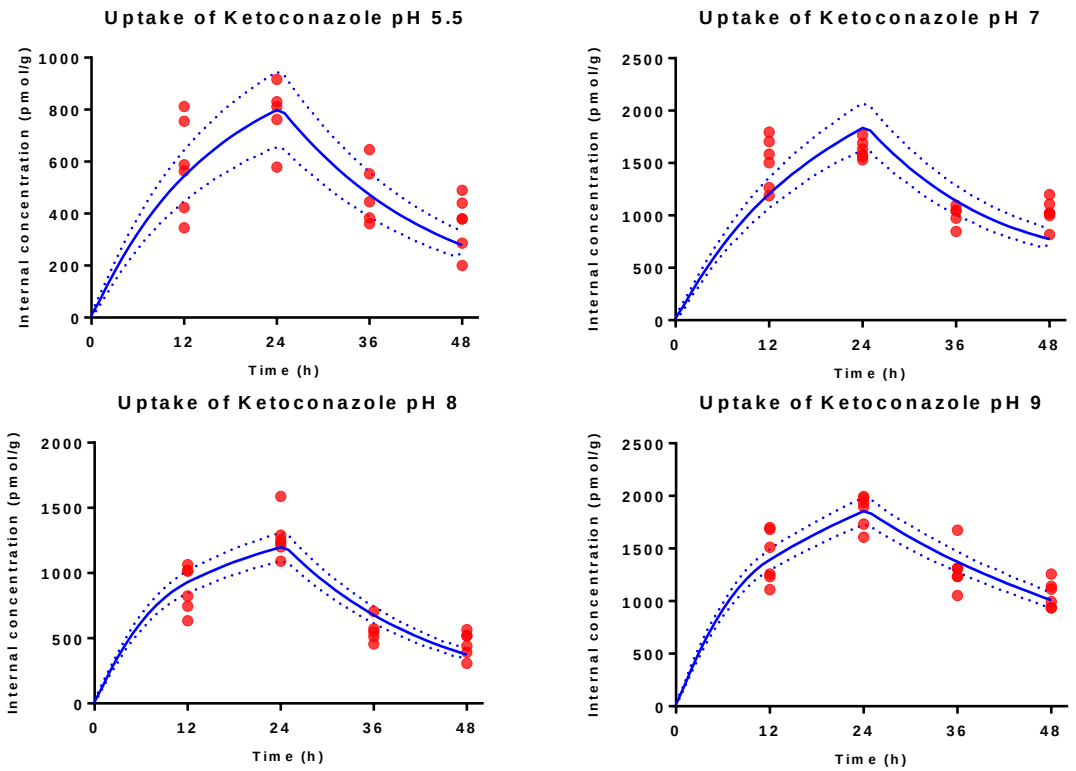


Figure 3. 10. Uptake (0-24-h) and depuration (24-48-h) graphs of ketoconazole at multiple pHs in *L. variegatus*. Red dots represent the internal measured concentrations (pmol/g wet weight). The thick line is the model fit of the toxicokinetic model and the dotted lines are 95% prediction intervals of the model. The concentrations are based on measured activity and it is assumed it is the parent compound.

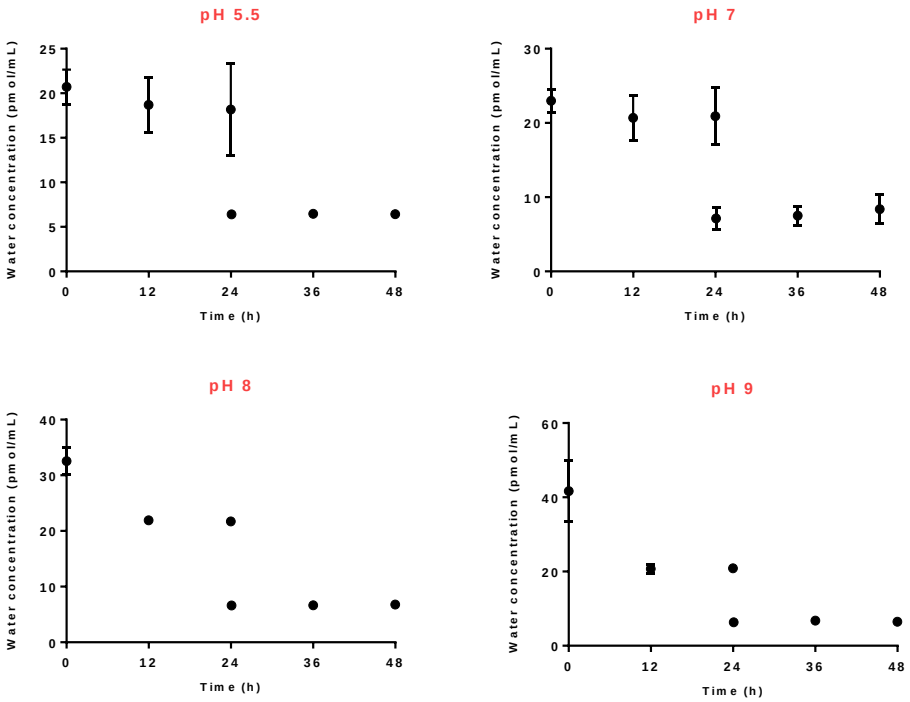


Figure 3. 11. Exposure water concentrations (pmol/mL) and 95% confidence intervals of ketoconazole at multiple pHs over 48 hours. The concentrations are based on measured activity and it is assumed it is the parent compound.

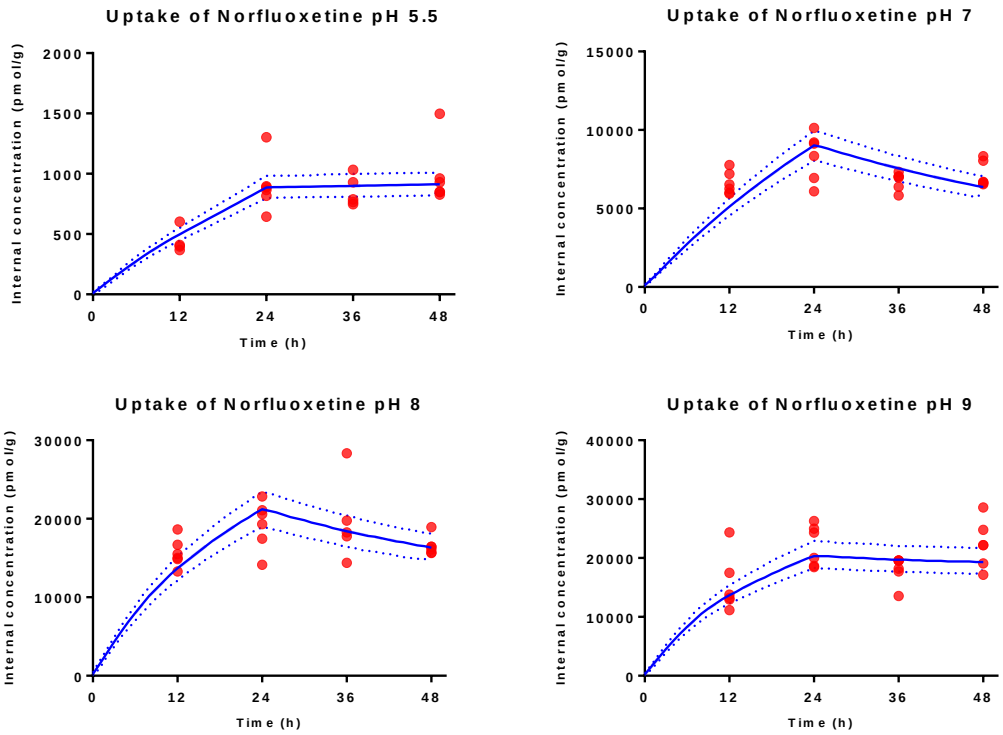


Figure 3. 12**.** Uptake (0-24-h) and depuration (24-48-h) graphs of norfluoxetine at multiple pHs in *L. variegatus*. Red dots represent the internal measured concentrations (pmol/g wet weight). The thick line is the model fit of the toxicokinetic model and the dotted lines are 95% prediction intervals of the model. The concentrations are based on measured activity and it is assumed it is the parent compound.

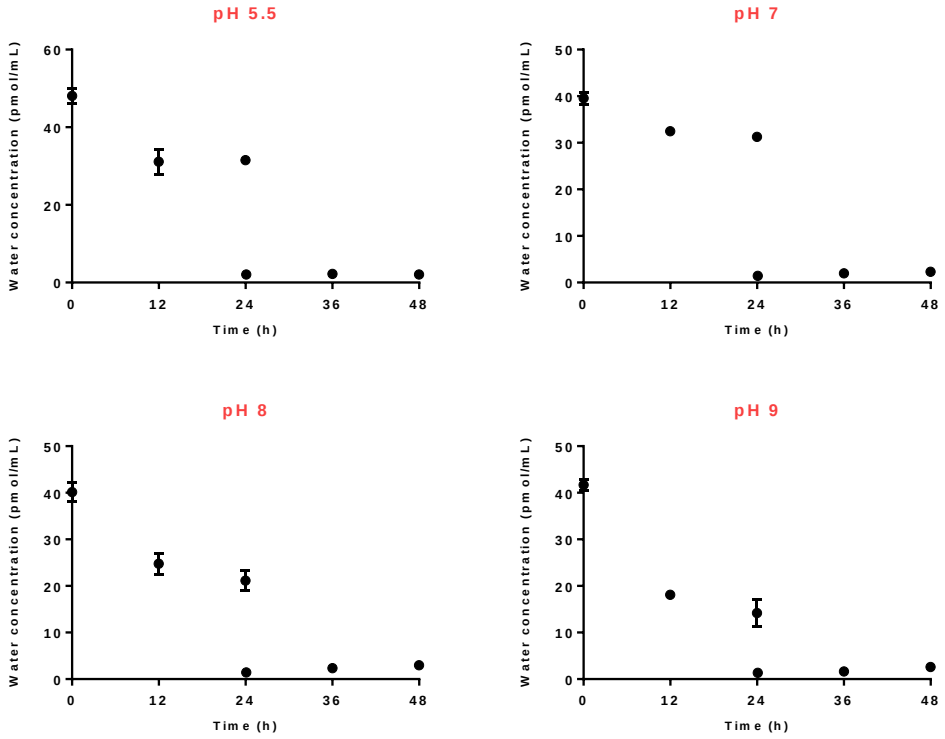


Figure 3. 13. Exposure water concentrations (pmol/mL) and 95% confidence intervals of norfluoxetine at multiple pHs over 48 hours. The concentrations are based on measured activity and it is assumed it is the parent compound.

API	pH	$f_{neutral}$ (%)	K_{in} (mL g^{-1} wet weight h^{-1}), mean + SD	K_{out} (h^{-1}), mean + SD	BCF (mL/g wet weight), mean + LCI, UCI
Amitriptyline	5.5 ± 0.3	0	7.27 ± 0.54	0.0498 ± 0.00544	146 (135-166.7)
Amitriptyline	7 ± 0.3	0.3	19.55 ± 0.84	0.0456 ± 0.0029	391.4 (367.4-432.9)
Amitriptyline	8 ± 0.3	4	20.83 ± 0.78	0.0320 ± 0.0022	653 (623.1-700)
Amitriptyline	9 ± 0.3	30	27.39 ± 1.36	0.0119 ± 0.0020	2284 (2151-2497)
Diclofenac	5.5 ± 0.3	97	14.48 ± 0.94	0.041 ± 0.0041	353.4 (326-399)
Diclofenac	7 ± 0.3	98	6.30 ± 0.56	0.076 ± 0.0089	81.42 (71.0-101.3)
Diclofenac	8 ± 0.3	99	1.647 ± 0.14	0.12 ± 0.0121	13.75 (12.38-16.16)
Diclofenac	9 ± 0.3	100	0.365 ± 0.03	0.096 ± 0.009	3.78 (3.31-4.27)
Ketoconazole	5.5 ± 0.3	15	4.41 ± 0.40	0.0048 ± 0.0065	93.04 (83.4-110.1)
Ketoconazole	7 ± 0.3	76	5.11 ± 0.24	0.0051 ± 0.0034	100.9 (95.2-111.3)
Ketoconazole	8 ± 0.3	97	5.11 ± 0.25	0.0051 ± 0.0035	101.1 (95.7-110.2)

Ketoconazole	9 ± 0.3	99	5.80 ± 0.20	0.0027 ± 0.0018	211.4 (202.6-225.9)
Norfluoxetine	5.5 ± 0.3	0	1.06 ± 0.005	$1 \times 10^{-7} \pm 0.001$	1.06×10^{7} (1×10^{7}-1.19×10^{7})
Norfluoxetine	7 ± 0.3	2	14.46 ± 0.77	0.0017 ± 0.0027	870.4 (817.7-958.9)
Norfluoxetine	8 ± 0.3	14	39.61 ± 2.09	0.0014 ± 0.0023	2902 (2724-3209)
Norfluoxetine	9 ± 0.3	61	43.88 ± 2.29	0.004 ± 0.0018	1.11×10^{4} (1.04×10^{4}-1.25×10^{4})

Table 3. 1. Mean of uptake and depuration constants (k_{in} and k_{out}) with standard deviations and mean values of BCF estimated with lower confidence interval (LCI) and upper confidence interval (UCI) at different pH ranges.

3.3.5 General considerations

The mechanism of uptake and depuration of APIs into sediment-dwelling invertebrates is poorly understood. In our study, we assumed that the main uptake pathway of the APIs was *via* the skin only through passive adsorption and that only the parent compound was taken up. However, inside the body, a compound is metabolized and the degree of metabolization depends on the physicochemical characteristics of each pharmaceutical such as water solubility and the organism chosen to test. In our study, due to the analytical method limitations, possible metabolites were not detected. Accounting for biotransformation products leads to a better estimation of uptake and depuration rates (k_{in} and k_{out}). In addition, metabolites have sometimes been found to have a greater bioaccumulation factor compared to the parent compounds (Ashauer *et al.,* 2012; (Miller *et al.,* 2017) and they could partially explain the difference in uptake of the four APIs into the worms. Therefore, in this study, the LSC measurements should to be interpreted carefully because they can under or over-estimate the total BCF due to the analysis of the parent compound only. More research about the possible metabolic pathways and metabolites resulting from the bioconcentration of APIs in non-target invertebrates is needed. Measuring the metabolites in non-target organisms is important in order to have accurate measurements of BCFs and better understand whether the toxicity is due to the parent compounds or the metabolites.

3.4 Conclusions

This study clearly demonstrated the key role of environmental parameters such as pH in characterizing uptake of ionisable pharmaceuticals at a laboratory scale covering a broad range of pH from 5.5 to 9. Amitriptyline and ketoconazole were found to be increasingly bioaccumulative at high pH, except norfluoxetine for which the highest BCF was measured at pH 5.5 and pH 9. The opposite was observed for diclofenac. Exposing the organisms to each API in water only, produced valuable insights into our understanding of the internal exposure of inveretebrates to APIs and possible potential toxic effects that those chemicals may pose to non-target organisms. Thus, we strongly recommend particular attention to pH when assessing the uptake of ionisable compounds into non-target organisms.

In this study, the role of metabolism was not investigated due to the measurement of the concentrations by the LSC which cannot distinguish between the parent and the metabolic compounds. In this way, a possible under or overestimation of the BCF could occur. Therefore, it is advisable to consider and analyse the biotransformation products for better estimating BCFs.

Finally, it is important to recognize that the present study evaluated uptake and depuration of only four compounds, three weak bases and one weak acid and more work is needed with a wider range of APIs in order to generate more quantitative data for a better evaluation of the risk that ionisable pharmaceuticals may cause to aquatic organisms.

L. variegatus lives mainly submerged in the sediment compartment where it burrows in the top layer. To further investigate the processes that influence the uptake of the ionisable APIs into sediment-dwelling invertebrates, a better understanding of the fate and mobility of these APIs in the sediment compartment is necessary. Thus, in the next chapter, experiments to understand the sorption behaviour of these APIs in different sediments will be presented.

Chapter 4. Sorption of ionisable active pharmaceuticals in four different types of sediments

4.1 Introduction

In the previous Chapter, the importance of pH in assessing the uptake of ionisable APIs into sediment-dwelling invertebrates was described. However, to fully understand the different pathways of uptake of ionisable APIs, the fate and the sorption behavior of these compounds have to be assessed. Therefore, in this Chapter, the degree of sorption and the mechanisms that influence the sorption of APIs onto sediment particles will be presented.

Knowledge of the sorption behvaiour of an API is essential in understanding the persistence of sediment-associated chemicals because it gives an indication of the mobility and the bioavailability of these chemicals between different compartments, for example, the water-sediment compartment. In addition, the bioavailable fraction of chemicals is important in determining the uptake and toxicity to non-target organisms. Once a chemical ends up in the sediment compartment, it could bind to the sediment particles being less available in the water column, but, still elicits negative effects to the sediment-dwelling organisms that are exposed *via* sediment particles (for example through ingestion of the particles) and the pore water (=interstitial water between the sediment particles) (Maund *et al.,* 2002). In the surface water, pharmaceuticals may undergo several natural processes such as biodegradation, volatilization, hydrolysis, photo-degradation and sorption onto the solid matrix (Martínez-Hernández *et al.,* 2014a; Baena-Nogueras *et al.,* 2017; Koumaki *et al.,* 2017b). For instance, the fate of four anti-inflammatories including diclofenac was investigated in the water-sediment system and it was found that biotic processes such as biodegradation were the main processes of removal of the compound from the water-sediment phases. Baena-Nogueras *et al.,* (2017) studied the disappearance of many PPCPs by hydrolysis, changes in pH, photodegradation and biodegradation and found that while some compounds

were susceptible to these processes, some chemicals (carbamazepine and amitriptyline) were more persistent.

For new chemicals that have to be authorized, sediment toxicity data are necessary. When experimental data are lacking, the Equilibrium Partition (EqP) proposed by Di Toro *et al.,* (1991) is used by regulators as the first screening for sediment quality criteria. It provides useful data in assessing the contamination of sediment-associated chemicals. For neutral chemicals, the EqP states that in an equilibrated sediment system, the chemical sorbed to the sediment organic carbon is in equilibrium with the pore water, thus, the exposure of this chemical toward benthic organisms is the same regardless of the exposure pathway and can be predicted based on the K_{oc} (= organic carbon-normalized sorption coefficient) of the compound (Fig. 4.1). The EqP also demonstrated that water-only exposure is equal to the sediment-pore water exposure by assuming that the chemical activity in each system is at the equilibrium.

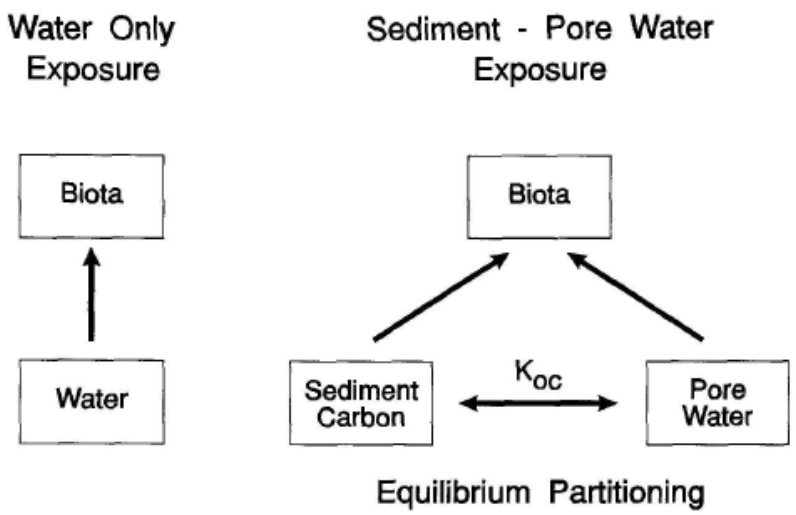


Figure 4. 1. Equilibrium partitioning illustrated by di Toro *et al.,* (1991)

Typically, the sorption behavior of chemicals is described by several sorption coefficients such as (K_d) and the Freundlich coefficient (K_f). They describe the distribution of a chemical between the water and the solid phase. These sorption coefficients differ between different soils/sediments depending on the chemical and the sorbent; and, for non-polar chemicals, it has been demonstrated that the main mechanism of sorption is through hydrophobic interactions with the organic content of the sediment (Karickhoff *et al.,* 1979; Chiou *et al.,* 1979; Karickhoff, 1981). Therefore, K_{oc} is often estimated with

linear regression analysis by using chemical descriptors, such as the $LogK_{ow}$, that reflects the hydrophobicity of the molecule (Sabljić *et al.,* 1995; Doucette, 2003).

Unlike neutral chemicals for which the hydrophobic interactions with the organic matter are the main mechanism of sorption (Delle Site, 2001); for ionisable chemicals, the differences in the sorption are more likely due to other mechanisms and the interactions between the pharmaceuticals' physicochemical properties ($LogK_{ow}$, pK_a) and the properties of the sediments (pH, cation exchange capacity, texture) (Kodešová *et al.,* 2015).

Research exploring the sorption of ionisable pharmaceuticals in the sediment compartment has recently increased (Stein *et al.,* 2008; Martínez-Hernández *et al.,* 2014; Styszko, 2016; Al-Khazrajy and Boxall, 2016; Kiecak *et al.,* 2019).

For example, Styszko, (2016) investigated the sorption of several pharmaceuticals both acids and bases in three different sediments and found that the main mechanisms of interactions between the APIs and the sediments were the hydrophobic and electrostatic interactions. Another study by (Al-Khazrajy and Boxall, (2016) assessed the sorption of five ionisable pharmaceuticals (1 acid and 4 bases) in ten types of sediments. The authors explored the possible sorption mechanisms involved for each compound and the main predictors that were likely to play a key role in determining the sorption. Both physicochemical and environmental predictors were identified such as $LogD_{ow}$, clay content, CEC and the organic content of the sediments. Their results also found that the main mechanisms of sorption for the four basic APIs were hydrophobic, electrochemical and hydrogen bonding interactions. The cation exchange was observed as the possible mechanism of sorption for the acidic compound.

The information reported in the abovementioned studies showed the diverse and complex processes involved in the sorption of ionisable pharmaceuticals for which the hydrophobic interactions are not the only mechanism of sorption as for neutrals. While the study of Al-Khazrajy and Boxall, (2016) is one of the latest in explaining some of these interactions, much work is needed to better understand the mobility and persistence of these APIs in the sediments.

Some studies have proposed predictive models for estimating the K_{oc} of neutral organic chemicals (Burkhard, 2000; Kipka and di Toro, 2011); however, few models have been

proposed for ionisable chemicals. For example, Franco and Trapp, (2008) proposed a model for estimating the K_{oc} in the soil for both bases and acids separately. Specifically, the model accounts for the $LogK_{ow}$ and the pK_a of the chemicals which are essential parameters to predict sorption. However, our knowledge about the sorption processes that drive the partitioning of ionisable chemicals between water and sediment is still very limited.

Therefore, the aim of this study was to explore the sorption behavior of two bases (amitriptyline and norfluoxetine) and one acid (diclofenac) in four different types of sediment and evaluate the influence of sediment properties on pharmaceutical sorption. Ketoconazole was not included in this study due to the dissipation of the API in the aqueous phase during the shaking.

With this study, we aim to bring new information about the partitioning behavior of ionisable pharmaceuticals in sediments and consequently the mobility and the available fraction of these chemicals that could be taken up by sediment-dwelling organisms. In addition, a separate test of biodegradation for diclofenac in the sediments was conducted to establish whether the dissipation of the API was due to the sorption and not to the activity of the microorganisms. Ultimately, an evaluation of predictive sorption models, proposed in the literature for ionisable compounds, was carried out using the resulting data.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Diclofenac (CAS 15307-86-5) was purchased from American Radiolabelled Chemicals, Inc. (UK); amitriptyline (CAS 64-17-5) was obtained from Merck & Co (New Jersey, USA) and norfluoxetine (CAS 57226-68-3) was obtained from Sanofi (Paris, France). Test chemicals were all 14-C labelled and they were selected to represent different physicochemical properties (see Table 1.2 in Chapter 1). Stock solutions were prepared in ethanol and stored at -20˚C. Calcium chloride was obtained from Sigma Aldrich (UK). Centrifuge PTFE tubes were purchased from Oak Ridge by Nalgene Nunc International.

4.2.2 Sediments sampling and characterization

Four sediments were collected in different rivers of England: Millington Beck (Millington, 53.967063,-0.718046), River Dove (Barnsley, 53.527113,-1.445819), Farndale Brook (Yorkshire Moors, 54.370489,-0.968043) and River Foss (Earswick, 54.007509,-1.060050). The sediments were sampled and sieved through a 2 mm sieve and stored at 4 ^{o}C. The sediments were then dried at 25 ± 1 °C. The chosen locations were selected based on previous information about the sediment properties from other studies and the accessibility to the site. The characterization of the sediments was carried out by the Forest Research (https://www.forestresearch.gov.uk/, Surrey, UK) for the following properties: pH (ISO 10390), Total Organic Carbon (TOC) (ISO 10694), Cation Exchange Capacity (CEC) (ISO 11260) (1994) and texture (Table 4.1).

4.2.3 Sorption studies

Sorption studies were carried out following the OECD guideline 106 "Adsorption-Desorption using a batch Equilibrium method" (OECD, 2000). The study consisted of an intial preliminary study in order to determine:

- the best solution/sediment ratio;
- the equilibration time and the amount of each API absorbed to the sediment at equilibrium;
- the stability of the chemical and potential absorption to the test vessels.

The main study was then performed to generate the sorption isotherms and to obtain the sorption coefficient of the tested APIs in the four sediments.

In the preliminary experiments, different sediment/solution ratios were tested for each API and sediment. Air-dried sediment (0.2 g, 0.4 g, 0.5 g and 1 g) was placed into test tubes and mixed with 3 mL, 5 mL and 40 mL of 0.01M of $CaCl_2$ overnight before spiking the pharmaceutical. Solution/ratios of 1:1, 1:5, 1:40 1:80, 1:100 and 1:200 were chosen. After 24 hours of shaking, chemicals were spiked at a concentration of 10 µg/L and the tubes were placed on the shaker in the control chamber at 20 ^{o}C ± 2 for 24 hours, 48 hours and 72 hours to assess the optimum time that is required for the API to reach an equilibrium between the sediment and the solution. Norfluoxetine studies were done

at 4˚C using glass jars. The use of the glass jars was necessary due to the absorption of the API of over 30 % to the surface of the PTFE tubes during the experiments to observe the stability of the compound. Duplicate tubes per each sediment, time and solution/ratio were chosen. Controls containing 0.01M of $CaCl_2$ and the test chemical were added to check the stability of the compounds and possible adsorption to the test vessels. To avoid photolysis, the entire test was run in the dark and the test tubes kept in a sealed container. The velocity of the shaker was set at 250 rpm.

At sampling, 1.5 mL of the supernatant was collected and pipetted into a microcentrifuge tube (VWR) and centrifuge for 5 minutes at 3500 rpm using a Micro Star 12 microcentrifuge (VWR). Then, 1 mL of the supernatant was collected for analysis and mixed with 10 mL of Ecoscint A (NationalDiagnostics). The measurements were conducted with a Liquid Scintillation Counter (LSC) from HIDEX 300SL. Samples were analyzed three times for 5 minutes and corrected for background activity using blank controls. The counting efficiency and color quenching were corrected using the external standard ratio method.

4.2.4 Main study

The main study was performed in order to determine the sorption coefficient of each API in the four sediments. The same conditions described above were used and are summarized in Table 4.2. Five different concentrations were chosen in order to generate the sorption isotherms and triplicates test tubes were used along with controls and one blank per sediment. The measurements of the samples were carried out using the same methodology as described above. Sorption isotherms were fitted to derive K_d using either the linear isotherm method and K_f (Freundlich coefficient) using the Freundlich isotherm methods.

The mass of the API absorbed to the sediment was determined by calculating the difference in concentration between the initial (C_i) and the concentration in solution at sampling time (C_{aq}) (Equation 1)

$$C_s = (C_i - C_{aq}) \times V / m_s \qquad \text{Equation 1}$$

V is the volume in the test tubes (L), C_i is the initial concentration of the compound; C_{aq} is the concentration of the API in the aqueous solution at the end of the sorption test and m_s is the mass of the sediment (Kg).

Then, the sorption isotherms were derived by using the linear and the Freundlich coefficients, respectively K_d and K_f.

In addition, the organic carbon-normalized sorption coefficient (K_{OC}) was calculated by using the K_d value as shown in the following Equation 2:

$$K_{oc} = K_d / f_{OC} \times 100 \qquad \text{Equation 2}$$

Where f_{OC} is the fraction of the organic content (%).

4.2.5 Biodegradation experiments

Diclofenac is known to be biodegradable (Koumaki *et al.,* 2017b), thus, in order to check whether its dissipation was due to sorption and not to the biodegradation, an additional equilibrium time experiment of diclofenac was carried out using sterilized sediments. Triplicates of sediments were autoclaved at 121 °C for 45 minutes (Dodgen *et al.,* 2014). During the sterilization process, some main characteristics of the sediments such as pH and Total carbon (TC) may have been changed. Therefore, triplicates of sterilized sediments were analyzed to determine their total carbon and pH and the results compared to the unsterilised air-dried sediments. The pH was assessed following the ISO 10390:2005, using a 1: 5 v/v of sediment and 0.01M of $CaCl_2$. The determination of the TC was carried out by the C/N analyzer (Vario Macro Elementar). Statistical analysis using a Kruskal-Wallis test was performed to compare the samples.

4.2.6 Evaluation of predictive models

The model developed by Franco & Trapp, (2008) was evaluated. The model predicts the $LogK_{oc}$ of chemicals and we calculated the K_{oc} for each API and sediment for acids (Equation 3) and bases (Equation 4) separately.

$$LogK_{oC} = Log[\varphi_n \times (10^{0.54 \times logKow + 1.11})] + [\varphi_{ion} \times (10^{0.11 \times logKow + 1.54})] \qquad \text{Equation 3}$$

$$LogK_{oC} = Log[\varphi_n \times (10^{0.37 \times logKow + 1.70})] + [\varphi_{ion} \times (10^{pka\ 0.65 \times logKow + f0.14})] \qquad \text{Equation 4}$$

Where: $LogK_{ow}$ is the octanol-water partition coefficient; pK_a is the dissociation constant; ƒ is the diffusion limiting factor and it is calculated as $K_{ow} / (K_{ow} + 1)$; φ_n and φ_{ion} are the fraction of the neutral and ionised part of the chemical and they were calculated using Equation 5 and 6 below:

$$\varphi n = 1/\ 1 + 10^{a\ (pH\ -pKa)} \qquad \text{Equation 5}$$

$$\varphi ion = 1 - \varphi n \qquad \text{Equation 6}$$

Properties	*Millington*	*Barnsley*	*Moors*	*Earswick*
Silt (%)	54	24	12	4
Sand (%)	23	58	78	91
Clay (%)	23	18	10	5
pH (H_2O)	7.31	7.52	6.43	7.78
TOC (%)	6.576	14.260	0.787	0.782
CEC (cmol+/Kg)	28.772	26.257	5.312	6.221
Tot Al^{3+} (mg/Kg)	1	1	2	0
Tot Fe^{2+} (mg/Kg)	1	0	0	0
Tot Ca^{2+} (mg/Kg)	5482	3064	728	1083
Tot K^{+} (mg/Kg)	131	115	47	50
Tot Mg^{2+} (mg/Kg)	95	223	146	63
Tot Na^{+} (mg/Kg)	38	1662	18	19
Tot Mn^{2+} (mg/Kg)	31	440	71	24

Table 4. 1. Properties of the four sediments used in the sorption studies.

Compound	*Experiment concentrations (ug/L)*	*Equilibrium time (h)*	*Temperature (˚C)*	*Millington solution/ratio (w/vol)*	*Earswick solution/ratio (w/vol)*	*Moors solution/ratio (w/vol)*	*Barnsley solution/ratio (w/vol)*	*Test vessels*
Amitriptyline	2, 5, 8, 11, 15	24 and 48 for Moors	20	1:200	1:40	1:100	1:200	centrifuge PTFE tubes
Norfluoxetine	2, 5, 8, 11, 15	24	4	1:200	1:40	1:80	1:200	glass jars
Diclofenac	2, 5, 8, 11, 15	24	20	1:5	1:1	1:5	1:5	centrifuge PTFE tubes

Table 4. 2. Summary of the laboratory conditions for the sorption study (OECD 106) of the three pharmaceuticals.

4.3 Results and discussion

4.3.1 Preliminary studies

In accordance with the OECD guideline 106, preliminary equilibrium time experiments were carried out for 48-h for diclofenac and 72-h for amitriptyline and norfluoxetine (Fig. 4.2). The three compounds reached the equilibrium between the sediment and the water within 24-h from the beginning of the test with the exception of amitriptyline in the Moors sediment. In this latter case, the API reached equilibrium within 48-h (Table 4.2).

In addition, a slight decrease in the sorption was noticed for diclofenac in the Barnsley sediment after 24-h. A possible explanation for the decline could be due to biodegradation. The sorption studies were carried out with air-dried sediments in order to maintain the sediment' properties. Hence, the decreased sorption of diclofenac during the test was probably due to the activity of the microorganisms present in the sediment. Different studies have demonstrated the dissipation of diclofenac *via* biodegradation by the microbial community of the water/sediment systems (Kunkel and Radke, 2008; Koumaki *et al.,* 2017). Therefore, an additional equilibrium test using autoclaved sediments was carried out to evaluate whether the presence of the microorganisms could be the factor responsible for the decrease in the sorption of the compound (Fig. 4.3). For this purpose, the sediments were autoclaved at 121 °C for 45 minutes and the main sediments' properties such as pH and Total Carbon were assessed by comparing the results with air-dried sediments (Figures C.1 and C.2). As can be seen, pH and Total Carbon showed no significant difference between the sediments treated in the autoclave and the sediments dried at 25 °C ($0.1 < p\text{-}value < 0.8$). By evaluating the sorption capacity of diclofenac in the autoclaved sediments (Fig. 4.3) and comparing the results with the air-dried sediments (Fig. 4.2), diclofenac was concluded to be stable and therefore we concluded that the activity of the microorganisms was negligible.

The optimum sediment/solution ratios are shown in Table 4.2. The ratios were chosen based on the criteria set up by the OECD 106 for which the sorption of the test substance should be preferably above 50 % and the concentration of the test substance in the water phase should be detectable by the analytical method.

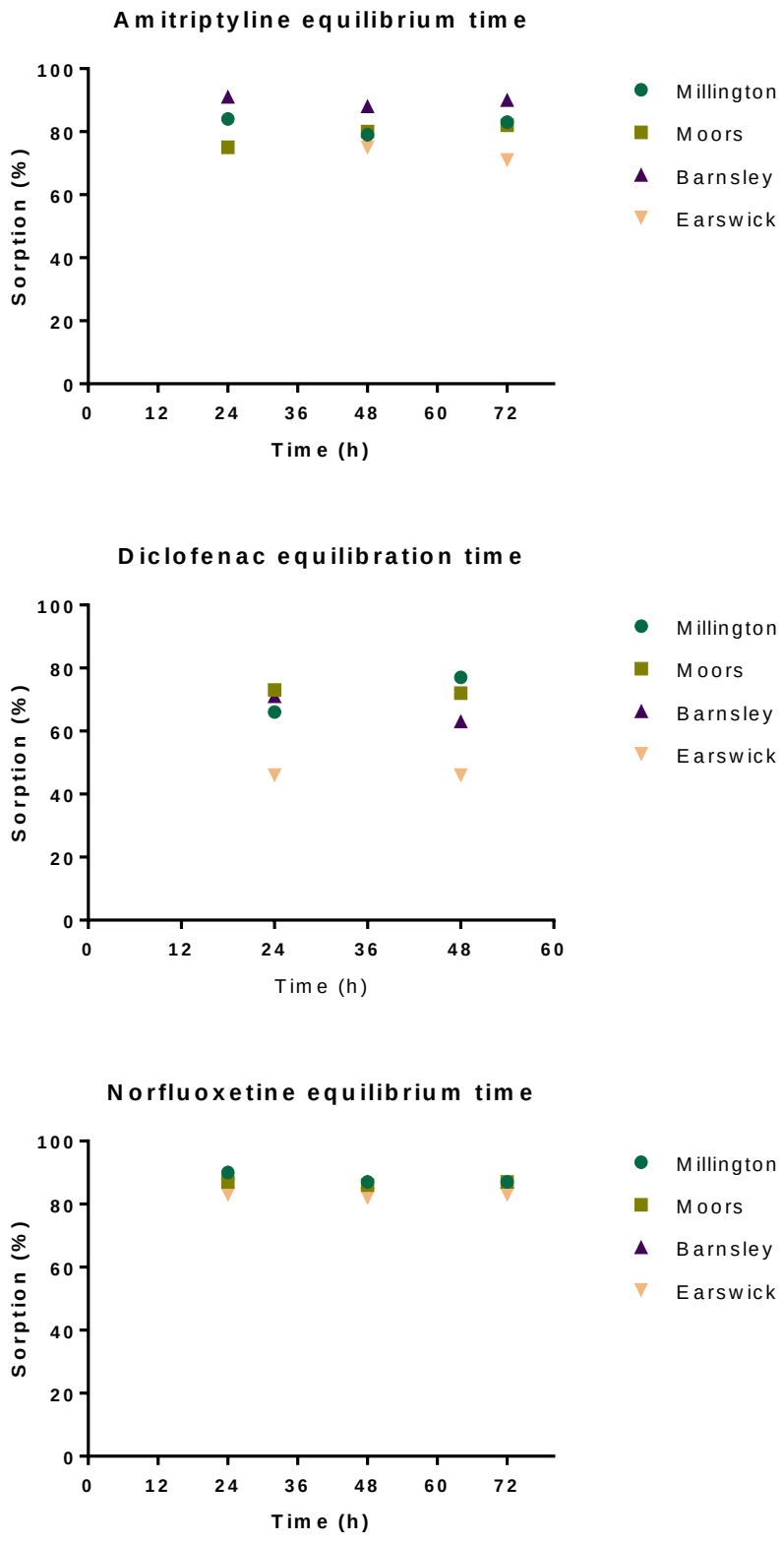


Figure 4. 2. Sorption behaviour of the three APIs using air-dried sediments over 48-h for diclofenac and 72-h for amitriptyline and norfluoxetine.

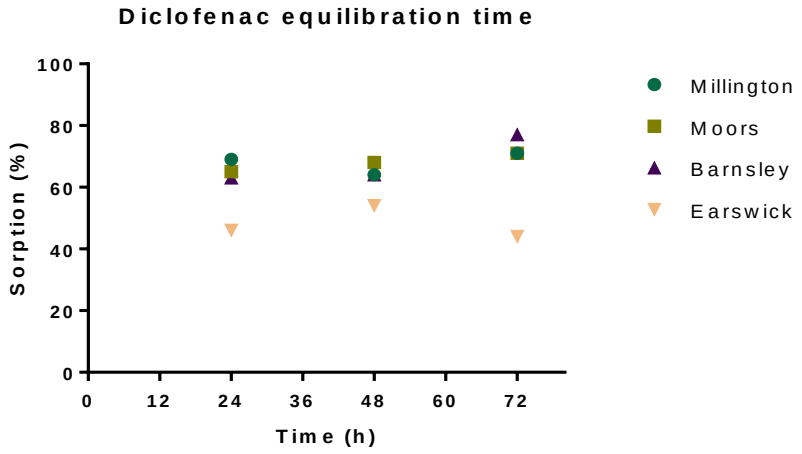


Figure 4. 3. Sorption behaviour of diclofenac using autoclaved sediments over 72-h.

4.3.2 Main studies

Along with the sediment beakers, the stability of each API was monitored throughout the duration of the test by measuring the controls. For all the APIs, the final concentration was measured and compared with the initial spiked concentrations. For diclofenac more than 95 % (μ = 96.4, σ = 2.5) of the remaining API was detected in the water; for amitriptyline more than 90 % (μ = 92.5, σ = 4.7) and for norfluoxetine more 95 % (μ = 96, σ = 4) was detected in the water phase after 24 h suggesting low degradation or absorption of the pharmaceuticals on the surface of the test tubes.

The K_f and the K_{oc} coefficients were obtained by plotting the Linear and the Freundlich isotherms (Figure 4.4 and 4.5). The Linear (R^2 = 0.678-0.995) and the Freundlich models (R^2 = 0.542-0.998) all adequately described the sorption of the three APIs at the 5 different concentrations (Table 4.3). The lowest R^2 calculated was for diclofenac in the Earswick sediment, 0.678 and 0.542 respectively for the two isotherms. Also, the 1/n parameter was reported (Table 4.3). This parameter shows the linearity of the model when the slope (n) is > 1; however, the n ranged between 0.462 and 0.826 demonstrating the non-linearity of the sorption isotherm with the data.

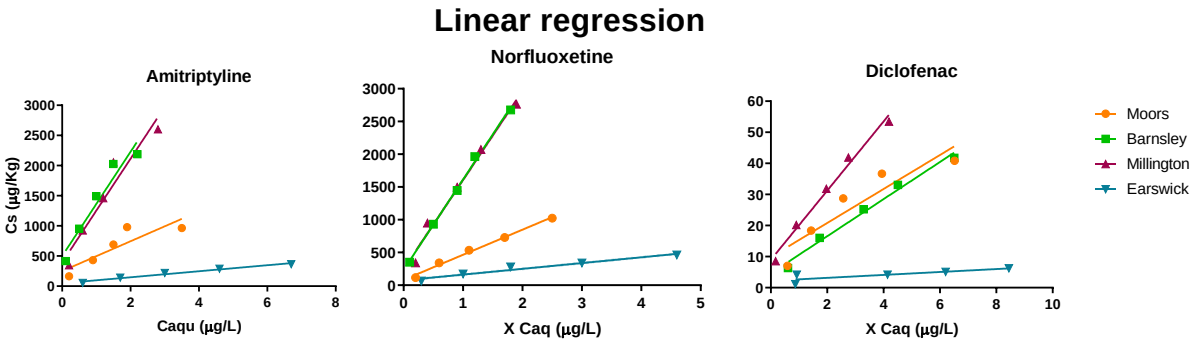


Figure 4. 4. Linear isotherms of the selected APIs in the sediments. Initial concentration ranged between 2 to 15 µg/L. Points represent the mean of three replicates.

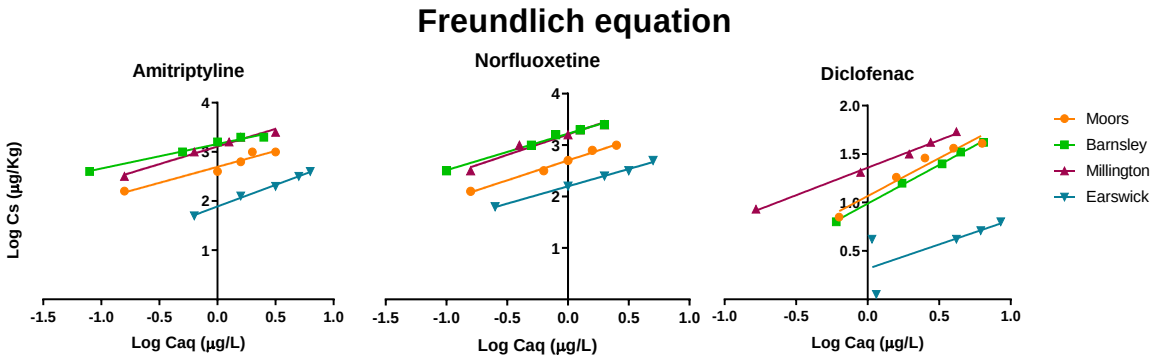


Figure 4. 5. Freundlich isotherms of the selected APIs in the four sediments. Initial concentration ranged between 2 to 15 µg/L. Points represent the mean of three replicates.

The K_f and K_{oc} of diclofenac, amitriptyline, and norfluoxetine are shown in Figure 4.6.

The sorption coefficient (K_f) for Moors sediment increased in the following order diclofenac < norfluoxetine < amitriptyline; for Millington, Barnsley and Earswick in the order of diclofenac < amitriptyline < norfluoxetine. The K_f value ranged from 2.07 (L/Kg) for diclofenac to 1681.12 (L/Kg) for norfluoxetine.

Regarding the K_{OC}, it increased in the order diclofenac < amitriptyline < norfluoxetine in the four sediments and it ranged from 41.8 (L/Kg) for diclofenac to 48131 (L/Kg) for norfluoxetine. A table that summarizes all the values is provided in Table 4.3. The pH of the sorption experiments was recorded (Fig. C.3) and this was found to be slightly acidic for Moors (5.52-6.16) while slightly alkaline for the rest of the sediments (6.87-7.62).

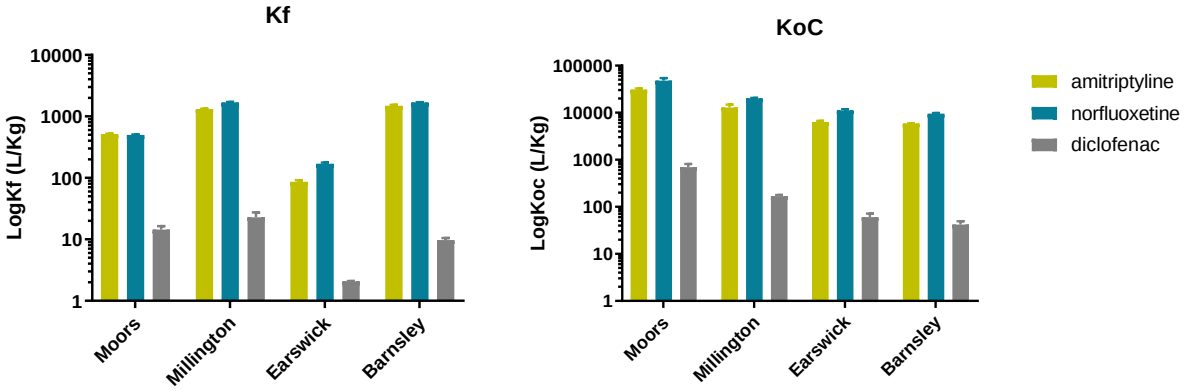


Figure 4. 6. Logarithmic K_f and K_{oc} values of the three APIs in the four types of sediments. The bars represent the mean of three replicates and the error bars represent the standard deviations.

Sediment	Compound	Linear			Freundlich		
		K_d L kg^{-1} (±SD)	Koc (±SD)	R^2	K_F µg $^{1-1/n}$ (cm^3) $^{1/n}$ g^{-1}	1/n	R^2
Moors	**Amitriptyline**	243.5(±12.9)	30946.6(±1668.8)	0.756	510.4	0.641	0.952
	Diclofenac	5.46(±0.9)	694.7(±118.6)	0.99	14.4	0.751	0.944
	Norfluoxetine	378.7(±48.2)	48131(±6124.9)	0.994	498.7	0.774	0.998
Millington	**Amitriptyline**	848.3(±131.2)	12901(±1996)	0.934	1315.2	0.741	0.989
	Diclofenac	11.1(±0.6)	169.2(±10.0)	0.985	22.7	0.569	0.995
	Norfluoxetine	1318.5(±5)	20050(±489)	0.983	1673.01	0.826	0.978
Barnsley	**Amitriptyline**	833.7(±9.4)	5846.5(±66.4)	0.936	1489.4	0.529	0.994
	Diclofenac	5.96(±1.05)	41.8(±7.4)	0.985	9.75	0.798	0.997
	Norfluoxetine	1348.3(±24.3)	9455.1(±376.9)	0.995	1681.1	0.703	0.996
Earswick	**Amitriptyline**	49.7(±2.5)	6365(±328.1)	0.972	86.4	0.7934	0.992
	Diclofenac	0.47(±0.09)	60.3(±11.3)	0.678	2.07	0.462	0.542
	Norfluoxetine	87.5(±5.3)	11191(±688.9)	0.962	169.2	0.682	0.993

Table 4. 3. Comparisons of the Kd and Koc measured for pharmaceuticals in the different sediments.

Comparing the results with the literature, the K_d and K_f values of amitriptyline in our study are approximately in the same range of the values reported by Al-Khazrajy & Boxall, (2016a) which were 8.78- 247.9 and 39.81-950.9 (L/Kg) respectively.

For norfluoxetine, sorption studies were only found for fluoxetine which is the parent compound. For example, Kwon and Armbrust, (2008) analysed the sorption behaviour of five selective serotonin reuptake inhibitors (SSRIs) including fluoxetine in two sediments. The authors reported high K_d and K_f values ranging from 785-1304 and 229-419 (L/Kg), close to those reported in our study (K_d 87.51-1348; K_f 169-1681, L/Kg).

On the other hand, sorption coefficients of diclofenac were much lower compared to the two bases amitriptyline and norfluoxetine. Even though diclofenac has a predicted $LogK_{ow}$ of 4.51, from previously reported studies where the sorption behavior of diclofenac has been characterized in sandy sediments, K_f values were reported to be low, ranging from 0.81 to 7.81 (L/Kg) (Scheytt *et al.,* 2005). Similar findings have been reported by Le Guet *et al.,* (2018) where low sorption coefficients (K_d and K_f) have been measured in the sediment: 9.9 and 7.5 (L/Kg) respectively.

The differences in pharmaceuticals sorption behavior are likely due to the different sediments properties and their interaction with the physicochemical properties of the pharmaceuticals.

For example, the highest K_f value for amitriptyline was observed for Millington and Barnsley sediments, 1315.2 and 1489.4 (L/Kg) respectively. In contrast, lower K_f values for Moors (510.4, L/Kg) and Earswick (86.34, L/Kg) were seen for these compounds. Differences in the sorption behavior of amitriptyline in Millington and Barnsley sediments compared to Moors and Earswick are likely due to the high organic content of the first two sediments. Al-Khazrajy and Boxall, (2016) previously suggested that hydrophobic interaction with the organic matter is the main mechanism of sorption for amitriptyline. In addition, they measured a sorption coefficient of 186.21 (L/Kg) for the Millington site while in this study, a higher sorption coefficient was measured. Differences in the measurements may be due to the different sampling season of the sediments which could potentially slightly change the environmental properties of the sampled sediment. In addition, a second mechanism of sorption was suggested between the cationic form of the API and the sediment through cation exchange interactions (Al-Khazrajy and Boxall; 2016; Bagnis *et al.,* 2018).

The sorption behavior of norfluoxetine is similar to amitriptyline with Millington and Barnsley sediments having the greatest K_f values as well as K_{oc}. Both APIs are bases possessing similar $LogK_{ow}$ values (4.92 for amitriptyline and 4.16 for norfluoxetine) suggesting a potential strong hydrophobic interaction with the organic content present in the sediments.

For norfluoxetine, in the Earswick sediment, the influence of pH might partially explain the low sorption behavior of the two bases. For example, a sorption study of three basic antimicrobial agents on two types of soils found low sorption coefficients at high pH (ter Laak *et al.,* 2006). Here, low K_f values in Earswick sediments were observed (86.34 L/Kg for amitriptyline and 169.28 L/Kg for norfluoxetine) where the pH measured of the sediment was alkaline (7.78). The influence of pH in determining the sorption behavior of fluoxetine was explained also by Kwon and Armbrust, (2008). The authors found a strong correlation between the pH and the sorption of five SSRIs, including fluoxetine, in two sediments and three soils. They suggested that a possible ionic binding between the positive API and the sediment could explain the mechanism of sorption.
In the Moors sediment, the K_f values of the two basic compounds was almost equal (e.g. 510.4 L/Kg for amitriptyline and 498.74 L/Kg for norfluoxetine). At the experimental pH (6.09-7.69), both the APIs were 100% ionic due to their pK_a values; which means that they were present in their cationic form and a possible interaction between the positive functional group and the sediment may have occurred. In fact, previous studies highlighted the contribution of the amine groups on the possible mechanism of sorption of the positively charged compounds (Martínez-Hernández *et al.,* 2014b; Bagnis *et al.,* 2018).

Significant variation in sorption on the four sediments was observed also for diclofenac. The K_f coefficient increased in the following order: Earswick < Barnsley < Moors < Millington. The reason may be due to the presence of the anionic form of the API in the sediment-water solutions during the batch sorption experiments. In fact, diclofenac has a pK_a of 3.9 and the majority of the compound is negatively charged in the pH range of the experiments (6.09-7.69). Thus, a possible electrostatic repulsion by the anions

present on the sediments surface may partially explain the low sorption behavior of this compound (Scheytt *et al.,* 2005; Krascsenits and Hiller, 2008).

4.3.3 Evaluation of predictive models

The evaluation of the Franco and Trapp, (2008) model for estimating the K_{oc} resulted in different prediction scenarios (Fig. 4.7). For amitriptyline a slight under-prediction for the Millington sediment was observed as well as the Moors sediment with the measured data being two times higher than the predicted values. In the Earswick sediment, a slight over-prediction is seen but it is worth noting that for the Barnsley sediment almost equal results between the predictions and the observed data were found. Regarding norfluoxetine, the model under-predicted the measured data for the Moors and Millington sediments, whereas, good predictions for the Eraswick and Barnsley sediments were observed. On the contrary, for diclofenac generally K_{oc} predictions higher than the measured values were observed.

The same model has been evaluated by Al-Khazrajy and Boxall, (2016) and the authors found that the model generally overestimated the sorption of the bases and under-estimated the sorption of the acids. The scenario of our study showed the opposite results. The model was able to predict quite well the bases but not the acidic diclofenac. The estimation of the K_{oc} for acids included in the model the $LogK_{ow}$ as the sole descriptor. This descriptor is important for neutral compounds for which hydrophobic partitioning with the organic matter has been recognized but it may not be appropriate for ionisable acids for which the best predictor for estimating sorption was found to be the LogD (=pH-corrected octanol-water partition coefficient) (Kah and Brown, 2007).
On the other hand, the model worked quite well for the bases and especially for amitriptyline. The slight under-prediction observed in our study for norfluoxetine was observed also by Karlsson *et al.,* (2013). In order to predict the sorption of basic compounds, the model accounts for the $logK_{ow}$ and the pK_a of the chemical. These two parameters as the only predictors for estimating the sorption of basic compounds may not be enough; in fact, it is well known that additional sorption processes such as cation exchange are an important mechanism of interaction that should be accounted (Schaffer *et al.,* 2012).

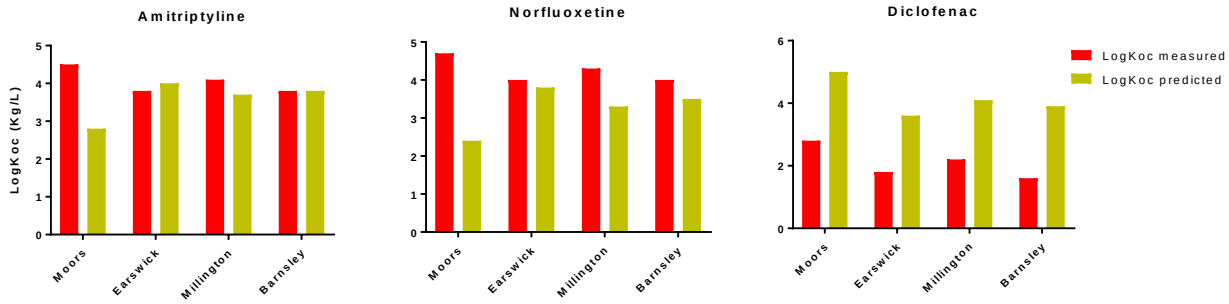


Figure 4. 7. Comparison of the predictive and measured data for estimating the K_{oc} using the Franco and Trapp, (2008) model for the study APIs in four sediments.

4.4 Conclusions

The dissipation of emerging environmental contaminants in surface water and sediments is due to different processes such as photodegradation, volatilization, biodegradation and sorption. In the present study, some of these processes were evaluated such as sorption of three APIs in the sediment compartment and the sorption coefficients K_f and K_{oc} were derived. The biodegradation experiment was limited to diclofenac only. Comparing the sorption results, differences between the sorption coefficients for the three APIs were seen. These differences are likely due to the interaction of the sediment properties (e.g. organic content, pH, etc.) with the physicochemical properties of the APIs (pK_a and $LogK_{ow}$).

In general, this study showed that the sorption behavior of two basic APIs amitriptyline and norfluoxetine on two sediments containing high organic content (Millington and Barnsley) was driven mainly by the interaction with the organic content. Conversely, for Earswick, the sorption behavior could be explained by the pH of the sediment, while Moors by the interaction of the positive charges of the two APIs with the surface of the sediments.

For diclofenac that is acidic at the testing pHs, the interaction between the anionic particles on the surface of the sediments and the anionic API could describe the mechanism of sorption.

Lastly, a predictive model for estimating the K_{oc} of ionisable chemicals was evaluated that it resulted in quite satisfactory predictions for the basic compounds and in particular for amitriptyline.

Furthermore, the study highlighted the complexity in describing the processes that drive the sorption of APIs in sediments and more research is needed with more pharmaceuticals to fully understand what influence the sorption of ionisable compounds.

The experiments of this Chapter highlighted the distribution of the studied APIs to four different types of sediments possessing diverse properties. Knowing the fraction of the API that is bound to the sediment is essential in order to assess the free fraction of the compound that is more likely to be taken up by non-target organisms such as the worm *Lumbriculus variegatus* that lives in this compartment. Therefore, in the next Chapter, these sediments will be used to investigate the sediments' properties that may influence the uptake of ionisable APIs into the sediment-dwelling worm *L. variegatus*.

Chapter 5. Influence of sediment properties on the uptake of ionisable APIs into the sediment-dwelling invertebrate *Lumbriculus variegatus*

5.1 Introduction

In the previous Chapters, the fate of the APIs in the study sediments and the uptake into sediment-dwelling worms from the water column were assessed. This chapter builds upon the findings of the previous chapters to explore the uptake of APIs from sediment into lumbricids.

In the past decade, different classes of pharmaceuticals have been frequently detected in sediments (Vazquez-Roig *et al.,* 2010; Silva *et al.,* 2011; Al-Khazrajy and Boxall, 2017). Once a chemical is bound to the sediment particles, it could be taken up into benthic invertebrates. Consequently, sediments are both a sink and a source of pharmaceuticals, settling on the bottom of the water column of rivers and lakes and then potentially moving into sediment-dwelling organisms (Liebig *et al.,* 2005; Henderson *et al.,* 2010; Camacho-Muñoz *et al.,* 2013). The three main routes of exposure to sediment-dwelling invertebrates such as *Lumbriculus variegatus* are: from the sediment particles themselves; the water overlying the sediment and the interstitial water present between the sediment particles (Gilroy *et al.,* 2012). Therefore, concerns over the impact of sediment-associated chemicals on benthic organisms have started to be assessed (Dussault *et al.,* 2008; Egeler *et al.,* 2010; Lucero *et al.,* 2015; Nieto *et al.,* 2017a). Different responses have been investigated such as reductions in growth, emergence, survival as well as oxidative stresses such as increased damage to the DNA (Lucero *et al.,* 2015). For example, recently a study conducted by Nieto *et al.,* (2017) on *Chironomus riparius* with two APIs (diclofenac and carbamazepine) found that the midges showed a decrease in growth and changes in sex ratio after 10 days of exposure at relatively low concentrations of the two chemicals (30 μg/g).

The combination between chemical and sediment properties and organisms traits is important in determining the uptake into benthic invertebrates (Leppänen and Kukkonen, 2000; Mäenpää *et al.,* 2008; Slootweg *et al.,* 2010; Meredith-Williams *et al.,* 2012; Du *et al.,* 2015; Karlsson *et al.,* 2016). For instance, sediment properties are important for determining the bioavailability of many pollutants because they regulate

their fate and presence in the water-sediment column, consequently, they affect the bioaccumulation potential to non-target organisms living in this column. For example, the organic carbon and pH of sediment have been recognized as important environmental properties in regulating the sorption and retention of APIs in the sediment systems (Al-Khazrajy and Boxall, 2016). Another study by Maënpaä¨ *et al.,* (2003) exposed *C. riparius* and *L. variegatus* to three pesticides spiked in four sediments and found an inverse relationship between the bioconcentration factor of the pesticides and the organic matter of the sediments suggesting that the organic material had a pronounced effect on the bioavailability of the chemicals potentially accumulated by the organisms. Also, the authors suggested that other sediment properties such as texture affected the accumulation of the chemicals in the organisms.

Previous studies have demonstrated that the uptake of chemicals could differ among different test organisms due to different biological traits (e.g. different habitat, ingestion, metabolism and sediment contact) (Meredith-Williams *et al.,* 2012; Rubach *et al.,* 2010; Rubach *et al.,* 2012; Sidney *et al.,* 2016; Carter *et al.,* 2016a). For example, Meredith-Williams *et al.,* (2012) evaluated the difference in the bioconcentration factor (BCF) of a range of pharmaceuticals in freshwater invertebrates: the shrimp *Gammarus pulex*, the insect *Notonecta glauca* and the snail *Planobarius corneus*. The observed differences in the BCFs between the species were likely due to the diverse biological traits such as the mode of respiration and habitat. Furthermore, Sidney *et al.,* (2016) exposed four freshwater benthic invertebrates (*C. riparius*, *H. Azteca*, *L. variegatus* and *S. corneum*) to polychlorinated biphenyls (PCBs) in sediments and found that the species traits explained differences in bioaccumulation among the organisms. For instance, *H. Azteca* was found to have the greatest BSAF probably due to the habitat. *H. Azteca* lives mainly in the water column and the authors suggested that the shrimp was primarily exposed to the contaminants present in the water compared to the other organisms that lived in the sediment such as *L. variegatus*.

The idea of combining physicochemical properties of the APIs and environmental parameters is essential in understanding how APIs are taken up by non-target organisms and to what extent they may elicit adverse effects on them. Research exploring the combination of these properties has increased lately. However, our current knowledge regarding the uptake of pharmaceuticals from the sediment compartment is still very limited in particular for ionisable APIs. Recently some research has assessed the uptake

of ionisable APIs into sediment-dwelling worms (Karlsson *et al.,* 2016; Karlsson *et al.,* 2017). Experiments involving exposure of lumbricids to diclofenac, fluoxetine and triclosan revealed that the worms accumulated the APIs to a different extent due to the ionisation of the compounds and their hydrophobicity (Karlsson *et al.,* 2016). In the second study, the authors proposed a novel modelling approach to estimate the internal concentrations of ionisable APIs from sediment studies. They exposed the worms to different environmentally relevant concentrations of diclofenac, fluoxetine and triclosan at pH values ranging from 5.5 to 8.5. The model accounts for the ionized and unionized species of the chemical and it assumes that the exposure of the worms to the chemicals derived from the pore water. This is based on the EqP theory for which, at equilibrium, the pore water exposure of chemicals bound to the sediments is assumed to provide the same exposure as the water-only exposure (Di Toro *et al.,* 1991). The authors observed 47 and 37-fold difference in the internal concentrations of the lumbricids at the highest and lowest pH for these two compounds.

However, the study did not consider other environmental properties of the sediments that could influence the uptake such as the organic carbon. The last two mentioned studies have attempted to cover the gap between sediment uptake studies, particularly with sediment dwellers and ionisable APIs for which our knowledge is still very limited compared to fish species and other aquatic invertebrates. In respect to other aquatic invertebrates such as *Daphnia magna* or *Gammarus pulex*, there are not much data available in the literature regarding the uptake of ionisable APIs into *L. variegatus*. Exploring the effects of sediment characteristics that could potentially influence the accumulation of APIs in the worms will allow us to better estimate the internal exposure and thus the toxicity that these chemicals may have to the organisms and ultimately provide a better interpretation of the sediment bioaccumulation experiments.

Benthic organisms such as *L. variegatus* are important organisms dwelling in the sediment and *Lumbriculus* is one of the most common species used in bioaccumulation laboratory tests to assess xenobiotics (Maënpaä¨ *et al.,* 2003). Besides, oligochaetes are the prey of fishes thus, a trophic transfer could occur. They have been also found to bioaccumulate pharmaceuticals to a greater extent than higher trophic species in the food chain (Lagesson *et al.,* 2016).

Therefore, the main aim of this study was to evaluate the uptake and the depuration of three ionisable APIs (amitriptyline, norfluoxetine and diclofenac) in the sediment-

dwelling worm *L. variegatus* and to understand the degree of influence of sediment properties along with the physicochemical characteristics in determining the uptake. The results will give valuable insight in describing the difference in bioaccumulation of three ionic APIs in small invertebrates inhabiting the sediment. In addition, the results will bring new data for a better evaluation of the risk of benthic invertebrates.

5.2 Material and methods

5.2.1 Test organisms

L. variegatus were cultured as described in Chapter 3. One week before the beginning of the test, the organisms were transferred to small aquaria containing sediment (approx. 4 cm) and the APW for the acclimatization. The sediment was the same used to run the sorption experiments.

5.2.2 Test sediments

The sediments chosen for the experiments were the Moors and Barnsley sediments which were sampled from different locations in England. Their properties are fully described in Chapter 4.

5.2.3 Test chemicals

Radiolabelled 14-C amitriptyline (CAS 64-17-5), 14-C diclofenac (CAS 15307-86-5) and 14-C norfluoxetine (CAS 65277-42-1) were obtained from different suppliers as described in Chapter 3. All the radiochemicals were dissolved in ethanol and stored at -20 °C while the dosing stocks were prepared in APW the day before the beginning of the test in order to avoid any degradation of the compound.

PTFE centrifuge tubes were purchased from Oak Ridge by Nalgene Nunc International. Soluene-350 and Hionic Fluor were purchased from PerkinElmer (http://www.perkinelmer.com) and Ecoscint A from National Diagnostics (https://www.nationaldiagnostics.com). The solvents used in the extraction methods

were: methanol (HPLC grade, purity ≥99.9%); acetonitrile (≥ 99.9%); HLPC water and H_3PO_4 (ACS reagent, ≥ 85%) were all purchased from Sigma Aldrich (UK).
pH measurements were taken every 12 hours using a Mettler Toledo 51343104 InLab pH probe.

5.2.4 Evaluation of extraction methods for the test compounds from sediment

A range of potential solvents were assessed for the suitability for extracting the test materials from sediments. The six solvents chosen for evaluation of their suitability for use in extractions were: MeOH, ACN, 7:3 (v/v) ACN/H_2O; 0.1% H_3PO4 MeOH, 0.1% H_3PO_4 ACN and 0.1% H_3PO_4 7:3 (v/v) ACN/H_2O. For each assessment, triplicate PTFE tubes containing 3 g dw of sediment were spiked with approximately 0.010 MBq of each API. The tubes were then shaken in the dark for an hour to properly mixed the API with the sediment at 300 rpm. After that, the ethanol was left to evaporate for an hour and the extractions were then carried out. Test solvent (10 ml) was added to the tubes which were then left to shake in the dark for 1 h at 300 rpm. The samples were then centrifuged for 10 minutes at 2500 rpm and the solvent removed. The procedure was repeated twice and the solvent phases were pooled prior to the analysis.
Analysis of the extracts was carried out by LSC with 1 ml of the sample being taken and mixed with 10 ml of Ecoscint A (National Diagnostic).
Ultimately, the solvent extraction method that had the best recovery for each API and sediment was validated using triplicates samples at three concentrations of 0.075 µg/g, 0.15 µg/g and 0.225 µg/g.

5.2.5 Toxicokinetic experiments

Toxicokinetic experiments followed the same design as used by Karlsson *et al.,* (2016). Briefly, the system consisted of 3 g dw of sediment and 15 ml of APW. Each API was spiked into the sediment to give a final concentration of 10 µg/g in the water phase.
Vessels were then left to allow the test chemical to equilibrate between the overlying water and the sediment by shaking overnight at 300 rpm. After the equilibration time,

the tubes were positioned upright and the sediment was left to settle before adding the worms.

Twelve tubes containing 3 worms each were used during the uptake phase and six of these were sampled at 12 h and the remainders at 24 h. A further 12 tubes were set up to explore the depuration of the test chemical. Worms were exposed in these to the test chemical for 24 h after which time they were transferred to new tubes containing sediment. Six of these tubes were removed at 36 h after the start of the study and the remaining tubes were removed at 48 h.

Stability beakers without the worms and sediment controls (= tubes containing sediment, APW and worms with no API) were also prepared.

Exposures were performed at 20 °C in the dark to avoid photodegradation of the API. pH was measured with a Mettler Toledo 51343104 InLab pH probe.

At each time point, the worms were taken with a spatula and transferred in beakers containing 15 ml of APW for 6 h to purge their guts (Mount *et al.,* 1999). After 6 h, they were rinsed in water, dried on a tissue, weighted on a balance to measure the dry weight and frozen at -20 °C.

For the analysis, worms were dissolved in 2 ml of Soluene 350 (Perkin Elmer) overnight and then 10 ml of Ionic Fluor (Perkin Elmer) was added and the vials were ready to be analyzed by the LSC.

After the removal of the organisms from the tubes, the overlying water and sediment were centrifuged for 10 minutes at 3000 rpm and 1 ml of the water was taken in order to analyze the concentration of the API freely dissolved in the water column. The water was mixed with 10 ml of Ecoscint A, the pH was measured and the remaining water in the test tubes was disposed of.

Lastly, the sediment extraction was carried out by adding 10 ml of extraction solvent and shaking the tubes for 1 h at 300 rpm. Amitriptyline and norfluoxetine were extracted with 0.1 % H_3PO_4 7:3 ACN/H_2O for both sediments, while diclofenac with 0.1 % H_3PO_4 7:3 ACN/H_2O for Moors and 7:3 ACN/H_2O for Barnsley. The samples, after the shaking, were centrifuged for 5 minutes at 3000 rpm and the solvent was transferred to a glass vial. The operation was repeated twice and the solvents were pooled together. Again, 1 ml of solvent was mixed with 10 ml of Ecoscint A for subsequent analysis by LSC.

5.2.6 Pore water analysis

A separate test was carried out to determine the concentrations of each API in the sediment pore water of the system. Three test tubes containing 3 g dw of sediment and 15 ml of APW spiked at 10 µg/L of each API were prepared. The tubes were left to shake for 24 h in the dark at 300 rpm. At the end of the shaking, the sediment was transferred into disposable syringes containing 1 cm of glass wool at the bottom (Fig. 5.1). The syringes, contained in 50 ml Falcon tubes, were then centrifuged for 40 minutes twice and 500 µl of the extracted water was collected and pipetted into a 2 ml Eppendorf tubes. The Eppendorf tubes were centrifuged for 5 minutes and 300 µl of pore water was collected for analysis. The pH was measured using a Mettler Toledo 51343160 InLab micro pH probe (Fig. 5.1). Lastly, the pore water was mixed with 10 ml of Ecoscint A and ready for analysis.

Figure 5. 1. Pore water pH analysis (left figure) and Falcon tubes containing the syringes with 3g of the sediment ready for the extraction of the pore water (right figure).

Concentrations of the test chemicals in the pore water were also estimated based on the following formula suggested by Karlsson *et al.,* (2017):

$$C_{pw} = C_{sed} / \{[K_d \times (\%_{sed} / \%_{water}) \times \text{bulk density}] + 1\} \qquad \text{Equation 1}$$

Where C_{pw} is the concentration of the chemical in the pore water (pmol/mL) and C_{sed} is the concentration of the chemical in the sediment (pmol/g); K_d is the sorption coefficient; $\%_{water}$ and $\%_{sediment}$ are the % of moisture and sediment in the sediment samples.

For the bulk density, three replicates of wet sediment were weighted up to 80 mL in 100 mL beakers. The height and the diameter of the beaker were measured in order to obtain the radius and the ring density. The replicates were left in the over at 50 °C overnight and then the dry weight was measured.

5.2.7 Lipid analysis

Lipid content of the worms was measured following the method suggested by (Smedes, 1999). Triplicates test tubes containing approximately 100 mg of organisms were weighed (wet weight) and placed in a vial dried. After that, the tubes were placed in the lyophilizer for 2 h and then dried in the oven at 50 °C overnight, following which the dry weight was determined. The worms were transferred into pre-weighed vials and ground with a glass rod. In order to extract the lipids from the worms, 1.6 ml of 2-propanol (Sigma Aldrich), 2 ml of cyclohexane (Sigma Aldrich) and 2.2 ml of de-ionized water were added as solvents. The tubes were vortexed for 30 seconds, sonicated in a water bath for 5 minutes and centrifuged for 5 minutes at 3000 rpm. The upper layer was removed and pipetted into a pre-weighted brown glass vial and then the extraction procedure was repeated twice. The combined extracts were evaporated under a gentle stream of nitrogen and finally weighed on the balance to determine the lipid content by adjusting the results using an external liposome (1,2 – distearoyl-sn-glycerol-3-phosphocholine) and cyclohexane as controls.

5.2.8 Kinetic modelling analysis

Mean concentrations and standard deviations of the six replicates tubes were calculated for each time point. The uptake rate constant (k_{in}) and the depuration rate constant (k_{out}) were derived using Open Model (University of Nottingham, http://openmodel.info/ downloaded 5th May 2017). The accumulation was assumed to

follow the first-order one- compartment toxicokinetic uptake described by the following formula:

$$dC_{int} / d_t = (C_{sed} \times k_{in}) - (C_{int} \times k_{out}) \quad \text{Equation 2}$$

Where C_{int} is the organism internal concentration (pmol/g wet weight), C_{sed} is the water concentration of the analyte (pmol/g), k_{in} and k_{out} are the uptake (mL x g wet weight × h^{-1}) and depuration rate constant (h^{-1}) and t is time (hours).

The first order one-compartment toxicokinetic model is a reliable model that assumes a constant uptake and elimination per time. Therefore, the concentration of the compound into the body of an organism is proportional to the concentration in the water/sediment and the concentration of the compound eliminated is proportional to the concentration remained in the body.

Also, the kinetic biota accumulation factor (BAF_k) was calculated by setting the C_{sed} equal to 1 and by running the model until the equilibrium was reached. The Marquardt algorithm was first applied in order to find the general fit values of the uptake and elimination rates values, then, the Monte Carlo along with the Metropolis-Hastings algorithm was applied by running the model for 10000 iterations to find the best fit values.

The 95% confidence intervals around the fitted curves were derived (Ashauer *et al.,* 2010).

The biota-sediment accumulation factor (BSAF) was calculated following the OECD 305 by taking into account the lipid content of the worms and organic content of the sediment based on the formula below:

$$BSAF = BAF_k \times f_{OC} / f_{lip} \quad \text{Equation 3}$$

Where f_{OC} is the total organic content of the sediment (%) and f_{lip} is the lipid fraction of the worms (% wet weight).

5.2.9 Statistical analysis

Statistical analysis of the measured data was performed with Graphpad Prism (www.graphpad.com). For each API, differences of the concentrations over time in the stability vials containing sediment, water and the compound only were analyzed. Tests for normality were performed using the Shapiro-Wilk normality test. If the data passed the normality test, then, one-way ANOVA test was used to evaluate the differences in the concentrations between the treatment groups at 0 h, 24 h and 48 h.

5.3 Results and discussion

5.3.1 Evaluation of extraction solvents for the pharmaceuticals from the sediments

Extraction efficiencies varied by solvent, pharmaceutical and sediment type (Table 5.1). For example, the extraction efficiency of amitriptyline ranged between 0.2% with acetonitrile and 63% using the acidified 0.1 % H_3PO_4 and 7:3 ACN/H_2O. For diclofenac, efficiencies ranged between 3% with acetonitrile and 81% with 7:3 ACN/H_2O and for norfluoxetine efficiencies were in a range of 2% using acetonitrile and 65% with 0.1% H_3PO_4 7:3 ACN/H_2O (Table 5.1). Based on the preliminary studies, the solvents that resulted in the highest recoveries were used for the validation of the method and the results are shown in Table 5.2.

	Amitriptyline	
Moors	**Extraction solvent**	**Mean recovery (%)**
	MeOH	12
	ACN	0.2
	7:3 ACN/H_2O	21
	0.1% H_3PO_4 MeOH	20
	0.1% H_3PO_4 ACN	3
	0.1% H_3PO_4 7:3 ACN/H_2O	57
Barnsley	MeOH	51
	ACN	13
	7:3 ACN/H_2O	43
	0.1% H_3PO_4 MeOH	47
	0.1% H_3PO_4 ACN	20

	0.1% H_3PO_4 7:3 ACN/H_2O	63
	Diclofenac	
Moors	MeOH	11
	ACN	3
	7:3 ACN/H_2O	76
	0.1% H_3PO_4 MeOH	40
	0.1% H_3PO_4 ACN	20
	0.1% H_3PO_4 7:3 ACN/H_2O	77
Barnsley	MeOH	51
	ACN	10
	7:3 ACN/H_2O	81
	0.1% H_3PO_4 MeOH	9
	0.1% H_3PO_4 ACN	22
	0.1% H_3PO_4 7:3 ACN/H_2O	77
	Norfluoxetine	
Moors	MeOH	7
	ACN	2
	7:3 ACN/H_2O	41
	0.1% H_3PO_4 MeOH	13
	0.1% H_3PO_4 ACN	5
	0.1% H_3PO_4 7:3 ACN/H_2O	65
Barnsley	MeOH	20
	ACN	4
	7:3 ACN/H_2O	51
	0.1% H_3PO_4 MeOH	21
	0.1% H_3PO_4 ACN	6
	0.1% H_3PO_4 7:3 ACN/H_2O	58

Table 5. 1. Preliminary recoveries of the study compounds from the sediments using different extraction methods.

	Compound	**Extraction solvents**	**Mean recovery (%)**
Moors	Amitriptyline	0.1% H_3PO_4 7:3 ACN/H_2O	62 (1.53)
	Diclofenac	0.1% H_3PO^4 7:3 ACN/H_2O	81 (1.0)
	Norfluoxetine	0.1% H_3PO_4 7:3 ACN/H_2O	72 (2.08)
Barnsley	Amitriptyline	0.1% H_3PO_4 7:3 ACN/H_2O	64 (6.35)
	Diclofenac	7:3 ACN/H_2O	83 (3.5)
	Norfluoxetine	0.1% H_3PO_4 7:3 ACN/H_2O	72 (1.0)

Table 5. 2. Recoveries of the test compounds from the sediments for the validation of the extractions.

5.3.2 Lipid content of the organisms

The average wet weight of the worms was 25 mg which corresponded to 10-11 worms per vial (2.5 mg/worm). The mean wet weight (%) was 1.5 and the mean dry weight of the lipid content (%) was 1.7. The recoveries using the external liposome and the cyclohexane as controls were 99%. The liposome used was 1,2 – distearoyl-sn-glycerol-3-phosphocholine which is a major component of the biological membrane and its composition was found to be high in Oligochaetes (Bell *et al.,* 1994).

Assessing the lipid content of the worms is important because normally a positive correlation between bioconcentration and the lipid content of the organism is found; in particular for hydrophobic compounds that mainly partition into the lipid membrane of species (Mackay, 1982). Other studies have measured the lipid content of *L. variegatus*; for example (Liebig *et al.,* 2005) measured 8% dw. In their study, a higher dry weight of the worms than our measurements was reported and we believe that the difference in the lipid content of the worms depends on several factors such as the maturity of the organisms, the different suppliers and the different cultivation conditions.

5.3.3 Uptake and depurations experiments

Stability samples. The concentrations of the test APIs in the water phase and sediment and the pH of the overlying water in the vials without the worms were measured throughout the duration of the experiments (Figs. 5.2, 5.3, 5.4). The pH of the overlying water of the Moors sediment was slightly acidic (6.30-6.53) compared to the Barnsley sediment (7.14-8.15). Also, in both sediments, pH remained quite stable over the duration of the study. In the Moors sediments, concentrations of diclofenac remained stable (p-value > 0.05) while changes in the concentrations for the Barnsley sediments were observed (p-value < 0.05^{*}). Regarding the two bases, amitriptyline and norfluoxetine, concentrations were stable in the Moors sediment (p-value > 0.05). Whereas, in Barnsley sediment, after 24-h, a decrease in the sediment concentrations was observed (p-value < 0.001^{***}).

Looking at Figures 5.2 and 5.4, concentrations of amitriptyline and norfluoxetine were greater in the sediment than in the overlying water. For example, in the Moors sediment, the concentration of amitriptyline in the sediment compartment was 4-fold

higher than in the water column, as well as norfluoxetine. Regarding Barnsley, the same scenario as Moors was observed: the sediment concentrations of the two APIs were 3-fold higher than the concentrations detected in the water.

On the other hand, diclofenac seemed to be equally distributed between the sediment and the overlying water having half of the API adsorbed into the sediment and half dissolved in the aqueous phase.

The factors affecting the dissipation of pharmaceuticals particularly in sediments have not been well studied yet. For instance, the degradation of 6 pharmaceuticals including amitriptyline in 10 different types of sediments was investigated by Al-Khazrajy *et al.,* (2018). Amitriptyline was found to be persistent across the sediment types with an average half-life of 62 days. In addition, the authors evaluated the effect of the microbial activity using sterile and non-sterile sediments and a higher half-life was found under sterile conditions suggesting that the microbes contribute largely to the dissipation of amitriptyline. In our study, non-sterile sediments were used thus, it is possible that the degradation of amitriptyline may be due to the presence of the microorganisms. Our aim was to maintain the properties of the sediment similar to the sediment that we sampled in the field but further studies should be done to investigate which main factors could contribute to the dissipation of amitriptyline enabling us to better understand the fate of this highly prescribed antidepressant in the sediment.

Some data are available on the degradation of norfluoxetine in the sediment compartment. The fate of norfluoxetine and its parent compound, fluoxetine, was assessed by Kwon and Armbrust, (2006b) under different conditions to evaluate the possible influence of microbes, light and the water in the distribution of the API in the water-sediment column. According to the results, norfluoxetine rapidly adsorbed to the sediment and it was reported to be photolytically and hydrolytically stable and not degraded by the microbial activity. In our study, the test was performed in the dark and no contribution of the light was produced. Generally, the factors dominating the bioavailability and persistence of pharmaceuticals in sediments are unclear and little is known. Some attempts to explain the fate of these contaminants in the aquatic system are growing but due to the complexity of the diverse mechanisms that could be potentially involved, more research is needed.

Data regarding the persistence of diclofenac are limited, however, the fate of the API has been assessed in water-sediment systems under high and low flow velocity of the

overlying water (Kunkel and Radke, 2008). The authors reported a DT_{50} of 8.5 days under low flow conditions. Another fate laboratory test performed on diclofenac in a sediment river system found that diclofenac was stable under all biotic and abiotic conditions (Koumaki *et al.,* 2017). Similar to the findings of Koumaki *et al.,* (2017) study, diclofenac was found to be quite stable in both sediments over 48-h and we can therefore exclude the influence of biotic as well as abiotic degradation processes in the test.

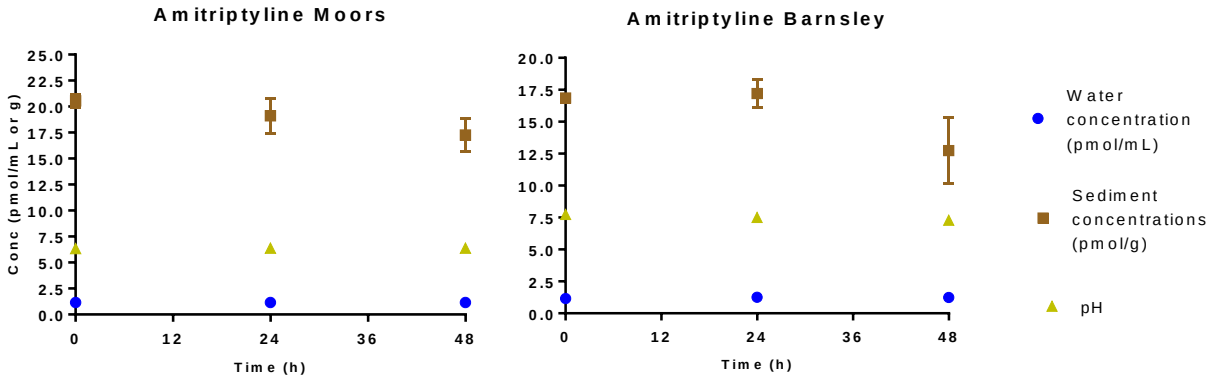


Figure 5. 2. Mean (±SD) of the measured water concentrations (blue dots), sediment concentrations (brown squares) and pH data (green triangles) of the stability beakers of amitriptyline in the two sediments.

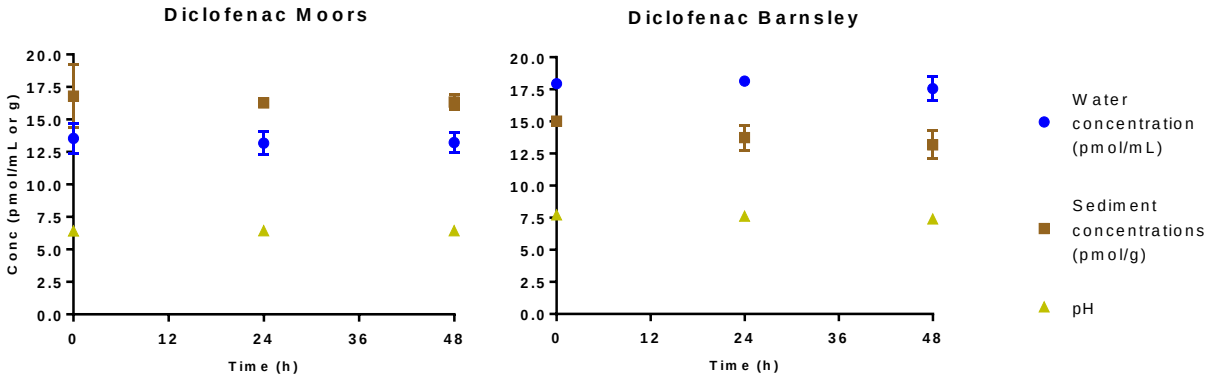


Figure 5. 3. Mean (±SD) of the measured water concentrations (blue dots), sediment concentrations (brown squares) and pH data (green triangles) of the stability beakers of diclofenac in the two sediments.

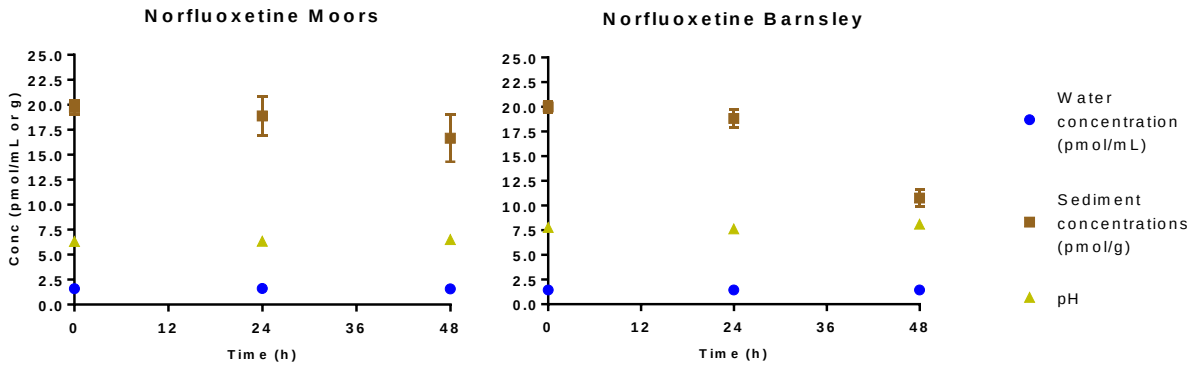


Figure 5. 4. Mean (±SD) of the measured water concentrations (blue dots), sediment concentrations (brown squares) and pH data (green triangles) of the stability beakers of norfluoxetine in the two sediments.

5.3.4 Toxicokinetic experiments

No mortality of the worms was observed during the experiments. The reproduction and growth of the organisms were limited by the short duration of the tests. The 14-C radioactivity of the three APIs was measured for the overlying water and sediment compartments. Also, the overlying water pH was monitored throughout the experiments and it tended to be alkaline for Barnsley (7.62- 8.15) and acidic for Moors (6.45-6.66). The concentrations in the sediment decreased over the 24-h of exposure possibly due to uptake of the API by the worms, whereas, the concentrations remained stable for the water phase.

The first order one-compartment toxicokinetic model was fitted to the experimental data (Figs. 5.5, 5.6, 5.7). For most datasets, the model seems to fit well in particular for amitriptyline, whereas for diclofenac and norfluoxetine different scenarios were observed. Specifically, the model underestimated the uptake rates of diclofenac in Barnsley and the depuration rates of diclofenac in Moors while it underestimated the uptake rates of norfluoxetine in the Barnsley sediment.

The kinetic BAFs, uptake, depuration rates and BSAFs of the three APIs are listed in Table 5.3 below. The kinetic BAF increased in the following order norfluoxetine < amitriptyline < diclofenac in the Moors sediment and norfluoxetine < diclofenac < amitriptyline in the Barnsley sediment.

The biota-sediment bioaccumulation factor (BSAF) increased in the following order norfluoxetine < amitriptyline < diclofenac for Moors and norfluoxetine < diclofenac < amitriptyline for Barnsley. The BSAF ranged from 5 g/mL for norfluoxetine to 201 g/mL for amitriptyline in the Barnsley sediment and from 13 g/mL for norfluoxetine to 17.2 g/mL for diclofenac in the Moors sediment.

Even though bioaccumulation is an important endpoint as part of the risk assessment of chemicals, not many studies have been focussed on the sediment bioaccumulation of pharmaceuticals, particularly into invertebrates. However, one study conducted by Karlsson *et al.,* (2016) reported the BSAF of diclofenac to be 0.57 L/Kg after 48-h of exposure into *L. variegatus* (Karlsson *et al.,* 2016). In this study, the BSAFs derived were higher by two orders of magnitude; 10.6 g/mL and 17.2 g/mL for the Barnsley and Moors sediments respectively. Although the organism used in the experiments was the same, differences in bioconcentration could be explained by the influence of the different sediment properties such as pH and organic matter. In the toxicokinetic experiments of Karlsson *et al.,* (2016), the pH was 7.67 and the organic carbon (OC) was 0.51 %. The two sediments chosen in our experiments possessed diverse properties (Barnsley pH = 7.52 and OC = 14.26 %; Moors pH 6.43 and OC = 0.787 %). Thus, differences in the accumulation are likely to be due to the different environmental characteristics. Also, a possible transformation process could have occurred into the worms leading to more hydrophilic metabolites that are excreted faster than the parent compound. This could explain the underestimation of the depuration rates in the Moors sediment. However, no studies regarding the metabolic pathway of diclofenac into sediment-dwelling invertebrates have been found in the literature. The metabolic pathway of diclofenac has been reported in fish species and several metabolites have been identified through glucuronidation (Kallio *et al.,* 2010; Lahti *et al.,* 2011). Recently, experiments investigating the metabolic pathway of diclofenac in marine mussels were studied (Bonnefille *et al.,* 2017; Świacka *et al.,* 2019). Bonnefille *et al.,* (2017) identified 13 metabolites of diclofenac in the marine organism *Mytilus galloprovincialis,* including 4'-hydroxy-diclofenac and 5-hydroxy-diclofenac that were found in another marine mussel (*Mytilus trossulus*) by Świacka *et al.,* (2019).

In the future experiments to detect the metabolites of APIs for invertebrates are necessary to have a better understanding of their influence in the uptake and

depuration process for a better estimation of the internal exposure, hence, potential toxicity to non-target species.

In addition, diclofenac is the only weak acid evaluated in this study. It possesses a pK_a of 3.9. In Chapter 3, the water-only uptake studies showed that at lower pH, diclofenac is accumulated more in the organisms. In this study, a higher BASF was observed for the Moors sediment compared to the Barnsley. Thus, we believe that the pH could be the property that may explain the accumulation differences of the worms among the two sediments.

The internal concentration of diclofenac has been reported in other aquatic invertebrates such as *Gammarus pulex* (Miller *et al.,* 2016). The authors estimated bioconcentration of 14 L/Kg after exposure of the gammarids to 48-h at 10 µg/L of the compound. In our study, the same exposure concentration was used and the results were in the same order of magnitude to that reported by Miller *et al.,* (2016). Slight differences in the uptake could be due to the different exposure time and species used in the toxicokinetic experiments, thus, different species traits such as locomotion, feeding habit, respiration (Ducrot *et al.,* 2005; Sidney *et al.,* 2016).

For amitriptyline, to the best of our knowledge, no data regarding sediment bioaccumulation into invertebrates have been published yet. However, the bioconcentration of amitriptyline has been assessed into a gilt-head bream (*Sparus aurata*) at two concentrations of 2 µg/L and 10 µ/L (Ziarrusta *et al.,* 2017). At the same concentrations used to expose the worms in our study (10 µg/L), the authors calculated a BCF 21 L/Kg which is slightly higher than the results reported in our study. They also noticed that amitriptyline was mainly accumulated in the brain which is not surprising as the brain should be its main target organ.

Understanding the relationship between the sediment characteristics and the physicochemical properties that affect the accumulation of APIs into non-target organisms is complex and not well studied. However, below, a possible explanation for the observations of uptake of the APIs into *L. variegatus* is also given for the two bases. The two studied study sediments had very different characteristics: Barnsley had high organic content (14.26%) and high pH (7.56); while Moors had low organic content (0.787%) and low pH (6.43). In this way, the two chosen sediments allow comparisons

between the sediment characteristics, physicochemical properties of the compounds and bioaccumulation.

Amitriptyline is a basic pharmaceutical (pK_a = 9.4, $LogK_{ow}$ = 4.92, see Chapter 1) and its fate in the sediment system depends on the speciation at the sediment pH. Based on the Henderson-Hasselbach equation, the API increases its neutrality with increasing pH. At pH 6.43 (the pH of Moors sediment), 0.11% of amitriptyline is in the neutral form, while at pH 7.52 (pH of the Barnsley sediment), 1.30% is in the neutral form. Based on our findings in Chapter 3, at high pH amitriptyline bioconcentration increases, and, similar results can be seen in this study when comparing the BSAFs of Barnsley and Moors (Table 5.3), i.e.: a higher BSAF of Barnsley was measured compared to Moors.

Amitriptyline has been found to be highly adsorbed to sediments containing high organic content suggesting that the hydrophobic interactions with the neutral part of the API are its dominant sorption mechanism (Al-Khazrajy and Boxall, 2016). Whereas, in our study, even though amitriptyline has a high affinity with the organic content, the pH seemed to have influenced greater uptake into the worms. Therefore, it is very important to account for the pH of the study medium because it may explain the differences in the bioaccumulation between sediments.

Norfluoxetine has not been well studied in invertebrate species yet, however, the uptake of fluoxetine and its major metabolite norfluoxetine has been investigated into a marine mussel *Mytilus galloprovincialis* (Silva *et al.,* 2016). The organisms were exposed to fluoxetine for 15 days and the metabolic products such as norfluoxetine were analyzed too. The pseudo-BCF calculated for norfluoxetine was found to be higher than fluoxetine at 15 days of exposure (124 L/Kg and 155 L/Kg for fluoxetine and norfluoxetine respectively). A possible explanation could be the polarity of fluoxetine compared to norfluoxetine: norfluoxetine has been found being more accumulative and hydrophobic than its parent compound (Gelsleichter and Szabo, 2013). Similar results were reported by different authors who exposed different fish species to fluoxetine and norfluoxetine to investigate the distribution and accumulation into non-target species (Brooks *et al.,* 2005; Nakamura *et al.,* 2008a; Paterson and Metcalfe, 2008b). In this study, norfluoxetine only was detected and the accumulation factors analyzed for the two sediments were much lower than those reported in the above-mentioned study of Silva *et al.,* (2016). Differences in the uptake could be due to the different exposure conditions, concentrations, test duration and the biological traits of the test species.

Norfluoxetine is also a basic pharmaceutical (pK_a = 10.01 and $LogK_{ow}$ = 4.16) and it increases the neutrality from 0.42% at pH 6.43 to 5% at pH 7.52. In the Moors sediment, a rapid uptake followed by a rapid depuration was observed whereas in Barnsley sediment the model over-estimated the uptake and under-estimated depuration. Also, the BSAF reduced with increasing of the sediments' organic content. These results contradict the amitriptyline' results, even if the two pharmaceuticals should show similar bioaccumulation patterns. In this case, the differences in the BSAFs of the two sediments could be explained by the different binding degree of the API to the organic material. We suggest that the bioavailability of norfluoxetine is due to the different power of binding to the organic material. Therefore, higher BSAF is observed for Moors sediment which contains relatively low organic content, consequently, a greater bioavailability of norfluoxetine in the pore water to be taken up by the organisms could explain the results. In addition, it has been suggested that sediments containing coarse particle size possess a higher degree of bioaccumulation than sediments having fine particle size (Maënpaä¨ *et al.,* 2003). This observation could be applied to the sediments of our study; in fact, the particles analyzed for Moors showed a high percentage of sand (78%), see Chapter 4, and a high BSAF was measured supporting the theory stated above.

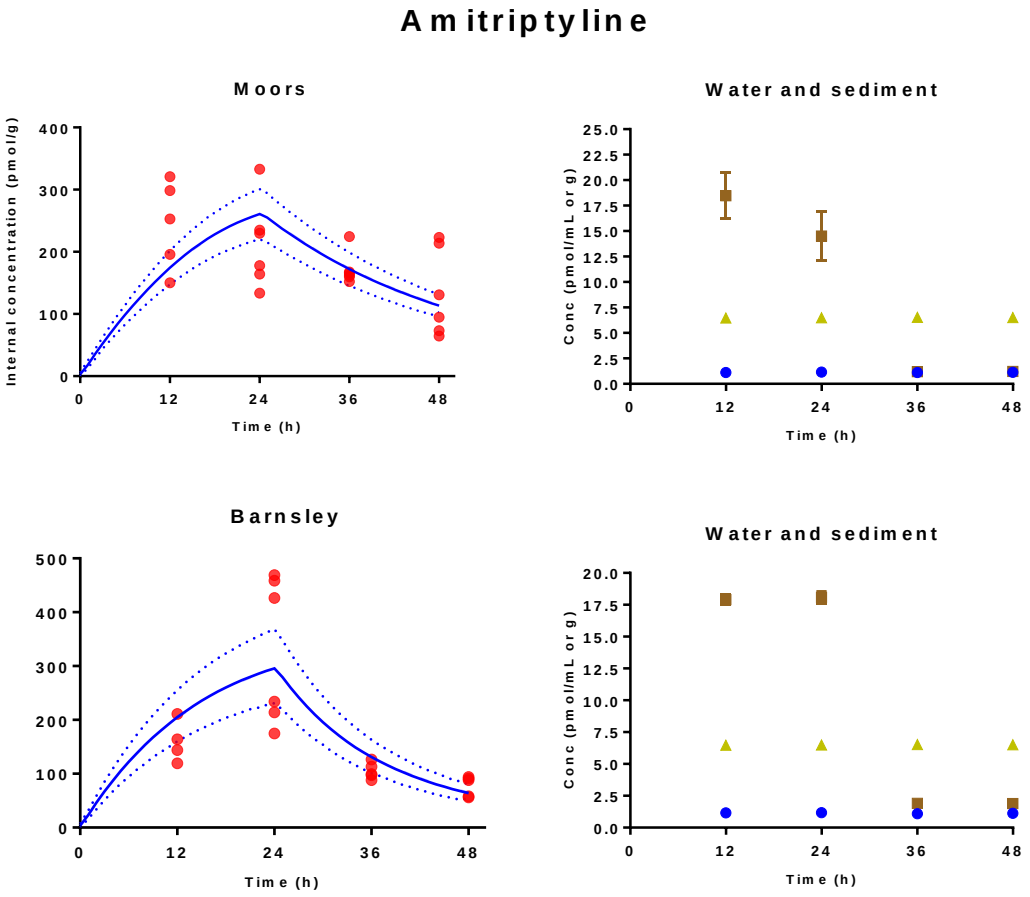


Figure 5. 5. Uptake and depuration of amitriptyline (right figure) in Moors and Barnsley sediments. Red dots represent the internal concentrations measured in the worms (pmol/g wet weight), the blue thick line represents the model fit curves and the dashed blue lines the 95% confidence interval of the model fit. The left figure shows the water concentrations (blue dots in pmol/mL), sediment concentrations (brown squares, pmol/g) and pH measurements of the overlying water (green triangles).

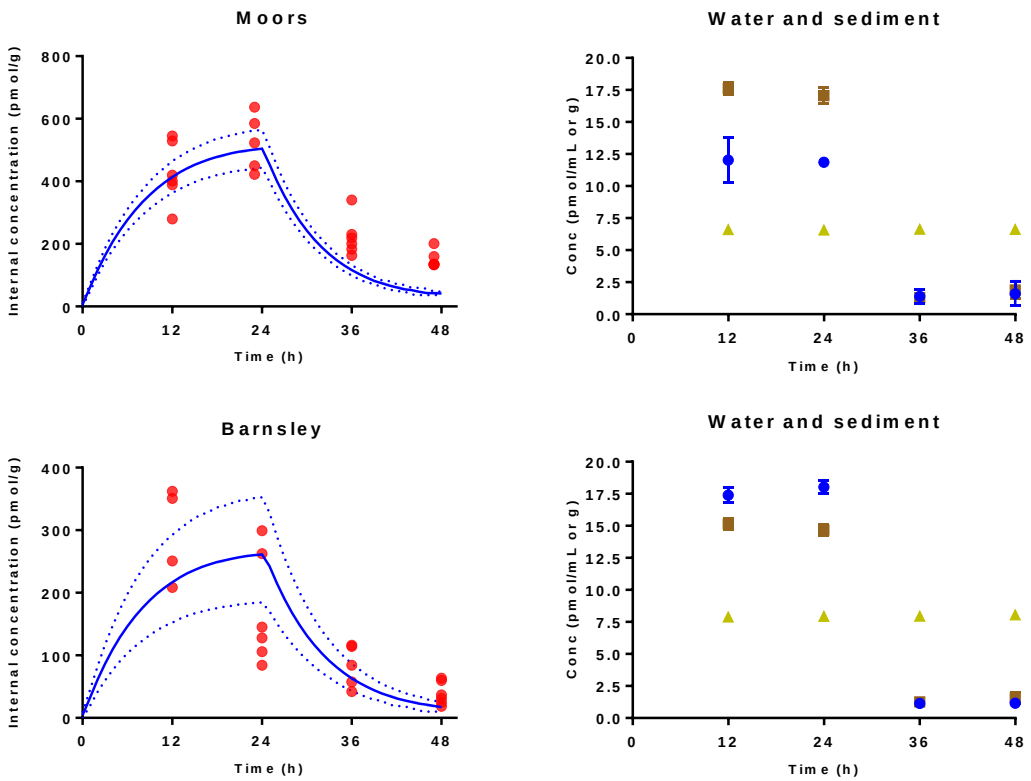


Figure 5. 6. Uptake and depuration of diclofenac (right figure) in Moors and Barnsley sediments. Red dots represent the internal concentrations measured in the worms (pmol/g wet weight), the blue thick line represents the model fit curves and the dashed blue lines the 95% confidence interval of the model fit. The left figure shows the water concentrations (blue dots in pmol/mL), sediment concentrations (brown squares, pmol/g) and pH measurements of the overlying water (green triangles).

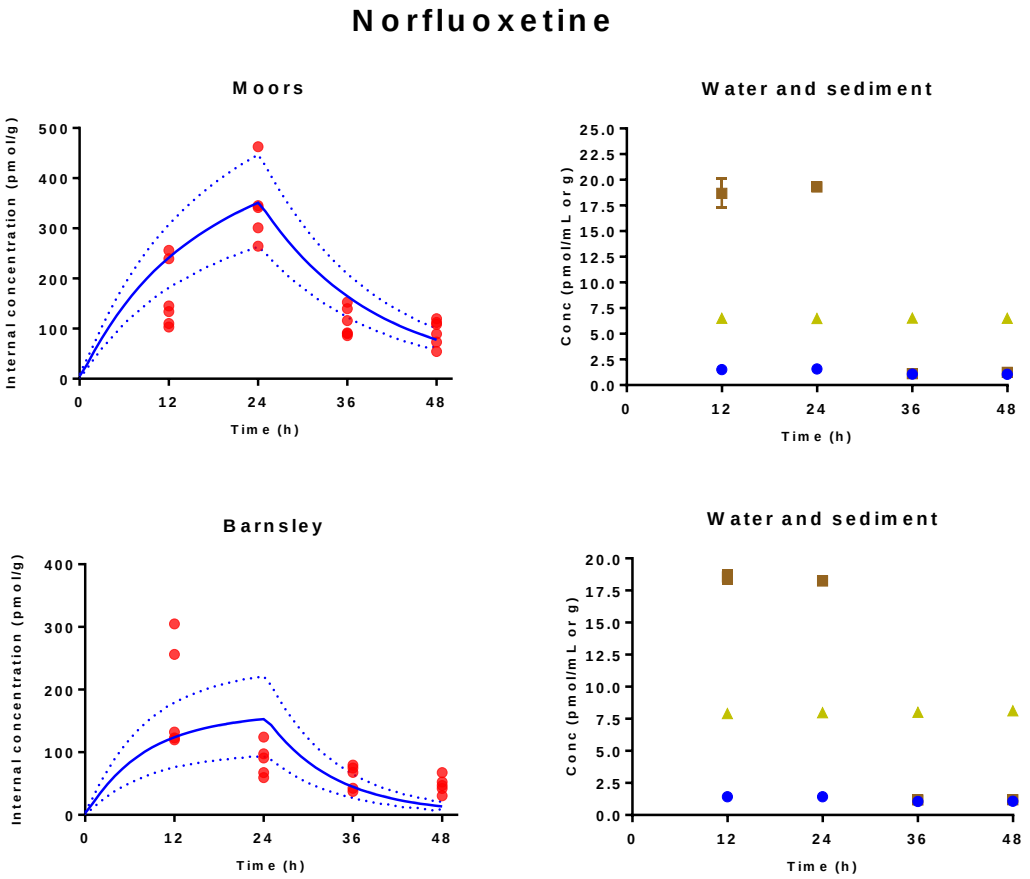


Figure 5. 7. Uptake and depuration of norfluoxetine (right figure) in Moors and Barnsley sediments. Red dots represent the internal concentrations measured in the worms (pmol/g wet weight), the blue thick line represents the model fit curves and the dashed blue lines the 95% confidence interval of the model fit. The left figure shows the water concentrations (blue dots in pmol/mL), sediment concentrations (brown squares, pmol/g) and pH measurements of the overlying water (green triangles).

API	Sediment	pH overlying water	pH sediment	K_{in} (ml × g × h^{-1})	K_{out} (h^{-1})	BAF_k (95% CI) (g/ml)	BSAF (g/ml)
Amitriptyline	Moors	6.50 (0.02)	6.43	0.98	0.03	27.5 (25.1- 31.73)	14.4
Amitriptyline	Barnsley	7.77 (0.04)	7.52	1.54	0.07	21.24 (18.6- 26.45)	201.9
Diclofenac	Moors	6.61 (0.02)	6.43	4.115	0.12	32.73 (30.54- 36.61)	17.2
Diclofenac	Barnsley	7.96 (0.06)	7.52	2.38	0.12	20.14 (16.47- 27.17)	10.6
Norfluoxetine	Moors	6.51 (0.01)	6.43	1.57	0.06	24.76	13.0

						(21.07- 31.54)	
Norfluoxetine	Barnsley	8.01 (0.09)	7.52	0.96	0.10	9.48 (7.17- 13.72)	5.0

Table 5. 3. Uptake and elimination rate constants, kinetic BAF and the sediment-biota accumulation factor (BASF) of *L. variegatus* exposed to the three API in the two sediments.

5.3.5 Limitations

In our study, we assumed that the radioactivity measured represented the parent compound only and either biotransformation products and metabolites were not quantified. This is mainly due to the limitation of the method, in particular, that the liquid scintillation counter detects the radioactivity without differentiates between parent compounds and metabolites. Thus, without considering the contribution of biotransformation products, an over or underestimation of the internal concentrations could occur. In fact, some studies have found higher internal concentrations of metabolites than parent compounds in invertebrates (Ashauer *et al.,* 2012; Miller *et al.,* 2017).

Lastly, we analyzed the total radioactivity absorbed through the skin and the respiratory system including the contribution of the diet. However, we did not consider the feeding route and the adsorption through the skin separately. In the future, further studies to understand the contribution of the uptake of sediment-associated APIs should be covered to evaluate whether it contributes significantly to the uptake. In the literature, contradictory results underlined a potentially significant difference in accumulation *via* food uptake. For example, in a study conducted by Karlsson *et al.,* (2016) of two pharmaceuticals and one personal care product on *L. variegatus* they found a negligible contribution of the diet for diclofenac and fluoxetine but a considerable difference in the uptake of triclosan between feeding and non-feeding worms. Similar to these findings, Ashauer *et al.,* (2010) assessed the food uptake of two pesticides attached to leaf discs on *G. pulex* and a food contribution of less than 2% was measured in assessing the whole bioaccumulation process leading the authors to conclude that the diet route was negligible.

5.3.6 Pore water analysis

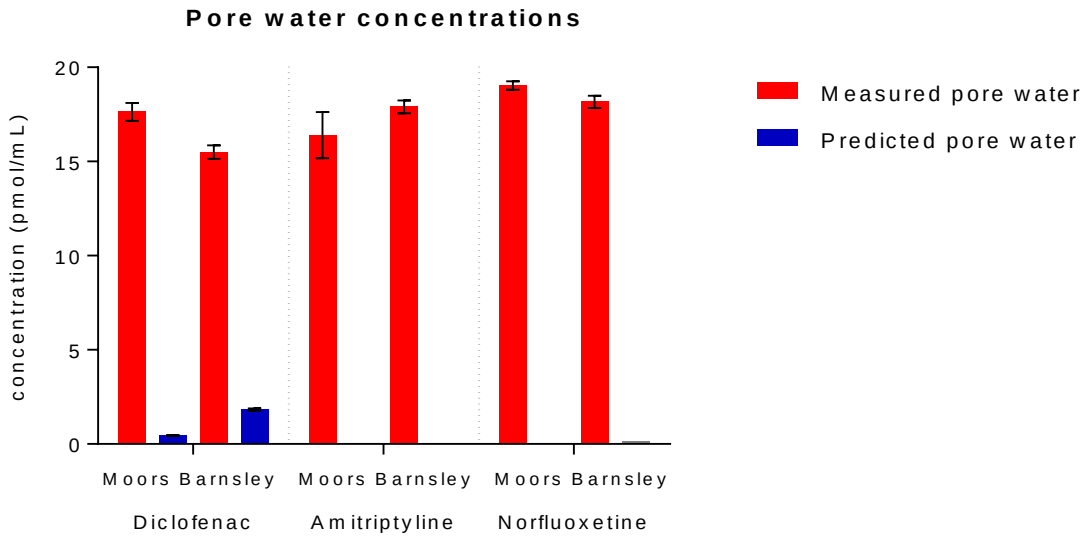


Figure 5. 8. Measured and predicted pore water concentrations of the three APIs in the two sediments. Bar charts represent the mean of three replicates with the standard deviations.

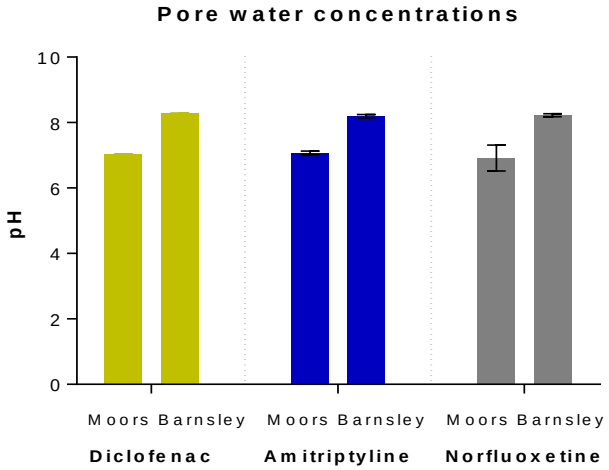


Figure 5. 9. pH measurements of the pore water of Moors and Barnsley sediments. Bar charts represent the mean of three replicates with the standard deviations.

A significant difference between the estimated and measured pore water concentrations were observed (Fig. 5.8). Generally, for all the APIs in the two sediments very low predictions have been obtained using the formula suggested by Karlsson *et al.,* (2017).

The estimated pore water concentrations were obtained by using the K_f values from the batch sorption experiments described in Chapter 4. Also, the bulk density was measured and it has been found to be 0.878 g/cm^3 for Moors and 0.450 g/cm^3 for Barnsley.

Based on these findings, the K_f value may not be appropriate for the estimation of the pore water concentration. We believe that the different conditions and water chemistry of the batch sorption studies and the sediment uptake studies of this chapter could have influenced the underestimation of the predicted pore water concentrations.

Pore water is an important parameter to consider because it could provide a better understanding of the route that contributes the most to the uptake of chemicals in sediment-dwelling worms (Di Toro *et al.,* 1991; Leppänen and & Kukkonen, 1998). However, measuring the pore water of API in the laboratory is challenging and to our best knowledge, not many studies have measured it. For instance, Carter *et al.,* (2016) measured the pore water concentrations of diclofenac, fluoxetine, and orlistat in different soil types and suggested that depending on the soil texture and the cation exchange capacity, the pore water concentrations of the compounds differed due to the soil characteristics and their interaction with the physicochemical properties of the chemicals.

Also, pH measurements of the pore water samples were taken (Fig 5.9), and the results show Barnsley sediment to be more alkaline than Moors, similar to the findings of the uptake and depuration experiments. pH is an important parameter when assessing the uptake of ionisable chemicals from the sediment into worms (Karlsson *et al.,* 2017). However, because of the lack of information about pore water concentrations and, in particular, pH, assessing the real risk that ionisable compounds may pose to benthic invertebrates is difficult. Therefore, we recommend more studies and data in order to establish a more reliable picture of which route is the main contributor to the uptake into benthic invertebrates. In this way, we will be able to evaluate the risk that APIs may pose to non-target organisms dwelling in the sediment compartment.

5.4 Conclusions

Toxicokinetic constants and the kinetic BAFs and BSAFs were derived from worms exposed to contaminated sediment. We demonstrated differences in bioaccumulation of the two basic APIs and one acid into *L. variegatus* and how uptake varies across two sediments possessing diverse properties. In addition, these experiments showed how sediment properties influence the bioavailability of the tested compounds. Among other properties, the pH and the organic content seemed to be the main descriptors to have influenced the uptake to the organisms. Also, the pore water concentrations were measured and compared to the predicted concentrations but a remarkable difference between the measured and predicted concentrations was found. We concluded that the K_f may not be appropriate in describing the pore water concentration of chemicals when assessing the uptake of sediment-dwelling invertebrates. However, analyzing entirely the mechanisms influencing the potential uptake into sediment-dwelling invertebrates is not simple due to the combination of environmental, physicochemical and biological factors that could be involved.

Therefore, we suggest more research regarding this topic, in particular, with more sediments to have a clear picture of the possible parameters that have to be considered for modelling approaches.

Chapter 6. General discussion and conclusion

6.1 Summary of the thesis and results

New data on the fate, uptake and elimination of ionisable active pharmaceutical ingredients in benthic invertebrates have been presented and discussed in this Ph.D. thesis. In the past 20 years, more attention has been dedicated to evaluating the behaviour of ionisable APIs in non-target organisms.

Bioaccumulation is a key criterion as part of the Environmental Risk Assessment process, therefore, many studies have been conducted on the uptake of pharmaceuticals in aquatic and terrestrial organisms like terrestrial earthworms (Carter *et al.,* 2016; Parelho *et al.,* 2018), aquatic invertebrates (Meredith-Williams *et al.,* 2012; Ding *et al.,* 2016), Hedgespeth *et al.,* 2018), sediment organisms (Karlsson *et al.,* 2016; Nieto *et al.,* 2017a) and plants (Li *et al.,* 2018; Kodešová *et al.,* 2019).

Although research regarding this topic is increasing, particularly assessing the uptake of APIs into aquatic invertebrates, the sediment compartment has not been well studied yet. Assessing the sediment uptake of ionisable APIs is important to understand the main routes of exposure of these chemicals into benthic invertebrates. Benthic species are key ecological organisms at the bottom of the food chain and prey of upper trophic organisms such as fishes. Thus, a potential biomagnification process could occur (Lagesson *et al.,* 2016; Haddad *et al.,* 2018). For example, Lagesson *et al.,* (2016) evaluated the trophic transfer of pharmaceuticals from bottom to top predators in the aquatic food web. The organisms chosen for the experiments were a fish species (common perch) and four invertebrates from different habitats and with different diets such as Zygoptera, Ephemeroptera, the waterlouse *Asellus aquaticus* and the snail Planorbidae. The authors found a higher accumulation of the APIs into the benthic species (*Asellus aquaticus* and the snail Planorbidae) than the fish. They suggested that the diet could explain the differences in the accumulation among species. The snail and the waterlouse feed on periphyton which has been found to accumulate pharmaceuticals (Du *et al.,* 2015). However, these studies did not consider environmental properties such as the pH that could influence the uptake among the different species.

Also, not many studies into understanding the trophic transfer of contaminants from prey to a predator have been proposed yet, specifically studies with *L. variegatus*. Further research on the trophic transfer of APIs in the aquatic food web is therefore needed, including sediment-dwelling worms. In this way, we will be able to understand the role of these organisms and the environmental properties that could affect the bioavailability of the APIs and ultimately the uptake and toxicity.

The studies of this Ph.D. thesis, therefore, have started to explore the fate and the uptake of ionisable APIs into sediment-dwelling invertebrates. Laboratory experiments have been conducted in order to elucidate which physicochemical properties and sediment characteristics are important to consider for assessing the bioconcentration and the risk that these chemicals may pose to benthic worms. The main findings of the thesis will be briefly summarized in the following paragraphs.

6.2 Main findings of the experimental chapters

Bioconcentration models available from the literature to calculate the BCF were evaluated (Chapter 2). The BCF models selected for this study included six developed specifically for fish species and two for invertebrates. The predicted and measured BCF were compared to evaluate whether the chosen models could suitably predict the BCF. We observed that all the models showed a low accuracy in predicting BCFs resulting in both over or under-estimation compared to measured values.

The evaluation of current BCF models was important in order to give a broad understanding of which physicochemical properties of chemicals, environmental parameters, and biological traits could be essential for a more accurate estimation of the bioconcentration. We believe that the differences between measured and predicted BCF estimations were due to different factors such as the different test conditions of the studies, different duration of the exposure and the different analytical methods used for the estimation of BCF. For example, for the fish species, the conditions of the experiments varied between the different studies. The majority of the tests performed the “flow-through” experimental design as recommended by the OECD 305, however, some studies performed static, semi-static or renewal experiments (Wang and Gardinali,

2013). The exposure concentrations and the duration could also explain the differences in the estimated BCF: for example, some studies analyzed the bioconcentration into fish species over shorter periods of time (7 days) (Wang and Gardinali, 2013) than others (42 days) (Steinbach *et al.,* 2016). Also, some studies evaluated the contribution of dietary uptake whereas others assessed adsorption through the skin only. Similar variability is seen in tests with with different studied involving: different duration of the test, different concentrations of the API spiked, different environmental conditions (water-only exposure or sediment exposure) and species traits such (Rubach *et al.,* 2012) and different measurements and reporting. For example, some studies reported the lipid content as wet weight and other as dry weight. Variability in the BCF can be attributable to the different lipid content measured among the organisms. Ultimately, the analytical method could lead to uncertainties in the estimation of the BCF. If the study used radiolabelled compounds, variability in determining the BCF could arise due to the fact that with that method is impossible to distinguish between the parent compound and metabolites. Therefore an overestimation could happen (Arnot and Gobas, 2006).

In general, the main findings in Chapter 2 underlined the need to improve BCF models for invertebrates. In fact, only two models were evaluated due to the fact that not many are available in the literature, that have specifically been developed for sediment-dwelling invertebrates. These models are necessary to predict the risk that ionisable APIs may pose to lower trophic organisms. Also, Chapter 2 showed a general overview of the difficulty in estimating the BCF using in-silico models.

In Chapter 3, water-only uptake studies were carried out. The main objective was to test the effect of the water pH on the uptake of ionisable APIs into sediment-dwelling worms. Depending on the degree of ionisation and the pH of the test medium, a compound can be fully or partially ionized and, consequently, its degree of bioaccumulation can vary. For instance, it is well known that the neutral part of a chemical is more bioaccumulative than the ionized counterpart (Anskjaer *et al.,* 2013).

Therefore, in order to test this hypothesis, the worms were exposed to environmentally relevant pH ranges between 5.5 and 9 in APW. Kinetic uptake and depuration rate constants and the BCF were derived (Table 6.1). Different uptake patterns were observed for weak bases and acids separately. For bases (amitriptyline, norfluoxetine

and ketoconazole), uptake increased with the increase in pH. Other studies have shown similar findings. For example, Bittner *et al.,* (2019) found that neutral species of basic antihistamines were taken up at a higher rate than the ionized species resulting in higher toxicity in Zebrafish. The same authors found that β-blockers such as metoprolol and propranolol were accumulated at higher pHs in embryos of zebrafish resulting in more toxic (Bittner *et al.,* 2018).

On the other hand, the BCF calculated for acidic compounds like diclofenac, showed an opposite uptake trend: higher uptake occurring at lower pH values. The results are consistent with other studies found in the literature. For example, the influence of the pH on the toxicity of triclosan to the microalgae *Chlorella ellipsoidea* exposed at different pHs showed that the compound, which is a weak acid, was more toxic at lower pH when present at its undissociated form (Khatikarn *et al.,* 2018).

These results clearly demonstrated the importance of water pH in determining the uptake and toxicity of ionisable organic chemicals. This is also important for regulatory purposes. For instance, when evaluating the potential bioaccumulation capacity of a chemical for the PBT assessment, more attention should be directed to the pH at which the substance is tested (Matthies *et al.,* 2016). For example, it could be useful to test the uptake of the molecule at the pH for which the molecule is expected to be more bioaccumulative. Moreover, based on the threshold values of the bioconcentration criteria for the chemical risk assessment, a substance is considered bioaccumulative (B) when the BCF > 2000 L/Kg and very bioaccumulative (vB) at BCF values > 5000 L/Kg. Therefore, amitriptyline at pH 9 and norfluoxetine at pH 8 would be classified as bioaccumulative and norfluoxetine at pH 5.5 and 9 as very bioaccumulative (EMA, 2018).

Because the tested organism is a sediment-dwelling species, the exposure of APIs from contaminated sediment has to be assessed. Hence, sorption studies were performed (Chapter 4) to understand the degree of sorption of these ionisable APIs to sediments and the available fraction freely dissolved in the water. For this purpose, batch equilibrium sorption studies were carried out with four sediments (Moors, Barnsley, Millington and Earswick). The sediments were selected based on their different properties such as different organic content, pH, CEC and texture. The sorption coefficients of amitriptyline, norfluoxetine and diclofenac increased in the order

diclofenac < norfluoxetine < amitriptyline in Moors sediment and diclofenac < amitriptyline < norfluoxetine in Barnsley, Millington and Earswick sediments. K_{OC} values varied greatly depending on the compound and the sediment. Diclofenac showed remarkably low sorption coefficients for the four sediments compared to the two bases. The K_{OC} ranged between 2.07 Kg/L in Earswick and 22.75 Kg/L in Millington. The sorption of diclofenac was measured in three sediments and low sorption coefficients were observed ranging from 4.5 Kg/L to 19.6 Kg/L as well (Styszko, 2016). Recently a study by Le Guet *et al.,* (2018) investigated the influence of the humic substances of sediments on the sorption behaviour of acidic and basic pharmaceuticals including diclofenac and found that humic substances facilitated the sorption of diclofenac onto the sediment, therefore an increase of the sorption was observed. In our study, the humic substances were not determined and we suggest to include this analysis in future sorption experiments to further evaluate the influence that humic substance may have on other pharmaceuticals.

Higher sorption coefficients were derived for the two bases amitriptyline and norfluoxetine. Amitriptyline was found to be highly absorbed to the two sediments possessing high TOC such as Millington and Barnsley. The same sorption behaviour was observed for norfluoxetine. Also, the contribution of the inorganic and organic components of different sediment types have been investigated by (Yamamoto *et al.,* 2018) and the authors found that the organic matter contributed the most in the adsorption of the basic APIs.

We concluded that the hydrophobic interactions between the sediment and the hydrophobicity of the two compounds were the main factors contributing to the sorption across the sediments.

Other processes potentially involved in the sorption of the bases and acids have been suggested by other authors. For example, for bases, cation exchange capacity was suggested being an important process of sorption to understand the mobility of the compounds in different sediments (Yamamoto *et al.,* 2009; Al-Khazrajy and Boxall, 2016). For acids, electrostatic repulsion between the sorbent and the sorbate could possible explain the low sorption behaviour (Kah *et al.,* 2017). A similar mechanism of sorption behaviour for diclofenac has been described on different river sediments by (Svahn and Björklund, 2015).

In Chapter 5, the uptake of the three ionisable APIs from sediment was investigated. The main objective was to combine the information derived from the previous chapters and have a comprehensive picture of the main sediment properties that influence the uptake of ionisable APIs into benthic worms. For this purpose, two sediments, Moors and Barnsley were selected in order to have sediment with high and low organic content and pH. Kinetic uptake, depuration rate constants and the kinetic bioaccumulation factor (BAF_k) were derived along with the biota-sediment accumulation factor (BSAF). Table 6.2 gives an overview of the sorption coefficients and the uptake parameters collected from Chapters 4 and 5. Different considerations regarding the possible factors influencing the uptake of the organisms will be argued.

For amitriptyline, a considerable higher BSAF was measured for Barnsley sediment than Moors. This might be due to the fact that the Barnsley sediment had a higher pH. Thus, the accumulative power of the compound increased with the increasing of the sediment pH. However, this was not the case of norfluoxetine where we found that the BSAF decreased with the increasing of the organic content of the sediments. As shown in Table 6.2, the BSAF was higher in the Moors sediment, which contained low organic content than Barnsley. Hence, we believe that in the Moors sediment, norfluoxetine was more freely bioavailable to be taken up by the worms and consequently more bioaccumulative.

Regarding diclofenac, higher BASF was found for the Moors sediment than Barnsley. Here again, the results are in agreement with chapter 3 where greater uptake of diclofenac was observed at lower pH values.

Assessing the uptake of sediment-associated chemicals is not easy due to the diverse composition of the sediment, the fate of the chemicals between the water and the sediment phase and the different physicochemical properties of the chemicals. The influence of sediment characteristics on the bioaccumulation of chemicals into *L. variegatus* has been argued by other authors who exposed the organisms to pesticides, (Mäenpää *et al.,* 2008) polycyclic aromatic hydrocarbons (Sheedy *et al.,* 1998) and pharmaceuticals (Liebig et al., 2005). Different sediment characteristics were suggested to influence the bioavailability of sediment-associated chemicals; for example, the bioaccumulation of sediment-associated herbicides in *L. variegatus* was investigated and the results showed that the bioaccumulation into the organisms was greater in the organisms exposed in the sediment containing less organic matter, thus, in the sediment

with the higher bioavailability of the compound to be taken up by the worms (Maënpaä¨*et al.,* 2003). The pH has been found to affect the uptake into the worms by a recent study published by Karlsson *et al.,* (2017) where differences in the uptake into the worms was observed between the higher pH (8.5) value and the lower pH value (5.5) of diclofenac and fluoxetine.

However, although *L. variegatus* is used a standard test organism for sediment toxicity tests (Egeler *et al.,* 2010; Gilroy *et al.,* 2012); uptake studies from the sediments of ionisable pharmaceuticals are still very limited and the study conducted in Chapter 5 is one of its kind in trying to interpret the influence of sediment characteristics on the bioaccumulation of ionisable sediment-bound pharmaceuticals.

Pore water concentrations of the APIs are another important source of uptake for benthic organisms (Leppänen and & Kukkonen, 1998; Sidney *et al.,* 2016). In our study, the pore water concentrations were estimated with a separate test from the sediment uptake experiments. The concentrations were compared to the predicted concentrations using a formula suggested by Karlsson *et al.,* (2017) and we noticed a remarkable difference between predicted and measured values. Pore water is an important exposure route, however, due to the difficulty of obtaining the data, the manipulation and the method extraction that could change the original matrix. The pore water should be analyzed in combination with the other exposure routes that could influence more the availability of sediment-associated chemicals (Chapman *et al.,* 2002). Therefore, we suggest a reliable method to estimate the pore water for bioaccumulation study in order to understand to what extent the pore water contributes largely to the uptake of APIs into the sediment-dwelling worms.

In conclusion, our experimental findings produced knowledge on the fate and the uptake of ionisable pharmaceuticals in the water-sediment compartment. We attempted to explain some environmental properties such as the pH and its influence on the uptake into the worms, the sorption behaviour of the APIs and the interactions that these APIs, possessing different physicochemical properties, had with some sediment characteristics toward the organisms.

Recently, a novel modelling uptake approach has been proposed to reliably estimate internal concentrations of ionisable APIs to the sediment-dwelling worm *L. variegatus* (Karlsson *et al.,* 2017).

In the next section, this model will be applied in order to test the applicability of this approach with the experimental data generated in this thesis and the wider implications will be discussed.

Compound	BCF $_{(L/Kg)}$ pH 5.5	BCF $_{(L/Kg)}$ pH 7	BCF $_{(L/Kg)}$ pH 8	BCF $_{(L/Kg)}$ pH 9
Amitriptyline	146	391	653	2284
Diclofenac	353	81	13	3.78
Norfluoxetine	1.06×10^7	870	2902	1.11×10^4
Ketoconazole	93	100	101	211

Table 6. 1. Summary of the BCFs at the different pHs of the four APIs measured in Chapter 3.

	Compound	K_F (mg/Kg) × (mg/L)	K_{OC} L/Kg	pH sediment	BAF_K (g/mL)	BSAF (g sediment/g worms lipid content)
Moors	Amitriptyline	510.4	30946.6	6.43	27.5	14.4
	Diclofenac	14.43	694.7	6.43	32.73	17.2
	Norfluoxetine	498.75	48131	6.43	24.76	13.0
Barnsley	Amitriptyline	1489.4	5846.5	7.52	21.24	201.9
	Diclofenac	9.75	41.8	7.52	20.14	10.6
	Norfluoxetine	1681.12	9455.1	7.52	9.48	5.0

Table 6. 2. Summary of the sorption coefficients and the uptake parameters for the three APIs measured in Chapter 4 and 5.

6.3 Applicability of the experimental data to a novel pH-dependent uptake modelling approach

The model proposed by Karlsson *et al.,* (2017) was developed to estimate the uptake of sediment-associated ionisable compounds to benthic invertebrates. The model is based on the first-order one-compartment toxicokinetic model where the uptake rates of the ionized ($k_{in\text{-}ion}$) and unionized part of the molecule ($k_{in\text{-}neu}$) and the depuration rate (k_{out}) have to be derived by using the experimental data of the water-only uptake studies (Chapter 3). These rates are shown in Table 6.3. First, to obtain these parameters, the fraction of ionisation of the chemical (ʃion) has to be estimated based on the pH and the pKa by using the equation of Henderson-Hasselbach. Then, the ionic uptake rate and depuration rate were derived by using the experimental data from the water-only uptake studies where each molecule was fully dissociated. For example, for amitriptyline and norfluoxetine at pH 5.5, 100% of the molecules are dissociated. For diclofenac, the dissociated uptake rate at pH 9 was derived first as at this pH value, the molecule is 100% unionized. Then, the data of the test with the most deviating pH were used to derive the other uptake rate.

Ultimately, we evaluated whether it was possible to estimate the internal concentrations into the worms (Figs. 6.1, 6.2, 6.3) by using the overlying water concentrations from the sediment-uptake studies (Chapter 5).

The goodness of fit between the modelled and measured internal concentrations was calculated using the Nash Sutcliffe value. If the value > 0, the fit is considered acceptable; if the value < 0, the fit is unacceptable.

	amitriptyiline	norfluoxetine	diclofenac
Measured water pH			
Water pH at 5.5	4.52-7.11	5.51-5.69	5.39-6.46
Water pH at 9	8.28-9.34	8.83-9.20	8.52-9.22
F_{ion} used for the modelling			
Water pH at 5.5	1	1	0.025
Water pH at 9	0.70	0.40	0
Rate constants derived by the model			
$K_{in\text{-}neu}$ ($L \times Kg^{-1} \times h^{-1}$)	229.5	66.2	0.37
$K_{in\text{-}ion}$ ($L \times Kg^{-1} \times h^{-1}$)	8.0	1.066	995
K_{out} (h^{-1})	0.096	1×10^{-7}	0.097

Table 6. 3. Water conditions, fraction of ionisation and the derived uptake and depurations constants of the three APIs by using the model proposed by Karlsson *et al.,* (2017).

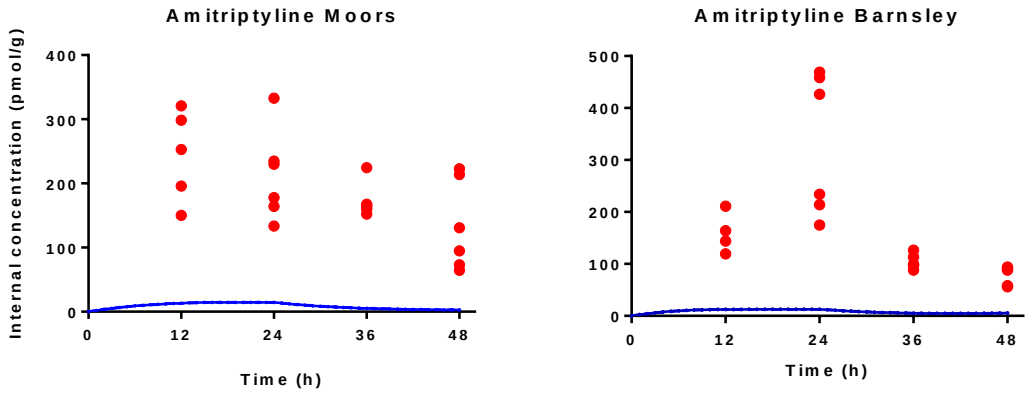


Figure 6. 1. Model evaluation of amitriptyline in sediment-exposed *Lumbriculus variegatus*. The red dots represent the internal concentrations measured in the worms (pmol/g wet weight). The blue thick line represents the model fit curves and the dashed blue lines the 95% confidence interval of the model fit.

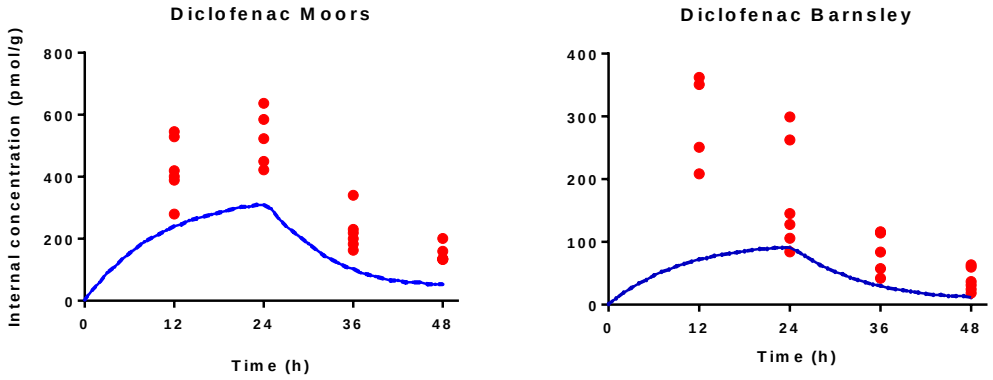


Figure 6. 2. Model evaluation of diclofenac in sediment-exposed *Lumbriculus variegatus*. The red dots represent the internal concentrations measured in the worms (pmol/g wet weight). The blue thick line represents the model fit curves and the dashed blue lines the 95% confidence interval of the model fit.

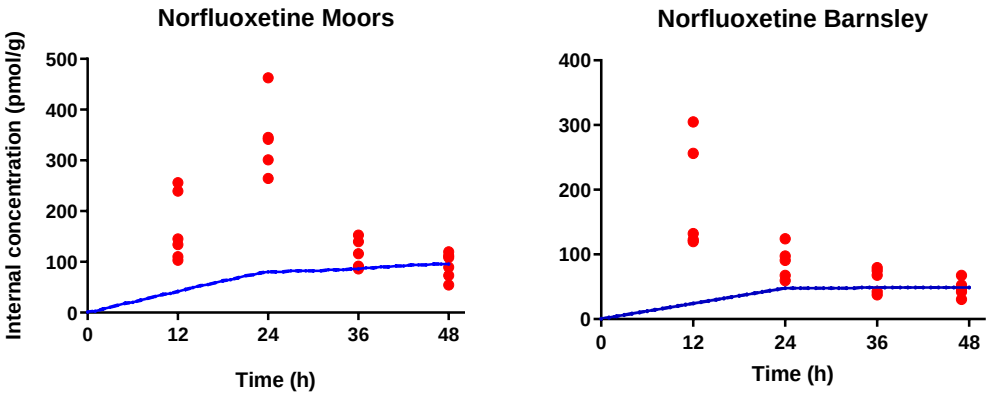


Figure 6. 3. Model evaluation of norfluoxetine in sediment-exposed *Lumbriculus variegatus*. The red dots represent the internal concentrations measured in the worms (pmol/g wet weight). The blue thick line represents the model fit curves and the dashed blue lines the 95% confidence interval of the model fit.

pH	amitriptyline	norfluoxetine	diclofenac
9	-13.7	0.22	0.65
7.52	-1.8	-1.0	-0.11
6.43	-6.4	-1.9	-0.12
5.5	0.93	0.83	-0.13

Table 6. 4. Nash Sutcliffe values for the model fit at the different pH values. The pH values 5.5 and 9 were analyzed from the water-only uptake tests (chapter 3). The pH values 7.52 and 6.43 were analyzed from the sediment uptake tests (chapter 5).

Even though the Nash Sutcliffe values are slightly negative (Table 6.4), comparisons of the model predictions with the measured internal concentrations into the worms showed that the model worked quite well for diclofenac and norfluoxetine. In fact, for diclofenac, the approach underpredicted the actual accumulation by a factor of 2 in Moors sediment but a reasonably good fit was found for Barnsley sediment. For norfluoxetine, the model underestimated the uptake data by a factor of 4 for the Moors sediment but a good fit for the depuration data was seen. However, amitriptyline results showed a remarkable underprediction for both sediments. These results may be explained by the sediment ingestion of the worms which was not investigated in this work. A previous study by Karlsson *et al.,* (2016) demonstrated the importance of the dietary uptake when comparing worms without the head and worms with the head. In the future, additional experiments with these compounds and the worms should be done to further understand the contribution of the ingestion of sediment particles. For example, using the method suggested by Karlsson *et al.,* (2016) it would be possible to better understand whether the particle ingestion is a major contribution to the uptake into the worms.

Although there is a mismatch between model predictions and measured observations of amitriptyline, the results of diclofenac and norfluoxetine are quite promising. In fact, with this approach, it is possible to predict the internal exposure to an acceptable degree of accuracy based on the concentrations of the API in the overlying water, the degree of ionisation and the water pH.

Diclofenac was also used in the model development by Karlsson *et al.,* (2017). The authors found that the comparisons between modelled predictions and measured data were less than a factor of 5. Similar results were reported for fluoxetine. Better predictions were observed for our results. However, in our study, we employed the model using the overlying water concentrations of the chemicals instead of the pore water concentrations as they suggested. Also, the different sediment characteristics may have contributed to those differences.

We tested the general applicability of the model with new data and sediments and the results seem quite promising. With this approach, it was possible to estimate the internal concentrations in the organisms with reasonably good predictions. In the future, we suggest to further explore the model with more molecules and sediments.

6.4 Implication for the Environmental Risk Assessment

Overall, the research presented in this thesis shows that APIs are taken up by non-target organisms and therefore they could potentially cause adverse effects to them. Bioconcentration is an essential parameter included in the PTB assessment of the European Medicine Agency (EMA) guidelines for medicinal products. However, experimental bioconcentration tests are required for fish species only and no tests with lower trophic species are considered. For this purpose, the OECD guideline 305 is recommended; however, the number of fish required, the time in the laboratory and the cost of the equipment is demanding. Therefore, the use of models to predict the bioconcentration has been proposed to overcome the use of the OECD guidelines. Some of them have been evaluated in Chapter 2. These models attempted to combine physicochemical, environmental parameters and biological traits to better estimate the accumulation of APIs in fishes and few in invertebrates. However, the majority of the model failed in predicting the BCF.

Recently, new modelling approaches have been proposed such as machine learning methods to extend the classical linear regression QSAR models and still trying to substitute the use of animals used to experimentally determined the BCF/BAF of a chemical. For example, machine learning methods were employed to predict the BCF in fish species by using classification trees and random forest on over 700 chemicals

(Strempel et al., 2013). The authors found a good prediction of bioaccumulation and with this novel approach they were able to understand also which physicochemical descriptors play a key role in determining the BCF in fish species. For instance, they found that the main important descriptors were the LogD (octanol-water partitioning distribution), the LogBioHL (logarithmic of the degradation kinetic by biotransformation half-life) and TPSA (polarity by topological polar surface area). More recently, the applicability of machine learning techniques has been extended to invertebrates (Miller et al., 2019). In this latter study, the authors applied machine learning models to one fish species (*Ciprinis carpio*) and a freshwater invertebrate (*Gammarus pulex*). Here again, they found good prediction performance and they also listed important descriptors such as MW (molecular weight), LogD, TPSA (topological polar surface area) and nN (numbers of nitrogens). The use of these new models is complex but advantageous. In fact, they can reliably estimate BCF, they specifically indicate the most important descriptors for a certain organism and they offer a rapid alternative to the linear relationship models-based.

In addition, these models could be integrated into the European guidelines and ultimately could be expanded to other species such as in this case to oligochaete. In fact, due to the lack of the data available for invertebrate species in the literature and the need of these data for regulators, the potential applicability of these novel approaches could offer an alternative due to their flexibility and the relatively low cost for first screening in the PTB assessment.

Risk assessment

In the European regulations such as the European Medicine Agency (EMA), the PTB assessment is included and in the case of substances possessing the $\log K_{ow} > 4.5$, the ECHA guideline should be followed for testing the bioaccumulation potential of substances in fish species and other aquatic organisms such as invertebrates (ECHA, 2017).

In Chapter 3, the role of the pH on the uptake of ionisable substances was investigated. The Bioconcentration factor (BCF) was observed to vary remarkably between the lowest pH value (pH =5.5) and the highest pH value (pH =9) for all the

APIs, Table 1. These changes are likely to affect the toxicity on benthic organisms and studies in the literature have demonstrated the relationship between the pH and toxicity to non-target species (Nakamura *et al.,* 2008b; Anskjaer *et al.,* 2013; Boström and Berglund, 2015). For example, recently, Scott *et al.,* (2019) evaluated the influence of pH on the fish species *Fundulus grandis* of three weak bases pharmaceuticals, carbamazepine, diltiazem, and diphenhydramine, at two pH values (8.3 and 6.7). They found a significant difference between the bioconcentration of the APIs into the fish between the two pH values suggesting the pH as a key parameter to consider when assessing the uptake of ionisable chemicals.

Therefore, for standard toxicity tests suggested by the European guidelines, we suggest that careful attention should be pointed to the pH values at which the organisms are exposed to the substance that it elicits the greatest uptake and therefore toxicity. Otherwise, an under or an over-estimation of the risk could occur. The data reported in Chapter 5 could be useful to interpret the transfer of the APIs to higher trophic levels and therefore assess the risk of secondary poisoning to predators such as fish species that feed on lumbricids in their diet (Diepens *et al.,* 2014).

In this chapter, we evaluated a new modelling approach that predicts the uptake of ionisable compounds into benthic invertebrates. The model is the first of its kind in trying to combine physicochemical properties and sediment characteristics for estimating the uptake into benthic invertebrates. The approach that we used was slightly different from the approach illustrated by the authors. However, the model worked quite well for some compounds (diclofenac and norfluoxetine). With this method, it was possible to predict the internal exposure within a factor of 2 for diclofenac and within a factor of 4 for norfluoxetine. We suggest that further improvement of the model should be done with more sediments. This could help in understanding if other sediment properties are involved in the uptake and, in a longer-term, the model could be an important tool in generating data on the uptake of ionisable compounds in other aquatic invertebrates.

6.5 Recommendations for future work

The research presented in this PhD thesis produced new insights into the uptake and fate of ionisable APIs into a sediment-dwelling worm *L. variegatus*.

Nevertheless, some areas of research need to be expanded and some questions have to be addressed in the future. In the following paragraphs, these questions will be briefly discussed.

1. In the current studies, a single species was used in order to have a clear picture of the relationship between physicochemical properties of the compounds (pK_a) and environmental properties (pH, OC). Although the range of molecules tested was limited, in the future, the species traits could be included in the analysis. In this way, we suggest additional species could be used enabling to evaluate the difference in uptake in different invertebrates (Rubach et al., 2012). For example, we suggest organisms that live in the water column only such as *Hyalella Azteca* or *Gammarus pulex* that have a different feeding habit and habitat. Another species that could be used is *Daphnia magna* which is easy to cultivate as the above mention organisms and for which there are available data in the literature. Also, the insect *Chironomus riparius* could be tested to evaluate other species traits such as mode of respiration.
2. The two main pathways of uptake of chemicals into benthic invertebrates are *via* the skin and the diet (Lappänen and & Kukkonen, 1998). It would be interesting to assess the diet exposure. Work is needed on a broader range of molecules to really understand relationships between properties and dietary uptake. This work is fundamental and it might be needed to understand the relationship that we can then integrate into models.
3. The experimental tests in this work used radiolabelled compounds and due to instrument limitations, we assumed the fate and uptake of the parent compounds only. The analysis of the metabolites has been omitted. Therefore, another future work would be the investigation of the transformation products of the chosen APIs by employing new techniques such as the TOF-MS.

4. This research demonstrated that linear regression models proposed in the literature failed in estimating the BCF. For benthic invertebrates that are submerged in the sediment, the environmental properties like pH and TOC have to be accounted for along with physicochemical properties as pK_a. The model proposed by Karlsson *et al.,* (2017) tried to link these factors but, because of the complexity of the uptake, a refinement of the model is needed. A possible solution could be the novel machine learning techniques that are rapid and cost-effective in determining the BCF and illustrating which factors are important to take into considerations. However, the need of data to run the models is necessary. For example, more BCF data on lumbricids on a large range of molecules would be required where these substances are tested at more than one pH value in order to estimate the bioconcentration at different pH ranges. Also, other physicochemical data could be selected such as the pK_a. For ionisable chemicals, it is an important parameter to take into consideration and few experimental measurements of the pK_a are available in the literature and the majority of the studies use estimated tools. If the pK_a could be measured, it would be a more reliable data.
5. In our research, the pore water concentrations were not measured along with the uptake experiments from sediments over time. This was due to the difficulty of properly handling the samples in the laboratory and the potential loss of part of the sediment. We also found a large difference between measured and predicted pore water concentrations. Thus, future work could be the development of an analytical method able to measure the pore water in the exposure beakers to better comprehend the contribution of the pore water as another route of exposure.
6. It would be worth assessing the impact that these compounds may have on the lumbricids. With this thesis, we demonstrated that the worms accumulate the APIs, hence, the internal concentrations could be linked with the effects. For example, the effects of ionisable compounds have been demonstrated in the literature on several invertebrate species (Nieto *et al.,* 2017b; Liu *et al.,* 2017; Nkoom *et al.,* 2019) and information on *L. variegatus* is needed to better understand the toxicity.

Appendices

Appendix A – Supporting Data for Chapter Two

Evaluation of models for estimating the bioconcentration factor of ionisable compounds

Name	CAS number	Class	LogKow	Reference	pKa	Reference
2,4-DB (4-(2,4-dichlorophenoxy)butyric acid	94-82-6	Herbicide	3.6	EPI Suite	7.8	(Kishino and Kobayashi, 1995)
Atenolol	29122-68-7	β-blocker	-0.03	EPI Suite	9.6	(Steinbach et al., 2014)
5-Fluorouracil	51-21-8	Anti-cancer	-0.81	EPI Suite	8.02	DrugBank
Asenapine maleate	85650-56-2	Antipsychotic	4.77	EPI Suite	8.6	iPiE database
Asunaprevir	630420-16-5	Anti-hepatitis C	3.42	iPiE database	4.8	iPiE database
Beclabuvir Hydrochloride	958002-36-3	Antiviral	6.11	EPI Suite	4.6/6.8	iPiE database
Bentazone	25057-89-0	Herbicide	2.34	EPI Suite	3.3	(Maënpaä¨ et al., 2003)

Buprenorphine	524885-79-7	Analgesic	3.11	iPiE database	8.3	iPiE database
Carvedilol	72956-09-3	β-blocker	3.05	Williams et al., 2012	8	(Meredith-Williams et al., 2012)
Ceritinib	1032900-25-6	Anticancer	5.1	EPI Suite	10.5	ACD I lab
Chloramphenicol	56-75-7	Antibiotic	0.92	EPI Suite	8.6	Karlsson et al., 2015
Dabrafenib mesylate	1195768-06-9	Anticancer	3.93	iPiE database	6.6	iPiE database
Daclatasvir Dihydrochloride	1009119-65-6	Antiviral	4.67	iPiE database	4.9/5.6	iPiE database
Dasanatib	302962-49-8	Anticancer	1.1	EPI Suite	3.1/6.8	iPiE database
Diphenhydramine	58-73-1	Anti-histamine	3.11	EPI Suite	9	Nichlos et al., 2015
Diazepam	439-14-5	Sedative	2.7	EPI Suite	3.4	DrugBank
Diltiazem	42399-41-7	Calcium channel blocker	2.79	EPI Suite	7.7	SRC, 2004

Diclofenac	15307-86-5	Nonsteroidal anti-inflammatory drug	4.02	EPI Suite	4.1	SPARC
Diclofenac sodium	15307-79-6	Nonsteroidal anti-inflammatory drug	4.02	EPI Suite	3.9	iPiE database
Eltrombopag	496775-61-2	Antithrombosis	6.15	EPI Suite	1.7/ 5.9	iPiE database
Fluoxetine	54910-89-3	Anti-depressant	4.65	EPI Suite	10.01	Nakamura et al., 2008
Formoterol	73573-87-2	β-antagonist	1.4	EPI Suite	9.81	DrugBank
Ibuprofen	15687-27-1	Nonsteroidal anti-inflammatory drug	3.79	EPI Suite	4.4	Syracuse Research Corporation
Ioxynil	1689-83-4	Herbicide	3.94	EPI Suite	3.96	Tomlin, 1994.
Imipramine	50-49-7	Anti-depressant	5.01	EPI Suite	9.2	DrugBank
Lapanitib	388082-78-9	Anticancer	5.29	EPI Suite	5.7	ACD I lab
Metoprolol	37350-58-6	β-blocker	1.69	EPI Suite	9.68	Escher et al., 2017

Mirtazapine	61337-67-5	Antidepressant	2.78	iPiE database	7	iPiE database
Moclobemide	71320-77-9	Antidepressant	1.16	EPI Suite	6.2	IPCS InCHEM database
Naproxen	22204-53-1	Anti-inflammatory	3.36	Karlsson et al., 2015	4.5	Karlsson et al., 2015
Nilotinib hydrochloride	923288-90-8	Anticancer	3.6	iPiE database	2.5/4.2	iPiE database
Norfluoxetine	54910-89-3	Anti-depressant	4.65	EPI Suite	9.8	DrugBank
Pentachlorophenol	87-86-5	Insecticide	4.74	EPI Suite	4.74	Ashauer et al., 2006
Posaconazole	171228-49-2	Antifungal	2.59	iPiE database	3.6/4.6	iPiE database
Propanolol	13013-17-7	β-blocker	2.6	EPI Suite	9.53	Ding et al., 2016
Ranitidine	66357-35-5	Anti-histamine	0.29	EPI Suite	8.08	DrugBank
Roxithromycin	80214-83-1	Antibiotic	2.75	EPI Suite	8.8	Ding et al., 2016
Salicylic acid	69-72-7	Anti-acne product	2.30	Karlsson et al., 2015	3.1	Karlsson et al., 2015

Sulfadiazine	68-35-9	Anti-bacterial	-0.09	Anskjær et al., 2013	6.5	Anskjær et al., 2013
Telcagepant	781649-09-0	Antinflammatory	3.44	EPI Suite	4.2	ACD I lab
Terbutaline	46719-29-3	β-antagonist	0.67	EPI Suite	9.76	DrugBank
Triclosan	3380-34-5	Anti-bacterial	5.42	Karlsson et al., 2015	8.1	SPARC

Table A. 1. List of the chemicals and their physicochemical characteristics recorded in the database for estimating the BCF.

Organisms	Water content (%)	Lipid weight (%) wet weight	Internal pH
Fish species			
Japanese medaka	85.2	4.3	7.5
Mosquito fish	65.97	1.56	7.5
Oncorhynchus mykiss	76.3	2	7.3
Fathed minnow	65.97	2.9	7.5
Lepomis macrochirus	71.3	4.02	7.5
Invertebrate species			
Gammarus pulex	80	1.34	8
Lumbriculus variegatus	62.8	1.2	8
Daphnia magna	97.8	0.94	8.44
Notonecta glauca	72.26	3.81	7.5
Planobarius corneus	76	2	7

Table A. 2. Specific biological traits of both fish and invertebrate species' order or class obtained from the open literature.

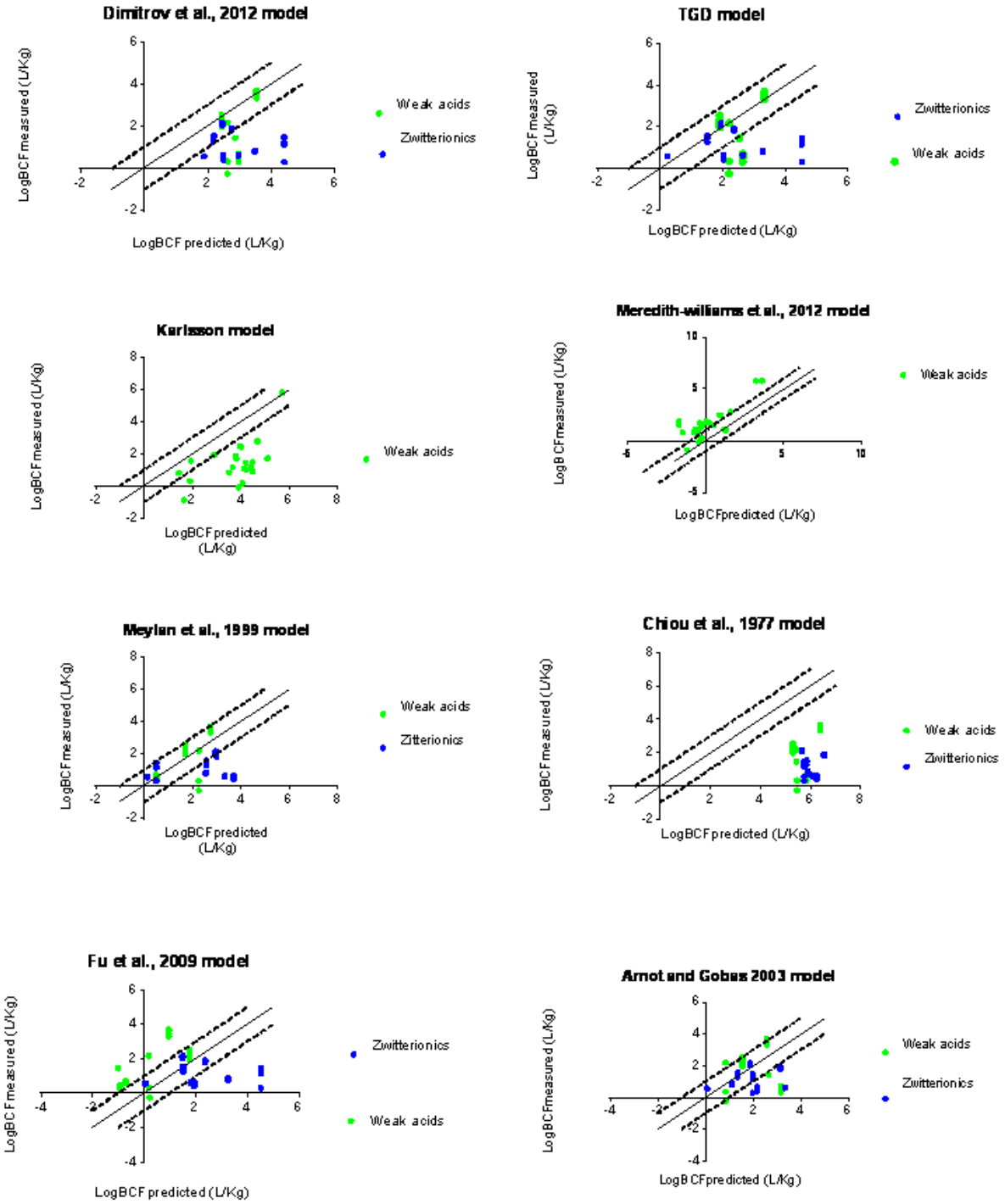


Figure A. 1. Models evaluated exploring the relationships of predicted BCF and measured BCF data for acids and zwitterions.

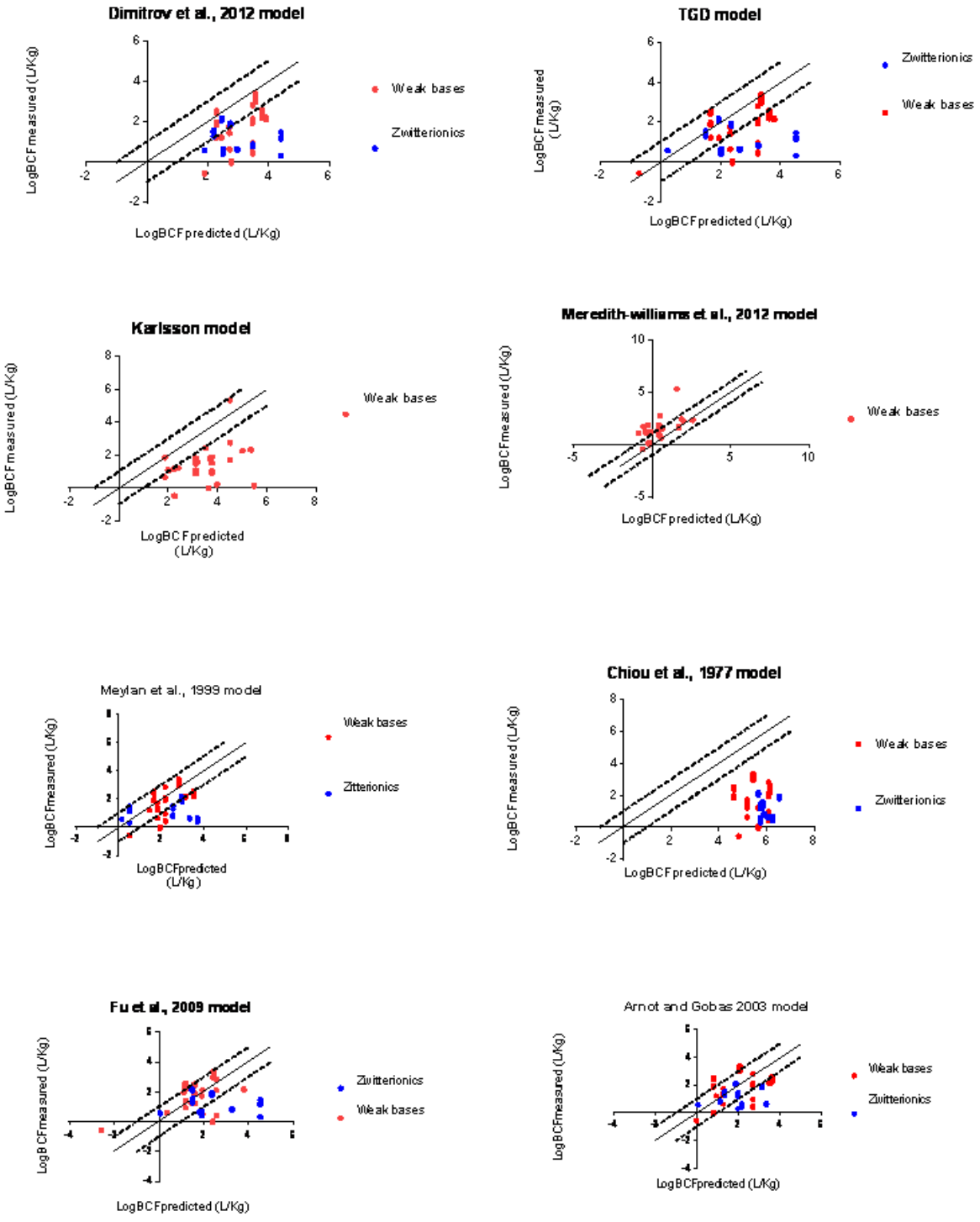


Figure A. 2. Models evaluated exploring the relationships of predicted BCF and measured BCF data for bases and zwitterions.

Appendix B – Supporting Data for Chapter Three

Influence of pH on the uptake of ionisable active pharmaceuticals ingredients (APIs) into the sediment-dwelling worm (*L. variegatus*)

Model code

The toxicokinetic model used is an OpenModel available from the University of Nottingham (https://www.nottingham.ac.uk/environmental-modelling/OpenModel.htm).

Initial:

- $C_{int} = 0$

Main:

- C_{water} = Water.$C_{water}(t)$
- C_{int}.rate = k_{in}x C_w – k_{out}x C_{int}

Indipendent variable options:

- Symbol: t
- Start: 0
- Stop: 48
- Output steplength: 1.

ODE Options:

- Method: Euler;
- Steptlength options: automatic.

Automatic Steplength:

- Error facto: 0.01
- Min steplength: 1E-12.

Merit function options:

- Squared deviations
- No weights

Settings for the Markov Chain Monte Carlo method

- Stopping rule: fixed iterations
- Fixed iterations: 10000
- No improvement steps: 1000
- Threshold no improvement: 0.01
- Maximum errors: 1000
- Maximum steps: 10000
- Proposing distribution: adapting proposal.

Data for the model

The dataset consists of concentration of the water (pmol/ml), concentrations in the worms (pmol/g wet weight) over time (hours) and mass of the worms (g).

Amitriptyline pH 5.5

Time (h)	C_{water} (pmol/ml)	Time (h)	C_{int} (pmol/g)	Mass of the worms(g)
0	39.15	12	2482.6	0.038
12	26.54	12	2483.6	0.028
24	22.09	12	1827.3	0.043
24.1	2.00	12	2097.3	0.034
36	6.33	12	2351.6	0.031
48	6.75	12	2851.9	0.037
		24	2607.1	0.023
		24	3333.1	0.026
		24	2239.2	0.043
		24	2260.7	0.051
		24	2208.6	0.035
		24	2705.8	0.043
		36	1720.3	0.039
		36	1196.1	0.046
		36	1514.1	0.020
		36	1425.7	0.037

		36	1279.3	0.03
		36	1957.2	0.03
		48	2604.9	0.025
		48	2615.9	0.029
		48	1360.2	0.023
		48	1697.6	0.048
		48	1344.5	0.032
		48	1352.5	0.029

Amitriptyline pH 7

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	33.19	12	5782.0	0.022
12	19.71	12	4594.7	0.027
24	19.05	12	4633.1	0.025
24.1	0.25	12	3841.6	0.034
36	5.21	12	4433.0	0.025
48	6.26	12	4812.8	0.025
		24	5652.8	0.021
		24	3983.5	0.030
		24	5711.1	0.024
		24	5916.5	0.020
		24	6918.8	0.019
		24	7528.5	0.014
		36	3539.9	0.025
		36	3763.6	0.021
		36	3007.7	0.014
		36	4160.4	0.016
		36	3951.1	0.024

		36	3761.6	0.015
		48	3181.1	0.017
		48	4726.1	0.018
		48	2677.1	0.021
		48	3267.1	0.020
		48	3567.1	0.021
		48	3945.4	0.022

Amitriptyline pH 8

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	37.93	12	5612.3	0.022
12	21.21	12	7193.8	0.021
24	17.94	12	7185.4	0.017
24.1	1.29	12	6244.0	0.021
36	4.14	12	5698.5	0.024
48	7.13	12	6086.8	0.020
		24	9758.8	0.015
		24	7286.5	0.023
		24	7715.8	0.022
		24	6234.7	0.026
		24	7847.4	0.021
		24	7877.3	0.025
		36	6331.1	0.020
		36	6431.6	0.019
		36	7451.7	0.014
		36	6604.0	0.017
		36	5232.6	0.026
		36	6080.4	0.023
		48	5679.8	0.018

		48	5343.6	0.020
		48	6083.0	0.015
		48	5606.6	0.013
		48	3662.6	0.029
		48	6086.4	0.019

Amitriptyline pH 9

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	40.01	12	8680.6	0.027
12	8.48	12	8584.8	0.033
24	3.65	12	7225.0	0.034
24.1	0.28	12	10072.3	0.025
36	2.00	12	7536.2	0.038
48	2.37	12	7445.0	0.036
		24	7409.0	0.043
		24	6894.7	0.050
		24	5948.8	0.059
		24	8769.0	0.036
		24	10160.3	0.031
		24	7424.6	0.037
		36	8675.2	0.040
		36	8486.2	0.038
		36	7762.2	0.045
		36	5769.3	0.055
		36	9022.4	0.037
		36	7515.9	0.044
		48	6370.5	0.048
		48	8714.0	0.034
		48	7130.4	0.046

		48	8534.7	0.036
		48	7409.8	0.045
		48	7322.8	0.038

Diclofenac pH 5.5

Time (h)	C_{water} (pmol/ml)	Time (h)	C_{int} (pmol/g)	Mass of the worms(g)
0	45.73	12	5486.84	0.026
12	21.67	12	3629.91	0.035
24	23.13	12	4106.37	0.051
24.1	1.02	12	3588.33	0.043
36	4.03	12	4810.00	0.030
48	6.17	12	3289.78	0.035
		24	7437.7	0.030
		24	5023.2	0.032
		24	5568.8	0.026
		24	6283.2	0.031
		24	7842.8	0.023
		24	5407.2	0.033
		36	2785.6	0.035
		36	4264.0	0.035
		36	4264.0	0.042
		36	2319.3	0.025
		36	5743.8	0.024
		36	2497.7	0.027
		48	3550.1	0.023
		48	2900.9	0.028
		48	2842.8	0.019
		48	2842.8	0.035
		48	3887.6	0.032

		48	3641.7	0.035

Diclofenac pH 7

Time (h)	C_{water} (pmol/ml)	Time (h)	C_{int} (pmol/g)	Mass of the worms(g)
0	33.20	12	1263.08	0.043
12	30.05	12	1521.15	0.047
24	28.73	12	1340.73	0.034
24.1	1.20	12	1698.06	0.041
36	3.47	12	905.49	0.042
48	4.68	12	1815.83	0.033
		24	2063.9	0.036
		24	2011.2	0.043
		24	3282.0	0.028
		24	1357.4	0.042
		24	2025.2	0.036
		24	2509.1	0.036
		36	716.9	0.03
		36	970.7	0.039
		36	970.7	0.023
		36	569.6	0.041
		36	766.3	0.051
		36	633.6	0.056
		48	572.8	0.024
		48	767.6	0.033
		48	1205.4	0.039
		48	1205.4	0.018
		48	304.1	0.036
		48	547.0	0.022

Diclofenac pH 8

Time (h)	C_{water} (pmol/ml)	Time (h)	C_{int} (pmol/g)	Mass of the worms(g)
0	34.07	12	427.6	0.036
12	28.70	12	272.9	0.032
24	28.12	12	324.2	0.041
24.1	3.06	12	454.3	0.037
36	3.41	12	359.2	0.037
48	2.92	12	391.5	0.045
		24	293.5	0.041
		24	324.6	0.041
		24	302.7	0.042
		24	374.5	0.050
		24	297.9	0.036
		24	300.9	0.037
		36	171.3	0.048
		36	121.2	0.052
		36	129.4	0.043
		36	126.0	0.029
		36	138.1	0.028
		36	145.2	0.034
		48	68.2	0.041
		48	130.3	0.029
		48	138.7	0.031
		48	145.5	0.034
		48	105.6	0.034
		48	125.8	0.037

Diclofenac pH 9

Time (h)	C_{water} (pmol/ml)	Time (h)	C_{int} (pmol/g)	Mass of the worms(g)

0	45.60	12	124.3	0.033
12	28.33	12	110.6	0.055
24	32.62	12	135.04	0.036
24.1	1.70	12	92.69	0.040
36	1.70	12	108.81	0.043
48	2.94	12	91.06	0.045
		24	96.27	0.052
		24	111.22	0.048
		24	70.1	0.034
		24	82.2	0.036
		24	74.0	0.032
		24	101.0	0.035
		36	32.0	0.021
		36	45.4	0.037
		36	47.3	0.028
		36	37.9	0.031
		36	52.9	0.043
		36	36.7	0.034
		48	30.1	0.031
		48	37.4	0.037
		48	32.6	0.031
		48	43.8	0.040
		48	37.3	0.027
		48	29.8	0.043

Ketoconazole pH 5.5

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	14.55	12	422.61	0.023
12	12.44	12	345.42	0.018

24	11.83	12	588.06	0.027
24.1	0.28	12	812.39	0.022
36	0.18	12	563.80	0.023
48	0.40	12	755.09	0.028
		24	830.16	0.027
		24	916.25	0.031
		24	579.05	0.026
		24	762.30	0.033
		24	811.56	0.034
		24	445.38	0.026
		36	383.68	0.031
		36	361.07	0.030
		36	646.43	0.033
		36	553.20	0.028
		36	489.71	0.036
		36	200.56	0.025
		48	379.69	0.029
		48	379.69	0.030
		48	440.63	0.026
		48	286.11	0.031
		48	422.61	0.027
		48	345.42	0.025

Ketoconazole pH 7

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	16.08	12	1584	0.026

12	14.25	12	1706	0.031
24	14.39	12	1188	0.023
24.1	0.43	12	1794	0.028
36	0.98	12	1504	0.029
48	2.02	12	1265	0.020
		24	1688	0.031
		24	1562	0.031
		24	1632	0.027
		24	1766	0.020
		24	1578	0.031
		24	1531	0.023
		36	1047	0.019
		36	847	0.025
		36	847	0.028
		36	1096	0.032
		36	1046	0.032
		36	973	0.025
		48	818	0.023
		48	1108	0.028
		48	1021	0.021
		48	1021	0.024
		48	998	0.026
		48	1199	0.024

Ketoconazole pH 8

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	26.56	12	1021.61	0.014

12	15.05	12	824.42	0.013
24	15.03	12	1013.65	0.013
24.1	0.08	12	745.50	0.015
36	0.04	12	1063.50	0.015
48	0.17	12	634.50	0.015
		24	1090.96	0.013
		24	1201.61	0.014
		24	1291.32	0.017
		24	1228.39	0.014
		24	1254.27	0.017
		24	1587.95	0.014
		36	572.68	0.014
		36	546.96	0.014
		36	546.96	0.02
		36	512.68	0.01
		36	705.75	0.012
		36	455.63	0.014
		48	565.78	0.016
		48	393.28	0.016
		48	519.26	0.017
		48	519.26	0.017
		48	308.04	0.014
		48	441.25	0.018

Ketoconazole pH 9

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	33.61	12	1698.52	0.032

12	14.39	12	1681.92	0.043
24	14.33	12	1512.13	0.034
24.1	0.12	12	1110.24	0.031
36	0.52	12	1256.93	0.022
48	0.26	12	1231.63	0.023
		24	1977.58	0.028
		24	1606.88	0.028
		24	1994.11	0.025
		24	1731.63	0.026
		24	1897.72	0.034
		24	1937.95	0.033
		36	1674.19	0.040
		36	1311.22	0.035
		36	1311.22	0.027
		36	1234.17	0.030
		36	1053.07	0.022
		36	1233.75	0.03
		48	1257.34	0.031
		48	1139.70	0.025
		48	935.95	0.032
		48	935.95	0.029
		48	1114.25	0.030
		48	994.25	0.030

Norfluoxetine pH 5.5

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	47.40	12	397.7	0.025

12	30.06	12	367.1	0.026
24	30.38	12	408.5	0.023
24.1	0.79	12	399.1	0.018
36	1.08	12	601.9	0.020
48	0.99	12	394.2	0.025
		24	887.8	0.022
		24	865.3	0.026
		24	644.1	0.028
		24	816.7	0.022
		24	895.3	0.021
		24	1303.1	0.017
		36	928.7	0.021
		36	747.0	0.023
		36	747.0	0.021
		36	787.3	0.019
		36	1032.3	0.026
		36	766.5	0.022
		48	1497.7	0.015
		48	960.0	0.018
		48	848.6	0.019
		48	848.6	0.019
		48	929.4	0.023
		48	827.3	0.029

Norfluoxetine pH 7

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	33.87	12	7762.6	0.015

12	31.31	12	7207.5	0.015
24	30.17	12	6253.2	0.018
24.1	0.51	12	6011.8	0.014
36	0.92	12	6528.0	0.017
48	1.24	12	5941.6	0.018
		24	10137.3	0.013
		24	8343.5	0.016
		24	6099.3	0.019
		24	6944.8	0.018
		24	9135.3	0.014
		24	9207.3	0.013
		36	7049.2	0.018
		36	6377.5	0.017
		36	6968.7	0.015
		36	5829.2	0.018
		36	7331.8	0.016
		36	7041.6	0.013
		48	6546.1	0.016
		48	6606.7	0.018
		48	6667.3	0.02
		48	6687.7	0.016
		48	8334.3	0.017
		48	8040.9	0.015

Norfluoxetine pH 8

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	38.85	12	14859.4	0.017

12	23.73	12	16677.7	0.014
24	19.99	12	15010.9	0.015
24.1	0.40	12	15522.5	0.017
36	1.20	12	13277.2	0.014
48	1.93	12	18636.5	0.014
		24	20595.3	0.017
		24	21094.1	0.016
		24	14135.1	0.018
		24	19316.1	0.015
		24	17470.0	0.021
		24	22846.7	0.016
		36	19762.2	0.013
		36	14395.8	0.015
		36	14395.8	0.011
		36	28334.1	0.015
		36	17765.9	0.020
		36	18263.8	0.021
		48	18931.5	0.017
		48	15636.3	0.018
		48	16435.4	0.013
		48	16435.4	0.017
		48	16099.7	0.017
		48	15680.8	0.016

Norfluoxetine pH 9

Time (h)	**C_{water} (pmol/ml)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass pf the worms(g)**
0	39.76	12	12956.7	0.032
12	16.96	12	11155.0	0.035

24	13.08	12	24359.4	0.017
24.1	0.26	12	13787.6	0.029
36	0.56	12	17479.2	0.023
48	1.50	12	13201.1	0.033
		24	26298.7	0.016
		24	18456.1	0.025
		24	24974.7	0.02
		24	20016.5	0.03
		24	18651.9	0.027
		24	24305.3	0.014
		36	17711.8	0.026
		36	19580.9	0.022
		36	19580.9	0.023
		36	18170.1	0.021
		36	13537.4	0.021
		36	19535.1	0.030
		48	19077.4	0.022
		48	28577.1	0.017
		48	22168.0	0.017
		48	22168.0	0.022
		48	17164.8	0.023
		48	24771.2	0.018

Mass balance calculations of 24 h uptake and 24 h depuration of the three APIs.

	Timepoint (h)	Tot mass of API in water (pmol)	Tot mass of API in the worms (pmol)	Tot mass of API in the whole system (pmol)	% of spiked API
Amitriptyline pH 5.5	0	374.7	0	374.69	100
	0	416.7	0	416.74	105.31
	0	520.5	0	520.46	119.02
	0	422.3	0	422.32	97.41
	0	328.9	0	328.94	79.72
	0	449.4	0	449.41	107.32
	12	267.5	94.34	361.84	96.57
	12	301.4	69.54	370.90	93.73
	12	288.3	78.57	366.92	83.91
	12	288.7	71.31	360.00	83.03
	12	241.2	72.90	314.15	76.13
	12	278.8	105.52	384.33	91.78
	24	210.2	59.96	270.198	72.11
	24	261.5	86.66	348.18	87.99
	24	222.3	96.29	318.63	72.86
	24	225.0	115.30	340.33	78.50
	24	237.5	77.30	314.84	76.30

	24	238.7	116.35	355.05	84.78
	36	84.0	67.09	151.051	36.1
	36	69.2	55.02	124.24	29.7
	36	64.5	30.28	94.83	22.6
	36	84.1	52.75	136.84	32.7
	36	64.7	38.38	103.09	24.6
	36	83.3	58.72	142.03	33.9
	48	82.4	65.12	147.53	35.2
	48	75.9	75.86	151.77	36.2
	48	52.4	31.28	83.64	20.0
	48	75.7	81.48	157.15	37.5
	48	68.3	43.02	111.30	26.6
	48	50.6	39.22	89.78	21.4
Amitriptyline pH 7	0	374.1	0	374.13	100
	0	353.7	0	353.72	97.20
	0	334.6	0	334.56	94.47
	0	328.8	0	328.83	94.54
	0	313.8	0	313.82	92.03
	0	334.0	0	333.98	98.28
	12	232.5	130.29	362.76	96.96
	12	211.3	127.14	338.48	93.01
	12	183.8	118.91	302.68	85.47
	12	178.6	133.70	312.29	89.79
	12	191.1	113.91	305.04	89.45
	12	202.1	123.40	325.53	95.79
	24	194.4	121.79	316.14	84.50
	24	158.0	122.59	280.61	77.11

	24	177.7	140.15	317.85	89.75
	24	207.3	121.41	328.74	94.52
	24	201.3	134.54	335.81	98.48
	24	222.0	108.48	330.50	97.25
	36	61.1	91.58	152.68	44.9
	36	52.9	82.12	135.01	39.7
	36	62.0	45.19	107.23	31.6
	36	55.8	69.65	125.49	36.9
	36	54.1	97.91	152.01	44.7
	36	42.6	59.50	102.07	30.0
	48	65.1	57.16	122.26	36.0
	48	61.2	88.15	149.31	43.9
	48	68.1	59.30	127.38	37.5
	48	62.0	68.42	130.44	38.4
	48	62.0	77.99	139.99	41.2
		57.0 48	89.88	146.91	43.2
Amitriptyline pH 8	0	327.4	0	327.38	100
	0	373.7	0	373.70	106.61
	0	399.1	0	399.12	108.83
	0	428.4	0	428.36	112.10
	0	445.6	0	445.63	112.86
	0	414.5	0	414.51	104.12
	12	210.7	123.47	334.16	102.07
	12	230.8	151.07	381.90	108.95
	12	204.4	122.15	326.59	89.05
	12	241.5	131.12	372.67	97.52
	12	238.5	136.76	375.30	95.05

	12	225.6	121.74	347.33	87.24
	24	193.3	146.38	339.647	103.75
	24	197.9	167.59	365.50	104.27
	24	190.8	169.75	360.60	98.33
	24	189.7	162.10	351.85	92.07
	24	184.9	164.80	349.68	88.56
	24	198.9	196.93	395.86	99.43
	36	41.8	126.62	168.455	42.3
	36	55.5	122.20	177.67	44.6
	36	57.1	104.32	161.41	40.5
	36	57.3	112.27	169.54	42.6
	36	55.1	458.00	513.15	128.9
	36	57.7	139.85	197.55	49.6
	48	73.9	102.24	176.13	44.2
	48	78.2	106.87	185.06	46.5
	48	75.5	91.25	166.77	41.9
	48	64.8	72.89	137.65	34.6
	48	75.2	106.21	181.42	45.6
	48	60.4	115.64	176.03	44.2
Amitriptyline pH 9	0	474.9	0	474.90	100
	0	413.5	0	413.54	93.09
	0	177.9	0	177.93	50.06
	0	428.1	0	428.15	114.59
	0	378.9	0	378.91	101.13
	0	404.2	0	404.17	106.47
	12	131.8	234.37	366.15	77.10
	12	98.3	283.30	381.60	85.90

	12	90.8	245.65	336.43	94.65
	12	127.9	251.81	379.72	101.63
	12	74.8	286.37	361.13	96.38
	12	51.5	268.02	319.55	84.18
	24	53.9	318.59	372.501	78.44
	24	35.8	344.73	380.52	85.66
	24	41.2	350.98	392.19	110.33
	24	49.1	315.68	364.79	97.63
	24	52.1	314.97	367.06	97.96
	24	52.8	274.71	327.47	86.27
	36	34.2	347.01	381.242	100.4
	36	27.2	322.48	349.70	92.1
	36	25.2	349.30	374.49	98.7
	36	29.2	317.31	346.55	91.3
	36	42.1	333.83	375.93	99.0
	36	28.1	330.70	358.78	94.5
	48	28.2	305.78	334.00	88.0
	48	51.0	296.28	347.24	91.5
	48	32.2	328.00	360.19	94.9
	48	38.2	307.25	345.40	91.0
	48	25.5	333.44	358.98	94.6
	48	30.1	278.27	308.39	81.2
	0	1022.6	0	1022.6	100
	0	992.1	0	992.07	98.48
	0	865.8	0	865.80	90.17
	0	924.1	0	924.05	97.15
	0	837.4	0	837.38	90.20

Diclofenac pH 5.5	0	993.3	0	993.33	105.76
	12	497.5	142.66	640.11	62.60
	12	447.2	127.05	574.21	57.00
	12	459.1	209.42	668.48	69.62
	12	481.4	154.30	635.66	66.83
	12	424.9	144.30	569.15	61.31
	12	432.2	115.14	547.31	58.27
	24	440.9	223.13	664.08	64.94
	24	496.5	160.74	657.27	65.25
	24	518.9	144.79	663.68	69.12
	24	478.0	194.78	672.82	70.74
	24	492.0	180.38	672.38	72.43
	24	491.0	178.44	669.46	71.28
	36	137.0	97.50	234.52	25.0
	36	118.0	149.24	267.22	28.5
	36	94.4	179.09	273.51	29.1
	36	99.6	57.98	157.58	16.8
	36	89.0	137.85	226.87	24.2
	36	88.3	67.44	155.75	16.6
	48	105.4	81.65	187.09	19.9
	48	129.4	81.22	210.66	22.4
	48	125.0	54.01	179.03	19.1
	48	119.0	99.50	218.51	23.3
	48	117.5	124.40	241.89	25.8
	48	144.6	127.46	272.06	29.0
	0	703.9	0	703.91	100
	0	687.2	0	687.16	98.80

Diclofenac pH 7	0	696.9	0	696.93	100.13
	0	653.4	0	653.40	95.34
	0	699.7	0	699.71	101.67
	0	688.6	0	688.64	100.05
	12	644.8	54.31	699.15	99.32
	12	618.8	71.49	690.31	99.25
	12	647.1	45.58	692.71	99.53
	12	581.8	69.62	651.46	95.05
	12	638.0	38.03	675.99	98.22
	12	622.1	59.92	682.07	99.10
	24	566.5	74.30	640.810	91.04
	24	594.7	86.48	681.19	97.94
	24	593.7	91.90	685.57	98.50
	24	629.6	57.01	686.58	100.18
	24	614.2	72.91	687.09	99.84
	24	581.8	90.33	672.16	97.66
	36	99.8	21.51	121.324	17.6
	36	98.6	37.86	136.48	19.8
	36	99.8	22.33	122.09	17.7
	36	79.0	23.35	102.39	14.9
	36	89.8	39.08	128.92	18.7
	36	82.1	35.48	117.57	17.1
	48	104.7	13.75	118.42	17.2
	48	95.6	25.33	120.90	17.6
	48	121.4	47.01	168.37	24.5
	48	91.9	21.70	113.55	16.5
	48	82.9	10.95	93.80	13.6

	48	65.3	12.03	77.38	11.2
Diclofenac pH 8	0	967.0	0	967.04	100
	0	1027.9	0	1027.94	103.05
	0	965.2	0	965.18	97.82
	0	1005.8	0	1005.81	101.44
	0	948.2	0	948.22	96.48
	0	947.4	0	947.41	96.98
	12	894.5	15.39	909.90	94.09
	12	851.2	8.73	859.93	86.21
	12	839.9	13.29	853.22	86.47
	12	825.4	16.81	842.20	84.94
	12	830.6	13.29	843.85	85.86
	12	815.8	17.62	833.41	85.31
	24	887.8	12.03	899.838	93.05
	24	846.4	13.31	859.76	86.19
	24	870.3	12.71	882.99	89.49
	24	836.0	18.73	854.76	86.21
	24	889.2	10.72	899.90	91.56
	24	873.0	11.13	884.16	90.50
	36	158.4	8.22	166.608	17.1
	36	109.0	6.30	115.34	11.8
	36	122.9	5.56	128.49	13.2
	36	113.1	3.65	116.73	11.9
	36	145.7	3.87	149.57	15.3
	36	162.5	4.94	167.44	17.1
	48	69.3	2.80	72.10	7.4
	48	50.8	3.78	54.53	5.6

	48	49.6	4.30	53.88	5.5
	48	44.7	4.95	49.65	5.1
	48	37.4	3.59	41.01	4.2
	48	41.4	4.65	46.03	4.7
Diclofenac pH 9	0	1025.2	0	1025.18	100
	0	900.1	0	900.11	93.50
	0	215.9	0	215.89	30.25
	0	976.4	0	976.36	125.27
	0	892.2	0	892.20	111.25
	0	1080.4	0	1080.44	127.36
	12	694.3	4.10	698.410	68.13
	12	690.7	6.08	696.79	72.38
	12	146.0	4.86	150.88	21.14
	12	643.3	3.71	646.96	83.01
	12	694.6	4.68	699.31	87.20
	12	672.2	4.10	676.32	79.72
	24	673.2	5.01	678.206	66.15
	24	661.4	5.34	666.76	69.26
	24	681.1	2.38	683.44	95.76
	24	691.7	2.96	694.65	89.13
	24	676.8	2.37	679.17	84.69
	24	677.3	3.54	680.83	80.25
	36	70.6	0.67	71.254	8.4
	36	40.2	1.68	41.88	4.9
	36	72.6	1.32	73.92	8.7
	36	60.8	1.17	61.94	7.3
	36	74.9	2.28	77.17	9.1

	36	32.5	1.25	33.76	4.0
	48	65.3	0.93	66.28	7.8
	48	52.5	1.38	53.91	6.4
	48	73.0	1.01	73.99	8.7
	48	58.5	1.75	60.22	7.1
	48	56.0	1.01	57.03	6.7
	48	47.7	1.28	48.96	5.8
Ketoconazole pH 5.5	0	412.5	0	412.4	100
	0	363.6	0	363.6	93.70
	0	447.6	0	447.5	109.73
	0	457.2	0	457.2	108.80
	0	379.8	0	379.80	92.15
	0	425.4	0	425.40	102.67
	12	423.9	9.72	433.62	105.12
	12	330.9	6.22	337.12	86.87
	12	360.9	15.88	376.78	92.37
	12	442.2	17.87	460.07	109.48
	12	287.1	12.97	300.07	72.81
	12	398.4	21.14	419.54	101.25
	24	442.2	13.28	455.48	110.42
	24	180.0	13.28	193.28	53.16
	24	395.7	16.49	412.19	106.22
	24	325.8	16.79	342.59	83.99
	24	416.4	19.06	435.46	105.66
	24	422.1	19.48	441.58	106.57
	36	133.2	11.58	144.78	34.94
	36	130.2	7.29	137.49	33.18

	36	133.2	8.06	141.26	34.09
	36	126.9	9.05	135.95	32.81
	36	118.5	13.83	132.33	31.94
	36	135.0	13.83	148.83	35.92
	48	130.5	8.33	138.83	33.50
	48	123.0	5.42	128.42	30.99
	48	125.7	9.49	135.19	32.63
	48	135.0	6.08	141.08	34.05
	48	127.5	7.05	134.55	32.47
	48	129.3		137.03	7.7333.07
Ketoconazole pH 7	0	473.7	0	473.67	100
	0	463.8	0	463.79	98.94
	0	452.1	0	452.09	97.60
	0	507.0	0	506.99	106.93
	0	421.8	0	421.80	90.97
	0	440.4	0	440.40	95.78
	12	531.3	41.19	572.49	120.85
	12	381.6	52.89	434.49	92.69
	12	382.8	27.32	410.11	88.54
	12	387.9	50.23	438.13	92.40
	12	408.3	43.61	451.91	97.46
	12	390.3	25.29	415.59	90.39
	24	359.7	52.33	412.04	86.98
	24	472.5	48.42	520.92	111.13
	24	354.0	44.07	398.07	85.94
	24	361.2	35.31	396.51	83.63
	24	532.8	48.93	581.73	125.46

	24	430.2	35.20	465.40	101.22
	36	138.0	19.89	157.89	33.64
	36	155.1	21.18	176.28	37.55
	36	136.5	23.72	160.22	34.13
	36	144.6	35.07	179.67	38.28
	36	196.5	33.47	229.96	48.99
	36	130.5	24.33	154.83	32.98
	48	235.5	18.82	254.33	54.18
	48	160.8	31.04	191.84	40.87
	48	157.2	21.45	178.65	38.06
	48	176.7	24.51	201.21	42.87
	48	130.2	25.95	156.15	33.27
	48	145.5	28.77	174.27	37.13
	0	702.0	0	702	100
	0	574.2	0	574.2	89.99
	0	652.5	0	652.5	101.49
	0	675.9	0	675.9	103.80
	0	618.6	0	618.60	95.96
	0	684.3	0	684.30	105.07
	12	429.0	14.30	443.30	63.15
	12	438.0	10.72	448.72	70.32
	12	449.1	13.18	462.28	71.91
	12	433.5	11.18	444.68	68.29
	12	441.3	15.95	457.25	70.93
	12	438.3	9.52	447.82	68.76
	24	435.6	14.18	449.78	64.07
	24	433.8	16.82	450.62	70.62

Ketoconazole pH 8	24	428.7	21.95	450.65	70.10
	24	436.5	17.20	453.70	69.68
	24	437.7	21.32	459.02	71.21
	24	434.1	17.47	451.57	69.34
	36	136.8	8.02	144.82	22.24
	36	125.1	7.66	132.76	20.39
	36	135.9	7.66	143.56	22.04
	36	136.5	10.25	146.75	22.53
	36	131.7	7.06	138.76	21.31
	36	132.6	5.47	138.07	21.20
	48	138.9	9.05	147.95	22.72
	48	152.4	6.29	158.69	24.37
	48	136.2	8.83	145.03	22.27
	48	131.1	8.83	139.93	21.49
	48	135.6	4.31	139.91	21.48
	48	120.0	7.94	127.94	19.65
Ketoconazole pH 9	0	953.7	0	953.70	100
	0	637.2	0	637.20	80.11
	0	769.5	0	769.50	97.80
	0	1073.4	0	1073.40	125.04
	0	753.3	0	753.30	89.95
	0	816.0	0	816.00	97.86
	12	402.9	54.35	457.25	47.95
	12	413.1	72.32	485.42	61.02
	12	397.5	51.41	448.91	57.06
	12	402.6	34.42	437.02	50.91
	12	408.0	27.65	435.65	52.02

	12	462.6	28.33	490.93	58.87
	24	433.8	55.37	489.17	51.29
	24	427.8	44.99	472.79	59.44
	24	411.0	49.85	460.85	58.57
	24	426.0	45.02	471.02	54.87
	24	403.5	64.52	468.02	55.89
	24	401.1	63.95	465.05	55.77
	36	137.1	66.97	204.07	24.47
	36	145.5	45.89	191.39	22.95
	36	139.8	35.40	175.20	21.01
	36	131.4	37.02	168.42	20.20
	36	126.3	23.17	149.47	17.92
	36	135.3	37.01	172.31	20.66
	48	132.6	38.98	171.58	20.58
	48	127.5	28.49	155.99	18.71
	48	133.2	29.95	163.15	19.57
	48	120.6	27.14	147.74	17.72
	48	138.9	33.43	172.33	20.67
	48	123.0	29.83	152.83	18.33
	0	1001.09	0	1001	100
	0	981.77	0	982	99.03
	0	1001.09	0	1001	100.65
	0	981.77	0	982	99.03
	0	1010.63	0	1011	101.54
	12	630.89	34.86	665.74	66.50
	12	500.89	34.94	535.82	54.05
	12	648.96	30.46	679.42	68.31

Norfluoxetine pH 5.5	12	647.03	18.74	665.78	67.15
	12	643.13	33.25	676.38	67.96
	12	666.77	34.58	701.35	70.47
	24	633.39	57.68	691.07	69.03
	24	617.34	78.04	695.39	70.14
	24	650.57	68.05	718.62	72.25
	24	627.86	53.28	681.14	68.70
	24	623.44	53.09	676.53	67.97
	24	628.39	50.27	678.66	68.19
	36	42.55	56.01	98.56	10.70
	36	39.06	53.38	92.45	10.04
	36	44.48	47.00	91.48	9.93
	36	38.91	38.22	77.12	8.37
	36	61.35	78.75	140.10	15.21
	36	39.22	50.17	89.39	9.70
	48	48.18	44.96	93.14	10.11
	48	40.63	42.01	82.63	8.97
	48	41.93	35.18	77.10	8.37
	48	34.64	41.53	76.16	8.27
	48	45.52	65.73	111.25	12.08
	48	36.98	92.59	129.57	14.06
	0	795.89	0	796	100
	0	766.25	0	766	98.10
	0	763.07	0	763	98.45
	0	784.58	0	785	100.92
	0	814.43	0	814	103.77
	0	825.05	0	825	104.23

Norfluoxetine pH 7	12	642.66	116.44	759.10	95.38
	12	657.34	108.11	765.46	98.00
	12	647.50	112.56	760.06	98.06
	12	665.47	84.17	749.63	96.42
	12	638.33	110.98	749.31	95.47
	12	646.30	106.95	753.25	95.97
	24	610.63	131.78	742.41	93.28
	24	615.99	133.50	749.48	95.96
	24	611.72	115.89	727.61	93.88
	24	629.48	125.01	754.49	97.05
	24	640.10	127.89	768.00	97.85
	24	642.19	119.69	761.88	97.07
	36	50.68	126.89	177.56	23.18
	36	38.39	108.42	146.80	19.16
	36	35.78	104.53	140.31	18.32
	36	38.18	104.93	143.10	18.68
	36	38.96	117.31	156.27	20.40
	36	37.19	91.54	128.73	16.81
	48	58.70	104.74	163.44	21.34
	48	40.63	118.92	159.55	20.83
	48	54.95	133.35	188.29	24.58
	48	41.88	107.00	148.88	19.44
	48	46.15	141.68	187.83	24.52
	48	35.99	120.61	156.60	20.44
	0	874.22	0	874	100
	0	797.34	0	797	95.40
	0	776.98	0	777	95.20

Norfluoxetine pH 8	0	817.86	0	818	100.15
	0	784.11	0	784	96.79
	0	768.44	0	768	95.68
	12	509.64	252.61	762.25	87.19
	12	562.60	233.49	796.09	95.25
	12	503.07	225.16	728.24	89.22
	12	452.50	263.88	716.38	87.73
	12	495.52	185.88	681.40	84.11
	12	447.24	260.91	708.15	87.41
	24	415.31	350.12	765.43	87.56
	24	407.86	337.51	745.37	89.18
	24	478.59	254.43	733.03	89.81
	24	468.28	289.74	758.02	92.83
	24	388.44	366.87	755.31	93.24
	24	377.29	365.55	742.84	91.70
	36	60.21	256.91	317.12	42.72
	36	44.95	215.94	260.89	35.14
	36	44.79	158.35	203.15	27.36
	36	52.24	425.01	477.25	64.29
	36	33.28	355.32	388.60	52.34
	36	44.64	383.54	428.17	57.67
	48	53.75	321.84	375.59	50.59
	48	36.72	281.45	318.17	42.86
	48	62.40	213.66	276.06	37.18
	48	73.39	279.40	352.79	47.52
	48	73.39	273.70	347.08	46.75
	48	60.00	250.89	310.89	41.88

Norfluoxetine pH 9	0	853.91	0	854	100
	0	850.16	0	850	99.78
	0	853.91	0	854	100.15
	0	808.80	0	809	96.09
	0	833.13	0	833	99.18
	0	803.80	0	804	96.38
	12	373.07	414.62	787.69	92.25
	12	370.78	390.43	761.21	89.34
	12	376.35	414.11	790.47	92.71
	12	346.98	399.84	746.82	88.73
	12	359.17	402.02	761.19	90.62
	12	346.20	435.64	781.84	93.08
	24	311.88	420.78	732.65	85.80
	24	261.88	461.40	723.28	84.89
	24	284.06	499.49	783.56	91.90
	24	211.61	600.50	812.11	96.49
	24	255.00	503.60	758.60	90.31
	24	376.30	340.27	716.58	85.31
	36	37.71	283.39	321.10	42.60
	36	36.46	489.52	525.98	69.78
	36	32.14	391.62	423.75	56.21
	36	33.70	545.10	578.80	76.78
	36	30.63	365.51	396.13	52.55
	36	27.86	273.49	301.36	39.98
	48	46.09	496.01	542.10	71.92
	48	40.89	628.70	669.58	88.83
	48	42.76	509.86	552.62	73.31

	48 48 48	55.42 70.73 54.17	465.53 360.46 743.14	520.95 431.19 797.30	69.11 57.20 105.77

Appendix C – Supporting Data for Chapter Four

Sorption of ionisable active pharmaceuticals in four different types of sediments

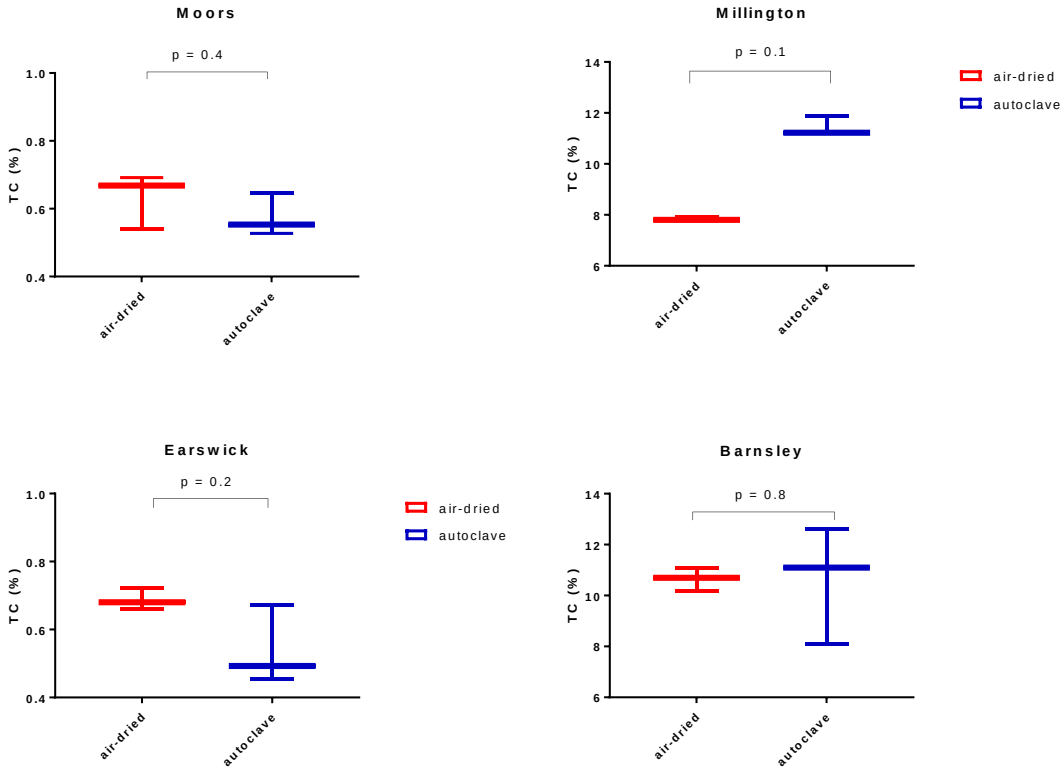


Figure C. 1. Total carbon analyzed of the four sediments air-dried and autoclaved.

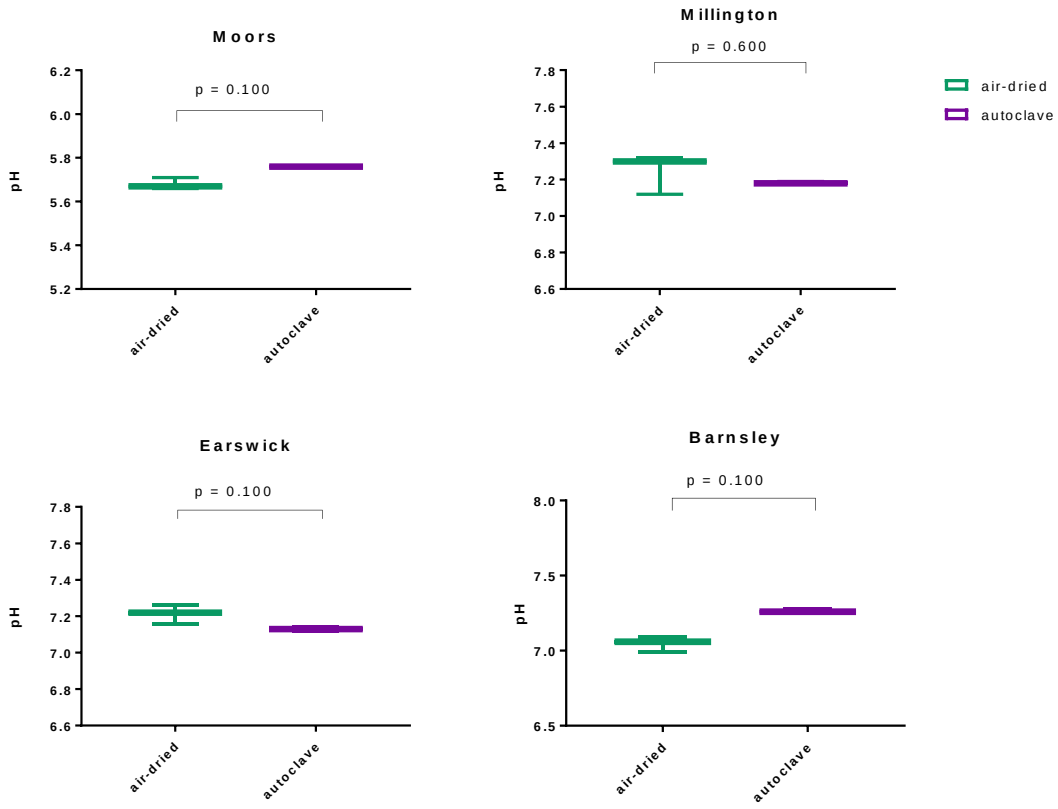


Figure C. 2. pH analyzed of the four sediments air-dried and autoclaved.

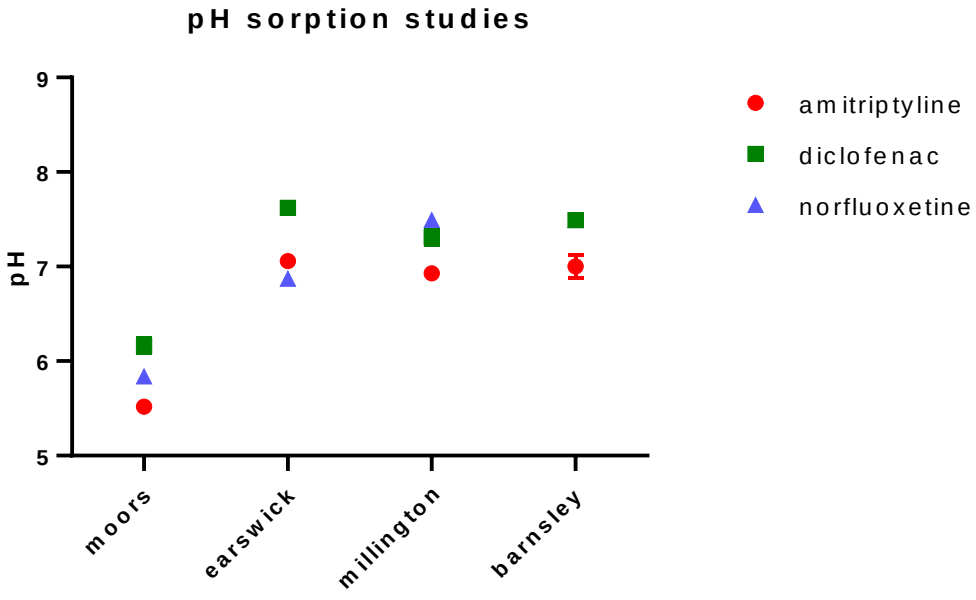


Figure C. 3. pH measurements of the four sediments during the sorption tests.

Appendix D – Supporting Data for Chapter Five

Influence of sediment properties on the uptake of ionisable APIs into a sediment-dwelling invertebrate *L. variegatus*

Data for the model

The dataset consists of concentrations of the water (pmol/mL), concentrations of the compounds in the sediment (pmol/), concentrations in the worms (pmol/g wet weight) over time (hours) and mass of the worms (g).

Amitriptyline Moors sediment

Time (h)	$C_{sediment}$ (pmol/g)	Time (h)	C_{water} (pmol/mL)	Time (h)	C_{int} (pmol/g)	Mass of the worms(g)
0	18.9	12	0.12	12	320.7	0.023
12	17.4	24	0.17	12	298.5	0.015
24	13.5	36	0.14	12	195.8	0.017
24.1	0.0	48	0.15	12	150.1	0.018
36	0.1			12	252.7	0.023
48	0.2			12	164.3	0.02
				24	234.8	0.013
				24	177.9	0.019
				24	229.7	0.014
				24	332.7	0.019
				24	133.6	0.017
				24	165.7	0.024
				36	167.5	0.012
				36	224.4	0.02
				36	159.9	0.016

				36	162.4	0.014
				36	152.2	0.016
				36	213.7	0.015
				48	73.2	0.011
				48	223.0	0.014
				48	64.5	0.014
				48	94.7	0.013
				48	130.7	0.017
				48		0.018

Amitriptyline Barnsley sediment

Time (h)	**$C_{sediment}$ (pmol/g)**	**Time (h)**	**C_{water} (pmol/mL)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	16	12	0.10	12	163.9	0.023
12	17	24	0.10	12	210.9	0.021
24	17	36	0.02	12	144.1	0.019
24.1	0	48	0.06	12	119.5	0.018
36	0.8			12	164.5	0.015
48	0.7			12	458.7	0.021
				24	469.0	0.023
				24	426.3	0.017
				24	214.1	0.018
				24	233.9	0.016
				24	174.8	0.019
				24	113.1	0.021
				36	97.3	0.018
				36	114.7	0.013

				36	99.3	0.014
				36	126.3	0.017
				36	88.1	0.017
				36	88.1	0.016
				48	58.1	0.018
				48	55.9	0.02
				48	90.9	0.014
				48	93.9	0.018

Diclofenac Moors sediment

Time (h)	**$C_{sediment}$ (pmol/g)**	**Time (h)**	**C_{water} (pmol/mL)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	15.7	12	10	12	419.9	0.016
12	16.5	24	10	12	279.7	0.01
24	16.0	36	0.0	12	389.2	0.021
24.1	0	48	1.60	12	401.0	0.016
36	0.2			12	529.0	0.012
48	0.8			12	545.3	0.013
				24	523.3	0.018
				24	636.8	0.011
				24	449.4	0.017
				24	422.3	0.018
				24	584.7	0.014
				24	340.2	0.017
				36	230.5	0.011
				36	200.5	0.015

				36	183.1	0.012
				36	163.2	0.015
				36	219.1	0.014
				36	200.8	0.015
				48	135.2	0.015
				48	159.5	0.015
				48	133.3	0.013
				48	135.0	0.017
				48	134.1	0.016

Diclofenac Barnsley sediment

Time (h)	**$C_{sediment}$ (pmol/g)**	**Time (h)**	**C_{water} (pmol/mL)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	14	12	16	12	250.7	0.022
12	14	24	17	12	362.1	0.018
24	13.4	36	0.1	12	208.6	0.017
24.1	0	48	1.15	12	350.7	0.02
36	0.01			12	145.1	0.019
48	0.3			12	127.8	0.016
				24	299.1	0.012
				24	262.6	0.019
				24	84.2	0.014
				24	105.8	0.012
				24	84.0	0.013
				24	114.3	0.017

				36	116.3	0.017
				36	42.3	0.016
				36	117.0	0.015
				36	57.5	0.019
				36	36.9	0.018
				36	24.6	0.018
				48	18.4	0.014
				48	59.7	0.018
				48	63.4	0.013
				48	31.2	0.015
				48		0.01

Norfluoxetine Moors sediment

Time (h)	**$C_{sediment}$ (pmol/g)**	**Time (h)**	**C_{water} (pmol/mL)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	18.7	12	0.6	12	145.1	0.022
12	17.6	24	0.7	12	133.8	0.021
24	18.2	36	0.2	12	256.2	0.015
24.1	0	48	0.13	12	239.4	0.019
36	0.1			12	110.0	0.016
48	0.2			12	103.1	0.019
				24	345.3	0.023
				24	462.6	0.024
				24	301.0	0.024
				24	264.4	0.021
				24	341.3	0.023

				24	152.7	0.024
				36	116.1	0.023
				36	91.6	0.012
				36	139.7	0.016
				36	89.8	0.011
				36	86.1	0.017
				36	112.2	0.018
				48	54.3	0.014
				48	108.6	0.018
				48	119.8	0.013
				48	89.2	0.012
				48	73.2	0.016

Norfluoxetine Barnsley sediment

Time (h)	**$C_{sediment}$ (pmol/g)**	**Time (h)**	**C_{water} (pmol/mL)**	**Time (h)**	**C_{int} (pmol/g)**	**Mass of the worms(g)**
0	19.0	12	0.4	12	304.7	0.017
12	17.4	24	0.4	12	256.1	0.023
24	17.2	36	0.0	12	132.3	0.02
24.1	0.0	48	0.02	12	119.8	0.021
36	0.18			12	122.7	0.016
48	0.19			12	97.5	0.024
				24	97.5	0.026
				24	124.1	0.02
				24	67.5	0.025
				24	90.9	0.027
				24	59.5	0.018

				24	67.9	0.024
				36	42.4	0.017
				36	79.4	0.02
				36	37.6	0.018
				36	74.9	0.02
				36	78.0	0.017
				36	67.6	0.018
				48	46.9	0.018
				48	42.1	0.023
				48	30.4	0.017
				48	52.6	0.018
				48		0.015

Mass balance calculations of 24 h uptake and 24 h depuration of the three APIs.

	Timepoint (h)	Tot mass of API in water (pmol)	Tot mass of API in sediment (pmol)	Tot mass of API in the worms (pmol)	Tot mass of API in the whole system (pmol)	% of spiked API
Amitriptyline Moors	12	17.5	55.13	7.38	80.0	110.15
	12	16.2	56.74	4.48	77.4	106.34
	12	16.8	42.59	3.33	62.7	91.83
	12	16.5	55.71	2.70	74.9	108.10
	12	16.3	60.27	5.81	82.4	116.39
	24	16.4	50.67	2.14	69.2	103.18
	24	17.3	44.95	4.46	66.7	103.14
	24	17.8	52.02	2.49	72.4	108.97
	24	18.0	34.93	4.36	57.3	90.96
	24	17.4	35.98	5.66	59.0	96.61
	24	17.4	42.77	3.21	63.4	106.12
	36	16.2	3.91	1.99	22.1	31.27
	36	15.5	3.41	3.35	22.2	31.41
	36	16.6	3.50	3.59	23.7	33.48
	36	18.2	3.80	2.24	24.3	34.32
	36	16.4	3.58	2.60	22.6	31.89
	36	18.0	3.39	2.28	23.7	33.47
	48	17.7	3.71	2.35	23.8	33.61
	48	17.1	3.66	1.02	21.8	30.81
	48	16.4	3.75	3.12	23.3	32.93
	48	17.2	3.54	0.84	21.6	30.50

	48	17.4	3.72	1.61	22.8	32.18
Amitriptyline Barnsley	12	17.9	52.28	3.77	74.0	105.37
	12	16.8	54.84	4.43	76.1	107.28
	12	16.7	54.54	2.74	74.0	104.11
	12	17.3	54.36	2.15	73.8	103.71
	12	18.3	54.28	2.47	75.1	105.04
	12	17.9	52.04	9.63	79.6	111.44
	24	18.2	54.22	10.79	83.2	114.90
	24	16.8	56.68	7.25	80.7	110.69
	24	17.2	52.43	3.85	73.5	102.34
	24	17.5	53.53	3.74	74.8	104.36
	24	19.5	54.49	3.32	77.3	107.17
	36	16.1	4.88	2.04	23.0	32.16
	36	17.3	6.02	1.27	24.6	34.42
	36	14.8	6.10	1.61	22.5	31.46
	36	18.1	5.83	1.69	25.6	35.88
	36	16.3	5.52	2.15	24.0	33.54
	36	15.4	6.09	1.41	22.9	32.02
	48	15.9	4.20	1.59	21.7	30.29
	48	17.6	6.71	1.16	25.4	35.58
	48	18.1	6.00	1.27	25.4	35.53
	48	15.3	4.52	1.69	21.6	30.15
	48	18.6	6.82	1.13	26.6	37.15
	12	229.2	53.27	6.72	289.2	102.38
	12	162.9	54.12	2.80	219.8	88.01
	12	171.9	51.15	8.17	231.2	96.01

Diclofenac Moors	12	169.1	52.25	6.42	227.8	96.53
	12	162.9	54.64	6.35	223.9	96.38
	12	186.5	51.59	7.09	245.2	105.12
	24	171.9	51.02	9.42	232.3	104.23
	24	176.7	53.21	7.00	236.9	104.63
	24	184.0	50.38	7.64	242.0	105.65
	24	178.4	52.86	7.60	238.8	104.02
	24	177.5	51.30	0.53	229.4	99.97
	36	18.0	4.15	5.78	27.9	11.97
	36	16.5	3.40	2.53	22.4	9.61
	36	16.2	3.77	3.01	23.0	9.87
	36	15.5	4.36	2.20	22.1	9.46
	36	34.3	3.72	2.45	40.5	17.34
	36	24.9	3.85	3.07	31.8	13.63
	48	23.5	4.03	3.01	30.6	13.10
	48	16.0	5.97	2.03	24.0	10.27
	48	18.7	8.70	2.39	29.8	12.79
	48	51.0	7.34	1.73	60.0	25.74
	48	16.5	3.65	2.30	22.5	9.63
	48	18.0	4.21	2.14	24.3	10.42

Diclofenac Barnsley	12	261.0	45.48	10.10	315.2	103.06
	12	258.8	46.23	4.26	297.0	98.52
	12	245.1	47.58	7.24	316.9	104.42
	12	265.4	44.24	3.96	314.3	103.11
	12	265.7	44.71	5.61	318.9	104.11
	12	268.9	44.39	1.74	319.5	100.55
	24	275.6	42.13	2.43	323.4	101.27
	24	278.0	43.00	4.19	312.0	98.90
	24	262.1	45.74	3.15	308.6	98.58
	24	260.4	44.98	1.10	316.5	100.96
	24	271.6	43.76	1.80	320.3	102.13
	25	273.9	44.58	1.43	21.2	6.91
	36	15.6	4.12	1.83	23.0	7.51
	36	17.4	3.79	1.74	23.1	7.54
	36	17.8	3.57	0.80	21.2	6.93
	36	16.4	3.96	2.11	23.1	7.54
	36	17.4	3.57	1.03	23.1	7.55
	36	18.5	3.57	0.52	24.7	8.07
	48	19.4	4.75	0.44	21.3	6.97
	48	16.5	4.36	0.24	21.6	7.06
	48	17.7	3.70	0.90	21.0	6.85
	48	15.9	4.16	0.63	20.7	6.77
	48	16.1	3.98	0.53	25.7	8.39

Norfluoxetine Moors	12	22.4	57.77	3.19	83.3	103.98
	12	23.1	51.19	2.81	77.1	99.85
	12	23.2	60.22	3.84	87.2	110.02
	12	23.8	59.33	4.55	87.7	109.25
	12	23.1	51.76	1.76	76.6	96.81
	24	24.8	56.66	7.94	89.4	109.75
	24	24.0	56.77	18.58	99.3	122.47
	24	18.5	57.85	11.10	87.4	109.95
	24	26.1	58.47	6.32	90.9	112.54
	24	24.3	59.68	6.08	90.0	110.56
	36	18.0	3.39	3.51	24.9	31.46
	36	16.5	3.22	1.39	21.1	26.65
	36	16.2	3.70	1.47	21.4	27.03
	36	15.5	3.21	1.54	20.3	25.58
	36	34.3	3.23	1.53	39.0	49.31
	36	24.9	3.29	1.55	29.7	37.53
	48	23.5	3.70	1.57	28.8	36.37
	48	16.0	3.89	0.98	20.8	26.30
	48	18.7	3.51	1.41	23.7	29.88
	48	51.0	3.82	1.44	56.2	71.02
	48	16.5	3.64	1.43	21.6	27.28
	48	18.0	3.77	1.17	22.9	28.92

Norfluoxetine Barnsley	12	21.2	53.10	2.53	76.8	103.41
	12	20.7	55.09	1.95	77.8	103.61
	12	21.9	55.66	3.10	80.7	106.28
	12	21.8	56.10	1.82	79.8	104.38
	12	20.3	56.67	1.64	78.6	102.69
	24	22.0	53.61	1.15	76.7	101.53
	24	21.1	55.13	0.85	77.1	101.56
	24	21.8	55.39	1.43	78.7	103.04
	24	20.9	54.30	0.75	75.9	99.83
	24	21.1	55.20	1.27	77.5	101.89
	36	14.8	3.90	1.15	19.8	25.90
	36	18.0	3.11	0.85	21.9	28.66
	36	16.8	3.63	1.43	21.8	28.52
	36	15.8	3.69	0.75	20.2	26.43
	36	14.5	3.84	1.27	19.6	25.57
	36	15.5	2.88	1.40	19.7	25.81
	48	15.9	3.47	1.22	20.6	26.95
	48	15.8	3.56	1.08	20.5	26.74
	48	15.9	3.78	0.72	20.4	26.60
	48	14.6	3.62	0.55	18.7	24.48
	48	17.3	3.52	0.79	21.6	28.20

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