UPTAKE OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS FROM SEDIMENTS INTO AQUATIC ORGANISMS

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Abstract

Over the past fifteen years there has been increasing interest in the environmental occurrence, fate and effects of substances used as pharmaceuticals or personal care products. While the understanding of the environmental fate and ecotoxicity of pharmaceuticals is now well developed, less information is available on the uptake of pharmaceuticals and personal care products into aquatic organisms and, in particular, into sediment-dwelling organisms. This study was therefore performed to develop an understanding of the factors and processes affecting the uptake of pharmaceuticals and personal care products into the sediment dwelling oligochaete worm, *Lumbriculus variegatus*. The study combined experimental studies into the distribution of a range of pharmaceuticals and personal care products in sediment-water systems and studies into the uptake of the study compounds under a range of conditions. The results were used to parameterize and evaluate a model for estimating uptake of pharmaceuticals and personal care products into benthic organisms.

Adsorption of the study compounds from water to sediment solids increased in the order diclofenac < chloramphenicol < salicylic acid < naproxen < caffeine < sulfamethazine < triclosan < fluoxetine. Comparison of the sorption results with estimations from available models for predicting sorption from chemical properties indicated that relationships developed for neutral organic chemicals were not appropriate for use on ionisable pharmaceuticals and personal care products. While predictive models, developed specifically for ionisable chemicals, produced improved predictions of sorption, even these predictions were not perfect.

Bioconcentration factors for the study compounds from water into *L. variegatus* were found to increase in the order chloramphenicol < diclofenac < salicylic acid < fluoxetine < naproxen < triclosan. The differences in bioconcentration factors could not be explained by differences in log Kow and log Dow which are descriptors that have previously been used to predict the uptake of neutral organic substances and ionisable substances in other species of invertebrates. There was also disagreement between the uptake measurements and predictions obtained from models developed for estimating the uptake of ionisable chemicals into aquatic organisms.

The uptake of four of the study compounds (caffeine, diclofenac, fluoxetine and triclosan) was further evaluated at different water pH values. For three of these compounds (diclofenac, fluoxetine and triclosan), the potential for metabolism by *L. variegatus* was also assessed as was the uptake and route of uptake from whole sediments. Uptake of diclofenac and fluoxetine was found to be highly sensitive to changes in pH with bioconcentration factors varying by over two orders of magnitude (diclofenac) and four orders of magnitude (fluoxetine) across three pH units. Tissue analysis indicated that while diclofenac is not metabolized by the worms, fluoxetine and triclosan are heavily metabolized. The whole sediment studies demonstrated that uptake of diclofenac and fluoxetine occurs primarily from the sediment pore-water whereas for triclosan, sediment ingestion provides a small contribution to the uptake.

Results from the different components of the study were used to parameterize and evaluate a model for estimating uptake of pharmaceuticals and personal care products from sediments into benthic organisms. Comparison of predictions from this model for diclofenac, fluoxetine and triclosan were compared to measurements from whole sediment studies. While the model was found to under-predict the uptake of triclosan, good predictions were obtained for diclofenac and fluoxetine. With further development and evaluation, the uptake modeling approach could provide a valuable tool for use in the risk assessment of ionisable compounds such as many pharmaceuticals and personal care products.

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Authors declaration

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With the exception of Chapter 4, the contents of this thesis have been produced solely by the candidate. Data on metabolism of diclofenac, fluoxetine and triclosan in chapter 4 was produced at CSIRO, Adelaide, Australia. The candidate was responsible for the experimental design, performance of the uptake study and the extraction of water and tissue samples. The LC-MS/MS analyses were performed by Dr Mike Williams at CSIRO.

CHAPTER 1

INTRODUCTION

1.1 Ionisable chemicals in the aquatic environment

Ionisable chemicals are chemicals that possess either weak acidic or basic functional groups that have the potential to protonate or deprotonate and thereby give positive or negative charges to the molecules. Ionisation of chemicals is described in more detail in Section 3.3. Ionisable chemicals are found ubiquitously amongst the different chemical categories used in our everyday life, including pharmaceuticals, personal care products, house hold chemicals and pesticides. For example, Franco *et al.* 2010 showed that out of a subsample of 1510 out of 17000 of the chemicals pre-registered and due for registration within the European chemical legislation REACH, 33 % were ionised at pH 7.

In addition to the chemicals pre-registered within REACH, a large proportion of substances used as pharmaceuticals and personal care products, which are commonly referred to as PPCP's, are ionisable (Daughton, 2001).The environmental fate and effects of human pharmaceuticals, personal care products and house hold chemicals has only recently come to the attention of the scientific community (Boxall *et al.* 2012). Nevertheless, extensive reviews addressing the environmental impact of these groups of chemicals have been produced over the last 15 years (Halling-Sorensen *et al*, 1998; Daughton and Ternes 1999; Monteiro and Boxall, 2010; Brausch and Rand, 2011). In the following sections, an overview is given of the environmental exposure and effects of mainly pharmaceuticals but also a few active ingredients in personal care products in the environment.

1.1.1 Environmental exposure routes

The major exposure route of down the drain chemicals into the aquatic environment is *via* the discharge of sewage waters from hospitals and households into the municipal waste water system (Carballa *et al.* 2004). Following use, the chemicals may be metabolized by the human body and then the parent compounds and any metabolites will be released to and transported *via* the sewerage system to a wastewater treatment plant. Once they enter the sewage treatment plants they can either associate with the sewage sludge or remain in the aqueous phase (Carballa *et al.* 2004). The fraction remaining in the aqueous phase can undergo degradation, either biotically (Onesios *et al.* 2009) or abiotically (Andreozzi *et al.* 2003) resulting in the formation of degradation products. A mixture of parent compounds, metabolites and degradation products will then be released into receiving waters (Stülten *et al.* 2008; Lee *et al*, 1998).

An additional exposure route that has recently been shown to be of major importance, predominantly in the developing world, is the intended and unintended discharge of substances from pharmaceutical manufacturing sites (Larsson *et al.* 2010). Veterinary pharmaceuticals may be released to the aquatic environment either directly, when used in aquaculture, or indirectly when manure and slurry from treated livestock is applied to land as a fertilizer (Boxall *et al.*, 2004). Following addition to land, veterinary pharmaceuticals can be transported to the aquatic environment *via* runoff from agricultural lands to adjacent streams and ditches (Boxall *et al.* 2004).

1.1.2 Occurrence of PPCPs in the aquatic environment

As a result of their continuous use by society and the fact that selected PPCPs are not removed by wastewater treatment, a range of PPCPs and their metabolites and transformation products have been detected in aquatic systems around the world (e.g.

Monteiro and Boxall, 2010; Brausch and Rand, 2011). Different classes of pharmaceuticals and active ingredients in personal care products are frequently detected in surface waters and sewage effluents at concentrations up to low µg/L levels; concentrations are slightly higher in effluents (Daughton and Ternes, 1999; Monteiro and Boxall, 2010; Brausch and Rand, 2011). Pharmaceuticals have also been detected in freshwater sediments at ng/g concentrations (Nilsen *et al.* (USGS); Vazquez-Roig *et al.* 2010). At manufacturing sites, the concentrations of pharmaceuticals can be much higher. For example, Larsson *et al.* (2007) measured concentrations of a range of broad spectrum antibiotics in waste water effluent in Hyderabad, India. The maximum detected concentration of ciprofloxacin, a fluoroquinolone antibiotic, was 31,000 µg/L which is higher than the highest therapeutic dose in human plasma and several orders of magnitude higher than concentrations where ecotoxicological effects have been reported in bacteria and aquatic plants.

1.1.3 Reported effects of PPCPs in the aquatic environment.

As many pharmaceuticals and active ingredients in personal care products are biologically active molecules, concerns have been raised over the potential impacts of PPCPs in surface waters on aquatic organisms (e.g. Halling-Sorensen *et al.* 1998; Daughton and Ternes, 1999; Fent *et al.* 2006). A range of pharmaceuticals have been shown to effect aquatic organisms at low concentrations. For example, several studies have reported effects on the reproduction of non-target organisms following exposure to ethinyl estradiol (EE2), the active ingredient in the contraceptive pill, at the individual level (Schultz *et al.* 2003; Nash *et al.* 2004) and also at the population level (Jobling *et al.* 2006; Kidd *et al.* 2007). EE2 has also shown to have effects not only related to reproductive endpoints but also on developmental endpoints. For example, Soares *et al.* (2009) observed effects on embryonic development in Zebra fish when exposed to low and environmentally relevant concentrations of EE2. Effects of pharmaceuticals with other mechanisms have also been reported. Porsbring *et al.* (2009) reported effects on sterol synthesis in marine microalgae when exposed to concentrations as

low as 50 pM of the fungicide clotrimazole. Exposure to diclofenac, a non-steroidal anti-inflammatory drug (NSAID), has been shown to alter the histology of the kidney (Schwaiger *et al.* 2004). Reported histological effects included hyaline droplet degeneration of the tubular epithelial cells and the occurrence of an interstitial nephritis. In the gills of fish, exposure to diclofenac results in necrosis of pillar cells leading to damage of the capillary wall within the secondary lamellae. (Schwaiger *et al.* 2004). Fluoxetine, a selective serotonin reuptake inhibiter which is used in the treatment of depression, has been shown to cause effects on behaviour such as decreased activity, aggression, and changes in avoidance behaviour in both vertebrates and invertebrates (De Lange *et al.* 2006; Perreault *et al.* 2003; Painter *et al.* 2009). The high use of antibiotics has led to effects that may have impacts on a global scale (Sarmah *et al.* 2006; Cabello 2006; Fricke *et al.* 2008). When bacteria are exposed to a selection pressure in the form of an antibiotic, a common response of the bacterial population is to develop resistance to the antibiotic (Wellington *et al.*, 2013). The genes associated with the resistance can then possibly be transferred *via* horizontal gene transfer into pathogenic bacteria, creating resistant pathogens which may have substantial ecological and economic outcomes (Kristiansson *et al.* 2011). However, the impact of antibiotics present in the aquatic environment on the frequency of resistance transfer is a topic of considerable debate. The information available to date suggests that the input of resistant bacteria into the environment from different sources seems to be the most important source of resistance in the environment and that exposure to antibiotics in the environment plays a limited role in the selection of resistance (Kummerer, 2009).

Pharmaceuticals have also been shown to have catastrophic effects in the terrestrial environment. For example, in the Indian subcontinent, the Oriental white-backed vulture was once one of the most common raptors (Ali and Ripley in Oaks *et al.* 2004). In the 1990s, population numbers declined by >95% (Pain *et al.* in Oaks *et al.* 2004). Since then, catastrophic declines in the population of two other species of vulture have also been observed (Pain *et al.* in Oaks *et al.* 2004). Oaks *et al.* (2004) showed a correlation between

renal failure, visceral gout and mortality in the vultures. The visceral gout was associated with residues of the anti-inflammatory drug diclofenac in the vulture tissues. The vultures are thought to have been exposed to diclofenac as a result of the consumption of carcasses of livestock that had been treated with diclofenac. The use of diclofenac in farm animals in the Indian subcontinent has now been banned and efforts are being made to re-establish the vulture populations.

1.1.4 Assessing risks of PPCPs in the environment and the importance of understanding chemical uptake.

A number of regions of the world require an environmental risk assessment for a PPCP to protect the natural environment from harm from these products. For example in Europe, the European Medicines Agency (EMA; previously called the European Medicines Evaluation Agency (EMEA)) requires an environmental risk assessment for all new human pharmaceuticals as part of the marketing authorization process. These assessments typically involve the performance of studies to assess the environmental mobility and persistence of a substance as well as ecotoxicological studies to assess the potential effects of the substance on aquatic and terrestrial organisms (EMEA, 2006). Similar requirements are in place in Europe for compounds used in veterinary medicine (VICH, 2000; VICH, 2005) or in personal care products (REACH, 2006).

In the risk assessment process, knowledge of the uptake of a substance from the environment into organisms as well as the factors determining uptake can be invaluable. An understanding of uptake allows an assessment of: the bioaccumulation potential of an organism; the potential for a substance to cause secondary poisoning; and can also help to support extrapolation of toxicity data from one species to another or from one environmental situation to another (Williams, 2005). For example, due to the fact that many organisms have the same receptors as humans, it has been suggested that pharmacological data could be used to inform the environmental risk assessment of a pharmaceutical product (Huggett *et al.*

2003; Berninger and Brooks 2010). The next section therefore provides an overview of how chemicals are taken up into organisms and reviews the current knowledge on the uptake of PPCPs into aquatic organisms

1.2 Uptake

A contaminant that accumulates in biota can under long term exposure and concentrate higher up in the food chain and as a consequence lead to secondary poisoning, an example is the decline of the populations of the Oriental white-backed vulture caused by the feeding on livestock treated with diclofenac, described in the previous section (Oaks *et al*, 2004). To better understand the effects caused by direct exposure or secondary poisoning from a contaminant in the aquatic environment it is therefore crucial to have knowledge about the uptake and depuration of contaminants in organisms. Due to many pharmaceuticals having enzyme and receptor based modes of actions, processes which are intensively studied during drug development, it has also been suggested to use pharmacological data in the environmental risk assessment process (Huggett *et al.* 2003; Berninger and Brooks 2010). However, to utilise this source of information, knowledge on how to estimate internal concentrations in non-target organisms is essential.

1.2.1 Bioconcentration, bioaccumulation and biomagnification.

In the current literature, the use of the terms bioconcentration, bioaccumulation and biomagnification are often used interchangeably. In this thesis the terms used are those defined in the Technical Guidance Document, TGD (2002). Bioconcentration is defined as the net result of uptake, distribution and elimination of a chemical from waterborne exposure. Bioconcentration is expressed in terms of the bioconcentration factor (BCF) which can be calculated either statically or dynamically. A static bioconcentration factor is the ratio between the concentration in the organism ($C_{organism}$) and the concentration in the surrounding

waters (C_{water}) at steady state. A dynamic bioconcentration factor can be calculated using uptake and depuration kinetics from the quotient of the uptake (K_{in}) and elimination (K_{out}) rate constants see Eq 1 (van Leeuwen *et al.* 2007).

$$BCF_{organism} = \frac{C_{organism}}{C_{water}} or \frac{K_{in}}{K_{out}} \quad \text{Eq 1.}$$

Bioaccumulation is the net result of the uptake, distribution and elimination of a chemical from all exposure routes i.e. water, sediment, soil, air and food. Biomagnification is the accumulation of a chemical *via* the food chain and can be defined by a biomagnification factor (BMF), which is the relative concentration in a predatory animal compared to the concentration in its prey:

$$BMF = \frac{C_{predator}}{C_{prey}} \quad \text{Eq 2.}$$

1.2.2. Uptake from sediment and sorption.

Sediment can act both as a sink through sorption of a chemical to particles and a source through resuspension of environmental contaminants. Sediment dwelling organisms have the potential to accumulate chemicals either passively *via* uptake from the water column or the pore water in the sediment or actively *via* ingestion of a food source. Therefore sediment associated contaminants may pose a threat to sediment dwelling organisms that are not predictable from concentrations in the water column alone. Measuring or estimating bioconcentration is a fairly standard procedure to assess the risks posed to sediment dwelling invertebrates by environmental contaminants and the uptake of contaminants *via* a food source is often discarded (Wenning *et al.* 2005) . However, for deposit feeding organisms, that live in and ingest sediment, to obtain nutrients from particles suspended in the sediment,

the risks from environmental contaminants may be underestimated if only uptake from pore water alone is considered. This is especially true for chemicals that strongly associate to sediment particles (Leppänen *et al.* 2000). There are examples of studies where the uptake of contaminants into sediment associated organisms has focused on the exposure from water only (Wildi *et al.* 1994; Artola-Garicano *et al.* 2003). However, most studies measuring uptake into sediment living organisms have exposed them to spiked sediment (Comber *et al.* 2007; Leppänen and Kukkonen 1998; Lu *et al.* 2004; Weston and Gulmann, 2000). Only a few studies have explored the importance of different uptakes routes of chemicals into sediment dwelling invertebrates and the results are inconclusive. Comber *et al.* (2007) and Leppänen and Kukkonen (1998) showed that uptake *via* ingestion of sediment into *Lumbriculus variegatus* was the main uptake route of uptake for some surfactants and polycyclic aromatic hydrocarbons whilst Lu *et al.* (2004) showed that the main uptake into oligochaetes is *via* the pore water. Weston and Gulmann (2000) showed that the importance of uptake routes into the polychaete *Abarenicola pacifica* is time dependent. To gain better understanding of the uptake of contaminants into sediment living organisms it is therefore crucial to have knowledge of the bioconcentration from the water exposure, the importance of the different uptake routes as well as the sorption behaviour of the chemical to sediment particles.

Sorption behaviour can be described by a solid/water distribution coefficient, Kd (mL/g). Kd is defined as the ratio of the concentration of a chemical in two different phases i.e. sediment/water, soil/water or sludge/water at equilibrium. Sorption is often normalised for organic carbon and expressed as a Koc value. The purpose of Koc is to reduce the variability in the sorption coefficient for a substance when applied to soils and sediments with varying organic carbon fraction. The formulae for calculating Kd and Koc are shown in equation 3 and 4.

$$K_d = \frac{C_s}{C_{aq}} \qquad \text{Eq 3.}$$

$$K_{oc} = \frac{K_d \cdot 100}{OC} \qquad \text{Eq 4.}$$

Where C_s (mg/g) is the concentration of a chemical in a solid phase i.e. soil/sediment/sludge and C_{aq} (mg/mL) is the concentration in the aqueous phase. The Koc assumes that there is only partitioning to the organic matter by hydrophobic interactions and may consequently not be a good descriptor for sorption of ionisable chemicals.

1.3. Equilibrium partitioning

1.3.1. Theory

To experimentally measure the environmental fate as well as toxicity to non-target organisms for all new chemicals that enter the market is a challenging and time consuming task. Equilibrium partitioning aims to predict the ratios of a chemical that will associate to soil or sediment particles and how much that will be freely dissolved in the pore water and consequently may be taken up into biota and possibly have an effect in the organism. A schematic figure of equilibrium partitioning is shown in Figure 1. By using equilibrium partitioning, variability in uptake between sediments is removed. Di Toro *et al* (1991) showed for neutral organic chemicals that by using pore water concentrations, to remove sediment variability, alongside effect data from water only studies, it is possible to predict the effects of these substances to sediment organisms. Di Toro also suggests that for neutral organics chemicals in an equilibrated sediment system, the effective exposure concentration is the same regardless of exposure route. However there are a few assumptions made when assessing risks of environmental contaminants in sediments using equilibrium partitioning (TGD; 2002):

- Sediment dwelling organisms are equally sensitive to the chemical as pelagic organisms.

- Concentrations in the sediment, the interstitial pore water and in the benthic organism are at thermodynamic equilibrium.

- Sediment-water partitioning can be measured or derived from chemical properties and sediment characteristics.

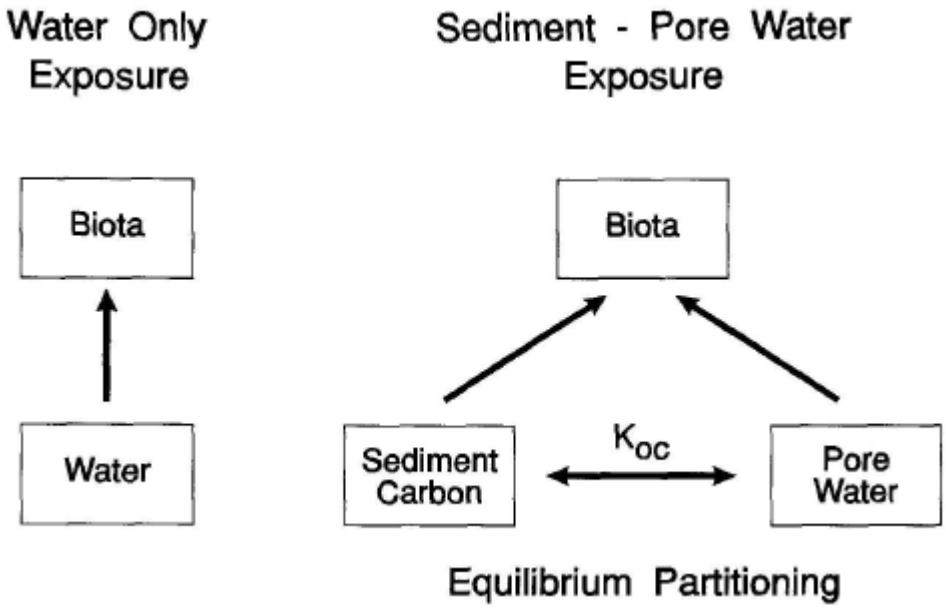


Figure 1. Diagram showing the exposure routes from a water only exposure (left) and a sediment system at equilibrium (right). Koc is the partition coefficient describing the distribution of a chemical between the pore water and the organic carbon fraction (di Toro *et al.* 1991).

1.3.2. Application of Equilibration Partitioning in Environmental risk assessment for chemicals in sediment.

In the Technical Guidance document, TGD, the European chemical bureau recommends the use of equilibrium partitioning when experimental data are missing for risk assessment of environmental contaminants in sediments. Chemicals that have a partition coefficient normalised for organic carbon, Koc < 1000 L/Kg are not likely to sorb to sediment, therefore

a trigger value for sediment effects assessment of log Koc or log Kow ≤ 3 is used to avoid extensive testing. To decide whether a chemical will have the potential to cause harmful effects in the environment a Risk quotient (RQ) is calculated by comparing a Predicted Environmental Concentration (PEC) with the Predicted No Effect Concentration (PNEC), the concentration below which unacceptable effects are not likely to occur. The RQ is calculated as follows:

$$RQ = \frac{PEC_{sed}}{PNEC_{sed}} \qquad \text{Eq 5.}$$

$$RQ = \frac{MEC_{sed}}{PNEC_{sed}} \qquad \text{Eq 6.}$$

If the PEC/PNEC is greater than 1, then there are indications of risks to non-target organisms in the sediment. If measured environmental concentrations are available, these can be used in calculating the RQ, shown in Eq 6. If measured (MEC) or predicted (PEC) sediment concentrations are absent the risk assessment for the aquatic compartment also has to cover the sediment compartment for substances with a log Kow < 5. For substances with a log Kow > 5 an assessment factor of 10 is applied since equilibration partitioning only considers exposure *via* the water. This is inconsistent with the recommendations in Di Toro *et al.* 1991 where it is stated that the exposure route is not of importance for a neutral chemical in an equilibrated sediment / water system.

For most chemicals that enter the market, sediment toxicity data are not available. Therefore equilibration partitioning is used as an initial screening approach to assess a potential risk of sediment associated chemicals. The Predicted No Effect Concentration (PNEC) for the sediment compartment is calculated as follows:

$$PNEC_{sed} = \frac{K_{susp-water}}{RHO_{susp}} \cdot PNEC_{water} \cdot 1000 \qquad \text{Eq 7.}$$

Table 1. Explanation of symbols

$PNEC_{water}$	Predicted No Effect Concentration in water	**[mg / l]**
RHO_{susp}	bulk density of wet suspended matter	**[kg / m^3]**
$K_{susp\ water}$	partition coefficient suspended matter water	**[m^3 / m^3]**
$PNEC_{sed}$	Predicted No Effect Concentration in sediment	**[mg / kg]**

If the first screening, using equilibration partitioning, indicates a potential risk, an experimental study with spiked sediment is recommended. When experimental data is scarce, assessment factors must be applied. The size of the assessment factors (1-1000) depends on the uncertainty of the data and the quantity of extrapolation needed, *i.e*. inter- and intra-laboratory variations of data, inter- and intra-species variations, short term to long term exposures and laboratory to field study extrapolations.

1.3.3. Equilibration Partitioning of Ionisable Chemicals, Dissociation and log D

Equilibrium partitioning can be applied to non-ionic organic chemicals, but the use for ionised chemicals is not recommended (TGD 2003; Di Toro *et al.* 1991). The reason is due to their chemistry. As stated previously, ionisable chemicals possess either weak acidic, basic or both acidic and basic functional groups which have the potential to dissociate in the natural environment depending on the pH. The dissociation of a weak acid is is described by equation 8 and illustrated in Figure 2. The dissociation of a weak base is described by equation 9. The degree of ionisation is described by the dissociation constant, K_a or K_b, and is defined in Eq 10 and 11. The measurement of the strength of an acid or a base in solution

is described by their pK_g or pK_b which is the pH at which the chemical is present in equal amounts of its neutral and ionic species (Figure 3).

$$HA \leftrightarrow A^- + H^+ \quad \text{Eq 8.}$$

$$HB^+ \leftrightarrow H^+ + B \quad \text{Eq 9.}$$

$$K_a = \frac{[A^-] \cdot [H^+]}{[HA]} \quad \text{Eq 10.}$$

$$K_b = \frac{[H^+] \cdot [B]}{[HB^+]} \quad \text{Eq 11.}$$

The protonation or deprotonation of a chemical with either acidic or basic or both acidic and basic functional groups can change the chemical properties and as a consequence also the fate and toxicity of the chemical in the environment (Franco *et al.* 2008). Several studies have reported a changing fate and toxicity of a chemical with a changing pH, see section 4.1.

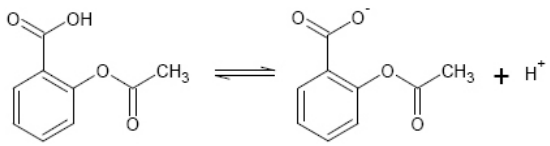


Figure 2. Ionisation of acetyl salicylic acid, a weak acid. Acetyl salicylic acid has a pKa of 3.1 (SPARC calculator) and is present predominantly in its neutral form (to the left) at pH < 3.5 and in its anionic form at pH > 3.5 (to the right).

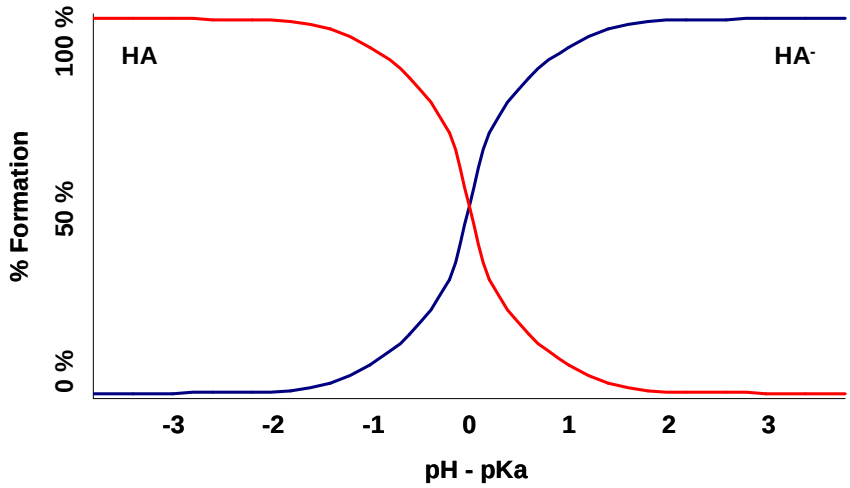


Figure 3. Dissociation of a weak acid. The neutral form is displayed in red and the dissociated anionic form is displayed in blue.

Lipophilicity of ionisable chemicals can be described by the Log D. Log D is defined as the ratio of the sum of the concentrations of all forms of the compound (ionised plus unionised) in each of the two phases e.g. octanol and water at a specific pH. Since Log D is pH dependent, one must specify the pH at which the log D was measured or calculated using Eq 12 (acids) or Eq 13 (bases).

$$\log D = \log Kow - \log(1+10^{pH-pKa}) \qquad \text{Eq 12.}$$

$$\log D = \log Kow - \log(1+10^{pKa-pH}) \qquad \text{Eq 13.}$$

1.4. What chemical, environmental and biological properties affect uptake of chemicals.

1.4.1. Chemical properties

1.4.1.1 Log Kow

It is generally recognised that the bioaccumulation of a chemical is related primarily to its lipophilicity and several studies support this (Mackay 1982, Chiou 1985). The Technical Guidance Document on risk assessment (2002) uses lipophilicity (expressed by the Log Kow) as a trigger value for secondary poisoning. A substance with a Log Kow > 3 is referred to as bioaccumulative and substances with a Log Kow > 4.5 as highly bioaccumulative and are subjects for higher tier testing (TGD, 2002). However, for chemicals that have the potential to ionise, e.g. pharmaceuticals, at environmentally relevant pH, Log D was suggested as a better descriptor for bioaccumulation in aquatic invertebrates (Meredith-Williams *et al.* 2012).

1.4.1.2 pKa

The pKa describes the degree of dissociation of a chemical at a particular pH value. If a chemical has a pKa in the range of environmentally relevant pH values it is very likely that will have an effect on the uptake into organisms due to protonation or deprotonation of ionisable chemicals, this is discussed further in section 4.2.1.

1.4.1.3 Molecular weight and steric effects

Molecular weight has been shown to influence the uptake of organic chemicals in soils. Topp *et al.* (1986) and Shaw and Connell (1980) showed that steric effects, e.g. due to chlorination, have an influence on the uptake of polychlorinated biphenyls (PCB's) in sea mullet. Lohner and Collins (1987) studied the uptake of six organochlorines in midge larvae (*Chironomus riparius*) and discovered a correlation between molecular weight, uptake constants and Log Kow.

1.4.2. Environmental properties

1.4.2.1 pH

Despite the variability of pH in waters, sediments and soils, only a few studies have investigated the effects pH have on the fate and toxicity of organic environmental pollutants. The bioconcentration of pyrene was studied in midge larvae (*Chironomus riparius*) at three different pH values, 4, 6 and 8. A correlation between an increased pH and an increased uptake was shown. However, exposing the midges at pH 4 induced an abnormal mucus secretion. Thus, it was hypothesized that this response to an acidified environment caused the reduced biconcentration (Wildi *et al.* 1994). Nakamura *et al.* (2008) investigated the toxicity and bioconcentration of fluoxetine in japanese medaka (*Oryzias latipes*) at three different pH values, 7, 8 and 9. They found that LC50 values ranged from 0.2 mg/L (pH 9) to 5.5 mg/L (pH 7). The difference in toxicity was explained by differences in bioconcentration at different pH values. BCF values ranged from 13 (pH 7) to 330 (pH 9), explained by a higher fraction of the lipophilic nonionised species at the higher pH. Another study investigated the sorption to natural sediments with varying pH (Zhang *et al.* 2010). The study showed that the sorption of the antibiotic, tetracycline, to sediment was dependent on a range of environmental factors and that sorption was facilitated at lower pH *via* a cation exchange mechanism.

1.4.2.2 Sediment characteristics

Several studies have investigated the influence of sediment characteristics on the sorption behaviour of chemicals. Organic matter has been shown to increase the sorption of phenolic chemicals (Isaacson and Frink 1984), as has the presence of cadmium and copper on the sorption of tetracycline to sediments and soils (Zhang *et al.* 2011). Particle size has also been shown to have an influence on the distribution of chemicals in sediments, hence also the bioavailabilty of organic xenobiotics (Kukkonen and Landrum 1996).

1.4.2.3 Food quality / quantity

Granberg *et al.* (2006) investigated the effects of sediment organic matter quality on the uptake of pyrene into the mud snail *Hydrobia ulvae* by using two different food sources, one high quality microalgae, *Tetraselmis sp* and a low quality lignin. Pyrene was accumulated into the organisms to a larger extent when a high quality food source was used compared to the low quality food. Gilek *et al.* (1996a) investigated the effects of algae concentration on the accumulation of PCBs in the Baltic mussel *Mytilus edulis* and observed a decreasing bioaccumulation when algae concentration increased.

1.4.2.4 Temperature

Uptake of a series of moderate to very hydrophobic PAHs was measured in *L. variegatus* at three different temperatures, 5, 12 and 24 ° C. Results showed that the uptake was an enthalpy driven process and that bioconcentration decreased with an increasing temperature (Muijs and Jonker 2009). The bioconcentration of anthracene was also measured in chironomids at three different temperatures, 16, 25 and 30 ° C. Results showed that anthracene was taken up to the greatest extent at 25 ° C, lesser at both 16 and 30 ° C (Gerould *et al.* 1983). Wang *et al.* (2011) saw increasing sorption behaviour with a decreasing temperature of five different PAHs to three different sorbents, two natural sediments and a treated inorganic fraction which consequently changes the bioavailability and the potential for uptake into biota.

1.4.3. Biological properties – size, feeding behaviour and metabolic pathways

The accumulation of environmental contaminants in aquatic organisms can vary between organisms due to several species specific traits, e.g. feeding behaviour, size and the presence or absence of metabolic pathways within an organism. The impact of ecological processes such as competition and predation on the uptake of environmental contaminants have been

less studied, however it is likely that they might influence the bioaccumulation into aquatic organisms.

1.4.3.1 Size

One can assume that an organism's body size will affect the bioconcentration of chemicals mainly as a result of two factors. A smaller organism has a higher surface/volume ratio and thus has the potential to accumulate chemicals passively to a higher extent than a larger organism (Arnot *et al*, 2012). However, a larger organism is more likely to have a longer gut which increases the gut passage time and the potential for accumulation *via* a food source. Ahrens *et al.* (2001) observed that larger individuals of *Nereis succinea* had a higher adsorption efficiency of food and sediment bound organic contaminants compared to smaller individuals which were explained due to a longer gut passage time. The influence of body size on the uptake, depuration, and bioaccumulation of polychlorinated biphenyl congeners was studied in the mussel, *Mytilus edulis,* by Gilek *et al.* (1996b). It was found that an increasing body weight resulted in a decreasing bioaccumulation that could not be explained simply by equilibrium partitioning.

1.4.3.2. Feeding behaviour

Gaskell *et al.* (2007) measured the bioaccumulation of DODMAC, a cationic surfactant, in four freshwater macroinvertebrates (*Asellus aquaticus*, *Chironomus riparius*, *Gammarus pulex*, *Lumbriculus variegatus*). *Chironomus* accumulated the DODMAC to the highest degree and the observed bioaccumulation pattern was *Chironomus* > *Gammarus* > *Asellus* = *Lumbriculus.* The results could not be explained only by gut passage time. Another study measured the bioaccumulation of PCBs and PAHs in three different marine species with different feeding behaviour: *Arenicola marina* which feeds by ingestion of sediments; *Macoma balthica*, a deposit feeder; and *Mytilus edulis*, a filter feeder. The contaminants were accumulated as follows: *Arenicola* > *Macoma* > *Mytilus* and the study concluded that the feeding does have an impact on the uptake behaviour (Kaag *et al.* 1997).

1.4.3.3. Ecological processes

In the study of Kaag *et al.* (1997), it was also observed that *Macoma balthica* accumulated the chemicals more than in the presence of *Mytilus edulis*. This was thought to be due to *M. edulis* preventing the blooming of phytoplankton which resulted in a decreased overlying food source (Kaag *et al.* 1997). Time could also be an important factor that might influence the uptake of chemicals into aquatic organisms. Several studies refer to bioconcentration and bioaccumulation factors as the ratio of the tissue concentration and the surrounding matrix. However, in many cases tests are not run until equilibrium and BCF and BAF should be referred to as pseudo BCF or BAF. Without equilibrium being reached, dynamic factors must be calculated based on uptake and depuration rates. Time can also influence the fraction of a contaminant that sorbs irreversibly to the sediment (Löffler *et al.* 2005). This is likely to influence the uptake of contaminants into sediment dwelling organisms.

1.5. A conceptual model for estimating uptake into sediment dwelling organisms

To estimate the uptake of sediment associated pharmaceuticals or active ingredient in personal care products into sediment dwelling organisms, there are some fundamental knowledge gaps that need to be filled. For example, we must have knowledge on the distribution of the contaminant between the sediment particles and the sediment porewater, e.g. by measuring a distribution coefficient such as K_d. We must also have knowledge on the degree of uptake and depuration from the pore water and of how environmental properties might affect this. For example, for ionisable chemicals, pH could be very important in determining the uptake of a compound. Additionally, we must have knowledge on the metabolism of the compound since metabolism can affect the uptake and depuration kinetics of contaminants. Last but not least, the additional uptake of contaminant *via* contaminated sediment must be addressed. A conceptual model illustrating the inter-linkages of all these process is shown in Figure 4.

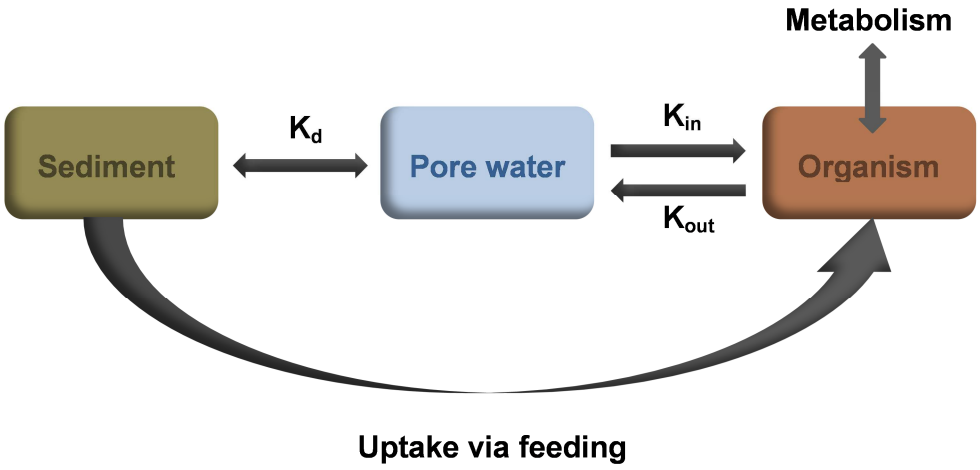


Figure 4 A conceptual model for estimating uptake into sediment dwelling organisms.

1.6. Aim and objectives of thesis.

The overall aim of this thesis was to develop an understanding of the factors and processes affecting the uptake of PPCPs into sediment dwelling organisms and to explore whether the conceptual framework described above is suitable for assessing the uptake of sediment-associated ionisable substances into benthic organisms. This was achieved using the following specific objectives:

1. To explore the sorption behaviour of ionisable compounds in natural sediment and to assess whether this behaviour can be predicted based on the physico-chemical properties of a substance (Chapter 2);

2. To explore the uptake and depuration behaviour of a range of ionisable compounds, in order to understand the relationships between uptake and the physicochemical properties of a molecule and to evaluate existing models for estimating uptake of ionisable substances into benthic organisms (Chapter 3);

3. To explore the effects of varying pH on the uptake of selected ionisable compounds from water into benthic invertebrates (Chapter 4);

4. To assess the degree of metabolism of selected ionisable compounds in benthic organisms (Chapter 4);

5. To determine the importance of feeding as a route of uptake for ionisable compounds (Chapter 5); and

6. To use the information, produced by Objectives 1-5, to model uptake of a selection of ionisable compounds from sediment into benthic organisms and to provide recommendations on how to better to assess the risks of sediment-associated ionisable substances (Chapter 6).

The work focused on the oligochaete worm, *Lumbriculus variegatus* and a range of compounds used as pharmaceuticals or ingredients in personal care products. An overview of *L. variegatus* and more detailed information on the study compounds is given in the following Sections.

1.7. Study organism

Lumbriculus variegatus

Phylum: Annelida

Class: Oligochaeta

Order: Lumbriculida

Family: Lumbriculidae

Genus sp: Lumbriculus variegatus

Common names: California blackworms; blackworms; mudworms

L. *variegatus* are found in North America and Europe. Preferred habitats are at the edges of ponds, lakes, slow flowing rivers or marshes where it feeds on decaying vegetation, microorganisms and sediment (Brinkhurst and Gelder 1991, Penttinen *et al.* 1996). *L. variegatus* inhabits both the sediment and water compartment simultaneously, dwelling with the head first in the sediment and keeping the tail in the water, where respiration and photoreception occurs (Penttinen *et al.* 1996). *L. variegatus* reproduces both sexually and asexually *via* autotomy into two fragments (Drewes and Fourtner, 1990). Both fragments can regenerate into two individuals. Sexual reproduction is very rarely observed in the laboratory, however asexual reproduction is commonly observed (Drewes and Brinkhurst 1990). Recent genetic work on the speciation of *L. variegatus* reveals that *L. variegatus* was found to consist of at least two distinct clades (I and II), both of which occur in Europe as

well as North America. The authors concluded that clades I–II are separately evolving lineages, and that they should be regarded as separate species (Gustavsson *et al.* 2009).

Lumbriculus variegatus and other benthic macroinvertebrates are the prey of various secondary consumers, e.g. crabs, bottom-feeding fish and birds eating sediment dwelling organisms. (Wootton, 1984; Wallace and Webster, 1996). Thus, transfer of accumulated contaminants to predators may occur and result in secondary poisoning.

L. variegatus has been commonly used for measuring bioaccumulation of sediment associated contaminants into sediment dwelling organisms. For instance, the uptake of PAHs into *Lumbriculus variegatus* has been well studied, and a number of publications on uptake routes, feeding behaviour, trophic transfer are available in the open literature (Kukkonen and Landrum, 1994; Leppanen, 1995; Conrad *et al.* 2002; Leppanen and Kukkonen, 2008; Navarro *et al.* 2013). Other groups of substances whose uptake has been studied in *L. variegatus* are chlorinated hydrocarbons, metals and surfactants (Phipps *et al.* 1993; Ankley *et al.* 1994, Comber *et al.* 2008). Although, more scarce, data on the bioaccumulation of PPCPs into *L. variegatus* are also available (Leibig *et al.* 2005; Higgins *et al.* 2009)

1.8. Study compounds

The study used a range of compounds used as either pharmaceuticals or as ingredients in personal care products. The compounds were selected to cover a range of physico-chemical properties and included acidic, neutral and basic substances. An overview of the structure and properties of the study compounds is given in Table 2 and an outline of the use, properties and environmental effects of the study compounds is given below

Caffeine

Caffeine (3,7-dihydro-1,3,7-trimethyl-1h-purine-2,6-dione) is a crystalline alkaloid that in humans act as a stimulant of the central nervous system. Caffeine has been reported to be the

most commonly consumed stimulant by humans (Lawrence *et al.* 2005) and human consumption has been estimated to average 70 mg caffeine/person/day (Buerge *et al.* 2003). Medicinally it is used as a cardiac, cerebral, and respiratory stimulant, and it also functions as a diuretic (Buerge *et al.* 2003). Caffeine mainly acts as an antagonizer to adenosine and increases activity in neurotransmission (Daly *et al.* 1981).

As a result of its high consumption, caffeine is a commonly detected substance in surface waters around the world (Kolpin *et al.* 2002; Weigel *et al.* 2002; Buerge *et al.* 2003; Metcalfe *et al.* 2003; Sankararamakrishnan and Guo 2005; Thomas and Foster 2005). Previous research has shown effects of caffeine on several non-target species in the environment with LC50 values of below 100 mg/L being reported for fish, aquatic invertebrates and insects (Moore *et al.* 2007). Bantle *et al.* (1994) found that relatively low caffeine concentrations in water affected Xenopus laevis egg development when exposed for 96 hours (LC50 = 0.22 to 0.37 mg/mL).

Chloramphenicol

Chloramphenicol (2,2-dichloro-N-[1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide) is a broad spectrum antibiotic that became available in 1949. Chloramphenicol prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome. It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit, preventing peptide bond formation (Jardetzky, 1963)

Chloramphenicol has been detected in both sewage effluents and surface waters (Stolker *et al.* 2004; Choi *et al.* 2008). Effects of chloramphenicol have been observed in fresh water and marine species of algae at high µg to low mg per liter concentrations (Campa-Cordova *et al.* 2006; Sanchez-Fortun *et al.* 2009). Concerns have also been raised over the potential for chloramphenicol residues in the environment to select for resistance in gram negative bacteria (Kelch and Lee, 1978).

Diclofenac

Diclofenac is a non-steroidal anti-inflammatory (NSAID) pharmaceutical which acts via inhibition of the prostaglandin synthesis pathway (Gan, 2010). Diclofenac is commonly detected in sewage treatment effluents and surface waters (Remberger *et al.* 2009; Buser *et al.* 1998). The most documented case of the environmental effects of diclofenac is the decline of vultures in the Indian subcontinent (Oaks *et al.* 2004). Diclofenac has also shown to have effects in the aquatic environment. For example, Schwaiger *et al.* (2004) reported histopathological alterations in rainbow trout following exposure to diclofenac.

Fluoxetine

Fluoxetine is a selective serotonin uptake inhibitor (SSRI); a class of anti-depressants that during the last decade has become one of the most prescribed pharmaceuticals (Vetulani et al 2010). Fluoxetine has been detected in surface water at low ng/L concentrations (Zorita *et al.* 2009). Several reports of toxic effects due to fluoxetine are available in the open literature. Brooks et al (2003), Laville *et al.* (2004) and Perreault *et al.* (2003) reported behavioral responses as well as physiological responses following exposure to fluoxetine.

Naproxen

Naproxen is a NSAID which acts by inhibiting the enzyme cyclooxygenase; an enzyme responsible for the biosynthesis of the prostaglandins. Prostaglandins are lipid compounds, derived enzymatically from fatty acids, which are secreted into the bloodstream, causing fever, inflammation, muscle contraction and which affect other processes in the human body (Segre, 1980). Naproxen has been detected in sewage effluents and surface waters across the globe (Tixier *et al.* 2003; Lee *et al.* 2003; Nakada *et al.*. 2005). Ecotoxicological effects of naproxen in the aquatic environment have been reported in e.g. fish, where naproxen exposure has been shown to influence oxidative metabolism in liver cells leading to oxidative damage. Exposure of mussels to naproxen results in reduced cell adherence and lipid peroxidation (Gagne *et al.* 2006a; Gagne *et al.* 2006b)

Salicylic acid

Salicylic acid is a NSAID that directly and irreversibly inhibits the activity of both types of cyclo-oxygenases (COX-1 and COX-2) to decrease the formation of precursors of prostaglandins and thromboxanes from arachidonic acid. Salicylic acid is also a key ingredient in many skin-care products for the treatment of acne, psoriasis, calluses, corns, keratosis pilaris, and warts (Drugbank). Acetyl salicylic acid, a precursor to salicylic acid, has been detected in drinking water, ground water and surface water (Stolker *et al.* 2004)

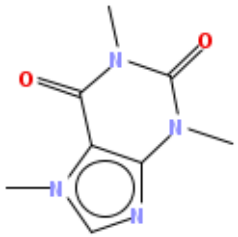
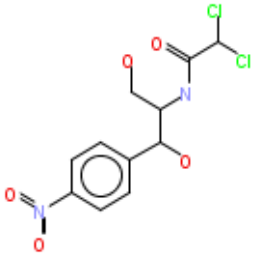
Sulfamethazine

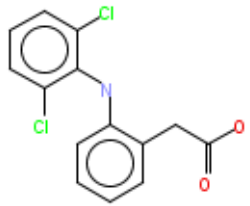
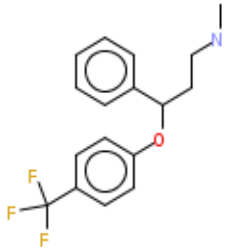
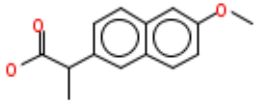
Sulfamethazine belongs to the antibiotic class of sulfonamides. Sulfonamides inhibit the enzymatic conversion of pteridine and p-aminobenzoic acid (PABA) to dihydropteroic acid by competing with PABA for binding to dihydrofolate synthetase, an intermediate of tetrahydrofolic acid (THF) synthesis. THF is required for the synthesis of purines (Drugbank). Sulfamethazine has been detected at ng/L concentrations in sewage effluents and surface waters. (Alder *et al.* in: Daughton and Jones-Lepp, 2011; Kolpin *et al.* 2002)

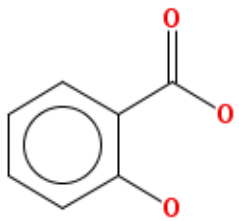
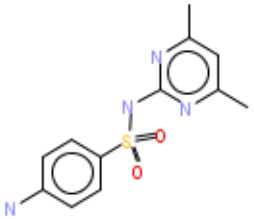
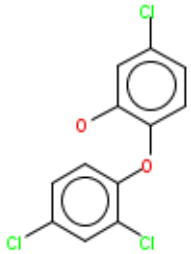
Triclosan

Triclosan is an antibacterial and antifungal agent. Triclosan acts as a biocide, with multiple cytoplasmic and membrane targets (Russel *et al.* 2004). Triclosan has been detected in sewage effluents, surface waters and sediments (Singer *et al.* 2002; Kolpin *et al.* 2002; Kookana *et al.* 2011). Effects of triclosan in the aquatic environment have been fairly well studied with a range of acute and chronic effects being reported in algae, macrophytes, daphnids and fish (Orvos *et al.* 2002; Kookana *et al.* 2011).

Table 2 Structures and properties of the pharmaceuticals and personal care products studied in the thesis.

Compound	Acid / Base	Cas	Chemical structure	Molecular weight (g/Mol)	Log Kow[1]	pKa[1]	Radioactive activity (GBq/mmol)
Caffeine	Weak Base	58-08-2		194.2	1.03	0.05	1.894
Chloramphenicol	Weak Acid	56-75-7		323.1	-0.02	8.6	2.220

Diclofenac	Weak Acid	15307-86-5		296.1	4.13	4.1	2.321
Fluoxetine	Weak Base	54910-89-3		309.3	4.16	10.1	2.035
Naproxen	Weak Acid	22204-53-1		230.3	3.36	4.5	2.035

Salicylic acid	Weak Acid	69-72-7		138.1	2.30	3.1	1.739
Sulfamethazine	Zwitterionic[1]	57-68-1		278.3	2.30	1.6; 6.0	0.303
Triclosan	Weak Acid	3380-34-5		289.6	5.42	8.1	2.431

[1] *Molecule includes both acidic and basic functional groups.*
[2] *Estimated using SPARC*

CHAPTER 2

SORPTION OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS TO AQUATIC SEDIMENTS

2.1 Introduction

To predict the fate and bioavailability of a chemical in aquatic sediment, an understanding of its sorption behaviour is vital. Sorption involve two processes: adsorption which is the process by which molecules of a substance attach onto the surface of a solid such as soil or sediment; and absorption which is the process by which molecules of a substance are taken into a solid phase (Schwarzenbach *et al.* 2002). Sorption behaviour can be described by a solid/water distribution coefficient, Kd (mL/g) which is defined as the ratio of the concentration of a chemical in the aqueous and solid phases of a system e.g. sediment/water, soil/water or sludge/water at equilibrium (Schwarzenbach *et al.* 2002).

The sorption behaviour of neutral organics in sediment-water systems has been well studied (Calvet, 1989). The main sorption mechanism for neutral organic chemicals is *via* weak (2-4 kJ/mol) hydrophobic interactions (*i.e.* Van der Waals forces) and results from interactions between organic matter in the sediment particles (OM) and the hydrophobic moieties of a chemical (Schwarzenbach *et al.* 2002). Factors that affect the sorption of neutral organics chemicals therefore include chemical properties such as hydrophobicity and environmental properties such as the organic carbon content of the sediment and the nature of the organic carbon (Karickhoff 1981; Briggs, 1981; Sabljic *et al.* 1995). As sorption of neutral organic compounds is so dependent on the organic carbon content of a solid matrix, sorption is often

expressed in terms of the organic-carbon normalized sorption coefficient (Hamaker and Thompson, 1972).

A range of predictive models is available for estimating Koc values for neutral organics (e.g. Karickhoff 1981; Bintein and Devillers, 1994; EPI suite, March, 2013). These models typically estimate sorption of a substance based on its octanol-water partition coefficient (e.g. Karickhoff 1981; Bintein and Devillers, 1994) or on molecular connectivity indices, which describe the shape and degree of branching of a molecule (EPI suite, March, 2013). As the models only need information on the chemical structure to run, predictions of sorption of neutral organic chemicals can be performed without the need for experimental testing.

Although we have a fairly good understanding of the factors and processes that influence the sorption of neutral organic chemicals in the environment, less is known about ionisable chemicals, especially in sediments. Whilst hydrophobic interaction are the main sorption mechanism for neutral chemicals the mechanisms involved in the sorption of ionisable chemicals are more complex and include not only hydrophobic interactions and Wan der Waals-interactions but also hydrogen bonding, covalent bonding and the formation of cation bridges (Tolls, 2001, Pan *et al.* 2009). Due to the complexity of the sorption behaviour of ionisable chemicals their sorption behaviour is likely to be not only influenced by chemical properties such as lipophilicity, polarity, charge and the degree of dissociation of a molecule (Karickhoff, 1981; Kah and Brown, 2006) but also by the sediment characteristics such as sediment pH, sediment and pore water organic carbon content and cation exchange capacity (Kah and Brown, 2006; Ter Laak *et al.* 2006) and the nature of the sediment components such as the type of humic acid present (Pan *et al.* 2009). The degree of sorption of an ionisable compound to sediment may also change with time (Conkle *et al.* 2012). Many compounds can also bind irreversibly to soils and sediments. These are referred to as non-extractable residues or bound residues (IUPAC 1984). Von Oepen *et al.* (1991) showed that polar chemicals are more likely to form bound residues than neutral chemicals since they

often contain OH^- or NH_2-groups, similar to those in humic substances, are more easily incorporated into humic substances.

Most studies exploring the sorption of ionisable chemicals to solid matrices have focused on pesticides in soils (Ogram *et al.* 1985; Gevao *et al.* 2000; Kah and Brown 2006). Over the past decade, more information on the fate and behaviour of PPCPs in soil has become available. Classes of PPCPs where Kd or Koc have been measured include antibiotics (ter Laak 2006; Tolls 2001; Thiele-Bruhn *et al.* 2003); estrogens (Yu and Huang, 2005); anticonvulsant and NSAIDs (Maoz and Chefetz, 2010). Pan *et al.* 2009 reviewed available studies on sorption of PPCPs in sediments, sludge, soils and other matrices. A majority of the reported K_d values ranged from 0 up to 500. Tetracycline and oxytetracycline showed to be highly sorptive with Kd values up to above 300 000 (Sassman and Lee, 2005). The sorption behaviour of pharmaceuticals in sediment has been less studied than in soil (Loffler 2005; Yamamoto 2009 ; Pan *et al.* 2009).

Due to the complexity of the sorption mechanisms for ionisable compounds and that sorption is affected by a range of soil and sediment properties, application of the predictive models for sorption of neutral organics to ionisable compounds can lead to either an over- or under-estimation of the sorption behaviour for polar compounds (Tolls, 2001). Several studies have attempted to develop improved approaches for predicting sorption of ionisable chemicals using different parameters than used for neutral organic substances (ter Laak *et al.* 2006; Franco *et al.* 2008). In addition, methods have also been proposed for estimating the sorption of pharmaceuticals based on pharmacological properties with correlations being derived between the drug volume of distribution (V_D) in humans and soil sorption coefficients (Williams *et al.* 2006; 2009).

As an understanding of sorption is essential to understand uptake of pharmaceuticals into sediment-dwelling organisms, this study therefore explored the partitioning behaviour of

eight PPCPs in a sediment-water system. The results of the studies were then used to evaluate whether a range of relationships, which have been developed for estimating the sorption behaviour of both neutral and ionisable compounds, are able to estimate the sorption of PPCPs in sediment systems.

2.2 Methods

2.2.1 Test chemicals

Test compounds were ^{14}C-labelled and had a specific activity ranging from 1.74 to 2.43 GBq $mmol^{-1}$. Chloramphenicol, diclofenac, naproxen and salicylic acid were obtained from Perkin Elmer (Boston, USA), fluoxetine was obtained from American Radiolabelled Chemicals (St Louis, USA), and triclosan was obtained from Unilever (Colworth, UK). Compounds were chosen to represent a wide range of chemical properties. Information on the structures and properties of the study compounds is given in Chapter 1 and data on the label position and specific activity of the study compounds is given in Table 3.

Table 3 Test chemicals, their specific activity and position of the ^{14}C label.

Test compound	Labelling	Specific activity [GBq/mmol]
caffeine	methyl-^{14}C	1.894
chloramphenicol	dichloroacetyl-1,2-^{14}C	2.220
diclofenac	U-^{14}C	2.321
fluoxetine	methyl-^{14}C	2.035
naproxen	methyl-^{14}C	2.035
salicylic acid	methyl-^{14}C	1.739
sulfamethazine	Phenyl-^{14}C	0.3034
triclosan	U-^{14}C	2.431

2.2.2 Test sediment

Sediment was sampled from the top 10 cm of the benthos at a river site near Buttercrambe, North Yorkshire, UK (SE 73499 58510) on the 5th of February, 2009 (sediment 1) and on the 9th of March, 2010 (sediment 2). After sampling, sediment was sieved (2 mm) and stored at 5 ± 1 ˚C. Studies were performed within six months of sediment collection. Sediment was analyzed by Laboratoire d'analyses des sols (INRA Arras, France) for the following properties: water content; clay (<2 μm), silt (2-50 μm) and sand (50-2000 μm) content; total organic carbon (ISO 10694); total nitrogen (ISO 10694); pH (ISO 10390); $CaCO_3$ content (ISO 10693); cation exchange capacity (NF X 31-130); and for Al, Ca, Fe, Mg, K and P. Sediment properties are listed in Table 4.

Table 4 Sediment properties

Parameter	Sediment 1	Sediment 2
pH:	7.33	7.67
OC [g/kg]	24.2	5.51
CEC [cmol+/kg]	15.8	4.65
Grain size [g/kg]		
- Clay:	124	42
- Silt:	157	27
- Sand:	719	931

2.2.3 Sorption studies

Test methods followed the OECD guideline Adsorption/Desorption using a Batch Equilibrium Method (OECD 106; 2000). Prior to the main study, a preliminary study was performed using sediment 1 to determine the optimal sediment/solution ratios, equilibration time and adsorption of the test substances to the surfaces of the test vessel. All tests were performed in the dark at 4 ºC to minimize degradation. The experimental methods for the preliminary study and the main study are described below.

2.2.3.1 Preliminary study

Water content was measured in the sediments before each experiment. In order for the sediment solution slurries to equilibrate, sediments (1, 2 or 4 g) were mixed with 20, 30, or 40 ml 0.01 M $CaCl_2$ in 50 ml centrifuge PTFE tubes (Oak Ridge centrifugation tube, FEP by Nalgene Nunc International) 18 h prior to spiking of the test chemicals. Three sediment solution ratios were used: 1:5, 1:10 and 1:20 or, for the chemicals known to sorb strongly to soil, ratios of 1:20, 1:30 and 1:40 were used. Samples were spiked with radiolabelled substances at concentrations ranging from 15 to 162 nmol/L and placed on a shaker (200 rpm) in the dark at 4 ºC for 72-96 hours. Controls containing only 20 ml 0.01M CaCl and the test chemicals were incubated as well in order to check for possible adsorption to test vessels. Three replicates of each ratio were taken for analysis at 2, 4, 8, 24, 48, 72 and 96 h. At sampling, tubes were centrifuged at 3500 g for 10 minutes (Hermle Z 513K Bench Top Centrifuge) and 1 mL of the supernatant was then taken and mixed with 10 mL Ecoscint A scintillation cocktail. Test substances remaining in the aqueous phase were then measured using a Liquid Scintillation Counting, LCS, (Liquid scintillation Counter LS 6500, Beckman Coulter Inc., Fullerton, USA). Samples were counted three times for 5 min. Counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method.

2.2.3.2 Main study

The main study was performed to investigate the sorption of all test compounds to sediment 1 and three compounds, diclofenac, fluoxetine and triclosan, to sediment 2. The study was performed under the same conditions as the preliminary study. Sediment-solution ratios were chosen to meet the criteria recommended in the OECD guidelines (OECD 106: 2000). The chosen sediment-solution ratios and equilibration times are shown in Table 5. Triplicate samples were left shaking at 200 rpm throughout the test and after equilibration, samples were taken and analysed as described in the preliminary study.

Table 5 Experimental setup for the main sorption study

Test compound	Applied radioactivity [Bq]	Concentration [nM]	Sediment : solution ratio	Equilibration time [h]
caffeine	982 ±19	25.9	1:5	48
chloramphenicol	1024±47	23.1	1:2	96
diclofenac	977±46	22.6	1:2	48
Fluoxetine	922±40	15.1	1:30	48
Naproxen	869±42	21.4	1:5	48
Salicylic acid	1015±14	29.2	1:5	48
Sulfamethazine	984±40	162	1:10	96
triclosan	1319±45	18.1	1:30	48

Estimation of sorption coefficients

The concentration of each PPCP adsorbed to the solid phase was calculated using Equation 14:

$$C_s = \frac{V(C_i - C_{aq})}{m_s} \quad \text{Eq 14}$$

Where: C_i is the concentration of pharmaceutical in the control treatment; C_{aq} is the concentration in the aqueous phase; V (mL) is the volume of solution in the suspension; and m_s is the mass of soil (g). Soil sorption coefficients (Kd (mL/g)) for each study chemical were then calculated using equation 15.

$$K_d = \frac{C_s}{C_{aq}} \quad \text{Eq 15}$$

2.2.4 Evaluation of predictive methods for estimating sorption of PPCPs

Six different models were evaluated to assess their suitability for estimating the sorption behaviour of PPCPs. The models are presented in Table 6. Chemical properties used as input parameters (i.e. pKa and log K_{ow}) were derived from SPARC (Carreira *et al.* 1994). Predicted K_{oc} values were then compared with experimental which were derived using Equation 16.

$$K_{OC} = \frac{K_d}{f_{oc}} \qquad \text{Eq 16}$$

Table 6 Relationships between chemical properties and sorption that were evaluated in the study.

Model			**Equation**
EPI suite	MCI		Log Koc = 0.5213·MCI + 0.60 + Σ Pf N
	Kow	Non-polar	Log Koc = 0.8679·log Kow – 0.0004
		Polar	Log Koc = 0.55313·log Kow + 0.9251 + Σ Pf N
Karickhoff			Log Koc = 0.989·log Kow- 0.346
Di Toro			log Koc = 0.00028 + 0.983·log Kow
Bintein & Devillers			log Kd= 0.93·logKow+1.09·log foc+0.32·CFa - 0.55·CFb + 0.25
MAMI	Acids		log Koc = log(φn ·100.54· log Kow+1.11 +φion ·100.11·log kow+1.54)
	Bases		log Koc = log(φn ·100.37· log Kow+1.70 + φion ·10pKa^(0.65) · f^(0.14))
	Amfoters		log Koc = log(φn ·100.50· log Kow + 1.13 + φ- ·100.11· log Pn+1.54 + φ+ ·10pKa^0.65 · f^ 0.14)
TGD	Hydrophobics		log Koc = 0.81 log Kow + 0.10

MCI – Molecular Connectivity Index; Σ P_f N - Sum of the products of all the applicable correction factorcoefficients (P_f)multiplied by the number of times that factor is accounted for (N); Kow – Octaol-water partitioning coefficient; foc – Fraction organic carbon in sediment; CFa / b – Correction factor to quantify the variation of dissociated acids and bases in the system*;* φn / ion – Fraction of neutral and ionic species*;* φ- /+ - Fraction of negatively and positively charged molecules*; f - is Kow/(Kow + 1).*

2.3 Results and discussion

2.3.1 Partitioning of PPCPs between water and sediment

Prior to the main experiment a set of preliminary experiments were performed in accordance with the OECD 106 guidelines (OECD 106). The preliminary study showed that, with the exception of sulfamethazine and chloramphenicol, all compounds reached equilibrium between the sediment and water phases within 48 hours (Figure 5). Sulfamethazine and chloramphenicol did not appear to have reached equilibrium by the last sampling time point. Studies with sulfamethazine and chloramphenicol were therefore run for 96 hours in the main study. According to the OECD 106 guideline, a sorption percentage above 20 % is a requirement whilst a percentage above 50% is preferable. This was achieved for all compounds in at least one of the sediment-solution ratios. All compounds, except for fluoxetine, showed an increasing proportion of sorbed chemical with increasing sediment:solution ratio. For chosen equilibration times and ratios, see table 3. Controls showed that compounds were stable and that no sorption to the test vessel occurred during the study.

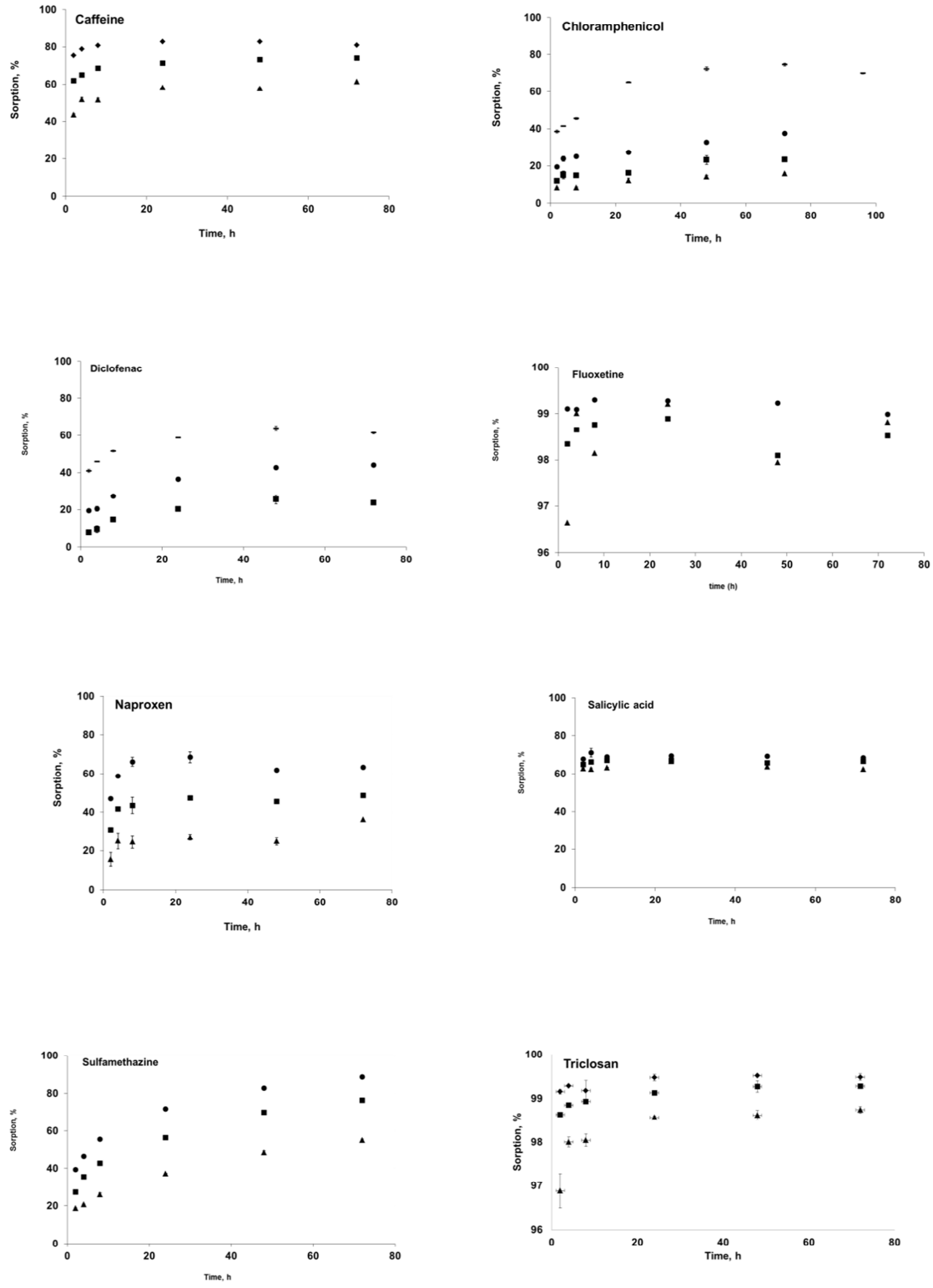


Figure 5. Sorption behaviour of the study compounds to sediment 1, Buttercrambe, in the preliminary test. Mean (n=3 ±SD) sorption of different sediment:solution ratios: dashes=1:2, circles=1:5, squares=1:10, triangles=1:20.

In the main experiment, sorption increased in sediment 1 in the following order: diclofenac < chloramphenicol < salicylic acid < naproxen < caffeine < sulfamethazine < triclosan < fluoxetine. Kd values ranged from 2.3 ml/g (diclofenac) to 1789.7 ml/g (fluoxetine) (Table 7). In sediment 2, sorption increased in the following order: diclofenac < triclosan < fluoxetine. Kd values ranged from 4.2 (diclofenac) to 422 (fluoxetine). Although Kd values for fluoxetine and triclosan were considerably higher than the other study compounds, these values are not exceptionally high in comparison with previously reported literature. Previously reported Kd values for triclosan are in the same range, 1272-1573 ml/g (Lin *et al.* 2011). Fluoxetine (pKa 10.1) is a highly sorptive chemical in environments below its pKa due to sorption of the positively charged species *via* cation exchange and charge transfer to organic matter and clay minerals. This mechanism has previously been suggested as the reason for high sorption observed for cationic pesticides (Kah and Brown, 2007). Previously reported Kd values of Fluoxetine confirms this. Kwon and Armbrust (2008) reported Kd values of 785-12 546 ml/g when investigating the sorption behaviour of fluoxetine in two sediments and three soils. In the soil where the highest Kd was estimated (12 546 ml/g), the organic carbon content was slightly lower than the organic carbon content of sediment 1 in the present study. However, the CEC was higher suggesting that ionic sorption is an important process in sorption of cationic substances. Based on the lipophilicity of diclofenac (log Kow 4.13), a higher sorption was expected than seen in the current study. The diclofenac results do however agree with previous data showing low sorption of diclofenac to natural sediments with reported Kd values ranging from 1.9 – 4.7 ml/g (Scheytt *et al.* 2005). A possible explanation for the low sorption of diclofenac is that the majority of the diclofenac in the sediment exposure is in anionic form and that the negatively charged species will thus be repelled by the negatively charged surfaces of the sediment. This mechanism has previously been suggested as the reason for low sorption of anionic pesticides (Kah and Brown, 2007). Kd values for caffeine, sulfamethazine and salicylic acid were within one order of magnitude of what has previously been reported in soils and

sediments (Dubus *et al.* 2001; Gao and Pedersen, 2005; Accinelli *et al.* 2007; Lin *et al.* 2010)

Table 7 Mean percentage of study compound sorbed to the sediment and the resulting sediment-water distribution coefficients. Standard deviations are shown in the parentheses.

	Sediment 1		Sediment 2	
Compound	**Sorption % (SD)**	**Kd (SD)**	**Sorption % (SD)**	**Kd (SD)**
Naproxen	72.8 (2.1)	13.4 (1.4)	-	-
Sulfamethazine	81.9 (0.5)	45.6 (1.1)	-	-
Chloramphenicol	72.9 (0.1)	5.6 (0.0)	-	-
Caffeine	73.4 (0.3)	27.7 (0.5)	-	-
Diclofenac	61.5 (2.4)	2.34 (0.2)	59.4 (1.3)	4.2 (0.2)
Salicylic acid	69.7 (0.8)	11.4 (0.4)	-	-
Fluoxetine	97.5 (0.1)	1789.7 (61.4)	93.7 (0.4)	422.5 (31.9)
Triclosan	97.1 (0.2)	1527.8 (120.7)	89.5 (0.8)	241.2 (20.0)

2.3.2 Evaluation of predictive models

The six models that were evaluated predicted the K_{oc} values for the study compounds with varying degrees of success (Figure 6 and 7). None of the models predicted K_{oc} to within one order of magnitude for all eight compounds. Models were divided into models using Kow to estimate Koc (Figure 6) and models including other descriptors more representative for ionisable organics (Figure 7).

The models using Kow to predict Koc worked fairly well for acidic compounds with the exception of chloramphenicol where all models underestimated Koc and diclofenac where all models overestimated the Koc. For the basic substances (caffeine and fluoxetine) all models underestimated the sorption to sediment. This would be expected since basic compounds can be present as cations and thus additional processes such as cation exchange would be involved in the sorption to soils and sediments (Vulava *et al.* 2000). For sulfamethazine,

which is the only zwitterionic, all models underestimated the sorption with approximately one order of magnitude.

The models including additional factors and processes other than lipophilic sorption predicted Koc slightly better than the models discussed above. Especially, the MAMI model and EPI Suite (MCI) that resulted in fair predictions of Koc for chloramphenicol, caffeine and diclofenac; the substances whose Koc values were poorly estimated by the Kow-based models.

None of the models evaluated against experimental data in this study predicted the Koc values within one order of magnitude. However, the MAMI model predicted the sorption fairly well for the majority of the study chemicals. It should also be noted that the MAMI model is not applicable for ionisable compounds with a pKa < 2 (caffeine).

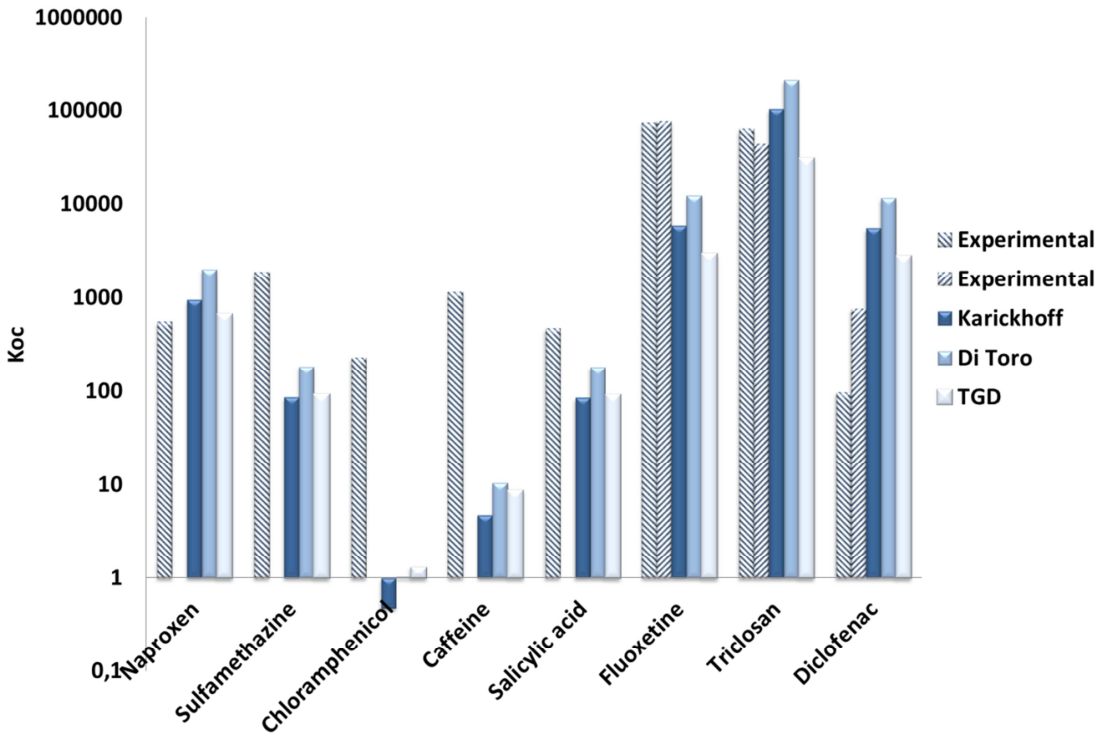


Figure 6 A comparison between experimental Koc values and estimated using models developed for neutral organics.

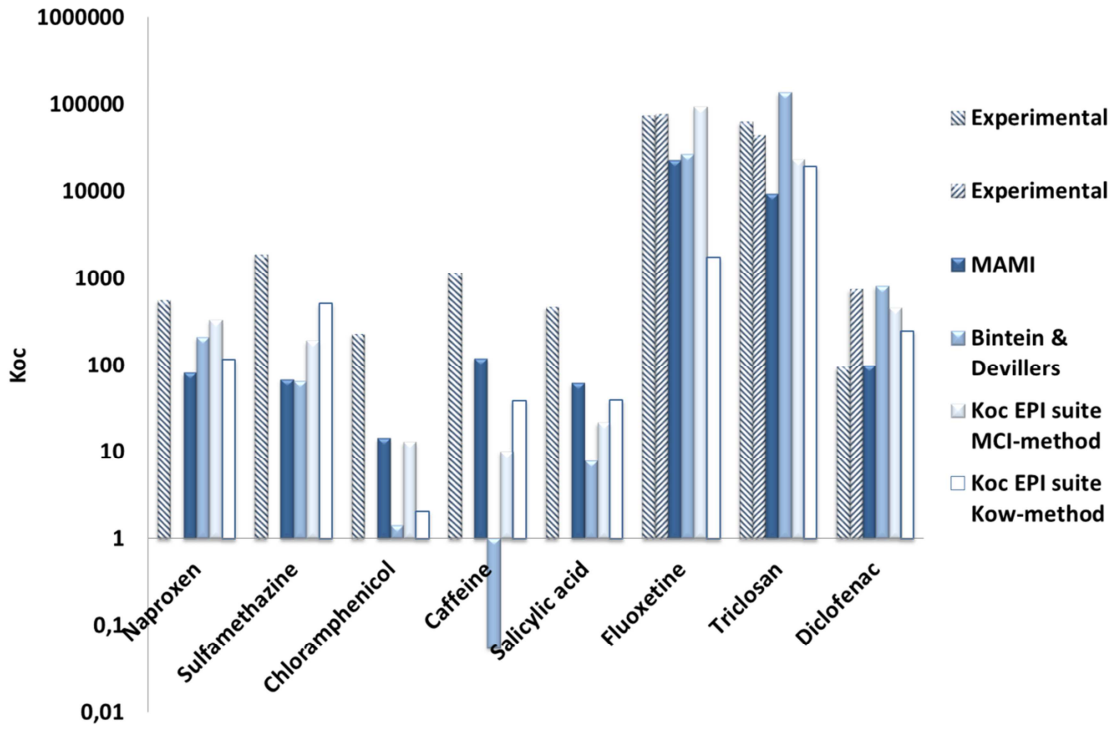


Figure 7 A comparison between experimental Koc values and estimated using models developed for ionisable organics.

2.4 Conclusions

This study has generated baseline data on the distribution behaviour of the study compounds in sediment-water systems. The experimental results in this chapter will be used in Chapter 6 to better understand the uptake from sediments into sediment dwelling invertebrates. Knowledge on the distribution of the study compounds are also valuable input in Chapter 5 where the importance of the different uptake routes into *L. variegatus* will be investigated.

Based on the results in this study it can be concluded that none of the predictive models for sorption is able to adequately predict the sorption behaviour of all of the compounds studied. Overall, the best predictions were made by the MAMI model which estimated the sorption of the study compounds with an error factor of maximum 25 whilst other models under- or overestimated the sorption of at least some of the compounds by several orders of magnitude.

It should also be noted that the MAMI model underestimated the sorption behaviour of all the study compounds except for diclofenac where the model predicted the Koc very well. If the main uptake route of chemicals to sediment dwelling organisms is *via* the pore water, the model will likely overestimate the exposure (and therefore risks) to such organisms.

CHAPTER 3

EFFECTS OF CHEMICALS PROPERTIES ON THE UPTAKE OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS INTO *LUMBRICULUS VARIEGATUS*

3.1 Introduction

An understanding of the internal concentration of a substance in an organism in the environment can provide valuable information for understanding the effects of chemicals on organisms and help in extrapolating from effects in standard laboratory studies to effects across the wider environment (e.g. Van Wezel *et al.*, 1995; Escher *et al.*, 2004). For example, for active pharmaceutical ingredients (APIs), it has been suggested that, by understanding internal concentrations in organisms in the natural environment as well as the presence or absence of the target receptors and pathways that the API is designed to interact with in humans, it may be possible to predict the potential effects of pharmaceuticals on the natural systems using preclinical and clinical pharmacological data, produced in the drug development process (e.g. Huggett *et al.*, 2003).

A number of studies have explored the uptake, depuration and metabolism of APIs and substances used in PCPs into aquatic and terrestrial organisms. Uptake has been shown in plants (e.g. Boxall *et al.*, 2006; Dolliver *et al.*, 2007; Kumar *et al.*, 2005), earthworms (Kinney *et al.*, 2008), and in fish and aquatic invertebrates (Dussault *et al.*, 2009; Mimeault *et al.*, 2005; Nakamura *et al.*, 2008; Paterson and Metcalfe, 2008; Ramirez *et al.*, 2009; Rendal *et al.*, 2011; Meredith-Williams *et al.*, 2012). For aquatic organisms, the degree of uptake is highly dependent on the traits (e.g. size and mode of respiration) of a test organism,

the physico-chemical properties (e.g. pH-corrected lipophilicity) of the test substance, and the characteristics of the test environment such as pH (e.g. Meredith-Williams *et al.*, 2012; Nakamura *et al.*, 2008; Kim *et al.*, 2010; Valenti *et al.*, 2009; Rendal *et al.*, 2011).

Several attempts have been made to predict the uptake of chemicals from water based on chemical properties (Mackay 1982, Chiou 1985, Veith *et al*. 1979). This work has focused mainly on neutral organic chemicals but more recently approaches for estimating uptake of ionisable organic chemicals have also been proposed (Trapp and Horobin, 2005; Neuwoehner and Escher, 2011). For neutral organic chemicals, most models predict the uptake into aquatic organisms based on the lipophilicity of the chemical i.e. based on a compounds' K_{ow} (Mackay 1982, Chiou 1985, Veith *et al.* 1979). For example, the European Commission Technical Guidance Document on Risk Assessment (TGD; 2003), suggests that uptake is estimated using the linear relationship between the lipophilicity of a chemical and the bioconcentration into organisms developed by Veith *et al.* (1979). The method is however not recommended for ionising substances since the water solubility of the ionisable form of a chemical can be orders of magnitude higher than the neutral species. Instead, the TGD suggests correcting the Log Kow in order to take only the neutral fraction of the compound into account at a given pH.

Other models have been suggested for estimating environmental fate and toxicity of ionisable chemicals. For example, Meredith-Williams *et al.* (2012) suggested that uptake of APIs into invertebrates (*Gammarus pulex* and *Notonecta glauca*) based on a compounds pH-corrected liposome-water partition coefficient (Log $D_{lip\text{-}water}$). Models have also been developed that predict the uptake of ionisable chemicals into both human cells (Trapp and Horobin 2005) and algae cells (Neuwoehner and Escher 2011). These models not only account for the uptake driven by hydrophobic interaction but also consider electrostatic interactions and ion trapping as important processes in the uptake of ionisable chemicals. These models have been applied to understand the toxicity of APIs. For example, Neuwoehner and Escher (2011) measured the toxicity of five basic pharmaceutical in

Scenedesmus vacuolatus at five pH values between 6.5 and 10 and found that the toxicity increased with an increasing pH. By using a toxico-kinetic ion trapping model, the authors showed that the differences in toxicity were explained by differences in uptake of the pharmaceuticals. What distinguishes this model to other models predicting the uptake into aquatic organisms (e.g. use of Log D or taking only the neutral form into consideration) is how it not only calculates the degree of dissociation outside the organism but also inside the cytosole. The dissociation inside the cell affects the depuration as well as uptake and sorption to organelles in the cell and will have an overall effect on the bioconcentration of ionisable chemicals in aquatic organisms. A similar approach has previously been published for the development of a model for selective accumulation of chemicals in tumour cells (Trapp and Horobin 2005).

While an increasing amount of data are now becoming available on uptake into organisms from the water column and into soil-dwelling organisms, an understanding of uptake of APIs and PCPs into sediment dwelling organisms is still less-well developed than for other chemical classes such as pesticides and neutral organic compounds. This study therefore explored the uptake and depuration of five APIs and one PCP, which covered a range of chemical properties (Table 8), into the sediment dwelling oligochaete, *Lumbriculus variegatus.* The study explored uptake from the water-phase as this uptake route is considered the main uptake route for many sediment-associated chemicals. The results of were used to explore the relationships between chemical properties and uptake and also to evaluate some of the existing models, described above, for estimating bioconcentration of neutral and ionisable substances.

3.2 Methods

3.2.1 Test organisms

Lumbriculus variegatus were reared in 20 L glass aquaria containing artificial pond water (APW, Naylor *et al.* 1989), at 20 ±2 °C, using a 16:8 h light:dark cycle. Shredded

unbleached tissue paper was used as a substrate and the culture water was renewed once a week. The cultures were fed with ground fish food (Tetramin, Tetra Werke, Melle, Germany) twice a week.

3.2.2 Lipid analysis of test organism

Three replicates containing approximately 25 mg of *L. variegatus* were weighed and dried overnight in an oven at 60 ° C. After the dry weights were determined, samples were transferred to a pre-weighed test tube and ground with a glass rod. 1.6 ml of 2-propanol, 2.0 ml of cyclohexane and 2.2 ml of deionised water were then added to the test tubes and the samples were vortexed for 30 s and sonicated for 5 minutes. After extraction, samples were centrifuged for 5 minutes at 3000 rpm and the upper cyclohexane layer, which contained the lipids, was transferred to a pre-weighed glass vial. The extraction was repeated a second time and the cyclohexane layer was added to the first extract. Extracts were concentrated to dryness under a gentle stream of nitrogen and the vials then weighed to determine the mass of lipid content in the tissues. Extraction recoveries were tested using a known amount of external liposome reference (1,2 – distearoyl-sn-glycero-3-phospocholine) dispersed in water.

3.2.3 Test chemicals

Test compounds were ^{14}C-labelled and had a specific activity between 1.74 and 2.43 GBq mmol^{-1}. Chloramphenicol, diclofenac, naproxen and salicylic acid were obtained from Perkin Elmer (Boston, USA), fluoxetine was obtained from American Radiolabelled Chemicals (St Louis, USA), and triclosan was obtained from Unilever (Colworth, UK). Compounds were chosen to represent a wide range of chemical properties. Compounds used are listed in Table 8. For further detailed information on the test chemicals, see Chapter 1.

Table 8. Test chemicals used in the uptake studies

Test compound	Labelling	Specific activity [GBq/mmol]
chloramphenicol	dichloroacetyl-1,2-^{14}C	2.220

diclofenac	U-^{14}C	2.321
Fluoxetine	methyl-^{14}C	2.035
Naproxen	methyl-^{14}C	2.035
Salicylic acid	methyl-^{14}C	1.739
triclosan	U-^{14}C	2.431

3.2.5 Uptake and depuration test

Uptake and depuration studies were carried out using a similar approach to that described by Ashauer *et al.* (2006). *L. variegatus* were acclimatized to the test conditions for 18 h in APW. For the uptake studies, animals were then exposed in groups of 10 animals contained in 40 ml APW to between 3 – 10 nmol/L of test compound for 3, 6, 12, 24 or 48 h. Exposure concentration were chosen to represent environmentally realistic concentrations and fall below the toxic threshold of the study organisms (exposure and effect concentrations are discussed in Chapter 1). For the depuration studies, groups of animals were exposed to the test chemical for 48 h after which time they were transferred to clean APW for 3, 6, 12, 24 or 48 h. Three replicates per time point were used, the study pH was set to 8.1±0.1 and the study temperature was 20 ±2 °C. Studies were performed in the dark to avoid photodegradation of the test compounds. Control beakers, containing APW and radio-labelled substances, were used to assess whether there was any sorption to the jars during the test period.

At each sampling time, 1 ml of test media was taken, placed into a 20 ml scintillation vial and 10 ml Ecoscint A scintillation cocktail (National Diagnostics) was added. Worms were rinsed in distilled water and blotted dry on a tissue before being put into a 20 ml scintillation vial. Worm samples were then weighed, 2 ml of tissue solubilizer (Soluene®-350, Perkin Elmer, Waltham, Massachusetts) was added to the vials and the vials were then left for 24 h to allow the worm tissue to dissolve completely. Prior to analysis, 10 ml of Hionic Fluor scintillation cocktail (Perkin Elmer) was added to the vials.

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

3.2.6 Derivation of uptake and depuration rate constants and bioconcentration factors

A first order one compartment model was used to estimate the uptake and depuration rates for each test compound. The change of concentration in the organism was estimated according to Branson *et al.* (1975) using Equation 17. The parameters were estimated using the software OpenModel (v. 1.2) downloaded on the 24th of June 2011). The model was parameterized using residual sum of squares with the Levenberg-Marquardt algorithm followed by Monte-Carlo Markov-Chain (MCMC) with the results from the Marquardt fit as input values. Confidence intervals were characterized by the 95% percentile of the simulated variables. Bioconcentration factors were calculated by setting the water concentration to 1 and by running the model until equilibrium was reached. Bioconcentration factors and their confidence intervals could then be read directly from the internal concentrations. The method is described in full in Ashauer *et al.* (2010).

$$\frac{dC_{org}}{dt} = K_{in} \cdot C_{water} - K_{out} \cdot C_{org} \qquad \text{Eq 17}$$

3.2.7 Evaluation of relationships between properties and uptake and existing models for estimating bioconcentration factors for ionic substances.

Linear regression analysis was used to explore relationships between bioconcentration factors (BCF) and Log Kow and Log Dow. Log Kow and pKa were calculated using the SPARC online calculator (archemcalc.com/sparc August 2011 release w4.6.1646-s4.6.1646). Log Dow for the mean experimental pH was derived from the Henderson Hasselbach equation (Henderson 1908). The fraction of the unionised and the ionised species was calculated using Eq 18 and the Log D was then estimated using Eq 19.

$$\alpha_{ion} = \alpha_{neutral} \cdot 10^{i(pH-pKa)} \quad \text{Eq 18}$$

$$D_{ow} = f_{ion} \cdot K_{ow(ion)} + f_{neutral} \cdot K_{ow(neutral)} \quad \text{Eq 19}$$

In addition, two existing models for predicting bioconcentration factors of ionic substances were evaluated by comparing predictions obtained using these models with experimental bioconcentration factors. The first approach followed the method described by Trapp and Horobin (2005) adapted to the experimental parameters in this study. The calculations were performed using Microsoft Excel 2010. A template for the use of the model was kindly sent by Stefan Trapp of the Danish Technical University. Parameter data for generic cells were applied as suggested in Trapp and Horobin (2005). The inserted outside pH values in the model were adjusted to correspond with the measured outside pH values in this study.

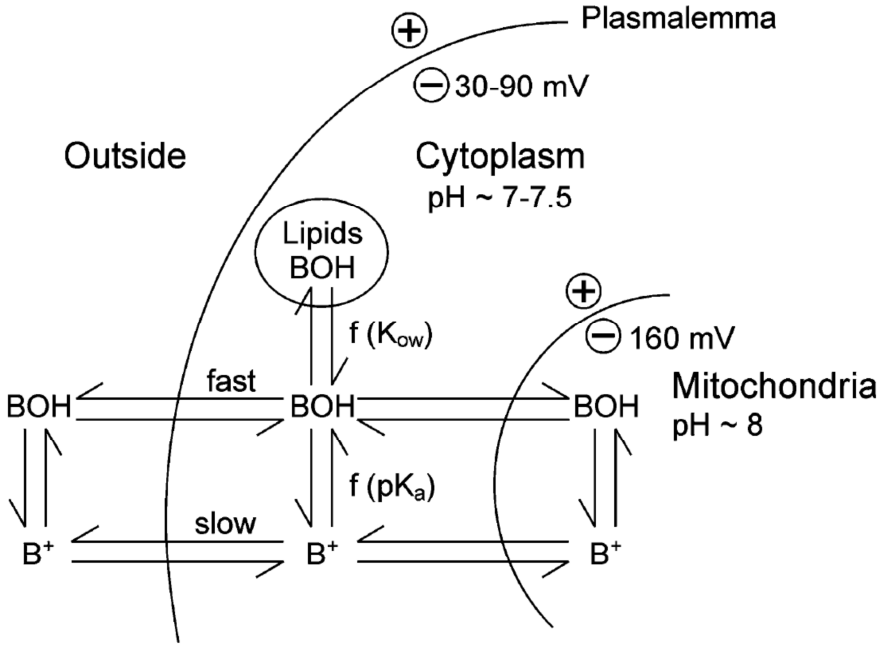


Figure 8 The cell. Compartments, molecular species, pH and charges in a cell shown for a weak base. From Trapp and Horobin (2005).

To predict the bioconcentration for ionic substances Neuwoehner and Escher (2011) developed a combined model using information on the fraction of dissociation, the lipophilicity of the different chemical species of a substance and the ion trapping model described in Neuwoehner *et al* (2011). The ion trapping model is described in Figure 9 and the combined model is described by Equations 20 and 21. Chemical properties used as input parameters in the model are displayed in Table 2 in Chapter 1. Lipid content was 1.2 % wet weight and measured as described above. pH values in the cytosol were adopted from Trapp and Horobin (2005) giving pH values in the cytosol for mammalian cells.

$$BCF = \frac{f_w \cdot (C_{HA,int} + C_{A^-,int}) + f_{lip} \cdot C_{lip,int}}{C_{HA,ext} + C_{A^-,ext}} \quad \text{Eq 20.}$$

Which is equal to:

$$= f_w \cdot \frac{1+10^{(pH_{int}-pKa)}}{1+10^{(pH_{ext}-pKa)}} + f_{lip} \cdot D_{lip-water} \quad \text{Eq 21.}$$

Where f_w is the water content of the organism, f_{lip} is the lipid content of the organism, C_{HA} and C_A^- is the concentration of the uncharged and charged species internally (int) and externally (ext) respectively. $D_{lip\text{-}water}$ is the liposome water partitioning coefficient corrected for pH which was calculated according to Equation 22 (Escher *et al.* 2009).

$$Log\ D_{lip-water} = 0.904 \cdot Log\ K_{ow} + 0.515 \quad \text{Eq 22.}$$

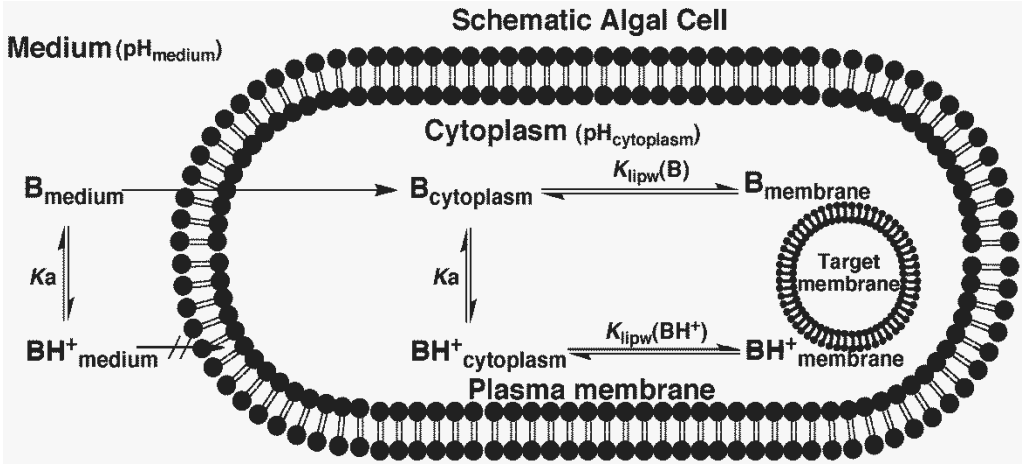


Figure 9 Ion trapping model by Neuwoehner and Escher (2011)

3.3 Results and discussion

3.3.1 Uptake and depuration in *L. variegatus*

No mortality was observed either in the treatments or in the controls during the uptake or depuration phases. The pH values during the exposure period ranged between 7.0 and 8.3, (Table 9). With the exception of salicylic acid, concentrations of all study compounds in control treatments, containing radioactive test compound and water only, were stable for the duration of the study (Figure 10). The concentration of salicylic acid at 48 h was 75 % of the initial start concentration. Salicylic acid has previously been reported as being degradable in constructed wet land systems designed for removal of pharmaceuticals and personal care products in waste water. Removal efficiencies were as high as 90% (Hijosa-Valsero *et al.* 2010; Reyes-Contreras *et al.* 2011). Degradation of salicylic acid in the environment has been reported to be temperature and pH dependant in waters (Alibrandi *et al.* 2003).

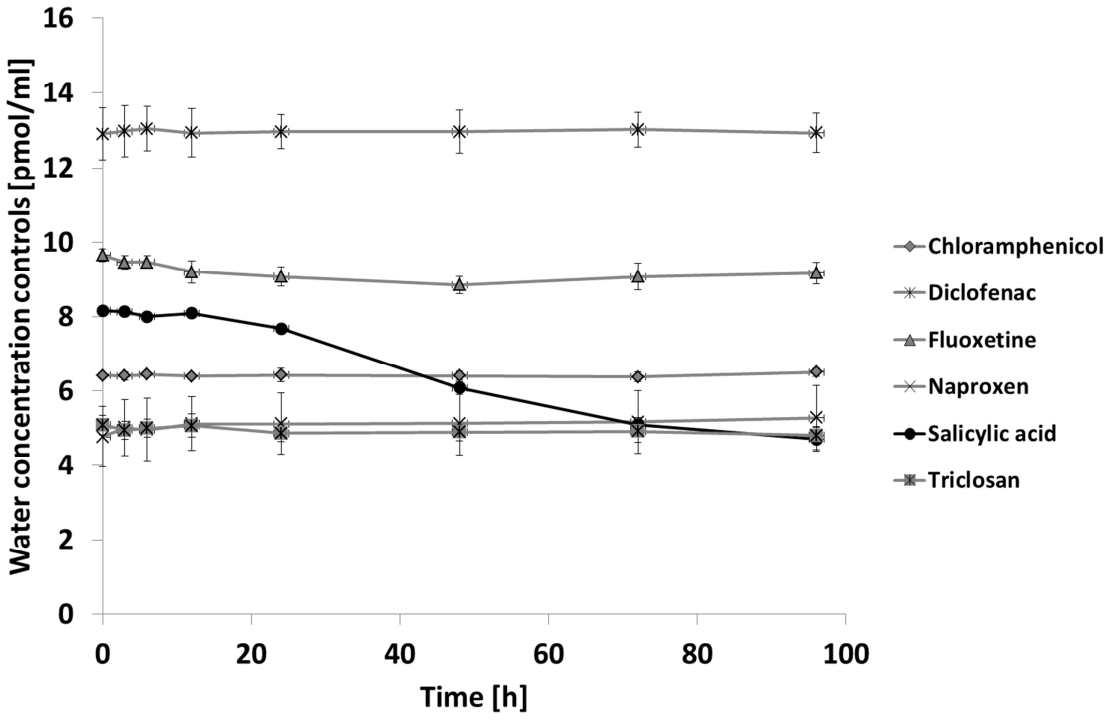


Figure 10 Mean concentrations (± 1 SE) of the study chemicals in the stability controls.

In the uptake beakers, concentrations of chloramphenicol, diclofenac and naproxen in the exposure solutions remained constant over the 48 h uptake phase while concentrations in treatments containing fluoxetine, salicylic acid and triclosan decreased during the 48h uptake phase (Figure 11a-f). For fluoxetine and triclosan, the reduction in concentration in the water column was explained by uptake of the compounds into the study organisms whilst for salicylic acid, reduction in the water columns is most likely due to abiotic degradation as a similar degree of reduction was also observed in the water only controls. A mass balance was performed and is presented in Table 1 in Appendix 1.

The first order one compartment model was successfully fitted to the uptake and depuration measurements for all compounds (Figure 11 a-f). The resulting uptake and depuration parameters and the bioconcentration factors are provided in Table 9. BCFs in the study ranged from 2 (chloramphenicol) to 700900 (triclosan) and increased in the order chloramphenicol < diclofenac < salicylic acid < fluoxetine < naproxen < triclosan.

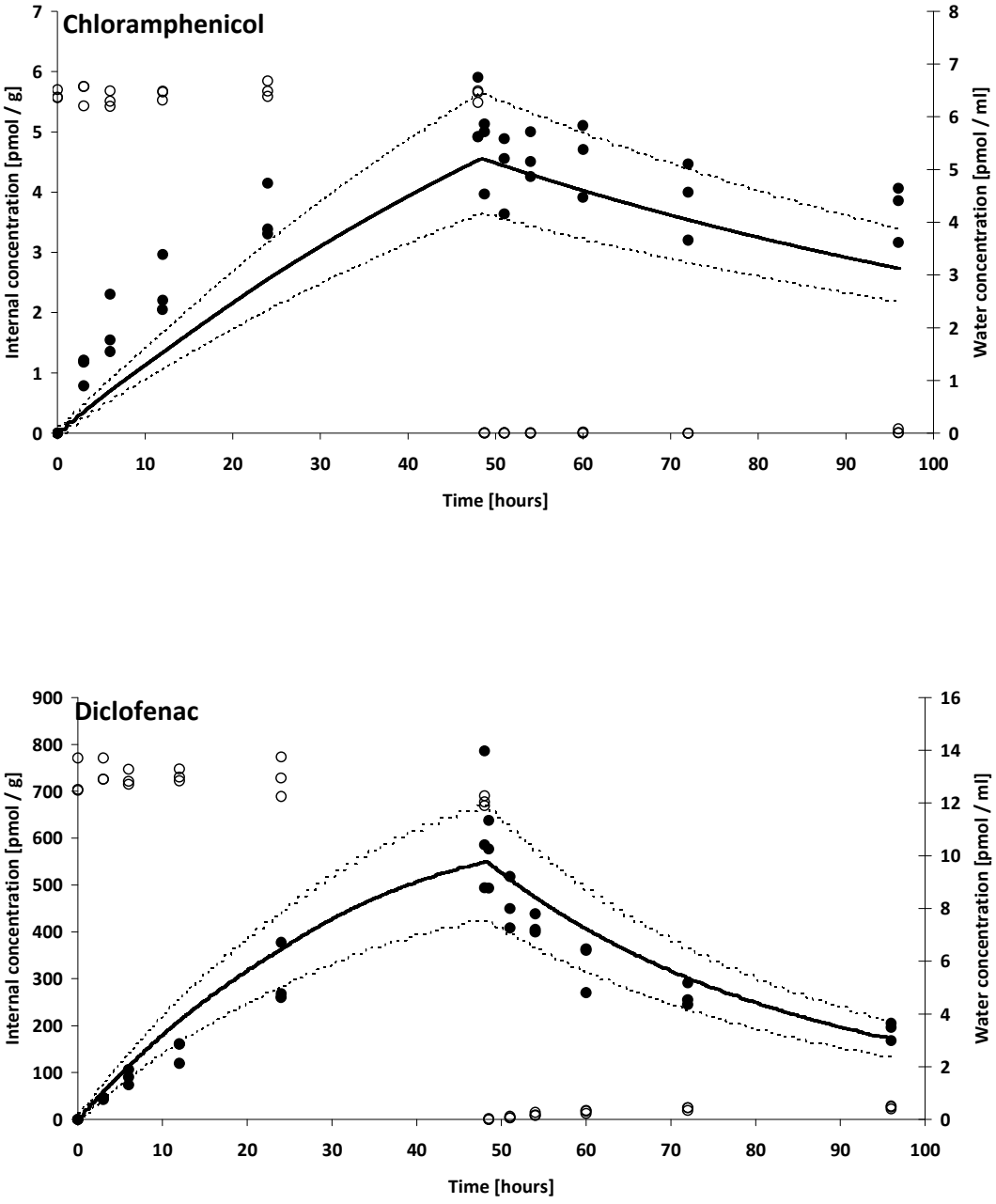
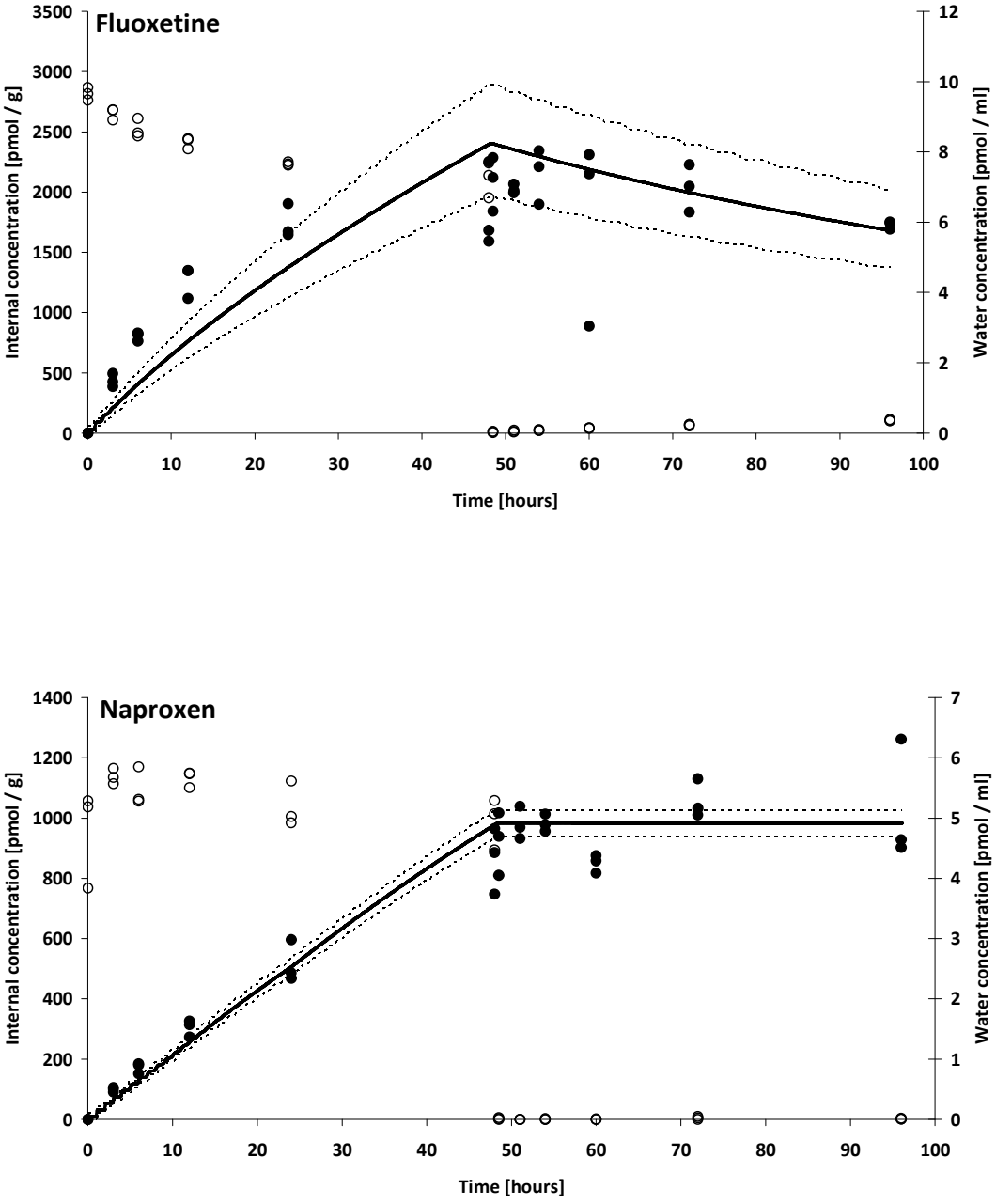
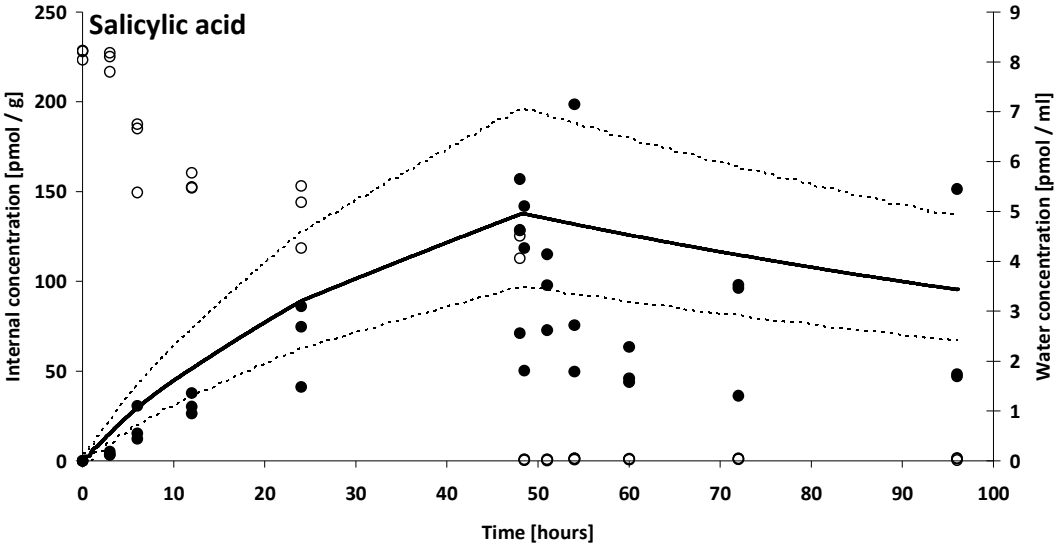
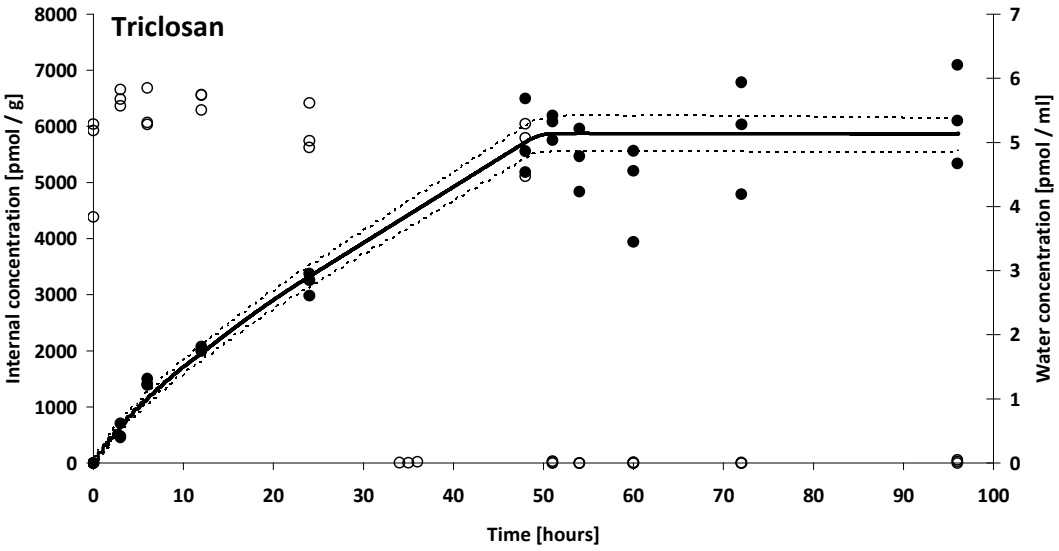


Figure 11 a,b. Uptake and depuration graphs for chloramphenicol and diclofenac into *L. variegatus.* Filled circles are measured tissue concentrations. Empty circles are measured water concentrations. Thick line is the Markov Chain Monte Carlo modelled mean and thin lines are 95% CIs using M.



Continue Figure 11 c,d. Uptake and depuration graphs for fluoxetine and naproxen into *L. variegatus*. Filled circles are measured tissue concentrations. Empty circles are measured water concentrations. Thick line is the Markov Chain Monte Carlo modelled mean and thin lines are 95% CIs.





Continue Figure 11 e,f. Uptake and depuration graphs for salicylic acid and triclosan into *L.variegatus*. Filled circles are measured tissue concentrations. Empty circles are measured water concentrations. Thick line is the Markov Chain Monte Carlo modelled mean and thin lines are 95% CIs.

Table 9. Uptake and depuration rate parameters along with water concentrations, pH intervals estimated BCF values and estimated time to equilibrium.

			Kin		Kout			Time to Eq [days]
Chemical	**Cw [nmol l^{-1}]**	**pH**	**RSS**	**Mean ± SD**	**RSS**	**Mean ± SD**	**BCF (LCI - UCI)**	
Chloramphenicol	6.42 ± 0.14	7.0 - 7.4	2.11E-02	1.87E-02 ± 1.99E-03	1.08E-02	1.25E-02 ± 2.58E-03	2 (1 – 2)	40
Diclofenac	12.4 ± 1.81	7.6 - 8.3	1.49E+00	1.58E+00 ± 1.55E-01	2.64E-02	2.63E-02 ± 3.80E-03	60 (46 – 73)	16
Fluoxetine	8.18 ± 0.73	7.5 - 7.6	7.20E+00	7.61E+00 ± 7.02E-01	8.35E-03	1.00E-02 ± 2.84E-03	911 (742 - 1097)	46
Naproxen	5.39 ± 0.39	7.2 - 7.3	3.81E+00	3.90E+00 ± 8.55E-02	5.40E-05	6.69E-04 ± 1.94E-04	72 240 (69 100 – 75 300)	5 948
Salicylic acid	5.85 ± 1.36	7.2 - 7.9	4.73E-01	6.55E-01 ± 1.20E-01	7.94E-03	1.97E-02 ± 6.45E-03	82 (65 – 99)	45
Triclosan	3.19 ± 0.79	7.9 - 8.3	4.52E+01	4.65E+01 ± 1.14E-00	6.64E-05	6.70E-04 ± 2.07E-04	700 900 (665 000 – 738 800)	4 875

The very high bioconcentration factors obtained for naproxen and triclosan are explained by the very slow or non-existent depuration of these compounds from the worms. Similar slow depuration has been found for APIs (fluoxetine and carvedilol) in the fresh water shrimp, *Gammarus pulex* (Meredith–Williams *et al.* 2011). Another possible explanation to the very high BCF values may be due to metabolism of the compounds. Metabolism of phenolic compounds has previously shown to have large impacts on the bioaccumulation kinetics (Ashauer *et al.* 2012). This could explain the very high BCF for triclosan. Naproxen has also shown to be easily degraded in fish (Brozinski *et al.* 2011).

Literature data on the uptake of pharmaceuticals and personal care products into fresh water organisms are limited. With the exception of fluoxetine and diclofenac, all measured BCF values in this study are greater than previously reported BCF values from the literature for other test organisms. Studies with fluoxetine have reported BCFs of 185,900 in aquatic invertebrates (Meredith-Williams *et al.*, 2012) and 8.8 – 260 in fish (Nakamura *et al.* 2008; Paterson and Metcalfe, 2008). For diclofenac, BCFs of <11, 12 – 2732 and 320 – 950 have been reported for plasma, liver and bile of rainbow trout (*Oncorhynchus mykiss*) respectively (Schwaiger *et al.* 2004; Kallio *et al.* 2010; Brown *et al.*, 2007). BCFs of 500 – 2300 have been obtained for naproxen in bile from *O. mykiss* (Brozinski *et al.* 2011) and BCFs of <2 – 56 have been reported in fish plasma (Brown *et al.*, 2007). Palenske *et al.* (2010) measured the uptake of triclosan in three different amphibian larvae. The highest reported BCF values were measured in *Bufo woodhousii woodhousii* and ranged between 243 and 740.

Differences in BCFs for *L. variegatus* and other species might be explained by differences in species traits (such as physiological and morphological traits, reproduction, ability to metabolise contaminants) and differences in the test conditions used in the different studies (e.g. pH or the presence/absence of food). Organism size has proven to be an important factor in determining the uptake of chemicals. Several studies have shown a negative correlation between size and bioconcentration (Hendriks *et al.* 2001; Rubach *et al.* 2010a; Meredith-Williams *et al.* 2012). *L. variegatus*, which is significantly smaller than the other

organisms where uptake of the study compounds have previously been explored, would therefore be anticipated to show greater uptake.

Respiratory strategy can also affect the bioaccumulation of compounds (Buchwalter *et al.* 2003; Baird and Van den Brink, 2007; Rubach *et al.* 2010a). Buchwalter *et al.* (2003) proposed that species with a relatively large exchange epithelial surface are more vulnerable to uptake of contaminants. *L. variegatus*, which uses diffusion alone as a respiratory strategy, would therefore be expected to be more vulnerable to uptake of contaminants than fish, amphibians and gammarids.

Whether there is a correlation between bioaccumulation of contaminants and lipid content is disputable. There are studies where a positive correlation has been shown (Hendriks *et al.* 2005) as well as studies where no such correlation was found (Rubach *et al.* 2010b; Meredith-Williams *et al.* 2012). The measured Lipid content in *L. variegatus* was 1.26 ± 0.08 % wet weight. (9.86 ± 0.63 % dry weight). Recoveries for the external liposome reference were 100 ± 0.6 %. Since *L. varieagatus* has a relatively low lipid content compared to e.g. *G. pules* (2.03%) and *N. glauca* (11.1%) (Meredith-Williams *et al.* 2012) a lower uptake might be expected, however this was not the case for fluoxetine which was the only compound tested for all three species. For this compound, uptake increased in the order *N. glauca* < *L. variegatus* < *G. pulex*.

L. variegatus showed very small tendencies to depurate the study compounds and for fluoxetine, naproxen and triclosan the depuration from the study organism was very slow (Figure 11c, d, f). A similar lack of depuration has previously been observed for fluoxetine and carvedilol in *G.* pulex. Since the study compounds have the potential to ionise, there is a possibility that the lack of depuration is due to the compounds being trapped in the organisms by ion trapping. Several studies have previously suggested ion trapping to be involved in the fate and effects of ionisable chemicals (Trapp and Horobin 2005; de Carvalho *et al.* 2007; Neuwoehner and Escher 2011). To what extent the chemicals is subject to ion trapping is dependant on the pKa and the pH in the test media. At the pH tested (see

Table 9), ion trapping alone could not explain the lack of depuration for fluoxetine, naproxen and triclosan. However, it is possible that if using an default internal cell pH of 7.5 (Neuwoehner and Escher 2011) ion trapping could interfere with the depuration of triclosan out of the organism since triclosan inside the cell is present at a higher proportion of its neutral species than it is outside the cell (mean test pH value = 8.1).

Another possible explanation to the lack of depuration out of the organisms is the lack of organic matter in the test vessels. The study compounds that displayed a very low depuration were the three compounds with the highest lipophilicity. It is possible that in a natural environment where organic matter is present, the body burden would be smaller due to a larger sorption of the chemical to organic matter which facilitates the depuration out of the test organism based on a fugacity model approach. McCarthy (1983) observed a reduction in the uptake and accumulation of polycyclic aromatic hydrocarbons in *Daphnia magna* with 97 % in the presence on natural organic matter in the form of humic acids.

When estimating a bioconcentration factor dynamically with modelled uptake and depuration parameters, a minor change in the uptake and depuration rate, either due to changing environmental conditions or lack of metabolism, can have large consequences. If there is no apparent depuration in the organism, the modelled time to reach equilibrium i.e. the state where the bioconcentration factor is reached can be very long. For the study compounds, the predicted equilibration time ranged from 16 days (diclofenac) up to 16 years (naproxen)(Table 9). Based on this knowledge one might question the use of bioconcentration factors, derived from kinetic experiments, in short lived organisms, especially for chemicals that tend to depurate insignificantly or very slowly.

Despite the many advantages of using radiolabelled chemicals in uptake studies (e.g. low detection limits, labour efficient) there are also shortcomings in the method. Since total radioactivity was measured, any possible metabolites formed in the organisms were not identified. Data on the metabolism of substances used as pharmaceuticals or in personal care products in aquatic invertebrates is scarce. The metabolism of fluoxetine and several other

APIs has been characterised in the fresh water shrimp *G. pulex* (Meredith-Williams *et al.* 2012). In these studies, no metabolism was seen for fluoxetine and most of the other pharmaceuticals tested.

3.3.2 Evaluation of predictive models for estimating bioconcentration

Several attempts have been made to develop methods for estimating uptake of APIs and PCPs based on chemical properties and environmental properties. For neutral compounds, uptake is strongly correlated with lipophilicity (Brock Neely *et al.* 1974, Mackay 1982, Barron 1990), hence, most models predicting bioconcentration are based on relationships with Log Kow. Relationships between the measured bioconcentration factors of the study compounds in *L. variegatus* and measures of lipophilicity were therefore investigated. In Figure 12 a and b, comparisons have been made between the measured bioconcentration factors, the lipophilicity (Log K_{ow}) and the lipophilicity corrected for pH (Log D_{ow}). Correlations between BCF and Log K_{ow} gave an $r^2 = 0.59$ ($p= 0.073$) and between BCF and Log D_{ow} gave an $r^2 = 0.50$ ($p= 115$) were found suggesting that additional processes are involved in the uptake of ionisable PPCPs.

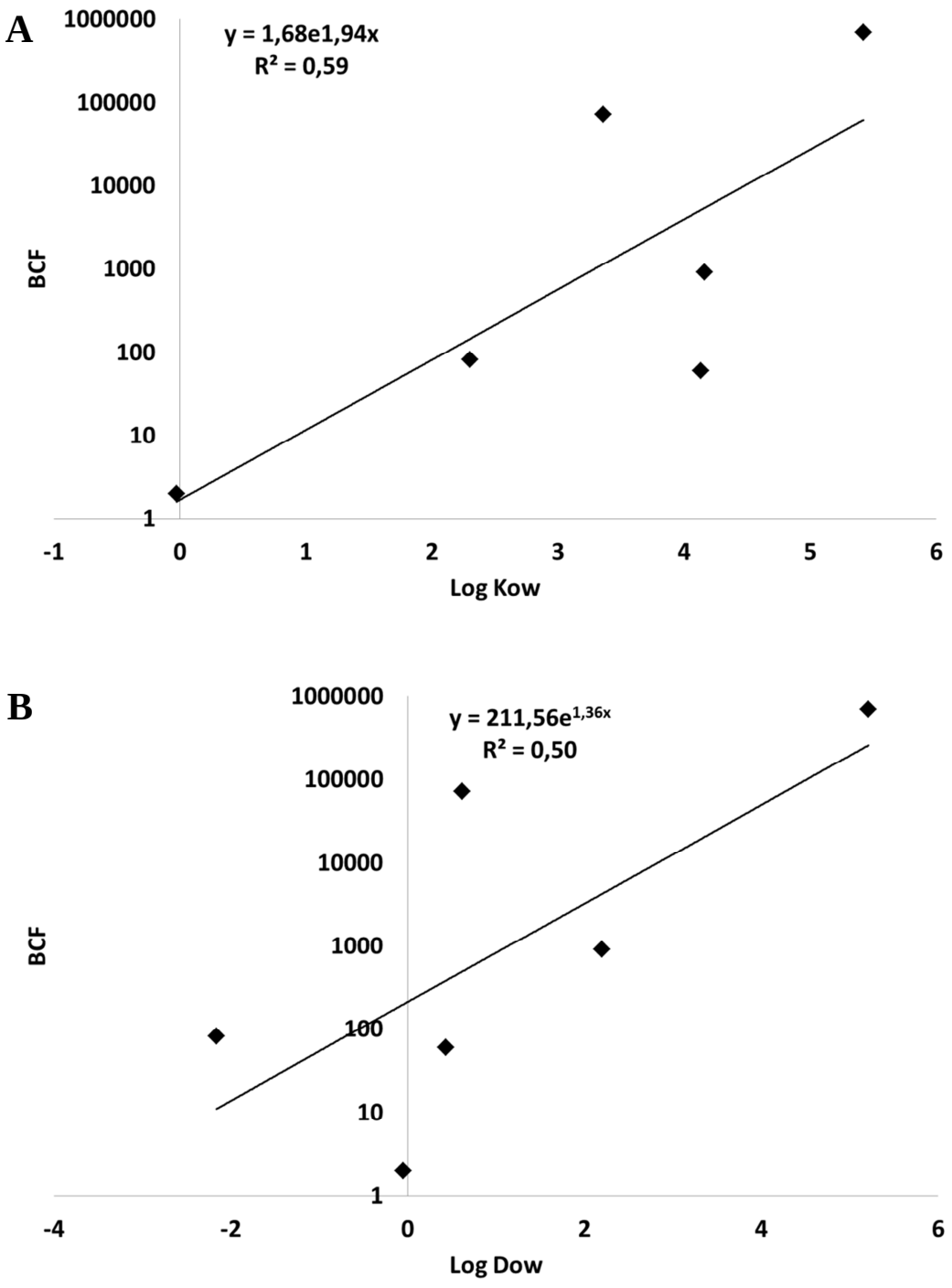


Figure 12 Correlations between bioconcentration (BCF) into *L. variegatus* and Log Kow (A) and Log Dow (B) for the study pharmaceuticals and personal care products.

In addition, there are two models that have been developed specifically to predict the uptake of ionisable chemicals into both human cells (Trapp and Horobin 2005) and algae cells (Neuwoehner *et al.* 2011) that not only accounts for the uptake driven by hydrophobic interaction but also account for effects of electrostatic interactions and ion trapping as these are believed to be important processes in the uptake of ionisable chemicals. The cell model developed by Trapp and Horobin has previously been evaluated successfully in fish (Fu *et al.* 2009). A comparison between experimental bioconcentartion factors in *L. variegatus* and bioconcentration factors estimated using the model by Trapp and Horobin (Figure 13) demonstrates that the cell model underestimated the bioconcentration for all six compounds. For two of the compounds, the BCF values were heavily underestimated (naproxen and triclosan). Possible explanations are that additional processes involved are not accounted for in the model and possibly also due to the metabolism of test compounds resulting in very high experimental bioconcentration factors (see discussion above).

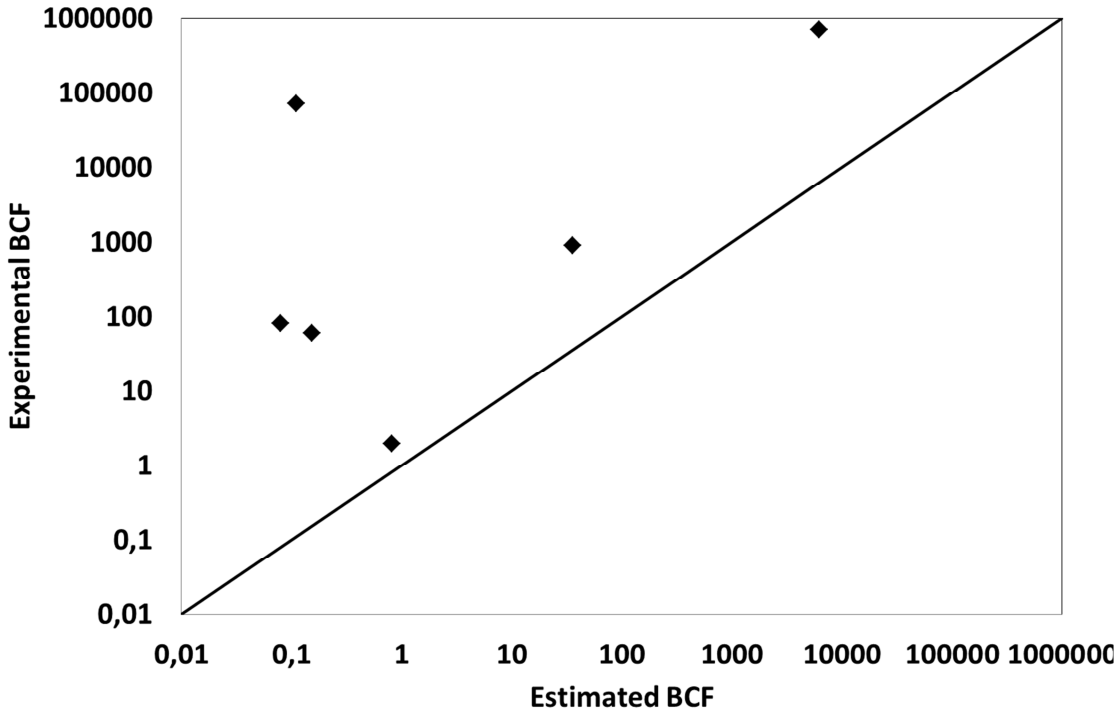


Figure 13 A comparison of the experimental BCF into *L. variegatus* and BCF predicted by the cell model by Trapp and Horobin (2005).

A similar pattern was observed in the evaluation of the combined model of Neuwoehner and Escher (2011). The model underestimated the bioconcentration of the test compounds into *L. variegatus*, especially for naproxen and triclosan (Figure 14). These results highlight the importance of understanding not only the dissociation and the varying lipophilicity of ionisable substances with varying pH values but also additional factors and processes such as environmental properties, electrostatic interactions, ion trapping and and metabolism of PPCPs.

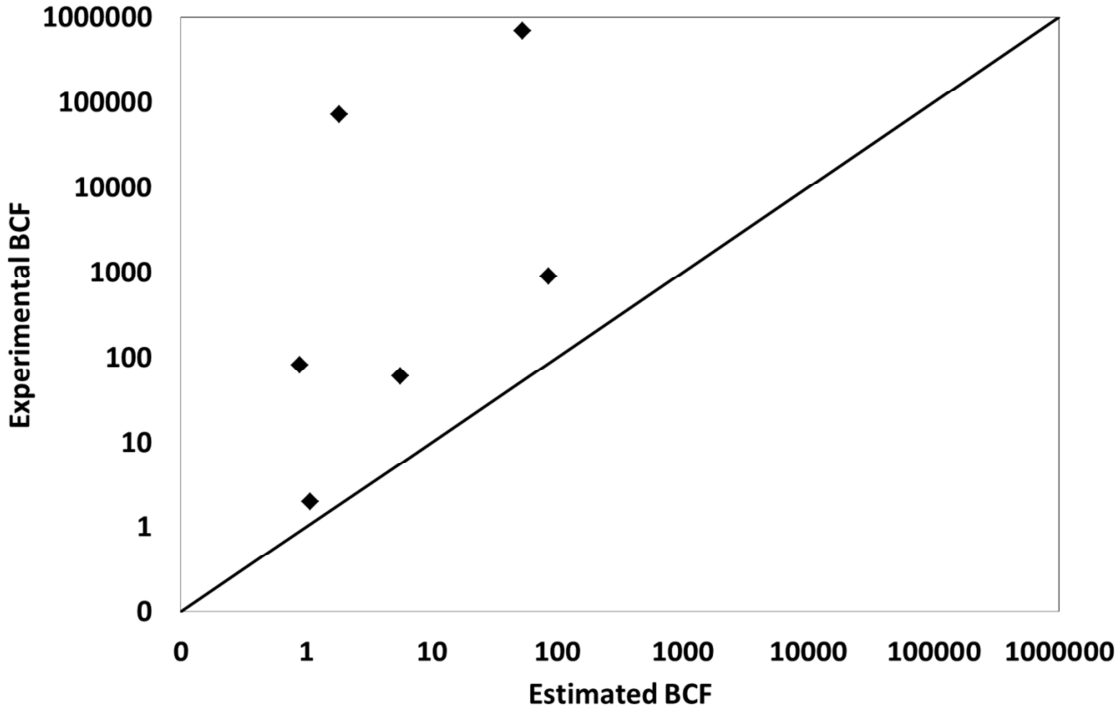


Figure 14 A comparison of the experimental BCF and BCF predicted by the cell model by Neuwoehner and Escher (2011).

3.4 Conclusions

Despite the knowledge available on what chemical properties affect bioconcentration of organic substances in the aquatic environment there is still a lot to discover. As for many neutral organic substances, there is a weak correlation between the lipophilicity of the study

compounds and the uptake into *Lumbriculus variegatus*. Since many PPCPs are ionisable substances, Log D_{ow} has been suggested to better described the fate and effects of ionisable substances. However, the correlation between K_{ow}, D_{ow} and BCF was not very strong suggesting that other mechanisms are involved in the bioconcentration of PPCPs. Due to the nature of ionisable substances, environmental pH is likely to have large impact on the uptake and depuration kinetics of such substances. Also, since metabolism has been shown to have an effect on the uptake and depuration kinetics of contaminants, it is crucial to have knowledge on the metabolism to accurately estimate the bioconcentration of chemicals into non-target organisms. Therefore, in the next chapter, the focus will be on exploring the importance of varying environmental pH for the bioconcentration of four of the study chemicals. In addition, the metabolism of three of the study chemicals in *Lumbriculus variegatus* will be investigated. These data will then be used to further assess the applicability of the available models for estimating uptake.

CHAPTER 4

EFFECTS OF pH AND METABOLISM ON THE UPTAKE OF IONISABLE CHEMICALS INTO *LUMBRICULUS VARIEGATUS*

4.1 Introduction

In the previous chapter, it was suggested that environmental factors such as pH and biotransformation have large effects on the uptake and thus also the toxicity of ionisable substances in aquatic organisms. Therefore, this chapter will focus on the effects of a varying pH on the uptake of three ionisable substances into *L. variegatus*. In addition, the Chapter describes an attempt to investigate whether metabolism of the study compounds is occurring in *L. variegatus.*

4.1.1 Effects of pH on fate and uptake of ionisable substances

As discussed previously, a large proportion of the chemicals that we use today are ionisable. For example, an evaluation of 1510 chemicals preregistered in REACH showed that 49 % were ionisable and at a pH of 7, 33 % of these chemicals would be in an ionised state (Franco *et al.*, 2010). The proportion of ionized chemicals within certain classes of chemicals is even higher. For example, between 84.6 and 95% of compounds used as active pharmaceutical ingredients (APIs) are thought to be ionisable (Manallack *et al.* 2007). As the degree of dissociation of an ionisable compound is affected by the pH of the environment in which it resides (Franco *et al.* 2009) and as ionized and unionized species of a compound behave differently in terms of their environmental fate and bioaccumulation into organisms,

the behaviour of a substance will be significantly affected by the pH of the environment in which it resides.

The pH of water bodies, soils and sediments in the natural environment can vary significantly. However, despite the known variability of pH values in surface waters (pH 2.2-9.8), sediments and soils (pH 3.4-7.6) (FOREGS-EuroGeoSurveys Geochemical Baseline Database) only a few studies have investigated the effects pH have on the fate and toxicity of ionisable organic environmental pollutants especially for PPCPs (Nakamura *et al.* 2008; Valenti *et al.* 2009). Nakamura *et al.* (2008) investigated the toxicity and bioconcentration of the weak base fluoxetine in japanese medaka (*Oryzias latipes*) at three different pH values, 7, 8 and 9. They found that LC50 values ranged from 0.2 mg/L (pH 9) to 5.5 mg/L (pH 7). The differences in toxicity were explained by differences in bioconcentration at different pH values which ranged from 13 (pH 7) to 330 (pH 9). The differences in BCF were explained by a higher fraction of the lipophilic non-ionised species being present at the higher pH value. For general chemicals and pesticides, the available data is somewhat more extensive. Research into the bioaccumulation and toxicity of the weak acidic compound pentachlorophenol was done on gold fish (*Carrasias auratus*). The bioconcentration factors at 1 h exposure to 0.1 ppm PCP-media at pH 5.5, 6, 7, 8, 9 and 10 were 131, 120, 56, 24, 12 and 2, respectively. Uptake and toxicity of four acidic sulfonylurea herbicides (metsulfuron-methyl, chlorsulfuron, triasulfuron and tribenuron-methyl) to the freshwater microalga *Chlorella fusca* have been investigated under different pH (Fahl *et al.* 1995). The bioconcentration of the four sulfonylureas in *Chlorella* did not exceed a factor of 9 at pH 6.0 but was significantly increased at pH 5.0, with a maximum value of 53 for chlorsulfuron. The authours suggested that the sulfonylureas penetrate the algal cell membranes primarily in their undissociated form and accumulate through an ion trapping mechanism. The influence of pH and humic acids on bioaccumulation and bioavailability of tributyltin chloride (TBT) was studied in *Daphnia*. Uptake rates and bioaccumulation of TBT in *Daphnia* were significantly higher at pH 8.0, where TBT predominates as neutral TBTOH

species compared to pH 6.0, where it predominates as the cationic species. The authors concluded that chemical speciation is an important factor for determining the bioavailability, and thus bioconcentration, of TBT (Fent and Looser, 1995)

The sorption of ionisable compounds to natural sediments can also be affected by pH (Zhang *et al.* 2010). For example, sorption of the antibiotic tetracycline to sediment depends on a range of environmental factors and sorption can be facilitated at lower pH values *via* a cation exchange mechanism (Zhang *et al.*, 2010). The soil sorption of three antimicrobial agents-sulfachloropyridazine, tylosin, and oxytetracycline, was investigated over a range of pH values. The sorption coefficients in two agricultural soils ranged from 1.5 to 1,800 L/kg and sorption coefficients were greater under acidic conditions (ter Laak *et al.* 2006). The effects of pH on the sorption of ionisable pesticides was investigated by Kah and Brown (2009) in nine temperate soils. They investigated the sorption behaviour of six acidic and four basic compounds. For the acidic substances, adsorption was negatively correlated with soil pH. However, for basic compounds, the behaviour was more complex, and approaches specific to each compound seemed to be required.

Difference in pH can also affect the behaviour of non-ionised substances. For example, the bioconcentration of pyrene was studied in midge larvae (*Chironomus riparius*) at three different pH, 4, 6 and 8 and uptake was found to increase with increasing pH. The differences were thought to be due to an abnormal mucus secretion at lower pH values which could have reduced the degree of biconcentration (Wildi *et al.* 1994).

4.1.2 Implications of metabolism for uptake of compounds

Metabolism of a compound is a key factor that affects the potential effects of a compound in non-target organisms (Lewis *et al.* 1998; Brooks and Huggett 2012). Metabolism or biotransformation is the process whereby a substance is changed from one chemical to another by a chemical reaction within an organism. Metabolism of xenobiotics normally

consists of two phases. Phase I reactions are generally reactions which modify the chemical by adding a functional structure. This allows the substance to "fit" into the Phase II enzyme so that it can become conjugated with another substance. The conjugated products are larger molecules than the substrate and generally polar in nature Thus, they can be readily excreted from the body. Metabolism of PPCPs in humans are typically detoxified *via* the cytochrome P450 system and then excreted as more polar compounds (Guengerich, 2001). Many of the phase I reactions typical of human metabolism are also abundant in the environment (Perez and Barcelo 2007). As a result, biotransformation products in environment have been detected in a number of organisms e.g. (Brooks *et al.* 2005; Lahti *et al.* 2011; Meredith-Williams *et al.* in preparation). However, in some organisms, human pharmaceutical target enzymes are not conserved. As a consequence, toxicity data that are generated from a species that lack the human target ortholog might not be protective for a species with a conserved target (Gunnarsson et al. 2012 in Brooks and Huggett; Connors et al. 2013).

Metabolism of a compound does not only decrease the sensitivity of organisms to environmental pollutants, it can also directly alter the toxicokinetics or bioconcentration of a chemical. A study by Ashauer *et al.* (2012) investigated the effects of metabolism of fifteen organic xenobiotics. The metabolite enrichment factors of 14 out of 19 identified metabolites were higher than the bioaccumulation factor of the parent compound. Thus, extrapolating toxicokinetics of chemicals in between organisms without knowledge of the metabolism is of low value.

4.1.3 Aim

This Chapter reports the results of a series of studies to understand the effects of changing environmental pH on the uptake of ionisable chemicals into *Lumbriculus variegatus*. The study looked at four of the compounds used in Chapter 2 and 3: caffeine, diclofenac, fluoxetine and triclosan. These were selected as they include a neutral compound, two acidic compounds and a basic compound. Investigations were also performed to understand whether these test compounds are metabolized by *L. variegatus* or not. These results were

than used to better understand and evaluate the model by Trapp and Horobin (2005) and Neuwoehner and Escher (2011) described in Chapter 3.

4.2 Methods

4.2.1 Oligochaete cultures

Cultures of *L. variegatus* were obtained from Blades biological (www.blades-bio.co.uk, Cowden,UK) and cultured using the method described in Chapter 3. Cultures used for the metabolism study were cultured at CSIRO (Adelaide, Australia), using a similar approach as described in Chapter 3.

4.2.2 Chemicals

The study explored the uptake of diclofenac (weak acid, CAS 15307-79-6), fluoxetine (weak base, CAS 59333-67-4) triclosan (weak acid, CAS 3380-34-5) and caffeine (neutral molecule, CAS 58-08-2. Caffeine, diclofenac, fluoexetine and triclosan were the same as used in Chapter 2 (See Table 3). Caffeine was used to determine whether or not the pH manipulations had any stress-related effect on uptake of neutral organic substances into *L. variegatus*. For the metabolism studies, non-radiolabelled compounds were used, these were purchased from Sigma Aldrich (Sydney, Australia) and had a purity of ≥ 98%. Further detailed information on the study chemicals is provided in Chapter 1.

4.2.3 Uptake and depuration at different pH values

Uptake and depuration studies were carried out using the general approach described in Chapter 3 (Section 3.2.5) but were performed using different exposure media with different pH values. Soft standard reference water (SRW) (40-48 mg / L as $CaCO_3$, total alkalinity = 30-35 mg/L as $CaCO_3$) was used throughout the tests and pH was set to 5.5, 7 and 8.5 using

chemical reagents as recommended for buffering according to USEPA (1975). KH_2PO_4 was substituted for NaH_2PO_4 for buffering the pH since KH_2PO_4 has previously been shown to be toxic to aquatic invertebrates (Fischer *et al.* 1991). Minor adjustments of pH during the test were made no more than every 24 h using either 0.1 M HCl or NaOH. pH was measured at each sampling point and values in test beakers were kept at the target pH value ± 0.3 throughout the test.

L. variegatus were acclimatized to the test conditions for 18 h in SRW. For the uptake studies, animals were then exposed in groups of 10 animals contained in 40 ml of SRW of which had been adjusted to pH 5.5, 7 or 8.5 to between 5 – 12 nmol l^{-1} of test compound for 3, 6, 12, 24 or 48 h. Caffeine studies were run at higher water concentrations (~55 nmol l^{-1}). All test concentrations were below toxicological thresholds. For the depuration studies, groups of animals were exposed to the test chemical for 48 h after which time they were transferred to pH-adjusted SRW for 3, 6, 12, 24 or 48 h. pH did not differ more than 0.3 pH-units throughout the test. Three replicates per time point and pH treatment were used. The study temperature was 20 ±2 °C and the beakers were kept in darkness throughout the test to minimize degradation of the test compound. Control beakers containing SRW and radio-labelled substances were used to monitor sorption to the jars.

4.2.4 Metabolism of diclofenac, fluoxetine and triclosan.

Alongside the uptake study using radiolabelled chemicals, a more simplistic uptake study was performed to facilitate the understanding of metabolism of diclofenac, fluoxetine and triclosan and to determine what effects it has on the uptake of the study chemicals in *L. variegatus*. The study was performed using the method described above with the exception that only uptake at one pH value and one time point was tested, i.e. 48 h. Test conditions such as nominal concentrations, test waters, temperature and dark/light conditions were chosen to mimic the parameters in the study with radiolabelled test compounds. pH throughout the test was kept at 7 ± 0.3.

4.2.5 Extraction and analyses

Samples from the pH studies were extracted and analysed using the methods described in Chapter 3 (section 3.2.5). For the metabolism studies, water samples were cleaned up and concentrated using solid phase extraction. Cartridges (HLB Waters) were conditioned with 2 x 4 ml of methanol and and 4 ml of MQ water before 50 ml samples were loaded onto the cartridges and allowed to flow through at a rate of 5 ml /min. Samples were then dried under vacuum for 30 minutes before put in a -18 ˚C freezer for storage until analysis. Prior to eluting, cartridges were defrosted for 30 min. Samples were eluted into a 15 ml test tube using 2 x 3 ml of methanol and 2 x 3 ml of dichloromethane. The eluted samples were evaporated under a gentle stream of nitrogen and reconstituted in 1 ml of methanol prior to analysis. Saples were kept at -18 °C prior to extractions, which were performed within two weeks of test termination. At sampling, samples were weighed in glass test tubes and frozen at -18 ˚C for a minimum of 24 hours before freeze dried for 48 hours. After freeze drying, dry weight of samples were determined before a glass rod was used to grind samples. Samples were extracted three times with 5 ml of methanol acidified with 0.1 M acetic acid. In the extraction procedure, samples were vortexed for 30 s and sonicated for 10 minutes before centrifuged for 45 minutes at 1500 rpm. Supernatants from the three extractions were pooled into a 15 ml test tube and evaporated under a gentle stream of nitrogen. Samples were reconstituted with 1 ml of methanol and filtered into an LCMS vial using a 0.2 µm hydrophilic PTFE filter.

Instrumental analysis was performed by high pressure liquid chromatography triple quadrupole-tandem mass spectrometry (HPLC-QqQ-MS/MS) using a TSQ Quantum Discovery Max, (ThermoFischer Scientific). Chromatographic separation was carried out with a Phenomenex C18 Kinetex column (2.1x100mm, 2.6 µm particle size) with solvents used for chromatographic separation were acetonitrile (A) and (B) 0.1% formic acid. Mobile phase conditions were as follows: 95% B (0-3 minutes), 20% B (4-5 minutes), 2% B (6-11

minutes) 95% B (12-15 minutes), with a total 15 minute run-time. Analysis of fluoxetine was undertaken in positive electrospray ionisation (ESI), while diclofenac and triclosan were analysed in negative ESI mode. Fluoxetine and diclofenac were identified by multiple reaction monitoring (MRM), monitoring two transitions per compounds (one for quantification and the other one for confirmation), while triclosan was identified using its parent ion only with the 37Cl isotope used as a confirmation ion. To account for recovery of analytes during extraction and from matrix interference during HPLC-QqQ-MS/MS analysis, stable isotopes of each analyte was spiked at an equivalent final concentration in solution of 100 µg/L. The stable isotopes used were fluoxetine d5, diclofenac d4 and triclosan C^{13}. Quantification of each analyte was undertaken by comparing the peak area ratio between that of the analyte and its respective isotope and with that in calibration standards prepared in 10% acetonitrile. A list of target compounds analysed and their corresponding MRM conditions are indicated in Table 10.

Table 10 Target compounds analysed and their corresponding MRM conditions. Details of the internal analytical standards are also provided.

Compound	Retention time (minutes)	Quantifying ions transition (Collision energy)	Confirmation ions transition (Collision energy)	LOQ (µg/L)
Fluoxetine	6.94	310 -> 44 (20 V)	310 -> 148 (20 V)	1
Fluoxetine d5	6.94	315 -> 44 (20 V)	315 -> 156 (20 V)	1
Diclofenac	7.86	294 -> 214 (20 V)	294 -> 250 (20 V)	1
Diclofenac d4	7.86	298 -> 217 (20 V)	298 -> 254 (20 V)	1
Triclosan	8.33	287 -> 287 (2 V)	289 -> 289 (2 V)	10
Triclosan $^{13}C_{13}$	8.33	300 -> 300 (2 V)	302 -> 302 (2 V)	10

4.2.6 Data analysis

Derivation of uptake and depuration rate constants and calculations of bioconcentration factor were performed using the method described in Chapter 3 (Section 3.2.6). Models fitted to the data are shown in Equation 23 and 24. The method is described in full in Ashauer *et al.* (2010). The caffeine data could only be fitted to a Levenberg-Marquardt algorithm and therefore no confidence intervals could be estimated around the bioconcentration factors. Equation 2 was used to calculate the caffeine bioconcentration factors.

$$\frac{dC_{org}}{dt} = K_{in} \cdot C_{water} - K_{out} \cdot C_{org} \quad \text{Eq 23}$$

$$BCF = \frac{K_{in}}{K_{out}} \quad \text{Eq 24}$$

To determine to effects metabolism have on the uptake, the internal concentrations was compared with the concentrations from the radiolabelled study. Bioconcentration factors were calculated using Equation 25.

$$BCF_{48h} = \frac{C_{org,48h}}{C_{water,48h}} \quad \text{Eq 25}$$

4.2.7 Evaluation of existing models for estimating bioconcentration factors for ionic substances.

The two models developed for ionizing substances described in Chapter 3 (Trapp and Horobin 2005; Nuewoehner and Escher, 2009) were evaluated by comparing estimated model bioconcentration and experimental data using the approach described in Chapter 3 (Section 3.2.7).

4.3 Results and discussion

4.3.1 Uptake and depuration in *Lumbriculus variegatus*

No mortality was observed in the treatments or in the controls. The pH values throughout the test were successfully kept at test pH ± 0.3 pH units in the treatments with a pH of 5.5 and 7 (Figure 15). However in the treatments with a pH of 8.5, pH decreased by up to one pH unit during the uptake and depuration phase. pH values in the test (Figure 15).

Concentrations of all study compounds in chemical controls containing radioactive test compound and water only were stable for the duration of the study. In the uptake beakers, concentrations of caffeine in water remained constant over the 48 h uptake phase whilst concentrations in the treatments containing triclosan decreased with approximately 80 % disappearing over the study period at all pH values (Figure 16B and 19B). Declines in concentrations of diclofenac and fluoxetine in the test solutions were pH-dependant. The diclofenac concentration in the pH 5.5 treatment decreased by approximately 30 % whilst the treatments at the higher pH values remained stable for the 48 h uptake phase (Figure 17B). The opposite pattern was observed for fluoxetine. In the treatments with pH 7 and 8.5 the concentration decreased with approximately 15 and 70 % respectively whilst the concentration in the pH 5.5 treatment remained stable (figure 16-19B). All losses of study compounds in the water phase could be explained by uptake into the study organisms. A mass balance was performed and is attached in Appendix 1.

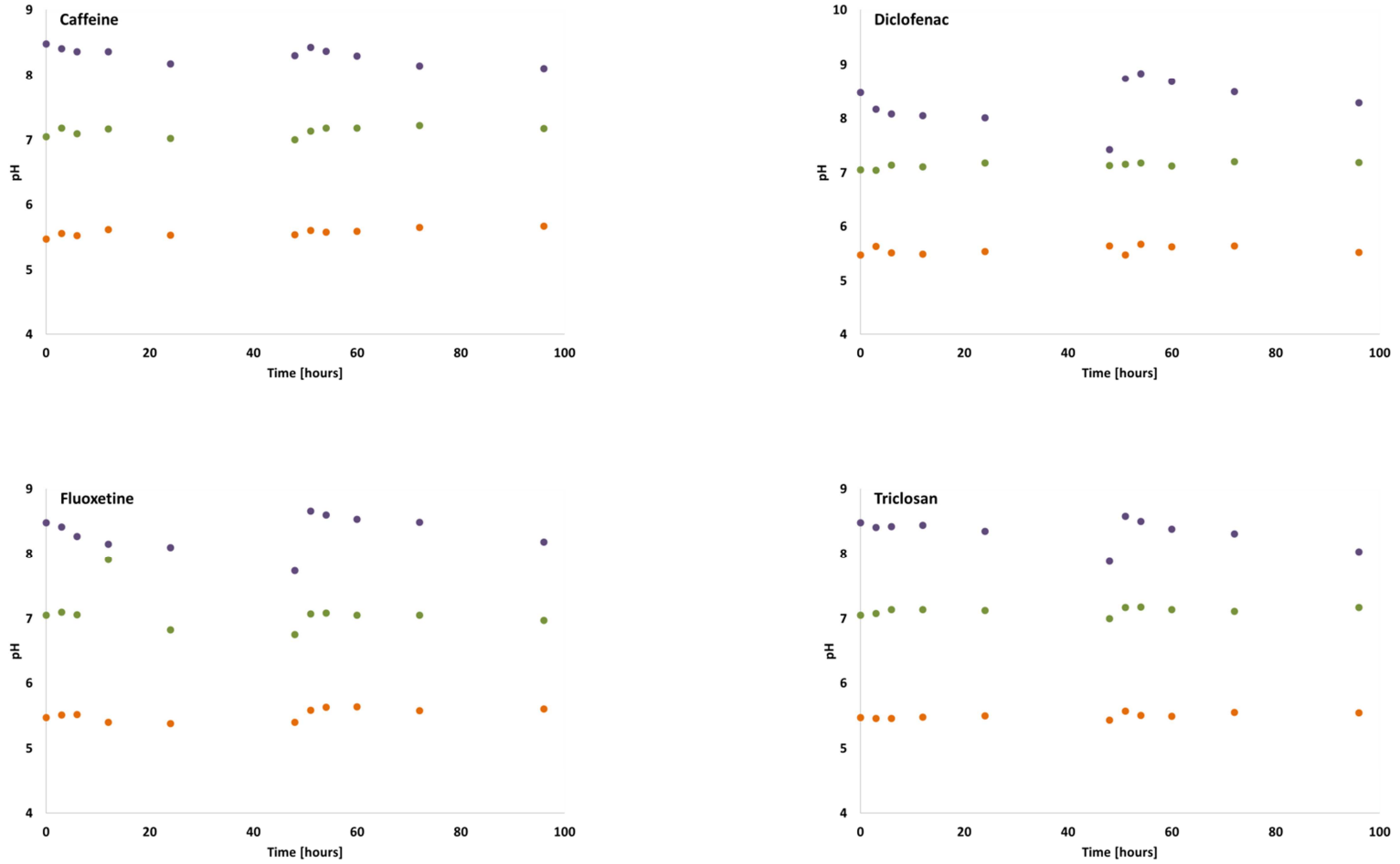


Figure 15 pH measurements for the different pH treatments (pH 5.5 - orange, 7.0 - green and 8.5 - blue) for the four study compounds

The first order two compartment model was successfully fitted to the uptake and depuration measurements for diclofenac, fluoxetine and triclosan (Figures 16-19) and the resulting uptake and depuration parameters and the bioconcentration factors are provided in Table 11. The MCMC model could not be fitted to the caffeine data using Open Model. A possible explanation for this could be due to the relatively low concentrations observed in the tissue compared to the surrounding media, something that has previously been observed when Open Model has been used to estimate uptake and depuration parameters in earthworms (Carter *et al.* unpublished). Therefore, the uptake and depuration parameters presented in Table 11 were estimated with a Marquardt fit only. However since the water concentration in the caffeine treatments remained stable and the internal concentrations in the worms reached equilibrium within the 48 hour uptake phase a static BCF could be calculated.

BCF values in the study ranged from 1 to 568400 at pH 5.5 and increased in the order caffeine < fluoxetine < diclofenac < triclosan. At pH 7, BCF values ranged from 1 to 646400 and increased in the order caffeine < diclofenac < fluoxetine < triclosan. At pH 8.5, BCF values ranged from 1 to 559300 and increased in the same order as for pH 7. All BCF values are presented in Table 11. The very high bioconcentration factors obtained for triclosan and fluoxetine (pH 8.5) are explained by a very slow or non-existent depuration of these compounds from the worms. Similar slow depuration was observed for fluoxetine and triclosan in *L. variegatus* in the experiments described in Chapter 3 (Section 3.3.1) and in previous uptake studies using the fresh water shrimp, *Gammarus pulex* (Meredith–Williams *et al.* 2012).

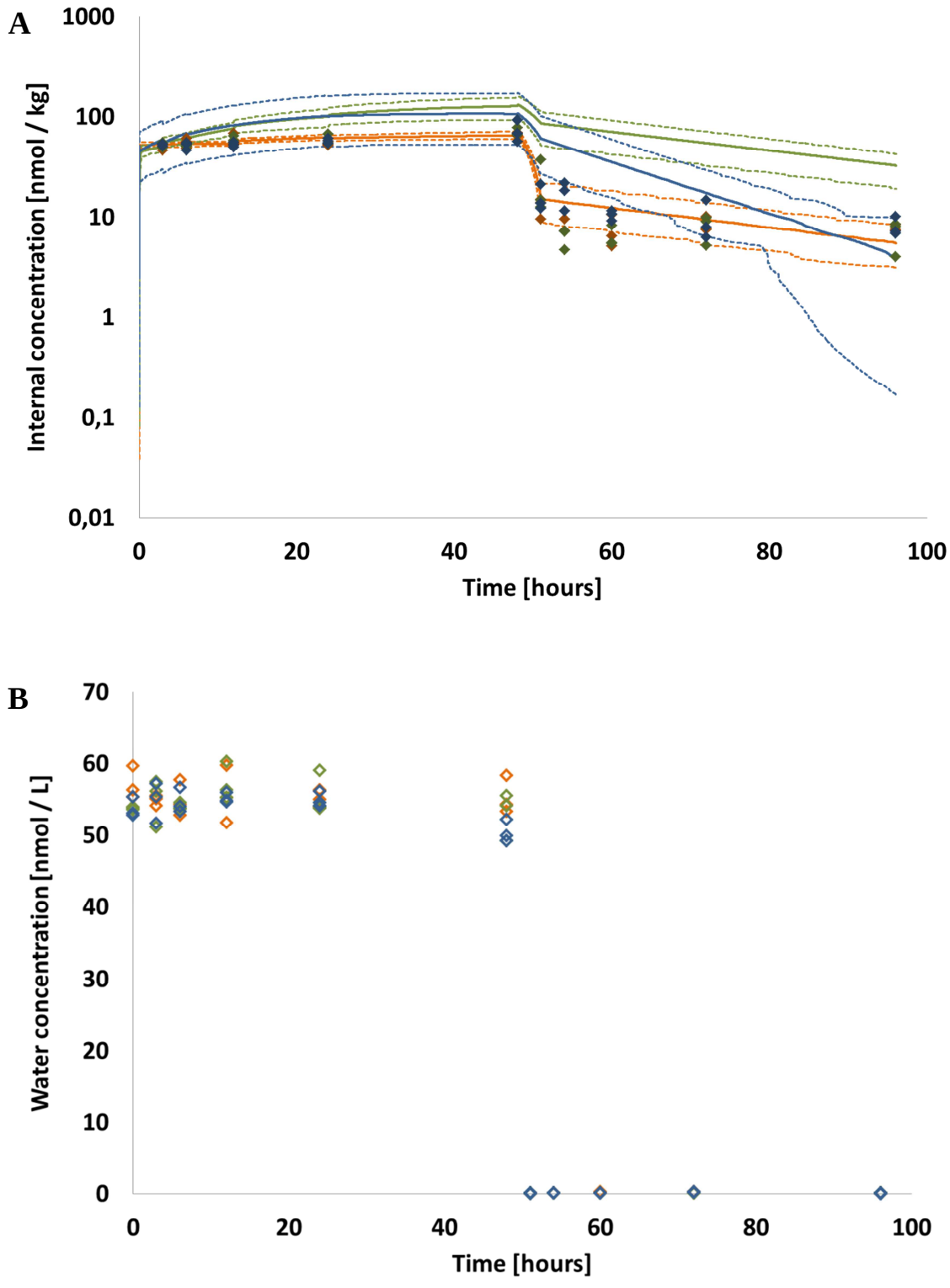


Figure 16 A- Uptake and depuration of caffeine at three different pH values; 5.5 (orange), 7 (green) and 8.5 (blue). Smooth lines represent the model fitted to the measured data (diamonds) and dotted lines represent the 95% confidence intervals. B- Corresponding water concentration.

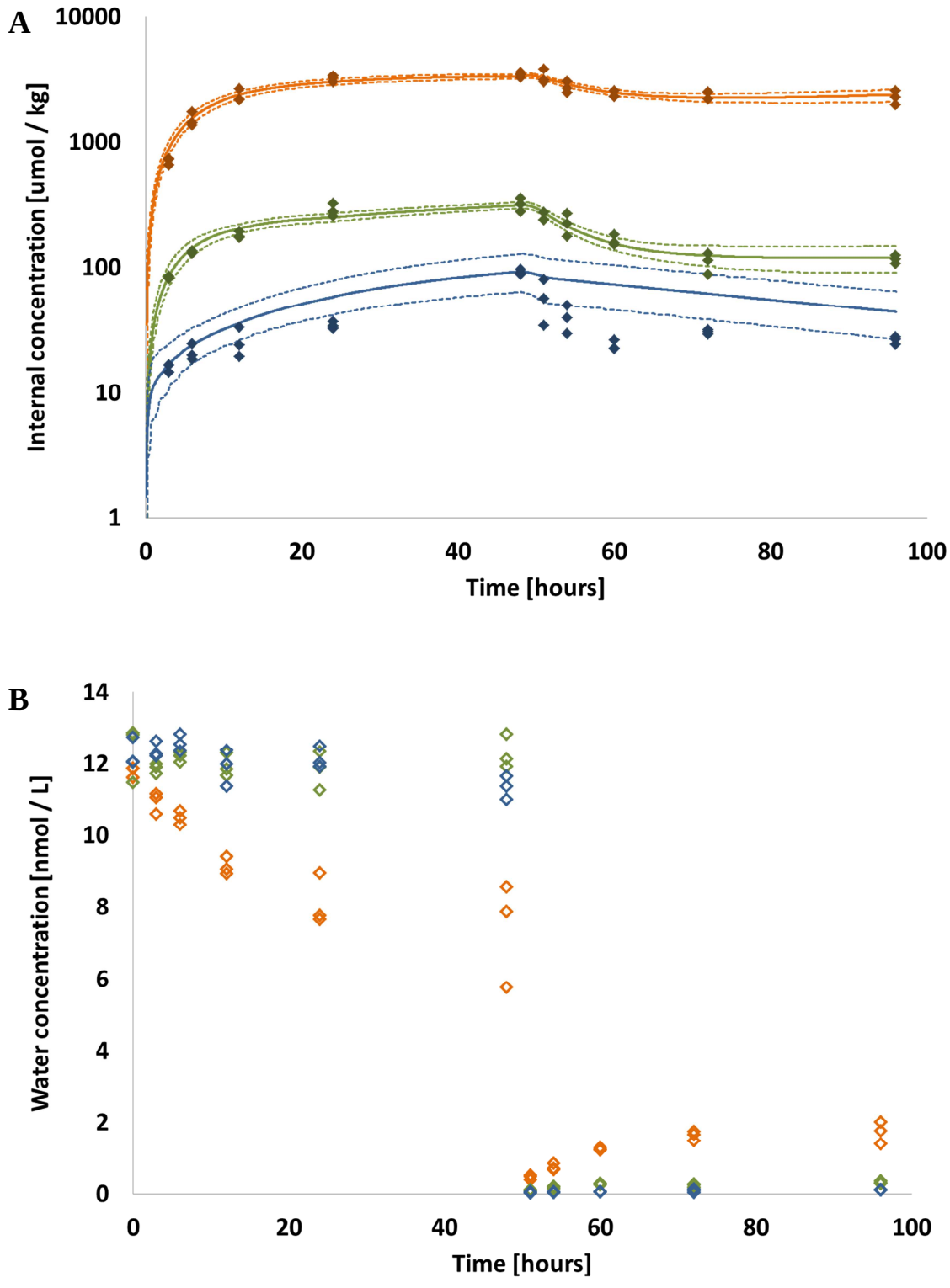


Figure 17 A- Uptake and depuration of diclofenac at three different pH values; 5.5 (orange), 7 (green) and 8.5 (blue). Smooth lines represent the model fitted to the measured data (diamonds) and dotted lines represent the 95% confidence intervals. B- Corresponding water concentrations.

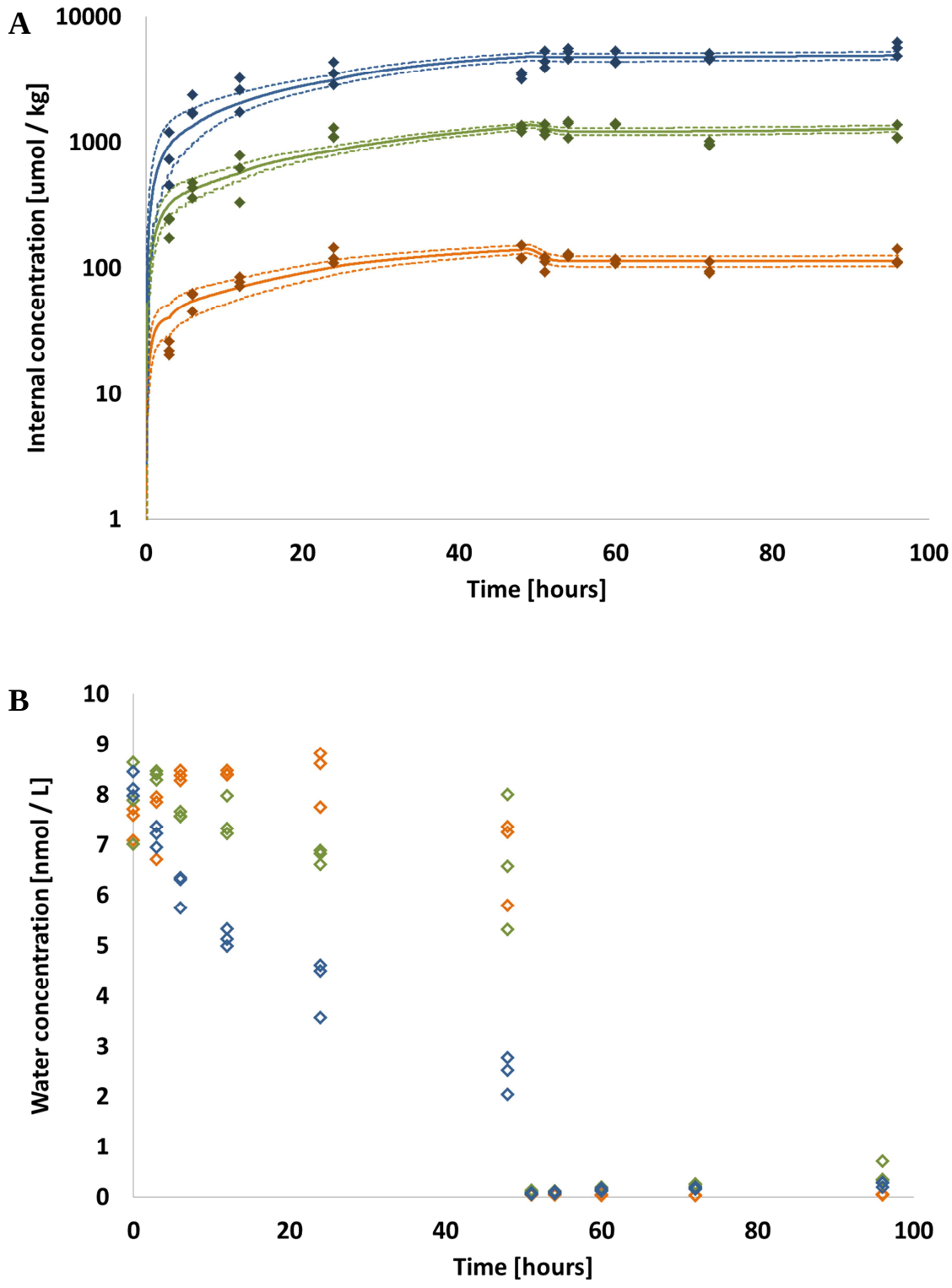


Figure 18 A- Uptake and depuration of fluoxetine at three different pH values; 5.5 (orange), 7 (green) and 8.5 (blue). Smooth lines represent the model fitted to the measured data (diamonds) and dotted lines represent the 95% confidence intervals. B- Corresponding water concentrations.

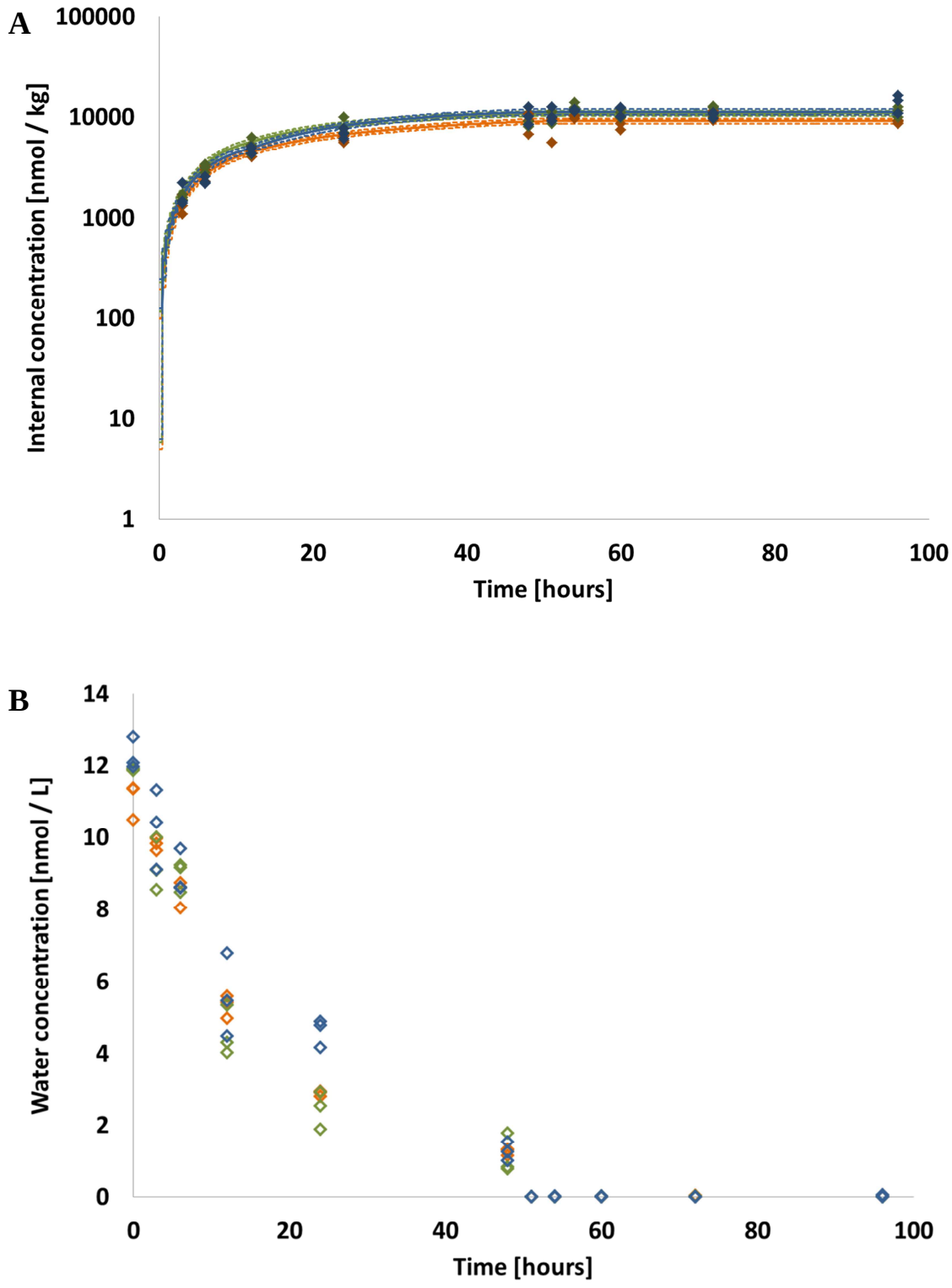


Figure 19 A- Uptake and depuration of triclosan at three different pH values; 5.5 (orange), 7 (green) and 8.5 (blue). Smooth lines represent the model fitted to the measured data (diamonds) and dotted lines represent the 95% confidence intervals. B- Corresponding water concentrations.

Table 11. Concentrations of test compound, uptake and depuration rate constants and bioconcentration factors with 95% confidence intervals.

			Kin		Kout		
	Cwater 0 h [nmol·L^{-1}]	**pH**	**RSS**	**Mean (n=3) ± SD**	**RSS**	**Mean (n=3) ± SD**	**BCF (LCI - UCI)**
Diclofenac	9.64 ± 1.71	5.5	2.01E+01	2.11E+01 ± 1.92E+00	3.39E-02	3.61E-02 ± 4.25E-03	623 (525 - 747)
	12.08 ± 0.44	7	1.80E+00	1.91E+00 ± 1.92E-01	6.43E-02	6.85E-02 ± 7.59E-03	30 (24 - 36)
	12.10 ± 0.50	8.5	2.43E-01	3.20E-01 ± 6.14E-02	3.93E-02	5.43E-02 ± 1.19E-02	8 (6 - 12)
Fluoxetine	7.82 ± 0.77	5.5	4.78E-01	5.39E-01 ± 6.39E-02	1.10E-02	1.50E-02 ± 3.88E-03	49 (39 - 61)
	7.45 ± 0.84	7	5.49E+00	5.80E+00 ± 4.70E-01	1.03E-02	1.20E-02 ± 2.40E-03	562 (482 - 665)
	5.54 ± 1.97	8.5	2.27E+01	2.33E+01 ± 9.33E-01	1.07E-04	7.05E-04 ± 2.10E-04	218 500 (200 900 – 236 600)
Triclosan	6.47 ± 3.71	5.5	4.50E+01	4.63E+01 ± 1.28E+00	8.14E-05	5.12E-04 ± 2.48E-04	568 400 (536 200 – 597 900)
	6.36 ± 4.04	7	5.67E+01	5.84E+01 ± 1.97E+00	9.03E-05	7.07E-04 ± 2.02E-04	646 400 (609 300 – 682 300)
	7.16 ± 3.88	8.5	4.70E+01	4.86E+01 ± 2.00E+00	1.06E-04	6.49E-04 ± 2.27E-04	559 300 (515 500 – 603 800)
Caffeine*	55.50 ± 2.20	5.5	1.23E+00	1.23E+00 ± 2.36E-01	1.18E+00	1.18E+00 ± 2.27E-01	1 (N.A.)
	54.76 ± 2.57	7	7.98E-01	7.98E-01 ± 1.35E-01	6.93E-01	6.93E-01 ± 1.20E-01	1 (N.A.)
	53.95 ± 2.20	8.5	6.67E-01	6.67E-01 ± 1.14E-01	6.19E-01	6.19E-01 ± 1.10E-01	1 (N.A.)

* BCF and confidence intervals could not be determined using the same approach as diclofenac, fluoxetine and triclosan. Instead, BCF was determined using the ratio of the uptake and depuration parameters Kin and Kout.

4.3.2 Effects of pH on the bioconcentration

As expected, pH had no effect on the uptake of caffeine (Figure 16 a). Therefore, any physiological response in the *L. variegatus* in response to pH that could affect the uptake behaviour can probably be excluded. At pH 5.5, 7 and 8.5 BCFs for diclofenac were 623, 30 and 8 respectively. The BCF values between the high and the low pH differed by a factor of approximately 80. Uptake of fluoxetine was also affected by pH. The BCF values calculated were 49, 562 and 219000 at pH 5.5, 7 and 8.5, a difference of approximately a factor of 4500 between the high and the low pH. The higher uptake at the higher pH value is consistent with previous data on bioconcentration of fluoxetine in fish (Nakamura *et al.* 2008). The BCF of fluoxetine in Japanese medaka, *Oryzias latipes* was reported at three different pH values, 7, 8 and 9 and BCF values were calculated to 8.8, 30 and 260. Changes in pH had no effect on the uptake of triclosan. Based on the pKa of triclosan (8.1) an effect due to a changing pH would have been expected since the proportion of neutral species increases at lower pH values. This could suggest that there are additional mechanisms involved in the limitations of the uptake and depuration of triclosan in *L. variegatus.* Although, to our knowledge, there is no available information in the open literature, possible explanations might include degradation or biotransformation mechanisms that are dependent on pH.

When correlating the uptake and depuration rate constants, Kin and Kout, with test pH, a clear negative correlation between Kin of diclofenac was observed whilst the correlation between fluoxetine uptake rates and test pH was positive (Figure 20 a and b). For triclosan, no such positive or negative correlation was observed (Figure 20 c). For diclofenac, no correlation between pH and Kout was observed indicating that the differences in uptake due to a varying pH is related to differences in uptake rather than differences in the depuration of the test chemicals. For fluoxetine, there was a negative correlation between Kout and pH (Figure 20). P-values for linear regressions are reported in Table 12.

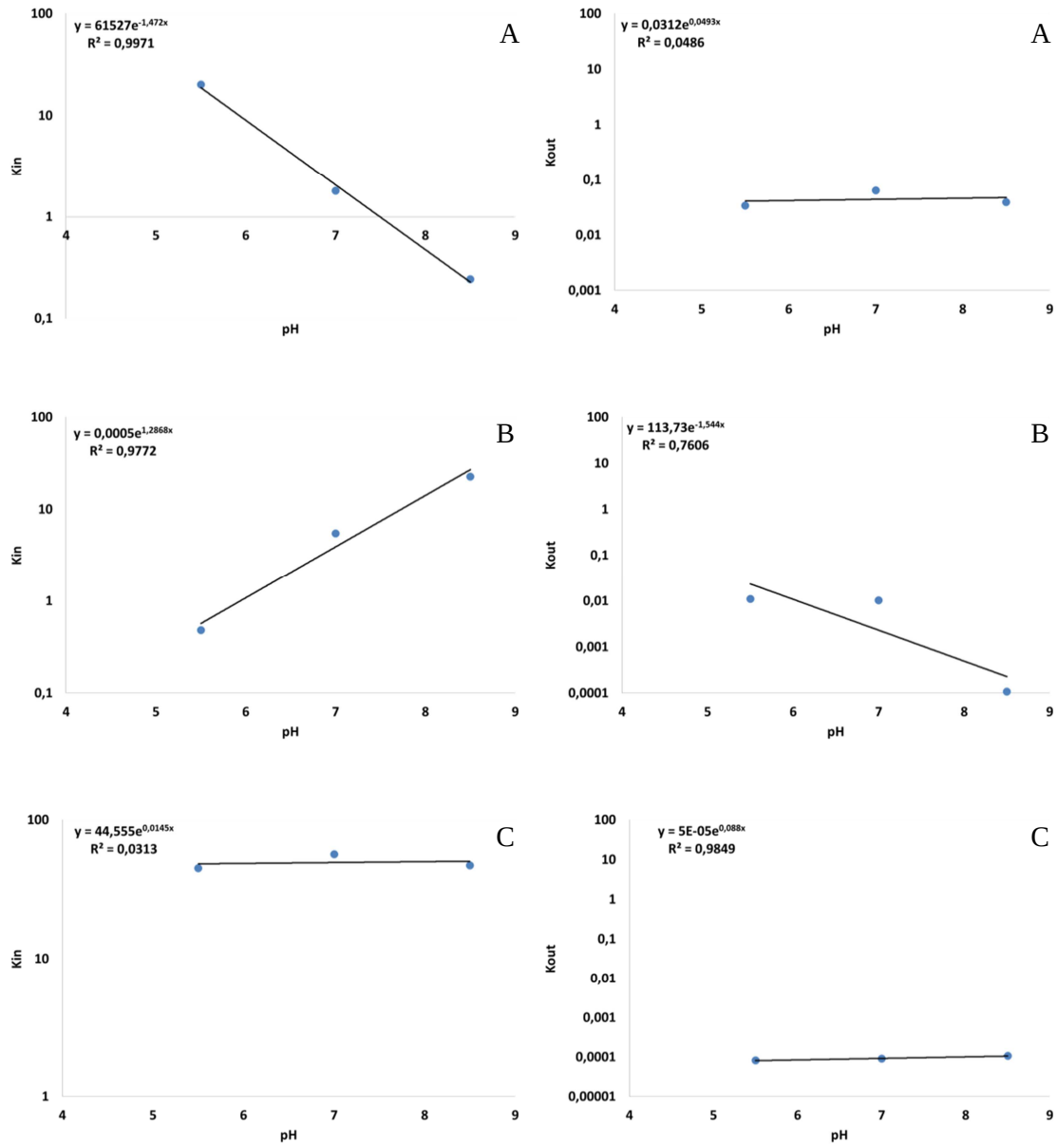


Figure 20 Uptake and depuration parameters (Kin and Kout) derived from studies n *L.variegatus* in correlation to test pH. A Diclofenac; B- Fluoxetine; C- Triclosan.

Table 12. Significance linear regression between pH and modelled uptake and depuration parameters for diclofenac, fluoxetine and triclosan into *L. variegatus*.

Test compound	Parameter	r^2	p-value
Diclofenac	K_{in}	0.997	0.034
	K_{out}	0.049	0.858
Fluoxetine	K_{in}	0.977	0.097
	K_{out}	0.761	0.325
Triclosan	K_{in}	0.031	0.887
	K_{out}	0.985	0.078

4.3.3 Metabolism

To investigate whether metabolism could have an effect on the uptake study a simplified non-radiolabeled study was run parallel to the radiolabelled study. Concentrations of the study compounds were determined at 0 h and 48 h and the internal tissue concentrations were measured at 48 h only. To determine the effects metabolism have on the uptake, the internal concentrations in the non-radiolabeled study was compared with the internal concentrations in the radiolabelled study and the bioconcentration factors at 48 hours were determined in both the radiolabelled study and the cold study. Results are presented in Table 13.

Water concentration for diclofenac and triclosan at 48 h were within 80-120% of the water concentrations at 0 hours. The concentration of fluoxetine at 48 h were approximately 65% of the concentration at 0 hours. In laboratory tests, fluoxetine has previously been shown to be hydrolytically and photolytically stable in aqueous solutions (Kwon *et al.* 2006). For diclofenac the estimated internal concentration was 210 ± 120 nmol /kg which is fairly close to the internal concentrations derived from the radiolabelled study (318 ± 39.0 nmol /kg) suggesting that only limited metabolism occurred in the worm tissue. Thus, metabolism should not have a significant effect on the uptake and depuration of diclofenac in *L. variegatus*. The concentration of fluoxetine detected in the worm tissue was low, 8.68 ± 0.74 nmol/kg. This is much lower than the internal concentrations detected when using radioactive analyses (1281 ± 73 nmol /kg). A possible explanation could be due to metabolism of fluoxetine in the worm tissue. The metabolism of fluoxetine and several other APIs has been characterised in the fresh water shrimp *Gammarus pulex* using Ultra Performance Liquid Chromatography (UPLC)-ToFMS (Meredith-Williams *et al.* In prep). In the study by Meredith-Williams *et al* no metabolites were detected in the tissue extracts. The metabolites of fluoxetine have also been detected in trout where norfluoxetine was detected at concentrations higher than the parent compound (Brooks et al. 2005; Chu and Metcalfe

2007). In humans, fluoxetine undergoes extensive metabolic conversion, leading to the active metabolite norfluoxetine and multiple other metabolites (Figure 21; Hiemke and Härtter 2000). However, studies with fish microsomes and fluoxetine indicates that hepatic metabolism of pharmaceuticals is much less than has been reported for mammals (Smith *et al.*, 2010).

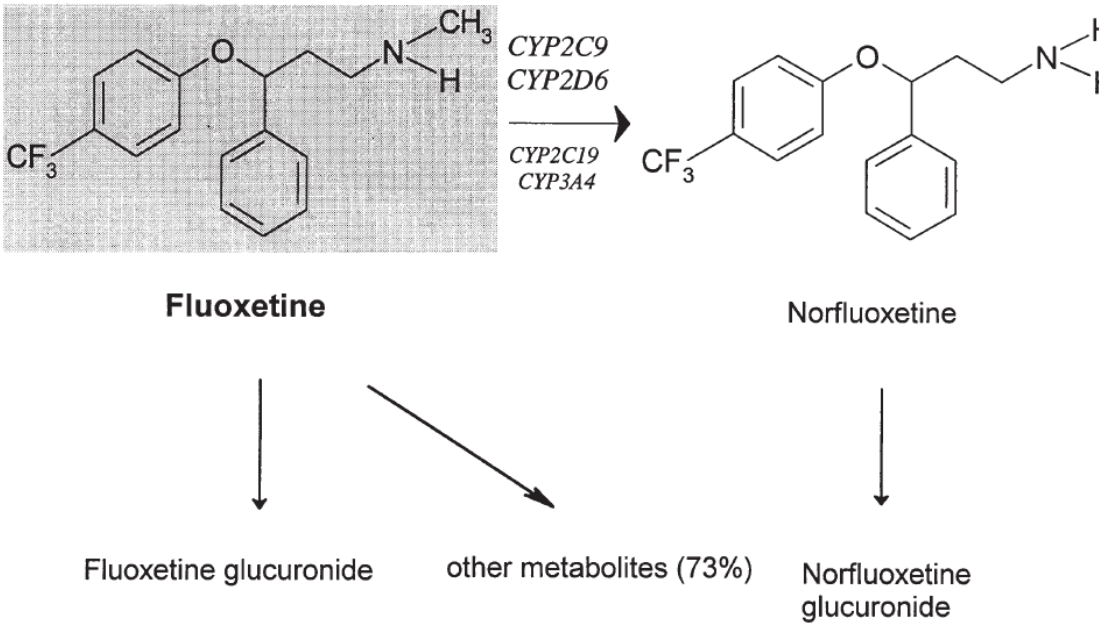


Figure 21. Simplified schematic of fluoxetine metabolism in humans (Hiemke and Härtter, 2000).

Results from the analyses of triclosan should be treated with caution since triclosan was only detected in the worm tissue of one of the replicates. As for fluoxetine, the concentration of triclosan was much lower in the tissue in the cold study (1.03 µmol/kg) than in the radiolabelled study (8.51 ± 0.47 µmol/kg). If this difference is due to metabolism of triclosan in the tissue, it could explain the very high bioconcentration factors observed for triclosan in the radiolabelled studies. Metabolism of phenolic compounds has previously been suggested to have an effect on the bioaccumulation kinetics into aquatic invertebrates (Ashauer *et al.*

2012). Available information on triclosan metabolism in aquatic organisms is scarce. In humans and other mammals, triclosan is extensively metabolized via glucuronide and sulfate conjugation (Fang *et al.* 2010). The metabolic pathway of triclosan is presented in Figure 22.

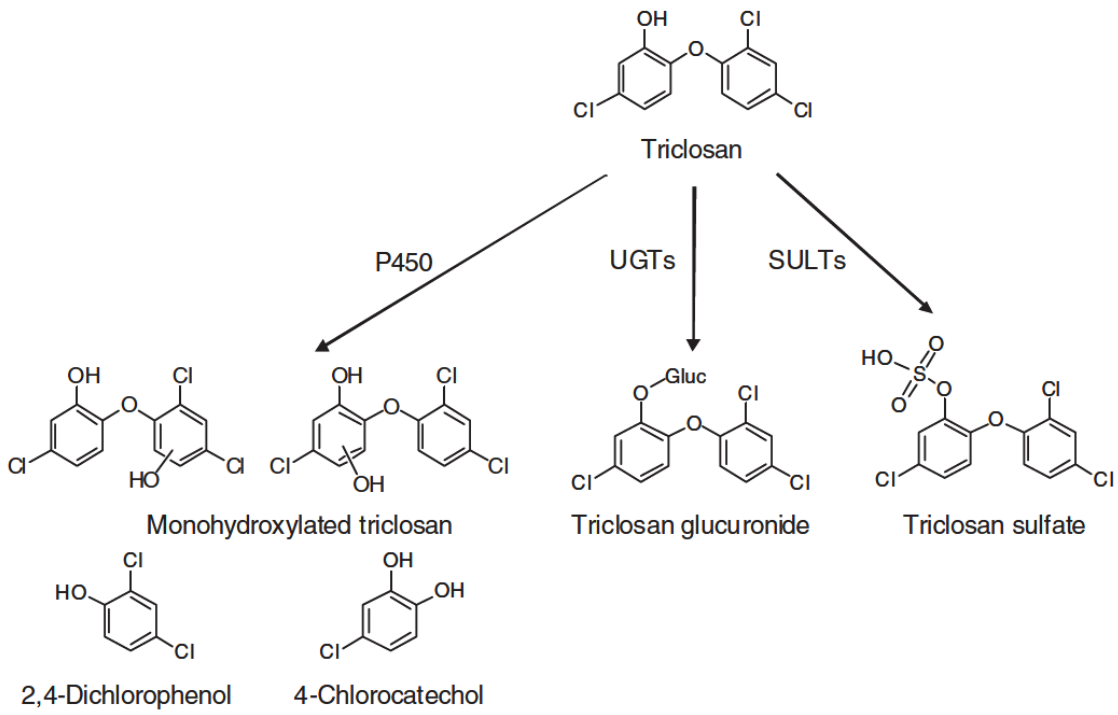


Figure 22. Simplified schematic of triclosan metabolism. P450; Cytochrome P450; UGTs; UDP-glucuronosyltransferases; SULTs; Sulfotranserases. Fang *et al.* (2010).

While metabolites and transformation products are usually less hazardous than the parent compound, some active pharmaceuticals ingredients are active mainly in the metabolized form e.g. fluoxetine (Sommi *et al.* 1987), in addition, some data for pesticides indicates that degradation products can be more toxic than the parent compound (Sinclair and Boxall 2003). Since degradates of environmental pollutants can either have a different toxicity to the parent compound or directly alter the toxicokinetics or bioconcentration of chemicals, it is crucial to have knowledge of the metabolism when assessing risks to non-target organisms from environmental pollutants.

Table 13 Test conditions and results from parallel study with cold test compounds.

	Cwater 48 h labelled study [nmol·l-1]	**Cwater 48 h [nmol·l-1]**	**Measured pH values**	**Temperature**	**Cinternal 48 h labelled study [nmol·kg-1]**	**Cinternal 48 h [nmol·kg-1]**	**Static BCF at 48 h radio-labelled study[1]**	**Static BCF at 48 h[1]**
Diclofenac	12.29±0.48	14.54±1.21	6.92	20±2 ˚C	318.2±39.0	210.0±120.0	25.9	14.4
Fluoxetine	6.62±1.35	5.35±0.47	6.85	20±2 ˚C	1 281±73.4	8.68±0.74	193	1.62
Triclosan	1.12±0.56	15.2±4.23	6.87	20±2 ˚C	8 514±466	1 033[2]	7 602	922

[1]*Chemical equilibrium was not reached in the tissue. BCF was calculated by dividing the average internal concentration with average water concentrations at 48 h.*
[2]*No standard deviations could be calculated since triclosan was only detected in one of the replicates.*

4.3.4 Evaluation of existing models for predicting bioconcentration of ionisable chemicals

The data from the pH studies were used to evaluate the models described in Chapter 3. Bioconcentration factors of all of the study compounds were underestimated by the cell model of the Trapp and Horobin (2005) (Figure 23a). The mismatch between model predictions and experimental results for triclosan and fluoxetine could be due to metabolism of the study compounds. However, metabolism would not explain the under-prediction of uptake for diclofenac. The cell model may not therefore be applicable to aquatic invertebrates. The Neuwoehner and Escher (2011) performed better with predictions of the bioconcentration factor for diclofenac, fluoxetine (pH 5.5 and pH 7) and caffeine being within one order of magnitude of the experimental bioconcentration factors. Predictions of bioconcentration factors for triclosan and fluoxetine (at pH 8.5) were greatly underestimated. The large differences of the predicted BCF and experimental BCF are likely to be due partly to metabolism of triclosan as discussed in the section above.

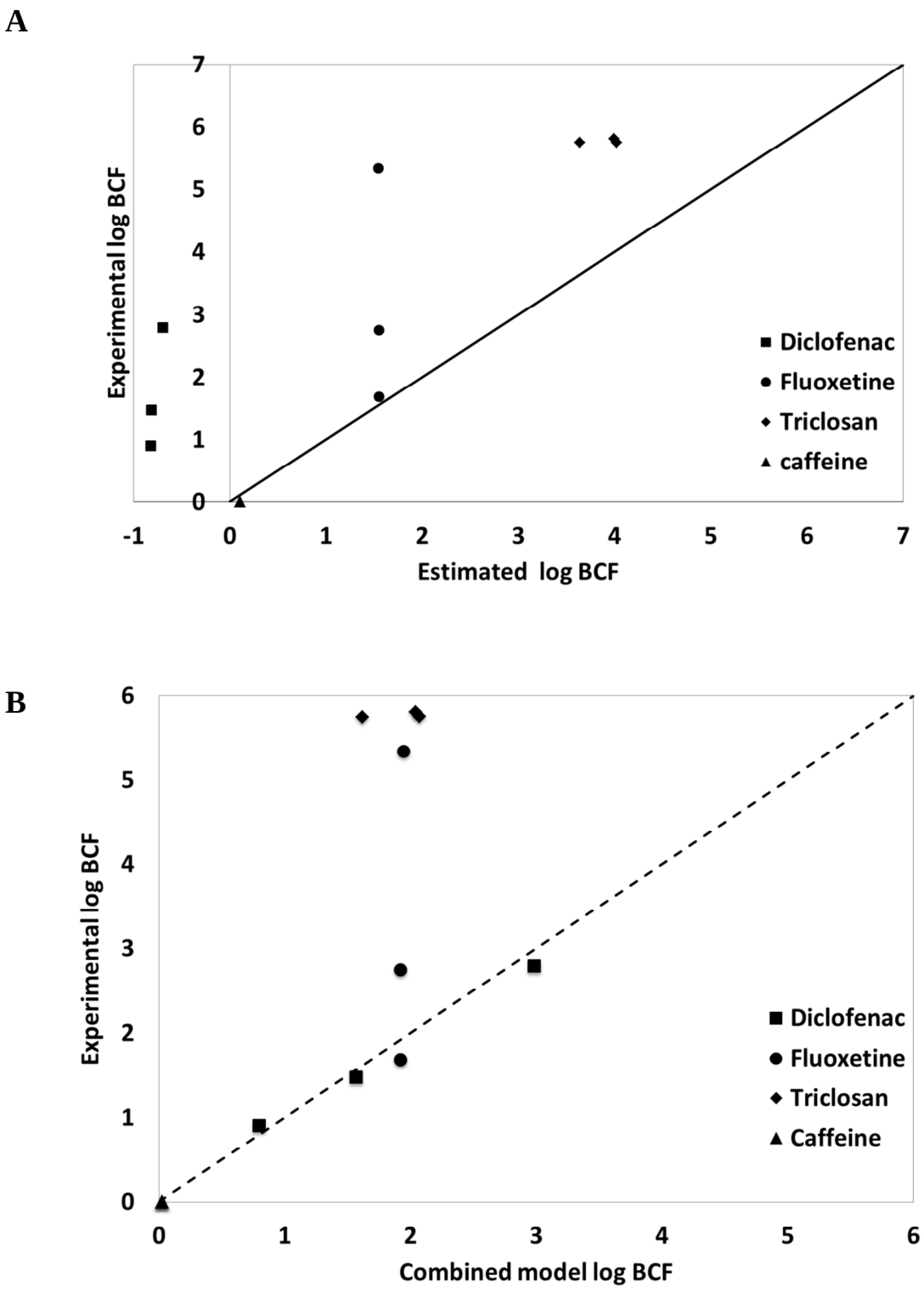


Figure 23. A comparison of the experimental BCF and BCF predicted by: A- the cell model by Trapp and Horobin (2005) and B- the combined model by Neuwoehner and Escher (2011).

In addition to the models described here for predicting bioconcentration of ionisable pollutants, recent work has developed a mechanistic mass balance bioconcentration model which has been parameterized for ionogenic organic chemicals (IOCs) in fish and evaluated against a compilation of empirical bioconcentration factors (BCFs). Key aspects of model development include revised methods to estimate the chemical absorption efficiency of IOCs at the respiratory surface (EW) and the use of distribution ratios to characterize the overall sorption capacity of the organism. Model prediction errors appear to be largely the result of uncertainties in the biotransformation rate constant (kM) estimates and the generic approaches for estimating sorption capacity (Armitage *et al.* 2013). Due to insufficient input parameter data available, evaluation of the model against experimental data on *L. variegatus* is not possible at this time.

4.4 Conclusions

The results from the studies described in this chapter highlight the importance of thoroughly investigating the impacts of environmental parameters such a pH as well as metabolism when trying to understand the uptake of ionisable chemicals in the environment. These factors and processes have a large impact on the bioconcentration of a compound. By altering the pH by two units, the BCF of diclofenac and fluoxetine were increased by a factor of nearly 80 and 4500 respectively! It can also be concluded that without knowledge on the metabolism of a substance, bioconcentration cannot be determined satisfactorily.

The main focus of the thesis so far has been on investigating the bioconcentration of ionisable chemiclas from the water phase. To further investigate what processes affect the bioaccumulation of sediment associated substances into sediment dwelling invertebrates, we must determine the potential uptake via ingestion of contaminated sediment. Therefore, in the next chapter, the focus will be on determining what the main uptake routes are for three of the study compounds (diclofenac, fluoxetine and triclosan) in *L. variegatus*.

CHAPTER 5

IMPORTANCE OF FEEDING FOR THE UPTAKE OF PPCPs INTO *LUMBRICULUS VARIEGATUS*

5.1 Introduction

The work described in previous chapters focused on understanding the uptake of pharmaceuticals and personal care products from the water phase into sediment dwelling organisms. In the real environment, these organisms will not only be exposed substances *via* the water phase but may also be exposed to chemicals associated with sediment particles. It is therefore important to have an understanding of the uptake of chemicals from whole sediments into benthic invertebrates. The risk assessments of sediment associated contaminants are likely to be more effective if they are built upon knowledge of from where and how organisms take up contaminants (Hare *et al.* 2003).

Previous research of the uptake of pharmaceuticals and personal care products from sediments into sediment-dwelling organisms is scarce. However, there are data on a few pharmaceuticals classes. Liebig *et al.* (2004) measured the bioaccumulation of a synthetic steroid 17α-ethinylestradiol (EE2) into *L. variegatus.* The accumulation factor normalised to worm lipid content and sediment TOC (AFlipid/OC) was 75 at the end of the uptake period, however, a steady state was not reached. There are also available studies on the anti-depressant fluoxetine. Bringolf *et al.* (2010) investigated the occurrence, distribution and bioaccumulation of fluoxetine near a municipal waste water treatment facility. They reported a BCF of 1347 ng/g and a Kd value of 178 ml/g 100 m downstream of the effluent discharge. From these figures a BSAF of 7.6 can be calculated. The occurrences of ten different anti-depressants were also investigated in surface waters, sediments and in brain tissue of native white suckers (*Catostomus commersoni*) (Schultz et al. 2010). The highest BSAF range from

approximately zero to ten with the highest values reported for the selective serotonin reuptake inhibitor sertraline.

Available research has shown that feeding as a route of bioaccumulation of sediment associated contaminants can be of major importance. Contaminated sediments can have direct toxic effects on aquatic life, such as the development of liver neoplasms and other liver lesions in bottom-feeding fish exposed to polynuclear aromatic hydrocarbons (PAHs) in sediments (Malins *et al.* 1987). This has also been shown in sediment dwelling invertebrates. Leppanen and Kukkonen (2004) showed that when *L. variegatus* was exposed to tetrabromo diphenylether both *via* contaminated water and contaminated water and sediment, the worms that did not ingest sediment had clearly lower influx rates than sediment-ingesting worms. Also, the estimated BSAF was statistically different from that of the sediment-ingesting *L. variegatus*. Both these findings support the significance of feeding behaviour in bioaccumulation.

In the Technical Guidance document, TGD, the European Chemical Bureau recommends the use of equilibrium partitioning when experimental data are missing for risk assessment of environmental contaminants in sediments. For substances with a log Kow > 5 an assessment factor of 10 is applied since equilibration partitioning only considers exposure *via* the water. (TGD; 2003). For deposit feeding organisms that live in and ingest sediment to obtain nutrients from particles suspended in the sediment the uptake *via* both pore water and ingestion of sediments are likely to contribute to the exposure of sediment-associated contaminants. It is known that the importance of different uptake routes is influenced by sediment and chemical characteristics and also the organisms themselves (Landrum and Robbins, 1990). For neutral compounds with log Kow < 5, the major route for the accumulation is pore water (Thomann *et al.* 1992; Belfroid *et al.* 1968). For more hydrophobic compounds, the contribution of ingested material in accumulation increases (Landrum *et al.* 1990). In addition to hydrophobic interactions affecting the distribution of chemicals, many of the active ingredients in pharmaceuticals and personal care products are

ionisable and will be affected by sorption mechanisms such as chemical dissociation and ionic sorption (Kah and Brown, 2007).

There is limited consistency across the literature regarding the importance of different uptakes routes of chemicals into sediment dwelling invertebrates. Comber *et al.* (2007) and Leppänen and Kukkonen (1998) showed that uptake of pyrene *via* ingestion of sediment into *Lumbriculus variegatus* was the main uptake route whilst Lu *et al.* (2004) showed that the main uptake of PAHs into a sediment dwelling oligochaete is *via* the pore water. Weston and Gulmann (2000) showed that the importance of uptake routes of benzo[a]pyrene (BaP) into the polychaete *Abarenicola pacifica* is time dependent.

The aim of the work described in this chapter therefore was to gain knowledge of the relative importance of the uptake of three PPCPs into a sediment-dwelling oligochaete, *Lumbriculus variegatus*, *via* pore water and *via* ingestion of contaminated sediments. Three PPCPs with relatively similar lipophilicity but different pKa values were used in the study, diclofenac, fluoxetine and triclosan. To determine the relative uptake *via* pore water and ingested sediment into *L. variegatus*, the approach described by Conrad *et al.* (2000) was used.

5.2 Method

5.2.1 Test sediment

Sediment was sampled from the top 10 cm of the benthos at a clean river site near Buttercrambe, North Yorkshire, UK (SE 73499 58510). The sediment was characterised using the methods described in Chapter 2 (Section 2.2.2). Sediment properties are listed in Table 13.

Table 14 Properties of the sediment used in the uptake studies with *L. Variegatus*

Parameter	Value
pH:	7.67
OC [g/kg]	5.51
CEC [cmol+/kg]	4.65
Grain size [g/kg]	
- Clay:	42
- Silt:	27
- Sand:	931

5.2.2 Test compounds

Three different substances commonly used as pharmaceuticals or personal care products, were chosen for the experiment, diclofenac, fluoxetine and triclosan. Purities for all study chemicals were > 98%. Further information on the test substances are found in Chapter 1 and Chapter 2. Acetonitrile (99.9 %), methanol (99.9 %) and phosphoric acid (99.9%) were obtained from Fisher Scientific (Loughborough, UK).

5.2.3 Oligochaete cultures

Cultures of *L. variegatus* were maintained using the methods described in Chapter 3, Section 3.2.1. Approximately two weeks prior to test initiation, tissue paper was replaced with sediment (depth of approximately 4 cm) identical to the sediment used in the uptake study to allow acclimation of the test organism to the test conditions.

5.2.4 Evaluation of extraction of test compounds from sediment.

Duplicate replicates of wet sediments (3.0 g dw corresponding to approximately 4.5 g ww) were spiked with approximately 1000 Bq and left to shake for 2 h. Samples were extracted twice by adding 10 ml of solvent; methanol, acetonitrile and a 7:3 mixture of acetonitrile: water. An additional set of acidified solvents were tested as well. Acidification was

performed using 0.1 % H_3PO_4. In total six different solvents. Samples were shaken at 250 rpm for 2 hours and then centrifuged at 3000 rpm for 10 min. (Hermle Z 513K Bench Top Centrifuge). The procedure was repeated and samples pooled prior to analyses. Samples were analysed with LSC using the method described above. The solvent resulting in the highest recovery for each test substance were evaluated using the same procedure with three replicates at three concentrations, 0.1, 0.5 and 1 µg/g. The solvents chosen for extraction of diclofenac, fluoxetine and triclosan were acidified methanol, an acidified 7:3 mixture of acetonitrile:water and methanol respectively. The average recovery, standard deviation and coefficient of variance were calculated.

5.2.5 Uptake studies

Prior to introduction of *L. variegatus* to the test system, 3 g (dw) of sediment, 15 ml of APW and test substances dissolved in 10 – 20 µL methanol were added to 50 ml centrifuge PTFE tubes (Oak Ridge centrifugation tube, FEP by Nalgene Nunc International). Chemicals were then equilibrated between the water and sediment phases by shaking for 2-12 hours at 300 rpm. Concentrations of test compounds ranged from 25 to 750 nmol kg^{-1} dry sediment. After equilibration, tubes were kept in an upright position in tube racks allowing the sediment to settle before adding the worms to the tubes.

Prior to treatment, half of the *L. variegatus* were cut in half with a razor blade according to the method of Conrad *et al.* (2000) to produce a set of 'non-feeding' worms. The uptake of the study compounds into either 'feeding' or 'non-feeding worms was then studied. Ten worms were added to each replicate. Three replicates were prepared for each timepoint and treatment. Tubes were then kept in the dark at 20 ±2 °C throughout the test. To determine the uptake rate, samples of water, sediment and worms were taken at 3, 6, 12, 24 and 48h from the beginning of exposure. To assess depuration depuration *L. variegatus* were exposed to the chemicals for 48 h and then transferred to tubes with clean sediment for sampling at

either 3, 6, 12, 24 or 48 h after transfer. At samplings, worms were retrieved from the test tubes using a modified spatula and transferred to 80 ml glass jars containing 40 ml artificial pond water to purge their guts of any remaining sediment. They were left there for 6 h as this timeframe had previously been reported as a sufficient time for *L. variegatus* to purge their guts (Mount *et al.*, 1999). After purging their guts, worms were rinsed in distilled water, blotted dry on a tissue, weighted and dissolved in 2 ml of Soluene®-350 (Perkin Elmer, Waltham, Massachussets) in a 20 ml glass scintillation vial. Worms were left to dissolve for 24 h before 10 ml Hionic Fluor scintillation cocktail (Perkin Elmer) was added prior to analyses.

Sediment and overlying water was centrifuged at 3000 rpm for 10 min (Hermle Z 513K Bench Top Centrifuge). A 1 ml aliquot of the supernatant was then sampled and placed into a 20 ml scintillation vial and 10 ml Ecoscint A was added. The remaining supernatant was disposed of and sediment samples were kept at -18 °C until extractions. Sediment samples were extracted within 14 d of collection by shaking the sediment with 10 ml solvent for 1 h at 300 rpm. Fluoxetine was extracted with an acidified mixture of acetonitrile:water (0.1 % H3PO4, 7:3), diclofenac was extracted with 0.1 % H_3PO_4 in methanol and triclosan was extracted with methanol. After shaking, the tubes were centrifuged at 3000 rpm and a 1 ml of aliquot was taken for analyses. Ecoscint A was used as a scintillant. Recoveries ranged between 85 to 105 %. Analyses were made using Liquid Scintillation Counting, LCS, (Liquid scintillation Counter LS 6500, Beckman Coulter Inc., Fullerton, USA). Samples were counted three times for 5 min. Counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method.

5.2.6 Data analysis

A Student's T-tests were used to determine the statistically significant differences between the uptake of the feeding worms and the non- feeding worms at each time point. Data were checked for normality using Shapiro-Wilk Test using the software SPSS Statistics v21.0.0. A first order one compartment model was used to estimate the uptake and depuration rates for each test compound. The change of concentration in the organism was estimated according to Branson *et al.* (1975) using Equation 1. The parameters were estimated using the software OpenModel (v. 1.2 downloaded on the 24th of June 2011). The model was parameterized using residual sum of squares with the Levenberg-Marquardt algorithm followed by Monte-Carlo Markov-Chain (MCMC) with the results from the Marquardt fit as input values. Confidence intervals were characterized by the 95% percentile of the simulated variables. Biota Sediment Accumulation Factors, BSAF were calculated by setting the sediment concentration to 26 and running the model until equilibrium. BSAF and their confidence intervals could then be read directly from the internal concentrations. The method is described in full in Ashauer *et al.* (2010).

$$\frac{dC_{int}}{dt} = C_{sed} \cdot K_1 - C_{int} \cdot K_2 \qquad \text{Eq. 26}$$

5.3 Results and Discussion

5.3.1 Analytical recoveries for sediment

The average recovery in percent were for diclofenac: 129%; for fluoxetine: 73.5 % and for Triclosan: 90.1 %. Results from the evaluation of the extraction method are presented in Table 16.

Table 14. Recoveries of study chemicals from screening of extraction methods from sediments

	Extraction solvent	Mean Recovery (%)
Diclofenac	H+ Methanol	115
	ACN	93
	H+ACN	111
Fluoxetine	ACN	60
	H+ Methanol	41
	ACN:H2O 7:3	65
	H+ACN:H2O 7:3	75
Triclosan	Methanol	92
	H+ Methanol	87
	ACN	90

Table 15 Recoveries of study chemicals from validating extractions from sediments

	Extraction solvent	Recovery (%)	St dev	CV
Diclofenac	H+ Methanol	129	13.6	10.5
Fluoxetine	H+ACN:H2O 7:3	73.5	2.34	3.2
Triclosan	Methanol	90.1	2.9	3.2

5.3.2 Uptake and depuration in *L. variegatus*

No mortality was observed either in the treatments or in the controls during the uptake or depuration phases. Concentrations of all study compounds in control treatments, containing radioactive test compound and water only, were stable for the duration of the study (Figure 25).

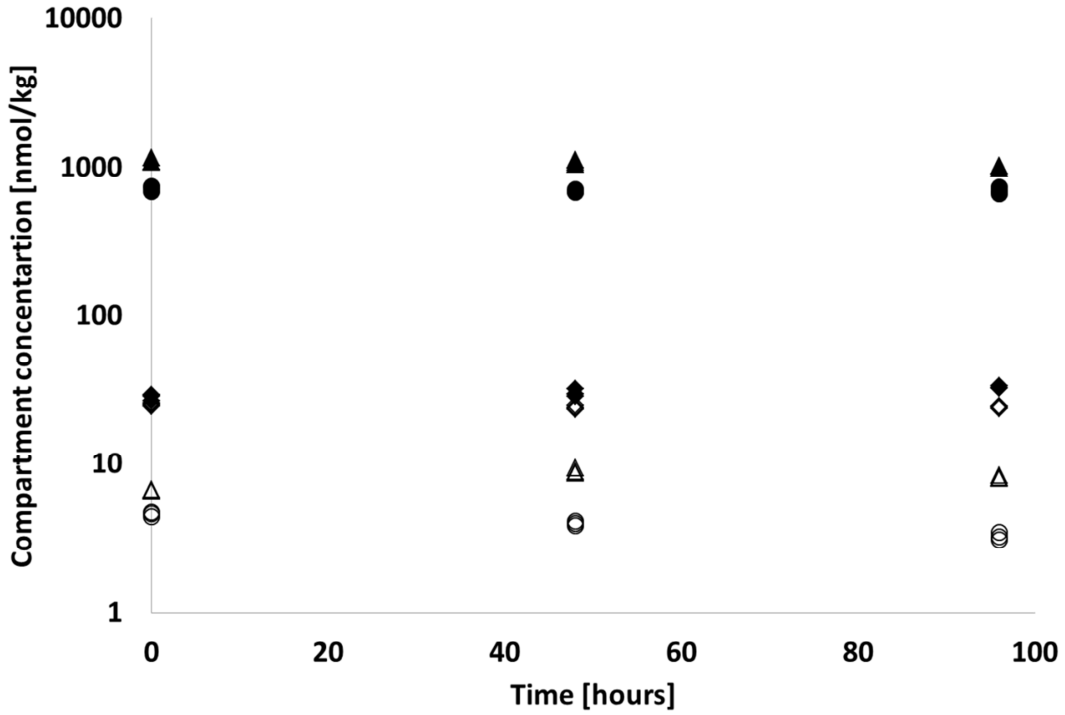


Figure 24 Concentration in the water and sediment compartments without worms present. Diclofenac in sediment (solid diamonds); in water (clear diamonds); Fluoxetine in sediment (solid triangles); in water (clear triangles); Triclosan in sediment (solid circles); in water (clear circles).

In the uptake beakers, concentrations of fluoxetine and diclofenac in the water phase remained constant over the 48 h uptake phase while concentrations in treatments containing triclosan decreased during the 48 h uptake phase (Figure 28-30). The reduction in concentration in the water column was explained by uptake of the compounds into the study organisms. These findings agree with the results on the water concentrations in the study on uptake into *L. variegatus* from water only in Chapter 3. The measured concentrations of diclofenac and fluoxetine remained stable in the sediment throughout the uptake phase whilst the concentration of triclosan decreased slightly over the 48 h uptake phase. This could be explained with a mass balance calculation by an uptake of triclosan into study organisms. Diclofenac has previously shown to be degradable in sediments both *via* biodegradation (Gröning *et al.* 2007), see Figure 30, and via photolysis (Buser et al, 1998; Petrovic and Barcelo, 2007).

However, due to the position of the radioactive labelling (U-ring labelled) of diclofenac in this study, degradation of diclofenac might occur, although not be observed. The fate of fluoxetine in waters and water-sediment systems was investigated under laboratory conditions by Kwon and Armbrust (2006). No evidence of degradation, biotic or abiotic, was found.

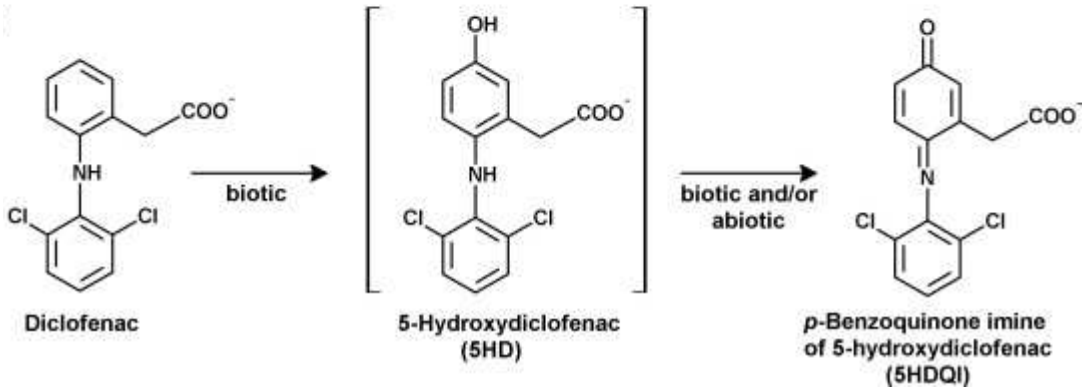


Figure 25. Suggested pathway for degradation of diclofenac in sediments. (Gröning *et al.* 2007)

Triclosan is known to be subject to photodegratdation (Singer *et al.* 2002; Aranami and Readman 2006; Buth *et al.* 2010). Half-lives of triclosan due to photodegradation was reported to be as low as 4 days (Aranami 2007). To avoid photodegradation of the study compounds, the study was performed in darkness. However, although all radioactivity from triclosan was recovered from mass balances, due to the position of the labelling (U-ring labelled) it is possible that some of the radioactivity recovered could derive from degradation products according to the figure below.

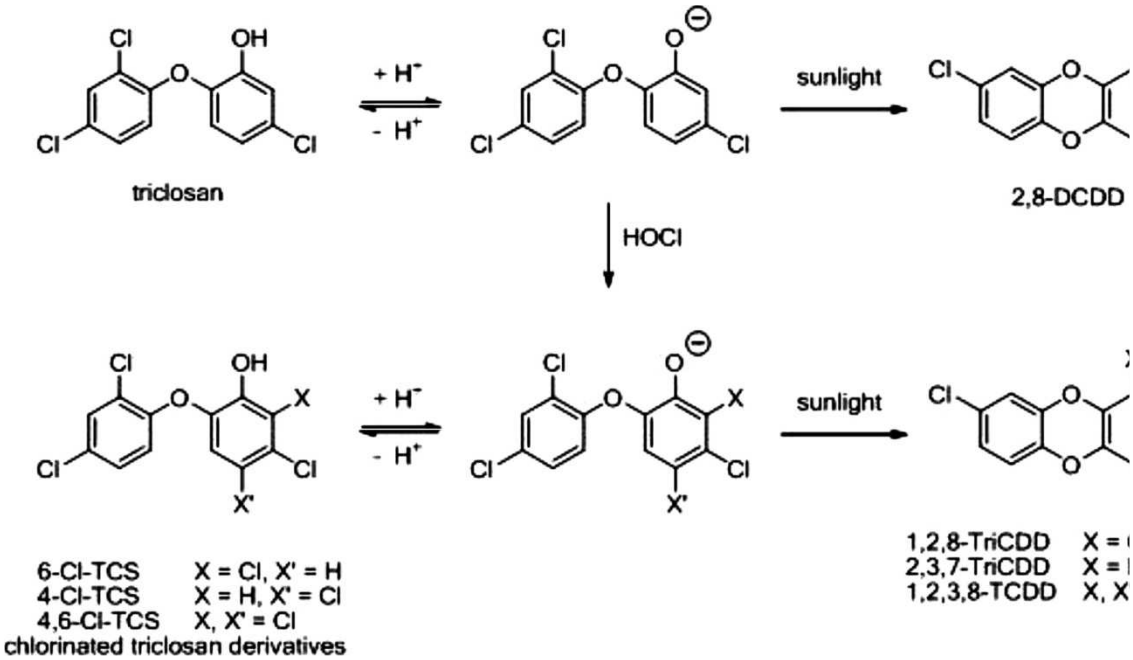


Figure 26. Chlorination and photochemical transformations of triclosan leading to polychlorinated dibenzo-p-dioxins (Buth *et al.* 2010).

The first order one compartment model was fitted to the uptake and depuration measurements with varying results. Overall, the model slightly underestimated the uptake and slightly overestimated the depuration of the study compounds (Figure 28-30). During the parameterisation for diclofenac, only a small number of accepted samples was generated which resulted in a coarse model fit. This phenomenon has previously been observed in Open Model for chemicals with a low uptake rate constants (e.g. caffeine, see Chapter 3). The resulting uptake and depuration parameters and the Biota Sediment Accumulation Factors are provided in Table 17.

The order of the biota sediment accumulation factor of the compounds was as follows: fluoxetine < diclofenac < triclosan. The BSAF ranged from 1.4 to 292 for the feeding worms and from 1.9 to 288 for the worms taken up study compounds only via the epidermis. Water and sediment concentrations, uptake and depuration rate constants and Biota Sediment Accumulation Factors, BSAF, are reported in Table 16. Although there are available data on uptake of PPCPs into aquatic organisms, not much work has been done using sediment organisms. To date, to the best of our knowledge, there is no data on the bioaccumulation of

diclofenac into sediment dwelling organisms. A BSAF of fluoxetine in the freshwater mussel *Elliptio complanata* has been measured at 4.5 in the effluent channel of a sewage treatment plant. The BSAF reported in the literature for fluoxetine are in line with the findings in this study.

Uptake of triclosan from sediments into aquatic organisms has been studied slightly more. Measured concentrations of triclosan in sediments and in a fresh water clam *Corbicula fluminea* resulted in BSAF (wet weight) of less than 1 (Edziyie, 2011). In addition, the BSAF (mass in tissue lipid OC / mass in sediment OC) of triclocarban, a similar compound was determined in *L. variegatus* to be 1.6 ± 0.6 (Higgins *et al.* 2009).. The values for triclosan are less than 2 orders of magnitude lower than the BSAF measured in this study. Possible explanations for the differences between the results of the current study and previous studies could be due to sediment characteristics, environmental pH (discussed in Chapter 1), metabolism of triclosan (discussed in Chapter 4) or degradation of triclosan into more lipophilic degradation products (see discussion above).

Table 16. Mean initial water and sediment concentrations, uptake and depuration parameters and BSAF values

Test Compound	Treatment	Water concentration (nmol/L)	Sediment concentration (nmol/kg)	Kin	Kout	BSAF (95% CI)
Diclofenac	Head	23.5	27.2	0.094	0.026	2.7 (2.1 - 3.0)
	No head	24.2	25.3	0.085	0.014	3.5 (3.2 - 3.9)
Fluoxetine	Head	5.3	736	0.044	0.002	1.4 (1.1 - 1.7)
	No head	5.6	759	0.031	0.002	1.9 (1.6 - 2.2)
Triclosan	Head	3.5	547	0.786	0.039	292 (248 - 356)
	No head	3.9	574	0.499	0.026	288 (245 - 363)

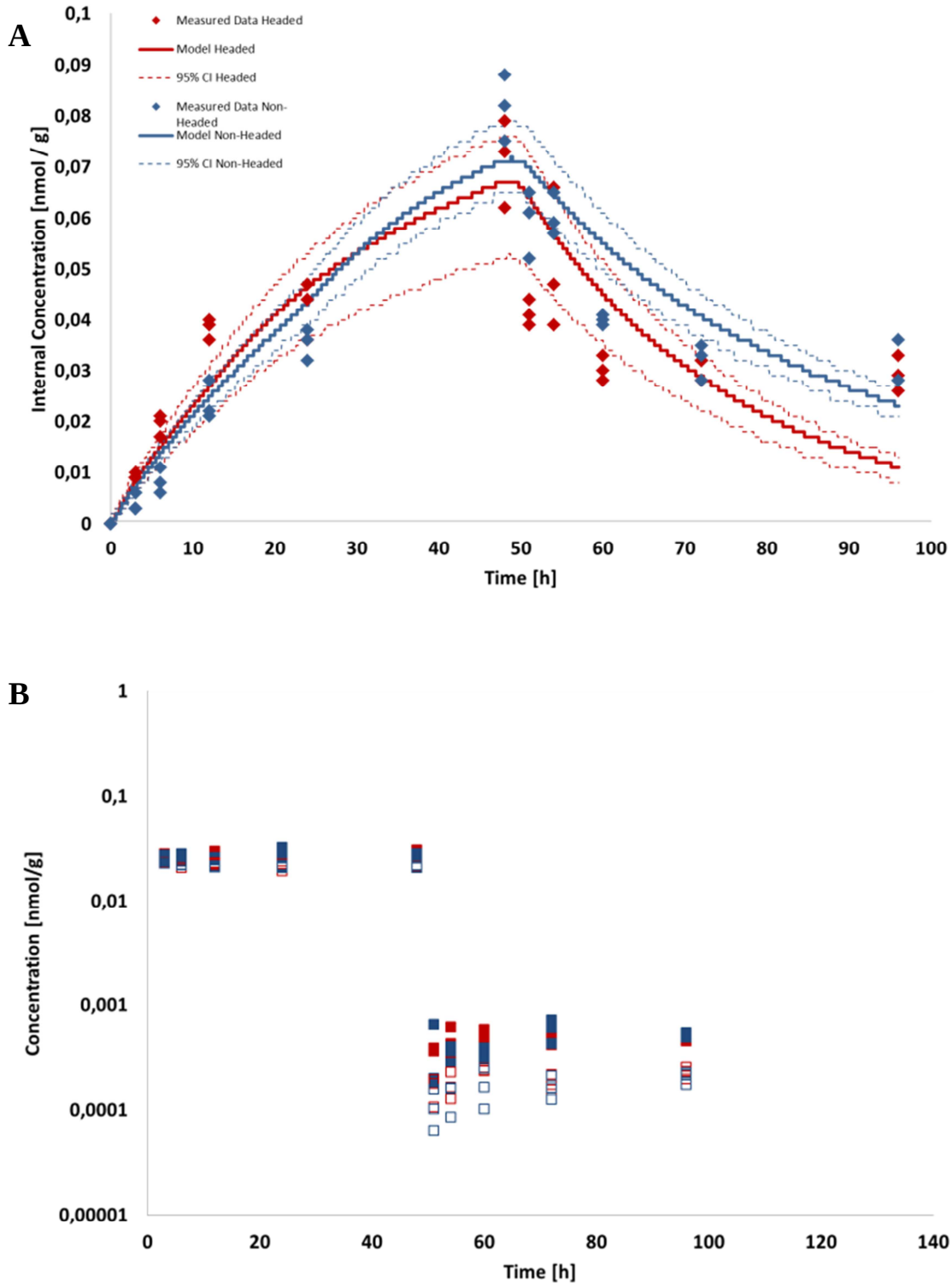


Figure 27. A- Uptake and depuration of diclofenac into *L. variegatus*. Red data represents feeding worms and blue data represents non-feeding worms. Solid line is the average model fit and broken lines are the 95% CI. B - Concentrations in sediment (filled squares) and water (open squares).

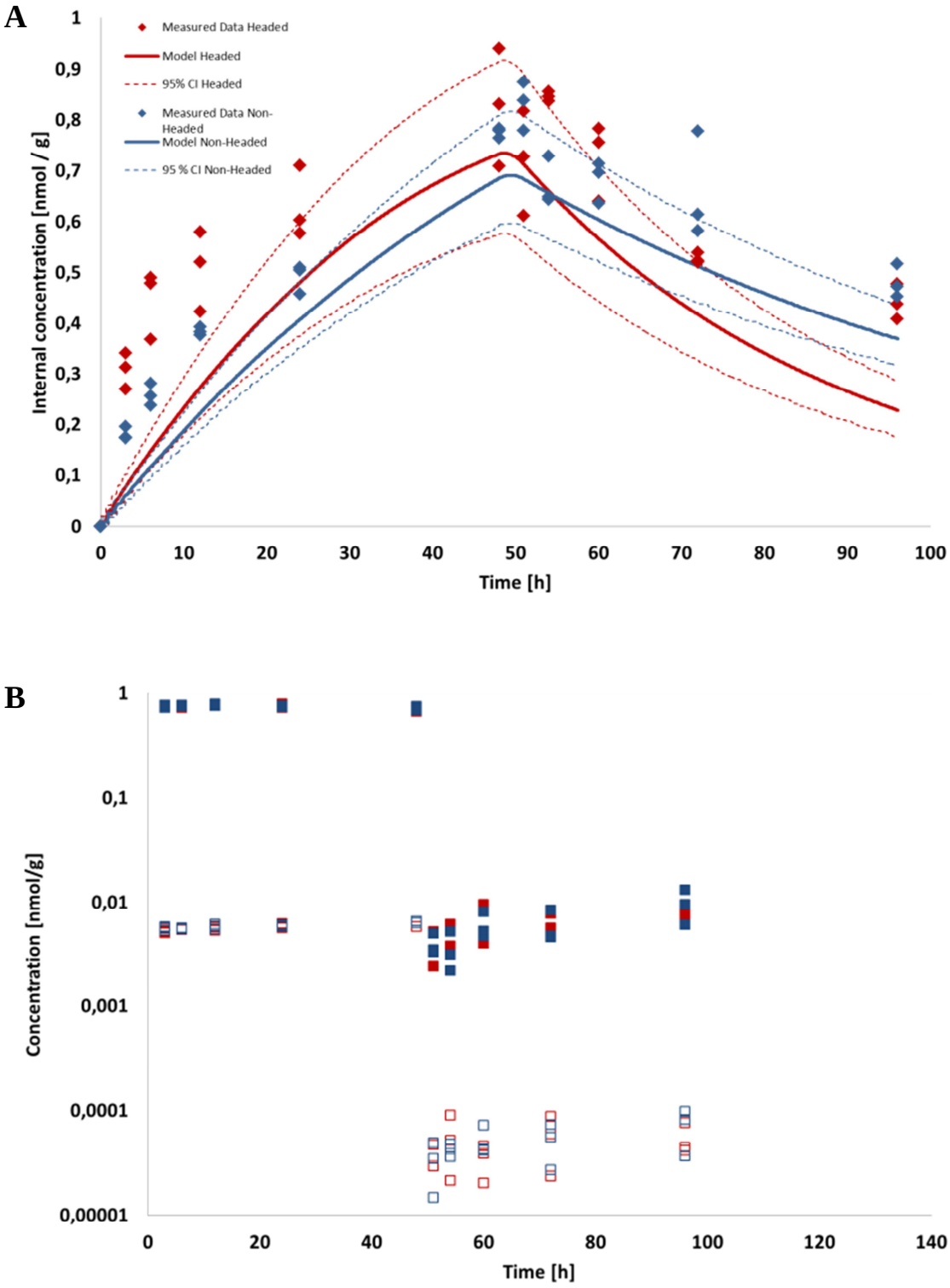


Figure 28A- Uptake and depuration of fluoxetine into *L. variegatus*. Red data represents feeding worms and blue data represents non-feeding worms. Solid line is the average model fit and broken lines are the 95% CI. B - Concentrations in sediment (filled squares) and water (open squares)

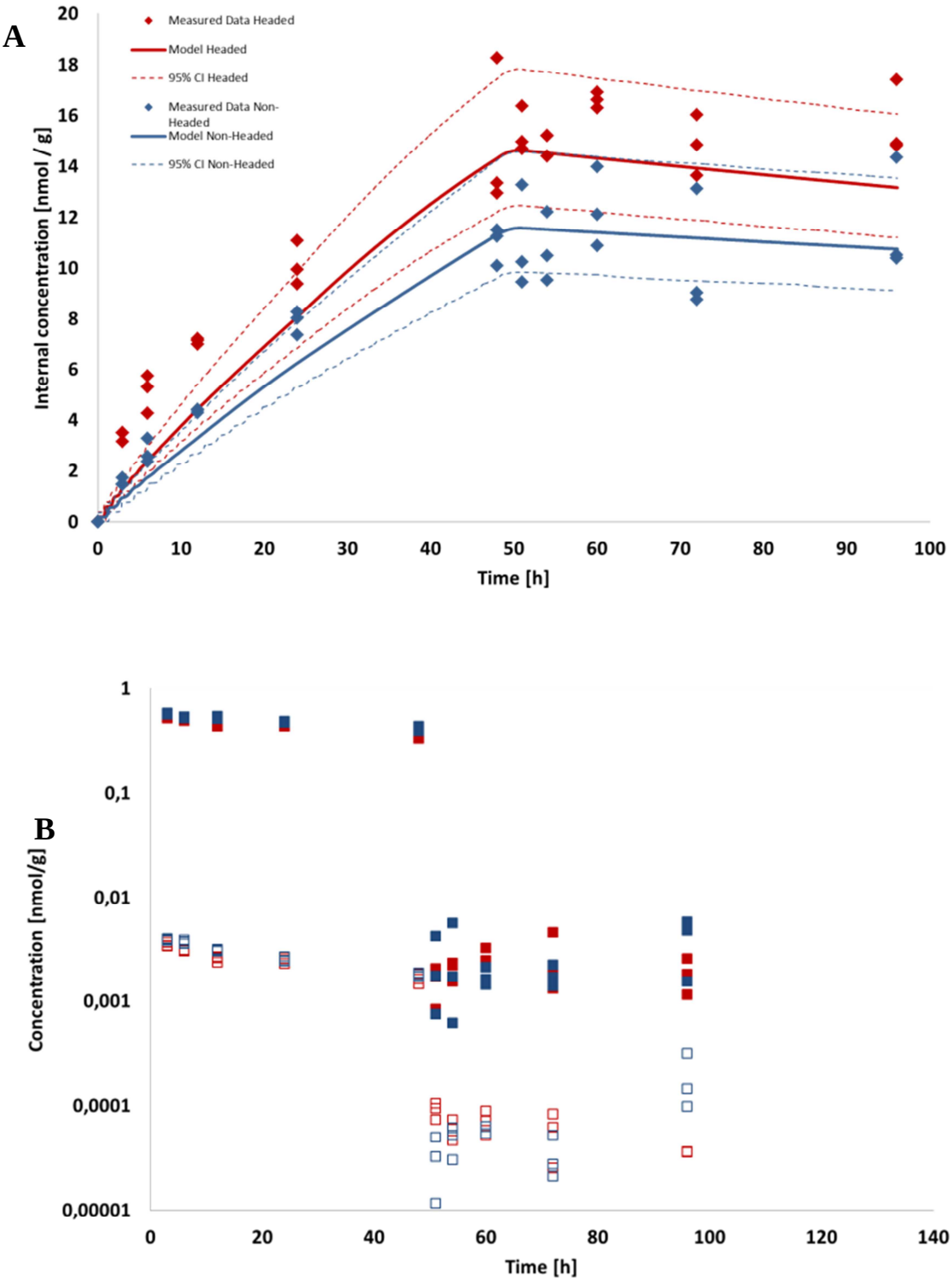


Figure 29A- Uptake and depuration of triclosan into *L. variegatus*. Red data represents feeding worms and blue data represents non-feeding worms. Solid line is the average model fit and broken lines are the 95% CI. B - Concentrations in sediment

5.3.3 The relative importance of feeding as an uptake route into *L. variegatus*

During the first 24 hours, the study compounds were generally taken up to a greater extent into the worms that bioaccumulated compounds both *via* the epidermis and *via* ingestion of contaminated sediment than in worms taking up the compound via the epidermis only. However, after the first 24 hours of the uptake phase and during the depuration phase, no statistically significant differences were observed between the worms ingesting sediment and the worms that did not. Consequently, there was no significant difference between the BSAF calculated for the non-feeding and feeding treatments. The BSAF for the worms taking up study compounds *via* the epidermis ranged from 1.9 to 288. The BSAF for the worms taking up study compounds both via the epidermis and ingestion of sediment ranged from 1.4 to 292

Hence, from these results in can be concluded that the uptake *via* ingestion of contaminated sediment is not of major importance for the uptake of the study compounds. This is in line with the results from Lu *et al* (2004) who concluded that the major uptake route of PAHs into sediment-dwelling oligochaetes is *via* the pore water. However, Comber *et al.* (2007) and Leppänen and Kukkonen (1998) obtained contradictory results and showed that uptake of pyrene *via* ingestion of sediment into *Lumbriculus variegatus* was the main uptake route.

Aging of sediment has previously been shown to have an effect on the bioavailability of sediment associated contaminants to sediment dwelling organisms. Leppänen *et al.* (2000) investigated the effects of sediment-chemical contact time on the bioaccumulation of two PAH into *L. variegatus*. The results were varying, the ratio of uptake via ingestion:uptake *via* epidermis increased for the more lipophilic compound (Benzo[a]pyrene) with an increasing sediment-chemical contact time whilst the ratio for the less lipophilic compound (pyrene) were stable. The authors suggested that these results were due to the bioavailability of the more lipophilic compound which changed to a greater extent due to sediment-chemical contact time than the less lipophilic compound. The author also concluded that the importance of feeding as a

route of bioaccumulation is dependent on sediment characteristics such as organic carbon and particle size distribution. These are all factors that might explain the lack of consistency among available data. In addition, feeding behaviour has also shown to have an impact on the bioaccumulation into sediment dwelling invertebrates. Gaskell *et al.* (2007) measured the bioaccumulation of DODMAC, a cationic surfactant, in four freshwater macroinvertebrates (*Asellus aquaticus*, *Chironomus riparius*, *Gammarus pulex*, *Lumbriculus variegatus*). *Chironomus* accumulated the DODMAC to the highest degree and the observed bioaccumulation pattern was *Chironomus* > *Gammarus* > *Asellus* = *Lumbriculus*. The results could not be explained only by gut passage time. Another study measured the bioaccumulation of PCB and PAH in three different marine species with different feeding behaviour, *Arenicola marina* which feeds by ingestion of sediments, *Macoma balthica*, a deposit feeder and *Mytilus edulis*, a filter feeder. The contaminants were accumulated as follows: *Arenicola* > *Macoma* > *Mytilus* and the study concluded the feeding does have an impact on the uptake behaviour (Kaag *et al.* 1997). Thus extrapolating results observed in this study to other sediment dwelling invertebrates is not recommended without knowledge on how the feeding behaviour affects the importance of feeding as a route of uptake into sediment dwelling organisms.

5.4 Conclusion

Determining the importance of different uptake routes into sediment dwelling organisms is a complicated task with many contributing factors affecting the uptake via feeding. The uptake of the three study chemicals into *L. variegatus* was shown to be mainly a result of the uptake *via* the epidermis. However, extrapolating in between different sediment types and different organisms with different feeding behaviour should be done cautiously. However, for the evaluation of the conceptual model of uptake of PPCPs into *L. variegatus* described in Chapter 1, feeding as a route of uptake appears to be negligible. In the next Chapter, this conceptual model will be evaluated using the results from Chapter 2-5.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 Introduction

Over the past 15 years that has been increasing interest in the occurrence, fate and effects of substances used as either pharmaceuticals or personal care products in the natural environment (Boxall *et al.*, 2012). A large amount of knowledge is now available on the levels of pharmaceuticals and personal care products in aquatic and terrestrial systems and their uptake and effects in aquatic and terrestrial organisms. A number of studies have explored the uptake, depuration and metabolism of pharmaceuticals and personal care products into aquatic and terrestrial organisms including plants (e.g. Boxall *et al.*, 2006; Dolliver *et al.*, 2007; Kumar *et al.*, 2005), earthworms (Kinney *et al.*, 2008), and in fish and aquatic invertebrates (Dussault *et al.*, 2009; Mimeault *et al.*, 2005; Nakamura *et al.*, 2008; Paterson and Metcalfe, 2008; Ramirez *et al.*, 2009; Rendal *et al.*, 2011; Meredith-Williams *et al.*, 2012). Studies with aquatic organisms have tended to focus on assessing uptake of substances from the water column and only limited work has been performed to understand the uptake of pharmaceuticals and personal care products from sediment; even though sediments have been shown to be a sink for selected compounds (Vazquez-Roig *et al.* 2010). The studies described in this thesis were therefore performed to develop an understanding of the potential for sediment-associated pharmaceuticals and personal care products to be taken up from sediments into benthic invertebrates. This Chapter begins with a brief summary of the findings of the different components of the thesis and then moves on to discuss the implications of the findings for understanding and managing the risks of pharmaceuticals and personal care products in the environment.

6.2 Key findings of the experimental chapters

A series of studies was performed to understand that fate and uptake of a range of substances used as pharmaceuticals or personal care products in sediment systems. In order that the study results could be applied more generally, study substances were selected to cover a range of physico-chemical properties and included acidic, neutral and basic substances. An overview of the results obtained for the study compounds is provided in Table 17.

It is generally recognised that an important route of uptake of many sediment-associated contaminants into benthic organisms occurs *via* the sediment pore water. Experimental studies therefore focused initially on understanding the distribution of pharmaceuticals between sediment and water and understanding uptake from the aqueous phase.

The sorption behaviour of eight of the study substances in sediment-water systems was explored using a batch equilibrium method (Chapter 2). Sorption was found to increase in the order diclofenac < chloramphenicol <salicyclic acid < naproxen < caffeine < sulfamethazine < triclosan < fluoxetine. Comparison of calculated K_{oc} values for the study compounds with a recommended trigger value (K_{oc} > 1000) for sediment risk assessment (Maund *et al.*, 1997) indicate that of the eight study compounds, only fluoxetine and triclosan are likely to be of concern in the sediment compartment.

Only limited data are available on the partitioning behaviour of compounds used as pharmaceuticals and personal care products in sediment-water systems. To experimentally assess the partitioning behaviour of all compounds used as pharmaceuticals or personal care products would be a mammoth task. Therefore, in Chapter 2, the sorption measurements for the study compounds were used to evaluate available predictive models for estimating sorption behaviour of organic compounds in sediments. Both 'traditional' models used for estimating sorption of neutral organic chemicals and models developed specifically for ionising substances were evaluated. None of the models tested were found to accurately

estimate sorption behaviour and in general models underestimated the sorption behaviour more frequently than they overestimated the sorption. Similar conclusions have been obtained in evaluation exercises for models for estimating sorption behaviour of pharmaceuticals in soils (Tolls, 2001). The mismatch between model predictions and sorption measurements probably reflects the complexity of the interactions that occur between ionisable compounds and sediment particles.

The uptake behaviour of six of the study compounds from the aqueous phase into *L. variegatus* was then explored. Bioconcentration factors (BCF) of the six substances ranged from 2 (chloramphenicol) to 700900 (triclosan). The very high BCF values for triclosan and naproxen resulted from extremely small depuration rates for these two substances. Comparison of the results with recommended trigger values for bioconcentration indicate that naproxen and triclosan would be classified as very bioaccumulative (ECHA, 2012). To try and explain the observed differences in the BCFs of the study compounds, the uptake data were compared to physic-chemical property data for the substances. Unlike, many other classes of organic contaminant, there was only a weak correlation between lipophilicity (Log K_{ow}) of the test substances and the BCF ($r^2 = 0.59$). Log D, which has been previously been suggested as a better descriptor for the fate and behaviour of ionisable (Nakamura *et al.*, 2008; Kim *et al.*, 2010; Valenti *et al.*, 2009; Meredith-Williams *et al.*, 2012), was also weakly correlated with uptake data ($r^2 = 0.50$).

The BCF results were also used to assess the performance of two models which had been specifically developed for estimating the uptake of ionising substances. Both of these models were found to underestimate the bioconcentration of the study compounds. One possible reason for the differences between the model predictions and measurements was that some of the study compounds were metabolised following uptake into the organism, this would not have been picked up in the radiolabelled uptake studies. In Chapter 4, the potential for selected study compounds to be metabolised was therefore assessed. The impact of environmental pH on uptake was also studied.

Table 17. Summary of sorption and uptake parameters for the study pharmaceuticals and personal care products

	Kd (l/kg)	Koc (l/kg)	BCF				BSAF
Compound			**APW**	**SW pH 5.5**	**SW pH 7.0**	**SW pH 8.5**	
Caffeine	27.7	113	-	1	1	1	-
Chloramphenicol	5.6	22.8	2	-	-	-	-
Diclofenac	2.34 – 4.2	17.1 - 42	60	623	30	8	2.7
Fluoxetine	423 – 1790	7303 - 7614	911	49	562	218500	1.4
Naproxen	13.4	547	72240	-	-	-	-
Salicyclic acid	11.4	46.5	82	-	-	-	-
Sulfamethazine	45.6	186	-	-	-	-	-
Triclosan	241.2 - 1528	4312 - 6234	700900	568400	646400	559300	292

Chapter 4 focused on three substances with similar lipophilicity but varying pKa values. As previous studied have indicated that pH changes can alter the physiology of an organism and affect uptake of neutral organic compounds (Wildi *et al.* 1994), caffeine was also employed as a neutral 'control' compound. There were distinct differences in the uptake of the weak acid diclofenac and the weak base fluoxetine at pH values of 5.5, 7 and 8.5 with BCF values differing by more than 2 (diclofenac) to 4 (fluoxetine) orders of magnitude across the pH range tested. These findings are significant as the pH range in natural waters across Europe is reported to range from 2.2 to 9.8 (FOREGS-EuroGeoSurveys Geochemical Baseline Database). Our data therefore indicate that there could be very large differences in the uptake (and also toxicity) of ionisable pharmaceuticals and personal care products across the European aquatic landscape.

Results from the studies in Chapter, where pH was much more tightly controlled than in Chapter 3, were correlated with uptake predictions obtained using the model of Neuwoehner and Escher (2011). However, uptake of triclosan was greatly underestimated by the model. Metabolism has previous been suggested as reason for unexpectedly high bioconcentrations (Aschauer *et al.* 2012). Significant metabolism of triclosan was confirmed by a simplified study using non-labelled substances ran parallel to the uptake and depuration study. While no attempt was made to characterise the transformation products of triclosan, previous studies have indicated that this compound can be metabolised to conjugates (glucuronidated triclosan and triclosan sulphate) and to hydroxyl triclosan, catechol and 2,4-dichlorophenol (Fang *et al.*, 2010). While the parent compound may have been metabolised in the worms, it is important not to overlook the potential risks of these transformation products as previous studies have demonstrated that transformation products of synthetic compounds can sometimes be more toxic than the parent compound and exhibit different fate characteristics (Sinclair and Boxall, 2003; Boxall *et al.*, 2004).

In Chapter 5, uptake of three of the study compounds from natural sediments was explored. The biota sediment accumulation factors for diclofenac and fluoxetine were low. The low BSAF for

fluoxetine, which had a high bioconcentration factor for water-based exposure, resulted from the compound having a high sediment sorption coefficient – uptake into the organism was offset by sorption to the sediment solids. Triclosan had a BSAF of around 300 indicating that this substance (and/or its metabolites) would be bioconcentrated from sediment into benthic organisms.

Chapter 5 also explored the relative importance of different routes of uptake from sediment into benthic organisms. Both feeding and non-feeding worms were exposed to diclofenac, fluoxetine and triclosan contaminated sediments and uptake and depuration was measured over 96 h. It was concluded that for diclofenac and fluoxetine, there was no difference in uptake in non-feeding worms and feeding worms. For triclosan, feeding worms accumulated slightly more of the study compound than the non-feeding organisms (although not statistically significant). These findings agree with previous studies where neutral compounds with log Kow values < 5, have been shown to be primarily accumulated from pore water (Thomann *et al.* 1992, Belfroid *et al.* 1995). For more hydrophobic compounds, the contribution of ingested material in accumulation may be more important (Landrum *et al.* 1989) so it is possible that feeding could be an important uptake route for other substances used as pharmaceuticals or in personal care products.

In the next sections, we discuss the implications of the findings of the work described in this thesis for the assessment and management of the risks of pharmaceuticals and personal care products in the natural environment.

6.3 Implications for regulatory assessment of uptake of pharmaceuticals and personal care products into benthic organisms

An assessment of the risks of sediment associated contaminants is required by many regulatory risk assessment schemes (e.g. REACH). In instances where experimental data are not available the use of the Equilibrium Partitioning approach is recommended (Di Toro *et al.*, 1991). In this approach, either measured sorption coefficients and bioconcentration factors or estimated sorption coefficients and bioconcentration factors (estimated based on Log K_{ow} values) are used alongside ecotoxicity data for pelagic organisms to estimate toxicity to benthic species. However, it is debatable whether this approach can be applied to ionisable substances (Di Toro *et al.*, 1991).

In the following section, we therefore use the data generated in the different Chapters in this thesis to evaluate whether it is possible to estimate uptake of ionisable substances into benthic organisms based on uptake data from aqueous exposures and batch sorption studies using the conceptual model described in Chapter 1 (Figure 31). To evaluate the model, estimations of tissue concentration over time are compared to uptake measurements from the whole sediment study.

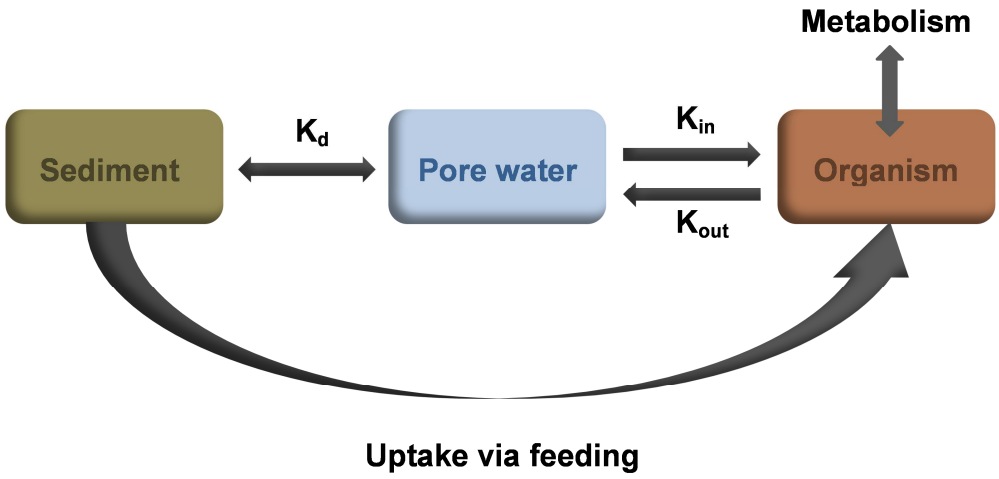


Figure 30 A conceptual model for estimating uptake into sediment dwelling organisms (repeated from Chapter 1).

For the model simulations we assumed that uptake *via* feeding is not important based on the fact that diclofenac and fluoxetine appeared to be only accumulated *via* the sediment pore water and that feeding only had a small effect on the uptake of triclosan (Chapter 5). As information was not available on rates of metabolism or on the concentrations of parent compounds and metabolites over time in the uptake studies, we used a whole residue approach (i.e. parent compound and metabolites are considered together).

For the modelling, concentrations in the sediment pore water were estimated from measured whole sediment concentrations using Equation 27. To estimate concentrations in the organism over time from the pore water concentrations, the one compartment first order model described in Chapter 3 was used. The modelling was performed using ModelMaker version 4.0 (developed by Cherwell Scientific Ltd).

$$BSAF_{modelled} = \frac{\frac{C_{sediment}}{K_d} \cdot \frac{K_{in}}{K_{out}}}{C_{sediment}} \qquad \text{Eq. 27}$$

The model was run using measured sediment concentrations of the study compounds (Chapter 5) and the sediment sorption coefficients for Sediment 2 which was the sediment used in the whole-sediment uptake studies (Chapter 2). Uptake and depuration rate constants for diclofenac and fluoxetine were estimated at pH 7.67 (the pH of the sediment used in the whole-sediment uptake studies) using the relationships between rate constants and pH described in Chapter 4 (Equations 28 and 29). As pH had a limited effect on the uptake and depuration of triclosan and the depuration of diclofenac and fluoxetine, a mean of the K_{in} and K_{out} values measured at pH 5.5, 7 and 8.5 was taken. A summary of the uptake and depuration rate constants used for the evaluation of the modelling is shown in Table19.

$$Kin_{DCF} = 61527 \cdot e^{-1.472 \cdot x} \quad \text{Eq. 28}$$

$$Kin_{FLX} = 0.0005 \cdot e^{1.2868 \cdot x} \quad \text{Eq. 29}$$

Table 19. Uptake and depuration rate constants used for the evaluation of the sediment uptake model.

Compound	**K_{in}**	**K_{out}**
Diclofenac	0.769	0.046
Fluoxetine	9.67	0.0071
Triclosan	49.6	0.000093

Figures 32-34. Modeled internal concentrations for fluoxetine and diclofenac agreed well with measured concentrations (Figures 32 and 33). For triclosan, however, modelled concentrations in the worms were lower than measured concentrations.

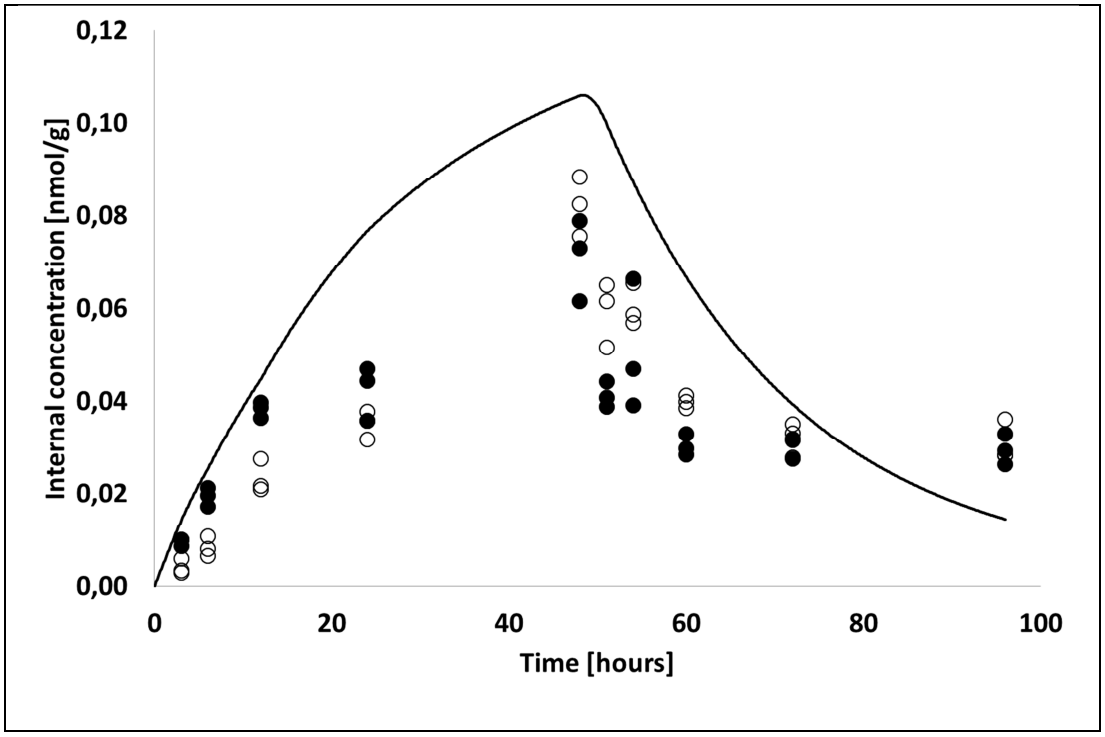


Figure 31 Modeled concentrations in comparison with measured concentrations of diclofenac over 96 hours. Smooth line is the modeled concentration whilst filled circles represent measured data. Empty circles represent non-feeding worms.

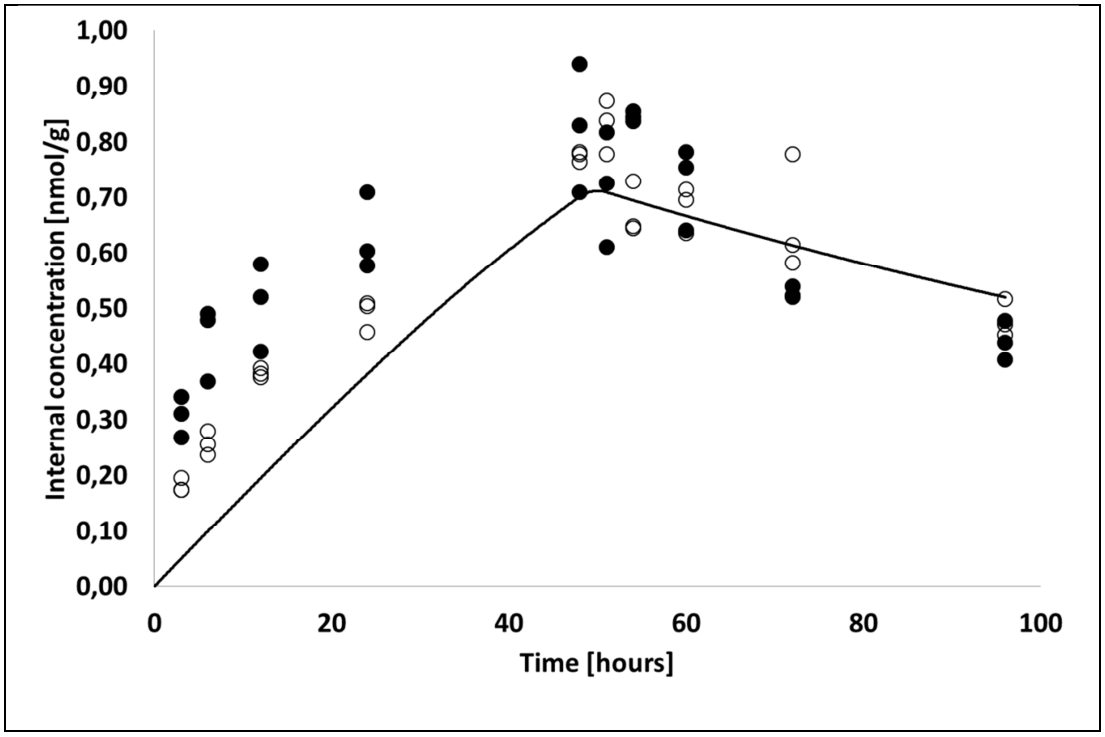


Figure 32 Modeled concentrations in comparison with measured concentrations of fluoxetine over 96 hours. Smooth line is the modeled concentration whilst filled circles represent measured data. Empty circles represent non-feeding worms.

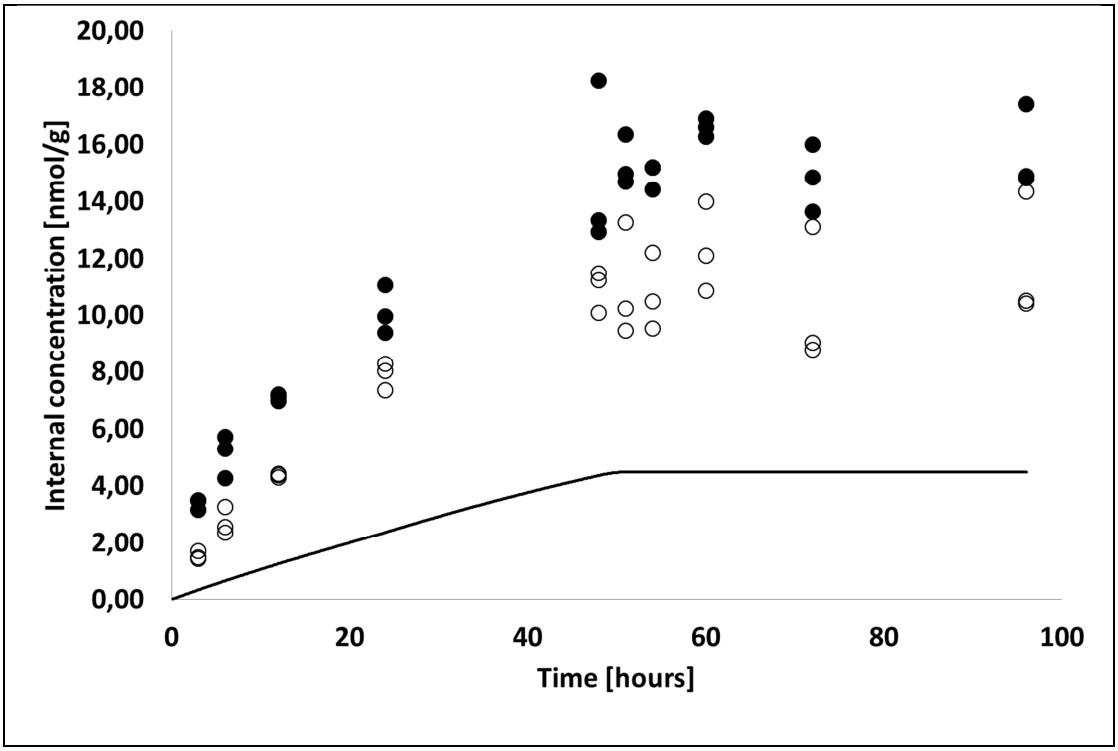


Figure 33 Modeled concentrations in comparison with measured concentrations of triclosan over 96 hours. Smooth line is the modeled concentration whilst filled circles represent measured data. Empty circles represent non-feeding worms.

In addition to a comparison of modelled and measured internal concentrations over time, BSAF values were also calculated for the feeding worms. This is because small variations in the uptake and depuration rates can have large implications for the BSAF at steady state. Modelled BSAFs for diclofenac and fluoxetine agreed well with values derived from the whole sediment studies (Table 20). For triclosan, the modelled BSAF was approximately one order of magnitude larger than the measured BSAF even though uptake over 96 h was underestimated by the model.

Table 20. BSAF values obtained using the model and derived from whole sediment studies.Substance

	C_{Sed} (nmol/kg)	K_d	C_{Water} (nmol/L)	BCF[a]	BSAF (Eq Part)	BSAF
Diclofenac	27.2	4.2	6.47	16.8	4.0	2.7
Fluoxetine	736	422.5	1.74	1 355	3.2	1.4
Triclosan	547	241.2	2.27	535470	2 220	292

A possible explanation for the large difference in BSAF for triclosan might be the increased depuration that was observed in the presence of sediment compared to the depuration in a water only system (Chapter 3 and 4). It is possible that in the presence of sediment where organic matter is present, the body burden over prolonged periods would be smaller due to a larger sorption of the chemical to organic matter which facilitates the depuration out of the test organism based on a fugacity model approach. McCarthy (1983) observed a reduction in the uptake and accumulation of PAH in D. magna with 97 % in the presence on natural organic matter in the form of humic acids.

While there was disagreement between model predictions and experimental observations for triclosan, the results for diclofenac and fluoxetine are very encouraging. These data indicate that if information is available on the sorption behaviour of an ionisable substance in sediment and on the relationships between pH and uptake and depuration from the water phase, it is possible to estimate internal exposure to a fair degree of accuracy. In the future, the model described here could be used to assess the spatial risks of substances such as diclofenac and fluoxetine to benthic organisms across the European landscape.

6.4 Implications for existing models for estimating sorption and bioconcentration

Many regulatory risk assessment schemes recommend the use of quantitative structure-activity relationships(QSARs) and quantitative structure-property relationships (QSPRs) for estimating the fate, uptake and toxicity of a chemical in the environment. For pharmaceuticals, for example, if experimental data are not available, the European Medicines Agency indicate that bioconcentration and soprtion can be predicted based on chemical properties (EMEA, 2006). The log Kow is typically used as the chemical descriptor of hydrophobicity in QSARs and

QSPRs for sorption, bioaccumulation and toxicity (Schwarzenbach *et al.* 2003; Nendza and Russom 1991; Hansch and Leo 1995) in many regulatory frameworks. The results of the current study clearly show that K_{ow}-based models are inappropriate for estimating the environmental properties and effects of ionisable substances such as many pharmaceuticals and personal care products.

The findings of this study also show that even models developed specifically for estimating the fate and effects of ionisable compounds (e.g. Trapp and Horobin , 2005; Franco *et al.*, 2008; Neuwoehner and Escher, 2011) may not perform well for compounds such as pharmaceuticals and personal care products. More work is therefore needed to develop and parameterise models for estimating the fate properties and uptake of ionisable compounds.

6.4 Implications for ecotoxicity testing of pharmaceuticals and personal care products

In Chapter 4, a clear negative correlation between the bioconcentration of the weak acid diclofenac and pH was observed as well as a positive correlation between the bioconcentration of the weak base fluoxetine and pH. Bioconcentration factors at the different pH values tested were found to vary by more than two orders of magnitude for diclofenac and four orders of magnitude for fluoxetine. These effects of pH on are not only likely to affect the uptake of the compounds but also the toxicity to organisms in the environment. Other studies also have demonstrated the importance of pH in determining the ecotoxicity of ionisable compounds. Both Nakamura *et al.* (2008) and Neuwoehner and Escher (2011) explained the pH dependant toxicity of fluoxetine to fish and green algae respectively based on differences in the uptake behaviour at different environmental pH values. In Japanese medaka the difference in toxicity ranged between 0.2 and 5.5 mg/L, a factor of > 25 at pH 7 and 9 respectively (Nakamura *et al.* 2008).

However, pH effects on toxicity are not currently considered in standard regulatory risk assessment schemes for organic compounds. Ecotoxicity tests are typically performed in standard media whose pH values range from 6.2 (OECD algal media) to 7.98 (EPA soft water)(Park *et al.*, submit). Therefore, when assessing the risks of an ionisable compound based on standard ecotoxicity test data, e.g. using the guideline on environmental risk assessment for medicinal products for human use (EMEA, 2006), there is a possibility that the risks to aquatic organisms could be greatly over- or underestimated. In the future, it may be appropriate to assess toxicity at environmentally relevant pH values where greatest uptake into an organism is expected. For example, for a weak base like fluoxetine, it may be appropriate to test at higher pH values (e.g. 8.5) whereas for a weak acid such as diclofenac, it may be appropriate to test at a lower pH value (e.g. 5.5).

The findings of this study also demonstrate the need to consider environmental metabolites of a substance in the risk assessment process. The importance of environmental metabolites has also been shown by Ashauer *et al.* (2012). Most pharmaceuticals are readily metabolized and/or excreted by humans, the reason why they have to be taken regularly (Jakoby *et al.* 1990). There is very little information available of the metabolism of pharmaceuticals in aquatic invertebrates, however it is rather well established that many of the detoxification enzyme responsible for the metabolism of e.g. pharmaceuticals in mammals are also present in fish (Goksør and Förlin, 1991). These metabolites could well affect organisms in the environment so it is important that their toxicity is established.

6.5 Conclusions

This study is one of the first to explore the factors and processes affecting the uptake of substances used as pharmaceuticals or in personal care products into benthic organisms. The

results show that distribution between sediment solids and pore water varies for different compounds. Differences are also seen in uptake from pore water into lumbricid worms. Unlike neutral organic chemicals, the differences in behaviour and uptake cannot be explained by a compounds' lipophilicity. Available models for predicting fate and uptake of ionisable compounds also do not appear to be at an advanced enough state to accurately predict the partitioning behaviour and uptake of pharmaceuticals and personal care products in aquatic organisms. Studies into the effects of pH on uptake demonstrate that this parameter is very important in determining the degree of uptake of some pharmaceuticals and personal care products and show that bioconcentration factors can differ by up to more than four orders of magnitude across a range of environmentally relevant pH values. Evaluation of a sediment uptake model demonstrates that for selected pharmaceuticals and personal care products, by combining information on uptake and depuration rate constants from water-only studies and on sorption behaviour from batch experiments, it is possible to estimate uptake into benthic organisms over time. The results of the model predictions are very promising and indicate that it may be possible to apply an approach, analogous to the equilibrium partitioning method, in the environmental risk assessment process for pharmaceuticals, personal care products and other ionisable compounds.

6.4 Recommendations for further research

While the work described in this thesis has generated novel information on the fate and uptake of compounds used as pharmaceuticals or personal care products in sediment environments, it also highlights a number of areas where future research is needed. In the future work should focus on the following aspects:

Development of new methods for estimating the sorption behaviour of ionisable compounds in sediments – This thesis has demonstrated that currently available models for estimating sorption of organic chemicals perform poorly for estimating sorption of pharmaceuticals and personal care products in sediment systems. There is a need to develop improved methods for estimating the sorption behaviour of these compounds to sediments which take into account the different interactions that occur between ionisable compounds and sediment solids. These relationships should be developed to also account for changes in sediment properties.

Development of new approaches to estimate the uptake of ionisable compounds in aquatic organisms – While models are available for estimating uptake of ionisable compounds into aquatic organisms, these did not perform well for the study compounds and *L. variegatus*. Future work should focus on understanding the mechanisms of uptake of ionisable organic compounds (both passive and active) as well as the metabolism of compounds within organisms with a view to developing and parameterising improved models for a range of species with different traits. These models should be able to account for the effects of differences in environmental parameters (e.g. pH) on uptake.

Understanding the formation and effects of metabolites of pharmaceuticals and personal care products – Data for triclosan indicate that this compound is extensively metabolised in the worms. Future uptake studies should ideally employ methods that allow the differentiation between parent compounds and transformation products of the parent compound (e.g. Radio HPLC, LC-MS, GC-MS) as well as the identification of metabolites (e.g. TOF-MS). Consideration should be given to assessing the hazard of any major metabolites and approaches developed to allow the risks of parent compound/metabolite mixtures to be assessed.

Understanding the linkages between internal residues of pharmaceuticals and personal care products and toxicity – This thesis has focused on uptake as an endpoint. In order to use this

information to understand risks in the environment, information is needed on the relationship between internal concentrations and effects (e.g. through the critical body residue approach). Questions that require answering include: do differences in uptake of an ionisable compound at different pH values correlate with differences in toxicity? and how do benthic organisms vary in terms of their sensitivity to exposure to an ionisable compound?

By addressing these issues, in the future it should be possible to develop a much better understanding of the overall risks of an ionisable compound across broad landscapes.

Appendix 1

Table 1. Mass balance calculations at 0-48 h from uptake study presented in Chapter 3.

	Timepoint (h)	Test compound	Concentration	Volume test	Sum recovery	Mean test	% of spiked
Naproxen	0	0	10.8	40	430.255		
	0	0	9.1	40	364.715	405.695	100
	0	0	10.6	40	422.115		
	3	5.6	11.9	40	479.8391667		
	3	5.4	11.39	40	458.8448333	468.4945	115
	3	4.8	11.59	40	466.7995		
	6	7.9	10.8	40	440.0726667		
	6	12.2	11.9	40	488.415	454.8419444	112
	6	6.2	10.7	40	436.0381667		
	12	11.5	11.675375	40	478.49		
	12	10.4	11.20370833	40	458.553	472.2323333	116
	12	12.1	11.68904167	40	479.654		
	24	19.5	10.010875	40	419.9176667		
	24	22.9	10.225375	40	431.8723333	445.421	110
	24	27.3	11.42954167	40	484.473		
	48	29.7	9.106375	40	393.9241667		
	48	35.7	10.31604167	40	448.3775	439.1561111	108
	48	44.5	10.76654167	40	475.1666667		
Fluoxetine	0	0	18.92675594	40	757.0702376		
	0	0	19.64754167	40	785.9016667	771.5045236	100
	0	0	19.28854167	40	771.5416667		
	3	25.75941667	18.70970833	40	774.14775		
	3	17.64841667	18.12304167	40	742.5700833	761.2859722	99
	3	19.06508333	18.701875	40	767.1400833		
	6	45.54841667	18.220875	40	774.3834167		
	6	49.32725	17.21754167	40	738.0289167	754.8048611	98
	6	57.12725	17.371875	40	752.00225		
	12	77.30941667	16.98304167	40	756.6310833		

	Timepoint (h)	Test compound	Concentration	Volume test	Sum recovery	Mean test	% of spiked
	12	0.00	10.76	40.00	430.26		97
	12	0.00	9.12	40.00	364.72	405.70	
	24	0.00	10.55	40.00	422.12		
	24	5.63	11.86	40.00	479.84		95
	24	5.35	11.34	40.00	458.84	468.49	
	48	4.78	11.55	40.00	466.80		
	48	7.90	10.80	40.00	440.07		91
	48	12.15	11.91	40.00	488.42	454.84	
	0	6.20	10.75	40.00	436.04		
	0	11.48	11.68	40.00	478.49		100
	0	10.40	11.20	40.00	458.55	472.23	
	3	12.09	11.69	40.00	479.65		
	3	19.48	10.01	40.00	419.92		91
	3	22.86	10.23	40.00	431.87	445.42	
	6	27.29	11.43	40.00	484.47		
Triclosan	6	29.67	9.11	40.00	393.92		91
	6	35.74	10.32	40.00	448.38	439.16	
	12	44.51	10.77	40.00	475.17		
	12	0.00	18.93	40.00	757.07		87
	12	0.00	19.65	40.00	785.90	771.50	
	24	0.00	19.29	40.00	771.54		
	24	25.76	18.71	40.00	774.15		100
	24	17.65	18.12	40.00	742.57	761.29	
	0	19.07	18.70	40.00	767.14		
	0	45.55	18.22	40.00	774.38		100
	0	49.33	17.22	40.00	738.03	754.80	
	3	57.13	17.37	40.00	752.00		
	3	77.31	16.98	40.00	756.63		101
Chloramphenicol	3	76.75	17.04	40.00	758.52	746.40	
	6	66.07	16.45	40.00	724.05		
	6	106.86	15.71	40.00	735.12		99
	6	112.77	15.51	40.00	733.03	734.32	
	12	111.58	15.58	40.00	734.82		
	12	105.68	15.64	40.00	731.40		100
	12	120.33	14.92	40.00	717.12	698.42	

	Timepoint (h)	Test compound	Concentration	Volume test	Sum recovery	Mean test	% of spiked
	24	101.59	13.63	40.00	646.74		
	24	0.00	12.64	40.00	505.58		102
	24	0.00	11.62	40.00	464.96	494.72	
	48	0.00	12.84	40.00	513.62		
	48	33.29	10.01	40.00	433.70		100
	48	57.22	10.83	40.00	490.34	450.99	
	0	47.42	9.54	40.00	428.94		
	0	140.75	7.76	40.00	451.17		100
	0	126.51	8.80	40.00	478.32	451.58	
	3	100.90	8.11	40.00	425.24		
	3	121.52	5.88	40.00	356.90		98
	3	155.38	8.86	40.00	509.62	428.35	
	6	148.68	6.75	40.00	418.52		
	6	299.76	5.22	40.00	508.64		77
Salicylic acid	6	254.71	6.09	40.00	498.22	495.47	
	12	270.15	5.24	40.00	479.56		
	12			40.00	0.00		69
	12			40.00	0.00	0.00	
	24			40.00	0.00		
	24	0.00	14.12	40.00	564.67		62
	24	0.00	14.46	40.00	578.39	569.62	
	48	0.00	14.15	40.00	565.82		
	48	0.23	14.60	40.00	584.40		55
	48	0.14	13.77	40.00	551.02	573.06	
	0	0.14	14.59	40.00	583.76		
	0	0.19	14.41	40.00	576.60		100
	0	0.22	13.97	40.00	559.07	562.05	
	3	0.24	13.76	40.00	550.50		
	3	0.35	14.02	40.00	561.08		85
Diclofenac	3	0.33	14.40	40.00	576.19	570.78	
	6	0.50	14.36	40.00	575.06		
	6	0.54	14.83	40.00	593.55		101
	6	0.35	14.18	40.00	567.49	579.15	
	12	0.52	14.40	40.00	576.40		
	12	0.72	14.35	40.00	574.59		102

	Timepoint (h)	Test compound	Concentration	Volume test	Sum recovery	Mean test	% of spiked
	12	0.80	13.92	40.00	557.66	569.88	
	24	0.81	14.41	40.00	577.39		
	24	0.00	14.31	40.00	572.31		103
	24	0.00	13.99	40.00	559.42	567.66	
	48	0.00	14.28	40.00	571.24		
	48	0.37	13.57	40.00	542.98		99
	48	0.24	14.10	40.00	564.04	558.79	

Table 2. Mass balance calculations at 0-48 h from uptake study presented in Chapter 4.

	pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
Diclofenac	5.5	0	0.00	26.96	40.00	1078.35		
		0	0.00	27.52	40.00	1100.96	1093.31	**100**
		0	0.00	27.52	40.00	1100.63		
		3	62.35	25.65	40.00	1088.51		
		3	79.38	24.60	40.00	1063.40	1084.34	**99**
		3	64.48	25.92	40.00	1101.12		
		6	115.60	24.80	40.00	1107.43		
		6	141.19	24.35	40.00	1115.13	1113.37	**102**
		6	161.39	23.90	40.00	1117.56		
		12	244.58	21.84	40.00	1117.99		
		12	245.01	21.01	40.00	1085.45	1108.62	**101**
		12	293.51	20.72	40.00	1122.44		
		24	377.32	17.75	40.00	1087.24		
		24	240.17	20.75	40.00	1070.09	1091.03	**100**
		24	395.77	18.00	40.00	1115.77		
		48	490.29	13.39	40.00	1026.05		
		48	340.41	18.23	40.00	1069.52	1075.10	**98**
		48	335.30	19.86	40.00	1129.74		
Diclofenac	7	0	0.00	29.74	40.00	1189.73		
		0	0.00	29.85	40.00	1193.96	1149.78	**100**
		0	0.00	26.64	40.00	1065.65		
		3	7.49	27.22	40.00	1096.29		
		3	8.82	27.82	40.00	1121.60	1110.50	**97**
		3	8.98	27.62	40.00	1113.62		
		6	15.78	27.93	40.00	1133.11		
		6	15.91	28.55	40.00	1157.72	1145.79	**100**
		6	12.37	28.35	40.00	1146.54		
		12	21.39	27.10	40.00	1105.20		
		12	21.72	27.49	40.00	1121.30	1129.94	**98**
		12	20.87	28.56	40.00	1163.34		
		24	26.88	26.16	40.00	1073.13		
		24	25.47	28.63	40.00	1170.71	1124.36	**98**
		24	24.60	27.62	40.00	1129.24		
		48	34.21	27.67	40.00	1141.17		

	pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
		48	23.99	29.78	40.00	1215.29	1172.87	**102**
		48	36.59	28.14	40.00	1162.14		
		0	0.00	29.57	40.00	1182.77		
		0	0.00	27.92	40.00	1116.97	1139.93	**100**
		0	0.00	28.00	40.00	1120.06		
		3	1.46	28.35	40.00	1135.38		
		3	1.27	28.49	40.00	1140.74	1150.20	**101**
		3	1.08	29.34	40.00	1174.48		
		6	1.12	29.77	40.00	1191.78		
		6	1.87	29.11	40.00	1166.43	1168.85	**103**
		6	1.15	28.68	40.00	1148.34		
Diclofenac	8.5	12	1.95	26.40	40.00	1057.89		
		12	1.63	28.73	40.00	1150.72	1107.53	**97**
		12	1.91	27.80	40.00	1113.99		
		24	3.55	27.65	40.00	1109.59		
		24	2.32	27.88	40.00	1117.66	1129.74	**99**
		24	2.39	28.99	40.00	1161.96		
		48	7.66	27.03	40.00	1088.72		
		48	8.34	25.52	40.00	1029.16	1059.70	**93**
		48	6.07	26.38	40.00	1061.21		
		0	0.00	15.67	40.00	626.95		
		0	0.00	14.40	40.00	576.00	606.79	**100**
		0	0.00	15.44	40.00	617.42		
		3	1.97	13.65	40.00	547.83		
		3	2.88	16.16	40.00	649.37	612.38	**101**
		3	1.21	15.97	40.00	639.96		
		6	7.31	17.05	40.00	689.16		
Fluoxetine	5.5	6	3.36	17.25	40.00	693.24	687.11	**113**
		6	5.83	16.83	40.00	678.94		
		12	5.85	17.09	40.00	689.46		
		12	8.65	17.06	40.00	690.91	693.83	**114**
		12	11.10	17.25	40.00	701.11		
		24	9.10	17.53	40.00	710.35		
		24	9.18	15.77	40.00	640.04	694.20	**114**
		24	14.45	17.94	40.00	732.23		

	pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
		48	12.32	11.78	40.00	483.70		
		48	14.76	14.98	40.00	613.83	567.05	**93**
		48	14.17	14.74	40.00	603.62		
		0	0.00	14.27	40.00	570.72		
		0	0.00	16.05	40.00	641.85	638.63	**100**
		0	0.00	17.58	40.00	703.32		
		3	19.13	17.09	40.00	702.73		
		3	21.38	16.86	40.00	695.69	703.50	**110**
		3	23.37	17.22	40.00	712.09		
		6	47.21	15.39	40.00	662.63		
		6	38.89	15.40	40.00	654.74	657.51	**103**
		6	31.84	15.58	40.00	655.16		
Fluoxetine	7	12	62.21	14.89	40.00	657.64		
		12	68.96	14.69	40.00	656.60	663.69	**104**
		12	28.03	16.22	40.00	676.84		
		24	112.59	13.45	40.00	650.43		
		24	130.55	13.86	40.00	684.96	665.15	**104**
		24	100.01	14.00	40.00	660.05		
		48	126.84	10.80	40.00	558.70		
		48	151.86	13.36	40.00	686.07	675.09	**106**
		48	129.12	16.28	40.00	780.50		
		0	0.00	16.22	40.00	648.92		
		0	0.00	16.51	40.00	660.37	665.72	**100**
		0	0.00	17.20	40.00	687.86		
		3	37.35	14.97	40.00	636.03		
		3	64.37	14.14	40.00	629.93	649.46	**98**
		3	95.26	14.68	40.00	682.41		
		6	168.41	11.68	40.00	635.55		
Fluoxetine	8.5	6	164.33	12.82	40.00	677.24	668.60	**100**
		6	176.70	12.91	40.00	693.02		
		12	303.24	10.13	40.00	708.62		
		12	234.24	10.83	40.00	667.44	659.01	**99**
		12	183.99	10.42	40.00	600.95		
		24	299.00	7.27	40.00	589.68		
		24	333.74	9.38	40.00	708.83	656.14	**99**

	pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
		24	303.66	9.16	40.00	669.92		
		48	341.62	4.13	40.00	506.96		
		48	421.99	5.10	40.00	626.04	590.29	**89**
		48	411.55	5.66	40.00	637.86		
		0	0.00	27.59	40.00	1103.74		
		0	0.00	25.51	40.00	1020.54	1076.24	**100**
		0	0.00	27.61	40.00	1104.44		
		3	181.74	24.21	40.00	1150.10		
		3	147.15	23.41	40.00	1083.38	1140.36	**106**
		3	231.79	23.90	40.00	1187.61		
		6	285.26	21.23	40.00	1134.33		
		6	369.64	20.92	40.00	1206.32	1162.10	**108**
		6	364.31	19.53	40.00	1145.66		
Triclosan	5.5	12	655.34	12.09	40.00	1138.88		
		12	571.41	13.59	40.00	1114.96	1138.51	**106**
		12	634.91	13.17	40.00	1161.70		
		24	879.83	7.06	40.00	1162.39		
		24	845.17	6.81	40.00	1117.69	1146.09	**106**
		24	872.65	7.14	40.00	1158.20		
		48	1036.72	3.17	40.00	1163.47		
		48	1013.47	2.77	40.00	1124.33	1111.61	**103**
		48	919.00	3.20	40.00	1047.03		
		0	0.00	28.83	40.00	1153.13		
		0	0.00	28.94	40.00	1157.46	1155.97	**100**
		0	0.00	28.93	40.00	1157.32		
		3	288.76	22.07	40.00	1171.59		
		3	322.68	20.75	40.00	1152.61	1170.56	**101**
		3	215.36	24.30	40.00	1187.47		
Triclosan	7	6	253.45	22.44	40.00	1151.14		
		6	395.63	20.61	40.00	1219.87	1183.43	**102**
		6	289.08	22.25	40.00	1179.28		
		12	795.15	10.47	40.00	1214.14		
		12	737.48	9.80	40.00	1129.52	1168.89	**101**
		12	643.08	13.00	40.00	1163.01		
		24	845.88	7.09	40.00	1129.36		

	pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
		24	961.64	4.58	40.00	1144.78	1143.31	**99**
		24	910.08	6.14	40.00	1155.77		
		48	1011.08	2.00	40.00	1090.97		
		48	979.29	4.31	40.00	1151.54	1133.66	**98**
		48	1083.74	1.87	40.00	1158.47		
		0	0.00	29.07	40.00	1162.94		
		0	0.00	29.33	40.00	1173.23	1194.02	**100**
		0	0.00	31.15	40.00	1245.90		
		3	205.11	25.34	40.00	1218.54		
		3	269.43	22.14	40.00	1154.99	1204.16	**101**
		3	138.37	27.51	40.00	1238.95		
		6	369.13	20.87	40.00	1203.93		
		6	235.69	23.54	40.00	1177.36	1167.05	**98**
		6	283.43	20.91	40.00	1119.85		
Triclosan	8.5	12	734.37	10.89	40.00	1169.80		
		12	685.61	13.30	40.00	1217.70	1191.02	**100**
		12	526.53	16.48	40.00	1185.55		
		24	761.62	11.86	40.00	1235.90		
		24	803.55	11.62	40.00	1268.30	1215.40	**102**
		24	736.31	10.14	40.00	1142.02		
		48	1080.64	2.43	40.00	1177.80		
		48	968.28	3.72	40.00	1117.26	1175.58	**98**
		48	1110.01	3.04	40.00	1231.66		
		0	0.00	106.71	40.00	4268.50		
		0	0.00	113.09	40.00	4523.57	4328.90	**100**
		0	0.00	104.87	40.00	4194.65		
		3	3.44	104.34	40.00	4177.07		
		3	5.48	102.58	40.00	4108.88	4165.50	**96**
		3	3.38	105.18	40.00	4210.55		
Caffeine	5.5	6	5.40	100.02	40.00	4006.28		
		6	6.05	109.32	40.00	4378.85	4169.05	**96**
		6	5.49	102.91	40.00	4122.02		
		12	4.36	98.13	40.00	3929.66		
		12	5.29	113.13	40.00	4530.38	4218.18	**97**
		12	5.29	104.73	40.00	4194.50		

	pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
		24	4.78	106.66	40.00	4271.37		
		24	6.58	102.21	40.00	4094.96	4180.08	**97**
		24	5.18	104.22	40.00	4173.90		
		48	7.16	101.09	40.00	4050.71		
		48	4.93	102.89	40.00	4120.58	4197.94	**97**
		48	4.39	110.45	40.00	4422.52		
		0	0.00	102.16	40.00	4086.56		
		0	0.00	101.58	40.00	4063.16	4074.01	**100**
		0	0.00	101.81	40.00	4072.31		
		3	4.85	96.96	40.00	3883.26		
		3	3.95	106.39	40.00	4259.57	4166.67	**102**
		3	4.65	108.81	40.00	4357.19		
		6	4.72	101.68	40.00	4071.81		
		6	5.21	101.88	40.00	4080.41	4096.09	**101**
		6	4.09	103.30	40.00	4136.04		
Caffeine	7	12	4.45	104.68	40.00	4191.60		
		12	6.26	106.78	40.00	4277.28	4347.85	**107**
		12	4.19	114.26	40.00	4574.67		
		24	6.09	101.88	40.00	4081.12		
		24	5.44	102.35	40.00	4099.47	4220.30	**104**
		24	7.02	111.83	40.00	4480.31		
		48	7.93	93.17	40.00	3734.77		
		48	8.75	102.57	40.00	4111.49	4020.55	**99**
		48	9.97	105.14	40.00	4215.38		
		0	0.00	100.53	40.00	4021.31		
		0	0.00	100.03	40.00	4001.03	4071.56	**100**
		0	0.00	104.81	40.00	4192.36		
		3	4.64	108.39	40.00	4340.20		
		3	4.02	104.95	40.00	4202.03	4153.67	**102**
Caffeine	8.5	3	3.59	97.88	40.00	3918.78		
		6	4.16	107.40	40.00	4300.25		
		6	3.77	101.07	40.00	4046.76	4148.80	**102**
		6	4.58	102.37	40.00	4099.39		
		12	4.53	103.86	40.00	4158.86		
		12	4.77	106.03	40.00	4246.15	4184.02	**103**

pH	Time	Test compound in	Concentration test	Volume test	Sum recovery	Mean test	% of
	12	4.89	103.55	40.00	4147.04		
	24	6.30	106.30	40.00	4258.26		
	24	4.56	102.62	40.00	4109.53	4169.21	**102**
	24	6.20	103.34	40.00	4139.84		
	48	8.60	94.57	40.00	3791.33		
	48	6.21	93.23	40.00	3735.28	3827.71	**94**
	48	4.24	98.81	40.00	3956.51		

Table 3. Mass balance calculations at 0-48 h from uptake study presented in Chapter 5.

Head	Time-point	Test compound	Concentration	Volume test	Bq /ml	Sum recovery	Mean test	% of
	0	0	50.07	20.00		1001.42		
	0	0	53.09	20.00		1061.72	1029.03	**100**
	0	0	51.20	20.00		1023.97		
	3	0.54	53.08	15.14	268.63	1072.99		
	3	0.72	55.53	15.15	269.85	1111.96	1092.79	**106**
	3	0.76	54.96	15.13	260.92	1093.43		
	6	1.62	48.65	15.15	255.43	994.08		
	6	1.30	55.39	15.15	279.75	1119.90	1057.28	**103**
	6	1.09	51.45	15.14	277.84	1057.86		
Diclofenac y	12	3.09	52.73	15.13	277.07	1077.93		
	12	2.95	54.19	15.14	282.63	1105.98	1082.76	**105**
	12	2.40	51.42	15.14	283.59	1064.37		
	24	3.54	52.72	15.13	287.02	1088.35		
	24	3.62	52.77	15.15	296.46	1099.65	1044.19	**101**
	24	3.02	44.85	15.13	263.10	944.56		
	48	4.70	49.37	15.13	291.95	1043.43		
	48	5.36	50.25	15.14	288.86	1055.10	1054.93	**103**
	48	4.25	50.04	15.13	305.00	1066.27		
	0	0.00	50.07	20.00	0.00	1001.42		
	0	0.00	53.09	20.00	0.00	1061.72	1029.03	**100**
	0	0.00	51.20	20.00	0.00	1023.97		
	3	0.22	59.42	15.13	278.82	1177.77		
	3	0.33	53.32	15.12	251.85	1058.54	1109.14	**108**
	3	0.15	55.60	15.14	249.02	1091.12		
	6	0.71	55.21	15.15	278.27	1115.15		
Diclofenac n	6	0.45	51.76	15.15	258.38	1042.74	1061.69	**103**
	6	0.75	50.94	15.15	254.90	1027.18		
	12	1.12	50.38	15.15	278.34	1042.43		
	12	1.71	48.71	15.14	264.89	1003.94	1015.87	**99**
	12	1.53	49.08	15.14	256.61	1001.25		
	24	2.38	51.38	15.15	270.73	1051.35		
	24	1.84	48.44	15.13	263.88	998.55	1033.06	**100**
	24	2.56	49.64	15.13	295.81	1049.29		

Head	Time-point	Test compound	Concentration	Volume test	Bq /ml	Sum recovery	Mean test	% of
	48	3.85	48.91	15.15	286.44	1031.11		
	48	4.91	48.45	15.14	286.49	1024.77	1044.86	**102**
	48	5.68	51.10	15.14	299.38	1078.70		
	0	0.00	304.74	15.00	0.00	4571.03		
	0	0.00	362.30	15.00	0.00	5434.53	5098.12	**100**
	0	0.00	352.59	15.00	0.00	5288.80		
	3	27.00	11.43	15.01	4524.06	4722.51		
	3	27.33	10.31	15.01	4566.44	4748.47	4738.29	**93**
	3	30.57	10.67	15.00	4553.18	4743.89		
	6	41.06	11.06	15.00	4611.72	4818.60		
	6	45.72	11.20	15.01	4617.99	4831.80	4787.60	**94**
	6	33.82	11.05	15.01	4512.74	4712.40		
Fluoxetine y	12	54.58	11.75	15.00	4715.71	4946.59		
	12	45.26	11.09	15.01	4782.11	4993.87	4945.88	**97**
	12	45.48	10.89	15.00	4688.29	4897.19		
	24	49.52	12.71	15.00	4763.02	5003.27		
	24	59.39	11.48	15.01	4507.42	4739.06	4952.72	**97**
	24	86.23	12.08	15.00	4848.46	5115.84		
	48	82.15	11.83	15.00	4421.59	4681.15		
	48	68.56	11.84	15.00	4450.65	4696.80	4588.69	**90**
	48	89.90	11.87	15.00	4120.10	4388.11		
	0	0.00	304.74	15.00	0.00	4571.03		
	0	0.00	362.30	15.00	0.00	5434.53	5098.12	**100**
	0	0.00	352.59	15.00	0.00	5288.80		
	3	22.08	11.81	14.99	4724.02	4923.21		
	3	20.52	11.56	15.01	4525.24	4719.21	4862.49	**95**
	3	18.61	10.90	15.00	4762.87	4945.05		
	6	35.37	11.15	14.99	4686.31	4888.82		
Fluoxetine n	6	33.31	11.26	15.01	4702.74	4904.98	4882.23	**96**
	6	33.63	11.48	15.00	4647.12	4852.90		
	12	46.27	12.07	15.00	4842.31	5069.59		
	12	41.04	11.30	15.00	4702.19	4912.75	4998.55	**98**
	12	35.66	12.49	15.00	4790.29	5013.32		
	24	78.83	12.22	15.00	4562.02	4824.17		
	24	67.98	12.33	15.01	4706.87	4959.79	4899.39	**96**

Head	Time-point	Test compound	Concentration	Volume test	Bq /ml	Sum recovery	Mean test	% of
	24	67.48	11.80	15.00	4669.73	4914.22		
	48	98.16	13.51	14.99	4439.04	4739.76		
	48	87.20	12.82	14.99	4559.03	4838.40	4725.53	**93**
	48	92.08	13.44	15.01	4304.69	4598.42		
	0	0.00	324.83	15.00	0.00	4872.38		
	0	0.00	322.30	15.00	0.00	4834.45	4807.27	**100**
	0	0.00	314.33	15.00	0.00	4714.98		
	3	526.37	8.97	16.14	4091.92	4763.02		
	3	601.42	8.36	16.14	4011.70	4748.08	4700.90	**98**
	3	550.65	8.22	16.16	3908.05	4591.61		
	6	870.56	7.41	16.15	3818.84	4809.04		
	6	843.16	7.63	16.15	3851.46	4817.84	4747.80	**99**
	6	870.65	7.42	16.15	3626.09	4616.51		
Triclosan y	12	1247.69	6.51	16.16	3562.35	4915.26		
	12	1403.40	5.75	16.16	3264.33	4760.73	4856.02	**101**
	12	1392.15	6.32	16.15	3397.80	4892.08		
	24	1475.95	6.00	16.14	3195.53	4768.38		
	24	1419.35	5.58	16.15	3332.28	4841.82	4814.27	**100**
	24	1248.38	6.24	16.16	3483.38	4832.61		
	48	2257.59	3.98	16.15	2503.09	4824.95		
	48	2332.85	3.55	16.16	2501.00	4891.22	4887.02	**102**
	48	2007.04	4.47	16.14	2865.77	4944.90		
	0	0.00	324.83	15.00	0.00	4872.38		
	0	0.00	322.30	15.00	0.00	4834.45	4807.27	**100**
	0	0.00	314.33	15.00	0.00	4714.98		
	3	232.83	9.69	16.16	4195.88	4585.23		
	3	246.84	9.37	16.14	4158.43	4556.59	4587.14	**95**
	3	161.46	9.55	16.14	4303.89	4619.59		
Triclosan n	6	417.13	9.08	16.16	3978.63	4542.44		
	6	616.24	9.43	16.16	3899.26	4667.79	4584.20	**95**
	6	409.74	8.79	16.15	3990.70	4542.38		
	12	529.66	7.51	16.16	3889.35	4540.45		
	12	736.51	7.41	16.16	3850.63	4706.89	4658.35	**97**
	12	622.35	7.75	16.14	3980.22	4727.70		
	24	1013.88	6.52	16.14	3545.35	4664.42		

Head	Time-point	Test compound	Concentration	Volume test	Bq /ml	Sum recovery	Mean test	% of
	24	1236.32	5.99	16.14	3577.29	4910.28	4805.78	**100**
	24	1241.17	5.95	16.16	3505.31	4842.64		
	48	1849.99	4.57	16.14	3205.38	5129.20		
	48	1727.95	4.39	16.15	2921.95	4720.82	4965.17	**103**
	48	1853.78	4.18	16.14	3124.21	5045.48		

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