Uptake and metabolism of pharmaceuticals in aquatic invertebrates

Melanie Jane Netherton

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Abstract

This thesis explored the uptake into the freshwater shrimp (*Gammarus pulex*) and the water boatman (*Notonecta glauca*) of key pharmaceuticals drawn from different therapeutic classes and covering a range of physico-chemical properties. For one compound, uptake was also assessed using the freshwater snail *Planobarius corneus*. In *G. pulex*, bioconcentration factors (BCFs) ranged from 4.6 – 185900 and increased in the order moclobemide < 5-fluoruracil < carbamazepine < diazepam < carvedilol < fluoxetine. In *N. glauca* BCFs ranged from 0.1 – 1.6 and increased in the order 5-fluorouracil < carbamazepine < moclobemide < diazepam < fluoxetine < carvedilol. For *P. corneus*, the BCF for carvedilol was 57.3.

The metabolism of the study pharmaceuticals in the shrimp was investigated. Diazepam was found to be metabolized by *G. pulex* and a metabolite was detected and tentatively identified as nordiazepam. For the other five study compounds no metabolites were observed and it was inferred that metabolism in *G. pulex* may not influence the BCF.

The influence of dietary uptake was explored in the test organisms with carvedilol and fluoxetine. It was found that uptake from water was the predominant route of exposure for *G. pulex* but the data for *N. glauca* was contrasting and the exposure from the food was predominant. In both organisms a combination of food and water exposure resulted in a higher uptake of the compounds. The differences in degree of uptake from water across the organisms may be due to differences in mode of respiration, behaviour and the pH of the test system. The differences in degree of uptake from food across the organisms may be due to differences in feeding strategies. The degree of uptake of pharmaceuticals within an organism was related to the hydrophobicity of the pharmaceuticals.

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This PhD is for my Dad

Authors Declaration

All work in thesis has been largely carried out by the author. Laura Carter (Fera) assisted with the sampling *Planorbarius corneus* in Chapter 2 section 2.2.5 as part of a BSc. dissertation. Eleanor Riches (Waters Corporation) assisted with method development for the UPLC –QTof-MS in Chapter 3. Chapters 2 to 4 have been written as papers for international peer-reviewed journals. The current publication status of the papers is presented in Table 0.1. All these papers have been reworked so that they can be presented in a consistent style and format in this thesis. For those papers that are published, copyright rests with the publishers.

Table 0.1 Status on the 30th of January 2012 of the papers presented in this thesis with respect to the publication process

Chapter	Title	Journal	Status
2	Uptake of Pharmaceuticals in Aquatic Invertebrates	Environmental Pollution	Accepted
3	The Metabolism of Pharmaceuticals in Aquatic Organisms	-	In prep
4	Uptake of pharmaceuticals through aquatic food chains	-	In Prep

Chapter 1 Uptake of pharmaceuticals in the environment: An introduction

1.1 Introduction

Pharmaceuticals are used extensively worldwide in therapeutics in human and veterinary medicine. They are a large and diverse group of chemicals with over 3000 active ingredients being used today with different physico-chemical properties and modes of action (Ayscough *et al*., 2000, Beausse, 2004, Richman and Castensson, 2008). Pharmaceutical substances can be used for treating or preventing disease and include antibiotics, anti-inflammatories, anti-epileptics, cardiovascular drugs, contraceptive hormones, lipid regulators and painkillers. Due to their extensive use, human and animal pharmaceuticals have been frequently detected in the natural environment and over the past two decades have become an important issue for environmental scientists and regulatory agencies (Kolpin *et al*., 2002, Kolpin *et al*., 2004, Crane *et al*., 2006, Kümmerer, 2010). This chapter provides an overview of the current published literature regarding the inputs to and fate of pharmaceuticals in the environment and discusses the available information on the uptake of pharmaceuticals in aquatic and terrestrial organisms and the factors and processes that influence uptake and accumulation. The Chapter also identifies major knowledge gaps for future research.

Human-use pharmaceuticals are released into the environment in emissions from the processes involved in manufacturing, in Sewage Treatment Works (STW) effluent and sewage sludge and by the disposal of expired or unwanted pharmaceuticals in household waste or down drains (Kümmerer, 2004; Figure 1.1). Veterinary pharmaceuticals enter the environment directly from excretion of faeces or urine onto pasture, the application of manure to agricultural land or from the waste water and surface run-off from farming facilities (Boxall *et al*., 2006, Sarmah *et al*., 2006, Fisher and Scott, 2008, Cunningham *et al*., 2010).

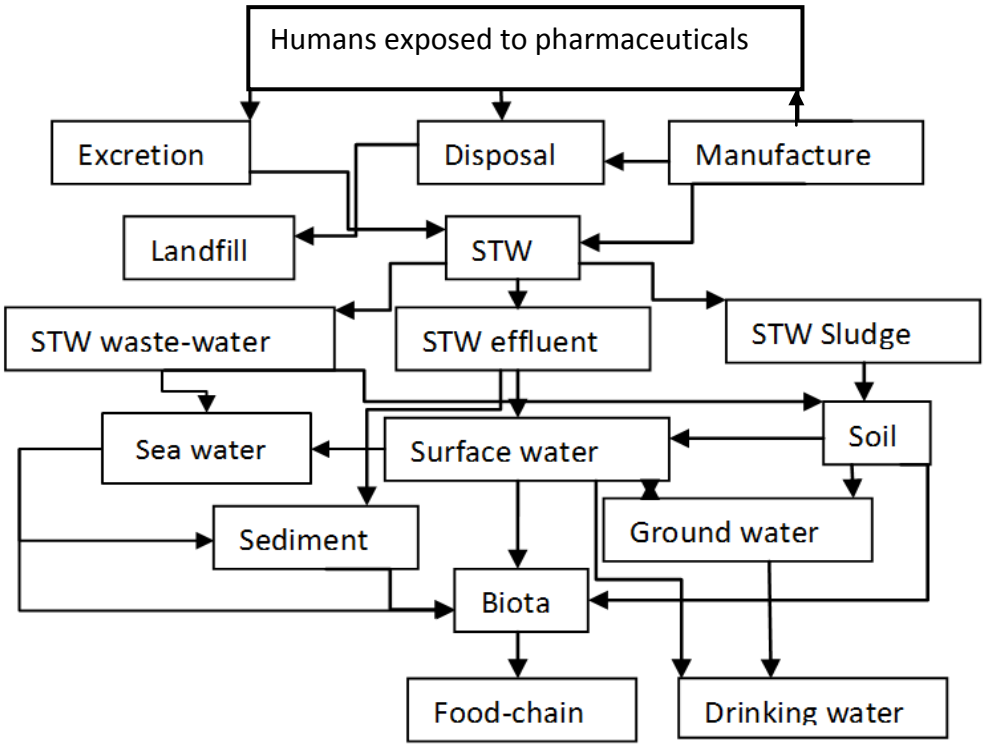


Figure 1.1 Pathways of human pharmaceuticals into the aquatic environment

STWs are thought to be an important source of pharmaceutical input into both the aquatic and terrestrial environments. STWs treat the wastewater from both the community and medical centres such as hospitals; which are thought to be a continual source of high concentrations of pharmaceuticals (Wilkinson, 2001, Kümmerer and Schuster, 2008). Many pharmaceuticals entering STWs may be largely removed from the effluent by microbial degradation or sorption to sludge and later transferred to fields as fertilizer. For example amoxicillin can be removed by up to 75 – 100 % depending on the season (Castiglioni *et al*., 2005). By contrast pharmaceuticals such as carbamazepine and diclofenac are removed to a lesser extent (< 10 % and 17 % removal respectively; Heberer, 2002). Removal by the STW may depend on the physicochemical properties of the pharmaceutical and may not be fully removed by the STW. Some of the more soluble substances may remain in STW effluents and sorptive pharmaceuticals may become associated with sewage sludge (Khan and Ongerth, 2002, Díaz-Cruz and Barceló, 2005, Jones-Lepp and Stevens, 2007, Radjenović *et al*., 2009, McClellan and Halden, 2010). Reported STW removal efficiencies can vary greatly (Carballa *et al*., 2004, Choi *et*

al., 2008, Ying *et al*., 2009, Monteiro and Boxall, 2010, Plósz *et al*., 2010) and the removal efficiency of the STW is reflected in the concentration that can be found in the sewage effluent which is released into fresh water. For a summary of the maximum concentrations in fresh waters near sewage effluent outflows the reader is referred to figure 1.2. The range of concentrations can be found in the original publication Monteiro and Boxall (2010).

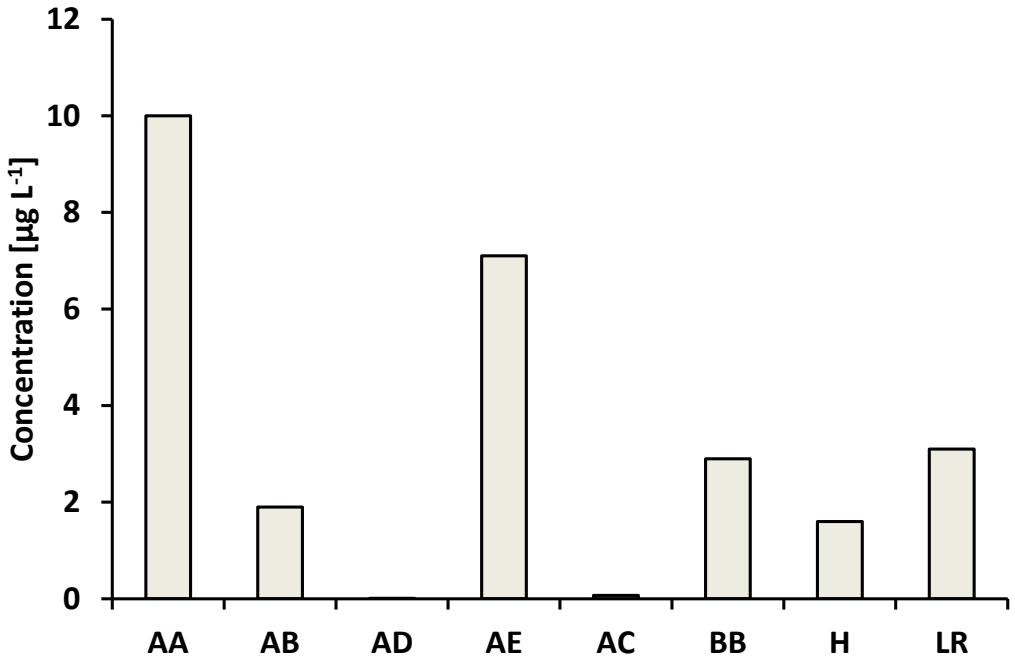


Figure 1.2 Maximum measured concentrations of pharmaceuticals; analgesics and anti-inflammatories (AA), antibacterials (AB), anti-depressants (AD) anti-epileptics (AE) anti-cancer (AC) beta-blockers (BB), hormones (H) and lipid regulators (LR) reported in fresh waters globally between 1985 and 2005 (n=3-43); for further information refer to Monteiro and Boxall (2010)

Appreciable amounts of wastewater from STWs are applied to land during irrigation and as a result pharmaceuticals can be deposited into the soil environment. A study in Germany showed that there were substantial amounts of pharmaceuticals, including antibiotics, beta-blockers and anti-epileptics released onto fields during irrigation with STW effluent (Ternes *et al*., 2007, Durán-Alvarez *et al*., 2009). Once applied to land, pharmaceuticals can penetrate soils to a depth of 30 cm and pharmaceuticals such as carbamazepine, acetaminophen and warfarin can

accumulate in the soil over time (Kinney *et al*., 2006, Xu *et al*., 2009). Pharmaceuticals such as ibuprofen have the potential to move down the soil profile with percolating water and therefore have the potential to impact ground water (Xu *et al*., 2009). The more hydrophobic pharmaceutical compounds such as the hormone 17α-ethinylestradiol tend to be absorbed into lipid fractions of the soil particles or associate with the organic matter via non-ionic van der Waals interactions (Ternes *et al*., 2004). Sorptive pharmaceuticals can enter the soil environment from the land application of manure from treated animals, the irrigation of land with STW effluent or from the inappropriate disposal of expired or unwanted pharmaceuticals to landfill (Holm *et al*., 1995, Boxall *et al*., 2002, Carlsson *et al*., 2006, Ternes *et al*., 2007, Schmitt and Römbke, 2008, Topp *et al*., 2008). Antibiotics residues have been detected in the soil environment at concentrations in the range of µg up to g kg^{-1} corresponding to concentrations found for pesticides (Thiele-Bruhn, 2003). Pharmaceuticals deposited in the soil compartment can be transported to fresh waters after rainfall events in surface runoff (e.g. Topp *et al*., 2008).

There is now a large body of data available that show significant environmental concentrations of pharmaceuticals in soils and fresh waters. This may be due to the recent development of sensitive and powerful analytical techniques has enabled detection and monitoring of pharmaceuticals in environmental systems (Ankley *et al*., 2007, Quinn *et al*., 2008). Several monitoring studies have taken place and pharmaceuticals have been observed in STW effluents, fresh waters (both freshwater and marine), groundwaters and in drinking water (Ternes, 1998, Zuccato *et al*., 2000, Kolpin *et al*., 2002, Ferrari *et al*., 2003, Ashton *et al*., 2004). Concentrations in the aquatic environment are reported to be in the nanogram to microgram per litre range in STW effluent, fresh waters and groundwater (Daughton and Ternes, 1999, Jørgensen and Halling-Sørensen, 2000, Kümmerer, 2001). Concentrations in the terrestrial environment are reported to be in the microgram to the milligram per kilogram range in soils (Monteiro and Boxall, 2010).

Pharmaceuticals detected in soils include antibiotics, anti-epileptics, anti-rheumatics, analgesics, anti-inflammatories, cardiovascular drugs, hormones, lipid regulators, sedatives, stimulants used for both human and animal medicines at concentrations in the range of 0.0006 – 2.9 mg kg−1 (Golet *et al*., 2002, Golet *et al*., 2003, Thiele-Bruhn, 2003, Pérez-Carrera *et al*., 2010, Vazquez-Roig *et al*., 2010, Wu *et al*., 2010a, Carr *et al*., 2011). Most pharmaceuticals found in monitoring studies were in soils amended with sludge from STWs or manure. However, 2.9 mg kg^{-1} of the

analgesic propyphenazone was found in soil beneath a landfill site in Croatia. Under these circumstances it would be expected that leaching to groundwater would occur depending on the soil and compounds properties (Ahel *et al*., 1998).

Monitoring studies completed for the aquatic environment give information about specific drugs. Analysis shows detections of pharmaceuticals in fresh waters, groundwater, seawater and potable water (Magnér *et al*., 2010, Prasse *et al*., 2010, Kleywegt *et al*., 2011). Compounds such as the analgesic acetaminophen have been detected at concentrations of 10 μg L−1 in the USA (Kolpin *et al*., 2002), however pharmaceuticals have generally been detected at lower concentrations. Pharmaceuticals such as caffeine, metoprolol, oxazepam and carbamazepine have been quantified, at concentrations ranging from 4 to 210 ng L−1 in seawater (Magnér *et al*., 2010). Following on from the detection of pharmaceuticals in the environment, exposure of non-target organisms to these bioactive compounds is an area of growing concern.

Even though pharmaceuticals are found at such low concentrations they are of environmental concern because they are continually released into the environment and their distribution is widespread, which may lead to biologically active concentrations in non-target species. They are designed specifically to be bio-available, readily transported across lipid membranes and to initiate a biological response to achieve a therapeutic effect at low exposure concentrations (Kümmerer, 2010). Pharmaceuticals have specific physico-chemical properties so that they withstand degradation in the body and perform their function; these very properties may lead to persistence in the environment (Kolpin *et al*., 2002, Ankley *et al*., 2007, Quinn *et al*., 2008). Genomic similarities and functionally equivalent mechanistic systems (for which the pharmaceutical is targeted) between organisms in the environment and humans and farm animals may lead to unwanted effects in non-target organisms (Huggett *et al*., 2005).

There are many studies reporting the ecotoxicological effects of pharmaceuticals, their metabolites and pharmaceutical mixtures in non-target organisms in the environment. The potency of pharmaceuticals in acute toxicity studies (e.g. Daphnia 48 h EC50 and fish 96 h EC50 studies) is generally low and acute effects are generally seen at concentrations many orders of magnitude higher than the low concentrations found in the environment (Cunningham *et al*., 2006). However, due to the chronic nature of the exposure, there is greater concern over longer-term chronic effects. Effects of pharmaceuticals on endpoints such as reproductive

function, growth, behaviour and immunological response have been demonstrated in plants, invertebrates, fish and birds (Brooks *et al*., 2003, Cleuvers, 2004, Flaherty and Dodson, 2005, Brain *et al*., 2006, Zurita *et al*., 2007, Markman *et al*., 2008, Quinn *et al*., 2008). Pharmaceuticals that are more likely to be of concern to the environment are 1) those used in the greatest quantities (e.g. anti-inflammatory pharmaceuticals), 2) those that are the most potent to humans and animals (e.g. hormones like ethinylestradiol) 3) that have clear toxic effects on taxonomic groups that occur in the environment (e.g. antibiotics), 4) those that could act through similar modes of action leading to possible additive effects (e.g. serotonin reuptake inhibitors) and 5) those that bioconcentrate or bioaccumulate (e.g. ethinylestradiol, Daughton and Brooks, 2011).

In the aquatic environment, several laboratory and field-based studies have demonstrated the oestrogenic effects of synthetic oestrogens, such as ethinylestradiol, at or below environmentally relevant concentrations (Jobling *et al*., 2002, Carlsson *et al*., 2006, Jobling *et al*., 2006, Ankley *et al*., 2007, Ji *et al*., 2010). Research has shown that ethinylestradiol can interact with vertebrate and invertebrate oestrogen receptors, which can influence several endpoints such as reproductive behaviour, physiology and morphology, possibly leading to reproductive impairment (Jobling *et al*., 2002, Scott and Sloman, 2004, Harris *et al*., 2011, Reyhanian *et al*., 2011). A notable example of this is a whole-lake experiment that showed that the addition of ethinylestradiol at environmentally relevant concentrations led to reproductive failure and a population level collapse of fathead minnow, *Pimephales promelas* (Kidd *et al*., 2007).

Other effects arising from pharmaceutical exposure in the terrestrial environment have also been observed. The non-steroidal anti-inflammatory drug (NSAID), diclofenac, can cause renal abnormalities and kidney failure in non-target organisms. It was responsible for the population crash of several species of the Oriental White Backed vulture in the Indian subcontinent (Oaks *et al*., 2004). The impact of diclofenac on vultures arose from an unusual route of exposure: dead livestock, formerly treated with diclofenac, which were left for scavengers to remove, which introduced a toxic amount of diclofenac into the diet of the vultures (Green *et al*., 2004, Oaks *et al*., 2004, Shultz *et al*., 2004, Swan *et al*., 2006). In a more recent study on ethinylestradiol, immunological changes in birds were attributed to exposure as a result of feeding on contaminated invertebrates inhabiting STW filter-beds (Markman *et al*., 2008). This particular study highlights that species at different trophic levels in the food-chain may be at risk from exposure to pharmaceuticals via

dietary uptake. However, while the toxic effects of some pharmaceuticals have now been studied, our understanding of the processes and factors influencing the uptake, distribution, metabolism and excretion of pharmaceuticals in organisms is relatively less developed. The uptake and bioaccumulation and or bioconcentration of pharmaceuticals has been previously reported in some aquatic organisms and agricultural crops (Mimeault *et al*., 2005, Nakamura *et al*., 2008, Paterson and Metcalfe, 2008, Redshaw *et al*., 2008, Boonsaner and Hawker, 2010, Fick *et al*., 2010, Karnjanapiboonwong *et al*., 2011). The potential transfer of pharmaceuticals through food-chains is of concern but largely unknown (Kolpin *et al*., 2002, Carlsson *et al*., 2006).

In the past five years there have been reports concentrations of pharmaceutical in the biological tissues of non-target organisms in the field. Laboratory research has shown the uptake, bioconcentration and elimination of pharmaceuticals in aquatic organisms. These data represent a limited number of pharmaceuticals in a limited number of organisms. The duration and frequency of exposure of non-target organisms to most pharmaceuticals is largely unknown (Daughton and Brooks, 2011). The following section reviews the current knowledge of the uptake of pharmaceuticals in non-target organisms. The section will also draw on knowledge gained from other chemical classes to discuss the factors and processes that are likely to affect the uptake of pharmaceuticals into organisms and within food chains.

1.2 Uptake and accumulation of pharmaceutical in non-target organisms

Due to the continual input of pharmaceuticals into the environment, non-target organisms are thought to be exposed to pharmaceuticals for the duration of their life-cycles (Kümmerer, 2004). Pharmaceuticals are more water soluble compared to some of the traditional environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), and as a consequence it has been assumed that they will not have the tendency to accumulate or biomagnify (Daughton and Brooks, 2011). However, during field monitoring and laboratory studies, pharmaceutical residues have been detected in biological tissues. While uptake of pharmaceuticals into terrestrial organisms has been demonstrated (Boxall *et al*., 2006, Redshaw *et al*., 2008, Boonsaner and Hawker, 2010, Herklotz *et al*., Wu *et al*., 2010a), uptake data available for aquatic organisms are mostly for fish (Brown *et al*., 2007, Fick *et al*., Kallio *et al*., 2010, Mehinto *et al*., 2010, Brozinski *et al*., 2011, Gomez *et al*.) with a few studies with aquatic plants

(Migliore *et al*., 2000, Pouliquen *et al*., 2009) and invertebrates (Lydy *et al*., 1994, Gomes *et al*., 2004). Several therapeutic groups have been detected in fish tissue, including anti-inflammatories and analgesics, anti-histamines and lipid regulators (Brooks *et al*., 2005, Ramirez *et al*., 2007, Ramirez *et al*., 2009, Fick *et al*., 2010). Measured concentrations in the fish body are often higher than the concentration found in the surrounding aquatic environment indicating that pharmaceutical compounds may have the potential to bioconcentrate (figure 1.3).

The bioconcentration of pharmaceutical residues in tissue is the net result of the rates of uptake, biotransformation and elimination of these substances by an organism (Nichols *et al*., 2009). Uptake of compounds into an organism can occur by a number of processes including filtration, passive or facilitated diffusion, active transport or phago/pinocytosis (Table 1.1; Timbrell, 2002). There are two major routes of uptake namely the exchange of substance between the surrounding environment (e.g. water for aquatic organisms and air for terrestrial organisms) and the respiratory surface and possibly dermal exchange or the uptake from an organism's food (McKim and Goeden, 1982, Barber, 2003, Barber, 2008, Powell *et al*., 2009). Organisms eliminate substances through biological processes such as respiratory exchange, metabolism and excretion via the faeces and the urine and growth dilution (Powell *et al*., 2009).

The degree of uptake of a compound can be described by the bioconcentration factor or the bioaccumulation factor. The bioconcentration factor (BCF) is the ratio between the concentration of contaminant in the tissue of the organism and the concentration in the water (Barber, 2003). BCF applies when the exposure is by water alone but the bioaccumulation factor (BAF) applies where exposure is from the food and the respired media (Barber, 2008, Powell *et al*., 2009). Due to the general physicochemical properties of pharmaceuticals such as their high water solubility and low hydrophobicity it is interesting that some have shown a tendency to bioconcentrate in tissue, however, for those substances where BCFs have been calculated, most have a BCF of less than 1000. According to REACH (Registration, Evaluation and Authorization of Chemicals) the regulatory framework for chemicals in the European Union (EU, 2006), a chemical is considered 'bioaccumulative' if the BCF is greater than 2000, and 'very bioaccumulative' if the BCF if greater than 5000. Therefore, only ibuprofen and the synthetic hormones; 17 α-ethinylestradiol and Levonorgestrel would be classed under REACH as bioaccumulative (Länge *et al*., 2001, Brown *et al*., 2007, Fick *et al*., 2010).

BCFs and tissue residue concentrations in specific tissues provide better information on the concentration of the contaminant at the target site compared water concentrations. They may give an indication of whether an adverse effect is likely to occur in the organism (Walker, 1990, Daughton and Brooks, 2011). In the following section the current information on the tissue concentrations of pharmaceuticals in aquatic organisms will be reviewed. The processes and factors involved in uptake, bioconcentration and elimination are discussed and knowledge gaps highlighted.

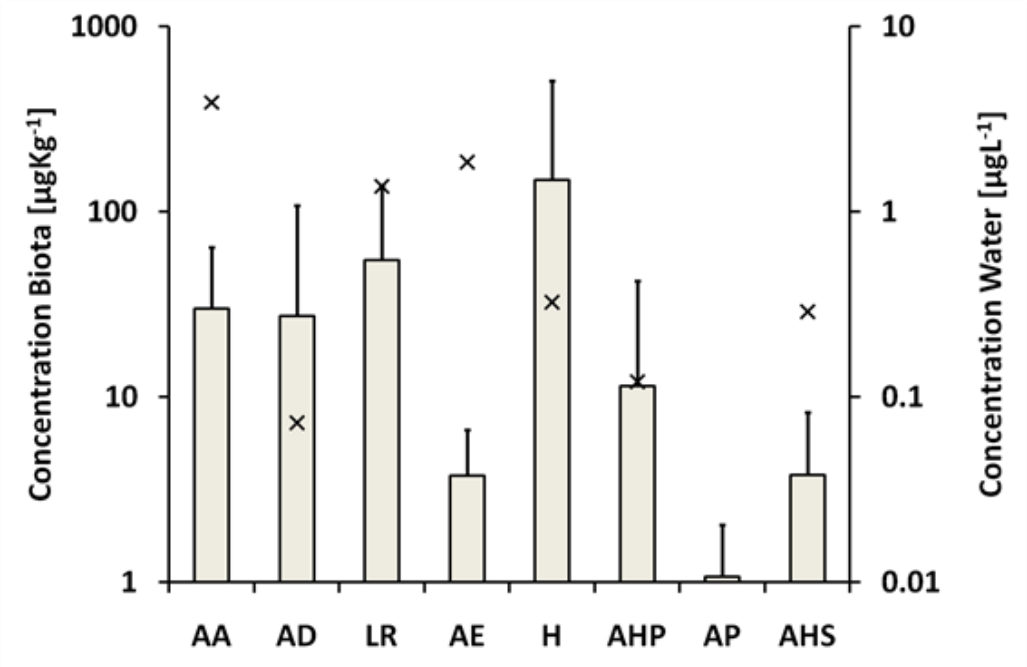


Figure 1.3 Mean concentration (µg kg^{-1}) of pharmaceuticals; analgesics and anti-inflammatories (AA), antidepressants (AD), lipid regulators (LR), anti-epileptics (AE), hormones (H), anti-hypertensives (AHP) anti-psychotic (AP) and anti-histamines (AHS) reported in wild fish tissues (bars with standard deviation; n=4-51) and reported concentrations (µgL^{-1}) in effluent and fresh waters (×)(Larsson *et al.*, 1999, Brooks *et al.*, 2005, Brown *et al.*, 2007, Chu and Metcalfe, 2007, Ramirez *et al.*, 2007, Kwon *et al.*, 2008, Nakamura *et al.*, 2008, Zhou *et al.*, 2008, Gelsleichter, 2009, Ramirez *et al.*, 2009, Al-Ansari *et al.*, 2010)

Table 1.1 Mechanisms through which uptake across membranes can occur (adapted from Timbrell (2002))

Uptake mechanism	
Filtration	Small molecules may pass through pores in membranes formed by protein molecules. Movement is down a concentration gradient
Passive diffusion	A first order process that describes movement of lipophilic compounds across a membrane down a concentration gradient. Factors influencing passive diffusion are described by Ficks Law: $$\boldsymbol{Rate\ of\ diffusion = KA(C_1 - C_2)}$$ Where K is the constant, A is the surface area, C2 is the concentration outside and C1 the concentration inside the membrane. Lipid solubility, distance, temperature and ionization potential and hence pH are factor in passive diffusion
Active transport	A mechanism generally for endogenous compounds such as nutrients that involves transport by a specific membrane carrier for which metabolic energy is required. This process occurs against concentration gradients but maybe saturated at high concentrations and analogue compounds may compete carriers
Facilitated diffusion	Unlike active transport no energy is required for this mechanism as it occurs across a concentration gradient. It is a carrier mediated transport and maybe saturated by high compound concentrations
Phagocytosis / pinocytosis	Invagination of the membrane to engulf compounds, droplets or particles, a mechanism where insoluble compounds can be absorbed

1.3 Occurrence of Pharmaceuticals in Aquatic Organisms

Table 1.2 (page 52) syntheses of the current knowledge of pharmaceutical residues in aquatic organisms, which will be discussed in the following sections by pharmaceutical class.

1.3.1 Analgesics and anti – inflammatory

Major analgesics such as aspirin and paracetamol are non-opioid analgesics used to control pain resulting from conditions such as headaches, toothaches, and mild rheumatism. Opioid analgesics, sometimes known as narcotic analgesics include codeine and more potent pharmaceuticals such as morphine and pethidine. The non-steroidal anti-inflammatory drugs are a large group of analgesics (NSAIDs) widely used for treating rheumatic conditions and include pharmaceuticals such as diclofenac and naproxen (BNF, 2011). They are one of the most frequently detected groups of pharmaceuticals in the aquatic environment (Mehinto *et al*. 2010).

In the field, Brown *et al* (2007), surveyed the concentrations of several NSAIDs including ibuprofen, diclofenac naproxen and ketoprofen in the plasma of rainbow trout, Oncorhynchus mykiss at three STWs. The steady state plasma concentrations were highest for ibuprofen (84 ng mL^{-1}) followed by naproxen (14 ng mL^{-1}) and diclofenac (12 ng mL^{-1}) but ketoprofen was not detected. Steady state concentrations in the plasma were reached after 2 days following exposure to 0.0045 ng mL^{-1} ibuprofen, 0.25 ng mL^{-1} naproxen and 2.32 ng mL^{-1} diclofenac. The main finding of this study was that the BCFs calculated for these pharmaceuticals in fish varied between sites despite the exposure scenarios being similar (ibuprofen BCF <63 – 18667, naproxen <2 – 35, diclofenac <5 - <11). Variations in environmental factors including temperature, dissolved organic material and the presence of surfactants were suggested to be responsible for the differences.

Fick *et al* (2010) exposed *O. mykiss* to effluent from three STWs in a semi-field study and detected analgesics and other pharmaceuticals including diclofenac, ibuprofen, ketoprofen, naproxen and tramadol in the plasma of the fish. In this study the NSAIDs had the highest concentrations in the fish tissues compared to those of any of the other pharmaceuticals measured. This was a direct result of the high concentrations in the effluent (e.g. ibuprofen, ketoprofen and naproxen were detected at a concentration 103 times higher than the hormone levonorgestrel or the anti-hypertensive verapamil). It was found that ibuprofen bioconcentrated the most followed by ketoprofen, diclofenac, naproxen and tramadol (e.g. BCFs were 58, 48, 29, 28 and 3.3 respectively). Ibuprofen has also been shown to bioconcentrate in adipose and muscle tissue of *O. mykiss* in a study by Zhang *et al*. (2010). In this study a mixture of pharmaceuticals, including several NSAIDs (e.g. naproxen, diclofenac and ibuprofen), was tested. Ibuprofen was detected at the highest concentrations of all the NSAIDs, ca 68 ng g^{-1} in the adipose tissue and ca 5 ng g^{-1}

in the muscle, with BCFs of 1.50 and 23.69 in the muscle and adipose tissue respectively. By contrast naproxen and diclofenac did not accumulate in tissue.

In the laboratory, research has been dominated by studies on diclofenac. Kallio *et al*. (2010) measured diclofenac in the bile of *O. mykiss* and identified several metabolites at environmentally relevant concentrations in waters (1.76 µg L^{-1}). Large variations in the concentrations of diclofenac and its metabolites were observed in the bile between individual fish. No parent diclofenac was detected but was detected up to a maximum concentration of 110 µg L^{-1} in some individual fish, this was 103 times greater than the exposure water containing 1.76 µg L^{-1}. The BCFs calculated for total diclofenac (diclofenac plus its metabolites divided by water concentration) ranged between 320 and 950. Schwaiger *et al*. (2004) investigated uptake of diclofenac into the liver of *O. mykiss* and obtained BCFs of ca 700 and 2732 after a four week exposure to 5 and 1 µgL^{-1} of diclofenac respectively. The BCF for diclofenac determined in the field by Brown *et al*. (2007) are much lower in comparison (BCF of 5). These reported differences may be due to the type of the tissue sampled: Brown *et al*., (2007) sampled the blood plasma whereas Kallio *et al*. (2010) studied the bile and Schwaiger *et al*. (2004) studied liver.

To summarise, some NSAIDs can bioaccumulate in fish tissues but there is inter- and intra-study variation in the literature reviewed. These variations are probably explained by differences in environmental conditions, exposure concentrations, the lipid content of the organism and the nature of the tissue sampled. Adverse effects such as gill and renal alterations in fish, and renal alterations in birds and mammals can occur at tissue concentrations found in fish (Hilton and Thomas, 2003, Ashton *et al*., 2004, Oaks *et al*., 2004, Schwaiger *et al*., 2004). The current literature shows that NSAIDs have the potential to cause toxic effects at the concentrations found in the environment.

1.3.2 Antibacterial drugs

Antibacterial drugs are widely used in modern medicine. They are pharmaceuticals which have a selective toxic action on single celled organisms and bacteria. Antibacterial drugs are used in human and veterinary medicine to treat bacterial infections and include compounds such as broad spectrum penicillins, beta – lactams and tetracyclines (Martin, 2010, Monteiro and Boxall, 2010).

Two studies (both laboratory and field) are available on the uptake of antibacterials from commercial fish farms into aquatic plants. In a field study the bryophyte, *Fontinalis antipyretica* was collected from sites in the vicinity of four fish farms and an STW to determine whether pharmaceuticals were present in plant tissues. Concentrations of oxolinic acid, florfenicol, flumequine and oxytetracycline at approximately 47, 120, 600 and 1000 ng g^{-1} respectively were detected in plant tissue (Pouliquen *et al*., 2009). Flumequine was found in 68 % of the plant samples tested and oxolinic acid was detected in 51 % of the samples.

In the laboratory, Migliore *et al*. (2000) exposed the euryhaline aquatic weed, *Lythrum salicaria* to high aqueous concentrations (5000, 1000, 500, 100 and 50 µg L^{-1}) of the flumequine. Measured concentrations in *L. salicaria* after 35 days exposure were 13,300, 8700, 700, 300 and 200 ng g^{-1} dry weight (pooled samples) respectively. These concentrations correlate in approximate BCFs in the range 2.6 to 4. It is not clear whether a steady state was reached in the experiment and therefore BCFs are not unequivocal. Although most of these exposure concentrations were relatively high, the lowest exposure concentration was equivalent to concentrations measured downstream of an intensive bass farm (Migliore *et al*., 2000).

1.3.3 Anti – depressants

Antidepressants are primarily used to treat psychomotor and physiological changes (BNF, 2011). There are several subclasses of antidepressants including tricyclic antidepressants, monoamine oxidase inhibitors, serotonin reuptake inhibitors (SRRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs; Martin, 2010). Clinical depression is a common illness affecting 15 % of people in the UK and therefore antidepressants are widely prescribed. SSRIs are the subclass of antidepressants that is most frequently dispensed in the UK with 231, 459, 000 prescriptions written in 2009 (NHS, 2011). SSRIs include citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine and sertraline. Due to their abundant application SSRIs are frequently detected in fresh waters and subsequently they are dominant type of antidepressant studied in the laboratory and in the field.

Citalopram is the most prescribed SSRI in the UK but has only been studied in the field in the USA on two occasions. It has been detected in the tissues of wild fish at low levels of detection (Gelsleichter, 2009, Schultz *et al*., 2010) and was found in

half of the juvenile bull sharks, *Carcharhinus leucas* sampled in the Caloosahatchee river (Florida, USA). Bull shark plasma concentrations were in the range of 0.25 – 0.57 ng mL^{-1} (Gelsleichter, 2009). In a study by Schultz *et al* (2010), mean concentrations of citalopram of between 0.01 and 0.07 ng g^{-1} were seen in the brain tissue of the white sucker fish, *Catostomus commersonii* (sampled from rivers in Colorado and Iowa). These levels were an order of magnitude lower than the plasma of the bull sharks.

Fluoxetine was the first SSRI developed and is the active ingredient in Prozac (Wong *et al*., 1995). It is probably the most well-known SSRI and is the most studied in fish by environmental scientists. Fluoxetine has been shown to accumulate in different tissues including the brain, liver and muscle and concentrations can differ over two orders of magnitude. Field surveys have detected fluoxetine and its major metabolite norfluoxetine in a number of species of fish in the US and Canada since 2005. Brooks *et al* (2005) was the first to report SSRIs in wild fish populations taken from an effluent dominated stream (Texas, USA). In this study fluoxetine was measured in the brain, liver and muscle of three species of fish; the Black crappie, *Pomoxis nigromaculatus*; the Blue gill, *Lepomis macrochirus*, and the Channel catfish, *Ictalurus punctatus*. Fluoxetine was found to accumulate in the brain of the three fish species with a maximum concentration of 1.58 ng g^{-1}. Fluoxetine was detected in the muscle tissue but this was the lowest concentration recorded (0.11 ng g^{-1}). These results were consistent with whole body concentrations of fluoxetine in the gizzard shad, *Dorosoma cepedianum* and the brown bullhead, *Ameiurus nebulosus* (0.16 – 1.02 and 0.14 – 0.31 ng g^{-1} respectively) sampled from Hamilton Harbour (Canada) by Chu and Metcalfe (2007). In a recent study by Schultz *et al* (2010) fluoxetine was one of the primary antidepressants measured in fish tissues and was detected in fish brain tissue of the white sucker, *Catostomus commersoni* at seven out of eight sites in Boulder Creek (Colorado, USA). Mean concentrations (± standard deviation) ranged from 0.02 ± 0.03 – 0.6 ± 0.06 ng g^{-1}. The highest recorded concentration of fluoxetine in a wild fish was reported by Ramirez *et al* (2009). This study was the most spatially diverse survey and fish were sampled in six states across the USA. Fluoxetine was measured in the livers of *C. commersoni* and the largemouth bass *Micropterus salmoides* in two states (Illinois and Pennsylvania), mean concentrations were 19 and 70 ng g^{-1} for *C. commersoni* and *M. salmoides* respectively. The highest concentration (80 ng g^{-1}) recorded in *C. commersoni* was at least 49 times than the concentrations reported in other studies. By contrast fluoxetine was not detected in four of the six sites sampled in the same

study. Also, fluoxetine was under the limit of quantification (0.030 ng g^{-1}) in the muscle of fish sampled at two sites downstream of STWs (Zhou *et al*., 2008). The authors suggested that recent upgrades at the STW may have enhanced the removal of fluoxetine from the water. Another possible contributory factor to the non-detection of fluoxetine was the selection of muscle tissue. Brooks *et al*. (2005), reported that of all tissues tested, muscle contained the lowest concentrations of fluoxetine.

Fluoxetine is one of the few antidepressants that have been studied in fish in the laboratory. BCFs for fluoxetine have been determined in two sub-species of Japanese Medaka, *Oryzias latipes* and *O. mykiss*. BCFs have been determined using different methods and using different tissues (muscle, adipose, whole body and liver tissues), at different exposure concentrations and at varying pH, which probably explains why the measured BCFs range over two orders of magnitude. Nakamura *et al*. (2008) determined BCFs for fluoxetine in *O. latipes* at different pH conditions. When exposed to 10 µg L^{-1} of fluoxetine at pH 7, 8 or 9, BCFs of 8.8, 30 and 260 in the whole body and 330, 580 and 3100 for the liver were obtained. This shows that the bioconcentration of fluoxetine, which is a basic ionisable compound, is strongly influenced by pH. The BCF increases with increasing pH indicating that fluoxetine uptake is greater when the compound is in the undissociated form. Similar results were found for norfluoxetine, a metabolite of fluoxetine (Figure 1.4).

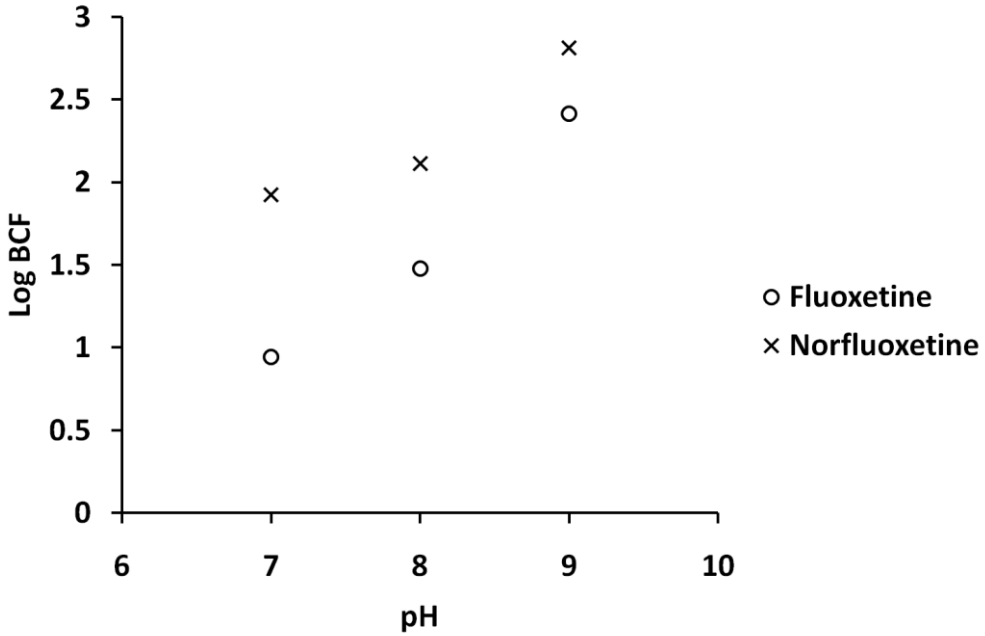


Figure 1.4 The bioconcentration of fluoxetine and its metabolite norfluoxetine into *Oryzias latipes* at differing pH values in freshwater. Data points are calculated log BCF values (adapted from Nakamura *et al*., 2008).

BCFs calculated for fluoxetine in muscle and adipose tissue of *O. mykiss*, were 58.98 ± 16.81 and 143.36 ± 21.50 respectively Zhang *et al*. (2010). *O. mykiss* were exposed to a mixture of pharmaceutical compounds for eight days (3 µg L^{-1} fluoxetine but at a total pharmaceutical concentration of 1.03 g L^{-1}). This study showed that fluoxetine uptake in both tissues increased continually throughout the exposure period but the rate of uptake differed between the tissue types. Uptake and clearance of fluoxetine has also been determined in *O. latipes* by Paterson and Metcalfe (2008). Fish were exposed to a mean concentration of 0.55 µg L^{-1} of fluoxetine for seven days and then transferred to clean water for twenty one days for clearance. The fish accumulated fluoxetine within five hours with a maximum concentration of 49.4 ± 6.4 ng g^{-1} observed in the whole body tissues at day 3 of the exposure. Two BCFs were estimated, a BCF of 74 was calculated from the mean exposure concentration and the concentration accumulated in the fish and a BCF of 80 was calculated from a modelled uptake constant of 5.9 ± 0.5 d^{-1} and a clearance constant of 0.074 ± 0.009 d^{-1}. The BCFs measured in this study were slightly higher

than those measured by Nakamura *et al*. (2008) for the same exposure pH range (7.2 – 7.5).

Fluvoxamine has only been reported once in fish in the plasma of *C. leucas* sampled in the Caloosahatchee River (Florida, USA). Although fluvoxamine was not detected in any of the water samples taken it was measured at low concentrations (0.15 – 0.90 ng mL^{-1}) in the plasma of four out of ten sharks sampled. This study shows that fluvoxamine may have some potential to bioaccumulate in fish tissues (Gelsleichter, 2009).

Although Paroxetine has been observed on several occasions in wild fish tissues, there is no laboratory data on the uptake of this compound. The concentrations measured in wild fish are fairly consistent across the different studies, which have included different species and tissues and have been performed at different study sites. The concentrations detected ranged between 0.11 to 0.58 ng g^{-1} (Chu and Metcalfe, 2007, Gelsleichter, 2009, Schultz *et al*., 2010).

The most recent detection was made by Schultz *et al*. (2010). Paroxetine was found in the brain tissue of *C. commersoni* at three out of eight sites. In this study mean concentrations of paroxetine ranged between 0.01 to 0.02 ng g^{-1}, with a maximum recorded concentration of 0.113 ng g^{-1}. Gelsleichter (2009) measured concentrations of 0.03-0.55 ng g^{-1} in the plasma of *C. leucas* and Chu and Metcalfe (2007) found paroxetine in half the fish collected from Hamilton Harbour in Canada at concentrations in the range of ND – 0.58 ng g^{-1}. In the laboratory there are currently few studies of the toxicokinetics of paroxetine. More investigation is needed into the uptake of paroxetine because it has been previously documented that paroxetine has a high binding affinity to the serotonin transporter in the plasma membrane of fish, and maybe actively transported across fish cell membrane. Paroxetine is known to influence the serotonin levels by inhibiting serotonin uptake in *O. mykiss* (Ferriere *et al*., 1996).

Sertraline, the most prescribed antidepressant in the US has been found in fresh waters at concentrations up to 37.5 ng L^{-1} and has subsequently been identified in wild fish. Sertraline uptake has been studied in the laboratory. Schultz *et al*. (2010) reported that sertraline concentrations (Cmax 4.24 ng g^{-1}) in the brain of *C. commersoni* were higher compared to other antidepressants surveyed including citalopram and venlafaxine. This is similar to the highest sertraline concentration (4.27 ng g^{-1}) found in the brain tissue of fish by Brooks *et al*. (2005). Brooks *et al*. (2005) also found that sertraline accumulates into the brain tissue more than in the

liver or in the muscle tissues. Ramirez *et al*. (2009) found that sertraline was regularly present in the liver tissue of seven different species of fish sampled in five different states in the U.S. The mean liver concentration ranged from 5 to 381 ng g^{-1}. This can be compared to the muscle tissue of these fish, where sertraline was found at only two of the sampling sites at much lower concentrations of 5 – 11 ng g^{-1}, 35 x lower than the liver tissue. Sertraline was also present at higher concentrations than any other antidepressant in the plasma of *C. leucas* (Gelsleichter, 2009). The concentrations (0.25 – 0.97 ng g^{-1}) were lower compared to those from other studies.

Fick *et al*. (2010) exposed *O. mykiss* in semi-field conditions to effluent from three STWs. Concentrations of sertraline were only quantifiable in the effluent from one STW at low concentrations (mean 8 ng L^{-1}). Despite this sertraline was detectable in fish plasma at fish from two of the STWs (mean concentrations were 1.2 ng g^{-1} and 1.1 ng g^{-1}). These findings resulted in fairly large BCFs for the two STW exposures > 240 and 138 determined using concentrations in the water and the plasma. However these BCFs were still lower than predicted by Fish Plasma Model (FPM; Huggett *et al*., 2003a).

Venlafaxine is an SNRI and 2,630,500 prescriptions were dispensed in the UK in 2011. Although it is more popular than sertraline in the UK it has rarely been studied in the environment (NHS, 2011). It has been detected by Gelsleichter (2009) in *C. leucas* at similar concentrations to other antidepressant detected in the sharks (ND – 0.32 ng g^{-1}). It has been reported to occur at low concentrations in the brain tissue (ND – 0.13 ng g^{-1}) of *C. commersoni* by Schultz *et al*. (2010). In this study, the authors suggested that the low levels of venlafaxine could be due to its low lipophilicity ($logK_{ow}$ -0.37).

In addition to looking for the parent pharmaceutical compound, a number of studies have explored the occurrence of metabolites of anti-depressants in aquatic organisms. The metabolites of fluoxetine and sertraline have been identified in fish; both norsertraline and norfluoxetine have accumulated in wild fish tissues and the biotransformation of fluoxetine to norfluoxetine in fish has been demonstrated in the laboratory. In general it has been shown that the metabolites of antidepressants such as norfluoxetine and norsertraline have longer half –lives than the parent compound which allows them to accumulate to a greater extent. This has been demonstrated in the field (Brooks *et al*., 2005, Chu and Metcalfe, 2007, Gelsleichter, 2009, Ramirez *et al*., 2009, Schultz *et al*., 2010) and in laboratory studies

(Nakamura *et al*., 2008, Paterson and Metcalfe, 2008). For example Schultz *et al*. (2010) observed maximum concentrations as high as 3.58 and 28.9 ng g^{-1} for norfluoxetine and norsertraline which were 2.5 and 7 times higher than the concentrations of the parent compounds fluoxetine and sertraline (C_{max} 1.68 and 4.24 ng g^{-1} respectively). In the laboratory, Paterson and Metcalfe (2008) showed that norfluoxetine was formed in fish after 5 hours exposure to 0.55 µg L^{-1} of fluoxetine. At 5 hours exposure, the norfluoxetine concentration was approximately 40 % of the parent fluoxetine concentration. The concentration of the metabolite was lower than that of the parent until after 7 days when norfluoxetine concentrations exceeded fluoxetine concentrations. The maximum concentration observed for norfluoxetine was 64.3 ng g^{-1} compared to 49.4 ng g^{-1} for the parent fluoxetine. A pseudo BCF of 74 was calculated using the measured concentration of norfluoxetine in the fish compared to the average fluoxetine concentration in the water. The detection of both parent and metabolite SSRIs in fish may indicate equivalent metabolic pathways between fish and mammals.

In general the literature suggests that tissue concentrations and BCFs for SSRIs and antidepressants can range over several orders of magnitude. Factors such as the analytical methods used, exposure concentration, pH, organisms differences and metabolic processes can influence the concentrations found. It is also important to recognise the anti-depressants do not necessarily occur in organisms as individual residues but can occur with a cocktail of other anti-depressants, other pharmaceuticals and other chemical classes (Schultz *et al*., 2010). It is possible that uptake, metabolism and clearance of mixtures will differ compared to single substances and this is an area where further research is required.

1.3.4 Anti-epileptics

Anti-epileptic medicines such as carbamazepine prevent or reduce the severity of seizures in various types of epilepsy. Some anti-epileptics prevent neurons firing rapidly (e.g. carbamazepine, lamotrigine). Others (e.g. barbiturates, benzodiazepine, vigabatrin) enhance the function of inhibitory synapses and some have multiple actions (BNF, 2009). Anti-epileptics are widely used and 14,011,600 prescriptions were dispensed in the UK in 2009 (NHS, 2011). Carbamazepine is one of the most highly dispensed anti-epileptic and is one of the more commonly found in final sewage effluent and surfaces waters (Monteiro and Boxall, 2010). Subsequently

carbamazepine has been studied in fish, invertebrates and algae in field and the laboratory.

Ramirez *et al*. (2007) detected carbamazepine at concentrations of 0.83 – 1.44 ng g^{-1} in muscles tissue of *O. mykiss* collected from Pecan Creek in Texas. Ramirez *et al*. (2009) also detected carbamazepine at 2.3 and 6 ng g^{-1} in the muscle and liver of *M. salmoides*. The maximum concentration of carbamazepine detected was 8 ng g^{-1} in the liver tissues. By contrast Zhou *et al*. (2009) did not detect carbamazepine in fish collected from three STW impacted rivers in Canada. Similarly Kwon *et al* 2009 did not detect carbamazepine in the liver tissue of turbot, Pleuronichthys verticalis collected from Californian coastal waters, a coastal not riverine study.

In a semi-field study (Fick *et al*., 2010) exposed *O. mykiss*, to STW effluent from three treatment plants in Sweden and showed accumulation of carbamazepine in their plasma. The mean concentration measured ranged between 0.3 and 1.0 ng g^{-1} and the calculated BCFs ranged from 0.8 – 4.2. These concentrations are in the range of those found by Ramirez *et al*. (2007) in the field. However, the BCF are slightly higher than the BAFs determined by Zhou *et al*. (2008). Zhou *et al*. (2008) carried out bioaccumulation experiments in fish muscle in the laboratory. BAFs of 0.44 and 0.22 were determined after 7 and 14 days exposure respectively. The lower BAF (following a longer exposure to carbamazepine) may reflect metabolic capacity of *O. mykiss* for carbamazepine.

Tissue specific accumulation has been identified for carbamazepine. Carbamazepine was shown to accumulate more in the adipose tissue than the muscle tissue (Zhang *et al*., 2010). Zhang *et al*. (2010) calculated a BCF of 0.52 for muscle tissue of *O. mykiss* which was similar to the BAF in the study by Zhou *et al*. (2008). Zhang *et al*. (2010) also calculated a BCF of 4.16 in the adipose tissue of *O. mykiss*. The adipose tissue in this study has a larger BCF than the muscle tissue which may be due to tissue specific accumulation, possibly a function of lipophilicity.

Carbamazepine has been studied over a wider diversity of taxonomic groups compared to other pharmaceuticals; in addition to fish, both invertebrates and algae have also been investigated. For example Lajeunesse *et al.* (2009) validated a method which allowed detection of carbamazepine in invertebrates and ^{measured} a concentration of 129 ± 57 ng g^{-1} in the crustacean, *Thamnocephalus platyurus*. This method was employed for the detection of the accumulation of carbamazepine in organisms at different trophic levels. Vernouillet *et al.* (2010) showed that the algae, *Pseudokirchneriella subcapitata* can accumulate carbamazepine to a concentration

of 10.2 ng g^{-1} when exposed to 0.15 ng mL-1. A crustacean, *T. platyurus* fed the exposed algae accumulated carbamazepine from the food to a maximum concentration of 128.6 ng g^{-1}. However, when a cnidarian, *Hydra attenuata* was fed the crustacean, negligible accumulation was observed. BAFs for *P. subcapitata* and *T. platyurus* were 2.2 and 12.6 respectively. In another study concerning algae, Andreozzi *et al*. (2002) found that carbamazepine was not accumulated by the algae, *Selenastrum capricornutum* and *Ankistrodesmus braunii* which contradict the findings of Vernouillet *et al*. (2010).

To date carbamazepine is the only antiepileptic compound to be studied in non-target organisms. So far research has shown that carbamazepine can accumulate in fish, invertebrates and algae and there is also the potential for it to be metabolised by non-target organisms. More work should be carried out into the potential accumulation of carbamazepine in algae as they are a primary food resource for many other organisms. Preliminary work shows that there may be the potential for transfer through the food-chain but again more research is needed.

1.3.5 Anti-histamine

Although detections of anti-histamine medicines are not well documented in fresh waters and sewage effluents, there have been a number of detections in the tissues of wild fish. Ramirez *et al*. (2007) found diphenhydramine present in the muscle tissue of fish sampled from Pecan Creek Texas with concentrations ranging between 0.66 and 1.32 ng g^{-1}. In a more recent study, Ramirez *et al*. (2009) conducted a survey of fish from Chicago, Dallas, Phoenix and West Chester. Mean concentrations of diphenhydramine were detected in all rivers at concentrations that ranged from 0.13 to 0.15 ng g^{-1} in the muscle and at 0.5 to 10 ng g^{-1} in the liver tissue. Zhou *et al*. (2008) detected diphenhydramine at concentrations of 0.032 ± 0.005 and 0.081.63 ± 0.008 ng g^{-1} in *C. commersoni* and the Johnny darter (*Etheostoma nigrum*) respectively. These concentrations were approximately one order of magnitude lower than those found by Ramirez *et al*. (2007), (2009). Zhou *et al*. (2008) did not detect more commonly found pharmaceuticals such as carbamazepine and fluoxetine, therefore the lower concentrations detected of diphenhydramine compared to other studies might be explained by recent upgrades at the STWs in the study area.

Other anti-histamine compounds have also been detected in fish tissues. In a semi-field study, Fick *et al*. (2010) exposed *O. mykiss* to STW effluent and reported detectable levels of meclizine in the plasma of the fish. Three exposures were maintained and each had effluent from three different STWs in Sweden. Meclizine was not detected in any of the exposure effluents in the study by Fick *et al*. (2010) but was found in fish tissues at concentrations of 0.1 to 0.7 ng g^{-1} in two out of three exposures. This may indicate bioaccumulation potential for meclizine in fish tissues. Minimum BCFs of > 200 and > 1400 were calculated using the LOQ for meclizine in the absence of measured water concentrations. These were both lower than the BCF of 2521 predicted by the FPM.

1.3.6 Cardiovascular drugs

Cardiovascular medicines are generally prescribed to treat high blood pressure angina, glaucoma and other related conditions, they include thiazide diuretics, the pyridazine angiotensin-converting enzyme (ACE) inhibitors, the calcium channel blockers, the beta blockers, and the angiotensin II receptor antagonists or ARBs (BNF, 2009).

The ACE inhibitor, cilazapril and the calcium channel blocker, diltiazem were detected by Fick *et al*. (2010) when *O. mykiss* were exposed to effluent from three different STWs in Sweden. Cilazapril was below the level of quantification in the three exposure effluents, however detectable concentrations were found in the fish plasma of 0.1 and 0.7 ng g^{-1}. This may indicate bioaccumulation and BCFs of > 100 and > 700 were calculated, which were 17 – 117 times greater than the predicted BCF of 6. The literature suggests that factors in addition to the log K_{ow} are important for bioaccumulation (Fick *et al*., 2010).

Diltiazem is the most commonly detected cardiovascular drug and has been reported in wild fish tissue by a number of researchers including Fick *et al*. (2010). Concentrations ranged from 0.002 to 0.7 ng g^{-1}, with a mean concentration of 0.9 ng g^{-1} in the plasma of fish and a calculated BCF of 24. There were three semi-field sites in the study and only one out of the three exposures showed bioaccumulation in the fish plasma, despite all having similar exposure concentrations. These results show high variation between the fish.

Diltiazem has been found at concentrations similar to those recorded by Fick *et al*. (2010) in wild fish in other studies. Ramirez *et al*. (2009) recorded diltiazem

concentrations ranging from 0.13 to 0.15 ng g^{-1} in the muscle and 0.3 to 0.7 ng g^{-1} in the liver tissue of *M. salmoides*. Ramirez *et al*. (2007) detected concentrations of between 0.11 and 0.27 ng g^{-1} in the muscle of *Lepomis* species. Zhou *et al*. (2008) measured diltiazem in fish at concentrations that were two orders of magnitude lower at 0.002 and 0.0056 ng g^{-1} in *C. commersoni* and *E. nigrum* respectively. Although these concentrations were lower than those found by other researchers, diltiazem was one of few pharmaceuticals detected by Zhou *et al*. (2008) most likely due to enhanced STW technology at this site. Therefore, it could be expected that the concentrations found by Zhou *et al*. (2008) are not typical for diltiazem in the field.

Beta blockers such as propranolol and atenolol have not been detected in wild fish tissues but have been studied in the laboratory (Winter *et al*., 2008, Owen *et al*., 2009, Ramirez *et al*., 2009). Owen *et al*. (2009) measured the uptake of propranolol into the plasma of the fish *O. mykiss* after exposure to 0.1, 1, 10, 100, 1000 and 10,000 µg L^{-1} for forty days. At the lowest exposure concentration, propranolol was measured at 0.94 ng g^{-1} (pooled sample) in the plasma of exposed fish. At the highest exposure concentration, plasma propranolol reached a mean concentration of 5,200 ± 1,333 ng g^{-1}. This study showed that propranolol plasma concentrations could reach similar concentrations to the exposure concentration; however this relationship was variable ranging between approximately 16 - 94 % of the exposure concentration. In this study the authors were able to predict plasma concentrations from the water concentrations using the mammalian fish leverage model (Huggett *et al*., 2004). A relationship between predicted and measured plasma concentration was obtained and 59 % of the measured concentration could be explained by the predicted values.

Winter *et al*. (2008) studied the plasma concentration of atenolol in fathead minnow, *Pimephales promelas*. Aqueous concentrations were 100, 320, 1000, 3200 and 10,000 µg L^{-1}. In the male fish atenolol concentrations ranged from 4.2 ng g^{-1} when exposed to 100 µg L^{-1}, to 164.3 ng g^{-1} when exposed at the highest concentration. For female fish concentrations ranged from < 0.5 ng g^{-1} when exposed to 100 µg L^{-1}, to 291 ng g^{-1} when exposed at the highest concentration. Plasma atenolol concentrations were between 1.8 and 6.2 % (males) and between 0 and 12.2 % (females) of the exposure concentrations. These plasma concentrations relative to the corresponding water concentrations were less than those found by Owen *et al*. (2009) which shows the variability of plasma concentrations between beta-blockers.

The literature for cardiovascular drugs shows that compounds such as cilazapril could potentially be bioaccumulative in fish and that accumulation may involve processes other than passive diffusion. However, at the concentrations found in the surfaces waters, cardiovascular drugs such as propranolol would be expected to give rise to only pg g^{-1} concentrations in fish plasma, which is several orders of magnitude lower than the therapeutic dose (Owen *et al*., 2009). Cardiovascular compounds are expected to occur in the environment in mixtures and additive effects for compounds with similar modes of action have to be considered.

1.3.7 Lipid regulators

Lipid regulating agents are medicines that lower the concentrations of lipoproteins, the agents that transport cholesterol and triglycerides, in blood (BNF, 2009). There are several types of lipid regulators including fibric acid derivatives (e.g. gemfibrozil), statins (e.g. simvastatin) and niacin (e.g. vitamin B). Bioaccumulation of lipid regulators in fish has been studied in the field and the laboratory. Ramirez *et al* (2009) found gemfibrozil in the liver tissue of *C. commersoni* and the Koi carp, *Cyprinus carpio* from the field. Liver concentrations were 27.1 and 70 ng g^{-1} for *C. commersoni* and *C. carpio* respectively. This was an order of magnitude less than the concentration found in *O. mykiss* in a semi-field study by Brown *et al* (2007) caged *O. mykiss* in the effluent outflow channel of Gryaab STW in Sweden for 16 days. Gemfibrozil accumulated to a concentration of 210 ng g^{-1} in the plasma of the fish. Fish were also caged at Gråbo and Spenshult STWs, but gemfibrozil was not detected in the fish at these sites. This may be a result of the exposure concentration as the gemfibrozil concentration was two times lower at Gråbo and Spenshult compared to Gryaab STW (e.g. 0.5 ng mL^{-1} compared to 1.0 ng mL^{-1} at Gryaab). Another lipid regulator, simvastatin was investigated in the field at a marine site by Kwon *et al* (2008). In this study simvastatin was below the limit of detection (< 12.3 ng g^{-1}) in all ten fish livers sampled.

Gemfibrozil is the only lipid regulating compound to have been studied in fish in the laboratory. In a study by Zhang *et al* (2010) the uptake of gemfibrozil was observed in *O. mykiss*. The fish were exposed to a mixture of pharmaceuticals. The total pharmaceutical concentration was 1.0 g L^{-1} and the individual compound concentration was 3 µg L^{-1}. The uptake of individual compounds was monitored in the adipose tissue and the muscle tissue of the fish. The concentration of gemfibrozil reached a maximum of ca 54 ng g^{-1} in the adipose and 0.8 ng g^{-1} in the

muscle tissue after 8 and 6 days respectively. BCFs of 20.75 in the adipose and 0.4 in the muscle tissue were calculated.

Another study that explored the bioconcentration of gemfibrozil in fish was conducted by Mimeault *et al*. (2005). Goldfish, *Carassius auratus* were exposed for fourteen days to both 1.5 and 1500 µg L^{-1} gemfibrozil. Plasma concentrations of the goldfish reached 170 ± 20 and 78,000 ± 5000 ng g^{-1} with corresponding BCFs of 89 and 16 at the low and high concentration exposures respectively. After the observation that gemfibrozil could bioconcentrate in goldfish, the authors investigated whether gemfibrozil could be taken up from tap water. Although gemfibrozil was not detected in the water used to hold the goldfish (dechlorinated tap water), it was detected at low concentrations in the control fish after 14 days. This highlights that the exposure concentration in the water is not indicative of the concentrations in the fish and that there is potential for lipid regulators to accumulate in fish tissues even at very low exposure concentrations (Mimeault *et al*., 2005).

1.3.8 Synthetic hormones

The bioconcentration of synthetic hormones and their metabolites has been documented in several different species of fish (in both field and laboratory studies) and invertebrates. In a study by Al-Ansari *et al*. (2010), 50 % of the Shorthead Redhorse Suckers (*Moxostoma macrolepidotum*) sampled downstream of a STW discharge were found to contain 17 α-ethinylestradiol in their bile. Houtman *et al*. (2004) reported 17 α-ethinylestradiol at concentrations of 17 ng mL^{-1} in the bile of wild bream (*Abramis brama*). It has also been documented that when *O. mykiss* are exposure to 100 % effluent from STWs the concentration of the synthetic hormone levonorgestrel in the plasma of the fish exceeded the human therapeutic concentration by a factor of 4 (Fick *et al*., 2010).

Oestrogens, androgens and progestins are known to act as reproductive pheromones in fish at nanogram per litre concentrations and have been linked to a multitude of reproductive abnormalities in fish such as the promotion of vitellogenin synthesis and development of secondary sexual characteristics. The synthetic hormone 17 α-ethinylestradiol and naturally occurring hormones such as 17 β-estradiol are thought to contribute to these effects (Sumpter and Jobling, 1995, Jobling *et al*., 1998, Houtman *et al*., 2004). As all oestrogenic chemicals act on the same receptors it is likely that their effects are additive manner and therefore will

cause pharmacological effects in fish downstream of STWs (Fick *et al*., 2010). It has been concluded in a number of papers that it is possible for synthetic hormones to accumulate (Larrson 1999; Houtmann *et al* 2004; Gelsleichter, 2009; Fick *et al* 2010), this is also supported and by the analyses in this paper. Also, it is likely that some will bioconcentrate to concentrations close to the human therapeutic levels (Fick *et al*., 2010).

1.3.9 Other pharmaceuticals

Anxiolytic compounds and their metabolites have been identified in final effluents and fresh waters (Monteiro and Boxall, 2010) and recently in fish (Kwon *et al*., 2008). Kwon *et al*. (2008) analysed the livers of marine fish, P. verticalis for a number of pharmaceuticals. The fish were collected from the sites near to ocean discharges of wastewater effluent. Diazepam was detected in the liver tissue of all ten fish samples. Concentrations of diazepam were in the range 23 to 45 ng g^{-1} in females and 58 to 110 ng g^{-1} for males. Oxazepam (an active metabolite of diazepam but also an pharmaceutical in its own right) was detected in all fish in the study by Fick *et al*. (2010). Despite the high exposure concentrations (701 to 881 ng L^{-1}) the concentration of oxazepam (0.2 to 0.7 ng g^{-1}) in fish plasma was low, with calculated BCFs of 0.7, 3.5 and 3.6.

Psycho-active pharmaceuticals are a large group of pharmaceuticals including antidepressants discussed earlier in section 1.3.3 on page 37. Anti – psychotic medicines used as tranquilizers in the treatment of schizophrenia and delirium (BNF, 2009). Fick *et al*. (2010) detected psycho-active pharmaceuticals in the plasma of *O. mykiss* when the fish were exposed to STW effluent. In the semi-field exposure, *O. mykiss* accumulated 1.2 ng g^{-1} of haloperidol and 0.3 – 2.4 ng g^{-1} of risperidone in the plasma. Haloperidol was present at detectable concentrations in the effluent at a maximum concentration of 374 ng L^{-1}, but risperidone was below the detection limit in the effluent and therefore may be accumulating in fish.

Memantine is drug used for the treatment of dementia specifically for Alzheimer's disease and was also detected in the fish by Fick *et al*. (2010). Plasma concentrations of 2.3 ng g^{-1} in *O. mykiss* were recorded after exposure to 14 ng L^{-1} of memantine. The concentration of memantine in fresh waters in the U.K. will be lower than the USA and as the National Institute for Clinical Excellence does not recommend its use and it contributes only 4 % of the total medicines prescribed for dementia (NICE, 2009). Other psycho active pharmaceuticals such as caffeine and paraxanthine as well as those from other classes have not been detected in fish tissues (Ramirez *et al*., 2009).

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
Analgesic & Anti-inflammatory						
Fish						
ibuprofen	*Oncorhynchus mykiss*	laboratory	Muscle	ca 5	1.50 ± 0.25	Zhang *et al.,* 2010
ibuprofen	*Oncorhynchus mykiss*	laboratory	adipose	ca 68	23.69 ± 2.23	Zhang *et al.,* 2010
ketoprofen	*Oncorhynchus mykiss*	laboratory	plasma	15 – 107	3.5 – 48	Fick *et al.,* 2010
naproxen	*Oncorhynchus mykiss*	field	plasma	< LOQ – 14	< 2 – 56	Brown *et al.,* 2007
naproxen	*Oncorhynchus mykiss*	laboratory	plasma	33 – 46	22 – 28	Fick *et al.,* 2010
tramadol	*Oncorhynchus mykiss*	laboratory	plasma	1.1 – 1.9	2.3 – 3.3	Fick *et al.,* 2010
Analgesic & Anti-inflammatory Metabolites						
4'- hydroxydiclofenac	*Oncorhynchus mykiss*	laboratory	bile	20 – 70		Kallio *et al.,* 2010
5 - hydroxydiclofenac	*Oncorhynchus mykiss*	laboratory	bile	nd – 40		Kallio *et al.,* 2010
acyl glucuronide of diclofenac	*Oncorhynchus mykiss*	laboratory	bile	nd – 220		Kallio *et al.,* 2010
acyl glucuronide	*Oncorhynchus mykiss*	laboratory	Bile	ca 290 – ca 620		Kallio *et al.,* 2010

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
Analgesic & Anti-inflammatory						
Fish						
acyl glucuronide of 5- hydroxydiclofenac	*Oncorhynchus mykiss*	laboratory	bile	ca 90 – ca 400		Kallio *et al.,* 2010
acyl glucuronide of 3'- hydroxydiclofenac	*Oncorhynchus mykiss*	laboratory	bile	nd – ca 120		Kallio *et al.,* 2010
ether glucuronide of 4'- hydroxydiclofenac	*Oncorhynchus mykiss*	laboratory	bile	nd – ca 40		Kallio *et al.,* 2010
sulphate conjugate of 5 - hydroxydiclofenac	*Oncorhynchus mykiss*	laboratory	bile	ca 20 – ca 50		Kallio *et al.,* 2010
Anti-biotic						
Plants						
florfenicol	*Fontinalis antipyretica*	field	whole body	ca 120		Pouliquen *et al.,* 2009
flumequine	*Fontinalis antipyretica*	field	whole body	ca 600		Pouliquen *et al.,* 2009
flumequine	*Lythrum salicaria*	laboratory	whole body	200 – 13300		Migloire *et al.,* 2000

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Anti-biotic			
			Plants			
oxolinic acid	*Fontinalis antipyretica*	field	whole body	47		Pouliquen *et al.,* 2009
oxytetracyline	*Fontinalis antipyretica*	field	whole body	ca 1000		Pouliquen *et al.,* 2009
			Anti-depressant			
			Fish			
citalopram	*Carcharhinus leucas*	field	plasma	ND – 0.57		Gelsleichter 2009
citalopram	*Catostomus commersonii*	field	brain	ND – 0.21		Schultz *et al.,* 2010
fluoxetine	Three fish species	field	brain	1.58 ± 0.74		Brook *et al.,* 2005
fluoxetine	Three fish species	field	liver	1.34 ± 0.65		Brook *et al.,* 2005
fluoxetine	Three fish species	field	muscle	0.11 ± 0.03		Brook *et al.,* 2005
fluoxetine	*Dorosoma cepedianum*	field	whole body	0.16 – 1.02		Chu and Metcalfe 2007
fluoxetine	*Ameiurus nebulosus*	field	whole body	0.14 – 0.31		Chu and Metcalfe 2007
fluoxetine	*Micropterus salmoides*	field	liver	19		Ramirez *et al.,* 2009
fluoxetine	*Catostomus commersonii*	field	liver	70		Ramirez *et al.,* 2009

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Anti-depressant			
			Fish			
fluoxetine	*Catostomus commersonii*	field	brain	ND – 1.65		Schultz *et al.,* 2010
fluoxetine	*Oryzias latipes*	laboratory	whole body		8.8	Nakamura *et al.,* 2008
fluoxetine	*Oryzias latipes*	laboratory	whole body		30	Nakamura *et al.,* 2008
fluoxetine	*Oryzias latipes*	laboratory	whole body		260	Nakamura *et al.,* 2008
fluoxetine	*Oryzias latipes*	laboratory	liver		330	Nakamura *et al.,* 2008
fluoxetine	*Oryzias latipes*	laboratory	liver		580	Nakamura *et al.,* 2008
fluoxetine	*Oryzias latipes*	laboratory	liver		3100	Nakamura *et al.,* 2008
fluoxetine	*Oryzias latipes*	laboratory	whole body	49.4 ± 6.4	74 – 80	Paterson and Metcalfe 2008
fluoxetine	*Oncorhynchus mykiss*	laboratory	muscle		58.98 ± 16.81	Zhang *et al.,* 2010
fluoxetine	*Oncorhynchus mykiss*	laboratory	adipose		143.36 ± 21.50	Zhang *et al.,* 2010
fluvoxamine	*Carcharhinus leucas*	field	plasma	ND – 0.9		Gelsleichter 2009
paroxetine	*Dorosoma cepedianum*	field	whole body	ND – 0.58		Chu and Metcalfe 2007
paroxetine	*Ameiurus nebulosus*	field	whole body	ND – 0.48		Chu and Metcalfe 2007

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Anti-depressant			
			Fish			
paroxetine	*Carcharhinus leucas*	field	plasma	ND – 0.55		Gelsleichter 2009
paroxetine	*Catostomus commersonii*	field	brain	ND – 0.11		Schultz *et al.,* 2010
sertraline	Three fish species	field	brain	4.27 ± 1.4		Brooks *et al.,* 2005
sertraline	Three fish species	field	liver	3.59 ± 1.67		Brooks *et al.,* 2005
sertraline	Three fish species	field	muscle	0.34 ± 0.09		Brooks *et al.,* 2005
sertraline	*Carcharhinus leucas*	field	plasma	ND – 0.97		Gelsleichter 2009
sertraline	*Cyprinus carpio*	field	muscle	5.0		Ramirez *et al.,* 2009
sertraline	*Catostomus commersonii*	field	muscle	11		Ramirez *et al.,* 2009
sertraline	*Micropterus salmoides*	field	liver	84		Ramirez *et al.,* 2009
sertraline	*Ictiobus bubalus*	field	liver	27		Ramirez *et al.,* 2009
sertraline	*Amia calva*	field	liver	21		Ramirez *et al.,* 2009
sertraline	*Cyprinus carpio*	field	liver	71		Ramirez *et al.,* 2009
sertraline	*Catostomus commersonii*	field	liver	381		Ramirez *et al.,* 2009

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Anti-depressant			
			Fish			
sertraline	*Catostomus commersonii*	field	brain	ND – 4.24		Schultz *et al.,* 2010
sertraline	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 1.2	138 – > 240	Fick *et al.,* 2010
venlafaxine	*Carcharhinus leucas*	field	plasma	ND – 0.56		Gelsleichter 2009
venlafaxine	*Catostomus commersonii*	field	brain	ND – 1.12		Schultz *et al.,* 2010
			Anti-depressant metabolite			
norfluoxetine	*Dorosoma cepedianum*	field	whole body	ND – 1.08		Chu and Metcalfe 2007
norfluoxetine	*Ameiurus nebulosus*	field	whole body	ND – 0.19		Chu and Metcalfe 2007
norfluoxetine	*Carcharhinus leucas*	field	plasma	ND – 4.08		Gelsleichter 2009
norfluoxetine	Three fish species	field	brain	8.86 ± 5.9		Brooks *et al.,* 2005
norfluoxetine	Three fish species	field	liver	10.27 ± 5.73		Brooks *et al.,* 2005
norfluoxetine	Three fish species	field	muscle	1.07 ± 0.41		Brooks *et al.,* 2005
norfluoxetine	*Lepomis sp.*	field	muscle	3.49 – 5.14		Ramirez *et al.,* 2007
norfluoxetine	*Micropterus salmoides*	field	muscle	3.2		Ramirez *et al.,* 2009

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Anti-depressant metabolite			
			Fish			
norfluoxetine	*Ictiobus bubalus*	field	liver	37		Ramirez *et al.,* 2009
norfluoxetine	*Cyprinus carpio*	field	muscle	4.0		Ramirez *et al.,* 2009
norfluoxetine	*Catostomus commersonii*	field	muscle	3.9		Ramirez *et al.,* 2009
norfluoxetine	*Micropterus salmoides*	field	liver	73		Ramirez *et al.,* 2009
norfluoxetine	*Amia calva*	field	liver	57		Ramirez *et al.,* 2009
norfluoxetine	*Cyprinus carpio*	field	liver	33		Ramirez *et al.,* 2009
norfluoxetine	*Catostomus commersonii*	field	liver	38		Ramirez *et al.,* 2009
norfluoxetine	*Catostomus commersonii*	field	brain	ND – 3.57		Schultz *et al.,* 2010
norsertraline	Three fish species	field	liver	12.94 ± 10.45		Brooks *et al.,* 2005
norsertraline	Three fish species	field	brain	15.6 ± 14.3		Brooks *et al.,* 2005
norsertraline	Three fish species	field	muscle	0.69 ± 0.59		Brooks *et al.,* 2005
norsertraline	*Catostomus commersonii*	field	brain	ND – 28.9		Schultz *et al.,* 2010

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Anti-epileptic			
			Fish			
carbamazepine	*Lepomis sp.*	field	muscle	0.83 – 1.44		Ramirez *et al.,* 2007
carbamazepine	*Micropterus salmoides*	field	muscle	2.3		Ramirez *et al.,* 2009
carbamazepine	*Micropterus salmoides*	field	liver	6		Ramirez *et al.,* 2009
carbamazepine	*Oncorhynchus mykiss*	laboratory	plasma	0.3 – 1.0	0.8 – 4.2	Fick *et al.,* 2010
carbamazepine	*Oncorhynchus mykiss*	laboratory	muscle		0.52 ± 0.11	Zhang *et al.,* 2010
carbamazepine	*Oncorhynchus mykiss*	laboratory	adipose		4.16 ± 0.87	Zhang *et al.,* 2010
carbamazepine	*Oncorhynchus mykiss*	laboratory	muscle		0.22 – 0.44	Zhou *et al.,* 2008
			Algae			
carbamazepine	*Psuedokirchneriella subcapita*	laboratory	whole body	10.2 mgg	2.2	Vernouillet *et al.,* 2010
			Invertebrate			
carbamazepine	*Thamnocephalus platyurus*	laboratory	whole body	128.6 mgg	12.6	Vernouillet *et al.,* 2010

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Fish			
			Anti-histamine			
diphenhydramine	*Lepomis sp.*	field	muscle	0.66 – 1.32		Ramirez *et al.,* 2007
diphenhydramine	*Micropterus salmoides*	field	muscle	1.4		Ramirez *et al.,* 2009
diphenhydramine	*Cyprinus carpio*	field	muscle	1.2		Ramirez *et al.,* 2009
diphenhydramine	*Catostomus commersoni*	field	muscle	1.7		Ramirez *et al.,* 2009
diphenhydramine	*Micropterus salmoides*	field	liver	7		Ramirez *et al.,* 2009
diphenhydramine	*Ictiobus bubalus*	field	liver	0.5		Ramirez *et al.,* 2009
diphenhydramine	*Cyprinus carpio*	field	liver	7		Ramirez *et al.,* 2009
diphenhydramine	*Catostomus commersoni*	field	liver	10		Ramirez *et al.,* 2009
diphenhydramine	*Catostomus commersoni*	field	muscle	0.032 ± 0.005		Zhou *et al.,* 2008
diphenhydramine	*Etheostoma nigrum*	field	muscle	0.081.63 ± 0.008		Zhou *et al.,* 2008
meclozine	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 0.7	> 200 – > 1400	Fick *et al.,* 2010
orphenadrine	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 0.9	< 100	Fick *et al.,* 2010

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Fish			
			Anti-hypertensive			
atenolol	*Pimephales promelas*	laboratory	plasma	4.2 – 291		Winter *et al.,* 2008
cilazapril	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 0.7	> 100 – > 700	Fick *et al.,* 2010
diltiazem	*Lepomis sp.*	field	muscle	0.11 – 0.27		Ramirez *et al.,* 2007
diltiazem	*Micropterus salmoides*	field	muscle	0.13		Ramirez *et al.,* 2009
diltiazem	*Catostomus commersoni*	field	muscle	0.15		Ramirez *et al.,* 2009
diltiazem	*Micropterus salmoides*	field	liver	0.7		Ramirez *et al.,* 2009
diltiazem	*Cyprinus carpio*	field	liver	0.3		Ramirez *et al.,* 2009
diltiazem	*Catostomus commersoni*	field	liver	0.7		Ramirez *et al.,* 2009
diltiazem	*Catostomus commersoni*	field	muscle	0.002 ± 0.0006		Zhou *et al.,* 2008
diltiazem	*Etheostoma nigrum*	field	muscle	0.0056 ± 0.0004		Zhou *et al.,* 2008
diltiazem	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 0.9	24	Fick *et al.,* 2010
propanalol	*Oncorhynchus mykiss*	laboratory	plasma	0.94-5200 ± 1333		Owen *et al.,* 2009
verapamil	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 0.7	175	Fick *et al.,* 2010

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Fish			
			Lipid regulators			
gemfibrozil	*Oncorhynchus mykiss*	field	plasma	< LOQ – 210	2 – 199	Brown *et al.,* 2007
gemfibrozil	*Cyprinus carpio*	field	liver	70		Ramirez *et al.,* 2009
gemfibrozil	*Catostomus commersoni*	field	liver	27.1		Ramirez *et al.,* 2009
gemfibrozil	*Carassius aratus*	laboratory	plasma	170 ± 20 – 78000 ± 5000	16 – 89	Mimeault *et al.,* 2005
gemfibrozil	*Oncorhynchus mykiss*	laboratory	muscle		0.4 ± 0.21	Zhang *et al.,* 2010
gemfibrozil	*Oncorhynchus mykiss*	laboratory	adipose		20.75 ± 3.74	Zhang *et al.,* 2010
			Synthetic Hormones			
17 *a*-ethynylestradiol	*Moxostoma macrolepidotum*	field	whole body	0.48 – 2.30		Al-Ansari *et al.,* 2010
17 *a*-ethynylestradiol	*Carcharhinus leucas*	field	plasma	ND – 3.79		Gelsleichter 2009
17 *a*-ethynylestradiol	*Abramis brama*	field	bile	17		Houtman *et al.,* 2004
17 *a*-ethynylestradiol	*Oncorhynchus mykiss*	field	bile	300 – 1200		Larsson *et al.,* 1999

Table 1.2 Tissue concentration, bioconcentration and bioaccumulation literature data (1999-2010) for pharmaceuticals in aquatic species

Compound	Species	Study type	Tissue type	Tissue concentration (ng g^{-1})	BAF/BCF	Reference
			Fish			
			Synthetic Hormones			
17 *a*-ethynylestradiol	*Oncorhynchus mykiss*	laboratory	bile	360000		Larsson *et al.,* 1999
17 *a*-ethynylestradiol	*Rutilis rutilis*	laboratory	bile	59000 – 2388000		Flores-Valverde *et al.,* 2010
17 *a*-ethynylestradiol	*Pimephales promelas*	laboratory	whole body	< 0.38 – 31	610 – < 2400	Lange *et al.,* 2001
levonorgestrel	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 12	> 8500 – 12000	Fick *et al.,* 2010
			Other Pharmaceuticals			
diazepam	*Pleuronichthys verticalus*	field	liver	23 - 110		Kwon *et al.,* 2009
haloperidol	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 1.2	3.2	Fick *et al.,* 2010
memantine	*Oncorhynchus mykiss*	laboratory	plasma	< LOQ – 2.3	164	Fick *et al.,* 2010
oxazepam	*Oncorhynchus mykiss*	laboratory	plasma	0.2 – 0.7	0.7 – 3.6	Fick *et al.,* 2010
risperidone	*Oncorhynchus mykiss*	laboratory	plasma	0.3 – 2.4	> 60 – > 480	Fick *et al.,* 2010

1.4 Factors influencing the uptake and bioconcentration of pharmaceuticals in aquatic organisms

There are many factors and processes that influence uptake and bioconcentration of pharmaceuticals in aquatic organisms, these can be environmental, chemical and biological (table 1.3). There are some data available for pharmaceuticals (see previous section) however information on factors and processes will be drawn from a variety of chemical classes and included in the following sections.

Table 1.3 Summary of the factors that can influence uptake and bioconcentration in aquatic organisms

Factors influencing uptake		
Environmental	Chemical	Biological
Climate – temperature, rainfall, season	Chemical form	Size
Water quality – pH, dissolved oxygen, hardness, organic matter content	Ionization potential	Growth
	Steric properties –size & shape	Life stage
	Hydrophobicity	Lipid content
	Bioavailability	Metabolic processes
		Behaviour
		Disease status

1.4.1 Environmental factors

Uptake and elimination are strongly influenced by local environmental conditions; factors such as climate (e.g. season, rainfall and temperature) and water quality (hardness, dissolved oxygen and pH) can heavily impact upon bioconcentration. Season can influence bioconcentration by both changing the exposure conditions (e.g. concentration) and by altering the organisms biological processes involved in uptake (e.g. growth).

Passive diffusion, which is thought to be the main process for uptake is dictated by the exposure concentration, the amount of pharmaceutical loaded into the fresh water along with hydrological processes such as flow rates and dilution can change

the exposure concentration throughout the year (Ashton *et al.*, 2004, Gelsleichter, 2009). Greater flow conditions in rivers and streams will increase the dilution of a chemical contaminant, reducing its concentration in the water and thus decreasing the uptake of pharmaceuticals compounds into organisms (Vieno *et al.*, 2005, Powell *et al.*, 2009). By contrast low flows will increase the proportion of compounds in effluent dominated streams (Loraine and Pettigrove, 2005, Peng *et al.*, 2008). In one study by Kolpin *et al.* (2004) antibacterial drugs and other prescription pharmaceuticals were frequently detected during low flow conditions but not during high flow conditions. In winter time, rivers affected by the presence of snow and ice can have high concentrations of pharmaceuticals due to low flow conditions in addition to decreased removal efficiency of STWs (Vieno *et al.*, 2005, Daneshvar *et al.*, 2010). Daneshvar *et al.* (2010) found that concentrations of ibuprofen, naproxen, diclofenac, and ketoprofen in effluent were at their highest (31 to 1,852 ng L^{-1}) during winter, whilst bezafibrate, a lipid regulator was observed at higher concentrations in spring. Peng *et al.* (2008) found the detection frequencies of twenty pharmaceuticals and personal care products increased in periods of low flow where rainfall was less. Some hormonally active compounds such as 17α-ethinylestradiol and estriol were only detected in low flow conditions.

The temperature of fresh water is dependent on the season and has several impacts on the exposure of organisms to compounds. Temperature can influence partitioning of compounds between the aqueous and the particulate phases in water (Borgå *et al.*, 2010, Haftka *et al.*, 2010, Hallanger *et al.*, 2011). Partitioning of compounds to particulate and dissolved matter is intimately linked to the bioavailability of a compound for uptake by aquatic organisms (Böhm and Düring, 2010, Borgå *et al.*, 2010). Reduced bioavailability due to increased partitioning to the particulate phase of the water has been demonstrated for a number of compounds including PCBs (Jota and Hassett, 1991, Akkanen and Kukkonen, 2003, Böhm and Düring, 2010, Haftka *et al.*, 2010, Ruotsalainen *et al.*, 2010). Belden *et al.* (2007) demonstrated that partitioning of ciprofloxacin an antibiotic occurs with both coarse particulate organic matter and fine particulate organic matter. The partitioning process is also influenced by the age, geochemical origin, and history of the organic material (Tang and Weber, 2006). Many organisms rely on the organic fraction in water as a food resource therefore the partitioning of a pharmaceutical to organic material may influence the route of uptake into an organism.

Climatic conditions not only influence the exposure concentration but the biological processes within the organism. When seasonality influences flow rates in rivers it

also influences an organism's physiology, biochemistry and behaviour. In wetter conditions there will be larger volumes of water in rivers and greater flow conditions. This may influence uptake by changing the respiration rates of fish, through directly affecting the compound exchange between the water and the respiratory surface (Powell *et al.*, 2009). Variation of temperature significantly affects the rates of feeding, growth, and lipid deposition in fish and this consequently affects the uptake and deposition of compounds such as pharmaceuticals (Brecken-Folse *et al.*, 1994). In general, an increase in the temperature will increase the rates of uptake and elimination (Barron, 1990, Nawaz and Kirk, 1996). Typically a 10 °C increase in constant temperature results in a 2-fold increase in uptake of pollutants such as PCBs, DDT, and methylmercury from food or water (Spigarelli *et al.*, 1983). Honkanen and Kukkonen (2006) found that the uptake rate of bisphenol-A into the common frog, *Rana temporaria* increased at higher temperatures compared to lower temperatures. A similar pattern was recorded in a study by Brown *et al.* (2007), where lower temperatures were thought to decrease the uptake of pharmaceuticals in the field. Difference in the uptake at different sites with similar exposure concentrations was attributed to lower ventilation and metabolic rates of the fish at lower temperatures (Brown *et al.*, 2007). The effects of temperature on uptake and bioconcentration are dependent on the organisms and the compound studied. Other temperature dependent processes involved in uptake include diffusion through cytosol, solvation, protein-binding, and changes in the permeability of tissue membranes because of changes to the lipid composition necessary to maintain membrane fluidity (Barron, 1990).

Water quality parameters such as water hardness, salinity, dissolved oxygen content and pH are thought to affect the uptake of compounds in organisms. It is currently thought that as water hardness increases the bioconcentration of compounds increases (Tolls *et al.*, 1994). An increase from low to intermediate water hardness increases the uptake rate of linear alkylbenzenesulfonate (a surfactant) in *O. mykiss* (Tolls *et al.*, 1999). This may be due to water hardness decreasing the partitioning efficiency of a variety of compounds to organic matter. As discussed previously the partitioning of compounds reduces their bioavailability and therefore bioconcentration and uptake from the water (Penttinen *et al.*, 1995, Tolls *et al.*, 1999, Akkanen and Kukkonen, 2001).

Water bodies that are in coastal and especially estuarine areas experience a variety of salinities (Jeon *et al.*, 2010). Salinity is an important factor that may influence the bioconcentration of a variety of compounds in organisms. Brecken-Folse *et al.*

(1994) found that bioconcentration of trichlorfon in Grass shrimps and Sheephead minnows tended to decrease with increasing salinity, but in the same study no effect was shown for nitrophenols. Jeon *et al.* (2010) found that uptake and elimination of polyfluorinated compounds increased with increasing salinity. As freshwater fish are hyperosmotic to the environment the diffusion of water may facilitate uptake of chemicals into to the organism (Barron, 1990).

Temporal depletion of oxygen content in fresh waters occurs frequently (Hattink *et al.*, 2005). Fish have developed mechanisms to manage hypoxia that involve changing physiological and behavioural responses to increase oxygen transport to the gills. The two main strategies are to increase ventilation rates and decrease metabolic activity to conserve energy when hypoxia is prolonged (Lloyd, 1961, McKim and Goeden, 1982). When compounds such as pharmaceuticals are present in the water, increasing ventilation rates and reducing metabolic activity may result in increased compound stress due to increased uptake (Lloyd, 1961). However the evidence surrounding this remains unclear (Brian *et al.*, 2008).

Pharmaceuticals are ionic compounds and uptake in organisms can be very sensitive to changes in environmental pH. The atmospheric transport of acidifying substances (e.g., CO_2, SO_2 and NO_2) and the effects of low pH on biota are well-known (Larsson *et al.*, 1991, Arnold *et al.*, 2009). Many organic compounds that are globally distributed are un-ionised. However many pharmaceuticals ionisable which means that their behaviour will be influenced by the pH of the environment. Studies with traditional ionisable pollutants such as pentachlorophenol show that bioconcentration is very different in acidified waters compared to non-acidified waters (Larsson *et al.*, 1991). The ionisation state of the compound is co-dependent on the chemistry of the pharmaceutical so this topic will be discussed in more detail in the next section.

There is currently limited information available on the influence of environmental fluctuations on uptake and bioconcentration of pharmaceuticals in the environment, this subject will become increasingly important in view of our changing climate (Arnold *et al.* 2009).

1.4.2 Chemical factors

Bioconcentration and uptake of compounds is generally assumed to be a thermodynamically driven process of exchange between the water and lipids of

organisms (Walker, 1990, Meylan *et al.*, 1999, Gatermann *et al.*, 2002, Barber, 2003). It has been reported that the uptake and elimination of both endogenous compounds (such as bile acids) and exogenous compounds (such as pharmaceuticals) can occur through carrier mediated transport (Yamazaki *et al.*, 1996); however, the exact uptake mechanism for pharmaceuticals is unknown. It is well known that physicochemical properties of the compound will profoundly affect the toxicokinetics of a substance (Arnot *et al.*, 2010). The factors that are important in determining the bioconcentration of the compound are the electronic, hydrophobic and steric factors (Barron, 1990).

In fish there are several anionic, cationic and neutral transporters that permit active and facilitated diffusion (Kallio *et al.*, 2010). As diffusion across cellular membranes is concentration dependent, it will be influenced by the compounds solubility and molecular form. Pharmaceuticals as a group of compounds vary in both of these properties; for example carvedilol has a solubility of 0.5 mg L^{-1}, diazepam has a solubility of 50 mg L^{-1} and the solubility of rifampicin can vary depending on its crystalline state by a factor of 8 (195 – 1576 mg L^{-1}). Various different salts are commonly used as anionic surfactants in pharmaceutical formulations which may have different physicochemical properties compared to the API itself. For example docusate sodium is used in pharmaceuticals formulations and is not readily biodegradable and with a log K_{ow} of 6 it is indicative of a bioaccumulative substance (Carlsson *et al.*, 2006). It has been theorized that surfactants may enhance the bioconcentration of ibuprofen in *O. mykiss* (Jones-Lepp and Stevens, 2007). Therefore, measuring and estimating the APIs potential to bioconcentrate or bioaccumulate in organisms, using single compound studies, may not reflect realistic exposure to pharmaceutical formulations.

Cellular membranes are comprised of phospholipids and therefore uptake of a substance is influenced by its lipophilicity: moderately lipophilic compounds readily diffuse across membranes and will be accumulate *via* aqueous uptake, but highly lipophilic compounds will accumulate *via* dietary uptake (Barber, 2008). Lipophilicity is generally expressed in terms of the octanol/water partition coefficient (K_{ow}) and has proved to be an invaluable parameter in bioaccumulation modelling (Nuutinen *et al.*, 2003b, Giulio and Hinton, 2008, Powell *et al.*, 2009, Borgå *et al.*, 2010). Linear relationships between lipophilicity and both uptake rate and bioconcentration have been developed (Álvarez-Muñoz *et al.*, 2010). Substances with high K_{ow} coefficients have high absorption and low elimination rates in a variety of organisms. A fair approximation of the bioaccumulative nature of a substance can be made using the

K_{ow} coefficients for compounds with log K_{ow} coefficients between 1 and 6 for non-ionic and non-metabolised substances (Meylan *et al.*, 1999). However for very lipophilic compounds the relationship breaks down as they often have lower BCFs and BAFs (Meylan *et al.*, 1999, Borgå *et al.*, 2010). The breakdown in the relationship is thought to be due to lack of compound in the aqueous elements of the lipid membrane and aqueous resistance limiting the diffusion (Arnot *et al.*, 2010).

Evidence suggests that non-ionised substances diffuse more easily across membranes than ionised substances, and there is some discussion in the literature as to whether K_{ow} is likely to be a good descriptor for the uptake of many pharmaceuticals which are ionisable (Daughton and Brooks, 2011). Zhang *et al.* (2010) found accumulation of gemfibrozil, ibuprofen, carbamazepine and fluoxetine in *O. mykiss* was a function of the K_{ow} but also suggested that for some compounds, such as fluoxetine, protein binding played an important role. The collation of data from the literature suggests that lipophilicity of a pharmaceuticals has a weak relationship with bioconcentration (R^2 = 0.39, figure 1.5). Therefore other factors and processes may influence bioconcentration. Wu *et al.* (2010b) suggested that uptake of ionisable compounds into plants is influenced by a combination of physicochemical properties including lipophilicity and the compounds dissociation constant (pK_a). The BCF is influenced by the pH of the exposure water if the compound has ionization potential. A good example of this pH dependence is presented by Nakamura *et al* (2008) where the extent of uptake of fluoxetine (pK_a 10) changes with the water alkalinity (figure 1.4, page 40).

The epithelium of organisms is more permeable to the non-ionised, neutral form of compounds. However, the internal pH of an organism is different from the external environment so that neutral compounds which diffuse easily into a cell can become ionised inside the cell and become trapped. This mechanism, known as ion-trapping, can lead to differences in bioconcentration depending on the pH (Lo and Hayton, 1981, Hayton and Schultz, 1991, Trapp *et al.*, 2010, Neuwoehner and Escher, 2011).

A better parameter for estimating uptake of ionisable compounds may be the $D_{ow.}$ The D_{ow} accounts for difference in partitioning of the neutral and ionic species of a molecule at a particular pH value. D_{ow} is calculated using a combination of the pK_a and the K_{ow} by using the Henderson Hasselbach equation (page 89; Po and Senozan, 2001).

Several other molecular parameters such as size and shape have been used to describe the process of passive diffusion across cellular membranes (Arnot *et al.*, 2010). Bioconcentration of polychlorinated naphthalene and biphenyls directly depend on their molecular size rather lipophilicity (Barron, 1990). Although lipophilic these compounds do not readily diffuse through lipid membranes due to their large size. It is thought that the tightly packed lipid membrane of organisms will act as a filter for molecules above a certain size (Arnot *et al.*, 2010). Structure-activity relationships have therefore been proposed that use molecular size to identify bioaccumulative compounds (Qin *et al.*, 2009, Nendza and Müller, 2010).

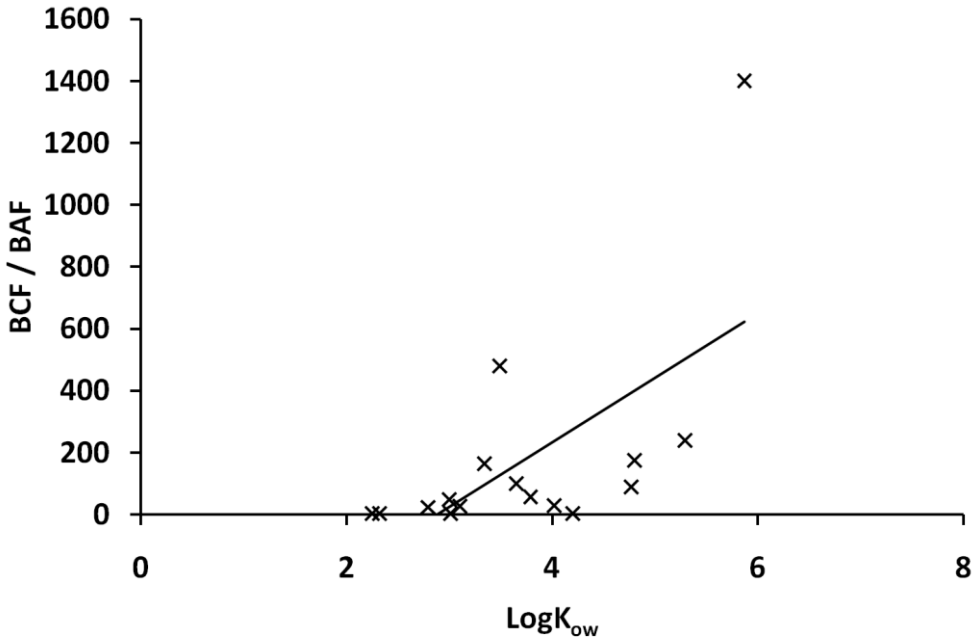


Figure 1.5. The linear relationship between pharmaceutical bioconcentration /bioaccumulation factors and octanol – water partition coefficient. Data for fish measured in the laboratory (n =16) ; regression relationship: BCF= 208.04 log K_{ow} - 598.03, R^2 = 0.39).

The structure of the lipid membrane may also limit the uptake of some molecules of long chain length and or large cross sectional area (Barron, 1990, Martin *et al.*, 2003). And steric properties such as maximum molecular length and maximum cross-sectional diameter will limit the diffusion of a compound (Dimitrov *et al.*, 2002, Sakuratani *et al.*, 2008, Dimitrova *et al.*, 2010, Louwen and Stedeford, 2011).

The physicochemical properties of pharmaceuticals will play an important role in the uptake, distribution and elimination of these compounds in biological tissues. Relationships already established for non-ionisable contaminants may not be appropriate to describe those for pharmaceuticals. Therefore, more research is needed to refine the prediction of bioaccumulation in these ionisable compounds.

1.4.3 Biological factors

The significance of biological factors should be taken into account when considering the bioconcentration of compounds (Leppänen, 1995). Diversity in biology and ecology play a fundamental role in uptake and elimination of contaminants (Larsson *et al.*, 1991, Li *et al.*, 2009, Rubach *et al.*, 2010, Rubach *et al.*, 2011). The bioconcentration of many aquatic contaminants is organism dependent (Davies and Dobbs, 1984, Barron, 1990). Different factors such as size, lipid content, respiration strategy, feeding strategy and habit as well as metabolic processes will all be controlling factors in uptake and bioconcentration (Barron, 1990).

Size has been recognized as important in determining physiology and anatomy and has been shown to influence processes such as uptake and bioconcentration (Barron, 1990). Organisms size has been negatively correlated with absorption and elimination rates of substances in both aquatic and terrestrial organisms (Hendriks and Heikens, 2001, Ashauer *et al.*, 2010). Hendriks and Heikens (2001) found significant relationships between the weight of an organism and internal concentrations of 14 compounds. However both positive and negative correlations were observed, and the small slopes of the relationships between wet weight and internal concentration indicated that the relationships were not ecotoxicologically relevant (Ashauer *et al.*, 2010).

The lipid content of the exposed organism plays a role in the bioconcentration of many contaminants because uptake is considered to be a partitioning process between lipids of the organism and the ambient water. Clear relationships between bioconcentration of neutral contaminants and lipid content have previously been established (Geyer *et al.*, 2000). This has been shown to be the case for pharmaceuticals as well. Recently Al-Ansari *et al.* (2010) found a significant positive correlation between the tissue concentration of the synthetic hormone 17 α-ethinylestradiol and lipid content of *Moxostoma macrolepidotum* the Red horse sucker fish. Also, Zhang *et al.* (2010) found that the higher lipid percentages in the

adipose tissue compared to the dorsal muscle of *O. mykiss* contributed to the differential concentrations of several pharmaceuticals.

The gill respiratory surface provides a relatively large exchange epithelium for the uptake of contaminants. Respiration strategies play an important role in providing vast differences among organisms in terms of physiological contact with the surrounding water (Buchwalter *et al.*, 2003). Buchwalter *et al.* (2002) found that respiratory strategy was a major determinant of chlorpyrifos in aquatic insects. Several studies have shown that organisms that have large gas exchange epitheliums are more susceptible to contaminant accumulation (Buchwalter *et al.*, 2003, Buchwalter *et al.*, 2004, Buchwalter *et al.*, 2008). Currently there is no data available for the impact of these traits on the uptake and bioconcentration of pharmaceuticals in non-target organisms.

Feeding strategy should be considered when studying bioaccumulation. Feeding habits such as feeding rate and food source are important regulating factors in bioaccumulation (Leppänen, 1995). Feeding habits have been shown to influence the accumulation pattern of chemicals such as PCBs (Zhou *et al.*, 1999, Lee *et al.*, 2000). Carnivorous and omnivorous fish were more susceptible to PCB bioaccumulation than filter feeders (Zhou *et al.*, 1999). This is also true for invertebrate organisms where different bioaccumulation patterns have been determined depending on the feeding type of the organisms. The feeding strategies studied have included engulfers, piercers, filter feeders and deposit feeders (Lee *et al.*, 2000, Brooks *et al.*, 2009).

1.4.3.1 Metabolism

Pharmaceuticals are absorbed and distributed from the site of adsorption to the site of metabolism. Metabolism is the conversion of one chemical entity to another in the body by enzymes (Rang *et al.*, 2007). Metabolic processes such as the nature and activity of detoxification enzymes and hence biotransformation routes can influence the toxicokinetics of contaminants. Drug metabolism in humans occurs mainly in the liver by the cytochrome P450 enzyme system (CYP – 450). The animal kingdom has evolved complex metabolic systems that can detoxify xenobiotics including pharmaceuticals (Rand and Dales, 2008). Metabolism involves two types of reaction known as phase I and phase 2 which will be discussed further in Chapter 3 (see page 114). After performing its therapeutic effect, the pharmaceutical is usually

metabolised to a more hydrophilic substance and excreted. Metabolism can substantially increase the elimination of substances by increasing solubility and creating conjugates that are substrates for active transport systems (Giulio and Hinton, 2008).

Our current knowledge of metabolism and detoxification pathways for pharmaceuticals in mammals is extensive due to preclinical and clinical data sets required for marketing a pharmaceutical product (Winter *et al.*, 2010) whilst less is known about metabolism in non-target organisms. Biotransformation of pharmaceuticals has been observed in the laboratory for aquatic organisms, mainly in fish (Paterson and Metcalfe, 2008, Kallio *et al.*, 2010). Schwaiger *et al.* (2004) first suggested that diclofenac could be metabolised in the liver of fish when *O. mykiss* were exposed to 1 μgL^{-1}, diclofenac showed ultra-structural cellular reactions in the liver. This was also suggested in a study by Hoeger *et al.* (2008) where the distribution kinetics of C-14 labeled diclofenac in fish was shown. This study demonstrated that diclofenac undergoes enterohepatic circulation and accumulates in the bile. The author concluded that diclofenac may have incomplete first pass metabolism which results in a higher accumulation. More recently Kallio *et al.* (2010) observed that diclofenac was metabolized in *O. mykiss* to acyl glucuronides of hydroxydiclofenac and one ether glucuronides of hydroxydiclofenac.

Metabolic studies in fish of the anti-depressant fluoxetine has shown that it can be metabolised to its *N*-demethylated derivative norfluoxetine (Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008, Smith *et al.*, 2010). Smith *et al.* (2010) showed that fluoxetine could be metabolised by several species of fish including rainbow trout, goldfish, zebrafish, and killifish. Generally killifish showed higher rates of metabolism and rainbow trout showed a lower capacity for metabolism of fluoxetine. However there was a lot of variability between individual samples and significance of the results could not be established. This study showed that fluoxetine was lost at a greater rate than norfluoxetine was formed, indicating that norfluoxetine may not be the main biotransformation product of fluoxetine in fish. Smith *et al.* (2010) also showed that CYP – 450 can be induced by pre-exposure to carbamazepine but unlike mammals may not be involved in metabolism of fluoxetine in fish.

Pharmaceuticals such as fluoxetine have metabolites with greater elimination half-lives than the parent compound. In mammals fluoxetine has an elimination half-live of 50 hours whereas its metabolite norfluoxetine requires several weeks to be eliminated in mammals (Giulio and Hinton, 2008). The length of elimination half-life

of the metabolite will influence the bioconcentration in the tissue. This is reflected in fish studies where the norfluoxetine bioconcentrates to a greater extent than the parent compound and elimination from the tissue is slower in fish (Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008). Patterson and Metcalfe (2009) showed that fluoxetine has an elimination half-life of 9.5 days in the Japanese Medaka, approximately 4.5 times longer than that of humans (Baldessarini, 2001). This probably indicates that fish have less capacity to metabolise and eliminate fluoxetine and this may be the major reason why this compound bioaccumulates in fish tissue (Paterson and Metcalfe, 2008, Smith *et al.*, 2010). Also pharmaceuticals such as fluoxetine and diclofenac may be metabolised to active metabolites (Baldessarini, 2001, Hoeger *et al.*, 2008). Liver and kidney pathology observed by some authors would certainly suggest that diclofenac could be excreted as a reactive metabolite (Schwaiger *et al.*, 2004, Hoeger *et al.*, 2008). Decreased liver function may also contribute to accumulation in tissues.

Field surveys have detected pharmaceutical biotransformation products such as norfluoxetine and norsertraline, the derivative of sertraline, in wild fish tissue. It is not clear whether these field observations are due to metabolism of the parent compound in fish or to the uptake of human biotransformation products from water. However, laboratory data for fluoxetine and norfluoxetine certainly suggest that detections of metabolites in fish tissue can be at least partly attributed to fish metabolism.

Currently there is limited information regarding biotransformation of pharmaceutical compounds in invertebrates or algae, however many organisms maybe capable of metabolising pharmaceutical contaminants. Invertebrates possess a suite of biotransformation enzymes in the digestive tissue (Livingstone, 1998) and have been shown to metabolise other contaminants (Nuutinen *et al.*, 2003a). More research is needed to elucidate which organisms are capable of metabolising pharmaceutical compounds.

Also, biotransformation rates for any organisms can be useful for predictive modelling of BCFs and BAFs for neutral compounds (Arnot, 2009), as there are biotransformation data available for pharmaceuticals from clinical data these should be used to enhance predictive models for bioconcentration.

1.5 Summary

In the past five years there has been increasing scientific interest in the occurrence of pharmaceuticals in the tissues of aquatic organisms and a number of studies have been published reporting uptake of pharmaceuticals into aquatic and terrestrial organisms. In this chapter the data currently available has been reviewed. Based on the previous Sections, it can be concluded that:

- There are several analytical techniques available for measuring ng g^{-1} concentrations of pharmaceuticals in a variety of biological tissues and there are many factors that affect uptake and bioaccumulation of compounds. These include environmental, chemical and biological factors which lead to inter and intra study variability in values such as tissue concentrations, BCFs and uptake rates.

- BCFs are reported and calculated in a number of ways, some are reported for whole body, some a specific tissue or on a lipid basis. BCFs are calculated using nominal concentrations or measured concentrations and if there is no detectable concentration BCFs will be calculated using the LOQ generating a minimum or maximum value, thus creating variation between studies

- There is data for several pharmaceutical classes however the most studied are hormones and antidepressants. The highest concentration of a pharmaceutical recorded in wild fish was the hormone 17 α - ethinylestradiol and the largest BCF recorded was for ibuprofen. Several pharmaceutical compounds at concentrations below the limits of detection in fresh waters and effluent were found in exposed fish, indicating their potential to bioaccumulate.

- Some data suggests that uptake of pharmaceuticals such as lipid regulators into fish is through passive diffusion over the gills. However, preliminary work shows that some pharmaceuticals such as the SSRIs may have the potential to be selectively accumulated into fish. Therefore facilitated diffusion or active transport may play a role in the uptake of some pharmaceuticals but more research must be completed to elucidate this.

- There are few data on the metabolism of pharmaceutical compounds in aquatic organisms. To date studies have shown that pharmaceuticals, including diclofenac, fluoxetine, sertraline and 17 α - ethinylestradiol, can be metabolized in fish to metabolites that have been characterized using human metabolic pathways. There is a great deal of unpublished information about the metabolism of pharmaceuticals in mammals generated by the pharmaceutical industry that could assist targeted screening approaches for pharmaceutical metabolites.

- There is contradiction in many studies as to whether traditional ways of predicting the uptake in aquatic organisms using the log K_{ow}, would be appropriate for pharmaceuticals compounds considering their ionsization potential, thus more work is needed to develop predictive power of traditional models

- Information for aquatic invertebrates and plants is very limited but preliminarily data indicate that they may accumulate pharmaceuticals. Also, information on trophic transfer is limited but it demonstrates that dietary uptake of pharmaceuticals may play a role in their accumulation in invertebrate organisms. Data indicates that humans may be potentially exposed to a number of pharmaceutical classes through consumption of contaminated fish, albeit at concentrations below therapeutic levels. Further data is required to assess the potential impact of pharmaceuticals on the food chain.

1.6 Recommendations for further research

There are currently large knowledge gaps and more research is needed into the uptake, metabolism and bioaccumulation of pharmaceutical compounds in aquatic organisms. Recommendations for further work therefore include:

- Assessment of a wider range of pharmaceuticals in order to develop improved models for estimating uptake - Much of the information gathered to date has been focused on particular compounds such as fluoxetine. By testing a wider range of compounds in a more systematic way, it should be

possible to develop relationships for predicting uptake based on the properties of a pharmaceutical.

- Most studies to assess the uptake of pharmaceuticals through food chains have focused on uptake in single organisms. In order to protect the wider environment, it would be beneficial to begin to understand those factors and processes affecting the uptake pharmaceuticals into, and through food chains.

- Integrating clinical data, available for pharmaceuticals, to better predict bioaccumulation and effects could therefore reduce the need for animal testing

- Assessment of environmental properties on uptake – Available data indicate that the uptake of pharmaceuticals is very dependent on the characteristics of the environment (e.g. pH, temperature). Further research is required to identify and assess the influence of environmental parameters on pharmaceutical uptake.

- Establishment of the effects of species traits on uptake – Most work to date has focused on fish with a few studies exploring uptake into invertebrates and algae. We should look at a wider range of organisms from different trophic levels and ultimately aim to develop trait-based models for assessing uptake into aquatic and terrestrial organisms.

1.7 Aims and objectives

This PhD project addresses many of the knowledge gaps described above. The understanding of those factors and processes affecting the uptake and pharmaceuticals in aquatic invertebrates will allow more accurate prediction of their bioconcentration in organisms in the aquatic environment. This will be invaluable for understanding the risks pharmaceuticals pose to the aquatic environment.

The core hypotheses behind this thesis were:

1. The uptake and clearance of pharmaceuticals in aquatic invertebrates are determined by the properties of the pharmaceutical, the traits of an aquatic invertebrate and the degree of metabolism within an organism;

2. Pharmaceuticals have the potential to biomagnify through aquatic food chains; and

3. The bioconcentration of pharmaceuticals into aquatic invertebrates is predictable based on the physicochemical properties of the pharmaceutical, knowledge of pharmacological behavior in humans and from the individual traits of the organism

In order to test these hypotheses, the following objectives were set:

1. To explore the influence of physicochemical properties of a variety of pharmaceuticals on uptake and clearance in the aquatic invertebrates

2. To compare uptake of these pharmaceuticals in aquatic invertebrates with contrasting biological traits

3. To develop models to predict the uptake of pharmaceuticals in aquatic invertebrates using data on physicochemical properties of the pharmaceutical, pharmacological behavior in humans and individual traits of the test organism

4. To develop methods to characterise the metabolism of pharmaceuticals in aquatic invertebrates and apply these methods to explore the metabolism of selected pharmaceuticals in aquatic invertebrates

5. To explore the uptake of selected pharmaceuticals from food and investigate biomagnification through a simple aquatic food chain

The thesis is based on laboratory studies using a set of model human pharmaceuticals namely: 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide. The selection of pharmaceuticals was based on their physicochemical properties including their octanol water partition coefficient,

disassociation constant and molecular weight. The aquatic invertebrates chosen were selected based on their size; lipid content, respiration strategy, feeding behaviour and environmental occurrence and availability. The organisms included in the studies were Gammarus pulex, Notonecta glauca, Lymnea stagnalis and Planorbarius corneus.

This thesis comprises five chapters. A brief description of each chapter is presented below.

Chapter 2 describes experiments exploring uptake and clearance of pharmaceuticals from water into the three study aquatic invertebrates. The resulting data are used to explore the relationships between pharmaceutical physic-chemical and pharmacological properties and uptake into invertebrates.

Chapter 3 describes experiments to characterize the metabolism of pharmaceuticals in *Gammarus pulex*. A Time of Flight Mass Spectrometry-based methodology was developed to detect and identify pharmaceutical and their metabolites in the organism. Where pharmaceutical metabolites were detected, uptake and clearance experiments were undertaken to determine the formation of metabolites over time and the influence on bioconcentration.

Chapter 4 describes experiments into the uptake of pharmaceuticals from the organisms' food using *Gammarus pulex* and *Notonecta glauca*. Uptake from the food and the water were compared to identify the predominant route of uptake for the organisms. Experiments were performed to evaluate the potential for trophic transfer and biomagnification of pharmaceuticals through a simple experimental food chain.

Chapter 5 synthesises the results of Chapters 2-4 and describes the broader implications of the reported findings in terms of the risks of pharmaceuticals to aquatic systems. Recommendations for further research in the area are also proposed.

Chapter 2 Uptake of pharmaceuticals in aquatic invertebrates

2.1 Introduction

Due to the large number of pharmaceuticals in use, there is increasing interest in the use of mammalian pharmakinetic data to identify whether effects on non-target organisms occur and which organisms are likely to be sensitive to a particular pharmaceutical (e.g. Huggett *et al.*, 2003a, Berninger and Brooks). In order for these approaches to work, it is essential that knowledge is available on the uptake of pharmaceuticals into non-target organisms. While the toxicity of many pharmaceuticals has been studied (e.g. Fent *et al.*, 2006), our understanding of the factors and processes influencing uptake and bioconcentration of pharmaceuticals is less well developed (See Chapter 1; Brooks *et al.*, 2009). Uptake of pharmaceuticals into non-target organisms has been previously reported in agricultural crops (e.g. Kumar *et al.*, 2005, Boxall *et al.*, 2006, Dolliver *et al.*, 2007) and in aquatic organisms (Mimeault *et al.*, 2005, Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008, Dussault *et al.*, 2009). For example, Dussault *et al.*, (2009) showed bioaccumulation of 17 α-ethinylestradiol in two benthic invertebrates (*Chironomus tentans* and *Hyalella azteca*). The uptake of the selective serotonin re-uptake inhibitor, fluoxetine, has also been shown in the Japanese fish Medaka (*Oryzias latipes*) (Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008).

Traditionally the octanol/water partition coefficient (K_{ow}) is used to estimate uptake of neutral organic compounds. However, K_{ow} is unlikely to be a good descriptor for the uptake of pharmaceuticals, many of which are ionisable. Uptake of pharmaceuticals can also be very sensitive to changes in pH of the environment due to their ionization potential. For example, Nakamura *et al.*, (2008) demonstrated that the extent of uptake of fluoxetine changes between pH 7 and pH 9. The pH-corrected octanol-water partition coefficient (D_{ow}) is likely to be a better parameter for estimating uptake of pharmaceuticals as it accounts for difference in partitioning of the neutral and ionic species of a molecule at a particular pH value (Kah and Brown, 2008, Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008). Estimations of uptake may be further improved using pH-corrected liposome–water partition coefficients

(D_{lipw}) as liposomes are more characteristic of biomembranes, which are predominantly composed of phospholipids, compared to octanol (Escher *et al.*, 2000, Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008). It has also been suggested that it may be possible to use pharmacological data to estimate the fate and effects of pharmaceuticals in the environment. For example, recent studies have shown that the Volume of Distribution (V_D) of a pharmaceutical, which is the distribution of the pharmaceutical in the human body, is potentially a good predictor of the sorption behaviour of a pharmaceutical in the natural environment (Williams *et al.*, 2009). V_D could also be used as a basis to predict uptake into aquatic organisms.

As discussed in Chapter 1, uptake of chemicals is not only influenced by physicochemical properties, but also by biological factors. Diversity in biology and ecology, such as taxon, size, life cycle, reproductive strategy and habitat preference, can affect uptake and can determine the sensitivity of organisms to contaminants (Usseglio-Polatera *et al.*, 2000, Baird and Van den Brink, 2007, Rubach *et al.*, 2010, Rubach *et al.*, 2010b). In a recent study, Rubach *et al.* (2010b) suggested that reproduction, respiration type/source of oxygen, feeding type and habitat are key biological traits in determining the sensitivity of aquatic organisms to pesticides. However, there is limited research on the influence of species traits in uptake and depuration studies with pharmaceuticals.

This chapter explores the relationship between pharmaceutical physicochemical properties and pharmacological properties and uptake, using pharmaceuticals from a range of therapeutic classes and with a range of physicochemical properties. The study focuses on two aquatic invertebrates: *Gammarus pulex* is a benthic crustacean and *Notonecta glauca* is a water column predator. For one pharmaceutical (carvedilol), uptake studies were also performed on the freshwater snail *Planorbarius corneus*, a grazing, pulmonate gastropod to explore organisms differences in uptake.

2.2 Materials and methods

2.2.1 Pharmaceutical compounds

Radiolabelled and unlabeled pharmaceuticals, 5-fluorouracil, carvedilol, diazepam and moclobemide were provided by F. Hoffman-la Roche Ltd (Basel, Switzerland), whilst carbamazepine and fluoxetine were obtained from Agriculture and Agri-Food Canada, London, Ontario. All radiolabelled pharmaceuticals used in uptake and depuration for *Gammarus pulex* were labelled with C-14 with the exception of the fluoxetine, which was labelled with 3H. Information on the chemical properties of the pharmaceuticals are provided in table 2.1 and table 2.2. These compounds are used extensively in medicine and may reach significant concentrations the aquatic environment. The annual number of prescriptions written in 2011 are as follows; 5-fluorouracil, 93,300; carbamazepine, 2,435,000; carvedilol, 496,000; diazepam, 54,148,400; fluoxetine, 5,384,300 and moclobemide, 23,600. They a diverse group with different physico – chemical properties so that any relationships between properties and uptake could be established (Table 2.1**)**

Table 2.1 Chemical properties of the radioactive study compounds

Pharmaceutical	CAS Number	Position of radiolabel	Chemical Purity [%]	Specific Activity [GBq mmol^{-1}]
5-fluorouracil	51-21-8	5-fluoro-1*H*,6[C-14]-pyrimidine	98.0	1.87
Carbamazepine	298-46-4	Carbonyl – [C-14]	99.9	0.83
Carvedilol	72956-09-3	2-[C14]-propanol	96.6	2.23
Diazepam	439-14-5	5-[C14]-phenyl	98.2	2.04
Fluoxetine	54910-89-3	*N*-methyl – [3H]	>99	2960
Moclobemide	71320-77-9	2-morpholin-4[C14]-ylethyl	98.6	1.02

Table 2.2 Physicochemical properties of the study pharmaceuticals

Pharmaceutical	Therapeutic class	Molecular weight [g mol^{-1}][a]	Water Solubility [mg l^{-1}] [a]	Log K_{ow} [b]	Acid/Base [c]	pK_a	V_D [l kg^{-1}][d]	Polar Surface Area[a]	Biological half-life [hours][d]	Plasma Binding [%][d]
5-fluorouracil	anti – cancer	130	> 1	- 0.81	weak acid	8.02[a]	1.4 ± 0.4	58.20	0.18 ± 0.07	8-12
Carbamazepine	anti – epileptic	236	18	2.25	neutral	7	1.5 ± 0.3	46.33	53 ± 41	94
Carvedilol	beta – blocker	406	0.6	3.05	weak base	8.00[d]	1.1 ± 0.3	75.74	15 ± 5	74 ± 3
Diazepam	anxyolitic agent	285	50	2.70	weak base	3.40 [a]	0.3 ± 0.12	32.67	2.2 ± 0.3	95
Fluoxetine	anti – depressant	309	50	4.65	weak base	10.01[e]	35 ± 21	21.26	43 ± 13	98.7 ± 0.2
Moclobemide	anti – depressant	269	-	1.16	weak base	6.20 [f]	1.4 ± 0.4	41.57	2-4[f]	50[f]

[a] DrugBank (http://www.drugbank.ca/) accessed 08/2011, [b] EPI suite V4.0,[c] Pubchem Compound NCBI (http ://pubchem.ncbi.nlm.nih.gov/) accessed 08/2011, [d] Bulter *et al.*, 2006, [e] Nakamura *et al.* (2007), [f] IPCS InCHEM database (http://www.inchem.org/) accessed 08/2011, [g] Specific Activity of compound

2.2.2 Test organisms

Gammarus pulex were collected from Bishop Wilton Beck, York, UK. (National Grid Reference (NGR) SE 797 553). *Notonecta glauca* were obtained from Blades Biological Ltd (Kent, UK). *G. pulex* were maintained in a 5 L aquarium in water collected from Bishop Wilton Beck (BWW) which was filtered to 1 µm using a Millipore vacuum filter. *N. glauca* were maintained in a 1.5 L aquarium in artificial pond water (APW; Naylor *et al.*, 1989). *Planorbarius corneus* were obtained from New Forest Koi (Hampshire, UK) and were maintained in a 15 L high density plastic tanks filled with APW. All organisms were kept at c. 20 °C under a natural light regime. *G. pulex* were fed rehydrated horse chestnut (*Aesculus hippocastanum*) leaves inoculated with the fungus *Cladosporium herbarum. N. glauca* were fed with adult *G. pulex* and *P. corneus* were fed with a mixture of duck weed, *Lemna minor* and algal tablets (King British, Pets at Home, London UK).

2.2.4 Toxicity assessment

Experiments were carried out between March 2007 and November 2008 to determine if any of the pharmaceuticals had toxic effects on *G. pulex* and *N. glauca* at the concentrations used in the uptake studies. The endpoint chosen for the toxicity tests was mortality. Exposure concentrations for *G. pulex* were based on limited data for another aquatic invertebrate, *D. magna* (table 2.3). At the time of the experimental design toxicity data was only available for diazepam and fluoxetine, however more data has now become available for the other pharmaceuticals (table 2.3).

G. pulex were exposed in 500 mL of BWW with 2, 4, 8, 16 and 32 µmol L^{-1} (or 0.25, 0.5, 1, 2 and 4 mg L^{-1}) of 5-fluorouracil; 1, 2, 4, 8 and 16 µmol L^{-1} (or 0.25, 0.5, 1, 2 and 4 mg L^{-1}) of carbamazepine; 0.5, 1, 2, 5 and 10 µmol L^{-1} (or 0.25, 0.5, 1, 2 and 4 mg L^{-1}) of carvedilol; 2, 4, 8, 14 and 28 µmol L^{-1} (or 0.5, 1, 2, 4 and 8 mg L^{-1}) of diazepam; 0.4, 0.8, 2, 4 and 6 µmol L^{-1} (or 0.1, 0.25, 0.5, 1 and 2 mg L^{-1}) of fluoxetine; and 8, 16, 32, 64 and 128 µmol L^{-1} (or 2.1, 4.3, 8.5, 17 and 34 mg L^{-1}) of moclobemide.

Table 2.3 Acute effective concentration µmol L^{-1} [mg L^{-1}] for *Daphnia magna* taken from published literature (1995 – 2012)

Pharmaceutical	EC_{50} *Daphnia magna* µmol L^{-1} [mgL^{-1}]	Endpoint	Reference
5-fluorouracil	276 [36]	Immobilisation	Zounkova´ *et al.* 2007 [a]
Carbamazepine	58 [>13.8]	Immobilisation	Ferrari *et al.* 2007 [a]
Carvedilol	[7.3]	Immobilisation	FASS.se 2012 [a]
Diazepam	[4.25]	Immobilisation	Liljus *et al.* 1995
Fluoxetine	[0.82]	Immobilisation	Brooks *et al.* 2003
Moclobemide	-	-	-

[a] Data available after the experiment was performed

Studies with *N. glauca* were carried out at a single concentration for each compound which was ten times that used in the uptake studies (8, 4, 2, 4, 3 and 4 µmol L^{-1} or 1 mg L^{-1}) 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide respectively). If toxicity was observed in the assessment for *N. glauca*, more concentrations would be tested. *G. pulex,* studies were carried out using three replicates of ten animals whereas five single animal replicates were used for the *N. glauca* studies. All organisms were exposed to test solution for 48 hours and the percentage survival was observed. The percentage survival was arcsine – square root transformed and the treatments were statistically compared to the controls using a one – way ANOVA with post – hoc Dunnett's test.

2.2.5 Uptake and depuration study

Uptake and depuration studies were carried out using the method of Ashauer *et al.* (2006) between July 2007 and February 2009. Test organisms were acclimatized to the test conditions for 24 hours without food under a specific photoperiod (12 hours light: 12 hours dark). With the exception of fluoxetine, a static exposure was used. Preliminary studies showed that fluoxetine adsorbed to the beakers and therefore a

static renewal approach was used for this compound (Appendix A). The static renewal approach for fluoxetine consisted of transferring the organisms in each beaker into fresh test solution every 24 hours. A sample of water (1 mL) was taken to determine the exposure concentration once the water had been renewed. Water quality parameters (pH, dissolved oxygen) were monitored throughout the experimental periods with a Symphony SB80BD bench top meter (VWR International LtD, UK).

All organisms were exposed to 0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 µmol L^{-1} (or 0.1 mg L^{-1}) of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide respectively. Uptake studies were run for 48 hours with the exception of *G. pulex* studies with fluoxetine and the *P. corneus* study with carvedilol. For fluoxetine and carvedilol in the crustacean and gastropod exposure period was extended to 72 hours. For the uptake studies, animals were exposed in 500 mL (*G. pulex*) or 150 mL (*N. glauca* and *P. corneus*) of test solution for 0, 3, 6, 12, 24 and 48 hours. For the 72 hour exposure period, animals were exposed for 0, 3, 6, 12, 24, 48 and 72 hours. For *G. pulex,* there were a minimum of three replicates of 12 animals where individual animals in each replicate separated by stainless steel cages to avoid cannibalism. One animal was taken from each replicate at each sampling point which left three sets of six animals at the end of the uptake study for use in the depuration study. For *N. glauca* and *P. corneus,* there were six sets of three replicates of single animals for each time point and a further six sets of three replicates to be used for the depuration studies. At each sampling point, 1 mL of the exposure water was taken to determine the pharmaceutical exposure concentration. After the exposure period the remaining organisms were transferred into water with no pharmaceutical present for the depuration test. The depuration test was run for the same period as the uptake test and samples were taken at the same time points.

The water sample was directly transferred to a scintillation vial and 10 mL of Ecoscint A (National Diagnostics, UK) were added. The sample was shaken to mix and left to settle and the radioactivity measured in the dark by liquid scintillation counting (LSC; Beckham LS6000 TA Liquid Scintillation Counter, Beckham Instruments Inc. USA).

Once removed from the beakers, each organism was rinsed in deionised water to remove any pharmaceutical residue from its surface. The tissue samples were then frozen at – 20 °C prior to analysis. Pharmaceuticals were extracted from *G. pulex*

and *N. glauca* using 2 mL of Soluene 350 (Perkin Elmer, UK) and 100 µL of hydrogen peroxide (30%; Sigma Aldrich, UK). The hydrogen peroxide bleached the tissue so that no colour quenching occurred during the analysis phase. The samples were then heated in a water bath for 24 hours until the tissue had dissolved. The *N. glauca* tissue sometimes took longer to bleach due to their dark colour.

Carvedilol was extracted from *P. corneus* using a Dionex 200 Accelerated Solvent Extractor (ASE) fitted with 33 ml stainless steel extraction cells. The gastropod whole body was removed from the shell, the tissue was placed in a mortar and pestle along with 6 g salt (Na_2SO_4) and c. 5.78 g of general purpose grade sand (Fisher Scientific, UK) and then homogenised into a fine powder. ASE cells were prepared with a filter paper, 1.35 g of hydromatrix (Varian Inc. California, USA) being placed at the bottom of the cell upon which the sample mixture was then added. To ensure the pharmaceutical was completely removed from the mortar and pestle, a further 34.68 g of sand was homogenised in the mortar and then transferred to the cell. Three ASE extractions with 100 % methanol as the carrier solvent were run for each sample. Once in the ASE, the samples were preheated for 5 minutes and extracted at 100 °C at a pressure of 1500 psi. The samples were transferred from the extraction vials into beakers and the solvent was allowed to evaporate off. Once evaporated, 2 mL of methanol were added to the beakers which were placed on an orbital shaker for ten minutes and then sonicated for five minutes to ensure no radioactivity remained sorbed to the beaker. The samples were transferred to scintillation vials. Using this method an extraction efficiency of 81 % was achieved.

All extracts were mixed with 10 mL Hionic Fluor scintillation cocktail (Perkin Elmer, UK) and the concentration of pharmaceuticals determined using LSC. Samples were counted three times for 5 minutes. Sample counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method.

2.2.6 Effect of pharmaceutical concentration on uptake

Carvedilol and fluoxetine have been shown to cause chronic effects on some aquatic invertebrates at low concentrations (De Lange *et al.*, 2006, Sánchez-

Argüello *et al.*, 2009, Guler and Ford, 2010) which might affect uptake and depuration rates. To assess whether the uptake and depuration rates of pharmaceuticals might be affected by the concentration of the test substances, an additional study was carried out on fluoxetine and carvedilol at a much lower concentration than used in the studies described above. Sets of six individual *G. pulex* were exposed to either 0.2 or 246 nmol L^{-1} of carvedilol and 0.3 or 323 nmol L^{-1} of fluoxetine. The lower concentrations of pharmaceuticals (0.2 and 0.3 nmol L-1 carvedilol and fluoxetine) were prepared with only radioactive pharmaceutical (5 Bq L^{-1}). However the higher concentrations (246 and 323 nmol L-1 carvedilol and fluoxetine) were prepared with radiolabeled (5 Bq L^{-1}) and non-radiolabelled pharmaceuticals. To evaluate whether any toxicity at the higher concentration would influence uptake into the organism; the uptake of radioactivity was statistically compared at both concentrations after 72 hours exposure. The experimental conditions and extraction methods were the same as described above.

2.2.7 Derivation of uptake and depuration rate constants and bioconcentration factors

A first-order one-compartment toxicokinetic model was fitted to the internal concentration data using individual replicates to estimate the parameters uptake and elimination rate constants (Ashauer *et al.*, 2006, Ashauer *et al.*, 2010, Rubach *et al.*, 2010). The toxicokinetic model is:

Equation 2.1 $$\frac{dC_{internal}}{dt} = \boldsymbol{k_{int}} \cdot \boldsymbol{C_{water}}\,(\boldsymbol{t}) - \boldsymbol{k_{out}} \cdot \boldsymbol{C_{internal}}(\boldsymbol{t})$$

Where t is time, $C_{internal}$ is the internal concentration [pmol g^{-1}], C_{water} is the concentration in the water [pmol mL^{-1}], and k_{int} and k_{out} are the uptake rate constant [mL g^{-1} d^{-1}] and the depuration rate constant [d^{-1}] respectively. The model was parameterized using least-squares minimization with the Levenberg-Marquardt algorithm followed by Monte-Carlo Markov-Chain (MCMC) to obtain a sample of parameter combinations that approximates the posterior distributions of the

parameters including their co-variances (Ashauer *et al.,* 2010). Subsequently we ran the model for all these parameter combinations and chose the 2.5th and 97.5th percentile as prediction intervals. Bioconcentration factors (BCFs) and their confidence intervals were calculated by setting C_{water} equal 1 and running the model beyond steady-state for all combinations of the parameter sample (Ashauer *et al.*, 2010). All simulations and parameter estimations were carried out using OpenModel (version 9th Dec 2009, http://www.nottingham.ac.uk/environmental-modelling/OpenModel.htm).

2.2.8 Development of relationships between pharmaceutical physico-chemical/pharmacological properties and uptake

Linear regression analysis was used to derive the relationships between V_D and BCFs; Log D_{lipw} and BCFs; solubility; molecular weight (MW) or polar surface area (PSA) and BCFs for *G. pulex* and *N. glauca*. V_D data were obtained from the literature. Log D_{lipw} values were estimated from predicted values of log K_{ow} obtained from the Kowin package (EPISuite40). Log D_{ow} for the pH of the experiment was initially derived from the log K_{ow} using the Henderson Hasselbach equation (Po and Senozan, 2001). Initially the fraction of ionised and unionised compounds at the test pH was determined using equation 2.2. The Log D_{ow} was then estimated using equation 2.3.

Equation 2.2 $$\propto \boldsymbol{ion} = \propto \boldsymbol{neutral} \cdot \mathbf{10}^{\boldsymbol{i(pH-pKa)}}$$

Equation 2.3 $$\boldsymbol{D_{ow}} = \boldsymbol{fion} \cdot \boldsymbol{K_{ow}}\,(\boldsymbol{ion}) + \boldsymbol{fneutral} \cdot \boldsymbol{K_{ow}}\,(\boldsymbol{neutral})$$

Where α_{ion} is the activity of the ion at a particular pH; $\alpha_{neutral}$ is the activity of the neutral species; f_{ion} is the fraction of the ion at the study pH; $f_{neutral}$ is the fraction of the neutral species at the pH. $K_{ow(ion)}$ and $K_{ow(neutral)}$ are the octanol water partition

coefficients for the neutral and ionic species respectively. It was assumed that the partition coefficient between octanol and water of the ionic species was 3.5 log units lower than the neutral species (Trapp and Horobin, 2005). Log D_{lipw} was then estimated equation 2.4 for non-polar compounds (i.e. carbamazepine) or equation 2.5 for polar compounds (i.e. all other compounds) (Escher *et al.*, 2009).

Equation 2.4 $$\boldsymbol{Log\ D_{lipw} = 1.05 \cdot Log\ D_{ow} - 0.32}$$

Equation 2.5 $$\boldsymbol{Log\ D_{lipw} = 0.90 \cdot Log\ D_{ow} + 0.52}$$

Solubility, MW and PSA were obtained from the online database DrugBank (Wishart *et al.*, 2008). The relationships between V_D; Log K_{ow}; Log D_{lipw}; solubility; MW and PSA of the pharmaceutical and bioconcentration were analysed by linear regression analysis, all statistical analysis was completed using Microsoft Excel and SPSS v17.0.0.

2.3 Results and discussion

2.3.1 Toxicity studies

There was no significant effect of any of the study pharmaceuticals on *G. pulex* mortality at any of the concentrations tested (Appendix B). At the highest test concentration (6 µmol L^{-1}) of fluoxetine, *G. pulex* survival was reduced to 70 %, however this was not significant compared to the controls (ANOVA p = 0.1). Similarly, all the studied pharmaceuticals had no effect on *N*. g*lauca* mortality (Appendix B). While the toxicity of carvedilol to *P. corneus* was not determined, data for another snail, *Lymnea stagnalis* indicate that the compound has no effect on the mortality of the snail at 2 µmol L^{-1} (Appendix B).

The results for carbamazepine and diazepam are in accordance with the published literature where EC_{50} values between 18 and > 351 µmol L^{-1} have been reported (Calleja *et al.*, 1994, Lilius *et al.*, 1995, Ferrari *et al.*, 2003, Nunes *et al.*, 2005, Quinn *et al.*, 2008). There are limited data from the literature to compare to 5-fluorouracil, moclobemide and carvedilol. The results for fluoxetine indicate that *G. pulex* is less sensitive than other crustaceans to fluoxetine. Studies with *Daphnia magna* have reported LC_{50} values as low as 2 µmol L^{-1} and chronic toxicity values of 0.1 µmol L^{-1} (Brooks *et al.*, 2003, Flaherty and Dodson, 2005). The sensitivity pattern follows the findings of Rubach *et al.* (2010) where Daphnids were the most sensitive organisms to organophosphates, followed by Gammarids and Notonectids.

2.3.2 Uptake, depuration and bioconcentration

The results from the toxicity tests indicated that there were no significant effects of any of the test pharmaceuticals on the survival of the organism, therefore 0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 µmol L^{-1} (or 0.1 mg L^{-1}) of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide were chosen as the exposure concentrations. These concentration were at least one tenth of the lowest concentrations tested in the toxicity test section 2.2.4, therefore it was assumed that toxicity would not influence the uptake of the compound into the organism at these exposure concentrations.

Analysis of exposure solutions confirmed that for diazepam, carvedilol, 5-fluorouracil, carbamazepine and moclobemide exposure concentrations remained constant throughout the uptake phase of the studies (figure 2.1 to figure 2.3). For fluoxetine, concentrations declined over time so a static renewal approach was required to maintain the exposure concentrations. The observed dissipation of fluoxetine contrasts with published data which indicate that the compound is both photolytically and hydrolytically stable in a range of aqueous media (Kwon and Armbrust, 2006), and the disappearance over time may be due to sorption of the compound onto the test vessels. A photolysis and sorption experiment was carried out and details can be found in Appendix A. Water quality parameters were measured throughout the experiments and are shown in table 2.4.

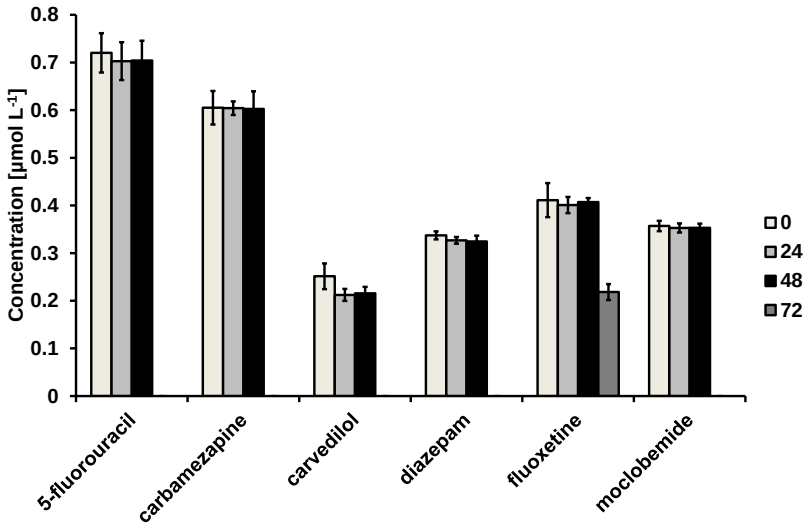


Figure 2.1 Mean measured water concentrations (± standard deviation) in *Gammarus pulex* uptake experiments. Nominal water concentration 0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 µmol L^{-1} (or 0.1 mg L^{-1}) of 5-fluorouracil (n=8), carbamazepine (n=8), carvedilol (n=8), diazepam (n=8), fluoxetine (n=3) and moclobemide (n=8)

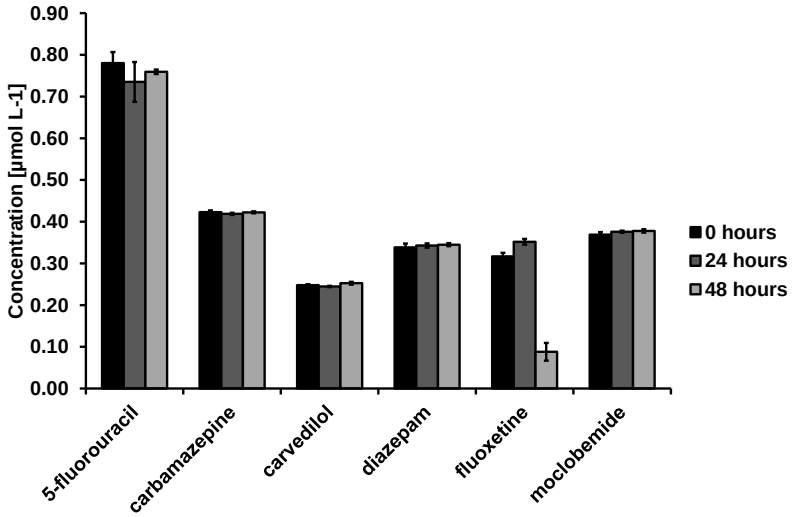


Figure 2.2 Mean measured water concentrations (± standard deviation) in *Notonecta glauca* uptake experiments (n=3). Nominal water concentration 0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 µmol L-1 (or 0.1 mg L-1) of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide

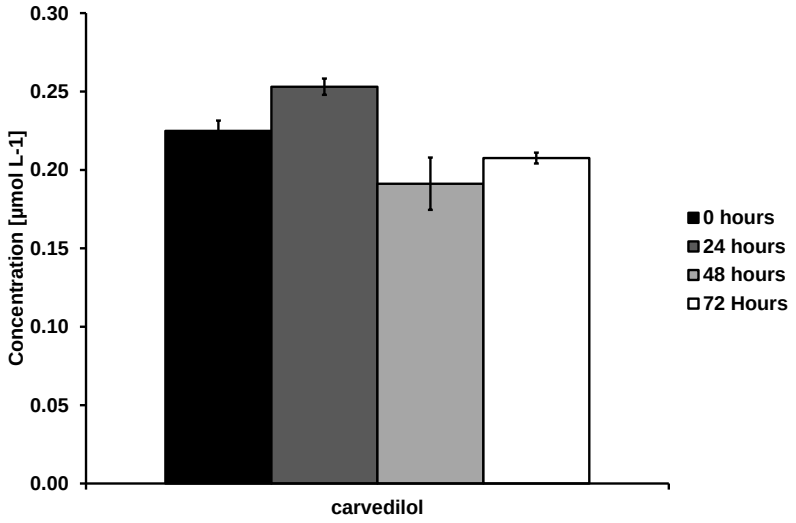


Figure 2.3 Mean measured water concentrations (± standard deviation) in *Planorbarius corneus* uptake experiments (n=3), nominal water concentration 0.2 µmol L-1 (or 0.1 mg L-1)

Table 2.4 Mean water quality parameters for uptake and depuration experiments with *Gammarus pulex, Notonecta, glauca* and *Planorbarius corneus.*

Species	Pharmaceutical	Dissolved O_2 [mgL^{-1}]	pH	Temperature (°C)
G. pulex	5-fluorouracil	8.49	8.61	12.10
	Carbamazepine	7.59	8.51	12.50
	Carvedilol	8.21	8.69	12.13
	Diazepam	8.17	8.93	11.59
	Fluoxetine	8.99	8.51	12.45
	Moclobemide	7.56	8.61	11.98
N. glauca	5-fluorouracil	8.31	7.94	20.19
	Carbamazepine	8.36	7.85	20.11
	Carvedilol	9.76	7.92	20.50
	Diazepam	7.54	7.94	19.96
	Fluoxetine	8.36	7.87	20.12
	Moclobemide	8.99	7.95	20.02
P. corneus	Carvedilol	7.66	7.93	20.15

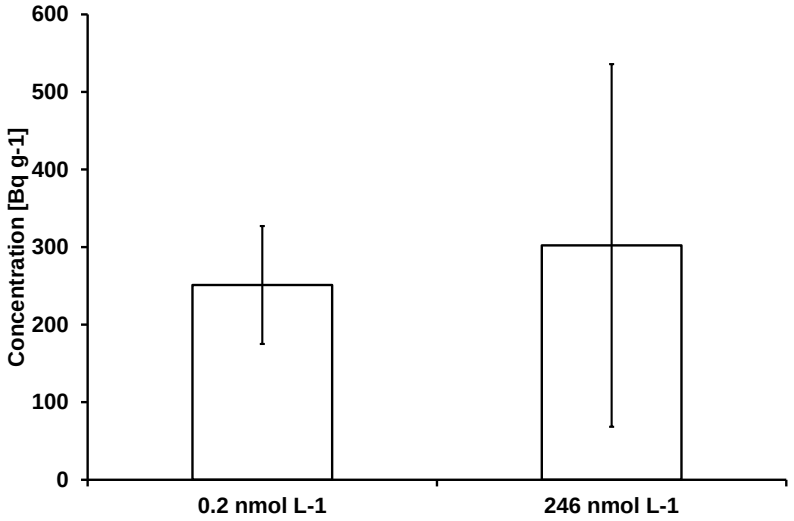


Figure 2.4 The mean measured internal concentration (± standard deviation) of Carvedilol in *Gammarus pulex* (n=6) at high (246 nmol L^{-1} or 0.1 mg L^{-1}) and low (0.2 nmol L^{-1} or 0.1µg L^{-1}) test concentrations

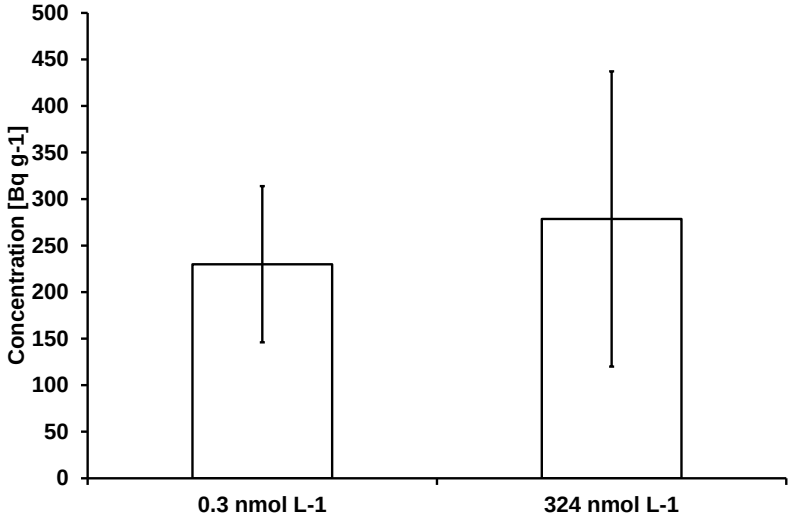


Figure 2.5 The mean measured internal concentration (± standard deviation) of Fluoxetine in *Gammarus pulex* (n=6) at high (324 nmol L^{-1} or 0.1 mg L^{-1}) and low (0.3 nmol L^{-1} or 0.1 µg L^{-1}) test concentrations

As carvedilol and fluoxetine have previously been shown to cause effects on invertebrates at low concentrations, a study was performed to assess the effect of concentration of these two pharmaceuticals on uptake (Brooks *et al* ,2003; Flaherty

and Dobson, 2005). There was no significant difference in the uptake of radioactivity at concentrations of 0.2 or 246 nmol L^{-1} of carvedilol and 0.3 or 323 nmol L^{-1} of fluoxetine. (t – test p = 0.62 and p = 0.34 for carvedilol and fluoxetine respectively; figures 2.4 and 2.5). The uptake and depuration rates and BCFs should therefore not be influenced by any toxicity occurring at the test concentration.

The first order-one compartment kinetic model was fitted to the uptake and depuration measurements for *G. pulex*, *P. corneus* and *N. glauca*. The experimental measurements and modelled uptake and depuration curves are shown in figure 2.6 to figure 2.12 and the rate constants and bioconcentration factors are provided in table 2.5. In *G. pulex*, BCFs ranged from 4.6 – 185900 and increased in the order moclobemide < 5-fluoruracil < carbamazepine < diazepam < carvedilol < fluoxetine. In *N. glauca* BCFs ranged from 0.1 – 1.6 and increased in the order 5-fluorouracil < carbamazepine < moclobemide < diazepam < fluoxetine < carvedilol. For *P. corneus* the BCF for carvedilol was 57.3. The largest BCF of 185900 was calculated for fluoxetine in *G. pulex.* The large BCF in *G. pulex* is due to the limited depuration in the animal (e.g. k_{out} of 0.0008 d^{-1}, table 2.5) and is over 700 times higher than those reported in the literature. Published data on the uptake of pharmaceuticals are limited, but data are available on uptake of fluoxetine into fish (*O. latipes*) which provides some data for comparison. Nakamura *et al.*, (2008) reported BCFs of 8.8 – 260 and Patterson and Metcalfe (2008) reported BCFs of 74 - 80. Environmental monitoring studies have also shown concentrations of fluoxetine in fish to be significantly higher than concentrations in the surrounding water column (Lajeunesse *et al.*, 2009, Schultz *et al.*, 2010).

The differences in the order of uptake between compounds in *G. pulex* and *N. glauca* may be partially explained by the ionisation state of the compounds in the test systems. The pH in the *N. glauca* uptake studies was 7.9, whereas in the *G. pulex* studies pH ranged from 8.5 – 8.9. Uptake of 5-fluorouracil, carvedilol and fluoxetine, which have pKa values in the range 8 – 10, would be sensitive to changes in pH in the range observed. However, in the experiments with carbamazepine, diazepam and moclobemide the same effects were observed where the effects of pH may not have been as important. Effects of pH on uptake of fluoxetine have been seen in fish in previous work, where uptake has increased from pH 7 to pH 9 (Nakamura *et al.*, 2008).

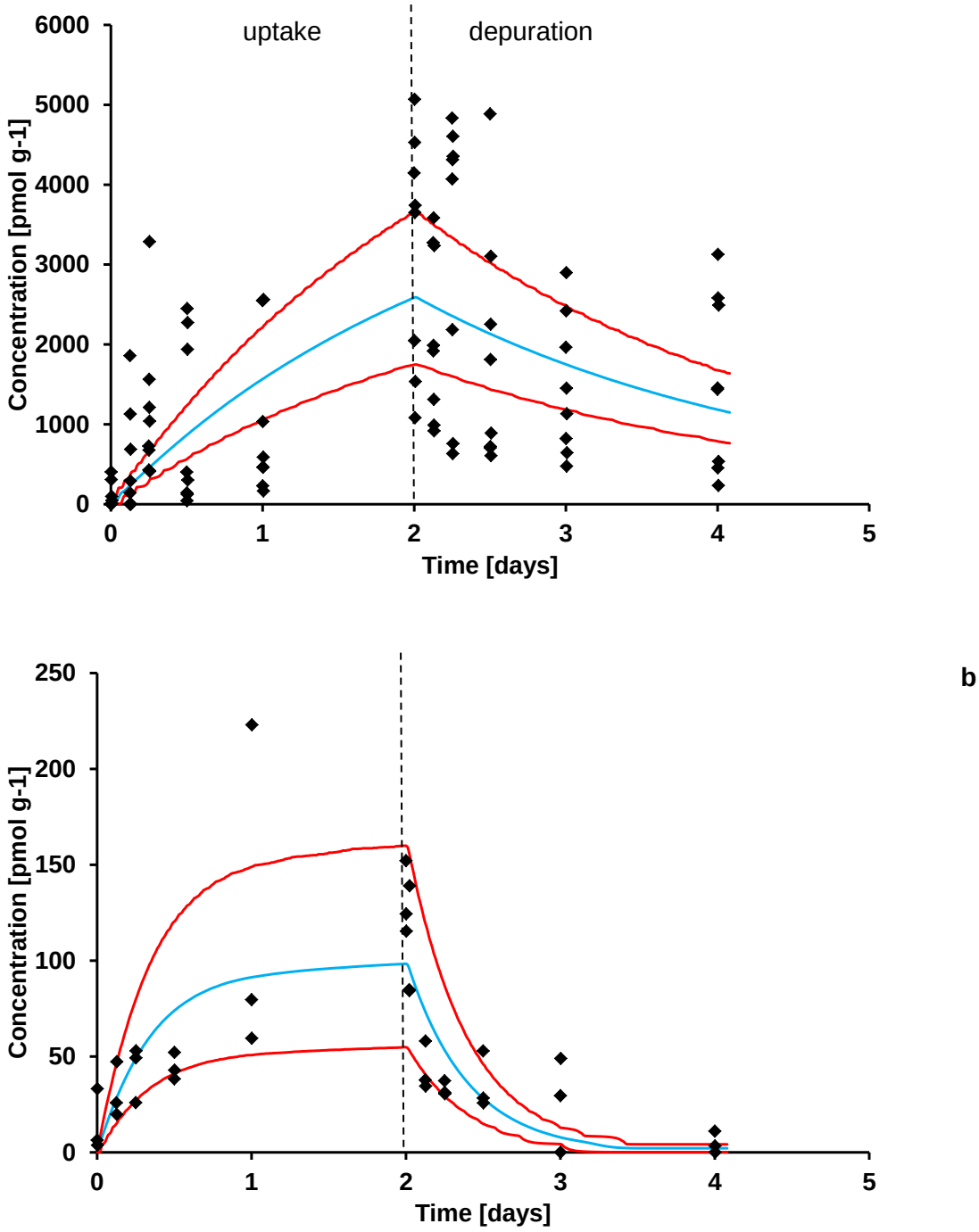


Figure 2.6 Measured internal wet weight concentrations in a) *Gammarus pulex* (n=7; September 2007) and b) *Notonecta glauca* (n=3; July 2009) exposed to 0.1 mg L^{-1} of 5-fluorouracil, providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater

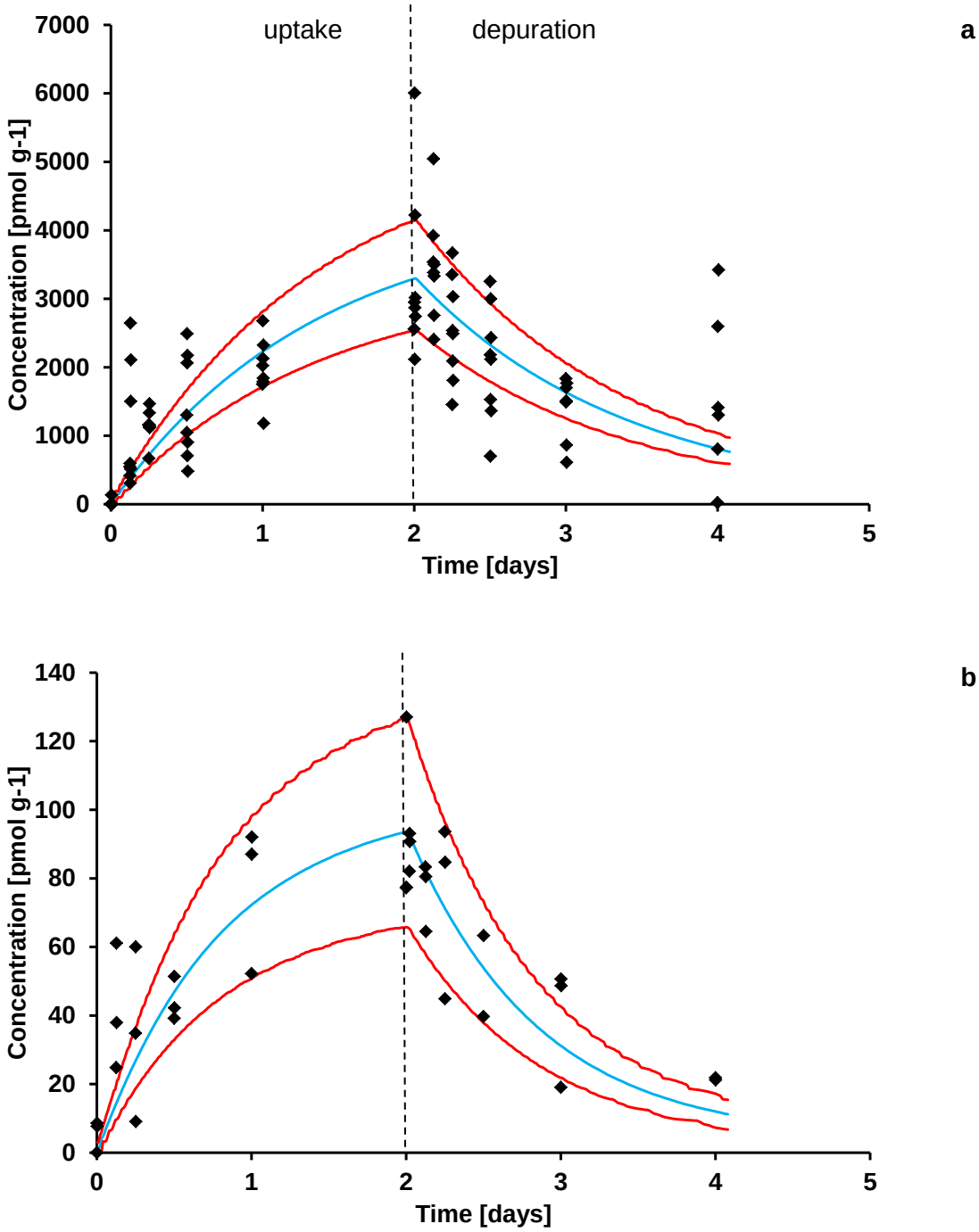


Figure 2.7 Measured internal wet weight concentrations in a) *Gammarus pulex* (n=7; September 2008) and b) *Notonecta glauca* (n=3; July 2009) exposed to 0.1 mg L^{-1} of carbamazepine, providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater

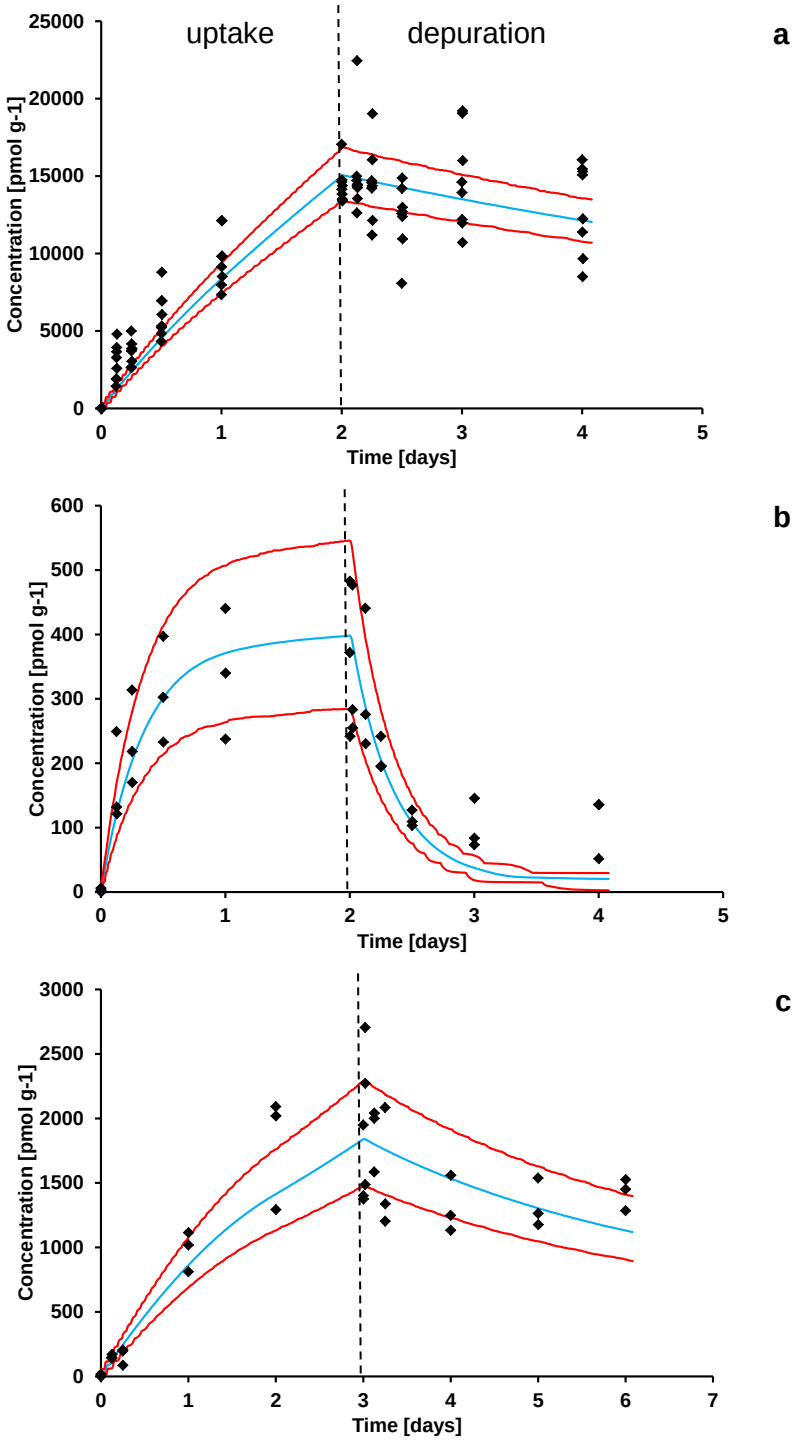


Figure 2.8 Measured internal wet weight concentrations in a) *Gammarus pulex* (n=7; July 2007), b) *Notonecta glauca* (n=3; July 2008) and c) *Planabarius corneus* (n=3; February 2009) exposed to 0.1 mg L^{-1} of carvedilol. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater

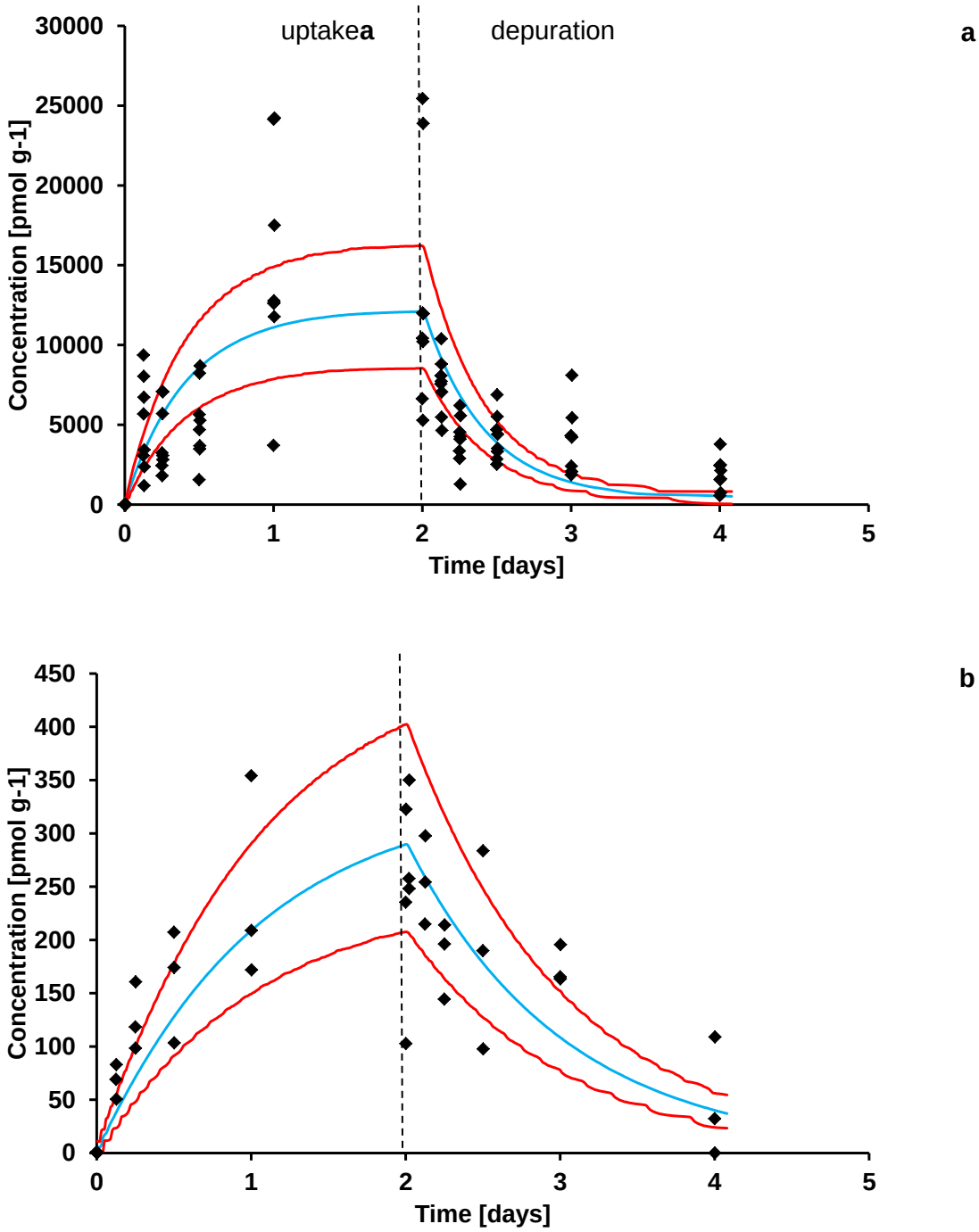


Figure 2.9 Measured internal wet weight concentrations in a) Gammarus pulex (n=7; August 2007) and b) Notonecta glauca (n=3; July 2009) exposed to 0.1 mg L-1 of diazepam, providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater

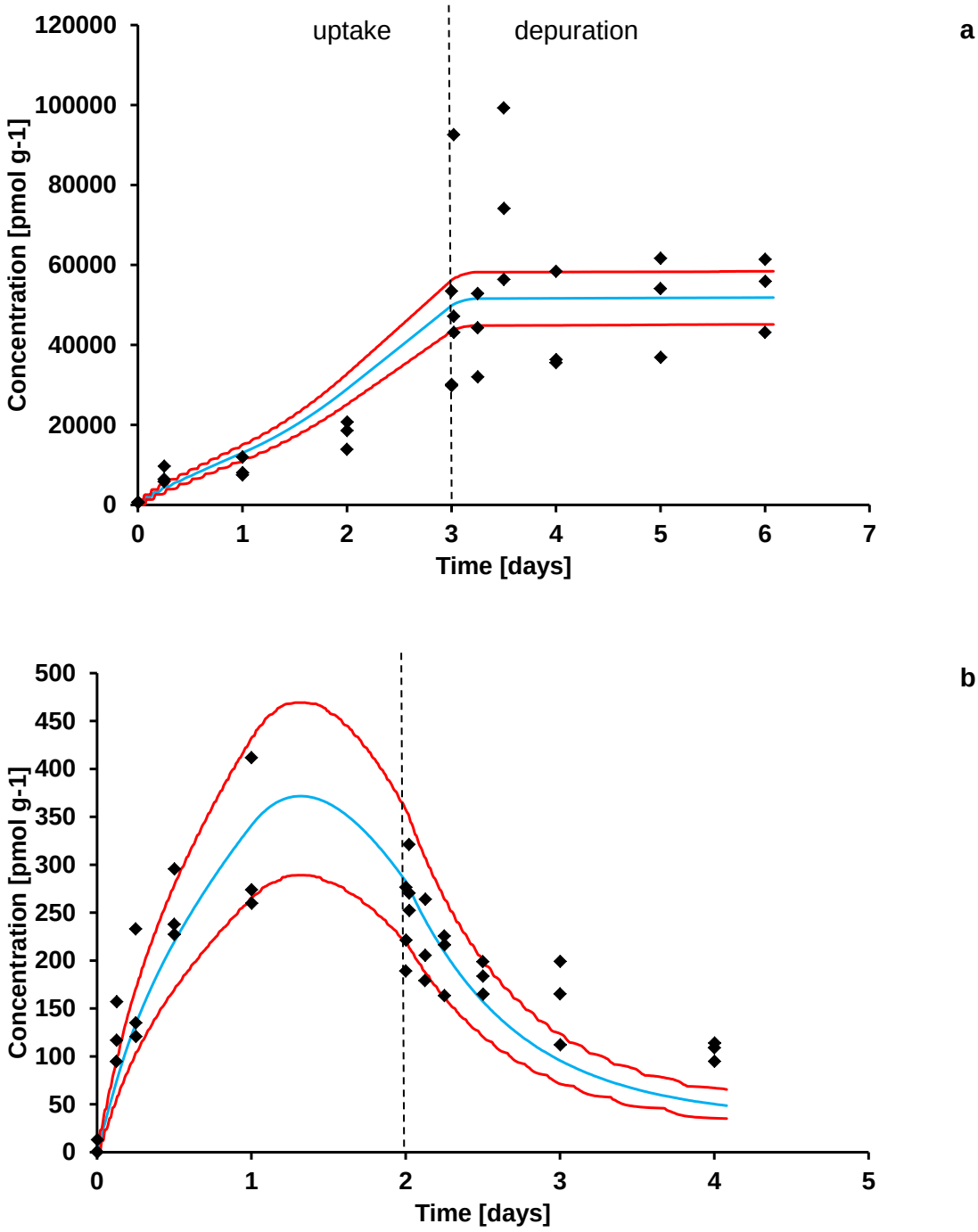


Figure 2.10 Measured internal wet weight concentrations in a) *Gammarus pulex* (n=3; June 2008) and b) *Notonecta glauca* (n=3; July 2009) exposed to 0.1 mg L^{-1} of fluoxetine, providing uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater

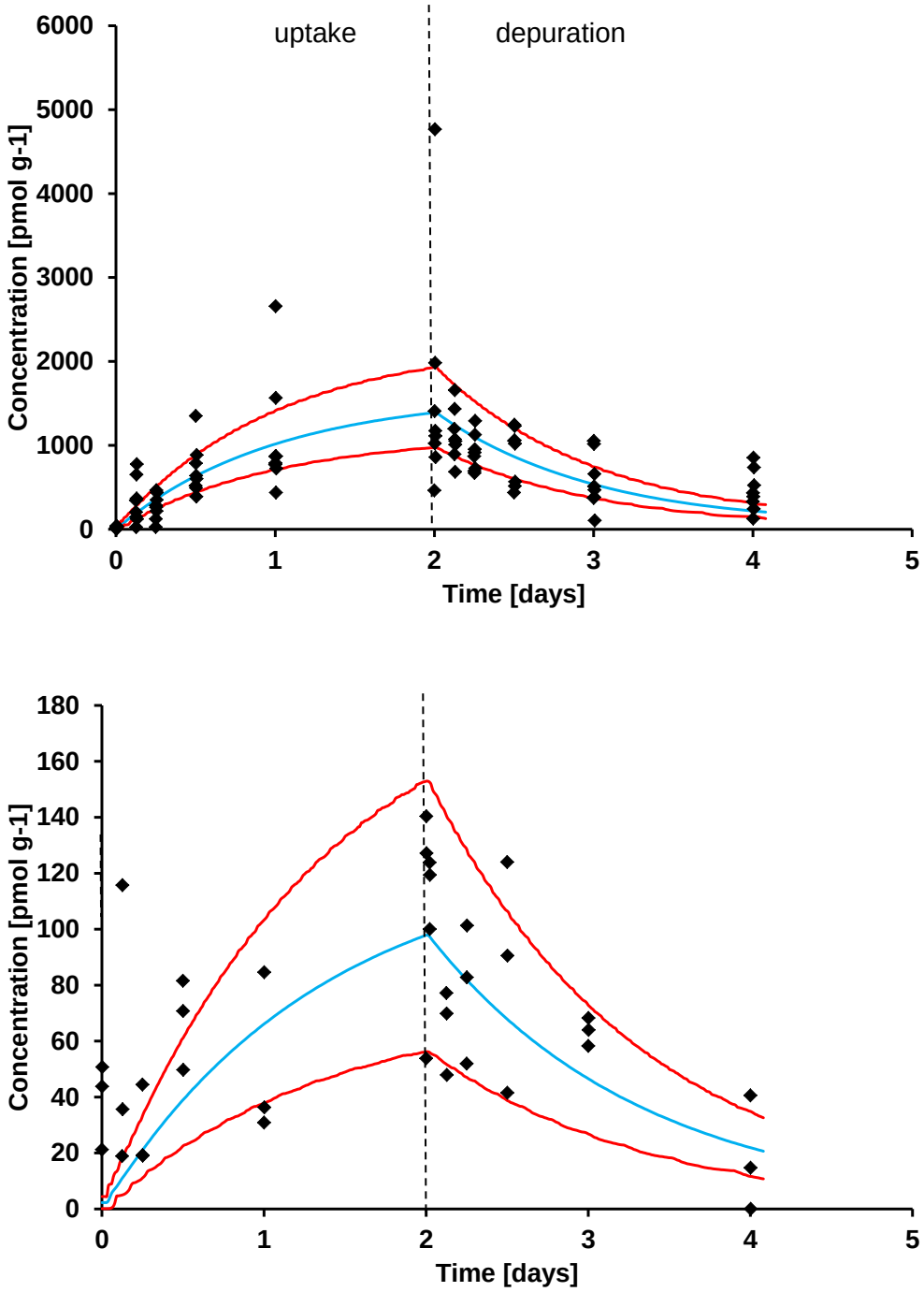


Figure 2.11 Measured internal wet weight concentrations in a) *Gammarus pulex* (n=7; September 2007) and b) *Notonecta glauca* (n=3; July 2009) exposed to 0.1 mg L^{-1} of moclobemide, providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater

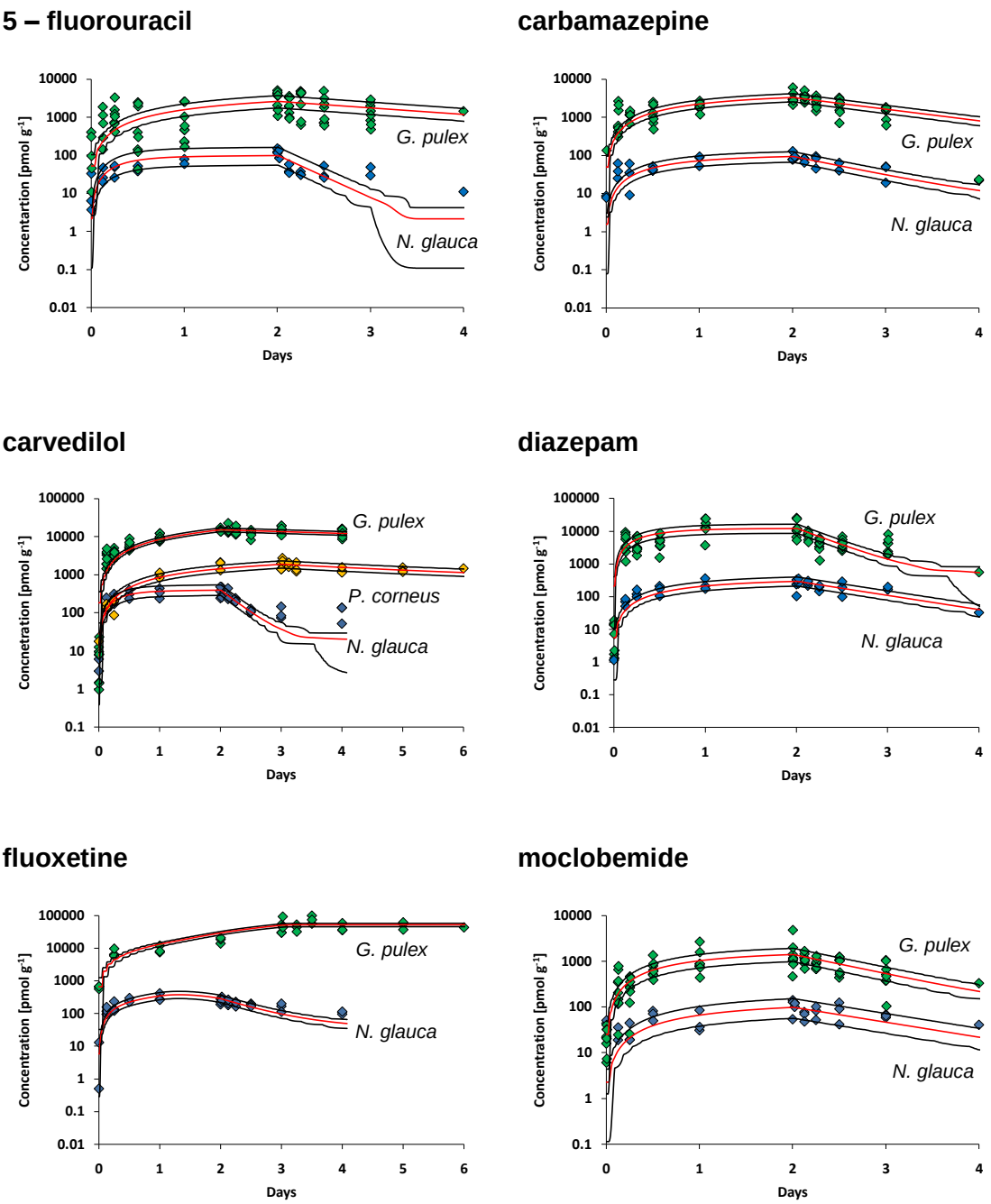


Figure 2.12 Measured internal wet weight concentrations on a log scale in *Gammarus pulex*, *Notonecta glauca* and *Planabarius corneus* exposed to 0.1 mg L^{-1} of test pharmaceuticals between July 2007 and February 2009. Diamonds are the measured concentration of individual replicates, the red line is the model fit and the black lines represent 95 % confidence intervals

Table 2.5 Modelled mean uptake and depuration rate constants (± standard deviation), Bioconcentration Factors and goodness of fit data (95th percentiles) for the uptake into *Gammarus pulex*, *Notonecta glauca* and *Planabarius corneus* exposed to 0.1 mg L^{-1} of test pharmaceuticals

Chemical	Species	Cw (µg l^{-1})[b]	k_{int} (L kg^{-1} d^{-1}) mean ± SD (ML) [a]	k_{out} (d^{-1}) mean ± SD (ML) [a]	BCF (L kg^{-1}) [95th percentiles]
5 – fluorouracil	*G. pulex*	92.22 ± 1.66	2.70 ± 0.53 (2.86)	0.37 ± 0.16 (0.42)	6.48 [4.37 - 9.17]
	N. glauca	100 ± 2.41	0.34 ± 0.09 (0.34)	2.52 ± 0.7 (2.6)	0.13 [0.07 - 0.21]
carbamazepine	*G. pulex*	142.68 ± 2.38	5.20 ± 0.66 (5.23)	0.72 ± 0.13 (0.73)	7.09 [5.47 - 8.94]
	N. glauca	100 ± 0.56	0.29 ± 0.048 (0.30)	1.18 ± 0.23 (1.20)	0.24 [0.17 - 0.33]
carvedilol	*G. pulex*	94.78 ± 3.10	38.9 ± 2.26 (39.3)	0.13 ± 0.039 (0.15)	270.8 [240.6 - 303.4]
	N. glauca	100 ± 1.03	4.72 ± 0.81 (4.62)	3.03 ± 0.52 (2.30)	1.6 [1.14 - 2.19]
	P. corneus	91.38 ± 0.91	12.39 ± 1.37 (12.68)	0.20 ± 0.056 (0.22)	57.3 [50.46 - 71.29]
diazepam	*G. pulex*	92.93 ± 1.16	91.3 ± 14.5 (90.2)	2.48 ± 0.40 (2.44)	37.47 [26.49 - 50.22]
	N. glauca	95.98 ± 1.82	0.97 ± 0.17 (0.96)	1.01 ± 0.22 (0.99)	0.98 [0.70 - 1.36]
fluoxetine	*G. pulex*	98.55 ± 16.54	51.6 ± 3.33 (51.8)	0.0008 ± 0.0008 (0.00028)	185900 [161800 - 209500]
	N. glauca	95.65 ± 7.27	1.95 ± 0.24 (1.94)	1.42 ± 0.18 (1.41)	1.39 [1.08 - 1.75]
moclobemide	*G. pulex*	95.01 ± 0.93	0.46 ± 0.81 (4.65)	0.98 ± 0.21 (1.00)	4.55 [3.19 - 6.32]
	N. glauca	100 ± 1.46	0.25 ± 0.062 (0.26)	0.70 ± 0.25 (0.75)	0.33 [0.19 - 0.52]

[a] Mean ± SD (Standard Deviation) of sample of posterior, ML (Maximum Likelihood parameter value)

2.3.3 Comparison of uptake in *G. pulex, N. glauca* and *P. corneus*

Uptake rates for all pharmaceuticals were greater in *G. pulex* than in *N. glauca* which can be clearly seen when transforming the uptake and depuration data onto a log scale (figure 2.12). The uptake rate constants were 8 (carvedilol) to 27 (fluoxetine) times greater in *G. pulex* than *N. glauca*. Similarly, for 5 – fluoruracil, carbamazepine, diazepam and moclobemide, depuration rates were higher in *G. pulex* than *N. glauca*. However, for fluoxetine and carvedilol, following the uptake phase, only limited depuration was seen in *G. pulex,* so that depuration rates were slower than in *N. glauca*. BCFs in *G. pulex* ranged from 15 (moclobemide) to 143000 (fluoxetine) times higher than in *N. glauca.* These findings are in agreement with those of Rubach *et al.,* (2010) who investigated the uptake of the pesticide chlorpyrifos into 15 aquatic arthropods covering a range of species traits, including *G. pulex* and another Notonectid, *Notonecta maculata*. Uptake of chlorpyrifos into *G. pulex* was found to be much faster than for the other organisms tested. The uptake and depuration rate constants and the BCFs were, 5, 17.9 and 2.5 times greater in *G. pulex* than *N. maculata,* respectively. Possible explanations for the difference observed in the uptake between the test species for carvedilol may include differences in: respiration strategy, locomotion and behaviour and differences in the temperature and pH of the test system (Rubach *et al.*, 2010a). Each of these is discussed in more detail below.

Respiration in *G. pulex* occurs through the gills directly from the water whereas *N. glauca* respires via a plastron which stores a thin layer of air for the insect to breathe (Felten *et al.*, 2008, Matthews and Seymour, 2010). Pulmonate molluscs use both cutaneous respiration through the skin and pulmonary respiration *via* a siphon from the surface to the mantle cavity (Jones, 1964). Uptake through the gills of aquatic organisms is a primary route of exposure to pharmaceuticals and other chemicals (Mimeault *et al.*, 2005, Felten *et al.*, 2008). The results of this study suggest that aquatic organisms that utilize plastron or pulmonary respiration may be less exposed to dissolved contaminants than those that breathe dissolved oxygen through gills. Both Baird and Van den Brink (2007) and Rubach *et al* (2010b) suggest that respiration type explained much of organisms sensitivity to contaminants.

Locomotion mode may also have influenced the uptake of pharmaceuticals into tissues. *G. pulex* is an epibenthic crustacean and was therefore fully submerged

throughout the exposure. *P. corneus,* also a benthic dwelling organism, only remains near the water surface when filling the lung (Jones, 1964). However, *N. glauca* is a surface swimmer and active flier (Usseglio-Polatera *et al.*, 2000). On one occasion in the present study a Notonectid was seen to have climbed up the side of the beaker before re-submerging itself. This type of avoidance behaviour has been previously recorded in response to a predator (Cook and Streams, 1984) and may well reduce the internal exposure concentration in the organism. The escape response of *N. glauca* was not quantified but may add to any variation in the data.

Other biological traits play a key role in influencing uptake and bioconcentration such as size, lipid content and the metabolic capacity of the organism (e.g. Hendriks and Heikens, 2001, Rubach *et al.*, 2010). In this study we found no correlations when comparing the wet weight or lipid content of any of the organisms and the internal concentration. Rubach *et al.*, (2010b) also found no relationship between lipid content and chlorpyrifos uptake in all of the 15 organisms they tested.

There is limited information available on the biotransformation of the study pharmaceuticals in aquatic organisms. However, information available for fish indicates that hepatic metabolism of pharmaceuticals such as fluoxetine is much less that for mammals (Smith *et al.*, 2010). It is possible that pharmaceuticals are also metabolised to different extents in aquatic invertebrates, however further work is required to confirm or discount this. Chapter 3 will describe a series of studies that explore the metabolism of the study pharmaceuticals in *G. pulex*.

2.3.4 Estimation methods for uptake of pharmaceuticals

There are over 3,000 pharmaceuticals in use today (Richman and Castensson, 2008). It is probably impractical to experimentally assess the environmental impact of all of these. One approach to estimating impacts is to measure internal concentrations of a pharmaceutical within an aquatic organism and to use data on plasma concentrations in humans alongside genomic information to estimate whether an effect is likely (e.g.Huggett *et al.*, 2003a). One of the key parameters needed in this approach is the bioconcentration factor; therefore the relationships between BCF and the $V_{D,}$ K_{ow}, $D_{lipw,}$ solubility, MW and the PSA of the pharmaceutical were explored using linear regression (figure 2.13 to figure 2.17 and

table 2.6. The best relationship for *G. pulex* was found between V_D and the bioconcentration factor. This supports previous work by Williams *et al.* (2009), where V_D provided a good estimation of the distribution of the pharmaceutical between water and solids. A strong relationship was also obtained between BCF and K_{ow}. Although pharmaceuticals are ionisable the data shows that D_{lipw} did not describe bioconcentration better than K_{ow} for *G. pulex* when using a linear relationship. For *N. glauca* however, the linear relationships obtained proved stronger and more significant than those found for *G. pulex*. PSA showed the best relationship with bioconcentration followed by D_{lipw}; MW; K_{ow} and V_D.

PSA describes the surface of the molecule in terms of its polar atoms and the molecules ability to permeate cells via active transport (Ertl *et al.*, 2000). The relationships found between BCF and PSA were strong however to obtain the best fit carvedilol was excluded from the analysis. This may imply that some pharmaceuticals are transported actively while some are not, however much more research will be needed to elucidate this hypothesis. For both animals no relationships were found between BCF and solubility.

The linear regression fitted to the data between BCF and PSA, V_D, K_{ow}, and D_{lipw} show that some of the relationships were not significant (table 2.6). Other nonlinear analyses such as exponential regression may be more appropriate and provide a better fit to the observed data (Appendix C), however more compounds should be tested to provide a more complete picture. These relationships could be invaluable in the future in prioritising testing requirements for pharmaceuticals in environmental systems.

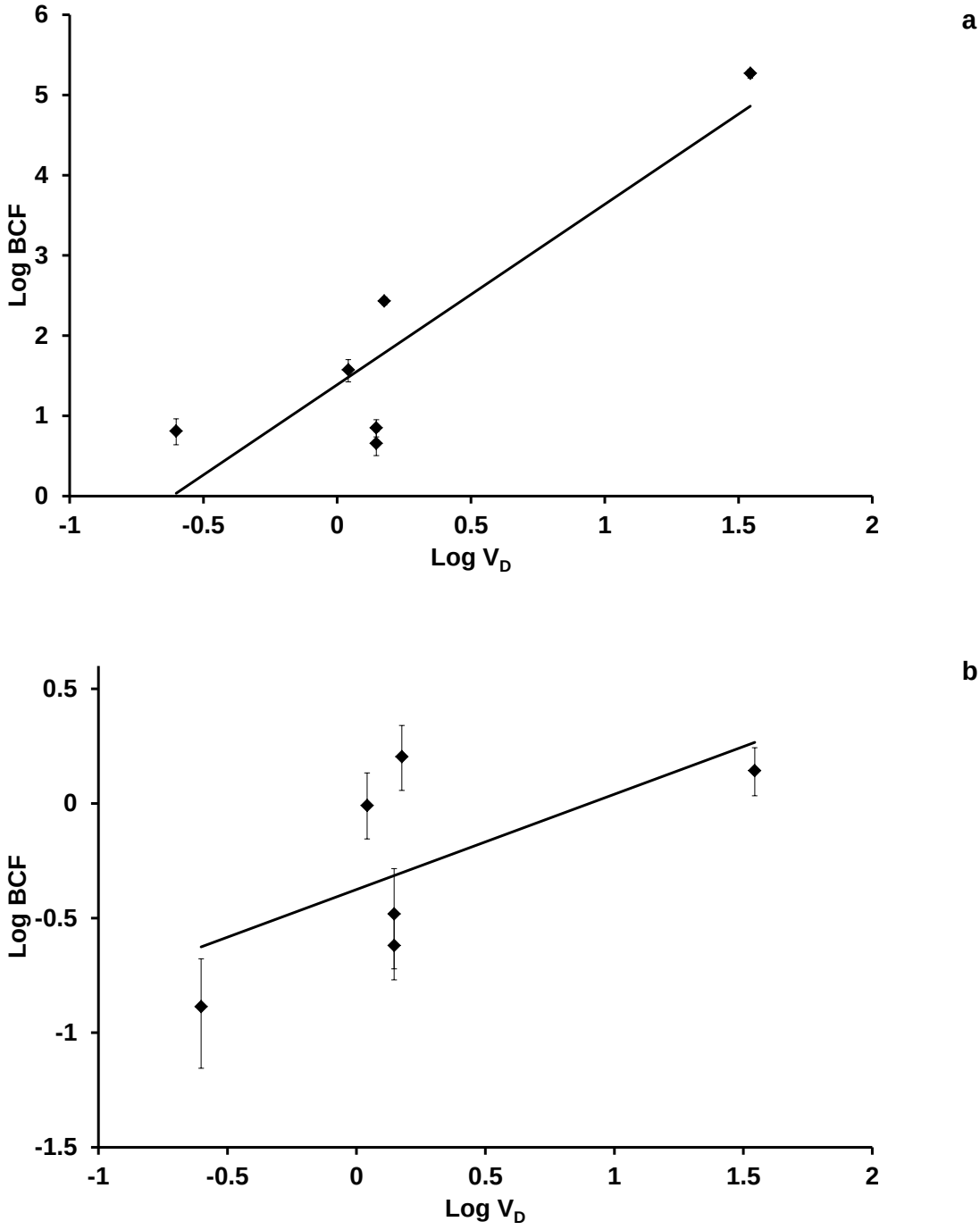


Figure 2.13 Relationships between bioconcentration factors (± 95th percentile) for the study pharmaceuticals and Volume of Distribution (V_D) for a) *Gammarus pulex* and b) *Notonecta glauca.* Diamonds are data points and the lines are linear regression

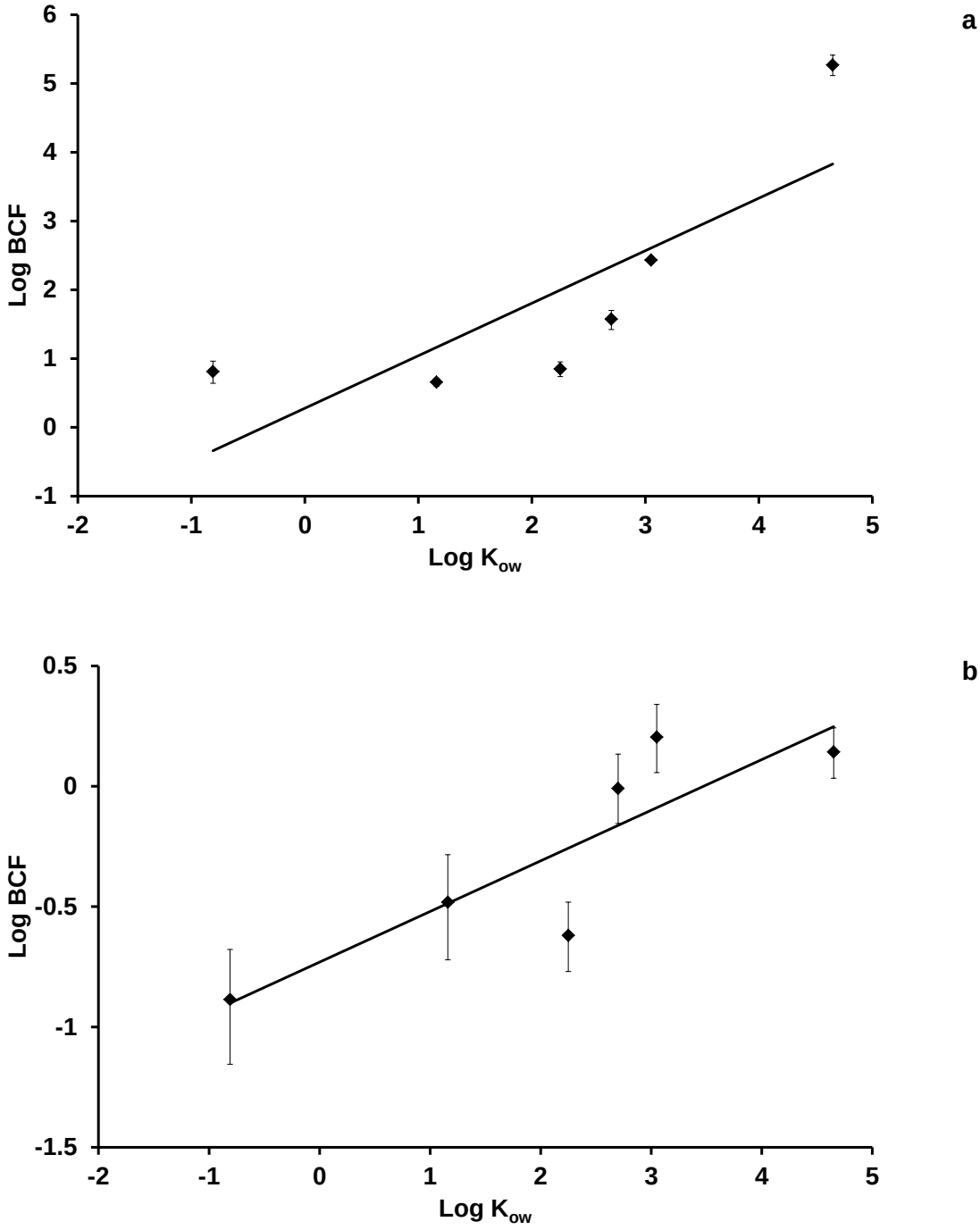


Figure 2.14 Relationships between bioconcentration factors (± 95th percentile) for the study pharmaceuticals and Log K_{ow} for a) *Gammarus pulex* and b) *Notonecta glauca.* Diamonds are data points and the lines are linear regression

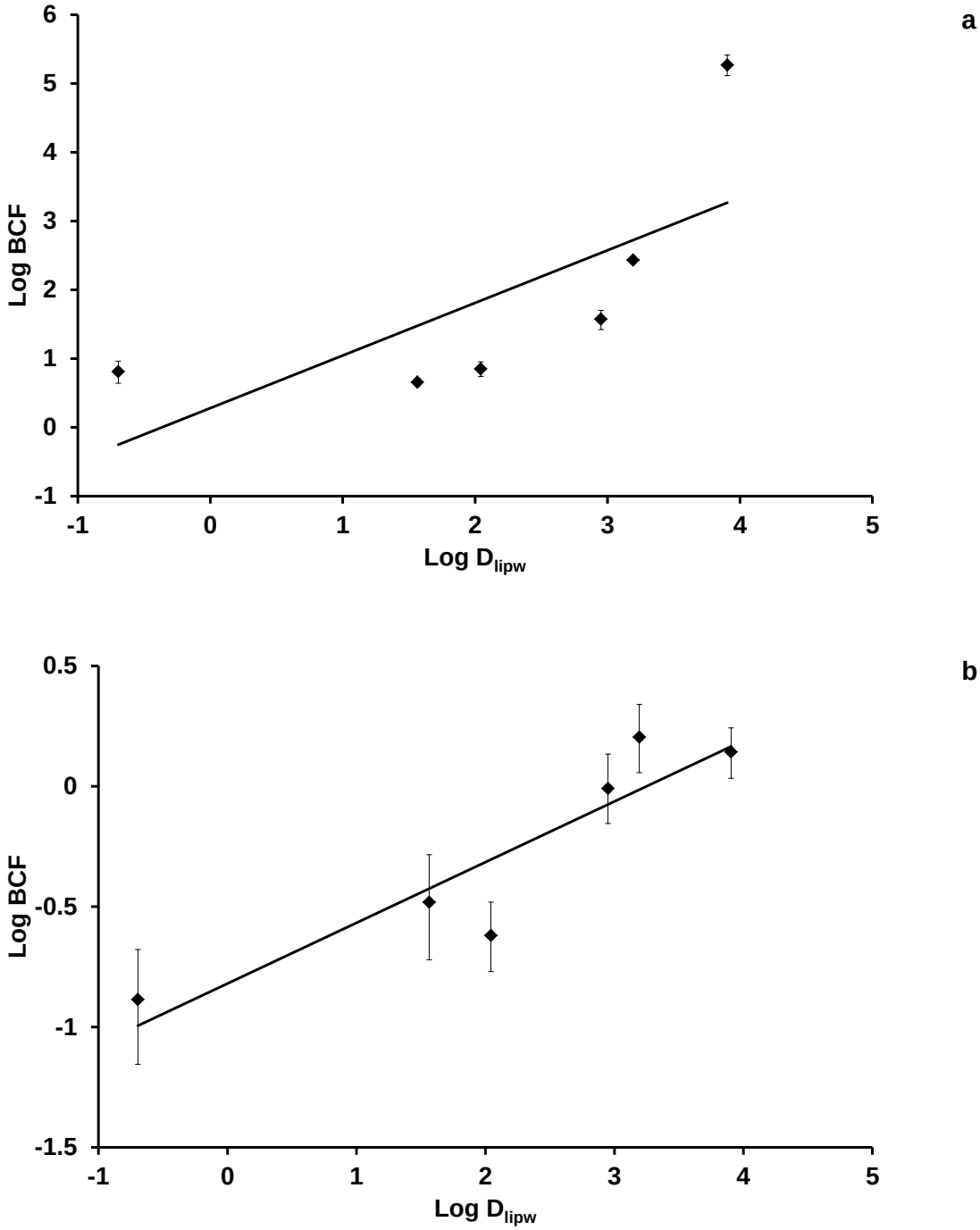


Figure 2.15 Relationships between bioconcentration factors (± 95th percentile) for the study pharmaceuticals and Log D$_{lipw}$ for a) *Gammarus pulex* and b) *Notonecta glauca.* Diamonds are data points and the lines are linear regression

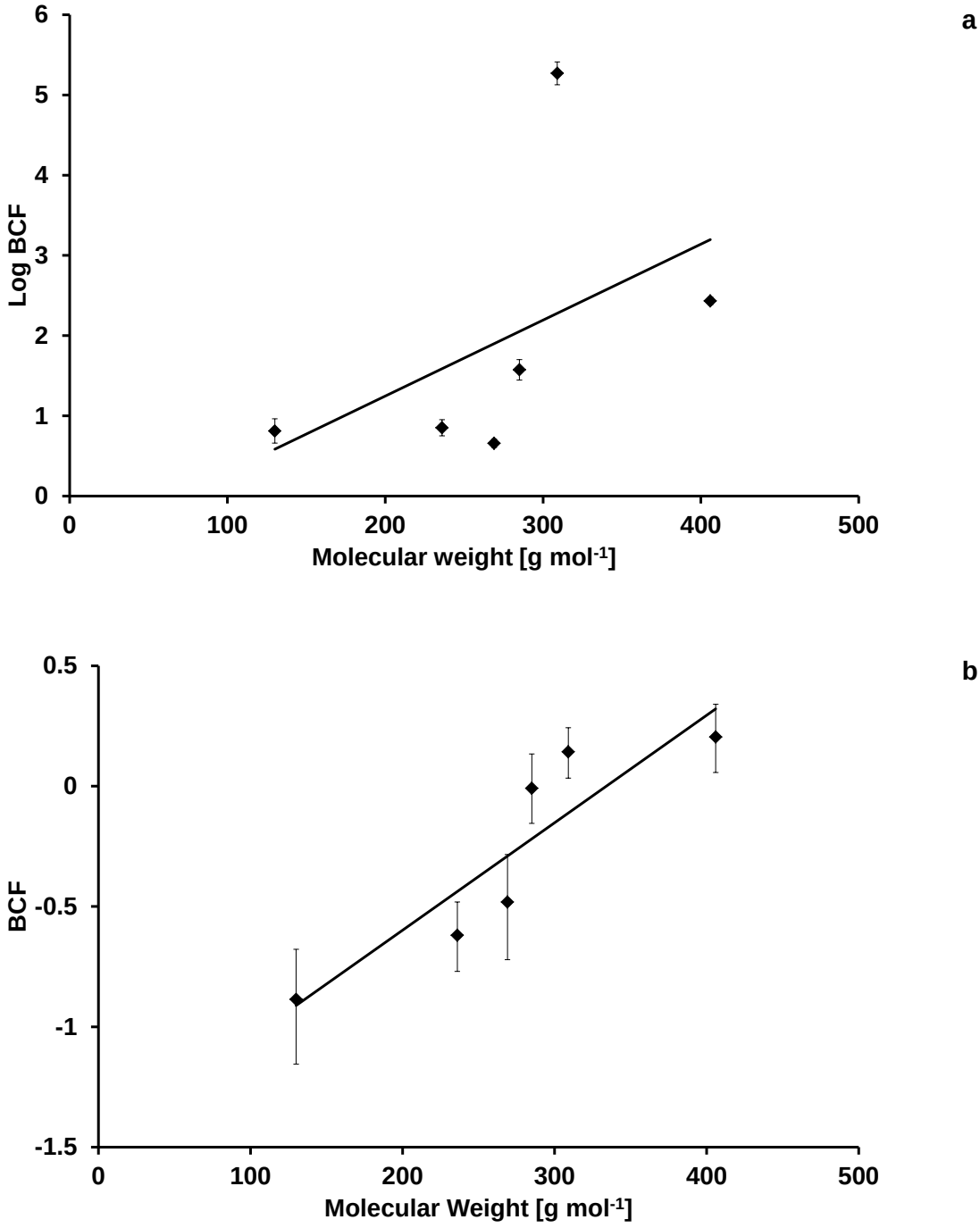


Figure 2.16 Relationships between bioconcentration factors (± 95th percentile) for the study pharmaceuticals and molecular weight for a) *Gammarus pulex* and b) *Notonecta glauca.* Diamonds are data points and the lines are linear regression

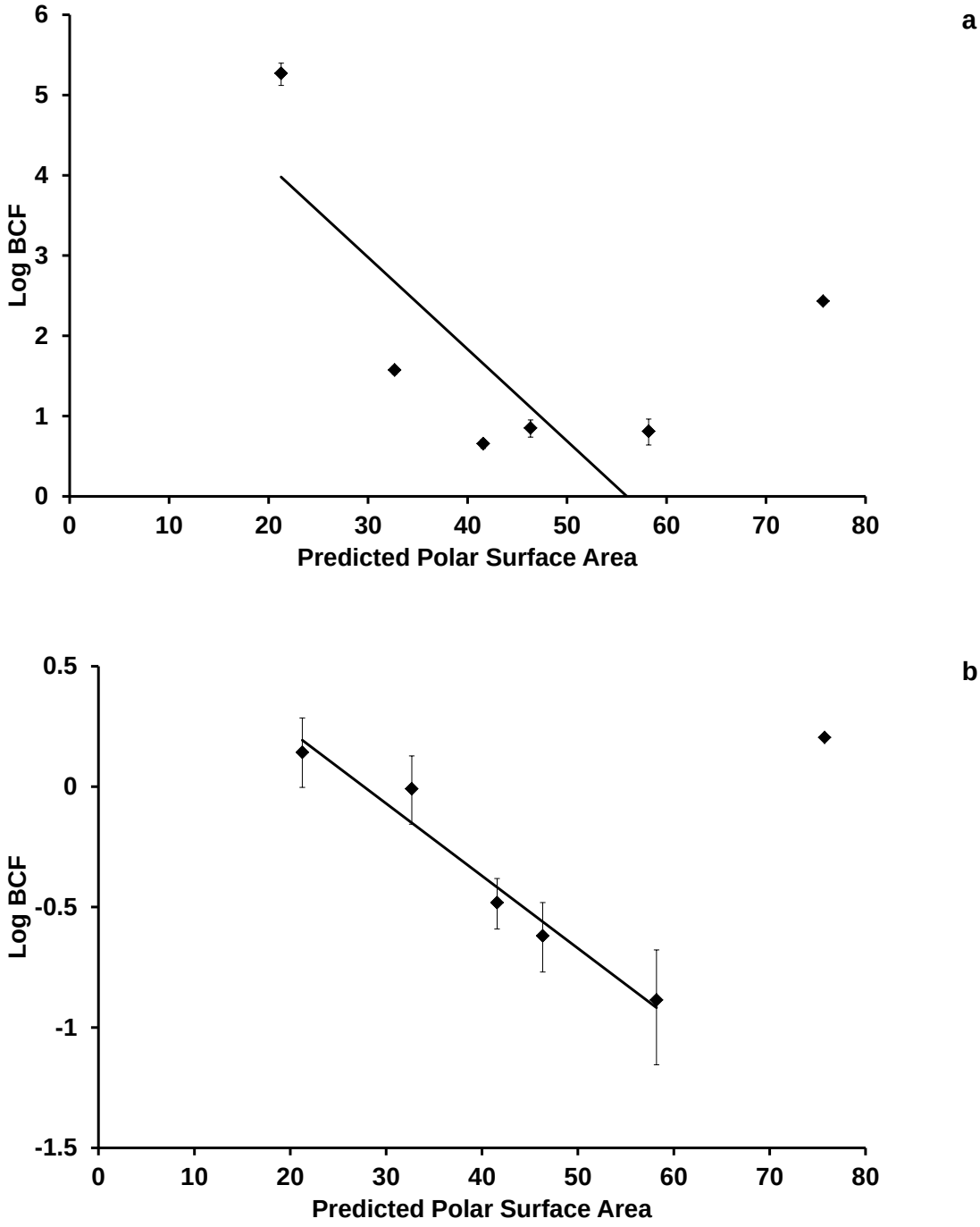


Figure 2.17 Relationships between bioconcentration factors for the study pharmaceuticals and Predicted Polar Surface Area for a) *Gammarus pulex* and b) *Nontonecta glauca*. Diamonds are data points and the lines are linear regression

Table 2.6 Regression analysis of the relationships between bioconcentration factors calculated for the study pharmaceuticals and physicochemical properties

Organism	Parameter for Predicting BCF	Regression	R^2	Equation	P value	Figure
G. pulex	Log V_D	linear	0.80	BCF= 2.25 x Log V_D + 1.39	0.06	2.13 a
N. glauca		linear	0.42	BCF= 0.42 x Log V_D – 0.38	0.67	2.13 b
G. pulex	Log K_{ow}	linear	0.64	BCF= 0.76 x Log K_{ow} + 0.28	0.06	2.14 a
N. glauca		linear	0.75	BCF = 0.21 x Log K_{ow} - 0.73	0.03	2.14 b
G. pulex	Log D_{lipw}	linear	0.50	BCF= 0.76 x Log D_{lipw} + 0.29	0.12	2.15 a
N. glauca		linear	0.84	BCF = 0.25 x Log D_{lipw} - 0.82	0.01	2.15 b
G. pulex	MW[a]	linear	0.24	BCF = 0.009 x MW – 0.65	0.33	2.16 a
N. glauca		linear	0.81	BCF = 0.004 x MW – 1.49	0.02	2.16 b
G. pulex	PSA [b]	linear [c]	0.67	BCF= -0.11 x PSA + 6.41	0.09	2.17 a
N. glauca		linear [c]	0.96	BCF= -0.03 x PSA + 0.83	0.004	2.17 b

[a]MW Molecular weight; [b] PSA Polar Surface Area; [c] linear relationship excludes carvedilol

2.4 Conclusions

The present study has shown that pharmaceuticals can be taken up from the ambient water into the tissues of aquatic invertebrates. The extent of uptake is dependent on the physico-chemical properties of the pharmaceutical and may be influenced by the test species itself. Uptake into an individual invertebrate species can be estimated based on the pH-corrected liposome water partition coefficient of a pharmaceutical. When combined with information on predicted exposure concentrations, receptor conservation within an organism and pharmacological information, these relationships are likely to be invaluable in identifying pharmaceuticals which are likely to pose the greatest concern in aquatic systems. Further work is now needed on a broader range of organisms covering a wide range of traits and with different levels of metabolic activity. Ultimately, we should aim to be developing relationships that combine physico-chemical properties and the biological traits in order to understand the internal exposure within an organism. By combining these relationships with information on drug potency and conservation of drug receptors within aquatic organisms we should be much better placed to establish the broad risks of pharmaceuticals to aquatic ecosystems

Chapter 3 The metabolism of pharmaceuticals in *Gammarus pulex*

3.1 Introduction

As discussed in Chapter 1 the fate of pharmaceuticals in crustaceans such as *G. pulex* is determined by the processes of uptake, metabolism and elimination. Radiolabelled studies such as those described in Chapter 2 enable the measurement of internal concentrations, uptake and elimination of pharmaceuticals in small tissue samples; but they do not provide information on the metabolism of the compound. The BCF determination should be based on the parent compound and should not include metabolites. Uncertainty in BCFs calculated for radiolabelled compounds arises because there is no differentiation between radiolabelled parent and radiolabelled metabolites (Arnot and Gobas, 2006). An over estimation of a BCF determined with radiolabelled compounds can occur when the parent is metabolized to a radiolabelled metabolite. By contrast an underestimate of the BCF can occur if the radiolabelled metabolite is eliminated to the water resulting in an over estimation of the radiolabelled parent compound in the water (Arnot and Gobas, 2006). Also degradation of the parent compound in the water during the exposure can contribute to error in calculation of the BCF. This demonstrates the need for an approach that is able to measure parent and any transformation products individually when investigating metabolism.

The metabolic fate of a compound can have an important influence on its accumulation potential in the organism. In mammals metabolism of compounds can be grouped into two phases; phase 1 reactions that introduce a functional group on the parent compound including oxidation, reduction and hydrolysis; and phase 2 reactions which lead to the covalent linkage between sulphate groups, sugars or peptides with polar elements such as –COOH, -OH, or –NH2 groups increasing the polarity of the compound. This allows the compound to be eliminated rapidly (Timbrell, 2002, Brouwer and Lee, 2007). For examples of the metabolic pathways of human pharmaceuticals in man the reader is referred to Figure 3.1.

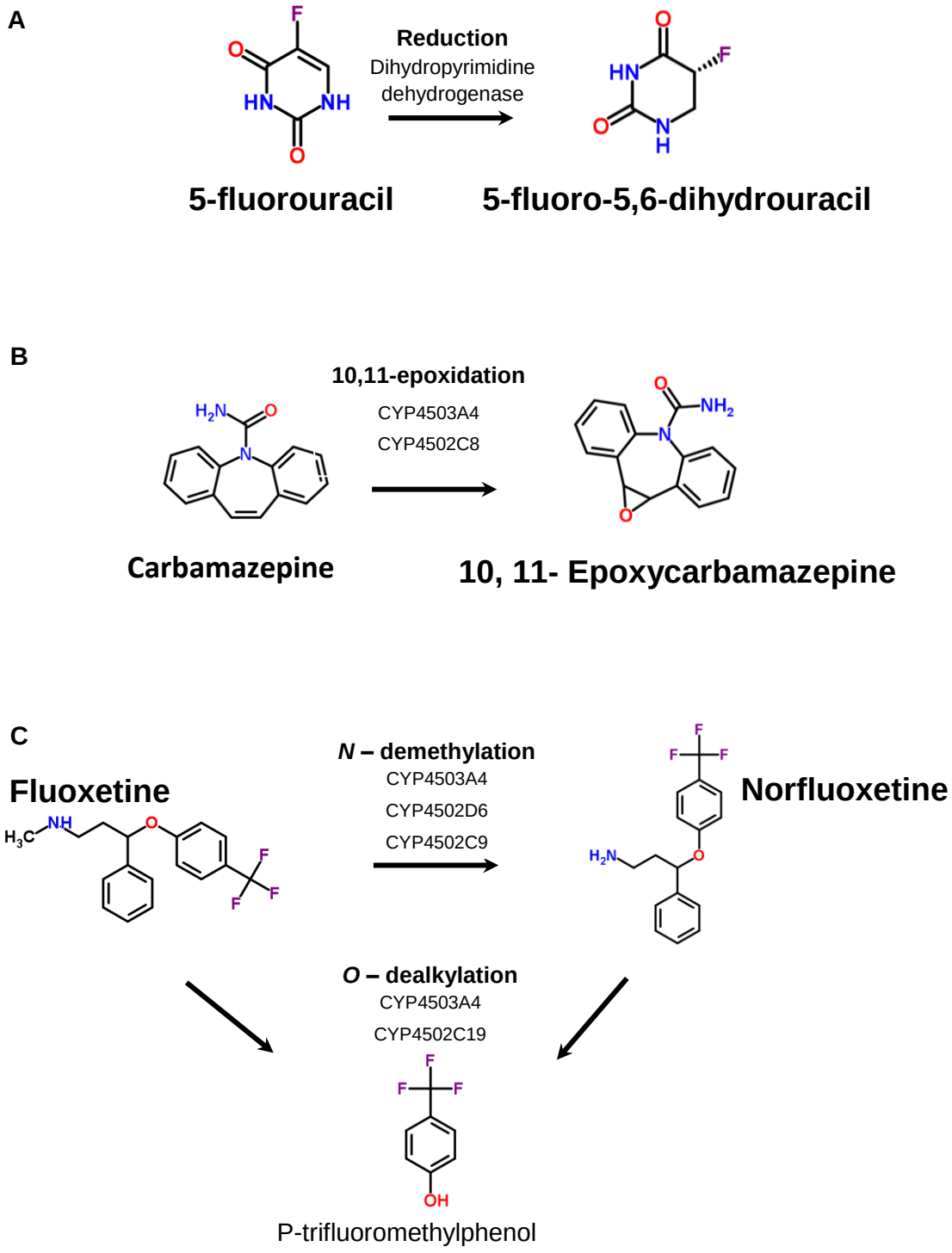


Figure 3.1 Metabolic pathways of human pharmaceuticals in man; A, 5-fluorouracil; B, carbamazepine and C, fluoxetine (Drug Bank http://www.drugbank.ca/)

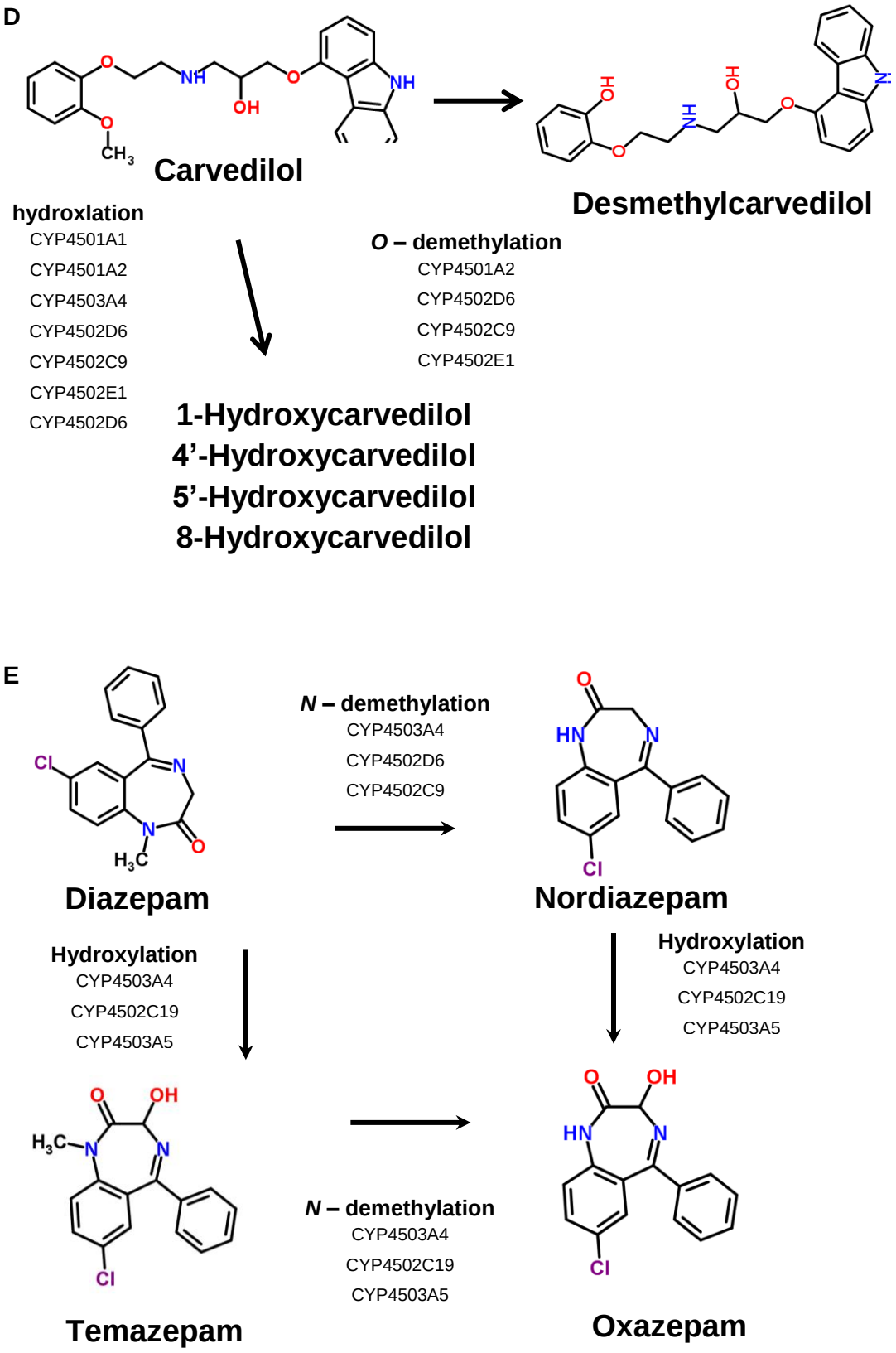


Figure 3.1 continued Metabolic pathways of human pharmaceuticals in man; D, carvedilol and E, diazepam (Drug Bank http://www.drugbank.ca/)

The majority of phase 1 oxidation reactions are catalysed by the cytochrome P450 enzymes (CYP-450) which are widely distributed across the animal kingdom (Wilkinson, 2001). They may play an important role in the metabolism of compounds in many organisms including fish and crustaceans (James and Boyle, 1998).

Pharmaceuticals are engineered to act on a particular target such as enzymes, ion channels and receptors (Winter *et al.* 2009). Many of these particular targets could be well-conserved through evolutionary lines. There are complete genomes mapped for several aquatic species including the *Gasterosteus aculeatus* (Three-spined stickleback), *Danio rerio* (zebrafish) and *Daphnia pulex* (water flea). Work by Gunnasson *et al.* 2008 evaluated the conservation of different functional drug targets and found that in *D. pulex* 61 % were derived from a common ancestral protein to those found in man. Also manual curation of the *D. pulex* genome has identified 75 functional CYP-450 genes. The genes for processing CYP-450 2, 3, 4, enzymes identified in the *Daphnia pulex* genome are similar to those CYP-450 genes identified insects and vertebrates (Baldwin *et al.,* 2009). Many of these CYP-450 enzymes are used for metabolising pharmaceuticals, suggesting that it may be possible to predict the metabolism and effects of pharmaceuticals in non-target species. These predictions could be based on mammalian metabolic pathways and known interactions between pharmaceuticals and drug targets in man (Gunnasson *et al.* 2008). For example the identification of norfluoxetine in fish after exposure to fluoxetine by Paterson and Metcalfe (2008) could have been predicted from known metabolic pathways of fluoxetine in man (figure 3.1C). Other known metabolites of human pharmaceuticals have also been identified by a number of authors in fish including metabolites of diclofenac, sertraline and 17 α-ethinylestradiol (Brooks *et al.*, 2005, Flores and Hill, 2008, Kallio *et al.*, 2010).

For invertebrates, research into metabolism of pharmaceuticals is limited, however they have been shown to metabolise other environmental contaminants (Nuutinen *et al.*, 2003b). For example, it is known that in crustaceans the hepatopancreas accumulates many of the compounds transported by the haemolymph from the site of uptake and performs many of functions that are associated with the liver in mammals such as the metabolism of xenobiotic compounds (Brouwer and Lee, 2007). Invertebrates possess a suite of metabolic enzymes and have been shown to metabolise environmental contaminants (Livingstone, 1998, Nuutinen *et al.*, 2003b), such as polyaromatic hydrocarbons, pesticides and steroids. For example, steroid hydroxylated metabolites and conjugates have been shown to be formed after exposure to progesterone and testosterone (Summavielle *et al.*, 2003). There is

evidence to suggests that pharmaceuticals can also be metabolized by CYP-450 enzymes in crustaceans for example oxidized metabolites of erythromycin and sulfadimethoxine, pharmaceuticals commonly used in aquaculture, were found in the American lobster, *Homarus americanus* (James, 1990a, James, 1990b, James and Boyle, 1998).

Research into the metabolism of pharmaceuticals in non-target organisms is important as it has implications for the environmental risk assessment of these compounds. Knowledge of the metabolic capacity of non-target organisms is necessary for accurate calculation of BCFs and the toxicity of the compound to the organism. In Phase 1 metabolism the reactions are catabolic and the metabolites formed are often chemically more reactive (Rand and Dales, 2008). Active metabolites such as norfluoxetine contribute to the overall potency of the parent compound and can have longer elimination half-lives (Paterson and Metcalfe, 2008, Straub, 2008). This will lead to an increased potential for bioaccumulation of active metabolites compared to the parent compound only, leading to increased potential of toxic effects on non-target organisms (Paterson and Metcalfe, 2008).

New methods have to be developed in order to study the metabolism of pharmaceuticals in aquatic invertebrates as when studying radiolabelled compounds there is no differentiation between radiolabelled parent and radiolabelled metabolites. The use of mass spectrometry methods has increased enormously in recent years due to improved mass resolution for small molecules (m/z <800), selectivity, throughput of analysis and greater affordability. Mass spectrometry techniques gaining in popularity include tandem quadrupole (QqQ-MS), ion trap (IT-MS) and accurate mass measurement methods such as. time-of-flight (Tof-MS) and LTQ-Orbitrap-MS (Gilbert *et al.*, 2009). These techniques have been shown to be useful tools in the structural identification of small molecules in the pharmaceutical industry (Pérez-Carrera *et al.*, 2010) and are beginning to be applied to the identification of 'unknowns', such as pharmaceutical-transformation products, in the environment (Radjenović *et al.*, 2009).

The research described in this Chapter is among the first to investigate the metabolism of human-pharmaceuticals in Crustacea. The objective of the research was to detect and identify any metabolites formed after exposure of *Gammarus pulex* to pharmaceuticals. The research involved:

- The development and evaluation of a simple solvent extraction procedure suitable to isolate analytes from *Gammarus pulex* and

compatible with ultra-performance liquid chromatography – quadrupole – time of flight mass spectrometry (UPLC-QTof-MS)

- The optimization of UPLC-QTof-MS parameters (including software protocols) for the detection, quantification and identification of parent compounds and metabolites present in the tissues of *Gammarus pulex* exposed to human pharmaceuticals
- To repeat pharmacokinetic experiments using non-labelled pharmaceuticals to allow measurements by the optimized UPLC-QTof-MS methods
- To compare the results for uptake and depuration of parent and metabolite compounds obtained from radio-labelled experiments and measurements using LSC, with those obtained using non labeled pharmaceuticals measured by UPLC-QTof-MS

3.2 Materials and methods

3.2.1 Experimental organisms, exposure water and handling

Gammarus pulex (both males and females) were collected from Bishop Wilton Beck, a small stream northeast of York U.K. (E479600, N455000) in February 2008 for metabolite identification studies and September 2009 for uptake and depuration studies. In the laboratory the animals were maintained in a 5 L aquarium in APW and kept at ca. 20 °C under a natural light regime (Naylor *et al.* 1989). Animals were fed a diet of rehydrated horse-chestnut leaves (*Aesculus hippocastanum*) inoculated with the fungus *Cladosporium herbarum* prior to use.

3.2.2 Pharmaceuticals compounds

Radiolabelled and unlabelled pharmaceuticals 5-fluorouracil, carvedilol, diazepam, nordiazepam and moclobemide (Figure 3.2) were provided by F. Hoffman-la Roche Ltd (Basel, Switzerland), whilst carbamazepine and fluoxetine were obtained from Agriculture and Agri-Food Canada, London, Ontario and Sigma-Aldrich Company Ltd.UK. Information on the chemical properties of the pharmaceuticals is provided in table 2.1 and table 2.2.

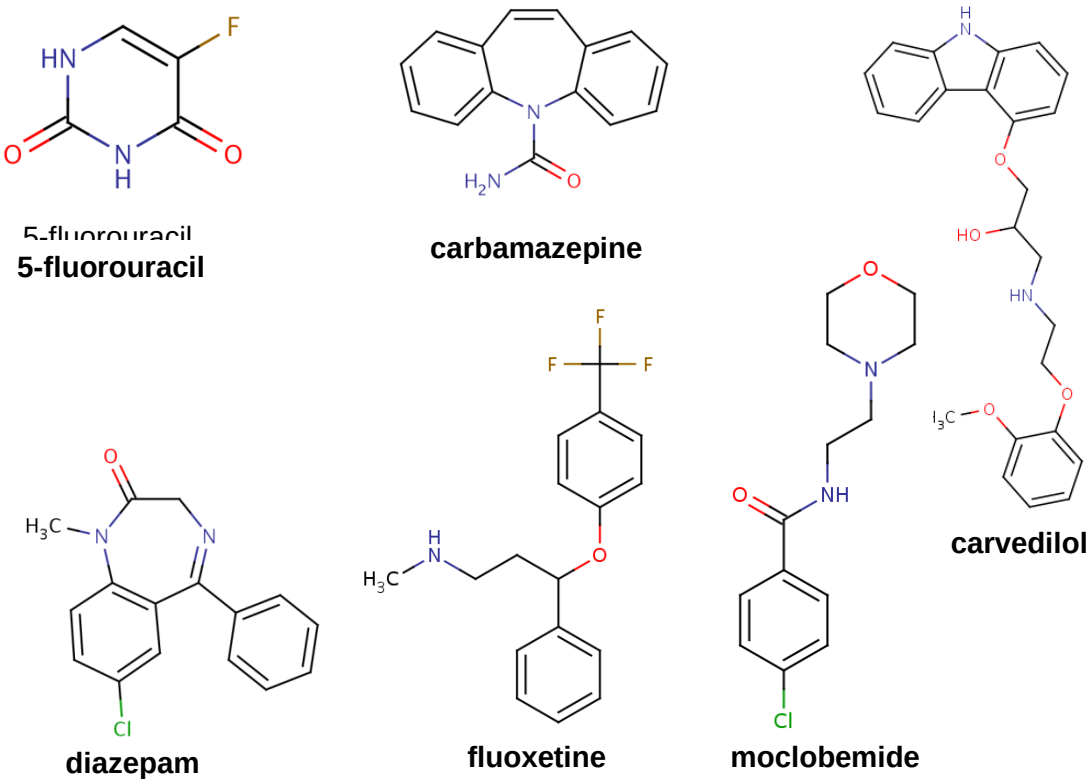


Figure 3.2 Molecular structures of the test pharmaceuticals

3.2.3 Experimental procedure

3.2.3.1 Preparation of stock standard solutions

Standard stock solutions of pharmaceuticals (10 µg mL^{-1}) were prepared by dissolving the compound in methanol at room temperature. All stocks were stored in the dark at - 20 °C before use in the metabolism studies.

3.2.3.2 Development and evaluation of the solvent extraction procedure using radiolabelled compounds

An alternative simple and rapid extraction procedure was required that avoided the use of Soluene in order to be compatible with UPLC-QTof-MS. The extraction procedure needed to provide effective extraction of parent and metabolite

compounds, but with minimal clean-up in order to avoid any losses of analytes of interest. It was therefore decided to employ a simple solvent extraction.

It was not permitted to analyse radio-labelled compounds using the UPLC-QTof-MS instrumentation available to the project. Therefore, the initial evaluation of the methanol extraction was undertaken by spiking radio-labelled pharmaceuticals onto homogenized *Gammarus pulex* tissues and then determination of the 'recovery' of spiked analytes by LSC. The spiking approach does not determine the 'extraction efficiency' of incurred or bound residues but simply indicates any losses of analytes occurring during extraction and clean-up.

Five non-treated ('blank') *G. pulex* samples (0.24 ± 0.04 g mean ± standard deviation, n=5) were ground with methanol (1 mL) using a pestle and mortar until the mixture was visibly homogeneous. The homogenized tissue was spiked with 10 µL of each radioactive stock solution. The spiking solutions added were then allowed to permeate the sample for approximately two minutes.

The spiked homogenate was transferred to a Teflon centrifuge tube using methanol (2 x 2 mL) to rinse the pestle and mortar. The final volume of the extract was approximately 5 mL. The extract was shaken on a rotary shaker, 200 MOT for 10 minutes then centrifuged at 3500 rpm at 5 °C for 15 minutes. The supernatant of each sample was filtered through a 0.2 µm PTFE 13 mm minisart filter (Sigma-Aldrich Company Ltd. Dorset, UK) and the filtrate evaporated to dryness in the fume hood overnight. The resulting residue was made up with methanol (1 mL), with vortex mixing (Clifton cyclone, 1min) and sonication (15 minutes) to aid dissolution of the matrix. A sub-sample (300 µL) was taken for LSC analysis and the remaining extract was stored at -20 °C.

To evaluate the recovery of analyte, the concentration in the extract were analysed against radioactive pharmaceutical standards prepared in methanol. The final extracts and solvent standards were mixed with Hionic fluor scintillant (Perkin Elmer, USA; 10 mL) and left to stand (approx. 15 min). The radioactivity in the samples was measured by LSC using the methods described in section 2.2.5. The radioactivity of the residues obtained from extracts of *G. pulex* was compared to the radioactivity in the corresponding solvent standard to determine the recovery of the pharmaceutical analytes from the sample.

3.2.3.3 Preliminary screening experiments for the detection and identification of metabolites using UPLC-Tof-MS

Firstly, small-scale experiments were undertaken for all of the test compounds to evaluate the method and also to provide *G. pulex* tissue that could be screened for the presence of metabolites. Three replicate vessels containing ten animals in 500 mL of APW (pH 8.78 ± 0.14, dissolved oxygen 7.59 ± 0.4 mg L-1 at 12 °C mean ± standard deviation, n=3) were prepared for each pharmaceutical treatment, and one set of replicates used as the solvent control (0.1% Methanol). The vessels were equilibrated to the experimental conditions for 48 hours prior to the start of the experiments. The vessels were then spiked with the individual non-labelled pharmaceutical (0.5 mg L-1) and *G. pulex* were exposed for 48 hours. After the exposure period, the *G. pulex* were removed, rinsed in deionised water and frozen at - 20 °C before extraction with methanol (see Chapter 2) and analysis by UPLC-QTof.

3.2.3.4 Preparation of standard solutions in solvent, pond water and *G. pulex* tissue for calibration of UPLC-QTof-MS

Three sets of calibration standards at concentrations of 5, 1, 0.5, 0.1, 0.05 and 0.01 µg mL^{-1} were prepared by serial dilution of standard stock solution into methanol, APW or extracted *G. pulex* (matrix). The UPLC-Tof-MS responses for these standards were compared to evaluate MS suppression effects. Also, the stability of the compounds in the calibration standards prepared 6 months previously was tested by reanalysing samples against freshly prepared stock solutions by UPLC-QTof-MS.

3.2.3.5 Large-scale-pharmacokinetic experiment using non-labelled diazepam

Uptake and depuration studies were carried out using the method as described in section 2.2.5 adapted from Ashauer *et al.* (2006). Test species were acclimatized to the test conditions for 24 hours without food under a specific photoperiod (12 hours light: 12 hours dark). A static exposure was used and water quality parameters (pH, dissolved oxygen) were monitored throughout the experimental periods using a Symphony SB80BD bench top meter (VWR International LtD, UK). *G. pulex* were exposed to 2 µmol L^{-1} (0.5 mg L^{-1}) of diazepam.

Animals were exposed to 500 mL of test solution for 0, 3, 6, 12, 24 and 48 hours. Each individual pharmaceutical experiment comprised five replicates of twenty animals with the individual animals separated by stainless steel cages to avoid cannibalism. After 48 hours exposure to diazepam the remaining organisms were transferred to clean non-treated water in order to study depuration. The depuration study was run for the same time period as the uptake study with samples collected at the equivalent time points. At each sampling point, two animals were taken from each replicate and pooled (n = 10). The animals were rinsed in deionised water to remove any pharmaceutical residue from their surface and weighed. The samples were then frozen at – 20 °C prior to analysis. Sample of exposure water (1 mL) were collected at each time point to enable determination of the diazepam exposure concentration by UPLC-Tof MS analysis.

3.2.3.6 Instrumentation: Liquid chromatography

Chromatographic separation was performed using an ACQUITY® UPLC® system operating with a ACQUITY® UPLC® HSS C18, 2.1 x 50 mm, 1.8 mm column (Waters, Manchester, U.K.). Mobile phase A comprised of 0.1 % formic acid in water and mobile phase B comprised of 0.1 % formic acid in methanol. A linear gradient was applied: 0 min: 100 % A; 6 min: 0 % A; 8 min: 0 % A; 8.1 min: 100 % A; 10 min: 100 % A. The flow rate was set to 0.6 mL min^{-1}, the column temperature to 40 °C and sample extract temperature to 5 °C. The injection volume was 5 µL using a 5 mL partial loop with no overfill.

3.2.3.7 Instrumentation: Mass spectrometry

The mass spectrometry was performed using a Waters-Xevo™ QTof-MS (Waters, Manchester, U.K.) operated in MS^E mode to allow acquisition of low energy precursor ion data and high energy fragment ion data in a single run. The MSE low energy was set at 6.0 and higher energy 25 – 30 V. The electrospray ionisation interface was operated in either positive mode or negative mode. The capillary voltage was maintained at 0.8 kV and sample cone voltage at 10 V. The source temperature was 120 °C and desolvation temperature was 450 °C. Nitrogen was used both for the desolvation gas, 800 L $hour^{-1}$ and the cone gas, 5 L hour-1. Cumulated spectra (m/z: 50 – 1000) were taken every 0.1 seconds. A LockSpray™ interface was used to correct the mass axis and the Lock spray compound was

Leucine enkephalin which has a two point lock mass (m/z 278.1141 and m/z 556.2771).

3.2.3.8 UPLC –QTof-MS analysis

Four replicate injections were made of each of the extracts collected from the treated and non-treated experiments. To prevent carry-over of analyte into the following analysis methanol was injected after treated extracts. Mass spectra were acquired using the UPLC-Tof-MS conditions described in Sections 3.2.3.6 and 3.2.3.7. Data acquisition and processing were performed using MassLynx V4.1 Software (Waters, Manchester).

3.2.3.9 UPLC-QTof-MS post acquisition data analysis

The data was evaluated automatically using MetaboLynx XS proprietary software (Waters, Manchester UK) and using MassLynx V4.1 software to manually reverse search the total ion chromatogram for the exact monoisotopic mass of the formulae proposed by chemical modelling. An overview of data processing scheme is given in Figure 3.3.

Post-acquisition data processing software MetaboLynx XS V4.0 (Waters, Manchester) was applied to both the full scan and product ion data. This software uses peak detection algorithms and complex statistical analysis to profile differences between the control samples (non-treated) and treated samples. Extracts are normally injected multiple times (n = at least 3) to improve the reliability. In theory, any differences are due to the treatments, but in practice the detection of significant differences (metabolites at trace concentrations) in complex matrices is extremely challenging. There is a risk that this automated non-targeted approach may fail to detect all of the metabolites.

To minimize the risk of non detection a targeted approach (manual reverse searching) was also undertaken. The individual Tof MS (full range acquisition) mass chromatograms were reversed searched using the exact monisotopic data for all of the possible psedomolecular ions, adduct ions, and metabolite structures (predicted from chemical modelling; Table 3.1).

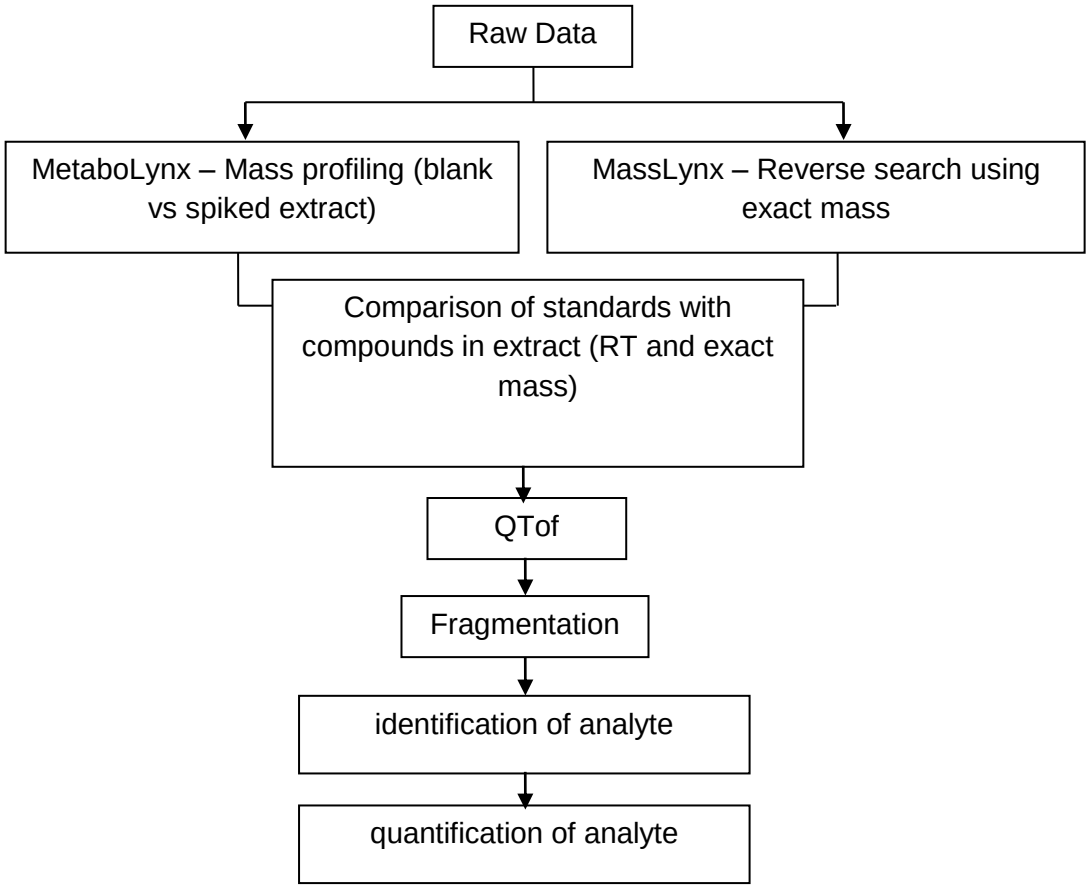


Figure 3.3 Sequence of the analysis of extracts using UPLC-QTof-MS

The accurate mass of any suspect peaks (potential metabolites) from acquisition of Tof data over the full mass range provides the possible empirical formulae, but not the chemical structure and hence identity of the compound. Unequivocal identification requires the availability of the standard reference material and MS/MS fragmentation information. The identification of parents and metabolites is usually verified by confirming that the retention time, Tof spectra (including isotope pattern) and MS/MS product ion spectra of peaks in the sample extracts match with those from the analysis of the standard reference materials.

The MetaboLynx software incorporates a 'dealkylation tool' to facilitate characterisation of fragments of the parent molecule by systematically identifying likely bond metabolic cleavages to predict the fragments produced during MS/MS.

Once the identification is verified quantification was undertaken using QuanLynx software (Waters, Manchester) to compare the response of the measured mass of a

component at a specified retention time with the corresponding response in matrix-matched calibration standards.

3.2.4 Derivation of uptake and depuration rate constants and bioconcentration factors

A first-order one-compartment toxicokinetic model was fitted to the internal concentration data using individual replicates to estimate the parameters uptake and elimination rate constants and the BCFs, the reader is referred to Chapter 2.

A pseudo BCF (Nakamura *et al.*, 2008) was also calculated for metabolite residues, with the concentration of metabolite measured in *G. pulex* after 48 hours exposure and the average water concentration of the parent pharmaceutical using Equation 3.1:

Equation 3.1

$$BCF = \frac{C_{internal}}{C_{water}}$$

3.3. Results

3.3.1 Extraction method performance

An extraction methodology was required that would be compatible with Tof-MS. The extraction procedure used in radiolabelled studies in Chapter 2 was not suitable, as Soluene 350 (Perkin Elmer, USA) is highly corrosive. However, the use of radio-labelled pharmaceuticals with LSC was employed to evaluate a simple extraction method. This approach was convenient because access to the Tof-MS Instrumentation was limited. Using methanol as extraction solvent the recovery of parent pharmaceutical compounds spiked into homogenized *G. pulex* tissue ranged from 82 – 97% and increased in the order fluoxetine < carvedilol < diazepam < carbamazepine < moclobemide < 5-fluorouracil (Figure 3.4).

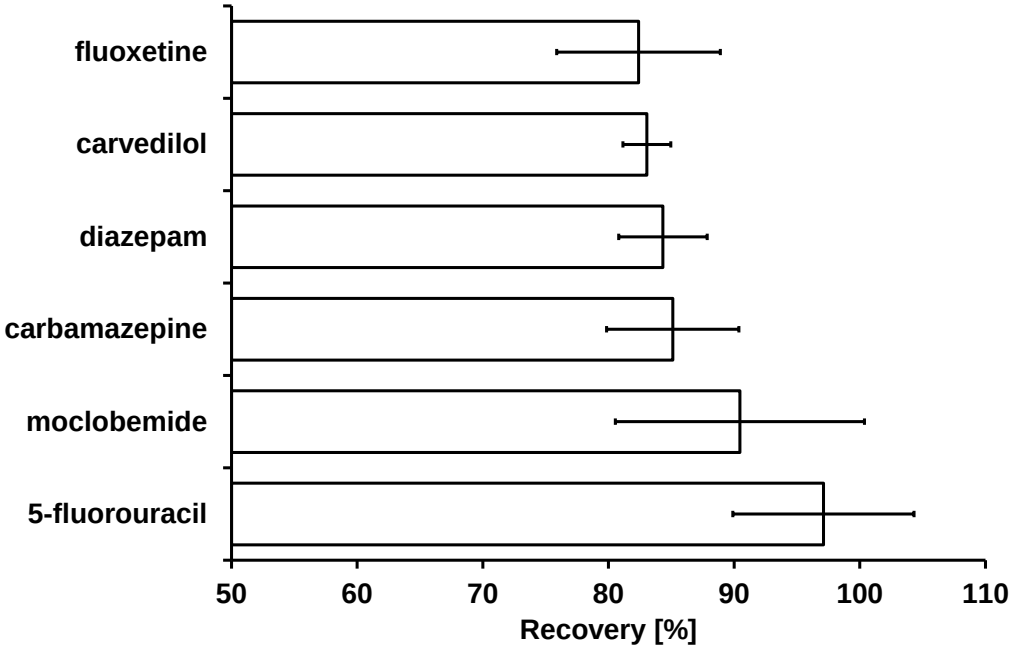


Figure 3.4 Radiolabelled recoveries of incurred residues of test pharmaceuticals from *Gammarus pulex* tissues (n=5). Mean percentage recovery (± standard deviation) of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide

Table 3.1 Target metabolites of test pharmaceuticals identified for post target screening, the exact monisotopic data predicted from chemical modelling

	Exact Mass $[M+H]^+$						
Metabolite	**5-fluorouracil**	**carbamazepine**	**carvedilol**	**diazepam**	**fluoxetine**	**norfluoxetine**	**moclobemide**
Hydroxylation/Sulphur oxidation (1)	147.02005	253.09715	423.19145	301.07383	326.13624	312.12059	285.1006
Dihydroxylation/Sulphur oxidation (2)	163.01496	269.09207	439.18636	317.06875	342.13115	328.1155	301.19551
Dehydroxylation	115.03022	221.10732	391.20162	269.084	294.14641	280.13076	253.11077
Demethylation	117.00948	223.08659	393.18088	271.06327	296.12568	282.11003	255.09003
Glucuronidation	307.05722	413.13433	583.22862	461.11101	486.17341	472.15776	445.13777
Acetylation	173.0357	279.1128	449.2071	327.08948	352.15189	338.13624	311.11625
Sulfation	210.98195	317.05905	487.15335	365.03573	390.09814	376.08249	349.0625
Dehydrogenation	129.00948	235.08659	405.18088	283.06327	308.12568	294.11003	267.09003
Oxidation (1)	145.0044	251.0815	421.1758	299.05818	324.12059	310.10494	283.08495
Oxidation (2)	160.99931	267.07642	437.17071	315.0531	340.1155	326.09985	299.07986
Hydration	149.0357	255.1128	425.2071	303.08948	328.15189	314.13624	287.11625
Reduction	133.04078	239.11789	409.21218	287.09457	312.15698	298.14133	271.12133
Glycosylation	293.07796	399.15505	569.24936	447.13174	472.19415	458.1785	431.1585
Methylation	145.04078	251.11789	421.21218	299.09457	324.15698	310.14133	283.12133

Table 3.1 continued: Target metabolites of test pharmaceuticals identified for post target screening, the exact monisotopic data predicted from chemical modelling

	Exact Mass $[M+H]^+$						
Metabolite	**5-fluorouracil**	**carbamazepine**	**carvedilol**	**diazepam**	**fluoxetine**	**norfluoxetine**	**moclobemide**
Carbamoyl glucuronidation	351.07086	457.14797	627.24226	505.12464	530.18705	516.1714	489.15141
Glycine conjugation	188.0466	294.1237	464.218	342.10038	367.16279	353.14714	326.12714
Taurine conjugation	236.01358	342.09069	512.18498	390.06737	415.12977	401.11412	374.09413
Glutamine conjugation	259.08371	365.16082	535.25511	413.13749	438.1999	424.18425	397.16426
Proline conjugation	228.0779	334.155	504.2493	382.13168	407.19409	393.17844	366.15844
Serine conjugation	218.05716	324.13427	494.22856	372.11095	397.17335	383.1577	356.13771
Arginine conjugation	287.12624	393.20335	563.29764	441.18003	466.24244	452.22679	425.20679
Histidine conjugation	268.08404	374.16115	544.25545	422.13783	447.20024	433.18459	406.16459
Aspartic acid conjugation	246.05208	352.12918	522.22348	400.10586	425.16827	411.15262	384.13262
N-acetylcysteine conjugation	276.04488	382.12199	552.21628	430.09867	455.16107	441.14542	414.12543
Gluthathione conjugation	438.10894	544.18605	714.28034	592.16272	617.22513	603.20948	576.18949
Fluorine displacement	129.02947	NA	NA	NA	308.14566	294.13001	NA
Chlorine displacement	NA	NA	NA	267.1128	NA	NA	251.13957

As methanol extraction provided satisfactory recovery for the purpose of detection and identification, no other organic solvents were tested. The presence of water in the tissues may have improved the recovery of the more polar compounds. The use of higher proportions of water or combinations of solvent (e.g. methanol/acetone) could be considered in future work and may be necessary if different pharmaceutical compounds are evaluated.

The experiment undertaken only measures recovery of compounds spiked into the matrix and gives no information of the extraction efficiency of incurred residues. However, an indication of extraction efficiency of incurred residues was obtained by comparing results from radiolabelled studies with the results from methanol extraction and UPLC-QTof-MS analysis. These are outlined later in the text.

3.3.2 Matrix suppression and standard stability of diazepam and nordiazepam

Three sets of standards were prepared in solvent, APW and *G. pulex* extracts to assess any suppression (lowering of the detector response) of the Tof-MS signal. The results indicate that the APW and *G. pulex* matrices suppress the response of diazepam and nordiazepam compared to the solvent standards (Figure 3.5). This observation can be explained by the increased concentration of matrix co-extractives providing competition for charge during the electrospray ionisation process. As a result less analyte ions enter the mass spectrometer so the response is decreased.

Consequently calibration standards were prepared in appropriate blank matrix (instead of pure solvent) in order to provide accurate quantification of the concentrations of diazepam and nordiazepam. The concentrations of diazepam and nordiazepam were stable in samples that had been stored at -20 ºC for 6 months.

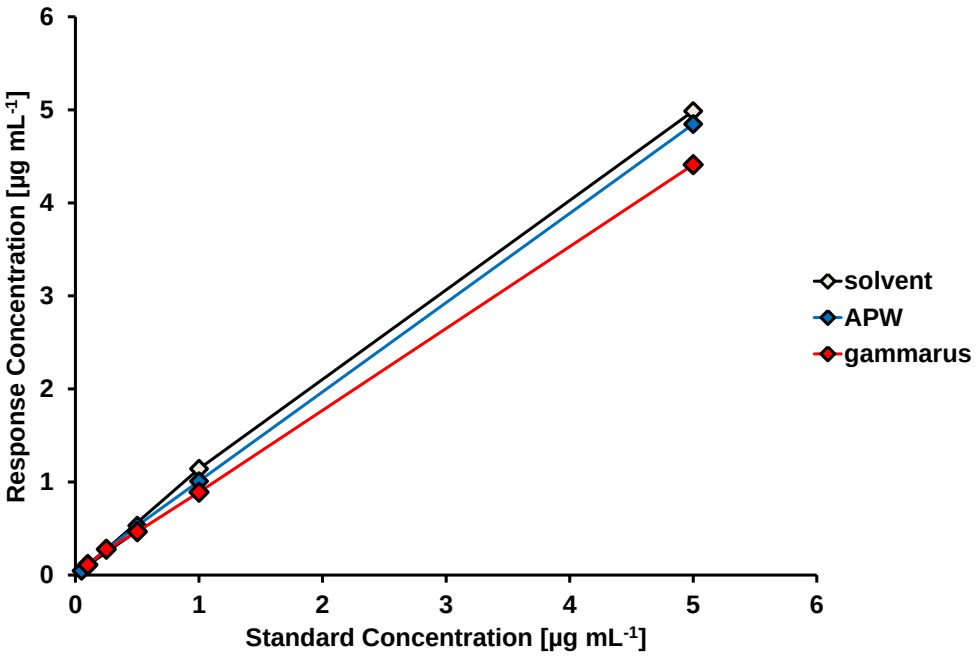


Figure 3.5 Suppression effects on the UPLC-QTof-MS response of nordiazepam in the matrix analysed tested at concentrations of 0.01, 0.05, 0.1, 0.5, 1 and 5 µg mL^{-1} of nordiazepam (white diamonds are methanol solvent standards, blue diamonds are artificial pond water (APW) standards and red diamonds are *Gammarus pulex* extracts; n=3)

3.3.3 Screening and identification of parent and metabolite pharmaceutical residues

The chromatographic separation of the parent compounds is shown in Figure 3.6. Moclobemide, carvedilol, carbamazepine fluoxetine and diazepam gave best response in the positive ion mode, whilst 5-fluorouracil gave best response in the negative ion mode. The use of polarity switching enabled all of the compounds to be analysed in a single analysis.

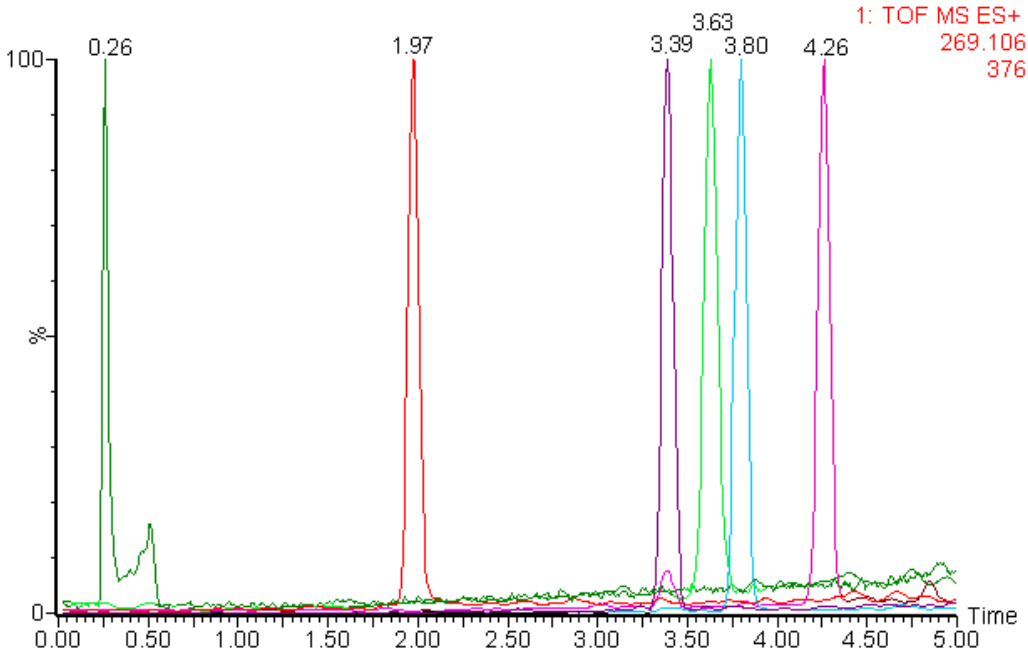


Figure 3.6 Extracted mass chromatograms of test pharmaceuticals from extracts of *Gammarus pulex* after 48 hours exposure to 0.5 mg L^{-1}; Compound identities and retention times (minutes) are: 5-fluorouracil (0.26), moclobemide (1.97) carvedilol (3.39) carbamazepine (3.63) fluoxetine (3.80) and diazepam (4.26) using UPLC-QTof-MS

Since Tof-MS acquires 'all of the data all of the time' each peak is the combined response of a number of ions related to the analyte e.g. psudomolecular species, adducts, fragments etc. In practice the predominant species were the protonated $[M+H]^+$ species in the positive mode and deprotonated $[M-H]^-$ species in the negative mode. Typical values for the measured accurate mass and the calculated exact mass for these species is given in Table 3.2.

3.3.3.1 UPLC-QTof-MS post acquisition data analysis

Processing the Tof-MS data either by MetaboLynx software or by reverse searching using exact mass (Table 3.1) did not detect any metabolites in the samples of *G. pulex* exposed to 5-fluorouracil, carbamazepine, carvedilol, fluoxetine or moclobemide. However, a compound with m/z 271.0638 possibly corresponding to the demethylated compound (nordiazepam) was detected in the *G. pulex* exposed to diazepam (Figure 3.7 and Figure 3.8). Nordiazepam the primary metabolite of

diazepam in mammals was not detected in the exposure water suggesting that diazepam was metabolized by *G. pulex*. Agreement of retention time (RT: 3.96), elemental composition, and isotope pattern (Figure 3.9) for this compound with standards prepared in matrix and solvent provides evidence of the identity as nordiazepam.

Table 3.2 Measured masses of pharmaceutical residues found in *Gammarus pulex* tissues compared to exact masses of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide using UPLC-QTof-MS

Compound	Molecular mass [g mol^{-1}]	Exact mass		Measured mass of incurred residues	
		$[M+H]^+$	$[M-H]^-$	$[M+H]^+$	$[M-H]^-$
5-fluorouracil	130.08	131.0257	129.0100	-	129.0103
Carbamazepine	236.27	237.1028	235.0871	237.1043	-
Carvedilol	406.47	407.1971	405.1814	407.1975	-
Diazepam	284.75	285.0795	283.0638	285.0806	-
Fluoxetine	309.33	310.1419	308.1262	310.1417	-
Moclobemide	268.74	269.1057	267.0900	269.1069	-

The measured accurate mass of nordiazepam in *G. pulex* tissues was m/z 271.0638 which was 0 ppm different to the exact mass of nordiazepam m/z 271.0638 in the solvent standard. Since nordiazepam contains a chlorine atom the isotope pattern CL35/Cl37 is very characteristic and the software calculated i-fit (isotope fit) of 0. The agreement of the isotope pattern with the reference standard provides further evidence to support nordiazepam as the identity of the compound.

For diazepam, the fragments generated by QTof (MSE experiments) were the same for the fragmentation of ions of the solvent standard and *G. pulex* extract. Extracted ion chromatograms for each of the fragment ions detected in the high energy MSE spectrum confirm that the fragment ions are related to the selected [M+H]+ precursor ion, m/z 285.0795, due to their identical retention times to the precursor ion (Figure 3.10).

Unfortunately the MS^E collision energy was too low to be able to differentiate the nordiazepam fragments from the matrix. Although retention time and the elemental composition data of nordiazepam provide convincing evidence, unequivocal confirmation requires UPLC-QTof-MS/MS fragmentation data. These studies are still in progress.

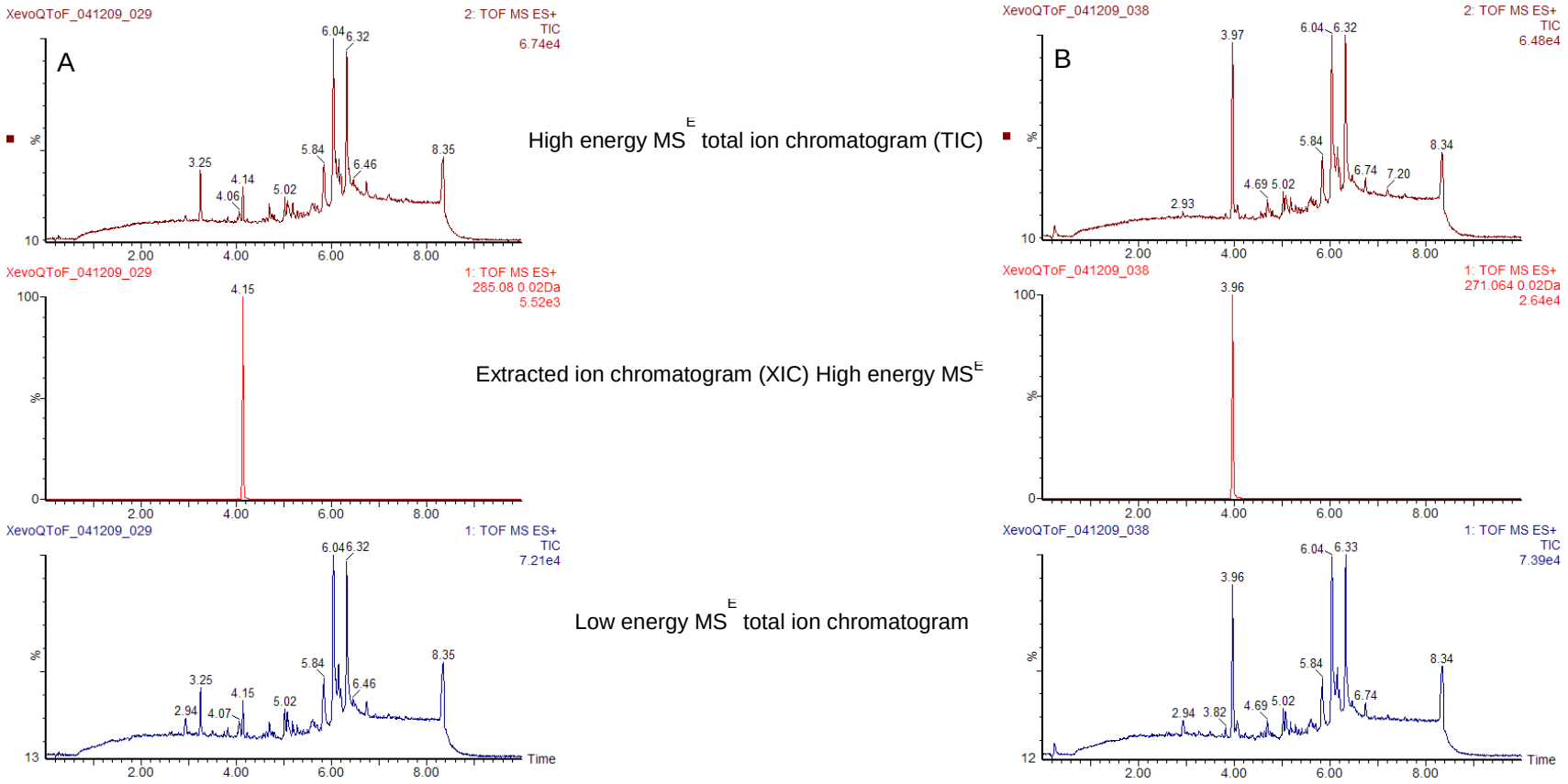


Figure 3.7 Total ion chromatograms with high and low energies and extracted ion chromatograms for a) diazepam and b) nordiazepam in methanol solvent standards using UPLC-QTof-MS

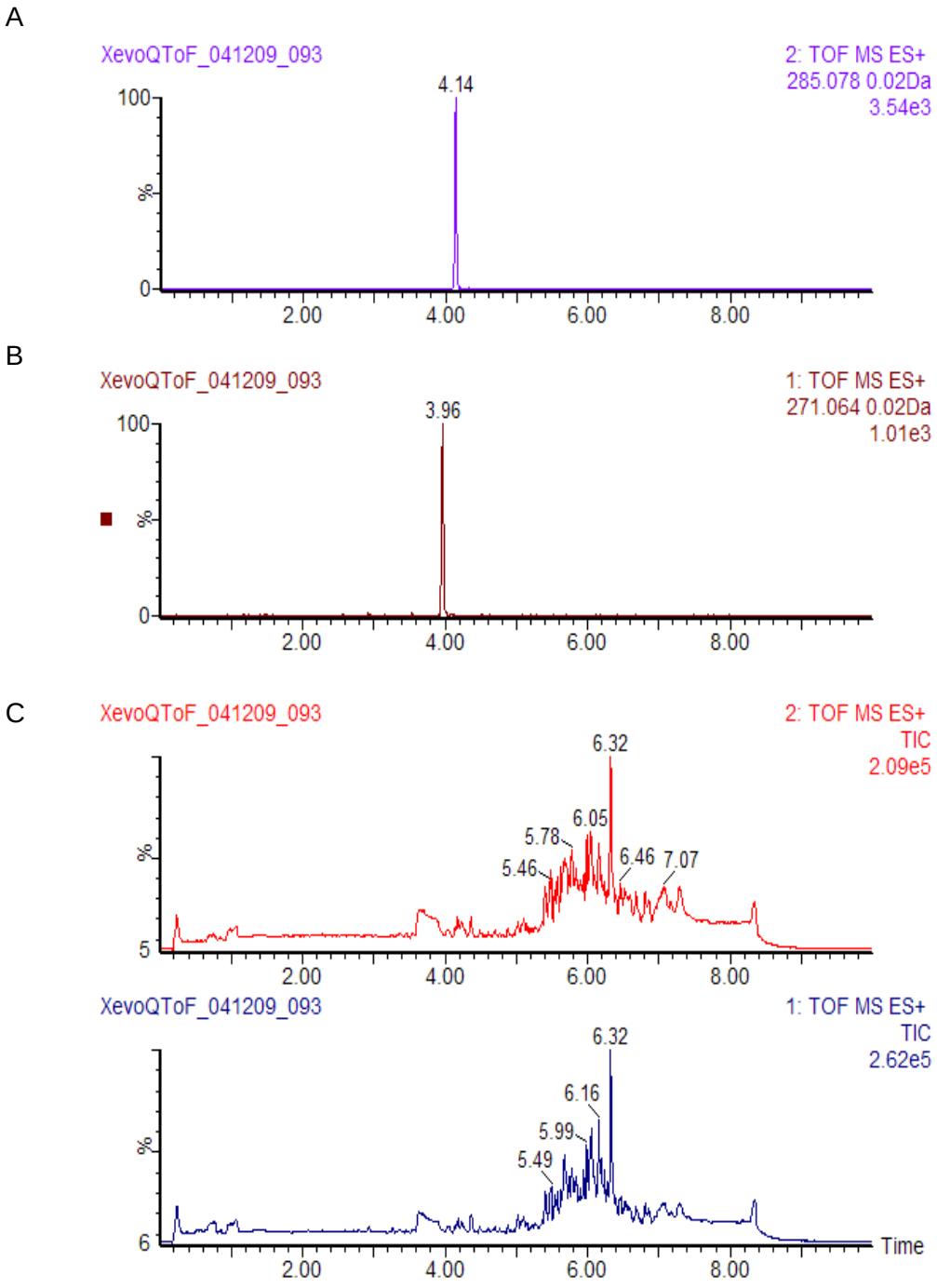
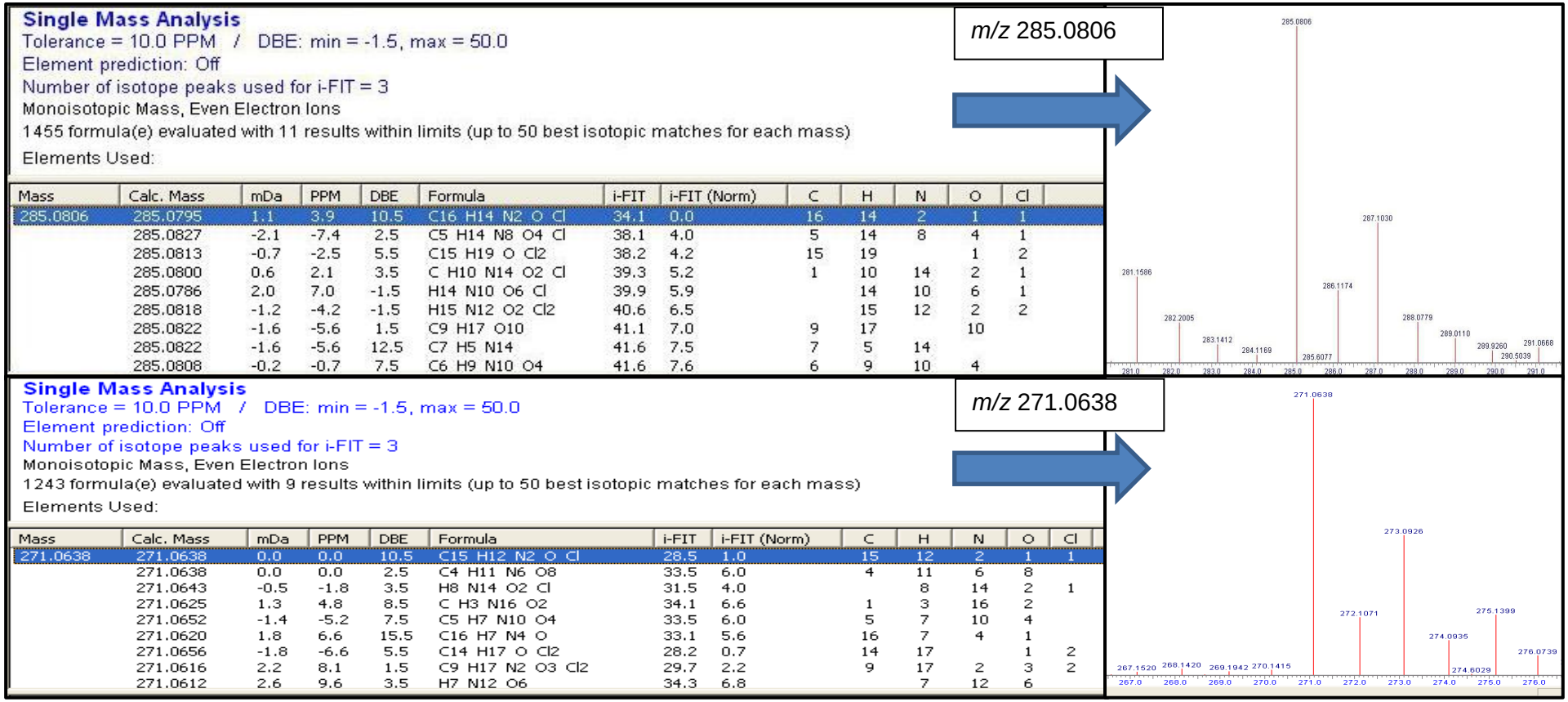


Figure 3.8 Extracted ion chromatograms for a) diazepam and b) nordiazepam and c) MS^E total ion chromatogram with high and low energies in *Gammarus pulex* samples after 48 hours exposure to diazepam (0.5 mg L^{-1}) using UPLC-QTof-MS



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT (Norm)	C	H	N	O	Cl
285.0806	285.0795	1.1	3.9	10.5	C16 H14 N2 O Cl	34.1	0.0	16	14	2	1	1
	285.0827	-2.1	-7.4	2.5	C5 H14 N8 O4 Cl	38.1	4.0	5	14	8	4	1
	285.0813	-0.7	-2.5	5.5	C15 H19 O Cl2	38.2	4.2	15	19		1	2
	285.0800	0.6	2.1	3.5	C H10 N14 O2 Cl	39.3	5.2	1	10	14	2	1
	285.0786	2.0	7.0	-1.5	H14 N10 O6 Cl	39.9	5.9		14	10	6	1
	285.0818	-1.2	-4.2	-1.5	H15 N12 O2 Cl2	40.6	6.5		15	12	2	2
	285.0822	-1.6	-5.6	1.5	C9 H17 O10	41.1	7.0	9	17		10	
	285.0822	-1.6	-5.6	12.5	C7 H5 N14	41.6	7.5	7	5	14		
	285.0808	-0.2	-0.7	7.5	C6 H9 N10 O4	41.6	7.6	6	9	10	4	

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT (Norm)	C	H	N	O	Cl
271.0638	271.0638	0.0	0.0	10.5	C15 H12 N2 O Cl	28.5	1.0	15	12	2	1	1
	271.0638	0.0	0.0	2.5	C4 H11 N6 O8	33.5	6.0	4	11	6	8	
	271.0643	-0.5	-1.8	3.5	H8 N14 O2 Cl	31.5	4.0		8	14	2	1
	271.0625	1.3	4.8	8.5	C H3 N16 O2	34.1	6.6	1	3	16	2	
	271.0652	-1.4	-5.2	7.5	C5 H7 N10 O4	33.5	6.0	5	7	10	4	
	271.0620	1.8	6.6	15.5	C16 H7 N4 O	33.1	5.6	16	7	4	1	
	271.0656	-1.8	-6.6	5.5	C14 H17 O Cl2	28.2	0.7	14	17		1	2
	271.0616	2.2	8.1	1.5	C9 H17 N2 O3 Cl2	29.7	2.2	9	17	2	3	2
	271.0612	2.6	9.6	3.5	H7 N12 O6	34.3	6.8		7	12	6	

Figure 3.9 MassLynx software V4.1 screen shot of elemental composition of a) diazepam *m/z* 285.0806 and b) nordiazepam *m/z* 271.0638 in *Gammarus pulex* tissues exposed to diazepam (0.5 mg L^{-1}) for 48 hours. Masses ranked by closest isotope fit (post – acquisition data from UPLC QTof MS)

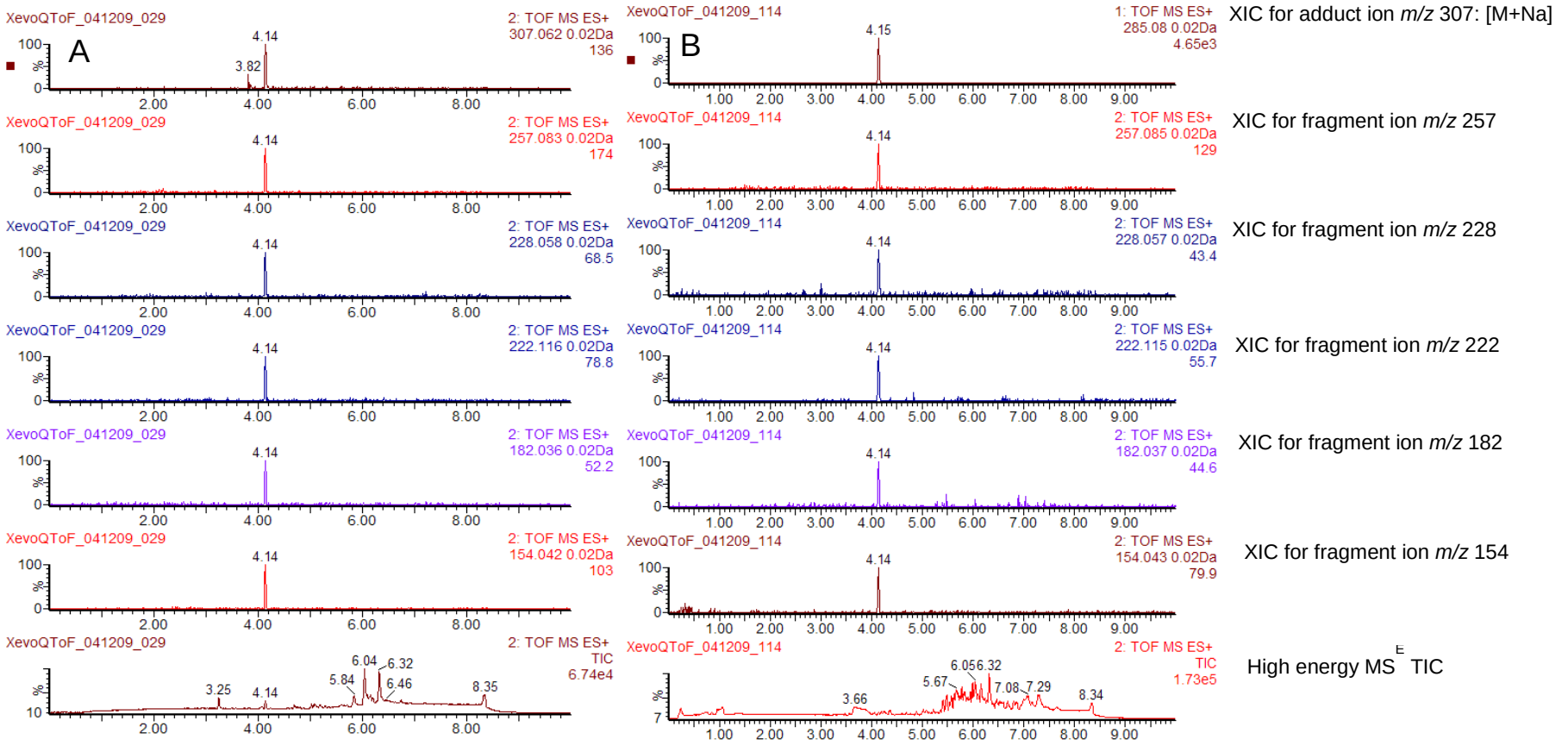


Figure 3.10 Extracted ion chromatograms precursor ion and fragment ions for diazepam in a) solvent standards and b) *Gammarus pulex* tissues in high energy MS^E spectrum (post – acquisition data from UPLC QTof MS)

3.3.4 Pharmacokinetic studies with non-labelled diazepam

The results from the metabolite detection and identification study indicated that diazepam was metabolized to nordiazepam by *G. pulex*. Therefore a pharmacokinetic study was performed to study the uptake and metabolism of diazepam over time. The water quality parameters remained constant during the study (Table 3.3). Water concentration of diazepam ranged between 90-85 % of the starting concentration and no nordiazepam was detected in the water throughout the study (Figure 3.11).

Diazepam is accumulated immediately in *G. pulex*. After 3 hours of exposure nordiazepam is detectable in the animal (Figure 3.12). Once the animals are transferred into clean water, diazepam is removed from the animal quickly but nordiazepam is continually detected until the end of the study. As diazepam is eliminated or metabolised, the ratio between parent and metabolites changes, increasing the percentage of nordiazepam in the organism (Figure 3.13). This is demonstrated by the concentration of diazepam decreasing to 3 % of its highest concentration, whilst nordiazepam persists longer in the organism. This may account for nordiazepam contributing a larger portion of the total pharmaceutical concentration in *G. pulex* after the depuration period. These results also demonstrate the value of being able to measure these compounds individually.

Table 3.3 Water quality parameters for pharmacokinetic study with *Gammarus pulex* exposed to 0.5 mg L^{-1} of Diazepam (mean values ± standard deviation; n=5)

pH	Dissolved Oxygen (mgL^{-1})	Temperature (ºC)
7.78 ± 0.20	9.45 ± 1.58	12.76 ± 0.45

3.3.5 Bioconcentration factors for diazepam and nordiazepam

BCFs were calculated using a one-compartment model for both total pharmaceutical (diazepam and nordiazepam) and diazepam in G pulex. The BCF for total pharmaceutical was 33.7 and the BCF for diazepam was 19.1. The pseudo BCF for nordiazepam calculated from the internal concentration and the diazepam water concentration at 48 hours was 1.36.

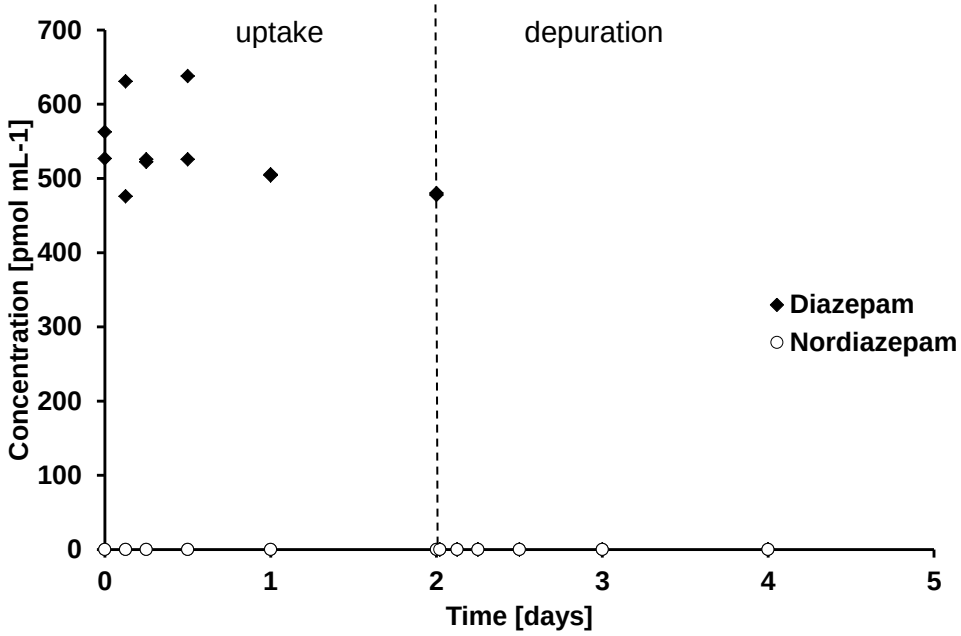


Figure 3.11 Measured concentration (n=2) of diazepam and nordiazepam in the exposure water during the uptake and depuration study using UPLC-QTof-MS

3.4 Discussion

The results obtained using UPLC-QTof-MS are consistent with the radiolabeled studies presented in Chapter 2 and confirm that *G. pulex* uptakes diazepam from the exposure water. Extraction with methanol recovered 82 – 97 % of the pharmaceuticals spiked into homogenized *G. pulex* matrix. These results are in agreement with the literature. Vernouillet *et al.* (2010) obtained 92 % mean recovery of carbamazepine from invertebrate tissues with a similar extraction methodology. All of the pharmaceuticals were detected by UPLC-Tof with good chromatographic separation, and excellent mass accuracy and stability.

The peak shape for the most polar compound 5-fluorouracil is relatively broad. This is probably due to column disturbance effects caused by the difference in polarity of methanol (injection solvent) and the initial mobile phase conditions (100% aqueous).

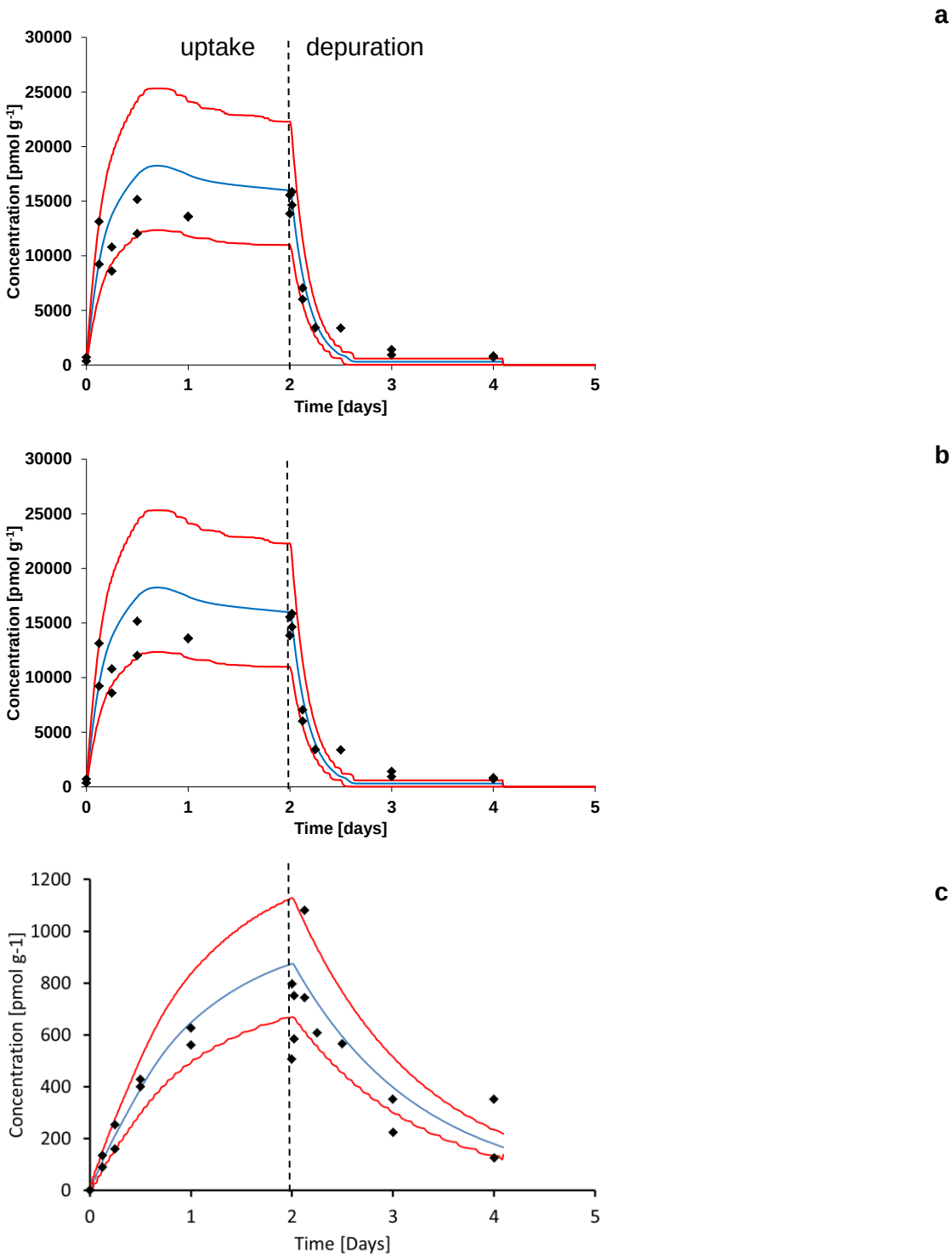


Figure 3.12 Measured internal wet weight concentrations in *Gammarus pulex* with UPLC-Tof-MS when exposed to 0.5 mg L^{-1} of diazepam (n=2; September 2008), providing an uptake and depuration curve over time of a) total diazepam and nordiazepam b) diazepam and c) nordiazepam. The diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals

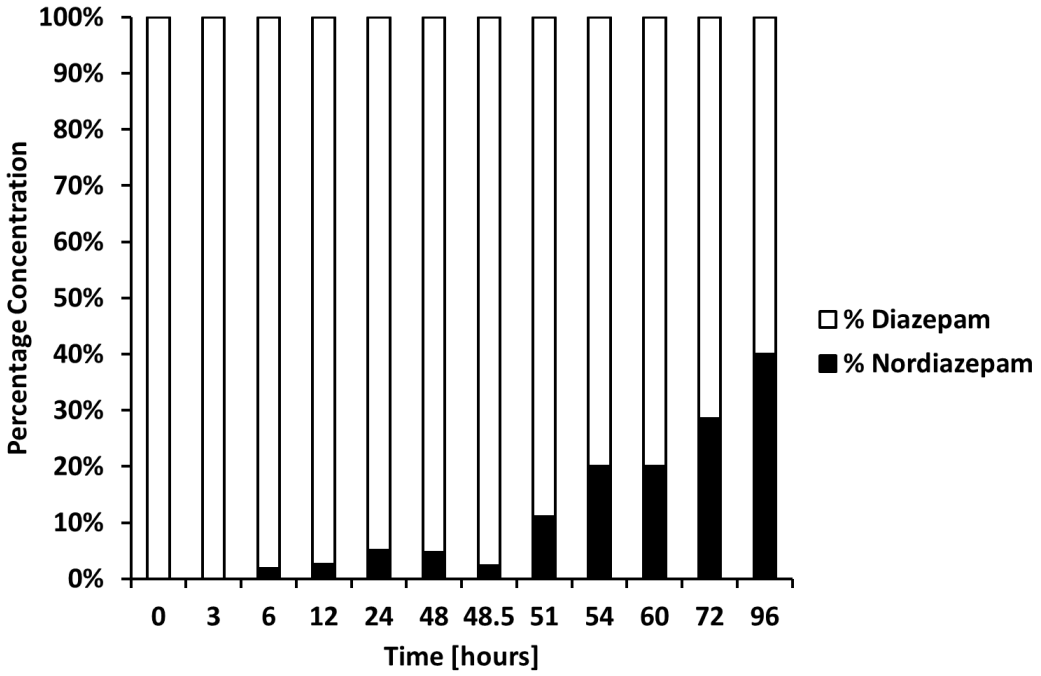


Figure 3.13 The percentage of nordiazepam metabolite detected compared to the parent diazepam detected in *Gammarus pulex* over 72 hours exposure (n=2) using UPLC-QTof-MS

If the study was specifically focused on 5-fluorouracil alone, then the peak shape could be improved by closer matching of the injection solvent with the initial mobile conditions; either by increasing the organic solvent composition at the start of the elution programme (at the expense of chromatographic resolution), or dilution the extract at the expense of sensitivity (Kaufmann, 2009). Because of the need for a multi-component method a compromise was necessary. Metabolites are typically more polar than the parent compounds and therefore would be expected to elute with shorter retention times (Zuehlke *et al.*, 2004).

No metabolite residues were detected for 5-fluorouracil, carbamazepine, carvedilol, fluoxetine and moclobemide, however it is possible that concentrations of metabolites do occur but below the lowest calibrated concentration (equivalent to 0.04 µg/g), either because losses during the extraction process or suppression by the matrix effects can occur when molecules coelute with the analyte of interest resulting in competition between the components and alteration of the ionization efficiency of the elecotrospray ion source (Paul, 2005). This has been observed for

pharmaceutical residues in complex environmental matrices previously (e.g. Zuehlke *et al.*, 2004).

However nordiazepam was detected and identified with evidence provided by UPLC-QTof–MS, The data which was acquired across the full mass range spectrum with high resolution and mass accuracy. The accurate mass of the major ion, the chlorine isotope pattern was in agreement with the same data obtained for reference standard materials. Product ion spectra produced from the MS/MS of m/z 285.0806 $[M+H]^+$ for diazepam provide unequivocal confirmation of the presence of diazepam in the Gammarus tissue. Unfortunately MS^E collision energy employed was too low to be able to differentiate the nordiazepam product ion fragments from the matrix. For completeness, further work to provide QTof MS/MS fragmentation data and better sensitivity for nordiazepam is in progress.

The concentration of diazepam and nordiazepam in the Gammarus after 48 hours exposure was quantified against matrix-matched. The detection of nordiazepam demonstrated that *G. pulex* will metabolise diazepam.

In mammals nordiazepam is formed after demethylation of diazepam in phase 1 metabolism (Straub, 2008). The pharmacokinetic study of diazepam showed that the uptake and metabolism of diazepam in *G. pulex* was similar to mammals. Preceding the application diazepam showed rapid uptake and the major phase one metabolite is nordiazepam was detected after only 3 hours and nordiazepam continued to be formed throughout the test. Diazepam was eliminated quickly from the organism however nordiazepam was eliminated more slowly. A contributing factor could be that nordiazepam has a longer elimination half-life and has a larger log K_{ow} (log of K_{ow} 3.89) compared to its parent diazepam (log of K_{ow} 2.70) (Charney *et al.*, 2001).

In mammals the important CYP - 450 enzymes involved in the metabolism of diazepam to nordiazepam are CYP3A4, CYP2C19, CYP2B6, CYP2C9 and CYP3A5 (Straub, 2008). This study is not conclusive that the CYP - 450 facilitates the metabolism of diazepam to nordiazepam in *G. pulex*, however nordiazepam was not detected in the water (Figure 3.11) and biotransformation of some compounds does not occur without initial oxygenation, which is usually dependent on the CYP - 450 enzymes (James, 1990a). There are other kinetic studies of pharmaceuticals that are used frequently in the aquaculture of crustacean species. In these studies demethylated metabolites of erythromycin and sulfadimethoxine were also found. The authors suggested that these metabolites might have been formed by CYP - 450 enzymes in the hepatopancreas (James and Boyle, 1998). Although no

definitive analysis was completed the demethylation of erythromycin in humans is facilitated by CYP - 450 3A enzyme (James and Boyle, 1998) which is similar to nordiazepam Figure 3.1.

Therefore both the current study and the study by James (1990a) could suggest that crustaceans such as *G. pulex* possess the CYP - 450 3A enzyme and this enzyme maybe used in the metabolism of pharmaceutical compounds such as diazepam in crustacea. However fluoxetine, carbamazepine and carvedilol are also processed by the same enzyme in mammals but no metabolites were identified in *G. pulex*. Although metabolites of these compounds were not identified, they may have been at concentrations below the sensitivity of the instrument or may not have been recovered by the extraction method. Therefore there is uncertainty over the metabolic pathway in this crustacean and further research may be required to clarify this hypothesis.

The pharmacokinetic study with diazepam is in agreement with the radiolabelled studies in Chapter 2. The BCF in this study for the total pharmaceutical (diazepam and nordiazepam) concentration was 33.7 compared to the BCF of 37.5 for the radiolabelled study. This study shows that the metabolism of diazepam to nordiazepam has an effect on the BCF. When calculating the parent compound alone the BCF for diazepam was 19.1. Therefore although radiolabelled studies can discern internal concentrations at low concentrations they provide information on total radioactive concentration and do not provide any information on the metabolism (Kaufmann, 2009). Radiolabelled studies may overestimate the BCF and internal concentration of the parent compound if a radiolabelled metabolite is equal to or more lipophilic than the parent (Arnot and Gobas, 2006). Also if a radiolabelled metabolite is more polar and excreted into the water there will be an overestimation in of the water concentration and underestimate the BCF (Arnot and Gobas, 2006).

Some pharmaceuticals like diazepam are biotransformed to metabolites that are as potent as the parent compound. Nordiazepam is an active metabolite and may account for some of the therapeutic action of the parent pharmaceutical (Gutstein and Akil, 2001, Sinclair and Boxall, 2003). Therefore it can be justified to consider the total pharmaceutical concentration of both parent and metabolite. This may not be the case for all pharmaceuticals as potentially some compounds will be biotransformed to more polar metabolites that can be excreted easily with no therapeutic effect (Sinclair and Boxall, 2003). In some cases the metabolite maybe

the active part of the compound where the parent compound is designed for optimal absorption into the organism and is metabolized into an active compound that elicits the therapeutic effect (Sinclair and Boxall, 2003). 5-fluorouracil requires metabolic reaction to the nucleotide 5'-monophosphate which is incorporated into ribonucleic acid, this metabolic sequence is vital for its anti-cancer activity (Calabresi and Chanbner, 2001).

Despite *G. pulex* being able to metabolise diazepam to nordiazepam, the BCFs and the internal concentrations were similar and no metabolites were detected for the five other pharmaceuticals. Therefore it is likely that the internal concentrations and the BCFs measured in Chapter 2 will reflect the measured internal concentrations and BCFs of *G. pulex* for the parent compounds tested. These data also confirm that extraction with methanol provides an extraction efficiency at least equivalent to extraction with Soluene 350 (Perkin Elmer, USA) when considering suppression effects of the matrix.

The identification and measurements of pharmaceutical metabolites in environmental matrices is challenging; however this study shows that UPLC coupled to a QTof is an excellent tool for the detection and identification of metabolites in complex animal matrices. Future studies on different compounds with different organisms will be necessary to validate this method for robust environmental screening.

3.5 Conclusions

It can be concluded from this work that:

- *G. pulex* is able to metabolise the pharmaceutical compound, diazepam. To the authors knowledge this is the first time that metabolism of a human pharmaceutical has been demonstrated in an aquatic invertebrate.
- The extraction technique proposed in this study needs to be refined and validated before application to the assessment of other pharmaceuticals and invertebrate species.
- The concentration of the test pharmaceuticals at which *G. pulex* were exposed in this study was high compared to those found in the environment. Additional research is required to test whether the extraction and detection

method for diazepam can measure metabolites at environmentally relevant concentrations.

- Metabolites should be considered in the risk assessment process as some have longer elimination half-lives compared to the parent compound and may influence the BCF. Also this study shows that BCFs calculated from radiolabelled studies ignore the formation of metabolites
- It could be implied from this work that CYP – 450 enzymes could be responsible for the metabolism of diazepam to nordiazepam, further work will be necessary to establish this conclusively. It may be possible to identify metabolites by looking at the metabolic pathways already identified in aquatic invertebrates.
- Human pharmacological information on metabolic pathways should be investigated in aquatic organisms to allow the prediction of active metabolites in non-target species.

Chapter 4 Uptake of pharmaceuticals through aquatic food chains

4.1 Introduction

As described in Chapter 1, a growing body of evidence shows that pharmaceuticals can accumulate in aquatic species (Brooks *et al.*, 2005, Brown *et al.*, 2007, Chu and Metcalfe, 2007, Ramirez *et al.*, 2007, Gelsleichter, 2009, Pouliquen *et al.*, 2009, Ramirez *et al.*, 2009, Fick *et al.*, 2010, Schultz *et al.*, 2010). A number of studies have also shown that even when pharmaceuticals are at concentrations below the limit of detection in the exposure water, they can be detected in fish; indicating that some pharmaceuticals may have the potential to bioaccumulate (Gelsleichter, 2009). It is thought that bioaccumulation of a chemical occurs largely by uptake from the aqueous phase. It is often considered to be the predominant route because of the large volume of water transferred across the respiratory surfaces of aquatic organisms (Gross-Sorokin *et al.*, 2003). Uptake of pharmaceuticals from the aqueous phase was explored in Chapter 2 of this thesis. The results tentatively indicated that all the aquatic invertebrates tested showed the potential to uptake pharmaceuticals from water and that the degree of uptake from the water can be influenced by species traits (e.g. organism size, mode of respiration and behaviour) and the chemicals properties (e.g. Log K_{ow} or Log D_{lipw}).

In its natural environment, an organism will not only uptake chemicals from the water but will also accumulate the chemicals associated with sediments or present in its food (Borgå *et al.*, 2004, Borgå *et al.*, 2010). Studying dietary uptake becomes increasingly important for more hydrophobic compounds (e.g. persistent organic pollutants and pesticides) and in REACH, the Log K_{ow} is used to distinguish if a chemical has the potential for bioaccumulation, biomagnification and trophic transfer (Gross-Sorokin *et al.*, 2003, Borgå *et al.*, 2004, Wilding and Maltby, 2006).

Trophic organization in aquatic systems can be very complex and invertebrates can be divided into groups based on their feeding mechanism and food type (Allan, 1996). Coarse particulate organic matter (CPOM) is an essential energy input in aquatic food chains and much of the energy support for lotic food chains is derived

from non-living sources of organic matter such as CPOM (Cummins, 1974). Studies have shown that in aquatic systems neutral chemicals and hormones can sorb to dissolved organic matter (Yamamoto *et al.*, 2003). Pharmaceuticals such as fluoxetine have a high affinity for organic matter, therefore it might be expected that these compounds may associate with sediments and detrital material (Ericson, 2007, Kwon *et al.*, 2008).

As sediments and detrital material such as CPOM are key elements in aquatic food chains there may be potential for detritivores feeding on CPOM to be indirectly exposed to pharmaceuticals from their food. Microbial communities that colonize CPOM may alter the adsorption of chemicals to CPOM and also, uptake or degrade these chemicals themselves (Schocken and Speedie, 1984). Therefore, organisms such as crustaceans *G. pulex* and *Asellus aquaticus* that use detrital matter, such as leaf litter and sediment, as a food resource could be potentially to exposed chemicals from consuming CPOM and the microbial communities belonging to it (Herbst, 1982, Fazi and Rossi, 2000, Swan and Palmer, 2006)

The relative importance of the route of uptake of chemicals from either food or water is unknown for many aquatic invertebrates and little is known about the factors that can influence accumulation. Feeding roles and feeding mechanisms may influence the dietary uptake of chemicals. The literature suggests that chemicals accumulation patterns can be related to feeding behaviour (Russell *et al.*, 1999). For example when considering the feeding strategies of a piercer, scraper and an engulfer; the piercer will only be exposed to the imbibed body fluids, a scrapper will only be exposed to the chemical adsorbed to the external surfaces of the prey but the engulfer will be exposed to the chemical accumulated in the whole prey organism (Brooks *et al.*, 2009).

Studying the accumulation of contaminants from the food is especially critical for understanding trophic transfer in food chains (Wang and Fisher, 1999) and is important for establishing accurate models for the fate of chemicals such as pharmaceuticals in the environment (Munger and Hare, 1997).

The chapter aims explore the accumulation of pharmaceuticals on CPOM and assessing the importance of feeding as an uptake route for pharmaceuticals compared to water exposure alone through a simple food chain. Using *G. pulex* and *N. glauca* this study will assess the influence of feeding strategy on the

accumulation pattern of pharmaceuticals; carvedilol and fluoxetine chosen as the study pharmaceuticals for their accumulative potential in the food source.

The specific objectives are to:

- Investigate the association of a range of pharmaceuticals with CPOM
- Determine the influence of physico-chemical properties of the compounds and the effect that the presence of the common aquatic fungus *Cladosporium herbarum* will have on the sorption.
- Investigate the impact of exposure route on accumulation, assessing the importance of feeding as an uptake route for pharmaceuticals compared to water exposure
- Ascertain the influence of feeding strategy on the dietary uptake of pharmaceuticals in *G. pulex* and *N. glauca*
- Establish whether pharmaceuticals can be transferred up a simple aquatic food chain using the CPOM as the primary trophic level, *G. pulex* as the consumer and *N. glauca* as a predator. Carvedilol and fluoxetine were chosen as the study pharmaceuticals for to their accumulative potential.
- Use previously derived uptake and depuration rate constants from Chapter 2 to estimate exposure at environmental relevant concentrations.

4.2 Materials and methods

4.2.1 Pharmaceutical compounds

Radiolabelled and unlabeled, 5-fluorouracil, carvedilol, diazepam and moclobemide were obtained from F. Hoffman-la Roche Ltd (Basel, Switzerland). Radiolabelled carbamazepine and fluoxetine were obtained from American Radiolabeled Chemicals Incorporated (Missouri, USA). All pharmaceuticals were labeled with ^{14}C,

chemical properties and specific activities of the pharmaceuticals are summarised (Table 2.1 and 2.2).

4.2.2 Study organisms

Horse-chestnut leaves (*Aesculus hippocastanum)* were collected in November 2008 from St. Mary's church yard Sand Hutton, North Yorkshire (NGR SE699583) after abscission. The leaves were inoculated with the fungus *Cladosporium herbarum* which was chosen as food source for the food uptake study with *G*. *pulex.* Mature *C. herbarum* cultures were obtained from the University of Sheffield (Sheffield, UK). Cultures were maintained at 22°C plated on sterile malt extract broth (MEB) plates comprising 3 % malt extract and 0.25 % mycological peptone in distilled water. The cultures were sub-cultured monthly. The leaves were then dried and stored at 20 °C in the dry prior to use.

Prior to each uptake study, the leaves were rehydrated and then cut into 2 cm diameter discs and placed into enriched water (Naylor *et al.,* 1989). The leaf discs were then autoclaved at 121 °C, 15 PSI (1.034 kPa) for 20 minutes to kill any microorganisms present (Naylor *et al.*, 1989). The prepared leaf discs were inoculated with mature *C. herbarum* at a standard ratio (fungi:leaves, 1:20). The inoculated flasks were agitated at 65 rpm on an orbital shaker for ten days before use.

G. pulex were collected from a field population at Bishop Wilton Beck and *N*. *glauca* were obtained from Blades Biological Ltd. (Kent, UK) in October 2009. All animals were maintained in the same conditions as Chapter 2, the reader is referred to section 2.2.2 of this thesis.

4.2.3 Interaction of pharmaceuticals with leaf material

To understand the association of pharmaceuticals on CPOM, the uptake of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide onto non-inoculated leaf discs and *C. herbarum* inoculated leaf discs was investigated.

Three replicates of either sterilised leaf discs or *C. herbarum* inoculated leaf discs were exposed for 72 hours to 200 mL of solutions of each of the test compounds in APW. All leaf discs were exposed to 0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 µmol L^{-1} (or 0.1 mg L^{-1}) of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide respectively. A static exposure was used for all pharmaceuticals except for fluoxetine where a static renewal exposure was employed as preliminary studies showed that fluoxetine adsorbed to the glassware. The static renewal approach for fluoxetine consisted of transferring the organisms in each beaker into fresh test solution every 24 hours. A sample of water (1 mL) was taken to determine the exposure concentration once the water had been renewed.

The leaf material was exposed at 20 °C with a dark/light cycle of 12 hours light: 12 hours dark. Following exposure, the concentration of the study pharmaceuticals in the water and leaf materials was determined by liquid scintillation counting (LSC). Water samples were transferred to scintillation vials and 10 mL of Ecoscint A (National Diagnostics) was then added. The sample was shaken to mix, left to settle and the radioactivity measured in the dark by LSC. LSC analysis was performed using a Beckham LS6000 TA Liquid Scintillation Counter (Beckham Instruments Inc. USA). Samples were counted three times for 5 minutes. Sample counts were corrected for background activity by using blank controls. Counting efficiency and colour quenching were corrected using the external standard ratio method.

Prior to analysis, leaf disc samples were rinsed with deionised water to remove any pharmaceutical residue from the surface. The leaf discs were then dissolved using 2 mL of Soluene 350 (Perkin Elmer, UK) a strong organic base, formulated with toluene, which has an excellent capacity for the solubilization of wet tissue. The tissue was also bleached with 100 µL of hydrogen peroxide, which was used to remove the tannins from the leaf tissue to prevent colour quenching during analysis. The rate of solubilization with Soluene 350 was increased by warming over 24 hours in a water bath at 60 °C to ensure that all the tissue had fully dissolved. Following solubilization and bleaching, the extracts were mixed with 10 mL Hionic Fluor scintillation cocktail (Perkin Elmer, UK) and the concentration of pharmaceuticals determined by LSC.

To understand the rates of adsorption/uptake and desorption/elimination of pharmaceuticals in detrital material, *C. herbarum* inoculated leaf discs were exposed at 20 °C in 200 ml of solutions of the study pharmaceuticals in APW. Concentrations

of the pharmaceuticals ranged from 230 (carvedilol) to 714 (5-fluorouracil) pmol mL^{-1}. There were twelve leaf discs in each replicate and three replicates per pharmaceutical. To assess the rate of sorption/uptake, one leaf disc was taken at either 0, 3, 6, 24, 48 or 72 hours after the start of the exposure. This left six leaf discs in each replicate at the end of the uptake study which were then transferred into clean APW in order to determine the rates of depuration. In the depuration study, samples of leaf material were obtained 0, 3, 6, 24, 48 and 72 h after transfer to the clean APW. The radioactivity in the water at each sampling time point and in the collected leaf samples was measured using the extraction and LSC method described above.

4.2.4 Uptake into aquatic invertebrates via water and food

Carvedilol and fluoxetine were selected for use in the uptake studies with invertebrates due to their accumulative behaviour on the leaf discs experiments. The experiments were carried out using components of a simple food chain namely inoculated leaf discs, *G. pulex* and *N. glauca*. In this food chain, *G. pulex* uses leaf material as its food source and *N. glauca* feeds on *G. pulex*. A systematic approach was used to assess the relative importance of water exposure and 'food' exposure in terms of uptake through the food chain. The studies therefore involved a) exposure of each component of the food chain to only APW containing the test pharmaceutical; b) exposure of each component of the food chain to 'food' containing the study pharmaceutical and clean APW; and c) combined exposure experiments where uptake was assessed from both the water phase and the 'food' phase.

For the water-only studies, inoculated leaf discs, *G. pulex* and *N. glauca* were exposed individually to 200 ml of either 2.5 or 21 pmol mL^{-1} (1 µg L^{-1}) of carvedilol and fluoxetine in APW. For *G. pulex* and *N. glauca* there were two sets of three replicates containing single animals. For the leaf discs there were eight replicates containing ten leaf discs – two leaf discs were used from each replicate to assess water uptake and the remainder were used in the 'food' uptake studies described below. Organisms were exposed at 12 °C for 72 hours. For the leaf discs, samples were taken immediately after exposure for analysis while for the *G. pulex* and *N. glauca*, only one set of replicates was taken. The other set were transferred to clean

water for either 6 h (*G. pulex*) or 12 h (*N. glauca*) to establish whether the study compounds are depurated during the gut purging times used in the 'food' only studies described below. Water samples (1 ml) were also taken at the end of the exposure period for analysis.

Dietary exposure was assessed in *G. pulex* and *N. glauca*. Three replicates of eight *G. pulex* in 500 mL of clean APW were fed eight of contaminated leaf discs obtained from the water-only uptake studies. Following 72 hours, one animal was removed from each replicate and frozen prior to analysis. A second animal from each replicate was transferred to clean APW for 6 hours to allow the animal gut to purge, after this time the animal and a water sample (1 ml) were taken and frozen for analysis. The remaining six animals were used as the 'food' source in the dietary and combined exposure studies with *N. glauca*. Six, single animal replicates of *N. glauca* were prepared in 200 mL of clean APW. Each of these *N. glauca* was fed with 3 of the *G. pulex* that had previously been exposed to the contaminated food. After 72 hours the animals were removed and water samples were obtained, three *N. glauca* were frozen for analysis and three animals were transferred into clean APW for 12 h to allow gut purging. Additional water samples were taken for analysis at the end of the purging period.

To determine the total uptake of pharmaceuticals from both dietary and water exposure into *G. pulex* and *N. glauca*, animals were exposed to both contaminated food and contaminated water. The water concentrations were the same as used in the water-only uptake studies and the food was prepared in the same way as for the 'food' uptake studies. Animals were exposed for 72 h and samples were taken for analysis with and without gut purging.

All samples were analysed by LSC using the methods described above. The extraction methods used for *G. pulex* and *N. glauca* were the same as used in the leaf experiments.

4.2.5 Data analysis

4.2.5.1 Determination of variance between inoculated and non-inoculated leaf discs

A Levene's test for Equality of Variance was used to determine whether there was equal variance between the concentrations of pharmaceuticals in the inoculated and non-inoculated leaf discs. If equal variance was found, the significance (at the 5% level) of the differences between these samples was determined using an independent *t*-test. A Mann Whitney *U* test was performed in instances where the variance between the samples was found to be unequal. All statistics were completed using SPSS Statistic V17.0.

4.2.5.2 Derivation of rate constants and food accumulation factors (FAF)

A first-order one-compartment toxicokinetic model was fitted to the total concentration on the leaf discs to estimate the parameters for uptake and elimination (Ashauer *et al.*, 2006, Ashauer *et al.*, 2010). All simulations and parameter estimations were carried out using OpenModel (version 9th Dec 2009, http://www.nottingham.ac.uk/environmental-modelling/OpenModel.htm) in accordance with Section 2.2.7 of this thesis.

4.2.5.3 Determination of trophic transfer efficiency

Transfer efficiencies between trophic layers were calculated using an adaptation of the method of Brooks *et al.* (2009). Transfer efficiencies are expressed as the percentage of pharmaceutical transferred from the food to the consumer using equation 4.1, where *p* is the body burden of the consumer after exposure and *b* is the concentration in the food.

Equation 4.1 $$TE = \frac{p}{b} \times 100$$

4.2.5.4 Determination of variance between exposure routes

A two-way analysis of variance (ANOVA) was performed on the *G. pulex* and *N. glauca* data to show any significant differences between the tissue concentration in the animals between the exposure routes and between chemicals. Further to this a least significant difference (LSD) test was performed on the route of exposure data to show which exposure routes were significantly different from one another. All statistics were completed using SPSS Statistic V17.0.

4.2.5.5 Comparison of predicted and measured uptake from the water

The concentration of pharmaceutical in the organism was predicted using previously derived uptake and elimination rate constants from Chapter 2 (table 2.5). Predicted concentrations and their 95 percent confidence intervals were calculated by setting C_{water} equal to either 2.5 or 21 pmol L^{-1} for carvedilol and fluoxetine and running the model for 72 hours for all combinations of the parameter sample (Ashauer *et al.*, 2010). All simulations and parameter estimations were carried according to Section 0.

4.3 Results and discussion

Understanding the accumulation of contaminants from the food is critical for explaining the trophic transfer of contaminants through food chains. The present study therefore used a systematic approach to assess the routes of uptake of pharmaceuticals into the component species of a simple food chain comprising CPOM, *G. pulex* and *N. glauca*.

4.3.1 Association of pharmaceuticals with leaf material

As CPOM is a key component at the base of aquatic food webs, initial studies focused on understanding the association of pharmaceuticals from water to non-

inoculated and fungi-inoculated leaf material. Analysis of the exposure solutions in these studies showed that for 5-fluorouracil, carbamazepine, carvedilol, diazepam fluoxetine and moclobemide showed that leaf discs were exposed to mean concentrations of 714, 418, 230, 345, 292 and 389 pmol mL^{-1} of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide (figure 4.1). In the fluoxetine studies, concentrations declined rapidly over time so therefore a semi static approached was required to maintain the exposure concentration. The dissipation of fluoxetine over time was primarily due to sorption to test vessels (Appendix A). Water quality parameters remained constant throughout the experiments (table 4.1).

Following 72 hours of exposure to the pharmaceuticals, the leaf discs inoculated with *Cladosporium* fungus were found to take up more pharmaceutical compared to the leaf discs without fungus (figure 4.2). The observed differences were only statistically significant for 5-fluorouracil, carbamazepine and moclobemide. Mean concentrations for the inoculated leaf discs ranged from 9700 – 149,000 pmol g^{-1} and for the non-inoculated leaf discs ranged from 1173 – 108,000 pmol g^{-1}. The leaf disc concentration for both inoculated and non-inoculated systems, increased in the order 5-fluorouracil < carbamazepine < moclobemide < diazepam < carvedilol < fluoxetine.

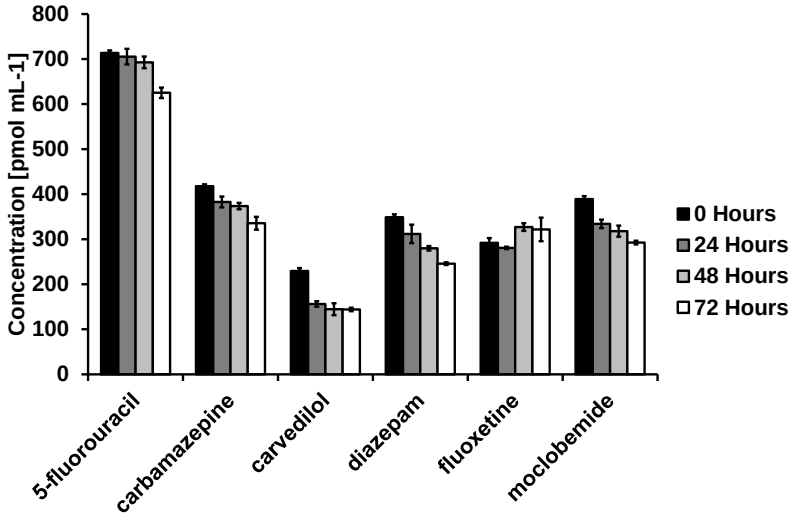


Figure 4.1 Mean measured water concentrations of the study pharmaceuticals (± standard deviation) for inoculated leaf disc uptake/sorption experiments over 72 hours exposure (n=3). Nominal concentration for pharmaceuticals were 0.8, 0.4, 0.2, 0.4, 0.3 and 0.4 µmol L^{-1} (or 0.1 mg L^{-1}) of 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide respectively

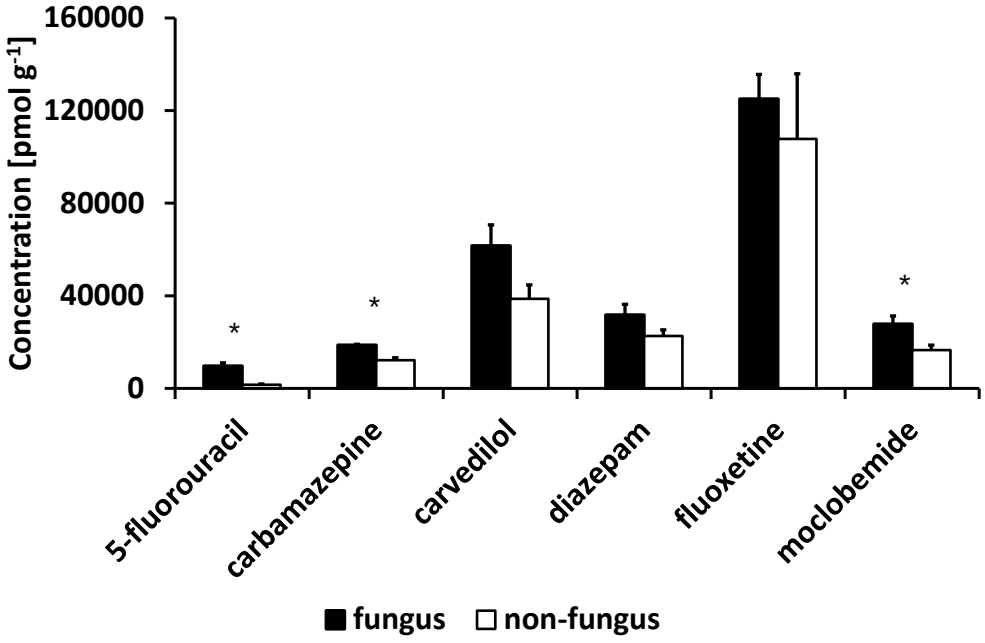


Figure 4.2 Mean sorption/accumulation (± standard deviation, n=3) of pharmaceuticals on leaf discs prepared with and without fungus. Nominal exposure concentration for pharmaceuticals was or 0.1 mg L^{-1} for 72 hours

Table 4.1 Mean water quality parameters for experiments with *Gammarus pulex, Notonecta glauca,* and *Cladosporium herbarum*

Experiment	Species	Pharmaceutical	Dissolved O_2 [mgL^{-1}]	pH	Temperature [°C]
Leaf sorption/uptake	Leaf disc inoculated with *C. herbarum*	5-fluorouracil	9.5	7.10	23.6
		carbamazepine	9.2	7.06	23.3
		carvedilol	7.1	7.76	23.3
		diazepam	7.8	7.72	22.9
		fluoxetine	9.2	7.45	22.9
		moclobemide	9.1	7.10	23.1
Water exposure	*G. pulex*	carvedilol	8.6	7.44	20.4
		fluoxetine	8.7	7.51	21.0
	N. glauca	carvedilol	9.3	7.81	20.7
		fluoxetine	8.6	7.55	20.6
Food exposure	*G. pulex*	carvedilol	9.1	7.23	20.6
		fluoxetine	9.0	7.50	20.9
	N. glauca	carvedilol	8.6	7.54	20.5
		fluoxetine	8.0	7.12	21.1
Food & water exposure	*G. pulex*	carvedilol	9.2	7.51	20.3
		fluoxetine	9.2	7.65	21.0
	N. glauca	carvedilol	8.8	7.47	20.4
		fluoxetine	9.0	7.52	21.0

The observed association of the pharmaceuticals with the leaf material may be due to adsorption onto the leaf and fungal surfaces or uptake into the biological material. The sorptive/accumulation potential of heavy metals and pesticides onto detrital material has been shown previously by Odum and Drifmeyer (1978). Filamentous fungi such as *C. herbarum* are known to have several potential accumulation sites for chemical contaminants, including carboxyl, sulphydryl, phosphate and hydroxyl groups on their cell walls as well as other ligands including the wall constituent, chitin (Rome and Gadd, 1987). Fungi also provide a larger surface area for the pharmaceuticals to adsorb to (Bolan, 1991). Surface area is thought to be one of the factors explaining higher bioaccumulation in other primary producers such as algae (Ellgehausen *et al.*, 1980). The presence of fungi can also affect the pH of a system, resulting in changes in the speciation and behaviour of chemicals compared to systems containing non fungi. These pH effects may be particularly important for pharmaceuticals, many of which are ionisable compounds.

The rate of sorption/uptake and depuration/desorption of the pharmaceuticals in inoculated leaf discs over time was also explored. Like the previous study, analysis of the exposure water showed that concentrations of all pharmaceuticals except fluoxetine remained within 10 % of the starting concentration over the study (figure 4.1). A first order – one compartment kinetic model was successfully fitted to the uptake/sorption and depuration/desorption measurements (figure 4.3 to figure 4.8). The rate constants and the resulting food accumulation factors (FAF) are provided in table 4.2. FAFs ranged from 24 – 1755 and increased in the order 5-fluorouracil < carbamazepine < diazepam < moclobemide < carvedilol < fluoxetine. As well as accumulating the most on the leaves, carvedilol and fluoxetine both showed lower desorption/depuration than the other compounds with modeled depuration/desorption constants of 0.18 and 0.22 d^{-1} respectively. Studies with aquatic organisms indicate that difference in uptake of pharmaceuticals can be explained by differences in the lipophilicity of the chemicals (Chapter 2). To explore whether this also holds true for leaf material, the relationship between accumulation into leaf material and Log K_{ow} and Log D_{lipw} (the pH-corrected liposome-water partition coefficient) was explored and positive relationships were obtained (figure 4.9 and 4.10).

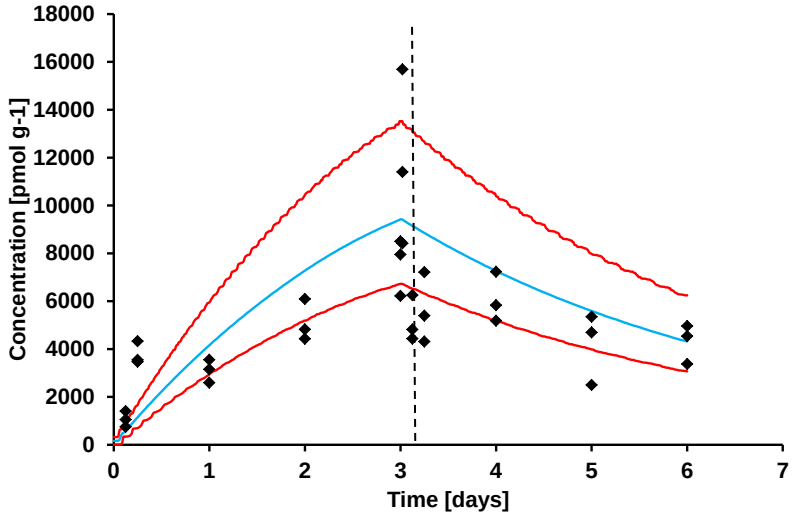


Figure 4.3 Measured internal wet weight concentrations of leaf discs exposed to 0.1 mg L^{-1} of 5-fluorouracil (n=3, May 2009), providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater.

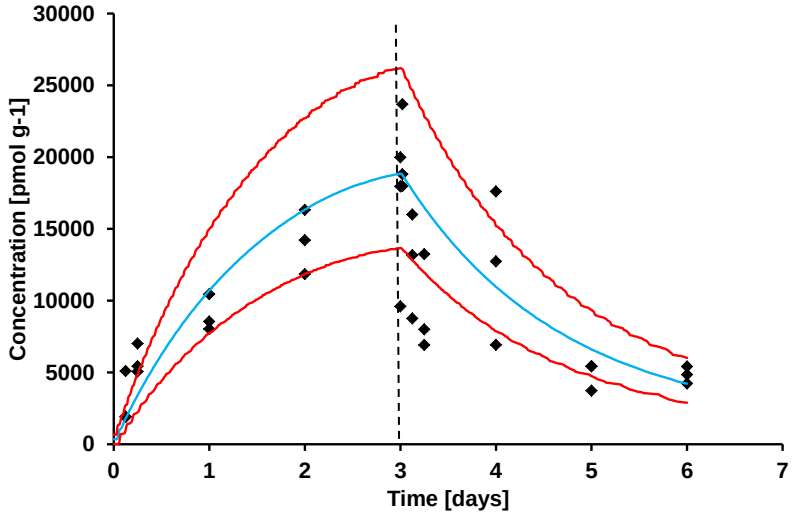


Figure 4.4 Measured internal wet weight concentrations of leaf discs exposed to 0.1 mg L^{-1} of carbamazepine (n=3, May 2009), providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater.

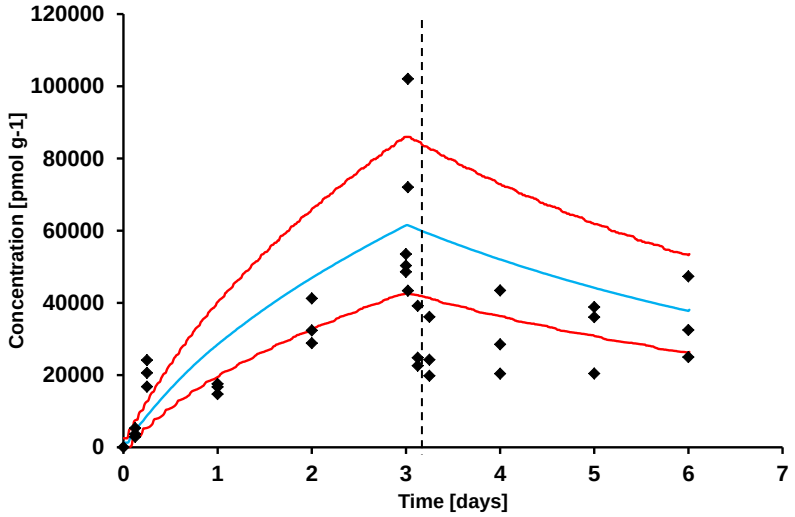


Figure 4.5 Measured internal wet weight concentrations of leaf discs exposed to 0.1 mg L^{-1} of carvedilol (n=3, May 2009), providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater.

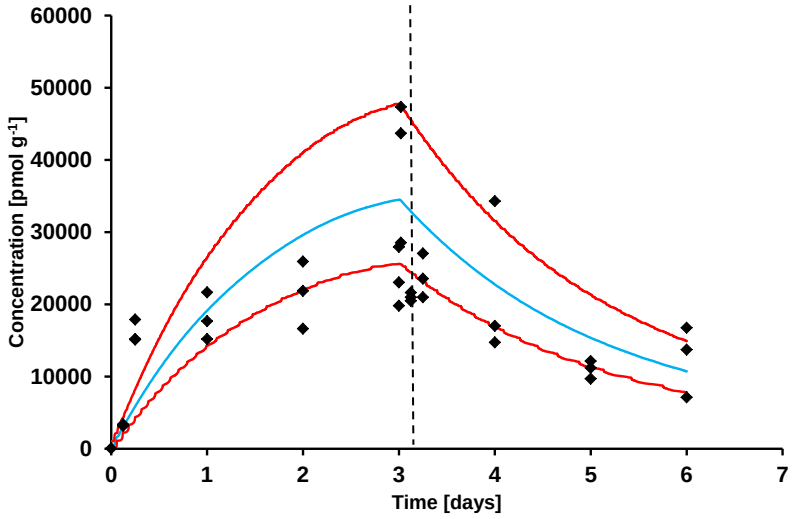


Figure 4.6 Measured internal wet weight concentrations of leaf discs exposed to 0.1 mg L^{-1} of diazepam (n=3, May 2009), providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater.

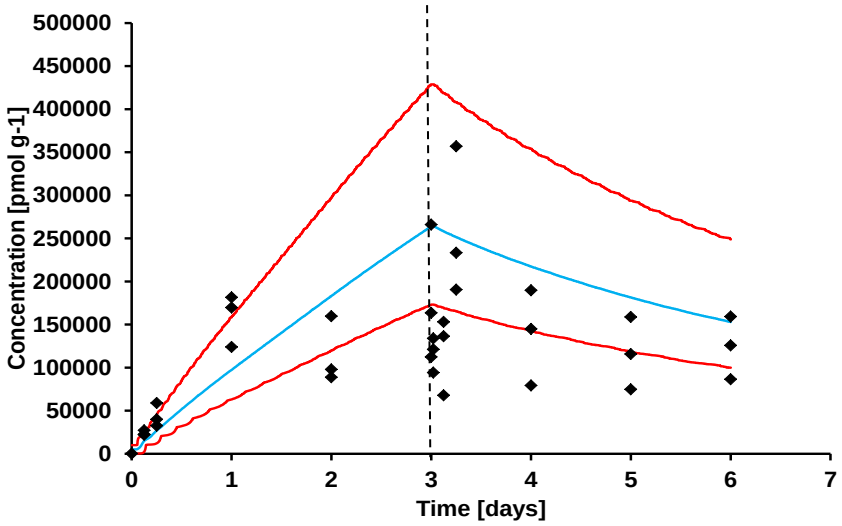


Figure 4.7 Measured internal wet weight concentrations of leaf discs exposed to 0.1 mg L^{-1} of fluoxetine (n=3, May 2009), providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater.

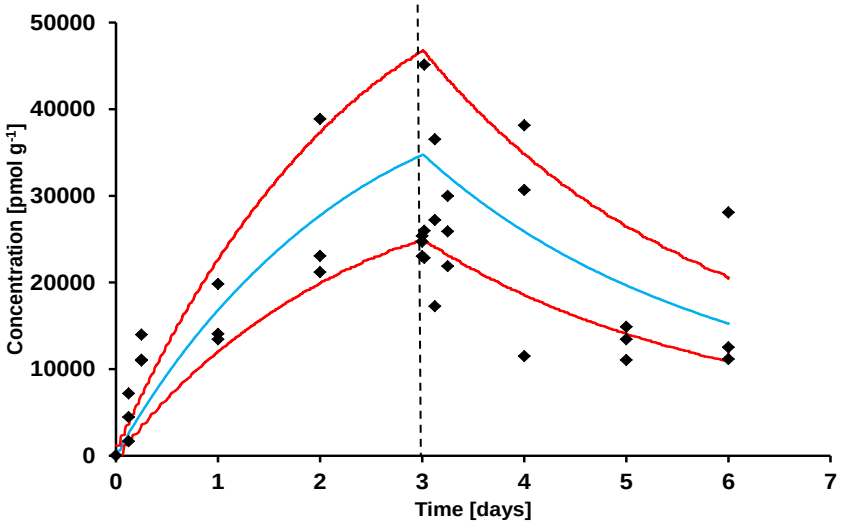


Figure 4.8 Measured internal wet weight concentrations of leaf discs exposed to 0.1 mg L^{-1} of moclobemide (n=3, May 2009), providing an uptake and depuration curve over time. Black diamonds are the measured concentration of individual replicates, the blue line is the model fit and the red lines represent 95 % confidence intervals. The dashed line represents change from exposure solution to freshwater.

Table 4.2 Modeled uptake/sorption and depuration/desorption rate constants, modeled uptake and depuration constants, FAF values and goodness of fit data for the accumulation/sorption of pharmaceuticals in fungus leaf discs

Chemical	C_w (pmol mL^{-1})	k_{int} (mL g-1 d-1) mean ± SD	kout (d-1) mean ± SD	FAF (L kg-1) [95th percentiles]
5 - fluorouracil	625.11 ± 11.69	5.48 ± 1.13	0.27 ± 0.12	24 [20 - 34]
carbamazepine	335.45 ± 14.29	30.46 ± 6.25	0.57 ± 0.16	63 [51 - 87]
carvedilol	144.02 ± 3.73	122.47 ± 30.47	0.18 ± 0.11	947 [766 - 1323]
diazepam	245.61 ± 3.04	60.58 ± 11.51	0.44 ± 0.12	166 [137 - 229]
fluoxetine	321.57 ± 26.21	258.73 ± 94.65	0.22 ± 0.2	1755 [1335 - 2845]
moclobemide	292.39 ± 4.11	47.85 ± 8.85	0.32 ± 0.10	176 [145 - 237]

Table 4.3 shows the significance of the linear relationships. Although both Log K_{ow} and Log D_{lipw} show good predictive power for FAFs, Log D_{lipw} has a more significant relationship and the regression model has a better fit. The relationship between Log D_{lipw} and FAF was highly significant (table 4.3). This type of model could be used to estimate the inputs of pharmaceuticals to the lowest trophic levels of aquatic food chains. In the future, these relationships may prove invaluable for environmental risk assessment and biological safety in identifying pharmaceuticals which are likely to pose the greatest concern in aquatic systems.

4.3.2 Uptake of pharmaceuticals into aquatic invertebrates from water and food

The relative importance of the route of uptake of chemicals from either food or water is unknown in most animals and little is known about the factors that can influence accumulation. However, this information is important for modelling the fate of chemicals in the environment (Munger and Hare, 1997). Studying the accumulation of contaminants from the food is especially critical for understanding trophic transfer in food chains (Wang and Fisher, 1999).

Carvedilol and fluoxetine were therefore chosen for use in studies to determine the relative importance of food uptake into invertebrates, as they showed the most association and the least desorption/depuration from the leaf discs in the uptake and depuration experiment. The invertebrate uptake studies involved water-only exposure, food-only exposures and combined water and food exposures.

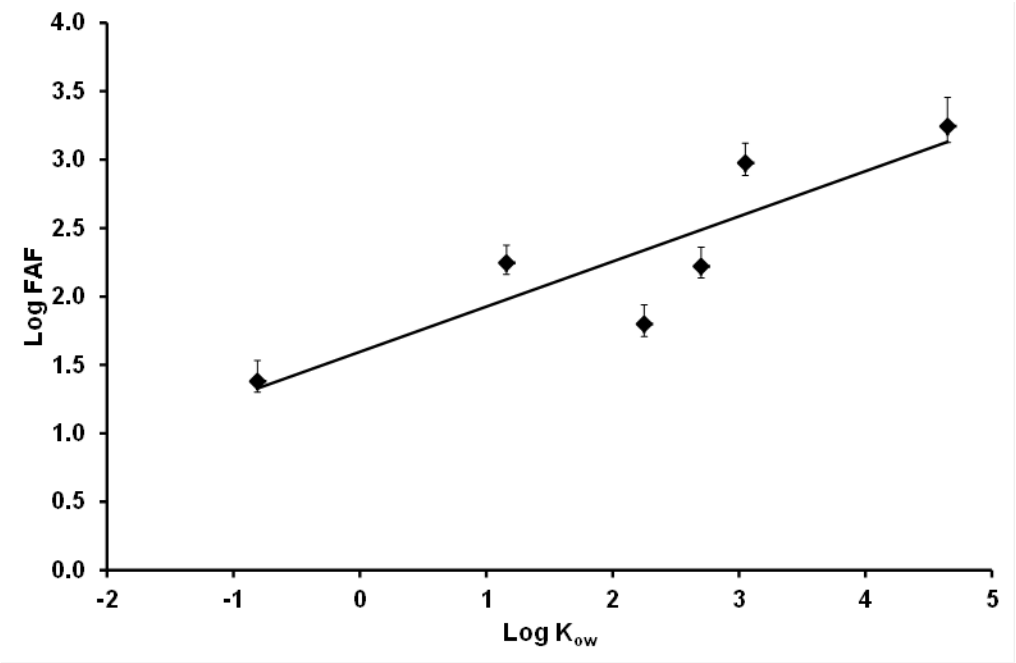


Figure 4.9 Relationships between food accumulation factors (± 95th percentile) and Log K_{ow} obtained from leaf discs sorption/uptake experiment with test pharmaceuticals; 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide. Diamonds are data points and the line is linear regression

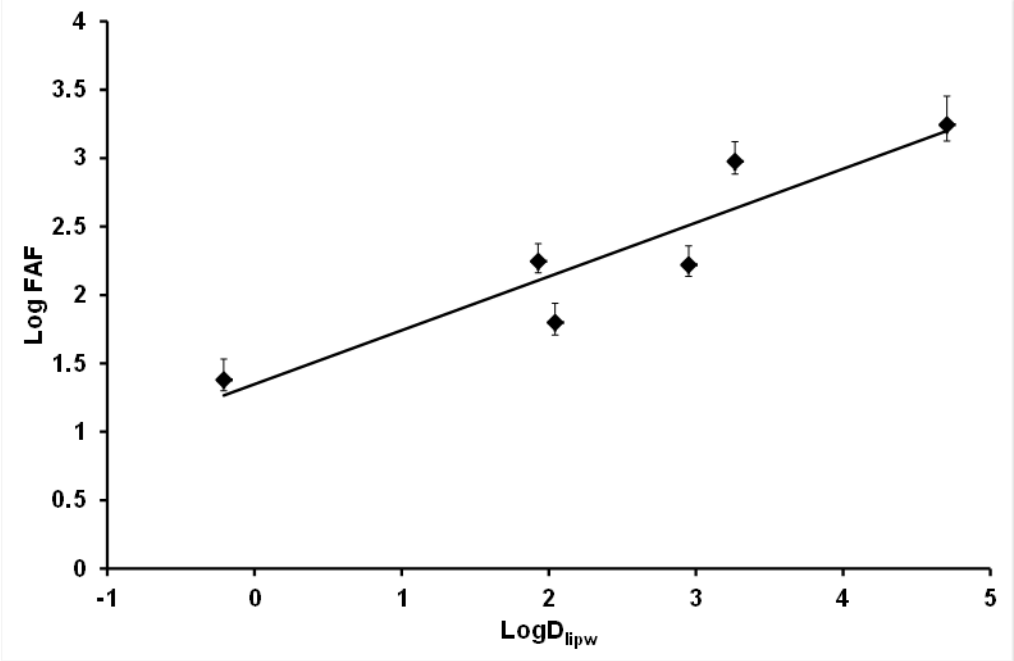


Figure 4.10 Relationships between food accumulation factors (± 95th percentile) and Log D_{lipw} obtained from leaf discs sorption/uptake experiment with test pharmaceuticals; 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide. Diamonds are data points and the line is linear regression

Table 4.3 Linear regression analysis of the relationships between Food Accumulation Factors (FAF) and lipophilicity of the test pharmaceuticals; 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide.

Parameter for Predicting FAF	R^2	Equation	P value	Figure
Log K_{ow}	0.76	FAF= 0.33 x Log K_{ow} + 1.60	0.02	4.09
Log D_{lipw}	0.85	FAF= 0.39 x Log D_{lipw} + 1.35	0.009	4.10

In order to avoid artifacts from the presence of food-associated pharmaceuticals in the gut of the animals, animals were allowed to depurate their gut contents for 6 h (*G. pulex*) or 24 h (*N. glauca*) prior to analysis. To assess whether this depuration period would have any effect on body residues of the compounds, animals were exposed to the study pharmaceuticals *via* water - half of these were analysed immediately after exposure and the other half were analysed following 6 h in clean water. No significant differences in the tissue concentrations of the depurated and non-depurated organisms were observed for both compounds in *G. pulex* and for carvedilol in *N. glauca* (for p values see supporting information). For fluoxetine in *N. glauca*, were significantly lower (≈ 56%) in the depurated organisms than the non-depurated organisms ($p = 0.05$). These observations are in agreement with the findings of Chapter 2 which showed that that *G. pulex* depurates fluoxetine and carvedilol very slowly and *N. glauca* depurates carvedilol slowly. The data presented below are therefore based on measurements following gut depuration.

Analysis of exposure solutions during the uptake studies showed that in the water only exposures, *G. pulex* were exposed to 2.72 ± 0.10 pmol mL^{-1} carvedilol and 21.80 ± 0.44 pmol mL^{-1} fluoxetine and there was no significant change in the concentration over 72 h ($p = 0.85$ and 0.2 respectively). In the *N. glauca* water-only studies, organisms were exposed to measured concentrations of 2.54 ± 0.09 pmol mL^{-1} carvedilol and 21.04 ± 0.51 pmol mL^{-1} and again there was no significant change in concentration over the 72 h test period ($p =0.3$ and 0.3 respectively). In the food-only exposures, both compounds were not detected in the aqueous phase at the start of the study for both test organisms but after 72 h, the concentrations for carvedilol of 0.09 pmol mL^{-1} and for fluoxetine of 1.51 pmol mL^{-1} were measured in the *G. pulex* studies and concentrations of 0.01 pmol mL^{-1} for carvedilol and 0.37 pmol mL^{-1} for fluoxetine were measured in the *N. glauca* studies. These observations are probably explained by depuration/desorption of the study compounds from the leaf discs or the invertebrates themselves.

Data on concentrations within the study organisms are summarized in figure 4.11. For both test chemicals, concentrations within the organisms decreased up the food chain (figure 4.12). In *G. pulex,* the simultaneous exposure through the food and the water resulted in a significantly higher concentration of both test compounds than the water only and food only exposures ($p < 0.004$; see Appendix C.2.3). Uptake from the water phase was significantly greater than uptake from the food phase ($p < 0.05$; see Appendix C.2.3). In *N. glauca*, simultaneous exposure to food and water

also resulted in higher mean tissue concentrations for both compounds than the water only or food only exposures but this was not significantly different ($p > 0.07$; see Appendix C.2.4). For carvedilol, uptake from water appeared to be more important than from food, whereas, for fluoxetine, uptake from food appeared to be more important than uptake from water.

From the food-only uptake results, it is possible to estimate assimilation factors (Table 4.4). *G. pulex* assimilated 3 % of the total carvedilol in the food compared to 6 % for fluoxetine and *N. glauca* assimilated 1.66 % of the total carvedilol and 5.52 % of the total fluoxetine. The assimilation efficiency of both organisms was much lower for these pharmaceuticals compared to assimilation efficiencies for neutral compounds. For example another aquatic amphipod *Diporeia* sp assimilated 7 – 33 % when exposed to benzo[a]pyrene in the food (Harkey *et al.*, 1994).*G. pulex* has also been shown to assimilate 82 % of 4 – Nonylphenol when fed dosed horse – chestnut leaves (Gross-Sorokin *et al.*, 2003). The results indicate that uptake of dissolved chemicals from the water may be dominant route for accumulation of the study compounds in *G. pulex*. This is possibly due to the respiration strategy of *G. pulex* and its reliance on dissolved oxygen. The gill ventilation rate of Gammarids is reported to be 6.0×10^{-3} L d^{-1} compared to the ingestion rate of 1.9×10^{-5} kg d^{-1} resulting in a larger amount of compound being passed over the gills compared to that of the gut (Morrison *et al.*, 1997).

The dietary exposure route is evident when in combination with the aqueous exposure because the accumulation in the tissue was significantly higher than the accumulation when solely exposed from the water. Consequently water only exposure would under estimate accumulation of pharmaceuticals in *G. pulex* if exposed from both aqueous and dietary routes discussed in section 4.3.3 of this thesis. This has been found before in *G. pulex* when exposed to zinc. A simultaneous exposure of zinc in the food and the water resulted in higher internal zinc concentrations than when exposed only in the water (Wilding and Maltby, 2006).

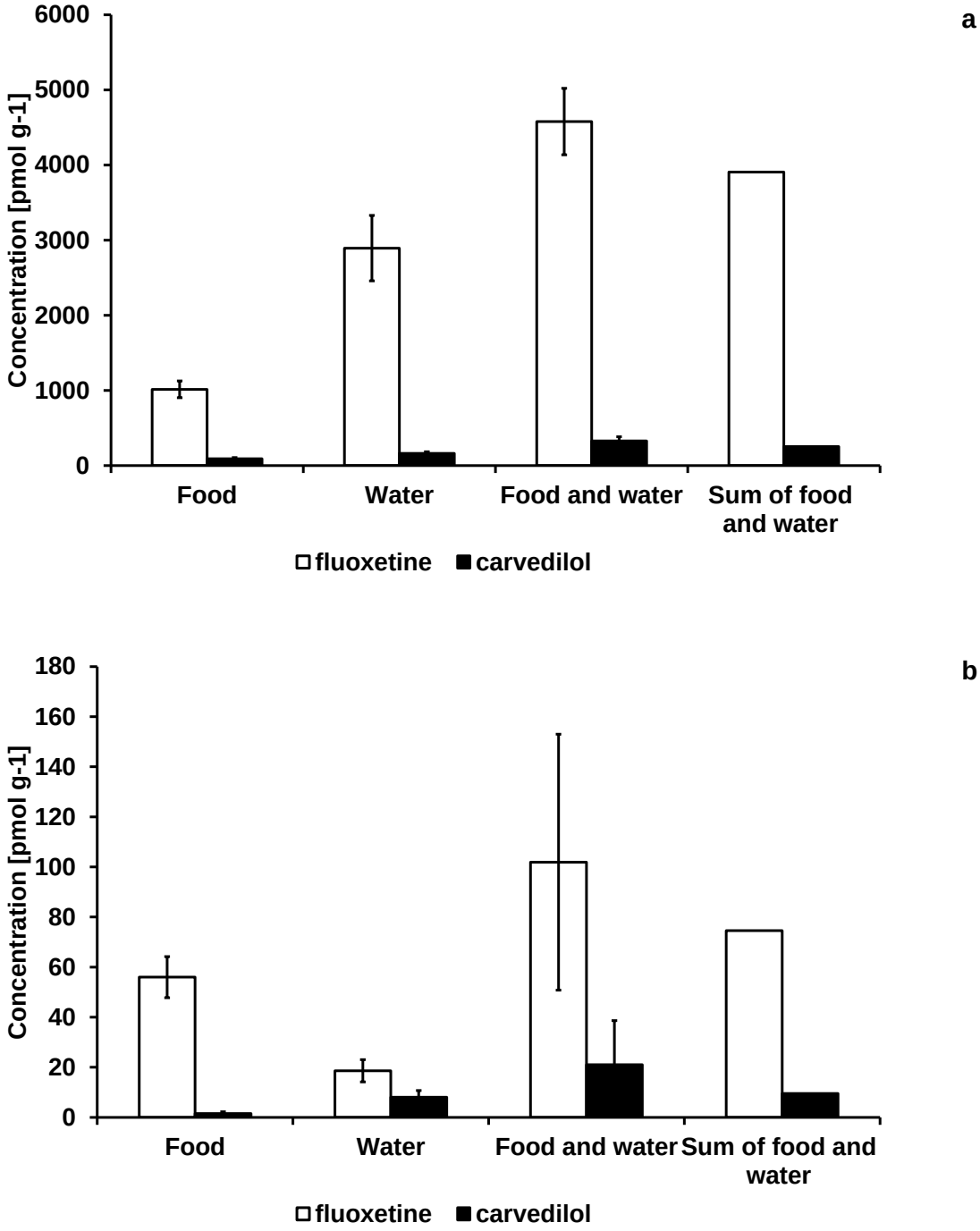


Figure 4.11 Mean whole body concentrations (wet weight ± standard deviation) of fluoxetine (white bars) and carvedilol (black bars) in a) *Gammarus pulex* (n=3) and b) *Notonecta glauca* (n=3) when exposed in the food, the water, and the food and water combined (exposure concentration 1 µg L^{-1}).

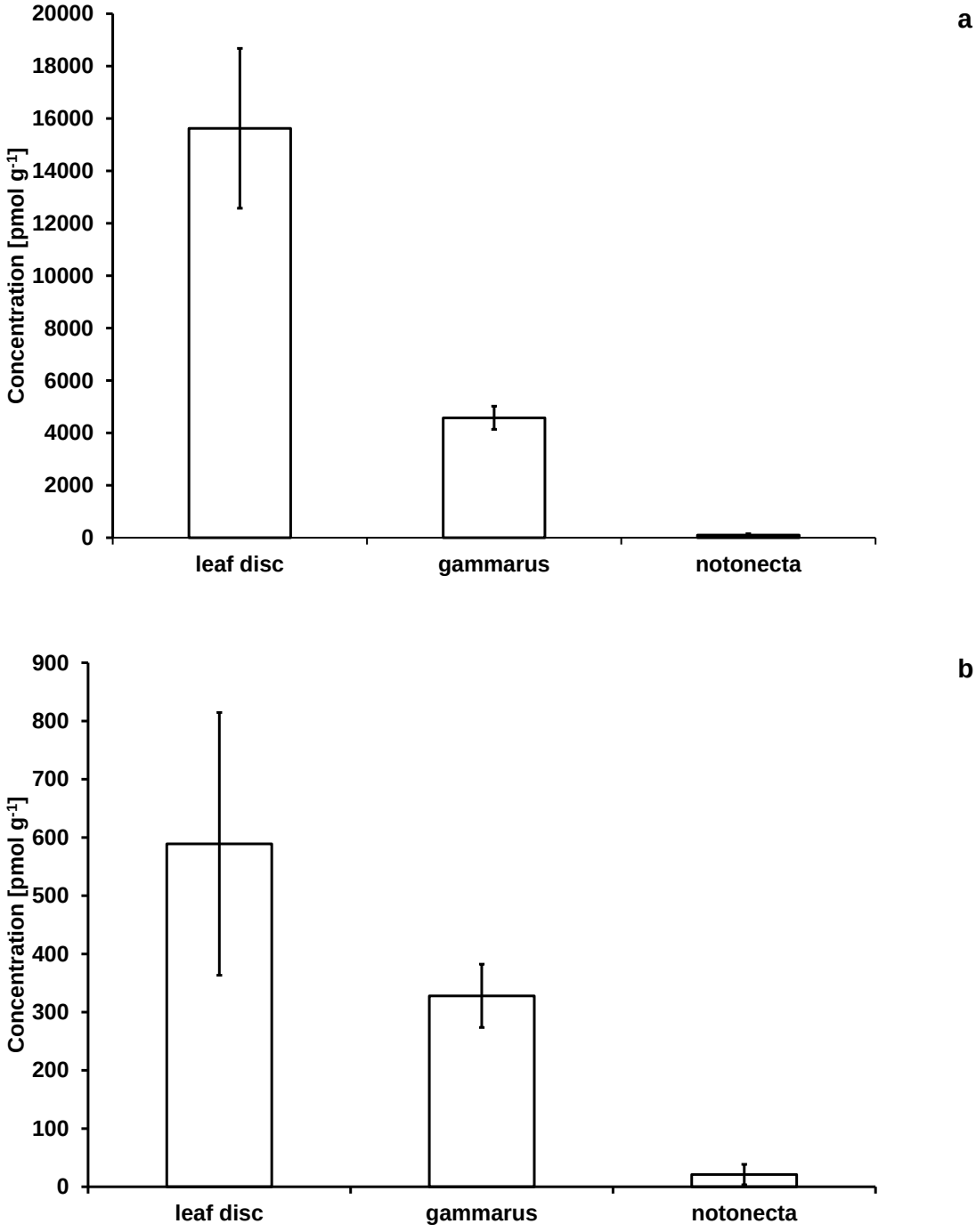


Figure 4.12 Mean whole body concentrations (wet weight ± standard deviation) of a) fluoxetine and b) carvedilol (exposure concentration of 1 µg L^{-1}) in a simple aquatic food chain with Leaf disc inoculated with *Cladosporium herbarum* (n=8), *Gammarus pulex* (n=3) and *Notonecta glauca* (n=3)

Table 4.4 Percentage assimilation of carvedilol and fluoxetine from the food source for *Gammarus pulex* and *Notonecta glauca*

	Assimilation from the food [%]	
	G. pulex	***N. glauca***
carvedilol	15.4	1.7
fluoxetine	6.5	5.5

N. glauca is a predator which pierces its prey, injecting it with paralytic enzymes, and sucking out the contents of the prey body (Peckarsky, 1982, Brooks *et al.*, 2009). Hence piercer predators will only be exposed to pharmaceutical accumulated in the internal tissues of the prey and any compound bound to the exoskeleton is discarded (Brooks *et al.*, 2009). Therefore the distribution of the contaminant in the food organism will influence the concentration accumulated in the predator (Arukwe *et al.*, 2000, Liu *et al.*, 2002, Heiden *et al.*, 2005, Gaskell *et al.*, 2007, Brooks *et al.*, 2009). Other types of feeding behaviours such as engulfers that consume the whole prey organism maybe exposed to larger concentrations.

N. glauca took up less from the water compared to that assimilated from the food. Uptake of pharmaceuticals from the water has been shown to be minimal compared to other species such as *G. pulex* in the present study and previous studies (Chapter 2). This is believed to be due to a combination of factors such as respiration strategy as *N. glauca* are not exposed to solutes as they rely on atmospheric oxygen for respiration (Matthews and Seymour, 2010). The present study shows that the food route of exposure to contaminants may be of more importance for some species than for others such as *N. glauca*.

This study also shows that pharmaceuticals can be transferred through a simple aquatic trophic chain. A previous study by Vernouillet *et al.* (2010) that carbamazepine can be taken up from water (10.2 mg L^{-1}) by algae and transferred through three trophic levels. However the top trophic level (*Hydra attenuata*) only showed trace concentrations of pharmaceutical in the tissues. In the case of the simple food chain tested in this study non biomagnification was observed despite the high bioaccumulative potential of the chosen compounds. Both Vernouillet *et al.*

(2010) and the current study show that certain species maybe indirectly exposed to pharmaceutical compounds through trophic interaction. Further study is warranted as little is known about what mechanisms maybe driving the uptake from the food. The kinetics of the uptake from the food would be needed to be studied to determine whether it is the rate of uptake or the extent that drives accumulation in predators. There are many factors that can affect the rate of diffusion across the gut such as conditions in the gut (Gaskell *et al.*, 2007) and chemical state (Cunningham, 2004). However distribution of the compound in the food item as well as the desorption efficiency from the food particle are important factors influencing the bioavailability of the compound to be absorbed (Liu *et al.*, 2002, Gaskell *et al.*, 2007). Also, contaminants bound to cell walls and membranes of the food item may not be available for accumulation in the predator (Reinfelder *et al.*, 1998).

4.3.3 Comparison of measured and predicted uptake in aquatic invertebrates

An attempt was made to assess the applicability of rate constants measured at higher exposure concentrations in the water to predict the uptake in organisms exposed at environmentally relevant concentrations. The rates constants provided from previous exposure scenarios (table 2.5; 0.1 mg L^{-1} of carvedilol and fluoxetine; see section 0) were therefore used to estimate the concentration in *G. pulex* and *N. glauca* after 72 hours at a lower concentration (1 µg L^{-1} of carvedilol and fluoxetine).

The whole body internal measured concentrations of pharmaceutical for *G. pulex* and *N. glauca* when exposed to 1 µg L^{-1} of carvedilol and fluoxetine has limited replication (n=3) and cannot be statistically compared to the predicted results from the one compartment model (the model for 1 µg L^{-1} ran 1000 iterations based on rate constants from Chapter 2). Despite the limitations of the data, the preliminary results indicate that the rate constants for uptake and depuration obtained in chapter 2 have the potential to be applicable for use at the lower water exposure concentration (1 µg L^{-1}). The data suggests that the predicted internal concentrations for both compounds are similar to the measured data in both *G. pulex* and *N. glauca* (figure 4.13 and 4.14).

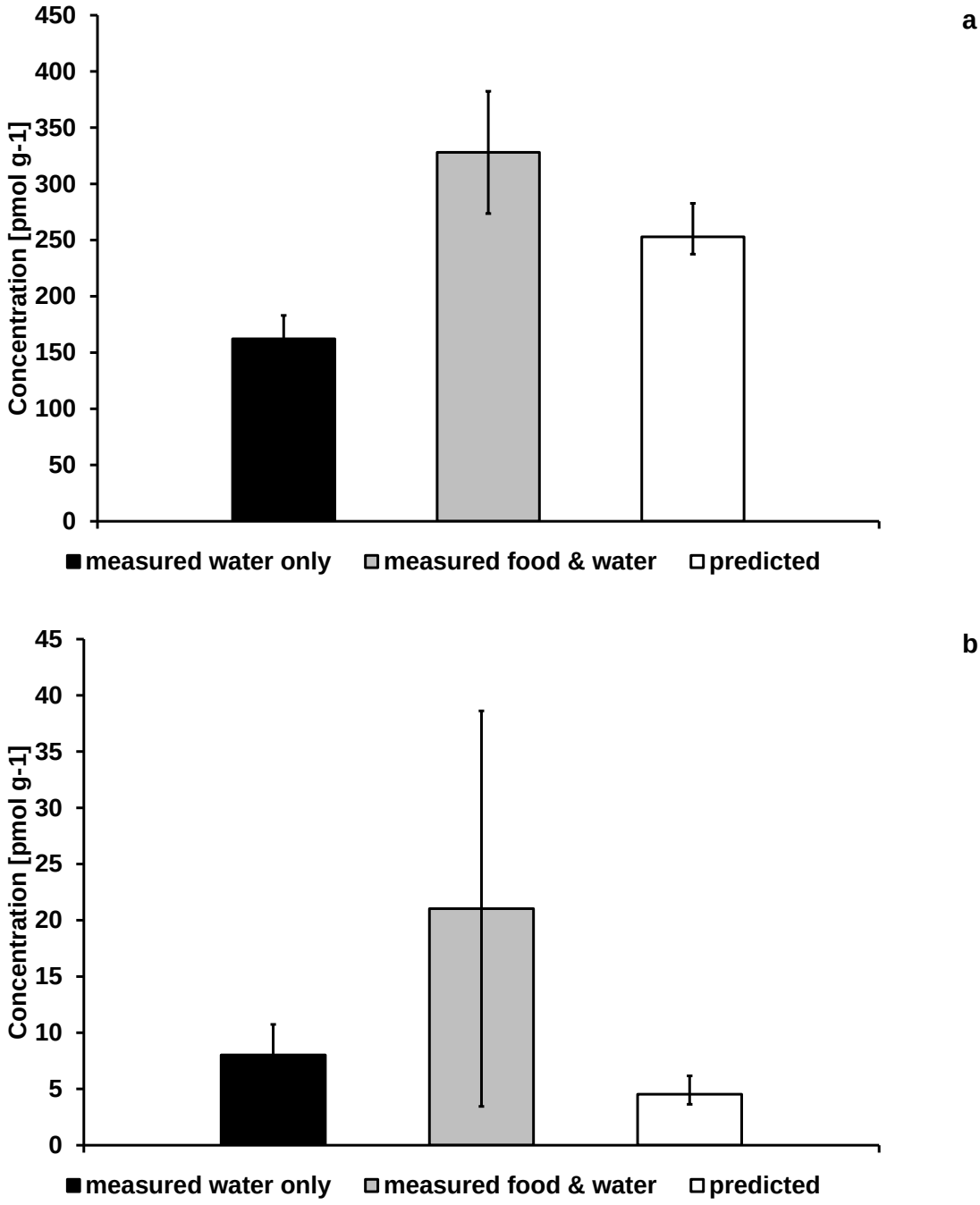


Figure 4.13 Mean whole body concentrations (wet weight ± standard deviation) in a) *Gammarus pulex* and b) *Notonecta glauca* when exposed to carvedilol in the water and in the food and water combined (exposure concentration 1 µg L^{-1}). Filled bars are measured values and open bars are the predicted values from a one compartment model and mean predicted concentration (see chapter 2; with 95th percentiles)

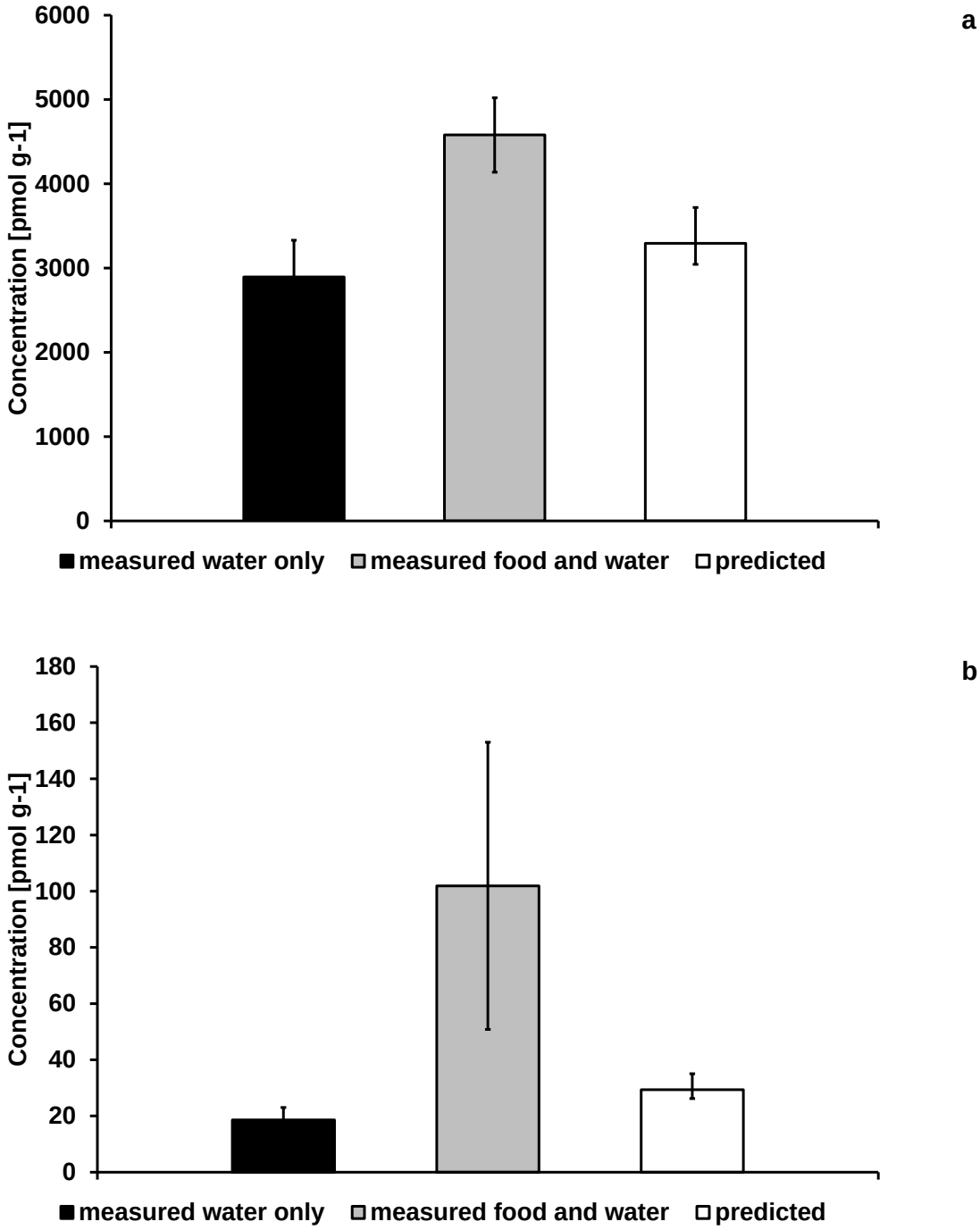


Figure 4.14 Mean whole body concentrations (wet weight ± standard deviation) in a) *Gammarus pulex* and b) *Notonecta glauca* when exposed to fluoxetine in the water and in the food and water combined (exposure concentration 1 µg L^{-1}). Filled bars are measured values and open bars are the predicted values from a one compartment model and mean predicted concentration (see chapter 2; with 95th percentiles)

However, the model does not account for exposure of organism to the pharmaceutical from the food and water combined. In both *G. pulex* and *N. glauca*, the internal measured concentrations of carvedilol and fluoxetine were higher than those predicted internal concentrations by the one compartment model.

This data suggests that the one-compartment model rate constants obtained in chapter 2 could provide a means to predict pharmaceutical concentrations in the aquatic invertebrate tested at lower concentrations. However, more replication is needed to determine the robustness of these findings and more research is needed to predict the uptake by invertebrates of pharmaceuticals from contaminated food.

4.4 Conclusions

The route of uptake has been previously shown to be an important factor that influences the extent of accumulation (Kraal *et al.*, 1995, Liu *et al.*, 2002, Mafra Jr *et al.*, 2010). There are many factors that can influence the accumulation of contaminants. For the food route factors include interspecies differences such as habitat, behaviour, feeding rate, gut passage time, desorption efficiency from the food, absorption efficiency of the gut, metabolism and elimination (Kaag *et al.*, 1997, Egeler *et al.*, 2001, Liu *et al.*, 2002, Gaskell *et al.*, 2007, Brooks *et al.*, 2009, Mafra Jr *et al.*, 2010). Also the distribution of the contaminant in the food item (Arukwe *et al.*, 2000, Brooks *et al.*, 2009) and the chemicals properties can effect accumulation in the predator (Kaag *et al.*, 1997, Egeler *et al.*, 2001, Mafra Jr *et al.*, 2010). Many of these factors also influence uptake from the water but aqueous exposure is dominated by exchange between the respiratory surface (Barber, 2003, Barber, 2008, Powell *et al.*, 2009) and thus factors such as respiratory strategy play a vital role in accumulation (Chapter 2, Buchwalter *et al.*, 2002, Rubach *et al.*, 2010).

The present study has shown that:

- Pharmaceuticals can accumulate on particulate matter and the accumulation is influenced by microorganisms present on the leaf

- The physicochemical properties of the pharmaceuticals affect the extent of the accumulation with the more lipophilic compounds accumulating the most

- The accumulation of pharmaceuticals in aquatic invertebrates may be influenced by the route of exposure and contaminated food may be an important route of exposure for some species

- Combining pharmaceutical exposure in both food and water, results in more accumulation than the water alone for both *N. glauca* and *G. pulex.* Therefore the inclusion of pharmaceutical uptake from the food may improve predictions of bioaccumulation and provide a more realistic exposure scenario

Chapter 5 General discussion and recommendations for future work

In the past five years there has been increasing scientific research into the occurrence of pharmaceuticals in the tissues of aquatic species and a number of studies have been published reporting uptake of pharmaceuticals into aquatic organisms (Daughton and Brooks, 2011). The majority of the studies to date have focused on fish (Brooks *et al.*, 2005, Brown *et al.*, 2007, Chu and Metcalfe, 2007, Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008, Gelsleichter, 2009, Ramirez *et al.*, 2009, Fick *et al.*, 2010, Kallio *et al.*, 2010, Schultz *et al.*, 2010, Zhang *et al.*, 2010); few studies exist concerning the fate of pharmaceuticals in aquatic invertebrates. It is important that we begin to understand the uptake of pharmaceuticals in aquatic invertebrates as these organisms play a very important role in aquatic food webs (Allan, 1996, Brooks *et al.*, 2009). At present, information is very limited and is generally restricted to pharmaceuticals that have been used in aquaculture (James, 1990b, James, 1990a, James and Boyle, 1998); however the limited data available indicate that aquatic invertebrates can accumulate pharmaceuticals (Vernouillet *et al.*, 2010). Therefore, this research explores those factors and processes affecting the uptake of human pharmaceuticals in aquatic invertebrates and an overview of the results can be found in table 5.1.

The core hypotheses investigated were:

- The uptake and depuration of pharmaceuticals in aquatic invertebrates are determined by the traits of an aquatic invertebrate, the degree of metabolism within an organism and by the properties of the pharmaceutical;

- Pharmaceuticals have the potential to biomagnify through aquatic food chains; and

- The bioconcentration of pharmaceuticals into aquatic invertebrates is predictable based on the physicochemical properties of the pharmaceutical, knowledge of pharmacological behavior in humans and from the individual traits of the organism

Each of the hypotheses investigated will be discussed in detail in the following section.

5.1 Discussion

Laboratory studies into the uptake and depuration of a variety of pharmaceuticals, covering a range of physico-chemical properties and therapeutic modes of action, showed that pharmaceuticals do have the potential to accumulate in aquatic invertebrates. Uptake and depuration data was modeled using a one compartment model to provide rate constants and BCFs. These BCFs were used to establish the accumulative potential of these compounds in invertebrates (table 5.1).

All of the compounds with the exception of fluoxetine would not be bioaccumulative under the European Medical Agency (EMEA) guidelines (EMEA, 2007). However fluoxetine would be prioritized for further screening due to the large Log BCF of 5.3. The BCF in *G. pulex* was 2 – 5 orders of magnitude higher than those found by other researchers (Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008, Zhang *et al.*, 2011). The large BCF in *G. pulex* is probably due to the limited depuration in the animal (kout of 0.00076 d^{-1}). Published data on the uptake of pharmaceuticals are limited; however data are available on fluoxetine in fish (Japanese madaka *O. latipes* and Rainbow trout, *O. mykiss*) which provides some data for comparison. Nakumara *et al.,* (2008) reported BCFs of 8.8 – 3100 and Patterson and Metcalfe (2008) reported BCFs of 74 – 80 and Zhang *et al.* (2010) reported BCFs of 143 – 22. Environmental monitoring studies have also shown concentrations of fluoxetine in fish to be significantly higher than concentrations in the surrounding water column (Lajeunesse *et al*., 2009; Schultz et al., 2010) also indicating potential for bioaccumulation.

BCFs for all compounds for the different species varied; *G. pulex* accumulated the most followed *N. glauca.* For carvedilol, *P. corneus* accumulated more than *N. glauca* but less than *G. pulex*. The results indicated that species traits such as respiration strategy, bahaviour and metabolism may play an important role in the accumulation patterns of compounds in organisms. These findings are in accordance with the published literature where the bioconcentration of compounds can be species dependent and influenced by the specific traits of an organism (Davies and Dobbs, 1984, Barron, 1990, Rubach *et al.*, 2010, Rubach *et al.*, 2010a, Rubach *et al.*, 2011). Studies have shown that bioconcentration of a compound can be predicted from the size and lipid content of an organism (Geyer *et al.*, 2000, Hendriks and Heikens, 2001, Ashauer *et al.*, 2010, Zhang *et al.*, 2010). However, this thesis and work completed by Rubach *et al.* (2010b) indicate that respiration strategy could potentially be more influential on bioconcentration, than size or lipid

content. Several other studies corroborate this hypothesis and show that aquatic organisms with large gas exchange epitheliums (such as *G. pulex*) will have larger BCFs when exposed to environmental contaminants in the surrounding media (Buchwalter *et al.*, 2002, Buchwalter *et al.*, 2003, Buchwalter *et al.*, 2004, Buchwalter *et al.*, 2008). However, in this thesis only one compound (carvedilol) was tested with all three species, therefore a firm conclusion with regards to the influence of species traits (such as respiration) on bioconcentration of pharmaceuticals cannot be drawn from this limited data set. Further research will be needed on a broader range of organisms and pharmaceuticals to determine the specific traits that are of most importance for the process of bioconcentartion. Ultimately this information could be used to develop relationships that can be combined with physicochemical properties in order to understand that internal exposure within an organism.

Extensive information on metabolism in humans is collected for preclinical and clinical trials for the marketing of pharmaceuticals (Winter *et al.*, 2010) however little is known about the metabolism in non-target organisms. This thesis explored the influence of metabolism in *G. pulex* on bioconcentration for all of the study pharmaceuticals and attempted to predict likely metabolic pathways from human data. Metabolism of diazepam was observed in *G. pulex,* and by using known metabolic pathways of diazepam for humans, nordiazepam (the major metabolite of diazepam in humans) was identified in the organism. Much of the current knowledge of metabolism is derived from fish studies which have shown that pharmaceuticals such as fluoxetine (Nakamura *et al.*, 2008, Paterson and Metcalfe, 2008, Smith *et al.*, 2010) and diclofenac (Kallio *et al.*, 2010) can be metabolized in non-target organisms.

Table 5.1 Synthesis of data obtained for six model compounds, 5-fluorouracil, carbamazepine, carvedilol, diazepam, fluoxetine and moclobemide –how the bioaccumulation factors (BCF) and biomagnification factors (BMF) correlate to EMEA 'Persistant, Bioaccumulative and Toxic' (PBT) assessment in *Gammarus pulex, Notonecta glauca and Planabarius corneus*

Compound	*G. pulex* BCF	*N. glauca* BCF	*P. corneus* BCF	*C. herbarum* FAF	Metabolism in *G. pulex*	Trophic transfer	*G. pulex* BMF	*N. glauca* BMF
5-fluorouracil	6.5	0.1	-	24	N	-	-	
carbemazepine	7.0	0.2	-	63	N	-	-	
carvedilol	270.8	1.6	57.3	947	N	**Y**	No biomagnification	No biomagnification
diazepam	37.5	1.0	-	166	**Y**	**-**	-	
fluoxetine	**185900**[a]	1.4	-	1755	N	**Y**	No biomagnification	No biomagnification
moclobemide	4.55	0.3	-	176	N	-	-	

[a]Very Bioaccumulative under EMEA PBT assessment; Y = Yes; N= No

The work in the current thesis is the first to identify human use pharmaceuticals in an aquatic invertebrates, however metabolites of the veterinary medicines erythromycin and sulfadimethoxine have been observed in invertebrate species important for aquaculture (James, 1990b, James, 1990a, James and Boyle, 1998). Diazepam was the only study pharmaceutical to be metabolized in *G. pulex* and the concentrations of nordiazepam were low, therefore the metabolism of pharmaceuticals in *G. pulex* was minimal and would not influence the bioconcentration of diazeoam. Therefore it can be concluded that the BCFs determined in Chapter 2 were reflective of the bioconcentration of the parent pharmaceuticals.

In the literature it has been shown that physicochemical properties of pharmaceuticals can influence BCFs, therefore the BCFs from the current thesis were compared to the physicochemical properties of the study pharmaceuticals. A summary of the data can be found in table 2.2 (Chapter 2, page 83). Although no significant linear relationships were found for *G. pulex,* Log K_{ow} provided the model of best fit, however for *N. glauca* Log D_{lipw} was shownto be the best model for this organism. The data in this thesis supports the current thinking that lipophilicity of the compound will influence it uptake and bioconcentration (Borgå *et al.*, 2004, Giulio and Hinton, 2008, Powell *et al.*, 2009). The literature suggests the relationship between log K_{ow} and bioconcentration is not straight forward. Compounds such as pharmaceuticals with ionization potential, can be dissociated to variable degrees according to their disassociation constant at ambient pH (Escher and Schwarzenbach, 2002). The relationship between log K_{ow} and bioconcentration is further complicated as log K_{ow} does not perfectly mimic the properties of the site of uptake – the biological membrane (Verhaar *et al.*, 1992, Verhaar *et al.*, 1996). Therefore researchers are starting to use Log D_{lipw} (a more realistic descriptor of lipid membranes) in quantitative structure activity relationships (QSARs) for ionisable compounds instead of log K_{ow} with good results (Escher and Schwarzenbach, 2002, Escher and Hermens, 2004, Escher *et al.*, 2009).

Table 5.2 Synthesis of significant relationships found between physicochemical properties and BCF in *Gammarus pulex* and *Notonecta glauca*

Parameters tested	*G. pulex*	*N. glauca*
Log K_{ow}	No	Yes
Log D_{lipw}	No	Yes
MW	No	Yes
PSA	No	Yes

To test the merit of using the physicochemical properties for predicting BCF, the model developed with the best fit in this thesis for *G. pulex* (Log K_{ow}; Equation 5.1) and *N. glauca* (Log D_{lipw}; Equation 5.2) are compared with BCFs predicted for fish in risk assessment using the Equation 5.3, taken from the technical guidance document (TGD; EC, 2003), and an equivalent BCF for fish (Equation 5.4; Log $P_{blood:water}$) predicted by the FPM (Huggett *et al.*, 2003b), a model for invertebrates presented by Arnot *et al*. (2006) (BCF_{ARNOT}; Equation 5.5) and the BCFs from the experimental data (table 5.3).

Equation 5.1 $$\boldsymbol{Log\ BCF_{Gam} = 0.76 \times Log\ K_{ow} + 0.28}$$

Equation 5.2 $$\boldsymbol{Log\ BCF_{Not} = 0.25 \times Log\ D_{lipw} - 0.82}$$

Equation 5.3 $$\boldsymbol{Log\ BCF_{TGD} = 0.85 \times Log\ K_{ow} - 0.70}$$

Equation 5.4 $$\boldsymbol{Log\ P_{blood:water} = 0.73 \times Log\ K_{ow} - 0.88}$$

Equation 5.5 $$\boldsymbol{Log\ BCF_{ARNOT} = -1.67 + 1.02 \times Log\ K_{ow}}$$

The predicted BCFs from the different models for BCF as well as the experimental data are synthesized in Table 5.3. The BCFs predicted by the FPM (Log $P_{blood:water}$ equivalent BCF) underestimated the bioconcentration in *G. pulex* for all compounds. Therefore the FPM would not be appropriate to use for these invertebrates. For 5-fluorouracil, all the models for *G. pulex* and *N. glauca* including those based on the experimental data underestimated the bioconcentration of the compound. For carbamazepine, all the models (with the exception of the Log $P_{blood:water}$ equivalent BCF) overestimated the experiment BCFs for both organisms. For carvedilol, the

model proposed in this thesis using Log K_{ow} predicted the concentration in *G. pulex* accurately but the other models underestimated the BCF. When looking at *N. glauca* all models overestimated the experimental BCF for carvedilol, with the exception of the model proposed in thesis using Log D_{lipw} where an underestimation was obtained. For diazepam, the BCF predicted from the TGD guidelines was accurate and the model proposed in this thesis overestimated the BCF in *G. pulex* and the model proposed by Arnot *et al.* (2006) underestimated the BCF. In *N. glauca* all the models overestimated the bioconcentration observed for diazepam. For moclobemide all of the models with the exception of the model proposed in this thesis for *G. pulex* underestimated the BCF, there were similar findings for *N. glauca.* Fluoxetine BCF was overestimated for *N. glauca* by all models however the models all underestimated the BCF for fluoxetine in *G. pulex.* This thesis has shown the difficulty in using a single parameter such as a physicochemical property to predict bioconcentration and it is likely that many factors will influence the BCF. An accurate model will need various parameters including those that are physicochemical as well as those that are biological to produce reliable results.

Table 5.3 Predicted bioconcentration factors using Technical Guidance document (BCF_{TGD}), Fish Plasma Model ($Log\ P_{blood:water}$), Arnot *et al.* (2006) (BCF_{ARNOT}) the models based on physicochemical properties with best fit from this thesis (BCF_{logkow} and $BCF_{logDlipw}$) and experimental data (BCF_{EXP}) using *Gammarus pulex, Notonecta glauca* and *Planabarus corneus*

Compound	Log BCF_{TGD}	Log $P_{blood:water}$	Log BCF_{arnot}	Log BCF_{logkow} *G. pulex*	Log $BCF_{logDlipw}$ *N. glauca*	Log BCF_{EXP} (*G. pulex*)	Log BCF_{EXP} (*N. glauca*)	Log BCF_{EXP} (*P. corneus*)
5-fluorouracil	-1.39	-1.47	-2.09	-0.33	-0.99	0.81	-0.89	
carbamazepine	1.21	0.76	1.04	1.97	-0.31	0.85	-0.62	
carvedilol	1.89	1.35	1.85	2.57	-0.02	2.43	0.20	1.76
diazepam	1.60	1.09	1.49	2.31	-0.08	1.57	-0.01	
fluoxetine	3.25	2.51	3.48	3.77	0.16	5.27	0.14	
moclobemide	0.29	-0.03	-0.08	1.15	-0.43	0.66	-0.48	

As fluoxetine and carvedilol were the most bioaccumulative compared to all the other study compounds, a series of laboratory studies explored the potential for trophic transfer and biomagnification in a simple aquatic food chain. With such a large BCF (5.3) obtained for fluoxetine in *G. pulex* it was thought that there could be potential biomagnification. This hypothesis was formulated because compounds such as DDT and it metabolites are well-known for biomagnification and they have BCFs in the range of 4.2-4.6 in aquatic invertebrates (Lotufo *et al.*, 2000).

The mean whole body concentrations measured in food chain studies from Chapter 4 were applied to the secondary poisoning assessment in the TGD. The secondary poisoning assessment derives a biomagnification factor (BMF); in order to determine whether a compound would have potential for biomagnification in an aquatic food chain. The BMF is is defined as the relative concentration in a predatory animal compared to the concentration in its prey (Equation 5.6; EC, 2003).

Equation 5.6 $$\boldsymbol{BMF = \frac{C_{predator}}{C_{prey}}}$$

Where $C_{predator}$ is the concentration in the predator and C_{prey} is the concentration in the prey.

The experimental BMFs calculated from the data in Chapter 4 were < 0.1, therefore the study compounds would not be of concern for secondary poisoning in aquatic food chains. This was in agreement with the experimental results where no biomagnification was observed (EC, 2003). These results are in concurrence with the limited literature on trophic transfer of pharmaceuticals where little or no trophic transfer has been observed in some studies (Vernouillet *et al.*, 2010). Although little information is available for pharmaceuticals, these results agree with studies with other compound groups (such as pesticides) and show that biomagnification does not occur for compounds with a Log K_{ow} < 5.5 (Russell *et al.*, 1999).

Although the BMF calculated suggested there is no potential risk for secondary poisoning, Chapter 4 identifies that biological traits and feeding strategy played an important role in accumulation patterns, which is in agreement with studies undertaken of neutral compounds where feeding relationships and behaviours have

been shown to control the exposure of chemicals in food chains (Russell *et al.*, 1999, Fisk *et al.*, 2001, Brooks *et al.*, 2009).

5.2 Effects assessment

For pharmaceuticals there a lot of data regarding their efficacy and potency from pharmacological safety information (Brooks *et al.*, 2005, Williams, 2005, Brown *et al.*, 2007). The FPM has been proposed for use in risk assessment of pharmaceuticals to determine potential for effects occurring in fish (Huggett *et al.*, 2003b, Huggett *et al.*, 2004). The principle of the model is that plasma concentrations needed to cause a therapeutic effect in humans, would be similar to the concentrations needed to cause an effect in fish. By using the physicochemical properties of the pharmaceutical and predicted environmental concentrations for surface water (PEC_{sw}) the model calculates the steady state plasma concentration in the fish (Log $P_{blood:water}$).

PEC_{sw} values were calculated for the study pharmaceuticals with equation 5.7 taken from the EMEA guidelines for the environmental risk assessment of medicinal products for human use (EMEA, 2007). The usage data required in this equation was taken from the National Health Service statistics for 2011 (NHS, 2011).

Equation 5.7 $$PEC_{Surface\ water} = \frac{DOSE_{ai} \times Fpen}{WASTEW_{inhab} \times DILUTION}$$

Where $DOSE_{ai}$ [mg inhabitants^{-1} day^{-1}] is the amount of pharmaceutical prescribed per person per day, F_{pen} is fraction of market penetration [default value], $WASTEW_{inhab}$ [L inh^{-1}d^{-1}] is the volume of waste water per inhabitant per day and DILUTION is the dilution factor applied.

The PEC_{sw} values for the test pharmaceuticals can be found in table 5.4. The PEC_{SW} calculated for the study compounds were very low (< 10 ng L^{-1}) when using the EMEA guidelines (table 5.4). Using the PEC_{sw} values for the test pharmaceuticals and the Log $P_{blood:water}$ (equation 5.4), the steady state plasma concentration in fish ($F_{ss}PC$) can be determined (equation 5.8).

Equation 5.8 $$F_{ss}PC = Log\ P_{blood:water} \times PEC_{sw}$$

Table 5.4 Predicted Environmental Concentration for surface water PEC_{SW} for the study pharmaceuticals

Chemical	PEC_{SW} [µg L^{-1}]
5-fluorouracil	0.00000006
carbamazepine	0.01
carvedilol	0.0001
diazepam	0.0002
fluoxetine	0.001
moclobemide	0.0001

The FPM can be adapted for aquatic invertebrates and used to predict the steady state concentration of a pharmaceutical in an organism. In accordance with the FPM, the experimentally derived BCFs from Chapter 2 (table 5.1) can be multiplied by the PEC_{sw} (Table 5.4) to determine the steady state plasma concentration in the aquatic invertebrate ($AI_{SS}PC$) at environmentally relevant concentrations (equation 5.9).

Equation 5.9 $$AI_{ss}PC = BCF \times PEC_{sw}$$

The predicted $AI_{SS}PC$ can then be compared the concentration known to produce a therapeutic effect in humans (H_TPC) to give an effect ratio (Equation 5.9) to estimate the probability of therapeutic effects occurring in the organisms (Huggett *et al.*, 2003b).

Equation 5.9 $$ER = \frac{H_TPC}{AI_{SS}PC}$$

At low ER values, the drug concentration in the aquatic invertebrate plasma is close to the level in human plasma required to give a therapeutic effect, thus effects in aquatic invertebrates are likely, provided the drug target is conserved in the invertebrate. At high ER values, the risk of target interactions is smaller. This model assumes that the pharmaceutical is evenly distributed in the plasma of the aquatic invertebrate and that the plasma of an aquatic invertebrate has similar properties to plasma in humans.

When comparing H_TPC and $AI_{SS}PC$ in *G. pulex*, *N. glauca* and *P. corneus* in the effect ratios were > 1, with the exception of fluoxetine for *G. pulex.* The effects ratios show that these compounds may not pose a risk to aquatic invertebrates at environmental concentrations. However, the effect ratio for fluoxetine was < 1, showing that plasma concentrations in *G. pulex* could potentially be large enough for effects to occur in the environment, providing that the drug receptors are present in the organism. Fluoxetine has been detected in surface waters at mean concentrations of 0.05 μgL^{-1} (greater than those predicted from the EMEA guidance; table 5.4), therefore a smaller effect ratio would be expected at these higher environmental concentrations (Monteiro and Boxall, 2009).

Table 5.5 Predicted Aquatic Invertebrate plasma concentration ($AI_{ss}PC$), experimental internal concentration (C_{int}) in *Gammarus pulex, Notonecta glauca* and *Planabarus corneus* with human therapeutic plasma concentration (H_TPC) and effects ratios (ER)

Compound	$AI_{SS}PC$ *G. pulex* [ng g^{-1}]	$AI_{SS}PC$ *N. glauca* [ng g^{-1}]	$AI_{SS}PC$ *P. corneus* [ng g^{-1}]	H_TPC [ng ml^{-1}]	ER [$AI_{SS}PC$] *G. pulex*	ER [$AI_{SS}PC$] *N. glauca*	ER [$AI_{SS}PC$] *P. corneus*
5-fluorouracil	<0.01	<0.01	N/A	1.5	3446092	223996000	N/A
carbamazepine	0.07	<0.01	N/A	9300	133445	4670560	N/A
carvedilol	0.02	0.00	<0.01	105	5394	912899	25491.1
diazepam	0.01	<0.01	N/A	300-400	51613-68817	1935488-2580651	N/A
fluoxetine	200.11	<0.001	N/A	200-531	0.99-2.65	132711-352348	N/A
moclobemide	<0.01	<0.01	N/A	54-161	20218-603508	3070019-9153207	N/A

H_TPC were all obtained from (Thummel and Shen, 2001)

These data show the importance of being able to measure or predict reliable internal concentrations in order to identify pharmaceuticals that may cause effects in the environment. It is important for risk assessment to know the mode of action of compounds to determine potential effects; for pharmaceuticals this information can be available from clinical data (Williams, 2005, Escher *et al.*, 2009). For example, fluoxetine is an SSRI that effects by inhibiting serotonin reuptake at presynaptic neuronal membranes in humans. In fish serotonin can regulate a wide range of behaviours including feeding activity, reproductive behavior and establishment of social hierarchies (Brooks *et al.*, 2003, Brooks *et al.*, 2005, Gaworecki and Klaine, 2008). Due to conservation of evolutionary lines it may be possible to predict likely effects occurring, by using human pharmacological data from clinical trials (Brooks *et al.*, 2005, Brown *et al.*, 2007, Owen *et al.*, 2009). However, in primary producers and invertebrates, mechanistic responses to pharmaceuticals and their metabolites are not well understood (Brooks *et al.*, 2003, Brooks *et al.*, 2005). Further research should be undertaken to identify common receptors in vertebrates and invertebrates to establish likely effects of pharmaceuticals in the environment, in order to prioritise compounds for risk assessment.

5.3 Implications for risk assessment

The EMEA guideline for environmental risk assessment of human pharmaceuticals (EMEA, 2007) refers to the TGD, and has set criteria to determine whether a substance is 'Persistent Bioaccumulative and Toxic' (PBT). If a substance;

- is highly adsorptive
- belongs to a class of substances known to have a potential to accumulate in living organisms
- has indications from structural features
- has no mitigating property such as hydrolysis (half-life less than 12 hours);

there is an indication of bioaccumulation potential (EC, 2003).

EMEA (2007) specifically requires a pharmaceutical to be screened for persistence, bioaccumulation and toxicity if it has a $\log K_{ow}$ above 4.5 (EMEA, 2007). Therefore

pharmaceuticals with a Log K_{ow} < 4.5 will not be screened. Under the current regulations, pharmaceuticals such as those studied in this research would not be screened for bioaccumulation; however results from this thesis indicate that aquatic invertebrates can uptake and accumulate these compounds.

In risk assessment the most important and widely accepted indicator for bioaccumulation potential is the Log K_{ow} (EC, 2003). There are however limitations to predicting BCF from models with Log K_{ow} as the main parameter. These models do not consider factors such as active transport, metabolism and the accumulation potential of any metabolites and special structural properties (e.g. ionization potential) (EC, 2003). The studies presented in this thesis reiterate that Log K_{ow} may not be the best predictor for uptake and bioconcentration in aquatic invertebrates, as some of the pharmaceuticals tested have potential for uptake and bioconcentration.

OECD No 305 is the current European guidance for characterising the bioconcentration potential of substances in aquatic species. It is a flow – through fish test. There is currently no guidance for experimentally determining bioconcentration in aquatic invertebrates (OECD, 1996). The data from this research shows that invertebrates have the potential to uptake pharmaceuticals to high concentrations. The use of fish data provide a simple ERA but may not be sufficient for safe-guarding against long-term effects on many other aquatic organisms, therefore a more diverse range of organisms should be used to determine bioconcentration. Being more inclusive and protecting a wider range of organisms would help fulfill the objectives of the water framework directives where a general requirement for ecological protection, and a general minimum chemical standard, was introduced to cover all surface waters (Kallis and Butler, 2001). An alternative route for risk assessment could involve investigating the mechanistic basis of species variability (Escher *et al.*, 2009). Applying knowledge about biological traits in combination with physicochemical properties (such as the Log K_{ow}) to prediction algorithms, may increase the reliability and robustness of models for a wider range species.

The findings of this thesis indicate that certain compounds such as fluoxetine may be high priority for risk assessment in aquatic invertebrates; however it is not considered an environmental risk under the current guidelines (EC, 2003, EMEA, 2007). To demonstrate the applicability of the models developed in chapter 2 of this thesis for risk assessment, the model for *G. pulex* will be applied to the top 50

pharmaceuticals in the UK (based on usage data NHS, 2011) to determine which pharmaceuticals could be of potential risk to aquatic invertebrates. The top 50 pharmaceuticals were ranked by their PEC_{sw} and then ranked by BCF, predicted using the *G. pulex* model.

The prioritization exercise shows that of the top 50 used pharmaceuticals in the UK, simvastatin; diclofenac; ibuprofen; flucloxacillin and naproxen would be the top 5 priority pharmaceuticals for risk assessment for aquatic invertebrates. Out of these top 5, simvastatin would be described as 'very bioaccumulative' and diclofenac would be described as 'bioaccumulative' under EMEA. As diclofenac has a $LogK_{ow}$ of 4.0 it would not be required to have a PBT assessment and further more a predicted BCF of 2.7 under the current system using the TGD (equation 5.3; page 180) would labelled it as non-bioaccumulative (EC, 2003, EMEA, 2007). Considering the known effects of diclofenac in fish and birds (Hilton and Thomas, 2003, Green *et al.*, 2004, Schwaiger *et al.*, 2004, Green *et al.*, 2007), and the incurred diclofenac residues in wild fish samples (Brown *et al.*, 2007, Fick *et al.*, 2010) this compound may warrant further investigation.

Table 5.6 Prioritisation approach using models based on physicochemical properties with best fit from this thesis for *Gammarus pulex* (BCF_{logkow}), top 50 pharmaceuticals ranked first by Predicted surface water concentration (PEC_{SW}) and then by BCF

Rank	Pharmaceutical	Theraputic class	Usage (kg)	$PEC_{SW}\mu gL^{-1}$	Log k_{ow}	BCF_{logkow} *G. pulex*
1	Simvastatin	Statin	43661	0.010	5.2	4.2
2	Diclofenac	NSAID	151297	0.034	4.0	3.3
3	Ibuprofen	NSAID	262553	0.058	3.8	3.1
4	Flucloxacillin	Penicillinase-resistant penicillins	42929	0.010	3.4	2.8
5	naproxen	NSAID	53082	0.012	3.1	2.6
6	Hydrocortisone	Corticosteroid	224823	0.050	1.6	1.5
7	Acetylsalicylic Acid	Salicylate analgesic/antiplatelet	94159	0.021	1.1	1.1
8	Paracetamol	Analgesic	1022268	0.227	0.3	0.5
9	Quinine	Antipyretic, antimalarial, NSAID, Analgesic	33384	0.007	3.3	2.7
10	Tramadol	Opioid analgesic	32577	0.007	3.0	2.5
11	ketoprofen	NSAID	39959	0.009	3.0	2.5
12	Diltiazem	Calcium channel blocker	24699	0.005	2.8	2.4
13	carbamazepine	Anticonvulsant	39069	0.009	2.3	2.0
14	Gliclazide	Sulphonylureas	29614	0.007	2.1	1.9
15	Penicillin V	Antibiotic	26785	0.006	1.9	1.7
16	Codeine	Opiate	39688	0.009	1.3	1.2
17	Ranitidine	H2-receptor antagonist	29791	0.007	0.3	0.5
18	Atenolol	Selective β1 receptor antagonist	27780	0.006	0.0	0.3
19	Allopurinol	Xanthine-oxidase inhibitor	29385	0.007	-0.6	-0.1

Table 5.6 Prioritisation approach using models based on physicochemical properties with best fit from this thesis for *Gammarus pulex* (BCF_{logkow}), top 50 pharmaceuticals ranked first by Predicted surface water concentration (PEC_{SW}) and then by BCF

Rank	Pharmaceutical	Theraputic class	Usage (kg)	$PEC_{SW}\mu gL^{-1}$	Log k_{ow}	BCF_{logkow} *G. pulex*
20	Oxytetracycline	Antibiotic (tetracycline)	21297	0.005	-2.9	-1.9
21	Amiodarone	Anti-arrhythmics	4247	0.001	8.8	6.9
22	Orlistat	Gastro-intestinal anti-obesity drugs	11434	0.003	8.2	6.4
23	Clotrimazole	Antifungal	4161	0.001	6.3	5.0
24	Mebeverine	Antispasmodic	20130	0.004	5.1	4.1
25	Verapamil	L-type Calcium channel blocker	6247	0.001	4.8	3.9
26	Fluoxetine	SSRI	4640	0.001	4.7	3.8
27	Dosulepin	Tricyclic antidepressant	4417	0.001	4.5	3.7
28	Losartan	Angiotensin-ii receptor antagonists	8612	0.002	4.0	3.3
29	Clopidogrel	Antiplatelet drugs	8493	0.002	3.8	3.1
30	Citalopram	SSRI	6943	0.002	3.7	3.1
31	Lansoprazole	Proton pump inhibitor	11661	0.003	3.7	3.0
32	Omeprazole	Proton pump inhibitor	12808	0.003	3.4	2.8
33	Ramipril	ACE inhibitor	4400	0.001	3.3	2.8
34	Venlafaxine	SNRI	8540	0.002	3.3	2.7
35	Quetiapine	Antipsychotic	5898	0.001	3.2	2.7
36	Valproic Acid	Anticonvulsant	8206	0.002	3.0	2.5
37	Fexofenadine	Antihistamine	6865	0.002	2.8	2.4
38	Propranolol	Beta blocker	7815	0.002	2.6	2.2

Table 5.6 Prioritisation approach using models based on physicochemical properties with best fit from this thesis for *Gammarus pulex* (BCF_{logkow}), top 50 pharmaceuticals ranked first by Predicted surface water concentration (PEC_{SW}) and then by BCF

Rank	Pharmaceutical	Theraputic class	Usage (kg)	$PEC_{SW}\mu gL^{-1}$	Log k_{ow}	BCF_{logkow} *G. pulex*
39	Nifedipine	Dihydropyridine calcium channel blocker	4010	0.001	2.5	2.2
40	Furosemide	Loop diuretic	15210	0.003	2.3	2.0
41	Salicylic Acid	Preparation for psoriasis	3809	0.001	2.2	2.0
42	Amlodipine	Calcium channel blocker	4243	0.001	2.1	1.8
43	Lidocaine	Antiarrhythmic	5712	0.001	1.7	1.5
44	Dihydrocodeine	Opioid analgesic	10529	0.002	1.5	1.4
45	Lamotrigine	Anticonvulsant	6502	0.001	1.0	1.0
46	trimethoprim	Bacteriostatic antibiotic	8836	0.002	0.7	0.8
47	Isosorbide Mononitrate	Nitrate	7800	0.002	-0.3	0.1
48	Lisinopril	ACE inhibitor	4718	0.001	-0.9	-0.4
49	Levodopa	Psychoactive drug	13773	0.003	-2.2	-1.4
50	Acamprosate	Psychoactive drug	3534	0.001	-2.9	-1.9

5.4 Conclusions

- There is increasing scientific research into the occurrence of pharmaceuticals in the tissues of fish; however limited studies exist on pharmaceuticals in aquatic invertebrates.

- The study presented in this thesis focuses on the uptake of human pharmaceuticals in aquatic invertebrates and the influences of physicochemical properties of the compound and the traits of the species.

- Modeling bioconcentration from physicochemical properties and pharmacokinetic parameters in aquatic invertebrates was shown to be difficult. However the refined models for predicting BCF for invertebrates species in thesis worked well compared to the alternative models available.

- To obtain precise prediction of bioconcentration in a particular invertebrate would either require detailed experimental data or new algorithms to be developed which consider the variability in biological, chemical and environmental factors.

- Biomagnifciation did not occur in the food chain tested however it is also important to recognise that exposure from contaminated food can contribute to uptake of pharmaceuticals in aquatic invertebrates.

- Accumulation patterns are associated with feeding behavior and feeding relationships may play an important role in controlling the exposure of pharmaceuticals in the aquatic environment.

5.5 Recommendations for future research

1 The study has only looked at a handful of pharmaceuticals. In order to corroborate the relationships between physico-chemical properties and uptake, additional pharmaceutical active ingredients should be studied.

2 This thesis has shown that $LogD_{lipw}$ can be used effectively in predicting BCF, however the test conditions were static. Further studies under different conditions to validate the QSAR for pH changes. Well characterized natural waters should be used to validate the system for the real world.

3 In this thesis only the influence of physicochemical and biological traits on BCF were explored. Therefore more research required to elucidate the influence of environmental factors such as dissolved organic carbon nitrates and suspended solids.

4 This thesis only assessed BCF using the water compartment. A real system would have a sediment compartment. Ultimately we should develop combined fate and uptake models for realistic environmental systems that can be used to characterise uptake distributions in the real environment.

5 Different patterns of accumulation were observed for *G. pulex* and *N. glauca*. This research proposes that species traits influence uptake of pharmaceuticals. It was thought that species traits including respiration and feeding strategy may be contributing factors to observed differences in uptake. Therefore further research is needed with more species of different traits to determine which traits and mechanisms have the greatest potential to accumulate pharmaceuticals.

6 In the food chain experiments no biomagnification was observed. The differences in uptake from the food were attributed to species traits. It is necessary to investigate the trophic transfer of pharmaceuticals with other species to confirm that biomagnification will not occur for compounds such as fluoxetine.

7 Further research is required to establish the difference in metabolism of pharmaceutical compounds in invertebrates in order to establish the influence on BCF.

8 Ultimately it would be advantageous to understand the ecological and physiological influences on BCF and to be able to integrate these along with the physicochemical parameters into modeling

9 As data from the above studies becomes available the developed knowledge should be used to determine new models for predicting uptake in aquatic organisms. These models will be invaluable for risk assessment of pharmaceuticals in the environment

Appendix A Sorption of Fluoxetine

A.1 Introduction

It has been shown to be hydrolytically and photolytically stable (Kwon and Armbrust, 2006), however it was observed to dissipate from the test water during the course of the study. It is known that fluoxetine adsorbs to soils and sediments (Kwon *et al.*, 2008, Monteiro, 2008). Its sorption potential varies greatly and is dependent on factors such as pH and radox status of the environment (Oakes *et al.*, 2010). Therefore sorption to the test vessels could potentially cause the dissipation of compound from the test system. To determine the fate of fluoxetine in the test system the aim of this preliminary studied was to determine the cause of the dissipation of fluoxetine from the test solution by performing a sorption experiment with the test beakers with different lighting conditions.

A.2 Materials and methods

A.2.1 Experimental procedure

Radiolabelled flouxetine was obtained from American Radiolabeled Chemicals Incorporated (Missouri, USA), the physicochemical properites are shown in table 2.2.

Test solutions were made up of radioactive fluoxetine (0.3 nmol L^{-1}) in APW. There were four treatments with three replicates each. Each replicate was a glass beaker containing 500 mL of test solution. Two treatments were kept in the dark by wrapping the test vessels in tinfoil. One of these treatments contained the steel cages used in Chapter 2 to separate *G. pulex.* The final two treatments were kept under a 12 hour light, 12 hour dark photoperiod one of these treatments also contained steel cages. Samples of water (1 mL) were taken from the test vessels at 0, 1, 2, 3 and 4 days when the exposure period was terminated.

The concentration of fluoxetine remaining in the water was determined by liquid scintillation counting (LSC). Water samples were transferred to scintillation vials and

10 mL of Ecoscint A (National Diagnostics) was then added. The sample was shaken to mix, left to settle and the radioactivity measured in the dark by LSC.

Environmental parameters pH and temperature were measured at the start and at the end of the exposure period with a Symphony SB80BD bench top meter (VWR International LtD, UK).

A.2.2 Data analysis

The determination of significant differences between the four treatments was calculated by an independent t-test with SPSS v.17.0.0.

A.3.1 Results and discussion

The concentration of fluoxetine in the test solution decreased over the exposure period in all four treatments (Figure 6.1). A independent t-test revealed that in three treatments (light; dark; light cages) the concentration of fluoxetine in the test solution was significantly different at the end of the exposure period compared to the concentration at the start ($p < 0.05$, Figure A.1). This was not the case for the treatment in the dark with cages ($p = 0.07$), however when considering the concentration of fluoxetine has significantly decreased after 72 hours compared to the start of the experiment ($p = 0.02$) it can be assumed that fluoxetine concentration is in decline in the test system.

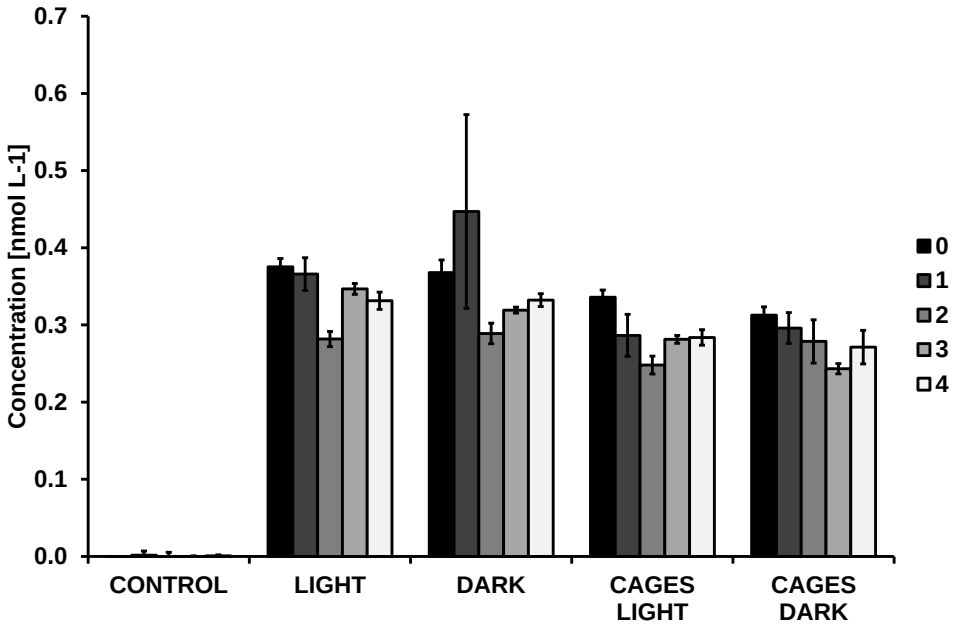


Figure A.1 Mean concentration (± standard deviation, n=3) of fluoxetine in the test solution at day 0, 1, 2, 3 and 4.

The overall trend in all treatments is that the fluoxetine concentration is declining. When considering the differences between the treatments, the data shows that fluoxetine is photolytically stable as there is no significant difference between the concentration in light and in the dark ($p = 0.94$). This is consistent with the literature as Kwon and Armbrust (2006) found that fluoxetine is nor susceptible to hydrolysis, photolysis and microbial degradation. However is it known that fluoxetine is prone to sorption in the environment (Kwon and Armbrust, 2006, Oakes *et al.*, 2010). Sorption to the cages in the test system could explain the significant difference ($P > 0.01$) between those treatments with and without cages. As fluoxetine is declining in all treatments it might be concluded that it is adsorbing to the glass beakers as well as the cages. Fluoxetine is an amine with a $pK_a > 9$ (Robertson *et al.*, 1988) therefore a larger fraction of the molecule will be positively charged (protonated; Yamamoto *et al.*, 2009) at the pH of the experiments (pH 8.42 – 8.42). This suggests that pH is unlikely influence the sorption behaviour of fluoxetine in this experiment.

A.4.1 Conclusions

It can be concluded from this preliminary experiment that fluoxetine is lost from the test solution over a four day time period. The exact process or processes that cause the loss of fluoxetine from the test solution is unknown, however the results indicate that sorption to the test vessels and cages play a more significant roles in fluoxetine dissipation than photolysis. For the proceeding experiments it can be concluded that a static renewal approach should be used to ensure a constant concentration of fluoxetine in the test solution.

Appendix B Toxicity of pharmaceuticals to the test organisms

	EC50 of test pharmaceuticals [µmol L^{1}]					
Species	5-fluorouracil	carbamazepine	carvedilol	diazepam	fluoxetine	moclobemide
Gammarus pulex	>31	>17	>10	>28	>6	>127
Notonecta glauca	>8	>4	>3	>4	>3	>4
Lymnea stagnalis			>3			

In all situations the EC_{50} values were above the highest concentration tested.

Appendix C Statistical Analysis

C.1 Statistical information for Chapter 2: Linear regression analysis using SPSS v17.0

C.1.1 LogK_{ow} and LogBCF for *Gammarus pulex*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.801[a]	.641	.551	1.18190

a. Predictors: (Constant), LogKow

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.641	7.142	1	4	.056

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	9.977	1	9.977	7.142	.056[a]
	Residual	5.588	4	1.397		
	Total	15.564	5			

a. Predictors: (Constant), LogKow

b. Dependent Variable: BCFgam

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	.278	.785		.355	.741
	LogKow	.764	.286	.801	2.672	.056

a. Dependent Variable: BCFgam

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-1.901	2.457
	LogKow	-.030	1.557

a. Dependent Variable: BCFgam

C.1.2 $LogD_{lipw}$ and LogBCF for *Gammarus pulex*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.706[a]	.498	.372	1.39793

a. Predictors: (Constant), LogDlipw

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.498	3.964	1	4	.117

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	7.747	1	7.747	3.964	.117[a]
	Residual	7.817	4	1.954		
	Total	15.564	5			

a. Predictors: (Constant), LogDlipw

b. Dependent Variable: BCFgam

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	.285	1.005		.283	.791
	LogDlipw	.764	.384	.706	1.991	.117

a. Dependent Variable: BCFgam

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-2.507	3.076
	LogDlipw	-.301	1.830

a. Dependent Variable: BCFgam

C.1.3 Molecular weight and LogBCF for *Gammarus pulex*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.486[a]	.236	.045	1.72435

a. Predictors: (Constant), MolecularW

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.236	1.235	1	4	.329

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	3.671	1	3.671	1.235	.329[a]
	Residual	11.894	4	2.973		
	Total	15.564	5			

a. Predictors: (Constant), MolecularW

b. Dependent Variable: BCFgam

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	-.647	2.426		-.267	.803
	MolecularW	.009	.009	.486	1.111	.329

a. Dependent Variable: BCFgam

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-7.383	6.089
	MolecularW	-.014	.033

a. Dependent Variable: BCFgam

C.1.4 Polar surface area and LogBCF for *Gammarus pulex*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.818[a]	.669	.558	1.29847

a. Predictors: (Constant), PolarSA

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.669	6.053	1	3	.091

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	10.206	1	10.206	6.053	.091[a]
	Residual	5.058	3	1.686		
	Total	15.264	4			

a. Predictors: (Constant), PolarSA

b. Dependent Variable: BCFGamlessCARV

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	6.413	1.950		3.289	.046
	PolarSA	-.114	.047	-.818	-2.460	.091

a. Dependent Variable: BCFGamlessCARV

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	.207	12.619
	PolarSA	-.263	.034

a. Dependent Variable: BCFGamlessCARV

This regression analysis excludes carvedilol and may only be appropriate for compounds with low PSAs.

C.1.5 Volume Distribution and LogBCF for *Gammarus pulex*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.896[a]	.803	.753	.87630

a. Predictors: (Constant), LOGVD

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.803	16.268	1	4	.016

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	12.493	1	12.493	16.268	.016[a]
	Residual	3.072	4	.768		
	Total	15.564	5			

a. Predictors: (Constant), LOGVD

b. Dependent Variable: LOGBCFGAM

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	1.389	.382		3.632	.022
	LOGVD	2.249	.557	.896	4.033	.016

a. Dependent Variable: LOGBCFGAM

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	.327	2.450
	LOGVD	.701	3.796

a. Dependent Variable: LOGBCFGAM

C.1.6 Comparision between linear and exponential relations between $LogK_{ow}$ and Log BCF for *Gammarus pulex*

Model Summary and Parameter Estimates

Dependent Variable:BCFgam

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	.641	7.142	1	4	.056	.278	.764
Exponential	.702	9.403	1	4	.037	.661	.361

The independent variable is LogKow.

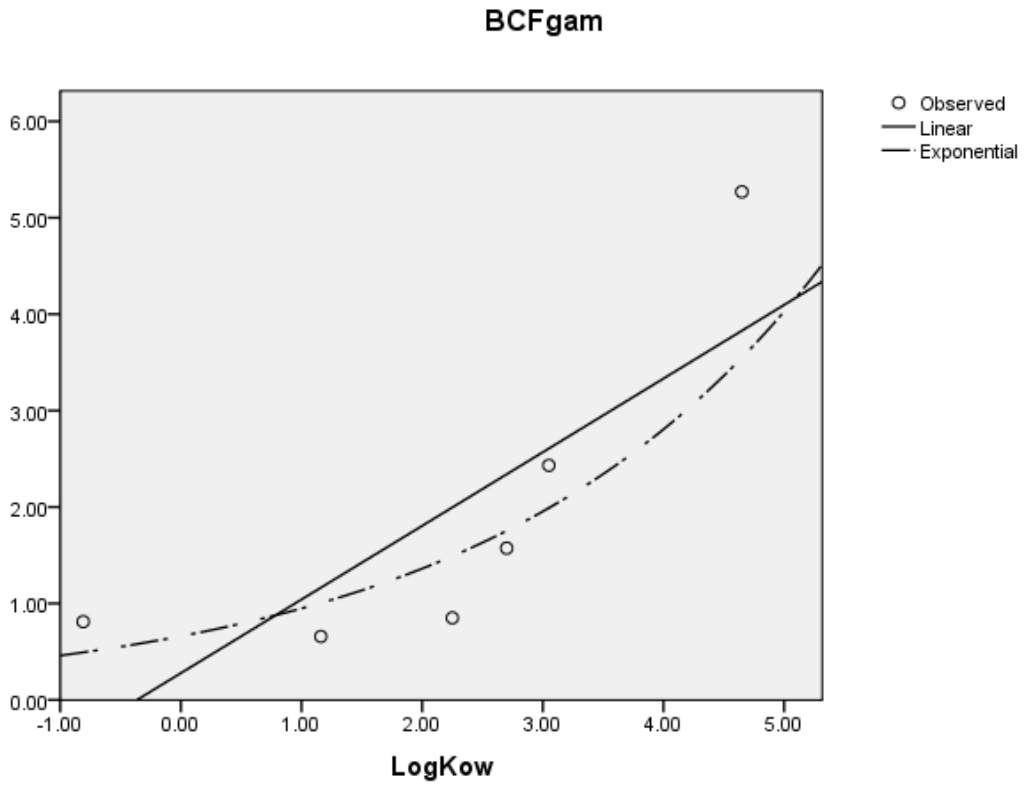


Figure C.1 Exponential and linear regression for the relationship between $LogK_{ow}$ and Log BCF

C.1.7 Volume Distribution and LogBCF for *Notonecta glauca*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.651[a]	.423	.279	.38159

a. Predictors: (Constant), LOGVD

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.423	2.937	1	4	.162

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.428	1	.428	2.937	.162[a]
	Residual	.582	4	.146		
	Total	1.010	5			

a. Predictors: (Constant), LOGVD

b. Dependent Variable: LOGBCFNOT

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	-.375	.166		-2.255	.087
	LOGVD	.416	.243	.651	1.714	.162

a. Dependent Variable: LOGBCFNOT

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-.838	.087
	LOGVD	-.258	1.090

a. Dependent Variable: LOGBCFNOT

C.1.8 $LogK_{ow}$ and LogBCF for *Notonecta glauca*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.866[a]	.750	.688	.25109

a. Predictors: (Constant), LogKow

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.750	12.022	1	4	.026

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.758	1	.758	12.022	.026[a]
	Residual	.252	4	.063		
	Total	1.010	5			

a. Predictors: (Constant), LogKow

b. Dependent Variable: BCFnot

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	-.731	.167		-4.383	.012
	LogKow	.210	.061	.866	3.467	.026

a. Dependent Variable: BCFnot

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-1.194	-.268
	LogKow	.042	.379

a. Dependent Variable: BCFnot

C.1.9 Molecular weight and LogBCF for *Notonecta glauca*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.899[a]	.809	.761	.21973

a. Predictors: (Constant), MolecularW

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.809	16.921	1	4	.015

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.817	1	.817	16.921	.015[a]
	Residual	.193	4	.048		
	Total	1.010	5			

a. Predictors: (Constant), MolecularW

b. Dependent Variable: BCFnot

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	-1.492	.309		-4.826	.008
	MolecularW	.004	.001	.899	4.114	.015

a. Dependent Variable: BCFnot

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-2.350	-.633
	MolecularW	.001	.007

a. Dependent Variable: BCFnot

C.1.10 Log D_{lipw} and LogBCF for *Notonecta glauca*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.914[a]	.835	.793	.20442

a. Predictors: (Constant), Log D_{lipw}

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.835	20.171	1	4	.011

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.843	1	.843	20.171	.011[a]
	Residual	.167	4	.042		
	Total	1.010	5			

a. Predictors: (Constant), Log D_{lipw}

b. Dependent Variable: BCFnot

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	-.818	.147		-5.567	.005
	D_{lipw}	.252	.056	.914	4.491	.011

a. Dependent Variable: BCFnot

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-1.227	-.410
	D_{lipw}	.096	.408

a. Dependent Variable: BCFnot

C.1.11 Polar surface area and LogBCF for *Gammarus pulex*

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.979[a]	.958	.944	.10168

a. Predictors: (Constant), PolarSA

Model Summary

Model	Change Statistics				
	R Square Change	F Change	df1	df2	Sig. F Change
1	.958	68.071	1	3	.004

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.704	1	.704	68.071	.004[a]
	Residual	.031	3	.010		
	Total	.735	4			

a. Predictors: (Constant), PolarSA

b. Dependent Variable: BCFNotLessCARV

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	.832	.153		5.449	.012
	PolarSA	-.030	.004	-.979	-8.251	.004

a. Dependent Variable: BCFNotLessCARV

Coefficients[a]

Model		95.0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	.346	1.318
	PolarSA	-.042	-.018

a. Dependent Variable: BCFNotLessCARV

C.2 Statistical Analysis for Chapter 4 using SPSS V17.0

C.2.1 Linear regression of FAF and Log Kow

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.871[a]	.758	.698	.38469

a. Predictors: (Constant), LOGKOW

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1.859	1	1.859	12.559	.024[a]
	Residual	.592	4	.148		
	Total	2.450	5			

a. Predictors: (Constant), LOGKOW

b. Dependent Variable: LOGFAF

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	1.598	.255		6.254	.003
	LOGKOW	.330	.093	.871	3.544	.024

a. Dependent Variable: LOGFAF

C.2.2 Linear regression of FAF and Log Dlipw

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.923[a]	.852	.815	.30124

a. Predictors: (Constant), LOGDLIPW

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	2.088	1	2.088	23.005	.009[a]
	Residual	.363	4	.091		
	Total	2.450	5			

a. Predictors: (Constant), LOGDLIPW

b. Dependent Variable: LOGFAF

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients		
		B	Std. Error	Beta	t	Sig.
1	(Constant)	1.350	.235		5.738	.005
	LOGDLIPW	.393	.082	.923	4.796	.009

a. Dependent Variable: LOGFAF

C.2.3 Statistical information for comparison of uptake/sorption of pharmaceuticals for fungus and non-fungus leaf discs:

Levene's test and independent t-test for all test pharmaceuticals

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
data_flu	Equal variances assumed	5.451	.042	5.700	10	.000	8109.64812	1422.84825	4939.34466	11279.95158
	Equal variances not assumed			5.700	5.655	.002	8109.64812	1422.84825	4575.94791	11643.34834
data_carb	Equal variances assumed	.293	.600	2.689	10	.023	5779.09738	2148.89101	991.06983	10567.12492
	Equal variances not assumed			2.689	7.681	.029	5779.09738	2148.89101	787.71644	10770.47832
data_carv	Equal variances assumed	.979	.346	2.116	10	.060	22923.18692	10835.72002	-1220.30185	47066.67569
	Equal variances not assumed			2.116	8.715	.064	22923.18692	10835.72002	-1711.73510	47558.10895
data_diaz	Equal variances assumed	2.766	.127	1.719	10	.116	9086.82736	5287.25112	-2693.90228	20867.55701
	Equal variances not assumed			1.719	8.010	.124	9086.82736	5287.25112	-3102.99661	21276.65134
data_fluox	Equal variances assumed	.632	.445	1.076	10	.307	40726.70784	37836.84402	-43579.03435	1.25032E5
	Equal variances not assumed			1.076	9.893	.307	40726.70784	37836.84402	-43702.51980	1.25156E5
data_moc	Equal variances assumed	.275	.611	2.742	10	.021	11310.14437	4124.64834	2119.85515	20500.43359
	Equal variances not assumed			2.742	8.400	.024	11310.14437	4124.64834	1876.99440	20743.29433

Mann Whitney *U* test for 5-fluorouracil

Ranks

	test	N	Mean Rank	Sum of Ranks
data_flu	fungus	6	9.50	57.00
	nonfungus	6	3.50	21.00
	Total	12		

Test Statistics[b]

	data_flu
Mann-Whitney U	.000
Wilcoxon W	21.000
Z	-2.882
Asymp. Sig. (2-tailed)	.004
Exact Sig. [2*(1-tailed Sig.)]	.002[a]

a. Not corrected for ties.

b. Grouping Variable: test

C.2.4 Analysis of variance comparing exposure route of pharmaceuticals for *Gammarus pulex*

Multiple Comparisons

data

LSD

(I) Route	(J) Route	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
food	water	-427.506*	104.3633	.001	-654.894	-200.118
	foodwater	-1108.372*	104.3633	.000	-1335.760	-880.984
water	food	427.506*	104.3633	.001	200.118	654.894
	foodwater	-680.866*	104.3633	.000	-908.254	-453.478
foodwater	food	1108.372*	104.3633	.000	880.984	1335.760
	water	680.866*	104.3633	.000	453.478	908.254

Based on observed means.

The error term is Mean Square(Error) = 32675.074.

*. The mean difference is significant at the 0.05 level.

C.2.5 Analysis of variance comparing exposure route of pharmaceuticals for *Notonecta glauca*

Multiple Comparisons

Data_not_directly

LSD

(I) test_type_route	(J) test_type_route	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
food	water	15.3876	19.48929	.442	-26.1528	56.9281
	foodwater	-31.1501	19.48929	.131	-72.6906	10.3903
water	food	-15.3876	19.48929	.442	-56.9281	26.1528
	foodwater	-46.5378*	19.48929	.071	-88.0782	-4.9973
foodwater	food	31.1501	19.48929	.131	-10.3903	72.6906
	water	46.5378*	19.48929	.071	4.9973	88.0782

Based on observed means.

The error term is Mean Square(Error) = 1139.497.

*. The mean difference is significant at the 0.05 level.

C.3 Statistical information for fluoxetine sorption test: T-test analysis using SPSS v17.0

C.3.1 Independent students t-test

Independent Samples Test

		t-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
Light_dark	Equal variances assumed	.944	-.00074	.00989
	Equal variances not assumed	.945	-.00074	.00989
Light_lightcages	Equal variances assumed	.011	.04768	.01065
	Equal variances not assumed	.011	.04768	.01065
Dark_lightcages	Equal variances assumed	.006	.04841	.00923
	Equal variances not assumed	.007	.04841	.00923
Light_Darkcages	Equal variances assumed	.020	.04009	.01062
	Equal variances not assumed	.020	.04009	.01062
dark_darkcages	Equal variances assumed	.011	.04082	.00920
	Equal variances not assumed	.012	.04082	.00920

Independent Samples Test

		t-test for Equality of Means	
		95% Confidence Interval of the Difference	
		Lower	Upper
Light_dark	Equal variances assumed	-.02821	.02673
	Equal variances not assumed	-.02913	.02766
Light_lightcages	Equal variances assumed	.01811	.07724
	Equal variances not assumed	.01796	.07739
Dark_lightcages	Equal variances assumed	.02279	.07403
	Equal variances not assumed	.02245	.07437
Light_Darkcages	Equal variances assumed	.01059	.06958
	Equal variances not assumed	.01043	.06974
dark_darkcages	Equal variances assumed	.01529	.06636
	Equal variances not assumed	.01497	.06668

Abbreviations

AA	Analgesics and Anti-inflammatories
AB	Anti-biotics
AC	Anti-cancer
ACE	Angiotensin-Converting Enzyme
AD	Anti-depressants
AE	Anti –epileptics
AHP	Anti – hypertensives
AP	Anti – psychotic
API	Active Pharmaceutical Ingredient
APW	Artificial Pond Water
ASE	Accelarated Solvent Extraction
BB	Beta-blockers
BCF	Bioconcentration Factor
BAF	Bioaccumulation Factor
BMF	Biomagnification Factor
Bq	Becquerel
BWW	Bishop Wilton Beck Water
C-14	Carbon 14 isotope
$C_{internal}$	Internal Concentration
C_{max}	Maximum Concentration
CO_2	Carbon Dioxide
C_{water}	Water Concentration
CYP – 450	Cytochrome P450 enzymes
D_{lipw}	Partition coefficient for liposome-water partition coefficient
D_{ow}	Partition coefficient for Octanol and Water corrected for pH
EC_{50}	Effective Concentration with 50 % mortality
f_{ion}	The fraction of the ionic species

$f_{neutral}$	The fraction of the neutral species
FPM	Fish Plasma Model
H	Hormones
H-3	Tritium isotope
HPLC	High Pressure Liquid Chromatograpy
HSS	High Strength Silica
i-Fit	Isotope fit
IT-MS	Ion Trap Mass Spectormetry
K_{int}	Uptake rate constant
K_{out}	Depuration rate constant
K_{ow}	Partition coefficient for Octanol and Water
LOD	Limit of Detection
LOQ	Limit of Quantification
LSC	Liquid Scintillation Counting
LR	Lipid Regulators
MCMC	Monte-Carlo Markov-Chain
MW	Molecular Weight
ND	Not Detected
NGR	National Grid Reference
NHS	National health Service
NICE	National Institute for Clinical Excellence
NO_2	Nitrogen Dioxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
PAH	Polycyclic Aromatic Hydrocarbons
PBT	Persistent Bioaccumulative and Toxic
PCB	Polychlorinated Biphenyls
PEC_{FW}	Predicted Environmental Concentration for Fresh Water
pK_a	Partition Coefficient
PSA	Polar Surface Area

PTFE	Polytetrafluoroethylene
QqQ-MS	Tandem Quadrupole Mass Spectrometry
QTof-MS	QuadrupoleTime of flight Mass Spectrometry
QTof-MS/MS	QuadrupoleTime of flight Tandem Mass Spectrometry
REACH	Registration, Evaluation and Authorisation of Chemicals
RPM	Revolutions Per Minute
RT	Retention Time
SA	Specific Activity
SNRI	Serotonin – Norepinephrine Reuptake Inhibitors
SO_2	Sulphur Dioxide
SSRI	Selective Serotonin Reuptake Inhibitors
STW	Sewage Treatment Works
Tof-MS	Time of flight Mass Spectrometry
UPLC	Ultra Performance Liquid Chromatography
UK	United Kingdom
USA	United States of America
V_D	Volume Distribution

Glossary

Absorption	the movement of a substance, such as a liquid or solute, across a cell membrane by means of diffusion or osmosis
Active transport	a mechanism generally for endogenous compounds such as nutrients that involves transport by a specific membrane carrier for which metabolic energy is required
Adipose tissue	tissue containing stored fat that serves as a source of energy
Benthic	relating to the benthic zone, which is the ecological region at the lowest level of a body of water such as an ocean or a lake, including the sediment surface and some sub-surface layers
Bioaccumulation	a process by which a substance is absorbed by an organism through all routes of exposure, i.e. through diet and ambient exposure
Bioconcentration	a process by which a substance is absorbed by an organism from the ambient environment only through its respiratory and dermal surfaces, i.e. diet is not included
Biomagnification	a process by which the thermodynamic activity of a substance in an organism exceeds that of its diet
Biotransformation	a series of chemical alterations of a substance within the body
Catabolism	destructive reactions, i.e. the breakdown of sugar to carbon dioxide and water
Co-variance	a measure of how two random variables change together
Electrospray	a type of ionisation that creates ions at atmospheric pressure

Elimination	a process of removal or of extrusion, especially of drug expulsion from the body
Enterohepatic circulation	refers to the circulation of bile from the liver
Epibenthic	the area of water overlying the sediment
Depuration	to eliminate substances or impurities from the body
Facilitated diffusion	a mechanism as it occurs across a concentration gradient for which no energy is required
Filtration	Small molecules passing through pores in membranes formed by protein molecules
Hepatopancreas	is an organ of the digestive tract of arthropods, mollucs and fish. It provides the functions which in mammals are provided separately by the liver and pancreas
Hydrophobic	tending to repel water
Hyperosmotic	pertaining to a solution that has a higher solute concentration than another solution
Ion	an atom or a group of atoms that has acquired a net electric charge by gaining or losing one or more electrons
Ionic	relating to or occurring in the form of ions
Ionised	a process where substance is converted totally or partly into ions
Ionisable	a substance that have a net electric charge by adding or removing one or more electrons
Isotope	atoms that contain the same number of protons but a different number of neutrons
Lentic	a body of still water, i.e. a pond or a lake
Lipophilic	having an affinity for lipids
Lotic	a body of moving water, i.e. a river or a stream

Metabolism	a process involving a set of chemical reactions that modifies a molecule into another for storage, or for immediate use in another reaction or as a by product
Microsome	vesicles in the endoplasmic reticulum
Neutral	relating to a substance that has neither positive nor negative electric charge
Oxidation	a process in which a portion of electronegative substituent is increased i.e., the increased portion of oxygen
Passive diffusion	a first order process that describes movement of lipophilic compounds across a membrane down a concentration gradient
Phagocytosis	invagination of the membrane to engulf particles, a mechanism where insoluble compounds can be absorbed
Pharmacodynamics	event consequent on interaction of the pharmaceutical with its receptor or site of action
Pharmacokinetic	the relationship between the time course of the pharmaceutical concentrations attained in different regions of an organism during and after dosing
Pharmacological	relating to the pharmacology of a compound
Pinocytosis	invagination of the membrane to engulf fluid together with its contents, a mechanism where insoluble compounds can be absorbed
Potable	safe to drink or drinkable
Predation	a biological interaction where a predator (an organism that is hunting) feeds on its prey (the organism that is attacked)

Quenching	a process that interferes with the transmission of light from a solution, i.e. colour, refraction of photons by chemical
Reduction	a process in which a portion of electronegative substituent is deceased i.e., the increased portion of hydrogen to decrease the partition of oxygen
Solvation	which there is some chemical association between the molecules of a solute and those of the solvent
Sorption	absorption and adsorption considered as a single process
Steric factors	relates to spatial arrangement of atoms in a molecule
Target site	a specific site of action for a substance, i.e. a receptor, ion channel or enzyme
Trophic transfer	the transfer of chemicals (xenobiotics or nutrients) from one organism to another through the biological levels of a food chain
Xenobiotic	a substance which is found in an organism or nature but which is not normally produced or expected to be present in it

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