The Fate and Effects of Human Pharmaceuticals in the Aquatic Environment

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Table of Contents

List of Figures

List of Tables

Abstract

There is relatively little known about the fate of human pharmaceuticals once they are released into the aquatic environment and what adverse impacts these compounds have on exposed aquatic organisms. Both of these factors are essential in defining the potential risk pharmaceuticals pose in the aquatic environment.

For this project up to 14 human therapeutic agents were selected as representative compounds to assess both their fate and effects within model aquatic systems. Considering sediments often serve as a repository for aquatic contaminants, the interaction of the selected pharmaceuticals with sediment was assessed. The sorption of the selected pharmaceuticals was found to be highly variable. Furthermore, the solution pH and ionic strength, due to Ca^{2+}, were found to exert a large degree of influence on the extent of sorption observed. These solution parameters, among others, may therefore make it difficult to predict the fate of pharmaceuticals, in terms of their association with sediments, using standardised assessment methods alone.

There is an extensive pool of knowledge on pharmaceuticals, in terms of their pharmacological profile, so their distribution within the human body (using the volume of distribution or V_D) was compared with their distribution within a sediment / water system (using the partition coefficient or K_d). The correlation between the V_D and K_d indicated this relationship provided a reasonable basis for estimating the distribution of drugs within the test sediment / water systems. This finding suggests that further exploration of the use of pharmacological data in understanding the potential fate of pharmaceuticals in aquatic systems is warranted.

The extent of the pharmaceuticals respective desorption values was also found to be highly variable within a standard test system. Further analysis on the desorption of carbamazepine, an anti-epileptic drug, was undertaken using an isotopic dilution technique. Observations from the isotopic dilution study indicated that both contact time with sediment and the quality of organic carbon could play an important role in the potential for sediments to irreversibly sorb carbamazepine present in aquatic systems. The desorption hysteresis observed for the other pharmaceuticals also indicates considerable effort is still required to address the issue of whether sediments can be a means of reducing exposure of pharmaceuticals to aquatic organisms (a "sink") or a means of increasing exposure to sediment-dependent organisms (a "source").

The necessity for further work on investigating the role that sorption with sediments may play in the fate and effects of human pharmaceuticals was highlighted by a series of ecotoxicological assays in both sediment and solution-only systems. Sediment-dwelling freshwater midges, *Chironomus tepperi*, were exposed to carbamazepine in both short- and long-term assays. Wet weight was found to be significantly reduced during short-term assays, while the development of *C. tepperi* larvae was found to be significantly inhibited when exposed to spiked sediment, over a longer exposure period. For these assays, the aqueous phase may have been a more important route of exposure of carbamazepine for the midges.

This study has indicated that sediments are likely to play an important role in the fate of pharmaceuticals and, subsequently, their effects. However, considerably more effort is required to assess the role sediments have and how this knowledge can be linked with current regulatory ecological risk assessments.

Abbreviations

ACR	acute:chronic ratio
ANOVA	analysis of variance
ASM	Australian statistics on medicines
ATC	anatomical therapeutic catagory
ATL	atenolol
ATP	adenosine triphosphate
BSA	bovine serum albumin
Ca	calcium
C_{aq}	aqueous concentration of drug
CAF	caffeine
CBZ	carbamazepine
CEC	cation exchange capacity
CH_3CN	acetonitrile
CHOOH	formic acid
CIM	cimetidine
C_s	solid phase concentration of drug
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CV	coefficient of variation
DCF	diclofenac
DDD	defined daily dose
DIL	diltiazem
DMI	desipramine
DPH	diphenhydramine
dpm	disintegrations per minute
E-value	isotopically exchangeable value
EC	electrical conductivity
EC50	concentration where 50% effect observed relative to control
EE2	17α-ethynylestradiol
EMEA	European medicines agency
ERA	ecological risk assessment
FDA	food and drug administration
FLX	fluoxetine
g	gravities
GC	gas chromatography
H	hysteresis index
HPLC	high pressure (or performance) liquid chromatography
I	ionic strength
IBU	ibuprofen
IMI	imipramine
K_d	partition coefficient
K_f	Freundlich coefficient
K_{OC}	partition coefficient normalised to organic carbon content of sediment
K_{OW}	octanol-water partition coefficient
LC50	concentration where 50% lethality observed relative to control
LOD	limit of detection
LOEC	lowest observable effect concentration
LOQ	limit of quantitation

LSC	liquid scintillation counting
MDL	method detection limit
MHW	moderately hard water
Milli-Q	ultrapure water (18.2 $M\Omega cm^{-1}$)
ML	minimum limit of detection
MS	mass spectrometry
NaCHOO	sodium formate
Na^+/K^+-ATPase	sodium/potassium adenosine triphosphatase
NMR	nuclear magnetic resonance
NSAID	non-steroidal antiinflammatory drug
OC	organic carbon
OECD	Organisation for Economic Cooperation and Development
OTC	over-the-counter
OUB	ouabain
PAC	paracetamol
PDA	poly diode array
PEC	predicted environmental concentration
P_i	inorganic phosphate
pK_a	acid dissociation constant
PRL	propranolol
PTFE	polytetrafluoroethylene
QA	quality assurance
QC	quality control
SEI	sucrose/ethylenediaminetetraacetic acid/imidazole
SPE	solid phase extraction
SSRI	selective serotonin re-uptake inhibitor
TGA	Therapeutic Goods Administration
UV	ultraviolet
V_D	volume of distribution
WHO	World Health Organisation
WWTP	wastewater treatment plant

Acknowledgements

Having come to the end of writing this thesis, I cannot remember a longer period of constant introspection, waging a meditative conflict with my egos and their various opinions of interpreting scientific information. I am not sure if my inner critic or narcissist would have emerged the victor but I have no doubt either of these scenarios would have eventuated if not for the guidance and support that I was able to rely on. So, to briefly turn my attention away from my angst-ridden musings, I would like to pay tribute to the following people who were an unfailing social web, who provided the necessary and highly appreciated guidance, advice, support and coping mechanisms, licit or otherwise.

However, I should start by acknowledging two institutions, CSIRO Land and Water and University of Adelaide Earth and Environmental Sciences, which provided me with the necessary infrastructure and support to undertake my experiments.

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and swoop in to save our experiments from imminent disaster, we could only be classified as merely cheap. Hai Doan, Colin Rivers, Sonia Grocke, Tasha Waller and Lester Smith embodied the labour and support that is necessary to keep a good scientific laboratory humming along. Their work played a critical role in supporting a number of experiments, especially when entering the murky waters of animal husbandry and toxicity assays; they are also a damn nice bunch of people! As for my fellow apprentices, I was infinitely fortunate to share offices and a laboratory with such a diverse, intelligent and funny group of people. To undertake a PhD project can be both highly enlightening and painful but having such a fantastic group of people to share the experience can make it both more enjoyable and less overwhelming. I would especially like to thank Dougal Currie, Therese McBeath, Mark Whatmuff, Dan Smith, Sam Stacey, Warwick Dougherty, Sean Mason and Laurence Jassogne who were often recipients, willing or otherwise, of my need for an outlet of humour, desperate last-minute requests for consumables from their likewise miniscule stockpiles and general banter that ranged from witty to delirious but always removed us from our immediate predicaments. They were, of course, very clever and committed people and I wish them all the best in their future, post-doctoral experiences.

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There were many other people who, no matter how small, played important roles throughout my project. These scientists were often involved in passing conversations with me at conferences or passing comments on my manuscripts or even hosting me in their laboratories for relatively fleeting moments. Perhaps these people will not read this thesis or even remember our encounters but I was always amazed at how warm and positive the majority of professional scientists have been towards scientists-in-training. It has made me feel very optimistic about my career choice and I look forward to reciprocating this role when I am given the opportunity.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in a university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Michael Williams _____

Publication arising from this thesis

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Chapter 1. General introduction

Human pharmaceuticals have been identified as an emerging class of contaminants of aquatic ecosystems within the last decade. Analytical methods for detecting pharmaceuticals at sub-part per billion levels in solution have become more sophisticated and affordable. These analytical methods have allowed numerous environmental surveys to detect an array of human pharmaceuticals in aquatic systems. However, approaches to understanding their fate once released into the aquatic environment or how their fate influences their potential for ecotoxicological impacts have not been well established. Defining the potential impact of pharmaceuticals at low levels in the aquatic environment represents an interesting challenge. The recent interest in human pharmaceuticals is indicative of a paradigm shift in environmental sciences towards defining the subtle effects in the functioning of aquatic organisms from low level, chronic exposure to biologically active agents (Daughton and Ternes 1999). Therefore, short-term toxicity assays with pharmaceuticals may not represent a suitable means of defining the ecological risk of these compounds. Long-term toxicity assays, where assessment of critical or even complete life-stages can lead to the elucidation of subtle yet significant effects, may be advantageous compared with short-term assays.

Draft guidelines for an ecological risk assessment (ERA) have been developed for new drug products by the European Medicines Agency (EMEA) (EMEA-CPMP 2005), while the Food and Drug Administration (FDA) in the USA has also released its own guidelines (FDA-CDER 1998). In both cases, the ERAs represent an assessment of the likely exposure concentrations to aquatic organisms and the

potential impact from these exposure levels. The FDA guidelines have cut-off limits based on a number of aquatic ecotoxicity studies, while the most current EMEA guidelines recommend assessing the factors that affect the fate of pharmaceuticals, such as sorption, in the risk assessment process. However, there is currently little data available, in terms of the fate and effects of pharmaceuticals in aquatic systems, to address even the ERA for drugs that are currently on the market, let alone for new drug products.

The objective of this thesis was to assess a number of factors that can influence the environmental fate and effects for a number of human pharmaceuticals, in an attempt to define areas of the ERA that might need further consideration. The central theme of this work has been the role that sediments can play in the fate and, therefore, effects of pharmaceuticals in aquatic systems. The chapters in this thesis have therefore been organised around the following themes:

- To give an indication of the present state of research on human pharmaceuticals in the aquatic environment, with respect to their fate and effects, through a review of available literature (Chapter 2).
- To select a number of human pharmaceuticals that were deemed to be a representative cross-section of drugs which are available for therapeutic use. Also, the development of an appropriate analytical method for the selected drugs is presented (Chapter 3).
- To assess the potential role that sediment can play in the fate of human pharmaceuticals in the aquatic environment, by conducting batch sorption / desorption experiments (Chapter 4).

- To determine the role that the pH and concentration of Ca^{2+} ions in solution can have on the extent of sorption of the selected pharmaceuticals (Chapter 5)
- To develop an approach based on the known extent of distribution of pharmaceuticals in humans to give an indication of the extent of distribution of pharmaceuticals within an aquatic system containing sediment. (Chapter 6).
- The use of an isotopic technique as an alternative approach to determining the extent of desorption of an anti-epileptic drug, carbamazepine, and comparing the results with those found in Chapter 4 (Chapter 7).
- To expose a sediment-dwelling freshwater midge, *Chironomus tepperi*, to carbamazepine in a system containing both solution-only and solution and sediment. Both the short-term and long-term toxicity of carbamazepine were determined for a number of effects, including an enzyme that was thought to have been specifically targeted based on the pharmacological properties of carbamazepine in humans (Chapter 8)
- To incorporate the findings of this study into recommendations that can be used to further develop the current ERA guidelines (Chapter 9).

Chapter 2. Literature Review

2.1 Pharmaceuticals in the aquatic environment

Pharmaceuticals as therapeutic agents have become an invaluable tool in the treatment and prevention of disease in animals. This not only includes humans but also animals which humans have become reliant on, such as farmed stock. Similarly, human societies depend on their surrounding ecosystems to provide services that are essential for their existence. These can include removal of waste, food production and supply of potable water, which a functioning ecosystem of adequate diversity can accommodate even with the input of anthropogenic stressors (Rapport *et al.* 1998). When such stressors have the potential to affect this diversity, then they become a matter of concern.

Pharmaceuticals have been detected in environmental water samples in studies up to 30 years ago, including the antidepressant imipramine (Jungclaus *et al.* 1978), the lipid lowering clofibric acid (Hignite and Azarnoff 1977) and a number of other classes of pharmaceuticals (Richardson and Bowron 1985). However, post-therapeutic fate and effects of pharmaceuticals have only recently become an issue of widespread environmental interest within the last decade. This has become increasingly apparent since more powerful analytical techniques have allowed their reliable detection and quantification (Barcelo 2003). Concerns relating to human and ecological health from their environmental contamination have followed (Daughton and Ternes 1999; Schiermeier 2003), particularly when the use of the anti-inflammatory diclofenac was found to have catastrophic consequences on the population of vultures in India and

Pakistan (Oaks *et al.* 2004). Despite the increasing awareness of the presence of pharmaceuticals, little is still known about their behaviour, in terms of their fate and effects, after their entry into the environment (Daughton and Ternes 1999; Schiermeier 2003).

2.1.1 Entry into the aquatic environment

Although many pharmaceuticals, such as antimicrobial agents, are used in both veterinary and human medicine, the routes into the environment are quite different (Halling-Sorensen *et al.* 1998; Heberer 2002). Veterinary medicines can contaminate terrestrial systems through livestock urine and manure, where leaching can lead to aquatic contamination (Jorgensen *et al.* 1998; Tolls 2001; Koschorreck *et al.* 2002). Direct contamination of the aquatic environment, through application in aquaculture, can lead to the exposure of aquatic and sediment dwelling organisms (Jacobsen and Berglind 1988).

By comparison, post-therapeutic entry into the environment of human medicines occurs predominantly via wastewater treatment plants (WWTPs) leading to contamination of aquatic systems, although biosolids and irrigation using recycled water is another potential pathway (Figure 2.1). As with veterinary medicines entering the terrestrial environment, sludge and treated water from WWTPs can also be applied to the land, as a means of irrigation or disposal. For this project, however, assessment of environmental behaviour was limited to human medicines in the aquatic environment for the purposes of consistency and simplification.

The majority of this review was undertaken prior to commencing this project, in 2003, in order to develop research questions. More recent references were included where it was considered appropriate, such as the updated EMEA guidelines. Also, more recent environmental surveys and toxicological references for human pharmaceuticals were included, as there were relatively few studies prior to the commencement of this project. Other literature from research after 2003 was included in subsequent chapters.

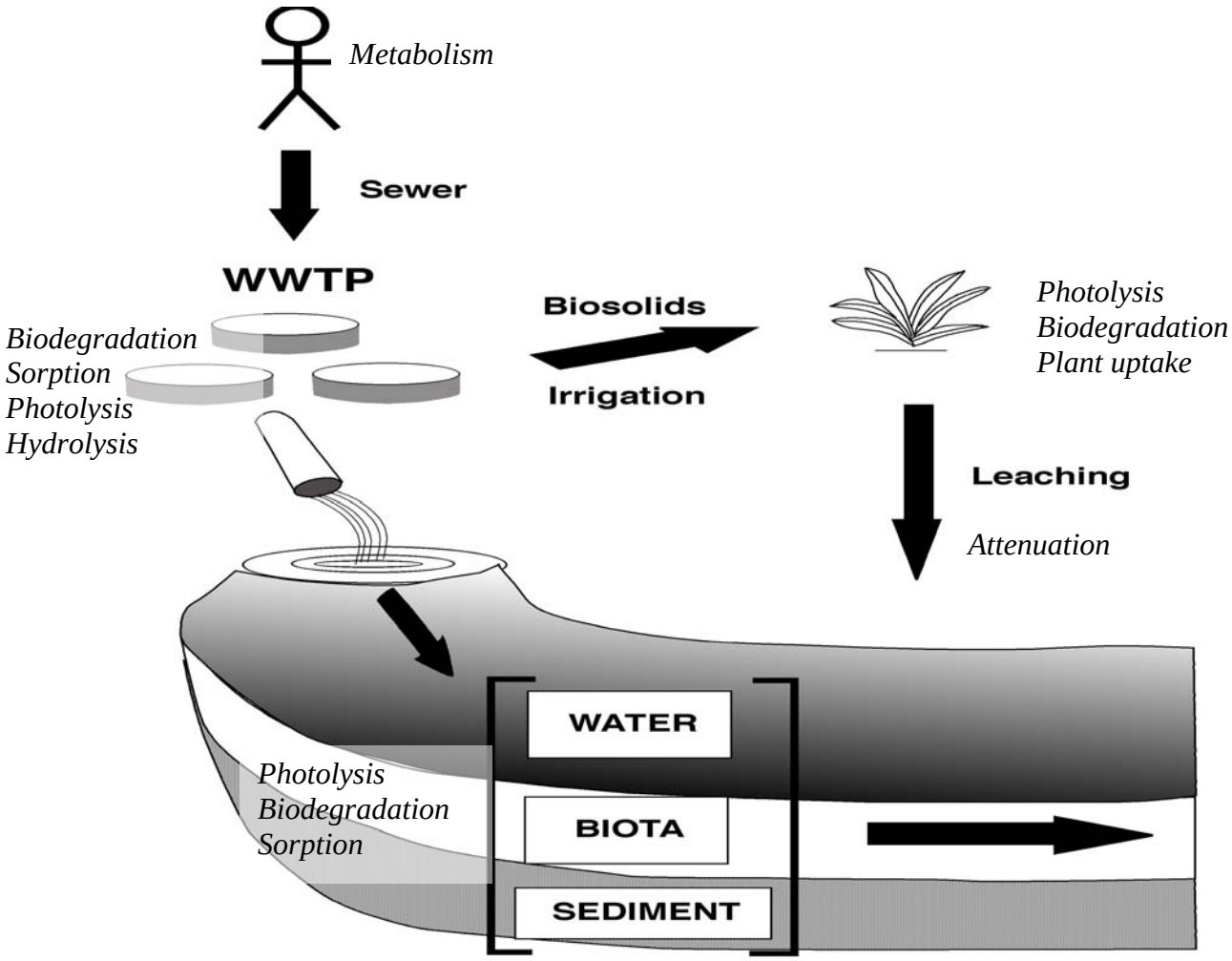


Figure 2.1: Conceptual overview of the entry of human pharmaceuticals into the aquatic environment showing partitioning into different compartments within an aquatic system. Italics indicate likely processes that can remove pharmaceuticals from system.

2.1.2 Use and classification of human medicines

Due to relatively few environmental surveys of pharmaceuticals being undertaken, therapeutic use statistics of medicines has been a useful tool in estimating the amount of pharmaceuticals that can enter the aquatic environment.

The use of medicines in Australia can be estimated using Australian Statistics on Medicines (ASM) data, published by the Drug Utilisation Sub-Committee of the Commonwealth Department of Health and Ageing (DUSC 2003). Data on prescriptions is collated yearly although medicines available without a prescription (over-the-counter or OTC) or prescribed in public hospitals are not considered in this data. The values obtained from these surveys should thus be taken as an indication of the patterns of medicine use in Australia and not as absolute values. The World Health Organisation (WHO) Collaborating Centre for Drug Statistics Methodology has developed the use of the defined daily dose (DDD) for estimating population drug use. The DDD value for a drug is the average amount of active drug that is used as a daily therapeutic dose for its major therapeutic use and thus gives an indication of the potency of the drug. Drugs are categorised by the Anatomical Therapeutic Chemical (ATC) grouping, which defines the major target organ, while sub-categories include more specific modes of action. The top 50 drugs summarised as the mass of pharmaceuticals prescribed in Australia in 2000, based on the number of prescriptions and DDD of each drug, are found in Table 2.1.

Estimating the amount of pharmaceuticals that enter the aquatic environment has been undertaken in a number of other countries, such as England (Jones *et al.* 2002), Italy

(Zuccato *et al.* 2005) and previously in Australia (Khan 2002). In the study by Zuccato *et al.* (2005) the amount reaching the environment was adjusted based on the extent of metabolism that the drugs are respectively expected to undergo in humans, prior to release. The number of prescriptions in the USA has been collated on the internet database, RxList (www.rxlist.com) and indicates the most commonly prescribed drugs on a yearly basis. An alternative approach by Stuer-Lauridsen *et al.* (2000) ranked drugs based on the number of DDDs used within the population in 1997.

Ranking drugs by mass has the advantage of estimating which drugs are likely to be detected in the aquatic system, due to the volume of use. Adjusting these estimates of mass in terms of the extent of drug metabolism during therapeutic use is likely to provide a more realistic estimate of drugs likely to be detected (Zuccato *et al.* 2005) Ranking drugs by the number of DDDs used within a population, as done by Stuer-Lauridsen *et al.* (2000), gives an indication of prescription numbers, as does using the ranking method used with RxList. However, if the DDD values are known, DDDs per population can be converted into mass of active drug prescribed per population. Furthermore, the DDD can be a useful indicator of the potency of the drug within humans and could give an indication of the potential for ecotoxicity of a drug. For example, the DDD of the synthetic estrogen, 17α-ethynylestradiol, is from low- to sub-mg levels per day (dependent on use) while the DDD of the non-steroidal antiinflammatory drug (NSAID) ibuprofen is 1200 mg per day (DUSC 2003). Ecotoxicity assays of 17α-ethynylestradiol show effects can occur in fish in low- to sub-ngL^{-1} aqueous concentrations (Larsson *et al.* 1999), while typical concentrations

of ibuprofen that cause toxicological responses are in above mgL^{-1} concentrations in a number of organisms (La Farre *et al.* 2001; Cleuvers 2003; Pascoe *et al.* 2003).

A comparison of the ranking systems in Australia, Denmark, England, Italy and the USA indicate that the ATC of drugs used and even the specific parent compounds are comparable between geographic locations (Table 2.2). The major difference between the ranking systems where prescription numbers were considered (Denmark and the USA) was that steroidal hormones, used for contraception, were included, whereas this class of drugs was not found in the ranking by mass (Table 2.2). Closer analysis indicates the DDDs of steroidal estrogens makes their annual prescription mass relatively insignificant, which is further suggested in various environmental surveys (Ternes *et al.* 1999; Baronti *et al.* 2000; Johnson *et al.* 2000).This could also be the case with drugs used to treat nervous disorders, such as depression and anxiety. For example, the DDD for the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine is 20 mg (DUSC 2003). Ecotoxicological assessments of fluoxetine have suggested low levels of fluoxetine in water and sediment can cause measurable effects (Fong *et al.* 1998; Brooks *et al.* 2003b), relative to other pharmaceuticals.

Table 2.1: Top 50 human medicines used in Australia in 2000 ranked according to mass prescribed

Drug	*ATC [b] group*	Therapeutic class	DDD [c] (mg)	Mass prescribed (kg/population) [d]
Paracetamol [a]	Nervous system	Analgesic	3000	232 773 [e]
Metformin HCl	Alimentary/metabolism	Antidiabetic	2000	127 873
Lactulose	Alimentary/metabolism	Laxative	6700	118 535
Amoxycillin	Antiinfective	Antibiotic	1000	52 244
Ranitidine HCl [a]	Alimentary/metabolism	Antacid	300	38 337
Valproate Na	Nervous system	Antiepileptic	1500	28 215
Cephalexin	Antiinfective	Antibiotic	2000	26 351
Naproxen [a]	Musculoskeletal	NSAID	500	25 514
Celecoxib	Musculoskeletal	NSAID	200	23 117
Allopurinol	Musculoskeletal	Antigout	400	20 251
Irbesartan	Cardiovascular system	Antihypertensive	150	18 845
Gemfibrozil	Cardiovascular system	Antihyperlipidemic	1200	17 178
Ibuprofen [a]	Musculoskeletal	NSAID	1200	15 768
Carbamazepine	Nervous system	Antiepileptic	1000	15 342
Aspirin [a]	Nervous system	NSAID	3000	14 891
Verapamil HCl	Cardiovascular system	Antihypertensive	240	12 134
Diltiazem HCl	Cardiovascular system	Antihypertensive	240	9689
Gliclazide	Alimentary/metabolism	Antidiabetic	160	9550
Erythromycin	Antiinfective	Antibiotic	1000	8406
Moclobemide	Nervous system	Antidepressant	300	8142
Chlorothiazide	Cardiovascular system	Diuretic	500	7738
Cefaclor	Antiinfective	Antibiotic	1000	7189
Metoprolol	Cardiovascular system	Antihypertensive	150	6354
Frusemide	Cardiovascular system	Diuretic	40	6204
Atenolol	Cardiovascular system	Antihypertensive	80	6161
Sertraline	Nervous system	Antidepressant	50	4734
Diclofenac [a]	Musculoskeletal	NSAID	100	4699
Codeine [a]	Nervous system	Analgesic	100	4671
Phenytoin	Nervous system	Antiepileptic	300	4432
Nizatidine [a]	Alimentary/metabolism	Antacid	300	4394
Theophylline	Respiratory system	Antiasthmatic	400	4133
Hydrochlorothiazide	Cardiovascular system	Diuretic	25	3897
Roxithromycin	Antiinfective	Antibiotic	300	3900
Ketoprofen [a]	Musculoskeletal	NSAID	150	3643
Venlafaxine	Nervous system	Antidepressant	100	3285
Simvastatin	Cardiovascular system	Antihyperlipidemic	15	3136
Isosorbide	Cardiovascular system	Vasodilator	40	3074
Atorvastatin	Cardiovascular system	Antihyperlipidemic	10	2760
Sotalol HCl	Cardiovascular system	Antihypertensive	160	2510
Doxycycline	Antiinfective	Antibiotic	100	2195
Captopril	Cardiovascular system	Antihypertensive	50	2191
Dothiepin HCl	Nervous system	Antidepressant	150	2137
Propranolol HCl	Cardiovascular system	Antihypertensive	160	2079
Amiodarone HCl	Cardiovascular system	Antiarrythmic	200	2077
Omeprazole [a]	Alimentary/metabolism	Antacid	20	2032
Amitriptyline HCl	Nervous system	Antidepressant	75	1576
Oxazepam	Nervous system	Anxiolytic	50	1239
Morphine	Nervous system	Analgesic	100	1205
Indomethacin	Musculoskeletal	NSAID	100	1159

[a] Also available without prescription including combinations
[b] Anatomical Therapeutic Chemical (DUSC 2003)
[c] Defined Daily Dose based on (DUSC 2003)
[d] Population of Australia in June, 2000 was 19 272 600
[e] Mass prescribed based on DDD per population prescribed

Common therapeutic targets in each country included treatments for pain management, cardiovascular disease, antibiotics and nervous system disorders (Table 2.2). Antibiotics, especially the penicillin-based amoxycillin, featured in all countries, except in Denmark. More than one class of antibiotic was used in each of the other countries. Drugs which are used to treat pain, such as the analgesics and NSAIDs, are also found on all the lists. A feature of a number of these drugs used for pain management is that they are available over the counter. The reliance on ranking systems based on prescription data may, therefore, underestimate the volume of such drugs entering the aquatic environment. Also, the drugs listed in Table 2.2 only represent a fraction of pharmaceuticals that are available for therapeutic use. Drugs that are not commonly used but have a high therapeutic potency, such as cytotoxic agents used to treat cancer, may not be flagged as a drug of interest in the aquatic environment.

However, the use of prescription data is an important indicator of drugs that may be present in the aquatic environment. Used alongside discretionary factors, such as the use of DDDs to estimate relative levels of drugs in the aquatic environment that have the potential to cause harm, prescription data is an important initial approach to determining the ecotoxicological risk of drugs. This data can be input into a structured approach to defining the risk of pharmaceuticals, based on the level of exposure to aquatic organisms and putting these levels of exposure in the context of toxicological assays. One such structured approach is an ecological risk assessment.

Table 2.2: The ranking of pharmaceuticals based on prescription data in a number of different countries. Anatomical and therapeutic categories are listed in italics

Rank	Australia (2000) [a]	Denmark (1997) [b]	England (2000) [a]	Italy (2001) [c]	USA (2000) [d]
1	Paracetamol *Nervous system/Analgesic*	Frusemide *Cardiovascular/Diuretic*	Paracetamol *Nervous system/Analgesic*	Amoxycillin *Antiinfective/Antibacterial*	Hydrocodone/Paracetamol *Nervous system/Analgesic*
2	Metformin HCl *Alimentary/Antidiabetic*	Paracetamol *Nervous system/Analgesic*	Metformin HCl *Alimentary/Antidiabetic*	Atenolol *Cardiovascular/β-blocker*	Atorvastatin *Cardiovascular/Antihyperlipidemic*
3	Lactulose *Alimentary/Laxative*	Aspirin *Blood/Platelet binding inhibitor*	Ibuprofen *Musculoskeletal/NSAID* [e]	Hydrochlorothiazide *Cardiovascular/Diuretic*	Conjugated estrogens *Genitourinary/Estrogen*
4	Amoxycillin *Antiinfective/Antibacterial*	Bendrofluazide *Cardiovascular/Diuretic*	Amoxycillin *Antiinfective/Antibacterial*	Ranitidine *Alimentary/Antacid*	Levothyroxine *Hormonal/Thyroid hormone*
5	Ranitidine HCl *Alimentary/Antacid*	Gestodene/estrogen *Genitourinary/Estrogen*	Valproate Na *Nervous system/Antiepileptic*	Clarithromycin *Antiinfective/Antibacterial*	Atenolol *Cardiovascular/β-blocker*
6	Valproate Na *Nervous system/Antiepileptic*	Aspirin (combined) *Nervous system/Analgesic*	Sulphasalazine *Alimentary/Antiinflammatory*	Ceftriaxone *Antiinfective/Antibacterial*	Frusemide *Cardiovascular/Diuretic*
7	Cephalexin *Antiinfective/Antibacterial*	Ibuprofen *Musculoskeletal/NSAID* [e]	Mesalazine *Alimentary/Antiinflammatory*	Frusemide *Cardiovascular/Diuretic*	Omeprazole *Alimentary/Antacid*
8	Naproxen *Musculoskeletal/NSAID* [e]	Lactic acid organisms *Alimentary/Antidiarrheal*	Carbamazepine *Nervous system/Antiepileptic*	Bezafibrate *Cardiovascular/Antihyperlipidemic*	Salbutamol *Respiratory/Adrenergic*
9	Celecoxib *Musculoskeletal/NSAID* [e]	Potassium chloride *Alimentary/Mineral supplement*	Ferrous sulphate *Blood/Antianaemic*	Ciprofloxacin *Antiinfective/Antibacterial*	Amlodipine *Cardiovascular/Ca^{2+}blocker*
10	Allopurinol *Musculoskeletal/Antigout*	Amlodipine *Cardiovascular/Ca^{2+}blocker*	Ranitidine HCl *Alimentary/Antacid*	Enalapril *Cardiovascular/ACE* [f] *inhibitor*	Alprazolam *Nervous system/Anxiolytic*
11	Irbesartan *Cardiovascular/ACE* [f] *inhibitor*	Budesonide *Hormonal/Corticosteroid*	Cimetidine *Alimentary/Antacid*	Spiramycin *Antiinfective/Antibacterial*	Propoxyphene *Nervous system/Analgesic*
12	Gemfibrozil *Cardiovascular/Antihyperlipidemic*	Terbutaline *Respiratory/Adrenergic*	Naproxen *Musculoskeletal/NSAID* [e]	Omeprazole *Alimentary/Antacid*	Metformin HCl *Alimentary/Antidiabetic*
13	Ibuprofen *Musculoskeletal/NSAID* [e]	Estradiol *Genitourinary/Estrogen*	Atenolol *Cardiovascular/β-blocker*	Erythromycin *Antiinfective/Antibacterial*	Cephalexin *Antiinfective/Antibacterial*
14	Carbamazepine *Nervous system/Antiepileptic*	Nitrazepam *Nervous system/Sedative*	Oxytetracycline *Antiinfective/Antibacterial*	Ibuprofen *Musculoskeletal/NSAID* [e]	Amoxycillin *Antiinfective/Antibacterial*

15	Aspirin *Various*	Desogestrel/estrogen *Genitourinary/Estrogen*	Erythromycin *Antiinfective/Antibacterial*	-	Loratidine *Respiratory/Antihistamine*
16	Verapamil HCl *Cardiovascular/Ca^{2+}blocker*	Enalapril *Cardiovascular/ACE [f] inhibitor*	Diclofenac Na *Musculoskeletal/NSAID [e]*	-	Hydrochlorothiazide *Cardiovascular/Diuretic*
17	Diltiazem HCl *Cardiovascular/Ca^{2+}blocker*	Diazepam *Nervous system/Anxiolytic*	Flucloxacillin *Antiinfective/Antibacterial*	-	Sertraline *Nervous system/Antidepressant*
18	Gliclazide *Alimentary/Antidiabetic*	Zopiclone *Nervous system/Sedative*	Phenoxymethylpenicillin *Antiinfective/Antibacterial*	-	Azithromycin *Antiinfective/Antibacterial*
19	Erythromycin *Antiinfective/Antibacterial*	Citalopram *Nervous system/Antidepressant*	Allopurinol *Musculoskeletal/Antigout*	-	Fluoxetine *Nervous system/Antidepressant*
20	Moclobemide *Nervous system/Antidepressant*	Salbutamol *Respiratory/Adrenergic*	Diltiazem HCl *Cardiovascular/Ca^{2+} blocker*	-	Ibuprofen *Musculoskeletal/NSAID [e]*
21	Chlorothiazide *Cardiovascular/Diuretic*	Xylometazolin *Respiratory/Decongestant*	Gliclazide *Alimentary/Antidiabetic*	-	Paroxetine *Nervous system/Antidepressant*
22	Cefaclor *Antiinfective/Antibacterial*	Digoxin *Cardiovascular/Cardiac glycoside*	Aspirin *Various*	-	Triamterene *Cardiovascular/Diuretic*
23	Metoprolol *Cardiovascular/β-blocker*	Hydrochlorothiazide *Cardiovascular/Diuretic*	Quinine sulphate *Antiparasitic/Antimalarial*	-	Celecoxib *Musculoskeletal/NSAID [e]*
24	Frusemide *Cardiovascular/Diuretic*	Hydrogen peroxide *Various*	Mebeverine HCl *Alimentary/ Antiinflammatory*	-	Paracetamol/Codeine *Nervous system/Analgesic*
25	Atenolol *Cardiovascular/β-blocker*	Ketoconazole *Antiinfective/Antimycotic*	Mefenamic acid *Musculoskeletal/NSAID [e]*	-	Lansoprazole *Alimentary/Antacid*

[a] Ranked by the mass of pharmaceuticals prescribed in Australia or England in 2000 (Jones *et al.* 2002; DUSC 2003)
[b] Ranked by number of defined daily doses (DDDs) prescribed within the population of Denmark for 1997 (Stuer-Lauridsen *et al.* 2000)
[c] Ranked by the mass of pharmaceuticals prescribed in Italy in 2001, adjusted for metabolism in humans (Zuccato *et al.* 2005)
[d] Ranked by the number of prescriptions in the USA in 2000 (www.rxlist.com/top200.htm)
[e] NSAID = non-steroidal antiinflammatory drug
[f] ACE = angiotensin-converting enzyme

2.2 Strategies for the risk assessment of human pharmaceuticals in the aquatic environment

2.2.1 Ecological risk assessment

When a contaminant of concern enters the environment, the potential for it to cause harm is determined through an ecological risk assessment. Risk assessment is based on the hazard, or potential of the contaminant to do harm, and the extent of exposure an organism has to the contaminant (Eason and O'Halloran 2002). The risk posed by the chemical is, therefore, the probability of harm occurring. Risk assessment is an iterative process, where an initial screening process is followed by more rigorous levels, or tiers, of assessment. The screening phase is usually highly conservative and a contaminant failing at this level indicates that there is enough uncertainty associated with its presence in the environment to warrant further investigation. Higher tiers of assessment use a number of parameters to reduce the uncertainty of risk to a level deemed acceptable to make a regulatory decision (Hill *et al.* 2000). Where there is little information available to reduce uncertainty to appropriate levels it has been proposed that the precautionary principle be used as a stop-gap measure (Santillo *et al.* 1998). For example, reduction in the use of a chemical of concern with unknown hazard or exposure levels would be an effective precautionary approach.

However, it has been argued that there is little scientific basis to the precautionary approach and, therefore, it does not support an evidence-based argument for regulatory decisions (Chapman 1999). As a compromise, Goldstein (1999) suggested

a combination of a precautionary approach and ecological risk assessment to mitigate potential harm caused by contaminants.

Daughton and Ternes (1999) have suggested a holistic stewardship role as a preferable approach, compared with regulatory compliance. Substitution with alternative therapies may be a viable option that benefits both the therapeutic requirements and environmental protection of human pharmaceuticals. For example, the anticancer agents ifosphamide and cyclophosphamide have been found to be resistant to biodegradation, which has implications for the environmental presence of these cytotoxic agents (Kummerer *et al.* 2000). The structurally related β-L-glucosylisophosphoramidmustard, on the other hand, is a therapeutically effective anticancer agent while being found to be inherently biodegradable, potentially reducing its environmental presence (Kummerer *et al.* 2000). Until efficient removal technologies are developed for WWTPs the use of such alternative therapies may be one of the few mitigating factors that are desirable.

Risk assessment of pharmaceuticals has been undertaken in terms of the potential for human health risk from exposure to pharmaceuticals in the environment. These studies all indicate that the human health risk in this instance is likely to be negligible (Christensen 1998; Schulman *et al.* 2002; Jones *et al.* 2004; Schwab *et al.* 2005). In terms of the potential risk associated with exposure of organisms to human pharmaceuticals, the level of uncertainty is much greater (Daughton and Ternes 1999). This uncertainty relates to the difference in exposure patterns between humans and, in particular, aquatic organisms. The release of human pharmaceuticals from WWTPs has been found to occur constantly over time (Heberer *et al.* 2002; Buerge *et al.* 2003) suggesting that aquatic organisms will also be constantly exposed over their

life-cycle (Halling-Sorensen *et al.* 1998; Daughton and Ternes 1999). Exposure to pharmaceuticals during critical developmental periods of an aquatic organism means there is less certainty relating to the potential effects at the single organism through to those at the population level.

The Food and Drug Administration (FDA) in the USA introduced a guidance document (FDA-CDER 1998), while the European Medicines Agency (EMEA) released a discussion paper (EMEA-CPMP 2001) for the ecological risk assessment (ERA) of new drug applications. These papers recommended that if the predicted or determined environmental concentration was below a threshold level, then a higher tier of assessment could be ignored. The threshold level recommended by the FDA paper is 1 μgL^{-1}, while the more conservative EMEA threshold level was 10 ngL^{-1}. Subsequent assessments required by the FDA, if the threshold value is breached, are reasonably detailed, with 3 tiers of assessment involving increasingly complex toxicity and physicochemical properties assessments. If critical values are exceeded at the final tier of assessment, then a case-by-case consultation with the FDA is required.

In contrast, the EMEA approach, while more conservative, only consisted of a screening level assessment, where crude predictions of environmental concentrations and effects were required. If the hazard quotient (the predicted environmental concentration over the predicted no-effect concentration) was greater than one, then consultation with the EMEA was required (Straub 2002). The FDA guidelines were adopted in 1998, while the EMEA guidelines were updated in 2003 with a more detailed discussion paper, including many of the proposals found in the FDA guidance document. These recommendations, including an assessment of the fate of human pharmaceuticals based on a number of standardised assessments of physicochemical

and toxicological assays, adopted a number of the recommendations included in the FDA guidance document (EMEA-CPMP 2003). These were included in a later draft guideline document (EMEA-CPMP 2005). Some of the more significant inclusions were the consideration of chronic toxicity testing and the effect of sediment interaction on the fate and effects of a drug (Laenge *et al.* 2006). Measured effects in chronic toxicity testing are different from those determined in short-term, or acute, toxicity testing. For example, effects such as reproductive or developmental success, compared with mortality, are likely to have greater relevance for human pharmaceuticals, considering their pattern of release into the aquatic environment (Lange and Dietrich 2002; Bound and Voulvoulis 2004). An overview of the risk assessment procedures are given for both the FDA and EMEA guidelines in Figure 2.2.

Two studies have used a risk assessment approach, based on the EMEA guidelines to determine the potential for ecotoxicological impacts of human pharmaceuticals. The potential risk of the top 25 pharmaceuticals by prescription mass was undertaken in the UK by Jones *et al.* (2002), while the potential risk of the top 20 drugs by population use was estimated in Denmark by Stuer-Lauridsen *et al.* (2000). Only 3 drugs, paracetamol, aspirin and ibuprofen, were common to both studies, which could reflect either the geographical specificity of risk assessments or the inherent differences in ranking systems used. A screening level assessment of the hazard quotient for paracetamol and ibuprofen showed that a higher tier assessment would be necessary for these compounds, while the Danish study indicated further assessment of aspirin would be necessary. Furthermore, only paracetamol and ibuprofen would require further assessment following FDA guidelines, as their predicted environmental concentrations (PECs) were above 1 μgL^{-1}. The Danish study found

that aspirin would also require further assessment following FDA guidelines. However, all 25 drugs in the UK study and 16 of the 20 drugs in the Danish study needed to be assessed in terms of their effects, since their PECs were greater than 10 ngL^{-1}.

In the UK study, the antibiotics amoxicillin and oxytetracycline and the NSAID mefenamic acid would also require further assessment, based on their respective hazard quotients (Jones *et al.* 2002). Based on FDA guidelines, however, only amoxycillin had a PEC that warranted further investigation. A study by Sanderson *et al.* (2003) also found that out of 72 drugs assessed, all 72 required further assessment using EMEA guidelines, while only 26 would require further assessment following FDA guidelines.

One limitation of these risk assessments, and others, was the lack of information available, particularly with respect to ecotoxicity data. When no environmental concentration data was available, estimates were made based upon models such as PhATE (Cunningham *et al.* 2004) and GREAT-ER (Schowanek and Webb 2002), or estimated using the FDA or EMEA guidelines (Stuer-Lauridsen *et al.* 2000; Jones *et al.* 2002; Brooks *et al.* 2003a). Where no toxicological data was available risk assessments relied on either toxicological models, such as ECOSAR (Jones *et al.* 2002; Sanderson *et al.* 2003), or acute toxicity data (Stuer-Lauridsen *et al.* 2000; Jones *et al.* 2002; Cunningham *et al.* 2004; Cleuvers 2005; Hernando *et al.* 2006).

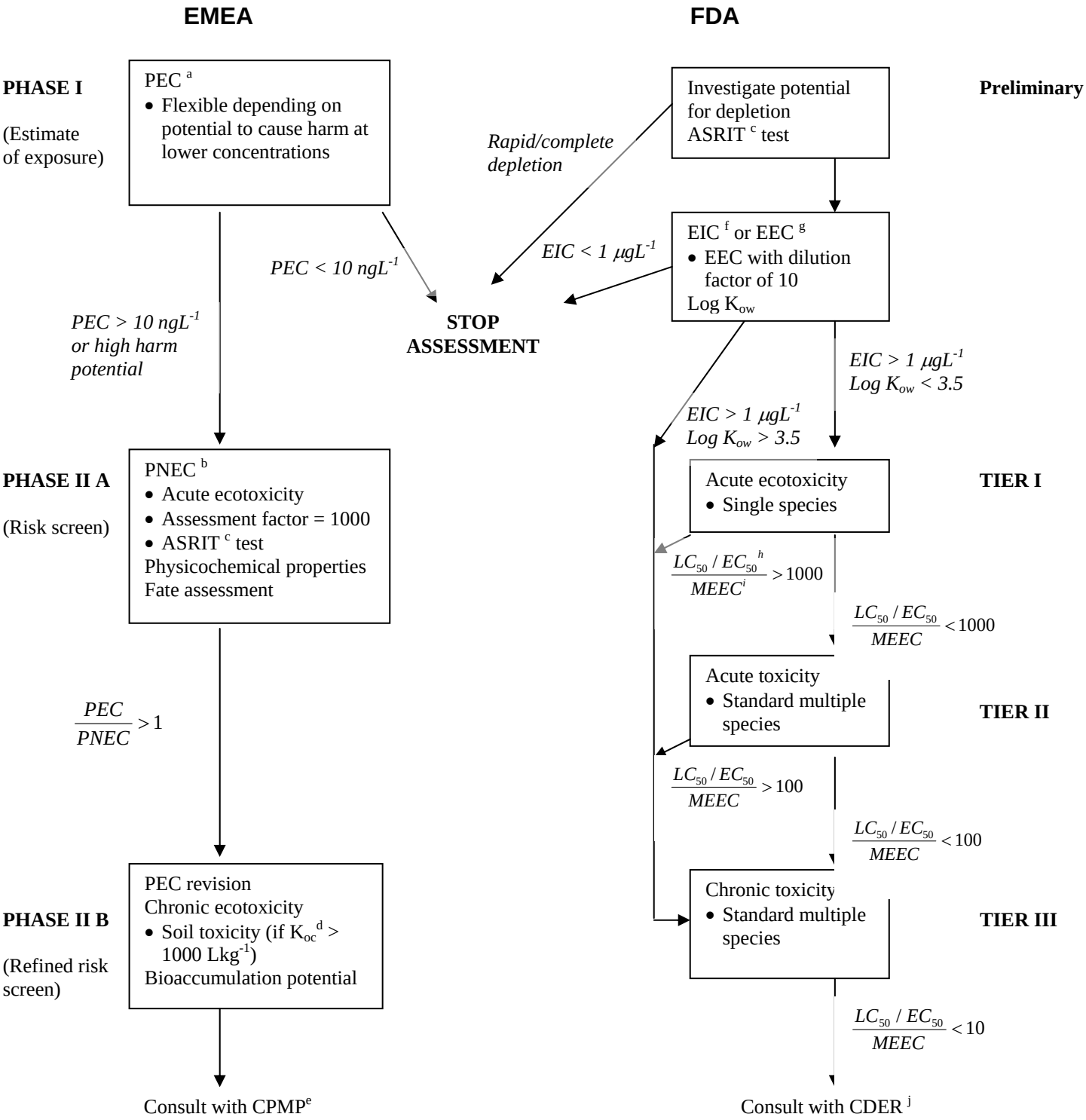


Figure 2.2: Sc ss for risk assessment of a new drug for the European Medicines Agency (EMEA) and Food and Drug Administration (FDA) (FDA-CDER 1998; Bound and Voulvoulis 2004; EMEA-CPMP 2005).

[a] Predicted Environmental Concentration
[b] Predicted No-Effect Concentration
[c] Activated Sludge Respiration Inhibition Test
[d] octanol/water partition coefficient
[e] Committee for Proprietry Medicinal Products
[f] Expected Introductory Concentration
[g] Expected Environmental Concentration
[h] Concentration where 50% of organisms show lethality/effect
[i] Maximum Expected Environmental Concentration
[j] Centre for Drug Evaluation and Research

While assessment factors are included when acute toxicity data is used, the use of acute toxicity testing may be problematic. For example, a more recent risk assessment of 6 pharmaceuticals in both Germany and France demonstrated that where acute toxicity testing was used no further assessment was required for 5 of the 6 drugs (Ferrari *et al.* 2004). However, when chronic toxicity assays were undertaken, further assessment was required for the anti-epileptic carbamazepine, the β-blocker propranolol and the antibiotic sulfamethoxazole. This conclusion was supported by a previous risk assessment of carbamazepine by Ferrari *et al.* (2003) using chronic toxicity data.

An acute : chronic ratio (ACR) is often used to account for the likelihood of effects occurring at lower concentrations following long-term exposure. For the risk assessment process, an assessment factor of 1000 is used as a conservative estimate that effects occur at levels 1000 times less after long-term exposure, compared with short-term exposures. However, the ACR is often highly variable, ranging from 6 for salicylic acid in *Daphnia magna* to 800 000 for 17α-ethynylestradiol in *Oncorhynchus mykiss* (Webb 2004). While the screening stage of a risk assessment is inherently conservative, the variable nature of the ACR may demonstrate the need for the appropriate use of input data (such as chronic toxicity assessment for human pharmaceuticals) for scientifically sound conclusions.

It is apparent that the basic requirements for an ecological risk assessment of human pharmaceuticals are an estimate of the exposure concentrations likely to occur in the aquatic environment and the implications these exposure concentrations may have for

organisms present within that system. The current guidelines of the EMEA and FDA are only applicable for new drug applications. However, most of the risk assessment studies undertaken have sought to validate these guidelines in terms of the potential risk posed by pharmaceuticals that are already approved for therapeutic use. The screening phase of these risk assessment studies can lead to variable conclusions. For example, the anti-epileptic agent carbamazepine had a hazard quotient of 47 in a study by Ferrari *et al.* (2003), while Jones *et al.* (2002) found a hazard quotient of 0.19.

More recent assessments of carbamazepine have also found a hazard quotient ranging from 0.0007 (Han *et al.* 2006) to a value >1 (Hernando *et al.* 2006). This may be associated with specific factors, such as geographical location of the studies. However, with the paucity of information available for these studies, the wide range of hazard quotients may also be related to the approaches undertaken for the assessments.

Since an ERA involves the estimate of risk based on an exposure assessment, the following sections will present an overview of previous work undertaken on determining concentrations of drugs in the aquatic environment. Also, ecotoxicity assays that have been undertaken to assess the concentrations of drugs that cause effects in aquatic organisms will be discussed.

Table 2.3: Overview of types of pharmaceuticals found in environmental surveys and their concentrations, grouped by their anatomical therapeutic chemical (ATC) code. Wastewater includes water sampled from wastewater treatment plants (WWTPs), while surface water and ground water were sampled post-WWTP

ATC group	Drug (therapeutic class)	Concentration (μgL^{-1})	Medium	Region	Reference
Alimentary & Metabolism (A)	Ranitidine (Antacid)	0.01	Surface water	USA	Kolpin *et al.* 2002
		0.04	Surface water	Italy	Calamari *et al.* 2003
	Metformin (Antidiabetic)	0.15	Surface water	USA	Kolpin *et al.* 2002
Blood (B)	Aspirin (Anticoagulant)	1.20	Wastewater	Brazil	Stumpf *et al.* 1999
		0.34	Surface water	Germany	Ternes 1998
Cardiovascular (C)	Propranolol (β-blocker)	0.59	Surface water	Germany	Ternes 1998
	Gemfibrozil (Antihyperlipidemic)	1.90	Wastewater	USA	Huggett *et al.* 2003b
		1.50	Wastewater	Australia	Khan 2002
		0.11	Surface water	Canada	Metcalfe *et al.* 2003b
Genitourinary (G)	17β-ethynylestradiol (Contraceptive)	0.002	Wastewater	Italy	Baronti *et al.* 2000
	Estradiol (Contraceptive)	0.02	Wastewater	Brazil	Ternes *et al.* 1999
		0.05	Wastewater	UK	Desbrow *et al.* 1998
		0.01	Wastewater	Europe	Johnson *et al.* 2000
Antiinfective (J)	Sulfamethoxazole (Antibiotic)	0.41	Surface water	Germany	Sacher *et al.* 2001
	Trimethoprim (Antibiotic)	0.47	Ground water	Germany	Hirsch *et al.* 1999
		0.71	Surface water	USA	Kolpin *et al.* 2002
		0.13	Surface water	Canada	Metcalfe *et al.* 2003b
Antineoplastic (L)	Tamoxifen (Antiestrogen)	0.04	Wastewater	England	Ashton *et al.* 2004
Musculoskeletal (M)	Diclofenac (NSAID)	1.03	Surface water	Germany	Heberer *et al.* 2002
	Ibuprofen (NSAID)	0.38	Ground water	Germany	Heberer *et al.* 1998
		7.80	Surface water	Norway	Weigel *et al.* 2004
		24.6	Wastewater	Canada	Metcalfe *et al.* 2003a
Nervous system (N)	Carbamazepine (Antiepileptic)	0.90	Surface water	Germany	Sacher *et al.* 2001
		1.20	Surface water	Europe	Andreozzi *et al.* 2003
	Fluoxetine (Antidepressant)	0.36	Ground water	Germany	Heberer *et al.* 2002
		0.01	Surface water	USA	Kolpin *et al.* 2002
		0.05	Surface water	Canada	Metcalfe *et al.* 2003b
Respiratory (R)	Diphenhydramine (Antihistamine)	0.02	Wastewater	USA	Kolpin *et al.* 2004
	Salbutamol (Adrenergic)	0.04	Surface water	Germany	Ternes 1998
		0.002	Surface water	Italy	Calamari *et al.* 2003

Table 2.4: Overview of pharmaceuticals used in ecotoxicological assays

ATC [a] group	Drug (therapeutic class)	Organism	Assay	Concentration	Reference
Alimentary & Metabolism (A)	Metformin (Antidiabetic)	*Daphnia magna*	48 h EC_{50}[b]	64,000 μgL^{-1}	Cleuvers 2003
	Ranitidine (Antacid)	*Daphnia*	EC_{50}	650, 000 μgL^{-1}	Webb 2004
Blood (B)	Aspirin (Anticoagulant)	*Hydra vulgaris*	7 d feeding	10-100 μgL^{-1}	Pascoe *et al.* 2003
		Daphnia magna	48 h EC_{50}	647,000 μgL^{-1}	Marques *et al.* 2004
Cardiovascular (C)	Propranolol (β-blocker)	*Ceriodaphnia dubia*	48 h LC_{50}[c]	800 μgL^{-1}	Huggett *et al.* 2002
		Oryzias latipes	4 week (plasma hormone)	>1 μgL^{-1}	Huggett *et al.* 2002
	Gemfibrozil (Antihyperlipidemic)	*Vibrio fischeri*	Microtox® EC_{50}	18800 μgL^{-1}	La Farre *et al.* 2001
		Daphnia magna	48 h EC_{50}	100,000 μgL^{-1}	Hernando *et al.* 2004
Antineoplastic (L)	Methotrexate	*Tetrahymena pyriformis*	48 h EC_{50}	45,000 μgL^{-1}	Henschel *et al.* 1997
Genitourinary (G)	17α-ethynylestradiol (Contraceptive)	*Dania rerio*	Multiple lifecycle reproduction	0.005 μgL^{-1}	Nash *et al.* 2004
Systemic hormone (H)	Prednisolone (Corticosteroid)	*Brachionus calyciflorus*	24 h LC_{50}	29,290 μgL^{-1}	Della Greca *et al.* 2004
Antiinfective (J)	Sulfamethoxazole (Antibiotic)	*Selenastrum capricornutum*	24 h EC_{50}	1530 μgL^{-1}	Eguchi *et al.* 2004
		Lemna gibba	7 d EC_{50}	81 μgL^{-1}	Brain *et al.* 2004
	Trimethoprim (Antibiotic)	*Selenastrum capricornutum*	24 h EC_{50}	80,300 μgL^{-1}	Eguchi *et al.* 2004
Musculoskeletal (M)	Diclofenac (NSAID)	*Ceriodaphnia dubia*	7 d EC_{50}	1000 μgL^{-1}	Ferrari *et al.* 2004
		Oncorhynchus mykiss	28 d LOEC[d]	1 μgL^{-1}	Triebskorn *et al.* 2004
	Ibuprofen (NSAID)	*Lemna minor*	7 d EC_{50}	22000 μgL^{-1}	Cleuvers 2003
Nervous system (N)	Carbamazepine (Antiepileptic)	Vero monkey (cell line)	72 h neutral red uptake	4490 μgL^{-1}	Jos *et al.* 2003
		Ceriodaphnia dubia	7 d LOEC	100 μgL^{-1}	Ferrari *et al.* 2003
	Fluoxetine (Antidepressant)	*Ceriodaphnia dubia*	8 d LOEC	45 μgL^{-1}	Henry *et al.* 2004
		Chironomus tentans	10 d LOEC	1300 μgkg^{-1}	Brooks *et al.* 2003a

[a] ATC = Anatomical Therapeutic Chemical
[b] EC_{50}= Concentration where effect is apparent in 50% of test organisms
[c] LC_{50} = Concentration where lethality is apparent in 50% of test organisms
[d] LOEC = Lowest concentration where an observable effect is apparent

2.2.2 Environmental surveys

Determining the concentration of pharmaceuticals in the environment is important for validating the risk assessment guidelines for new drug products. Concerns about the environmental impact of pharmaceuticals were based on surveys of the aquatic environment that detected a number of therapeutic agents present in aquatic samples (Halling-Sorensen *et al.* 1998). These surveys have included monitoring of WWTP influent and effluent, as well as open waters. Environmental surveys have become increasingly viable as more powerful analytical techniques, such as tandem mass spectrometry (MS/MS), become more commonly used (Barcelo 2003).

It is worth considering that these surveys can only target compounds *a priori*, meaning the drugs screened in surveys are dependent on the experimental aims of the investigators (Daughton 2003). Pharmaceuticals assessed in surveys are usually selected based on some or all of the following criteria; estimated volume of use in a population, land use characteristics, perceived resistance to degradation in WWTPs and in open water systems or concerns of potential ecotoxicology. Accordingly, commonly used drugs, reflecting the most prevalent ailments treated in a geographical location, are often targeted in environmental surveys. Some compounds that are assessed for this reason include NSAIDs (Musculoskeletal ATC group) such as ibuprofen, diclofenac and naproxen, cardiovascular treatments such as gemfibrozil, clofibric acid and propranolol (Cardiovascular ATC group), antibiotics such as erythromycin and sulfamethoxazole (General antiinfective ATC group).

The steroidal estrogen 17α-ethynylestradiol, used as a contraceptive (Genitourinary ATC group) is a drug selected for environmental surveys, due to its pharmacological potency, with particular attention being placed on studying the disruption of endocrine hormone function in recent years (Larsson *et al.* 1999). Environmental surveys of 17α−ethynylestradiol have determined its concentrations in WWTPs and surface waters to be below 10 ng/L, although the potential to cause disruption of endocrine function in fish may occur at levels below this concentration (Desbrow *et al.* 1998; Larsson *et al.* 1999; Ternes *et al.* 1999; Baronti *et al.* 2000; Johnson *et al.* 2000; Kolpin *et al.* 2002).

Some classes of drugs that are commonly prescribed also have associated concerns regarding their specific modes of action. In particular, the SSRI antidepressants (Nervous system ATC group), such as fluoxetine, have also been of interest in environmental surveys. Serotonin is found widely in biological organisms where it serves a diverse array of functions and SSRI drugs have been subsequently shown to affect functioning in invertebrates at μM levels (Fong *et al.* 1998; Uhler *et al.* 2000).

Drugs that are commonly prescribed that have subsequently been found to be resistant to degradation have also been targeted in environmental surveys. Clofibrate, belonging to the fibrates (Cardiovascular ATC group) which are commonly used in Europe for the regulation of blood lipid levels monitored in the environment used for treatment of cardiovascular disease, has been of particular interest due to its persistence in the environment (Stumpf *et al.* 1999; Ferrari *et al.* 2003). The resistance of clofibrate to

removal processes, such as degradation, is implicated in it being detected in the North Sea (Buser *et al.* 1998), as well as in public drinking water (Daughton and Ternes 1999). Carbamazepine, a commonly prescribed anti-epileptic (Nervous system ATC group), has also been found to be highly resistant to degradation in WWTPs and has been widely assessed for environmental surveys (Ternes 1998; Heberer *et al.* 2002; Metcalfe *et al.* 2003b; Clara *et al.* 2004a).

Geographic specificity is likely to occur between areas regarding the total volume of respective pharmaceuticals entering the environment (Daughton 2003). However, despite geographical variations in the usage patterns of pharmaceuticals, there is a reasonable degree of overlap in terms of drugs of interest in environmental surveys. The majority of environmental surveys have occurred in Europe and North America, while one study has been undertaken in Australia (Khan 2002). The survey of Australian WWTPs indicated that a number of drugs found commonly in European and North American surveys are also found entering Australian sewage system. For example, drugs that Khan screened for and detected in two Australian WWTPs included the NSAIDs ibuprofen, naproxen and ketoprofen and the antiepileptic carbamazepine. In the same study, Khan conducted a survey of a number of WWTPs in Berlin and found the same drugs within an order of magnitude of 1 μgL^{-1}.

A survey conducted in the USA by Kolpin *et al.* (2002) detected 11 drugs (not including antibiotics) that have been targeted in subsequent surveys, such as ibuprofen, fluoxetine and gemfibrozil. This was a broad survey, with 82 organic contaminants of 95 screened

for being detected in aquatic systems receiving wastewater. Despite the extensive nature of this survey, the compounds detected could only be selected prior to the survey being undertaken. The 11 pharmaceuticals detected (of the 15 screened for) are also only a small fraction of the thousands of drugs currently available as therapeutics (Pfluger and Dietrich 2001; TGA 2004). Furthermore, transformation of these therapeutics, either following ingestion by humans or following environmental discharge, can lead to the formation of compounds that maintain the biological activity of the parent compound. These transformation products are unlikely to be considered in environmental screening and, therefore, risk assessments.

The environmental surveys that have been undertaken have proved highly useful in providing a basis for the prediction of environmental concentrations of pharmaceuticals in general. However, the majority of these surveys have screened for pharmaceuticals only within aqueous samples, without determining how aqueous concentrations relate to the concentration of pharmaceuticals bound to solids. Distribution throughout an aquatic ecosystem may be an important consideration in terms of the overall fate and potential risk arising from sediment interaction.

2.2.3 Ecotoxicity Testing

To undertake a risk assessment of a potentially hazardous environmental contaminant requires a definition of the likely extent of exposure and the implications this has for organisms within the receiving system. Concerns relating to pharmaceuticals in the

aquatic environment were initially driven by reports that had detected drugs through advanced techniques in analytical chemistry. This is reflected in the literature, where relatively few studies have addressed the presence of pharmaceuticals in the environment in the context of the potential to cause effects.

A number of screening methods have been used to predict the potential toxicity of pharmaceuticals to aquatic organisms through models, such as ECOSAR (Jones *et al.* 2002) or EPIWIN (Sanderson *et al.* 2004), cell lines (Henschel *et al.* 1997; Jos *et al.* 2003) and microbial biosensors (La Farre *et al.* 2001; Ferrari *et al.* 2003). These assays represent rapid methods for defining the potential toxicity of a drug by excluding many of the factors that can complicate ecotoxicity assays. However, it is for this very reason that such assays have limitations.

2.2.3.1 Acute ecotoxicity testing

Ecotoxicological assays can be undertaken as short-term studies, measuring the acute effects on organisms. Acute assays usually involve measuring effects, or endpoints, such as mortality or indicators of short-term exposure, such as change in cellular function (Henschel *et al.* 1997; Schlenk 1999; Jos *et al.* 2003). The short-term nature of acute toxicity assays are an advantage, with less associated labour and confounding factors (such as growth of the organism and feeding interactions), making them a more common approach. A collation of 408 ecotoxicity studies by Webb (2004) had 360 of the assays conducted as short-term, or acute, exposures within water-only systems. This review by Webb (2004) showed the tests favoured a relatively limited range of organisms, with the

freshwater cladoceran, *Daphnia spp.*, especially used as a test organism. Fish and algae were also used, representing the major trophic levels. Other organisms that have been used for acute exposures include other invertebrates, such as *Ceriodaphnia dubia* (Huggett *et al.* 2002; Ferrari *et al.* 2003; Ferrari *et al.* 2004; Henry *et al.* 2004), and various algae, such as *Desmodesmus subspicatus* (Cleuvers 2003; Cleuvers 2004), and plants, such as *Lemna spp.* (Cleuvers 2003; Pomati *et al.* 2004; Richards *et al.* 2004).

Webb (2004) found that more than 100 pharmaceuticals were assayed in the acute studies, with general antiinfectives the most commonly represented ATC group, although antidepressants (Nervous system ATC group) were found to present the greatest extent of toxicity. Less commonly assessed drug classes included antihypertensives, antidepressants, antacids and NSAIDs. Antineoplastic drugs (Antineoplastic and immunomodulators ATC group), hypnotics (Nervous system ATC group), and other, various drugs for treating cardiovascular disease (Cardiovascular ATC group) also featured (Webb 2004). For all classes of drugs, effective concentrations were typically above 1 mgL^{-1} (Webb 2004), although a lowest observable effect concentration (LOEC) of 3 μgL^{-1} of the selective-serotonin reuptake inhibitor (SSRI) antidepressant fluvoxamine (Nervous system ATC group) has been observed to induce spawning in fingernail clams (Fong *et al.* 1998). Values for effective concentrations were also found below 0.1 mgL^{-1} for fluoxetine, another SSRI, in invertebrates and algae (Brooks *et al.* 2003a). However, a number of subsequent ecotoxicity assays of a diverse range of pharmaceuticals indicate acute toxicity is unlikely to occur below 1 mgL^{-1} (Table 2.4).

This value is a number of orders of magnitude above the aqueous concentrations of pharmaceuticals predicted or measured in environmental surveys (Table 2.3).

2.2.3.2 Chronic ecotoxicity testing

Long-term, or chronic ecotoxicity assays involve the exposure of organisms over relatively long periods, such as over entire life-cycles of the test organism, or during critical periods of the life-cycle, such as during developmental or reproductive periods (Daughton and Ternes 1999; Nash *et al.* 2004). The longer exposure periods often require a higher duty of care of the test organisms and can lead to the introduction of confounding factors that are necessary to support the organism, such as feeding. The use of chronic assays are thus less common and the number of pharmaceuticals that have been tested are far less compared with acute toxicity assays (Webb 2004). The 17α-ethynylestradiol has been commonly used (Webb 2004), with β-blockers (Cardiovascular ATC group), antidepressants and NSAIDs also being assessed (Huggett *et al.* 2002; Brooks *et al.* 2003b; Cleuvers 2003; Ferrari *et al.* 2004; Richards *et al.* 2004). With a lesser number of chronic exposure assays being undertaken, the range of organisms used for these was also less, although *Daphnia* spp. once again featured as an important test organism (Cleuvers 2003; Webb 2004). Other organisms included the rainbow trout, *Oncorhynchus mykiss*, and invertebrates, such as *Hyalella azteca*, *Ceriodaphnia dubia* and *Hydra vulgaris* (Huggett *et al.* 2002; Pascoe *et al.* 2003; Triebskorn *et al.* 2004; Webb 2004).

An important feature of the chronic toxicity assays demonstrate that effects occur at concentrations orders of magnitude lower compared with acute exposures, in an organism exposed to the same drug (Table 2.4). This demonstrates that long-term exposure is likely to be of greater concern for aquatic organisms. Also, different biological mechanisms may be affected after long-term exposure, which may give a more insightful indication of likely effects under environmental conditions. For example, exposure of Japanese medaka, *Oryzias latipes*, to the β-blocker propranolol did not cause mortality after 48 h of exposure or a significant reduction in growth rate after 2 weeks of exposure. Reproductive success, in terms of hatching and egg production, were also not affected, although plasma estradiol levels in females and plasma estradiol and testosterone levels in males were found to be significantly affected after the 2 weeks exposure period. However, after a 4-week exposure period, both hatching and egg production were found to be significantly reduced. These results indicate that effects on individual organisms, such as growth and mortality, may not be found during short-term assays, while effects that are likely to have an impact on the general population, such as reproductive success, can only be elucidated by longer-term exposure. The relevance of acute toxicity testing has previously been questioned within the parameters of an ecological risk assessment for pharmaceuticals, considering their pseudo-persistence within an aquatic ecosystem and how this may influence the ecotoxicological indicators within exposed populations (Halling-Sorensen *et al.* 1998; Daughton and Ternes 1999).

While acute exposure assays may have shortcomings, a number of important findings can be drawn from them. Short-term mixture toxicity assays, where a number of

pharmaceuticals present in solution can have an additive effect, have indicated that pharmaceuticals are likely to be of greater concern than as a single compound at equivalent concentrations (Cleuvers 2003; Della Greca *et al.* 2003; Della Greca *et al.* 2004; Richards *et al.* 2004). Acute assays have also indicated that transformation products of the parent drug may be equally or more potent than the parent drug (Della Greca *et al.* 2004). Finally, some species are comparatively more sensitive to some drugs but less so with others (Cleuvers 2003; Della Greca *et al.* 2004). Therefore, to more accurately define the likely risk posed by pharmaceuticals, it may be important to consider the effect of the presence of other pharmaceuticals, including likely physiological and environmental transformation products, has for a drug of interest. This should also be considered in a number of species, both within and between trophic levels, to ensure the more sensitive species are considered. Preliminary screening using cellular-level endpoints, or biomarkers, to determine whether bioavailable pharmaceuticals can interact with receptors would be one of the many useful tools for defining ecotoxicological risk of pharmaceuticals.

2.2.4 Use of pharmacological data

While the use of chronic toxicity assays in a number of trophic levels would be ideal, these tests are comparatively both labour and financially intensive. Also, the application of chronic toxicity assays for each new drug application would raise ethical concerns related to the excessive use of testing organisms (Lange and Dietrich 2002; Hutchinson *et al.* 2003). In humans, the specific receptor-level interactions of a drug and the steady-

state plasma levels that elucidate a therapeutic response must be well defined for a new drug application. This information on the effects, or pharmacodynamics, must be developed for every drug that exists as a therapeutic agent and, therefore, as a potential environmental contaminant. Therefore, as environmental contaminants, human pharmaceuticals are in a position where substantial information has already been generated on their mechanisms of biological action prior to environmental contamination (Lange and Dietrich 2002; Seiler 2002). Huggett *et al.* (2003) have proposed that the potential toxicity of human pharmaceuticals can be estimated based on the known receptor interactions of pharmaceuticals and human therapeutic plasma levels. A number of the receptors targeted in therapeutics can be conserved and found in other species, including invertebrates. While possessing common receptors may make the probability of effects higher, how these effects are expressed are less predictable. For example, serotonin has many important mood regulating functions in humans, while in invertebrates it regulates many other functions, such as reproductive processes (Fong *et al.* 1998; Lange and Dietrich 2002). The regulation of serotonin is strongly influenced by antidepressants, particularly the SSRI class of antidepressants (Baldessarini 2001).

Since the conservation of receptor function is likely to be most apparent in vertebrates, Huggett *et al.* (2003) have focussed on the presence of receptors in fish that may be predictably influenced following exposure to pharmaceuticals. Comparing the human therapeutic plasma levels with predicted or measured plasma concentrations in fish can give an indication of whether a toxicological response is likely to occur in the exposed fish (Huggett *et al.* 2004). This comparison generates an effect ratio, where a

comparatively higher fish plasma concentration leads to a decreased ratio value. The lower the effect ratio, the more likely an adverse response will be apparent. Following appropriate validation of drugs already present as environmental contaminants, this effect ratio may be another valuable tool in the screening stages of an ERA for pharmaceuticals. The validation of the effect ratio approach would, at least initially, require expert consultation from environmental and pharmaceutical industry scientists, as well as exposure of target organisms to pharmaceuticals of interest. However, the effect ratio approach has the potential to provide a greater degree of relevance to the current ERA screening process, which relies on acute toxicity data, while reducing the need for excessive chronic toxicity testing. The use of information that is available for all drugs approved as therapeutics is also an insightful method of addressing the paucity of environmental data relating to the effects of pharmaceuticals.

2.3 The fate of human pharmaceuticals in aquatic systems

Although more effort has been put into environmental surveys, compared with ecotoxicity assessments, there is still relatively little known about the fate of pharmaceuticals once they enter the aquatic environment. Environmental surveys give an important temporal and spatial insight into the concentrations of drugs in solution. Surveys based on grab samples are limited in that they indicate a certain pre-selected drug exists at a certain concentration in solution at a certain time, in a certain place. A number of environmental surveys have included more complex designs in terms of temporal or spatial sampling. For example, a number of surveys have attempted to

elucidate the extent of removal of drugs from WWTPs by determining concentrations at various points of the treatment process (Ternes 1998; Buser *et al.* 1999; Baronti *et al.* 2000; Heberer *et al.* 2002; Hilton and Thomas 2003; Metcalfe *et al.* 2003b). The majority of drugs are found to have a lower concentration in solution following passage through a treatment plant, indicating significant but variable removal of drugs within WWTPs. However, it is difficult to elucidate the exact mechanism of removal from these surveys. For example, depending on the treatment process undertaken in the WWTP, removal of pharmaceuticals by microbial transformation (Zwiener and Frimmel 2003), sorption to sludge (Khan 2002) or other processes (Pinkston and Sedlak 2004; Sires *et al.* 2004) can occur. Also, once a drug is discharged into the environment, a number of other processes can further reduce apparent concentrations of measured solutions, such as photodegradation (Boreen *et al.* 2003), dilution (EMEA-CPMP 2003) or sorption (Tixier *et al.* 2003). Transformation of drugs released in to the environment to by-products, especially less biologically active by-products, is a desirable environmental fate for pharmaceuticals. However, if removal in the environment is occurring through sorption to sediments, this does not necessarily imply that the environmental risk posed by pharmaceuticals is mitigated.

2.3.1 The role of sorption in environmental fate and effects

More recent evidence would suggest that sorption plays an important role in the fate of pharmaceuticals, both in WWTPs (Ternes *et al.* 2004) and once they are released into the aquatic environment (Furlong *et al.* 2004). One other notable feature of environmental

surveys and ecotoxicological testing has been a focus on the aqueous phase. That is, the extent of knowledge regarding the amount of interaction that pharmaceuticals have with sediments once they enter an aquatic ecosystem is very limited. It follows that our knowledge relating to how interaction with sediments can influence the ERA of pharmaceuticals is even less well understood.

A study by Brooks *et al.* (2003b) evaluated the toxicity of an SSRI antidepressant fluoxetine spiked to sediments. The freshwater midge, *Chironomus tentans*, and *Hyallela azteca* were added to sediments spiked with fluoxetine and exposed for 10 and 42 days, respectively. The lowest observable effect concentrations (LOECs) were found to be 1.3 $mgkg^{-1}$ for *C. tentans* and 5.6 $mgkg^{-1}$ for *H. azteca*, where effects on growth were found in both species. Previous environmental surveys have indicated that fluoxetine is either not detected (Boyd *et al.* 2003; Furlong *et al.* 2004) or in the low ngL^{-1} range, with a low frequency of detection (Kolpin *et al.* 2002). However, where fluoxetine was not detected in solutions, it was detected at a mean level of 1.84 $mgkg^{-1}$ in sediments (Furlong *et al.* 2004), which is a level of concern in relation to the study by Brooks *et al.* (2003b) for the sediment-dwelling *C. tentans* and *H. azteca*. That is, a pharmaceutical not being detected in an environmental survey does not necessarily preclude it from being present in an aquatic ecosystem. This may have important implications for the screening level of an ERA, which does not include an assessment of the potential for distribution into sediment or the implications sorption has for risk assessment until higher level assessment tiers (EMEA-CPMP 2005). While there is evidence that pharmaceuticals are

susceptible to degradation in the environment, their constant release into the environment should also be considered in terms of their interaction with sediments.

When organic contaminants are released into an aquatic system, equilibrium between the sediment and solution may take substantially longer than predicted due to the influence of slow sorption processes (Pignatello and Xing 1996; Warren *et al.* 2003). Slow sorption processes may be especially relevant for pharmaceuticals, as they are constantly infused into the aquatic environment. One important implication of slow sorption is that organic contaminants that bind to sediments will be re-released back into solution at a significantly lower rate, a process known as desorption hysteresis (Pignatello and Xing 1996). If slow sorption occurred for human pharmaceuticals, then the role of sediment as a reservoir for discharged pharmaceuticals is a worthwhile factor to consider. Furthermore, if pharmaceuticals are being retained by sediments then it is also worthwhile considering whether this can increase the exposure and, therefore, risk to organisms that interact with sediments.

In order to account for the potential to cause effects due to sediment interactions in the screening process for an ERA would require at least a predictive measure of how pharmaceuticals released into the aquatic environment will interact with sediments. Many available methods for predicting the fate of organic contaminants have been developed for neutral, lipophilic compounds, whereas pharmaceuticals generally have a moderate lipophilicity (based on their octanol / water partition coefficients) and are likely to be ionisable (Cunningham 2004).

Therefore other approaches to estimating their distribution to feed into a screening process would be desirable for pharmaceuticals, particularly if sorption was found to be an important process. Considering the approach that has been proposed in relating the potential for effects based on pharmacodynamic studies and the wealth of information already generated for pharmaceuticals for their marketing approval, it may be worth further exploring whether this information can be utilised within the screening tier of an ERA. The advantage of using such an approach is that if the sediment concentrations can be estimated in the context of estimated aqueous concentrations. Incorporating an estimate of sediment concentration would also enable a more focussed risk assessment at higher tiers, where decisions regarding ecotoxicity testing requirements could have a better basis.

2.4 Conclusions and summary

Based on this literature review, it is apparent that the ecological risk assessment undertaken by the FDA and EMEA are likely to be affected by the lack of information that is currently available for the environmental fate and effects of human pharmaceuticals. An increasing number of environmental surveys have found that a diverse array of classes of pharmaceuticals exist in aquatic ecosystems, following release from WWTPs. However, these surveys are limited by *a priori* targeting of drugs, spatial and temporal variations in concentrations. Also, the majority of environmental surveys have focused on the concentration of pharmaceuticals within the aqueous compartment of

an aquatic system. Ecotoxicological assessments of pharmaceuticals have been undertaken to a lesser extent than environmental surveys and, therefore, the number of drugs assayed and test organisms used have been even more limited. Furthermore, the majority of these assays have been undertaken as short-term studies, within the aqueous phase.

One factor that has thus far received limited consideration has been whether pharmaceuticals are likely to distribute into sediments within an aquatic ecosystem and how this distribution can influence the ultimate fate and potential risk within an aquatic ecosystem. That is, the partitioning to sediments within the risk assessment for pharmaceuticals has largely been ignored. The recent recommendations by the EMEA have included an assessment of the impact on fate and effects of pharmaceuticals due to sediment interaction. Interaction with sediments is not included within the screening tiers of an ERA even though this interaction may influence the decision to continue the assessment process.

Therefore, the research questions this thesis sought to address were as follows.

1. To determine whether sorption plays an important role in the fate of pharmaceuticals found within system containing water and sediment (Chapter 4).
2. To assess whether the physicochemical properties of pharmaceuticals can influence the extent of sorption and what role environmental factors have on the sorption process (Chapter 5).

3. To investigate whether sorption is easily reversible and what implications this may have for the role of sediment in accumulating pharmaceuticals (Chapters 4 and 7).
4. To investigate a novel approach for estimating the potential sorption of pharmaceuticals within an aquatic ecosystem, based on data available from pharmacological studies (Chapter 6).
5. To assess whether sorption of pharmaceuticals to sediments can lead to toxicological effects within a sediment-dwelling organism (Chapter 8).

Chapter 3. Analytical method development

3.1 Introduction

The determination of concentrations of human pharmaceuticals within model systems formed a significant basis for this project. Therefore, the development of robust analytical methods was essential for this work. Most analytical work relating to human pharmaceuticals in the aquatic environment has been developed for the detection and quantification (or quantitation) of these compounds in complex environmental matrices. These environmental matrices, especially relating to solids such as biosolids, soils and sediments, provide a distinct challenge as they can variously affect the response of analytical instruments. Furthermore, human pharmaceuticals are found in environmental samples in the part per billion to part per trillion (μgL^{-1} to ngL^{-1}) range. Development of sufficiently sensitive and selective instrumentation has fostered the recent effort into the assessment of environmental contamination by pharmaceuticals.

High pressure liquid chromatography (HPLC) was principally used throughout this project. Although gas chromatography (GC), coupled with either a flame ionisation detector (FID), nitrogen-phosphorous detector (NPD), electron capture detector (ECD) and mass spectrometry (MS), was available there were a number of reasons that meant HPLC was an advantageous technique. As a class of organic compounds, pharmaceuticals are generally polar in nature, as they contain functional groups that

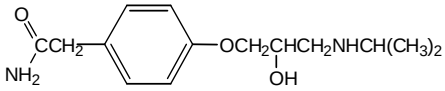
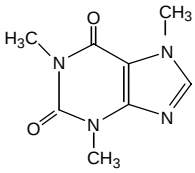
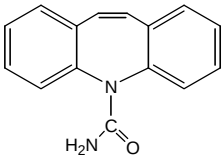
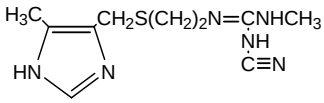
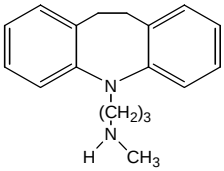
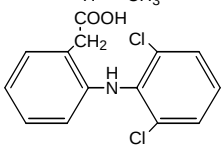
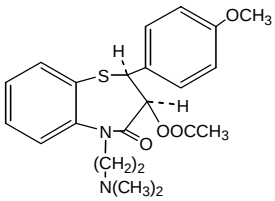
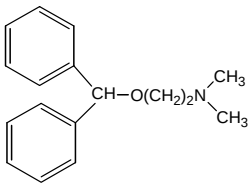
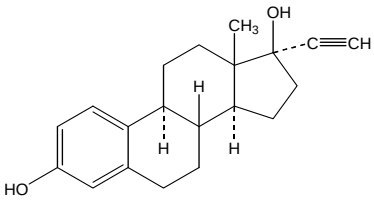
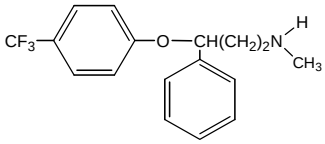
facilitate hydrogen bonding. Hydrogen bonding subsequently decreases the volatility of pharmaceuticals. The low volatility of pharmaceuticals tends to make them unstable for analysis using a method such as GC, as relatively higher temperatures are required to ensure they are in the gas phase. If GC methods are to be used, derivitisation of the compound is necessary to create a more volatile adduct amenable to reproducible GC analysis (Ollers *et al.* 2001; Khan 2002; Redderson and Heberer 2003). Derivitisation procedures use a number of different adducts, which require reaction with the pharmaceuticals to form adducts, thus adding substantially to sample preparation time. Furthermore, derivitisation agents are hazardous chemicals, with associated health risks. As HPLC techniques remain within the aqueous phase, such procedures are not necessary. HPLC has also been a technique which seems to be favoured by previous research on pharmaceuticals, meaning there is a substantial amount of literature for method development of human pharmaceuticals for HPLC.

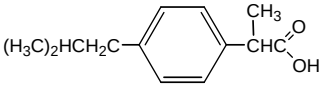
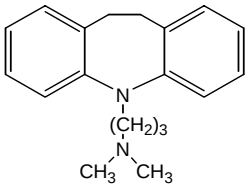
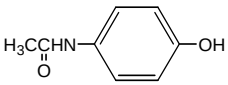
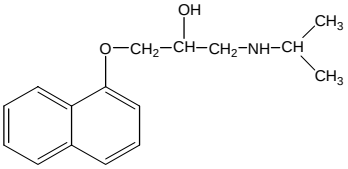
This chapter outlines the stages undertaken for the development of an analytical method for the selected pharmaceuticals by HPLC. Solid phase extraction (SPE), used for the concentrating and/or cleaning up of compounds prior to analysis, was also considered.

3.2 Test pharmaceuticals

As all pharmaceuticals used following therapeutic use have the potential to enter the aquatic environment, a prioritisation strategy was undertaken to form a practical number

Table 3.1: Structures and selected physicochemical properties of selected test pharmaceuticals.

Compound	Log K_{OW}[a,b]	pK_a[a,c]	Log S (mgL^{-1})[a,d]	Ionisation	Structure
Atenolol (ATL)	0.16	9.6	4.11	Base	
Caffeine (CAF)	-0.07	0.6	4.33	Base	
Carbamazepine (CBZ)	2.45	-	1.25	Neutral	
Cimetidine (CIM)	0.4	6.8	3.97	Base	
Desipramine HCl (DMI)	4.9	10.44	1.77	Base	
Diclofenac Na (DCF)	4.4	4.5	0.37	Acid	
Diltiazem HCl (DIL)	2.7	7.7	2.67	Base	
Diphenhydramine HCl (DPH)	3.27	9	3.49	Base	
17α ethynylestradiol (EE2)	3.67	-	1.05	Neutral	
Fluoxetine HCl (FLX)	4.05	10.5	1.78	Base	

Ibuprofen (IBU)	3.97	4.91	1.32	Acid	
Imipramine HCl (IMI)	4.8	9.5	1.26	Base	
Paracetamol (PAC)	0.51	9.3	4.15	Acid	
Propranolol HCl (PRL)	3.56	9.45	1.79	Base	

[a] Syracuse Research Corporation (SRC 2004)
[b] octanol / water partition coefficient
[c] acid dissociation constant
[d] water solubility

of pharmaceuticals to be used in the subsequent experiments (*see* Chapter 2). Current literature was assessed for target pharmaceuticals in environmental surveys, toxicity testing and risk assessments. Also, pharmaceutical databases, such as the Australian Statistics on Medicines database (DUSC 2004), were used to indicate pharmaceuticals of further research interest. The pharmaceuticals selected, their structures and some of their physicochemical properties are listed in Table 3.1.

The use of medicines was of major interest in selecting the test pharmaceuticals. While this could be estimated in Australia based on numbers of prescriptions (*see* Chapter 2) a number of commonly used drugs are also available over the counter (OTC) or by other means. For example, while the xanthine derivative caffeine is used therapeutically in treatments for migraines (DUSC 2004), the majority of its use derives from caffeinated beverages and food products (Buerge *et al.* 2003). Also, diphenhydramine (DPH) is

prescribed as an antihistamine but is commonly used in OTC formulations (Ferrer *et al.* 2004), while the NSAID ibuprofen (IBU) and the analgesic paracetamol (PAC) are both commonly used as OTC medications. Previous environmental surveys demonstrate the relatively high use of these therapeutic agents would warrant them as compounds of environmental interest. Ibuprofen, caffeine and paracetamol have all been found at low part per billion (or μgL^{-1}) concentrations in wastewater and surface water (Ternes 1998; Drewes *et al.* 2002; Kolpin *et al.* 2002; Ashton *et al.* 2004; Weigel *et al.* 2004). Diphenhydramine has only been found in low part per trillion (or ngL^{-1}) concentrations in wastewater (Kolpin *et al.* 2004), although it has been found in sediments at substantially higher concentrations than comparable concentrations in water (Ferrer *et al.* 2004; Furlong *et al.* 2004). Other selected pharmaceuticals, including atenolol (ATL), cimetidine (CIM), diclofenac (DCF), diltiazem (DIL) and fluoxetine (FLX), have high volumes of associated prescriptions ((DUSC 2004); *see also* Table 2.1).

The anti-epileptic carbamazepine (CBZ) has also been found at low part per billion levels in wastewater and surface water (Ternes 1998; Heberer *et al.* 2002; Andreozzi *et al.* 2003; Metcalfe *et al.* 2003a; Clara *et al.* 2004a; Ferrari *et al.* 2004). Also, a number of surveys of WWTPs have indicated that carbamazepine is resistant to removal during the treatment process (Ternes 1998; Drewes *et al.* 2002; Heberer 2002), with subsequent analysis further suggesting its resistance to degradation (Tixier *et al.* 2003; Clara *et al.* 2004a; Joss *et al.* 2005). Carbamazepine was selected due to this perceived persistence in aquatic systems, as well as an environmental risk assessment indicating carbamazepine may present an ecological risk (Ferrari *et al.* 2003). Carbamazepine elicits its effects in

humans through regulation of sodium (Na^+) channels (McNamara 2001). Regulation of Na^+ channels has been used to measure ecotoxicological effect in a number of studies, as it is critical process for cellular functioning (Emery *et al.* 1998; Cotou *et al.* 2001; Webb *et al.* 2001). This potential overlap of pharmacological response and ecological consequences also presents a strong argument for the selection of drugs of interest (Daughton and Ternes 1999; Seiler 2002; Huggett *et al.* 2003a).

Similarly, while not having a comparable prescription volume, the antidepressants imipramine and desipramine are modulators of serotonin and noradrenaline re-uptake in humans (Baldessarini 2001). The potency of selective serotonin reuptake inhibitors (SSRIs) in interfering with normal reproductive processes has been demonstrated in the fingernail clam, *Sphaerium striatinum* (Fong *et al.* 1998) or with cilia-mediated movement in the gastropod, *Physa elliptica* (Uhler *et al.* 2000). The steroidal estrogen 17α-ethynylestradiol (EE2) is a highly potent synthetic estrogen, with effects in aquatic organisms found to occur at low part per trillion levels (Nash *et al.* 2004). In environmental surveys, EE2 has been detected at levels which are above those that can lead to interference with normal hormonal processes, leading to “endocrine disruption” (Desbrow *et al.* 1998; Larsson *et al.* 1999).

One of the selected compounds, diphenhydramine (DPH), was not used in experiments, as it did not give a consistent HPLC response. The time of elution for DPH coincided with an interfering peak for all wavelengths. This interference may have been related to the gradient method selected, since the elution time of DPH coincided with a time when

the gradient ratio was decreased from 65 % buffer to 45 % buffer. After a number of batch sorption experiments (*see* Chapter 4), it was decided to select 17α-ethynylestradiol (EE2) and propranolol (PRL) to use within the established HPLC method, to increase the dataset of test pharmaceuticals.

Standards of test compounds were purchased from Sigma-Aldrich (NSW, Australia) and had a purity of ≥ 98 %. Stock solutions of respective pharmaceuticals were prepared by adding appropriate weight of free acid or base. For example, where the basic compounds were hydrochloride (HCl) salts, the weight of HCl was taken into account for the total mass. For all drugs, 1 gL^{-1} stock solutions were prepared in methanol and stored at -18 °C. Stock solutions were renewed every 6 months.

3.3 High pressure liquid chromatography (HPLC)

All HPLC-UV analysis was undertaken with an Agilent 1100 HPLC, with a polydiode array (PDA) detector. The mobile phase passed through a degasser and was mixed with a quaternary pump prior to injection. The software used for operating the HPLC system was ChemStation© (for LC 3D systems), Rev. B.01.03[204], Agilent Technologies (2001-2005).

3.3.1 Multi-residue analysis

As there was a mixture of pharmaceuticals, in terms of their physicochemical properties, a number of points needed to be considered. All of these factors relate to ensuring reproducible separation of compounds occurs. Selection of an appropriate stationary phase, or column, and the composition of mobile phase, which delivers the analytes to the detector, is of great importance. Also, the optimal wavelength of detection, when using ultraviolet (UV) detectors, ensured the method used for a compound is appropriate (Table 3.2).

3.3.1.1 Stationary phase

Reverse-phase chromatography, where hydrophobic interactions dictate the extent that an analyte partitions between the mobile and stationary phase, is the more commonly employed method of analysis for HPLC.

The affinity of the stationary phase in the column for each compound is likely to be different and hence lead to separation. The neutral compounds had varying water solubilities, while the ionisable compounds were either cationic or anionic when charged, where these charged functional groups were likely to influence the extent of column interactions. For example, stationary phases containing groups such as cyano (CN), hydroxyl (OH) and pentafluorophenyl (PFP) have been found to be selective for binding polar, cationic pharmaceuticals (Needham *et al.* 2000).

However, as a number of functional groups and physicochemical properties existed it was decided to use a column that did not have specificity for certain functional groups. The most commonly used columns are reversed-phase, hydrophobic silane groups, bonded with various lengths of carbon chains, such as the octadecylsilane (ODS), or C_{18}, column (Guiochon 1999; Watson 1999). The non-specific nature of this stationary phase is often suitable for separating compounds of either diverse or similar physicochemical characteristics (Aymard *et al.* 1997; Kolpin *et al.* 2002; Sun *et al.* 2003; Lam *et al.* 2004). The column used for separation of the selected pharmaceuticals was an Apollo C_{18} (250 x 4.6 mm, 5 μm) (Alltech), with an Alltima C_{18} guard column (7.5 x 4.6 mm; 5 μm) (Alltech).

3.3.1.2 Mobile phase

The other critical factor in HPLC method development, particularly when analytes of interest contain ionisable functional groups, is the mobile phase. A number of factors, such as pH, ionic strength and type of salts used, are essential considerations (Watson 1999).

With the use of the C_{18} column, pharmaceuticals with higher hydrophobicity (often estimated from octanol-water partition coefficient, or K_{ow}, values) or lower water solubility would be expected to interact with the stationary phase to a greater extent. As the hydrophobicity and water solubility of ionisable compounds are affected by the extent of charge, the effect of pH on ionisation must be considered. The performance and stability of C_{18} columns can be affected by the pH of mobile phase they are exposed to

(Watson 1999). The pK_a value of a compound is important to assess whether suitable resolution of chromatographic peaks will occur. For example, peak resolution will be best when a compound is either completely ionised or unionised. Also, the length of analysis needs to be taken into account when the pH of the mobile phase affects the extent of interaction that occurs between the stationary phase and compound. The use of a buffer solution is desirable, as it ensures pH values are consistent when using a particular method.

Based on the above factors, a 0.05 M sodium formate / formic acid (NaCHOO / CHOOH) at pH 3 was selected for use as the aqueous buffer for the mobile phase. At pH 3, it is expected that all compounds would be either completely ionised (in the case of the cationic drugs) or unionised (anionic drugs). Acetonitrile (CH_3CN) was used as the solvent component of the mobile phase, as this was found to give the best peak shape, compared with methanol. The formate buffer and CH_3CN were prepared as separate solutions and mixed during the run, via the quaternary pump.

In order to reduce the length of each analytical run, a gradient method was used. The gradient method changed the ratio of aqueous buffer and solvent over the time of the analytical run. The percentage of the NaCHOO / CHOOH buffer used was as follows; 0-4 min 90 %, 6-11 min 65 %, 12-15 min 45 %, 16-19 min 30 %, 22-25 min 90 %, giving a total run time of 25 min. Manual mixing of the buffer and CH_3CN confirmed there was no precipitation of the buffer at even the highest percentage of CH_3CN.

The flow rate of analysis was 1.2 $mLmin^{-1}$, with a typical operating pressure of 135-160 bar (from the highest to lowest ratio of CH_3CN). A 100 μL injection of sample was used for analysis.

3.3.1.3 Wavelength of analysis

The optimal wavelength of each compound was based principally on its wavelength of peak absorbance. The software for operating the HPLC was able to use a full wavelength scan, afforded by the PDA detector, to determine the peak absorbance for each compound. Another factor that needed to be considered was the resolution of the peak at the wavelength selected. A chromatogram of the drugs at 280 nm is shown in Figure 3.1.

Most drugs had their highest absorbance peak at wavelengths <215 nm, although due to other interferences absorbing below this wavelength, the quantifying peaks above this wavelength were found to be most appropriate. The second highest peak of absorbance was usually found to be suitable for quantification. There were, however, some exceptions to this. For example, while one peak absorbance of fluoxetine (FLX) was found to be 230 nm (Figure 3.2), the wavelength for quantification was found to be better resolved for the concentration range at 254 nm. While the sensitivity, or the response to a given concentration, was lower at the wavelength of 254 nm, there was also correspondingly less interference of the signal.

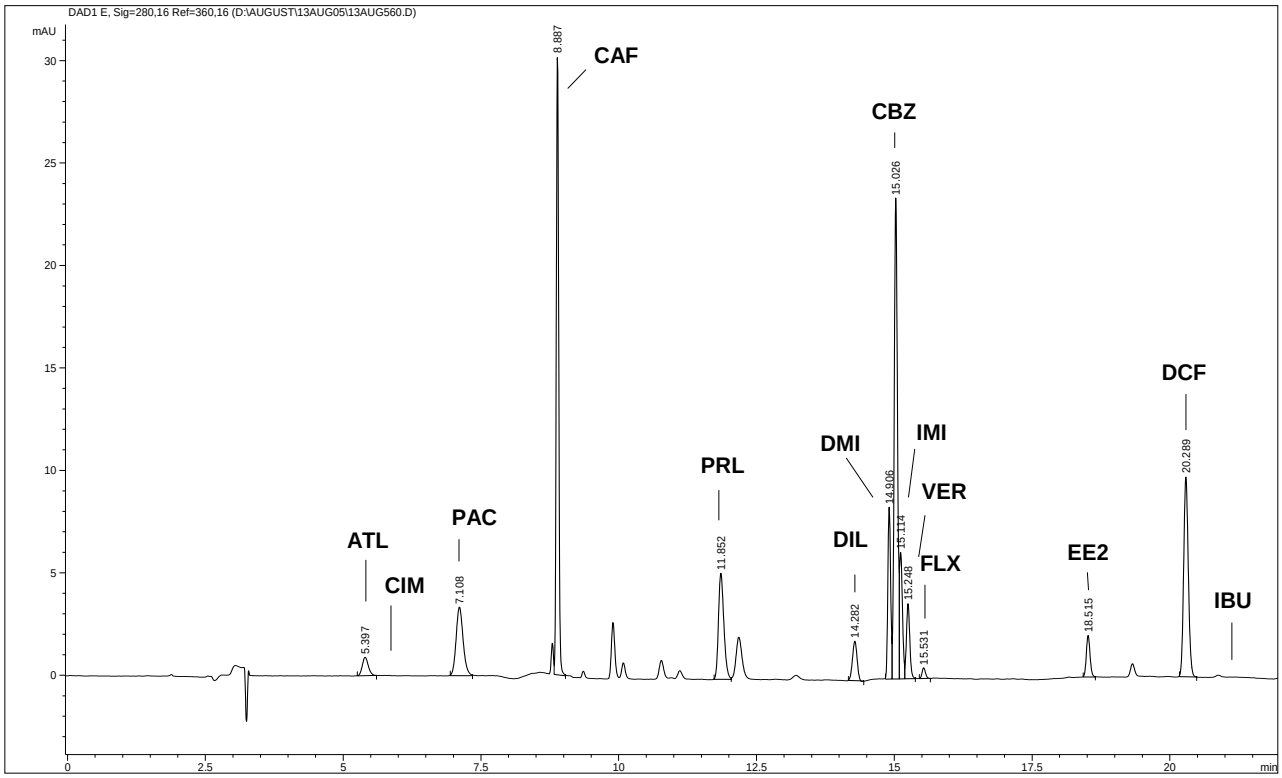


Figure 3.1: Chromatogram of a standard mixture of pharmaceuticals at 280 nm using HPLC-PDA

3.3.2 HPLC for carbamazepine (CBZ) analysis

For the experiments to determine the exchangeable (E) value of CBZ (*see* Chapter 7), HPLC analysis was undertaken using a slight modification of the multi-residue analysis. This was to take advantage of the fact there was only one compound for analysis, where separation was not necessary. To this end, a narrow-bore HPLC column was used, reducing the amount of mobile phase required and reducing the analytical time.

3.3.2.1 Stationary phase

As for the multi-residue analysis, a C_{18} stationary phase was used. The column used was an Alltima C_{18} (150 x 2.1, 3 μm) (Alltech), used in conjunction with an Alltima C_{18} guard column (7.5 x 2.1 mm; 3 μm) (Alltech).

Table 3.2: HPLC parameters for selected pharmaceuticals

Compound	Detection wavelength (nm)	Retention time (min)	Response factor [a]	LOD [b] (μgL^{-1})	LOQ [c] (μgL^{-1})	Interday %C.V. [d]
Atenolol	280	5.28	60	4	13	2.65
Caffeine	280	8.82	4.5	0.3	1	1.26
Carbamazepine	280	15.02	4.2	1	4	2.28
Cimetidine	230	5.66	4.8	17	51	20.07
Desipramine	280	14.9	12.5	2	6	10.10
Diclofenac	280	20.27	6.3	0.7	2	3.93
Diltiazem	280	14.35	25	8	23	9.86
Diphenhydramine	254	13.91	NA [e]	NA	NA	NA
17α ethynylestradiol	280	18.5	39	5	15	0.56
Fluoxetine	254	15.43	100	2	7	9.75
Ibuprofen	215	20.9	4.5	8	25	6.22
Imipramine	254	15.3	10	2	6	9.10
Paracetamol	254	7.06	3.3	1	4	5.57
Propranolol	290	11.96	10	0.9	3	5.46

[a] Response factor is equivalent to the peak area divided by the concentration of the analyte
[b] Limit of detection
[c] Limit of quantitation
[d] Interday percent coefficient of variation of response at 100 μgL^{-1} for 10 or more days
[e] Not applicable

3.3.2.2 Mobile phase

The mobile phase was also 0.05 M NaCHOO / CHOOH (pH 3), with CH_3CN used as the organic solvent. The ratio of aqueous and organic mobile phases was an isocratic (constant) mixture of 55 % buffer and 45 % CH_3CN. The mobile phase was passed through at a flow rate of 0.3 $mLmin^{-1}$, giving a typical pressure of 170 bar. A 50 μL injection of sample was used for analysis.

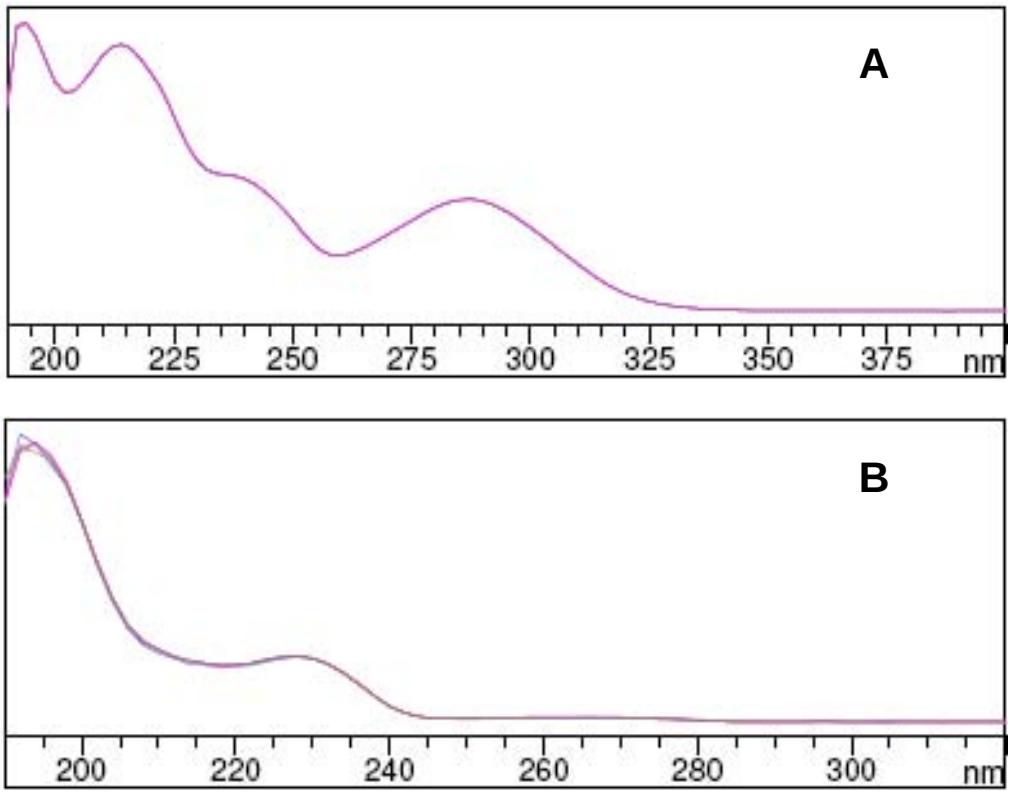


Figure 3.2: UV spectrum of (A) CBZ and (B) FLX showing relative absorbance intensity at various wavelengths of light

3.3.2.3 Wavelength of analysis

Spectral data for CBZ shows peak absorbances above 200 nm at 210, 240 and 285 nm (Figure 3.2). For multi-residue analysis of CBZ a quantifying wavelength of 280 nm was selected, as this was common to a number of the other analytes. For the determination of CBZ using the narrow bore column, a wavelength of 290 nm was used. While this means comparison of performance between the two methods is more difficult, 290 nm gave a chromatogram with less background interference for the narrow bore column method.

3.3.3 Quality assurance / quality control (QA/QC)

3.3.3.1 Calibration procedures

A calibration curve was generated for each compound by spiking an appropriate volume of the stock solution (in methanol) and made up to volume using 18.2 $M\Omega cm^{-1}$ water. Where a mixture of the drugs was used to prepare standards, the methanol was evaporated under a stream of N_2 and 0.1 % methanol (of the final volume) was added before making up to volume with 18.2 $M\Omega cm^{-1}$ water. The final volume of methanol, as a percentage of the final volume, was always < 0.1 %.

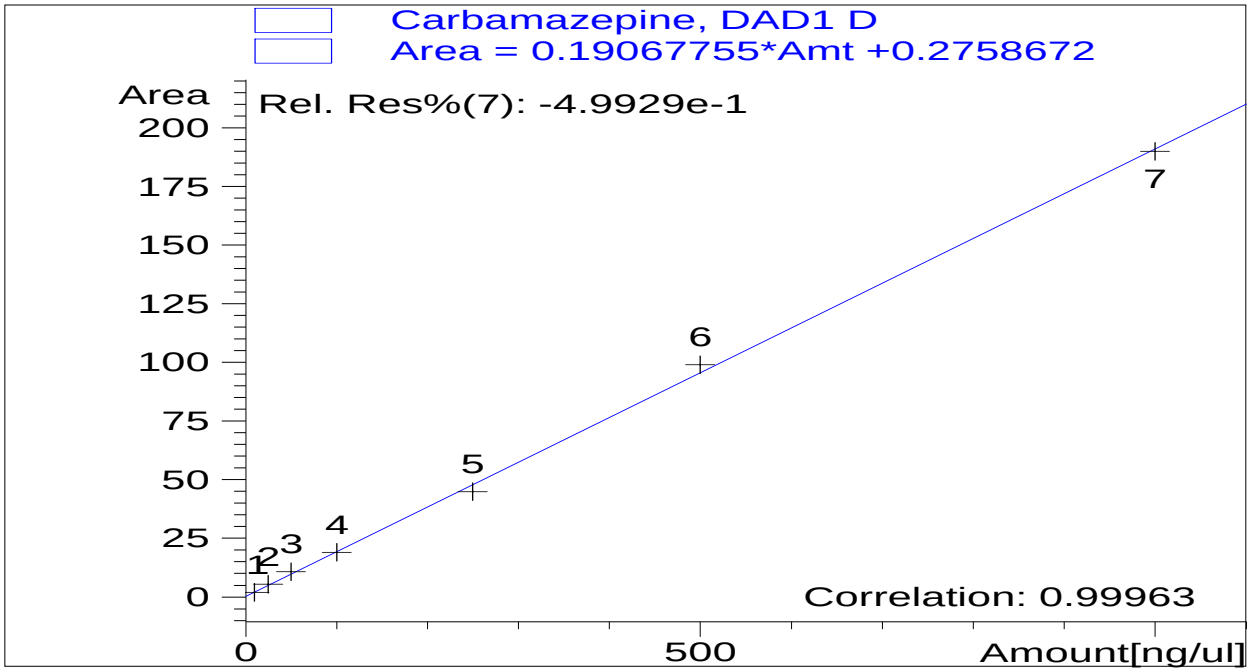


Figure 3.3: Representative calibration curve, depicting a typical response of CBZ for a concentration range from 10-1000 μgL^{-1}. Note that x-axis units were automatically generated by the software and should read pg/uL.

A set of calibration standards was then prepared from the solution with the highest concentration. If the concentration range was >1000 times less than the highest concentration solution, standards were prepared from the solution 1000 times less than the original highest concentration solution.

For all drugs, the maximum concentration used for linear calibration was 1000 μgL^{-1}, with the minimum concentrations around the limit of detection (Table 3.2). A typical calibration curve for CBZ, using the multi- and single residue analysis is shown in Figure 3.3.

3.3.3.2 Limit of detection / limit of quantification (LOD/LOQ)

The limit of detection (LOD), or the method detection limit (MDL), as defined by the United States Geological Survey is "*…the minimum concentration of a substance that can be identified, measured, and reported with a 99-percent confidence that the compound concentration is greater than zero.*" (Lee *et al.* 2002). The LOD / MDL can be defined as:

$$LOD = (S)(t_{(n-1,1-\alpha=0.99)}) \qquad (1)$$

where S is the standard deviation of replicate samples of analyte and $t_{(n-1,\ 1-\alpha=0.99)}$ is Student's *t*-value for the 99-percent confidence level with *n*-1 degrees of freedom for *n* replicates (USEPA 2004). As it is recommended to undertake 7 replicate sample, LOD is often defined as 3 ($t_{(6,\ 1-\alpha=0.99)}$ = 3.143) standard deviations of the signal from the lowest analyte concentration. The limit of quantification (LOQ), or minimum limit of detection (ML), is defined as 10 standard deviations of the signal from the lowest analyte concentration and is usually rounded to the nearest whole number nearest to 1,2 or 5 times 10^n, where *n* is an integer (USEPA 2004).

The lowest analyte concentration used was defined as the concentration above the spiked concentration where no signal was detected. The signal for the lowest analyte concentration detectable was confirmed using the peak purity spectral analysis function, to assess the respective signature wavelength of absorbance. The lowest analyte concentration, used to determine the LOD / LOQ, was spiked at a concentration no higher

than 5 times the estimated LOD (USEPA 2004). For the methods used, a series of standards were prepared as a calibration curve and a total of 8 injections were undertaken. The standard deviations of the signals for respective pharmaceuticals were then used for determination of LOD / LOQ.

Whenever the HPLC was used for analysis, external standards were injected for every 5 to 10 analytical samples. These external standards were usually spiked at concentrations between 100 and 250 μgL^{-1} and were used to assess intra- and inter-day variability for each compound.

The results for the LOD / LOQ, precision and description of the methods for each drug and for the two methods for CBZ are summarised in Table 3.2.

3.3.3.3 Matrix effects

When environmental solutions contain complex matrices, such as dissolved organic carbon, interference of the signal from the analyte of interest can occur. This interference can manifest itself as suppression or enhancement of the signal, leading to underestimation or overestimation of the actual concentration. This is particularly problematic when the analyte is in solution at concentrations close to its MDL.

To assess whether interference was likely to occur for the multiple residue analysis, a comparison of responses from each drug was determined for three different solutions. The solutions used were 18.2 $M\Omega cm^{-1}$ water, 10 mM $CaCl_2$ (prepared in 18.2 $M\Omega cm^{-1}$

water) and 10 mM $CaCl_2$, shaken with 2 mm sieved sediment for 24 h and centrifuged at 2000 *g* for 10 min. These 10 mM $CaCl_2$ was selected as it was the standard solution used for the batch sorption experiments.

Drugs were spiked at a concentration of 1000 μgL^{-1} by spiking equal volumes of respective 1 gL^{-1} (in methanol) drug stock solutions to a volumetric flask. The methanol was removed with high purity N_2 and methanol was then added to the volumetric flask to give a final methanol concentration of 0.1 %, in respective solutions. FLX seemed to be affected by $CaCl_2$ as a matrix, where its response was enhanced, relative to 18.2 $M\Omega cm^{-1}$ water (Figure 3.4). The response of DIL also seemed to be affected with the $CaCl_2$, when both neat and mixed with sediment. As all other drugs did not seem to have their response affected by the matrix, it was decided that the use of calibration standards in 18.2 $M\Omega cm^{-1}$ water was appropriate.

Statistical analysis of replicate HPLC-PDA responses in 18.2 $M\Omega cm^{-1}$ water, 10 mM $CaCl_2$ solution and 10 mM $CaCl_2$ solution mixed with sediments and filtered using a 0.45 μm filter was undertaken for each compound. A one-way analysis of variance (ANOVA) was used to determine whether there was a significant difference between responses for each compound, with Tukey's test ($\alpha = 0.05$) used as a multiple comparison test if a treatment was found to be significantly different. Toxstat (Version 3.4; Western EcoSystems Technology, Inc; WY, USA) was used to perform ANOVA and Tukey test.

A significant difference was found for PAC (sediment; $p = 0.01$), CAF (sediment and 18.2 MΩcm^{-1} water; $p = 0.03$), DIL (sediment and 10 mM $CaCl_2$; $p = 0.03$), IMI (18.2 MΩcm^{-1} water; $p = 0.001$). CBZ (10 mM $CaCl_2$; $p = 0.002$) and DCF (10 mM $CaCl_2$; $p = 0.003$). However, closer analysis indicates that there was less than 5 % difference between the responses for all compounds, where a significant difference was determined. This is within the acceptable range of analytical variability, even where it seemed a significant difference was apparent, such as for FLX (< 15 % variability) and DPH (< 7 % variability) (Figure 3.4).

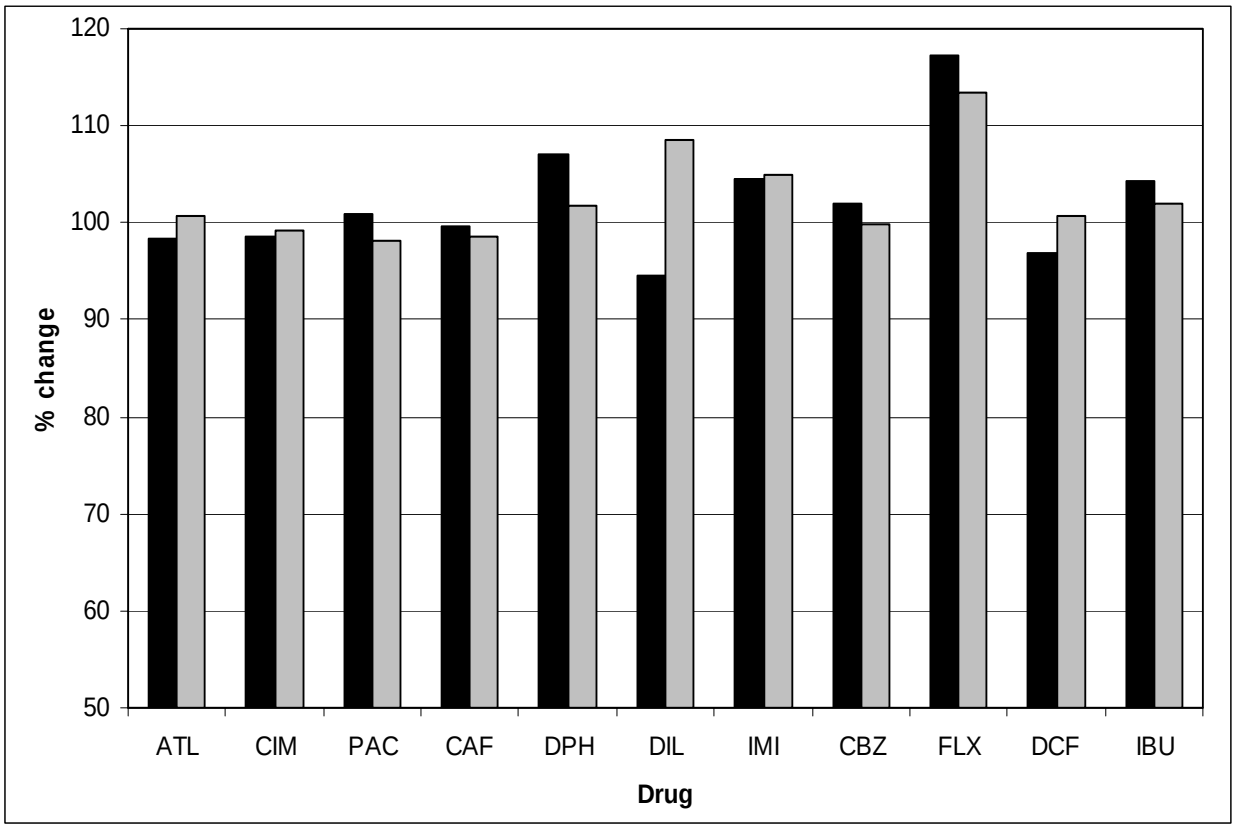


Figure 3.4: The percent change in peak area of pharmaceuticals spiked into 10 mM $CaCl_2$ (■) and 10 mM $CaCl_2$ mixed with sediment (□), relative to pharmaceuticals spiked in 18.2 MΩcm^{-}1 water

3.3.3.4 Sample analysis

A typical sample run would involve the generation of a calibration curve from the standards, followed by the analytical samples. An external standard, made from a mid-standard curve concentration (either 100 or 250 μgL^{-1} for each drug) of the calibration standards, was run every 5 to 10 samples, depending on the length of the analytical run.

If the integrated area under the curve value of the external standard was found to vary by more than 10% from the original value in the standard curve, the samples that the external standard bracketed were re-run. Prior to running these bracketing standards, a 18.2 $M\Omega cm^{-1}$ water control was run to ensure there was no carry-over of analytes from the column. A calibration curve was re-run following each analytical run and compared with the calibration curve run prior to the samples.

3.3.4 Clean-up procedures

HPLC analysis involved the use of analytes at high concentrations, relative to potential interfering matrices. This type of scenario cannot be expected when assessing contamination of human pharmaceuticals in the environment, as the concentration of pharmaceuticals is considerably lower and interfering matrices considerably higher. An important practical aspect of removing these matrices is to minimise their interaction with sensitive analytical equipment, increasing the lifespan of such equipment and decreasing the possibility of interference with analysis, particularly at lower concentrations.

3.3.4.1 Filtration

An important first step in preparing samples is often filtration of solutions. When filtration is used for environmental surveys glass fibre filters, of pore size greater than 0.45 μm in size, are commonly used (Hirsch *et al.* 1998; Kolpin *et al.* 2002; Hilton and Thomas 2003; Metcalfe *et al.* 2003a) This pore size is commonly defined as the cut-off for particulate matter. However, particulates such as humic acids are likely to be found in solution following filtration through these pore sizes (Lead and Wilkinson 2006). Centrifugation is another method that can remove matrices of a particular mass from solution. However, filtration and centrifugation are limited to removing matrices of a certain size and cannot separate matrices below their respective size and mass cut-off limits.

Centrifugation and filtration were used in this work for HPLC analysis of water samples following batch sorption experiments to not only reduce the amount of matrix within the analytical samples but also to provide an arbitrary cut-off between the aqueous and sediment-bound fractions.

The filters used for sample clean-up prior to analysis, following batch sorption experiments, were 0.45 μm nylon syringe filters with polypropylene housing (Biolab, Australia). The recovery efficiency was determined by filtering spiked control samples containing no sediment, with average recoveries ranging from 77 % for DMI to 101 % for ATL.

3.3.4.2 Solid phase extraction (SPE)

Solid phase extraction (SPE) allows the enrichment of analytes, along with removal of environmental matrices that can interfere with analytical detectors. The use of SPE has become increasingly important for the analysis of pharmaceuticals in the environment. SPE is preferable compared with other extraction techniques, such as liquid-liquid extraction, because of ease of use, reduced amounts of extraction solvents and higher specificity for polar compounds (Bakkali *et al.* 1999; Diaz-Cruz *et al.* 2003)

As with HPLC columns, SPE involves the binding of analytes from an aqueous solution onto a polymeric solid phase. The solid phase can either be selective for a particular analyte, such as cation exchange polymers, or non-selective, such as C_{18} polymers. While analytes bind to the solid phase, the majority of interfering matrices will pass through, especially when the SPE material is further cleaned.

For ionisable compounds, the pH of solution will affect their ability to interact with the SPE material (Bakkali *et al.* 1999). Therefore, as with HPLC columns, both the pH of the aqueous solution passing through the SPE material and the nature of the solid phase will be important factors to determine the extent of compound that can be retained. Other factors that can influence the efficiency of analyte retention are the concentration of analyte, the concentration of matrices such as salts, the flow rate of aqueous solution to the solid phase and the ratio of solvent used for eluting the analyte from the solid phase (Bakkali *et al.* 1999; Khan 2002).

To assess the efficiency of recovery from aqueous solution using SPE methods, 3 different solid phases were tested at pH 3 and 7. The pH value of solution was adjusted using buffer salts. Also, the absence of salts at pH 3 was undertaken to assess whether the presence of salts influences analyte recovery. The 3 stationary phases selected were C_{18} (Supelclean Envi-18™) (Supelco, USA), hydrophilic-lipophilic balance (Oasis HLB™) (Waters, USA) and mixed-mode cation exchange (Oasis MCX™) (Waters, USA). Supelclean cartridges contained 500 mg of packing material, while the HLB and MCX contained 60 mg. All cartridges had a 3 mL capacity.

The C_{18} solid phase material is similar to HPLC columns, with an octyldecylsilane functional group. The functional group for HLB solid phase is a mixture of *n*-divinylbenzene and *n*-vinylpyrrolidone copolymer. This copolymer gives a wide spectrum of retention selectivity and is therefore preferable to C_{18} for multi-residue analysis. However, both C_{18} and HLB SPE cartridges are commonly used for environmental sampling, where target pharmaceuticals contain a variety of functional groups (Drewes *et al.* 2002; Kolpin *et al.* 2002; Metcalfe *et al.* 2003b; Carballa *et al.* 2004; Ferrari *et al.* 2004; Lam *et al.* 2004). MCX solid phase contains the *n*-divinylbenzene and *n*-vinylpyrrolidone copolymer, with sulphonic acid groups substituted within the structure. This was used in conjunction with a Sep-Pak C_{18} (Waters, USA) cartridge, to ensure the acidic compounds IBU and DCF were retained.

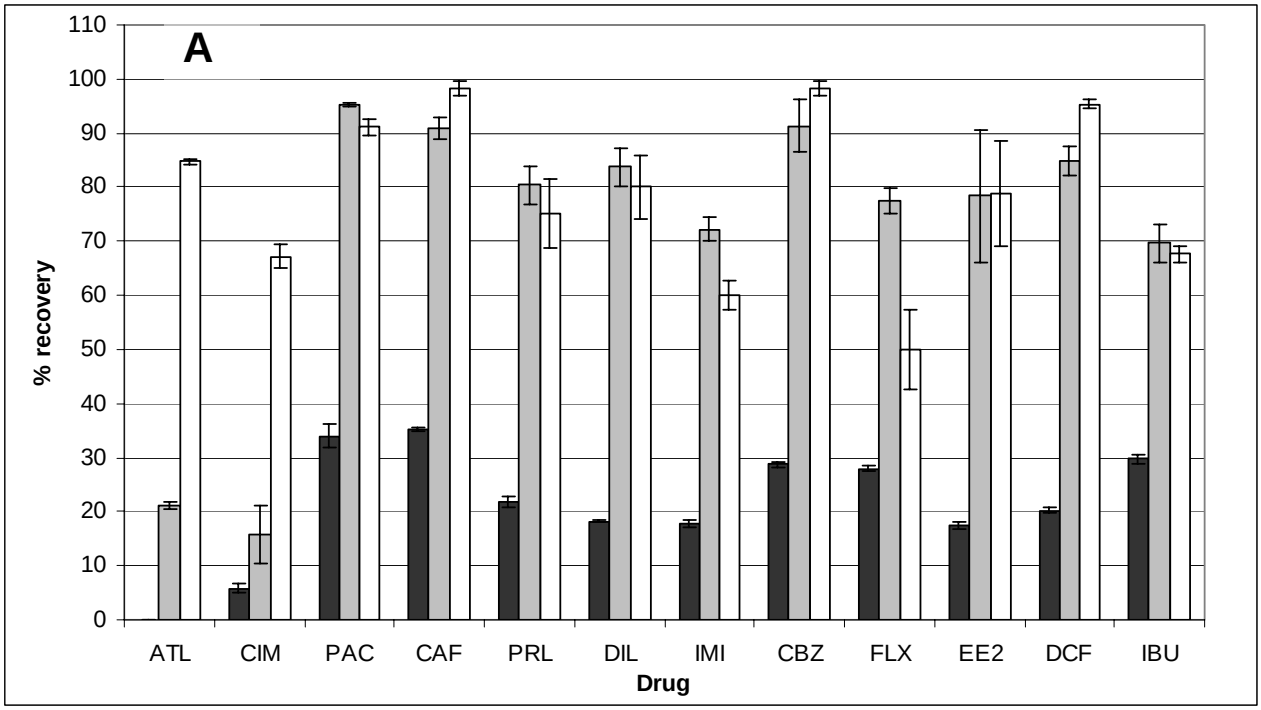
Due to the number of compounds that were ionisable, solutions were prepared at two different pH values, pH 3 and 7. The pH 3 buffer was comprised of 0.05 M NaH_2PO_3 / H_2PO_4, while the pH 7 buffer was comprised of 0.05 M Na_2HPO_3 / NaH_2PO_3. These pH values were used to represent a neutral, environmental sample, while the acidified sample was used to represent a sample preservation strategy. While sample preservation is usually undertaken with strong acids (such as HCl), pH was adjusted using phosphate buffers in 18.2 $M\Omega cm^{-1}$ water. The buffers were used for pH adjustment to maintain constant pH between replicates. The pH 3 treatment was also adjusted using H_3PO_4 alone, to represent a sample with minimal ionic strength.

Stock solutions of pharmaceuticals (1 gL^{-1} in methanol) were spiked to 500 mL of the respective solutions to give a final concentration of 100 μgL^{-1}. 100 mL of the solution was loaded onto each SPE cartridge at a flow rate of approximately 5 $mLmin^{-1}$. Prior to loading, SPE cartridges were conditioned with 3 mL methanol and 3 mL 18.2 $M\Omega cm^{-1}$ water. Following loading, cartridges were dried for 1 h under vacuum. Retained pharmaceuticals were eluted with 4x1 mL methanol for the C_{18} and HLB cartridges, while 4 x 1 mL methanol/ 5 % NH_4OH was used to elute the MCX / C_{18} columns.

Between each application of eluent, the cartridges were dried under vacuum. The collected eluent was then evaporated to dryness using high purity N_2 gas, while sitting on a hotplate set to 35 °C. After drying the eluent, 1 mL of HPLC buffer solution (0.05 M NaCHOO / CHOOH) was used to redissolve the collected extract for HPLC analysis.

The combination of MCX / C_{18} gave the most consistently high recoveries of the test pharmaceuticals (Figure 3.5). For most drugs, the recovery in the buffered solutions was equal or better than in the non-buffered solution. Also, there seemed to be little effect of pH of solution, with the exception of IBU. This effect was not found for the other acidic drug, DCF, which also has a comparably high K_{OW} value (Table 3.1). It would be expected at pH 7 both acidic drugs would be completely ionised, although this apparently only affected the retention of IBU, relative to the pH 3 treatments. The HLB cartridges gave similar recoveries to the MCX / C_{18} cartridges, with the exception of ATL and CIM, which had substantially lower recoveries in the pH 3 HLB treatments. The pH 3 solution not containing buffer also had a substantially lower recovery compared with the buffered solutions. There was no apparent effect of salt on the recoveries in the MCX / C_{18} cartridges.

The C_{18} cartridges on their own had lower recoveries for most of the pharmaceuticals, compared with the MCX combined with C_{18}. The effect of salt within solution was variable, where there was reduced recovery for ATL, CIM, CAF, PRL, FLX and IMI, while recovery was unchanged or enhanced slightly for the remainder.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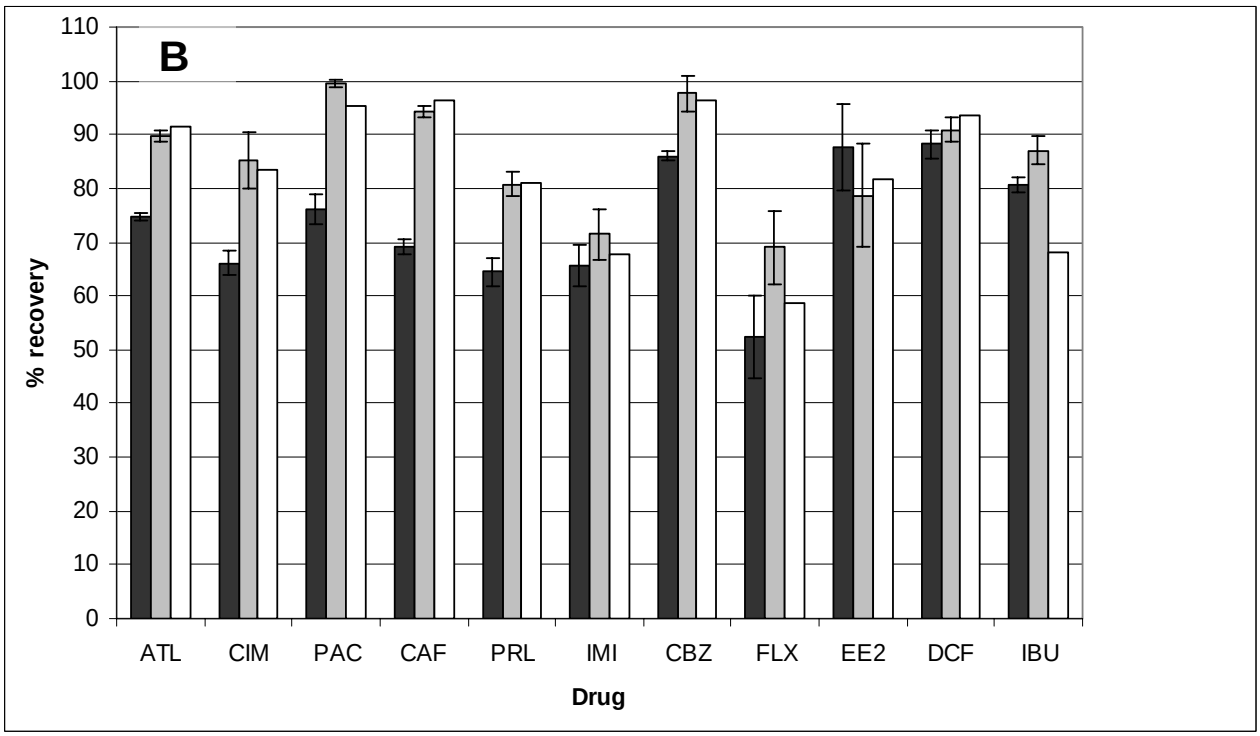
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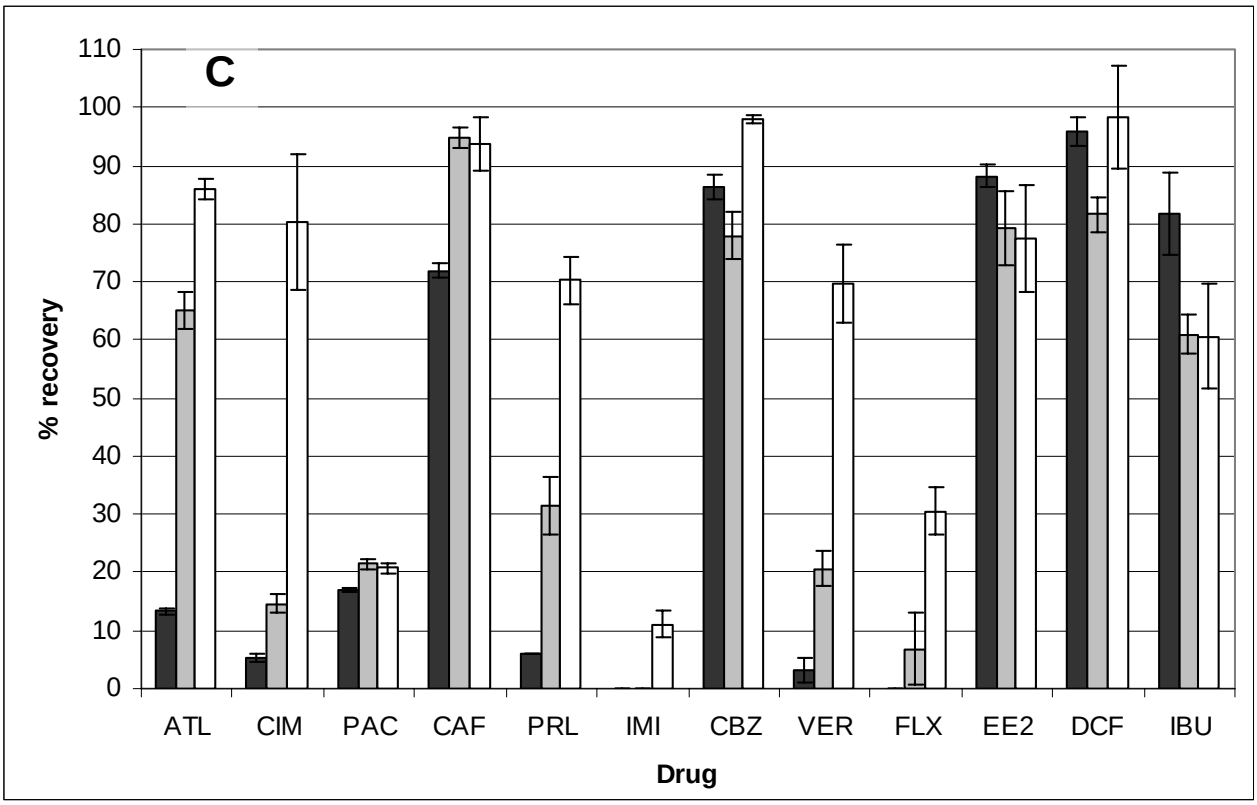


Figure 3.5: Recovery efficiency of pharmaceuticals for (A) HLB, (B) MCX/C_{18} and (C) C_{18} solid phase extraction matrices at pH 3 adjusted with H_3PO_4 (■), pH adjusted with NaH_2PO_4/H_3PO_4 (▨) and pH 7 adjusted with $NaHPO_4/H_3PO_4$ (□).

3.4 Conclusions

The pharmaceuticals selected for this project were based on a number of factors, including their range of physicochemical and pharmacological properties and the potential for their presence in the aquatic environment based on their extent of use. A multi-residue analysis undertaken with HPLC was developed for the selected pharmaceuticals. The HPLC method was found to be sufficiently robust for use as an analytical tool for the experiments conducted for this thesis. Extraction methods were also developed in the case where they were required for sample preparation and clean-up.

Chapter 4. Sorption and desorption of human pharmaceuticals

4.1 Introduction

Interaction of organic contaminants with solids is an important factor when considering their environmental fate. Transport, biodegradation and bioavailability of organic contaminants are all highly dependent on the extent that they are sorbed to solids in the environment (James and Kleinow 1994; Pignatello and Xing 1996; Standley 1997; Huang *et al.* 2003; Warren *et al.* 2003). The extent of sorption of an organic compound is dependent on the molecular structure, ionisation state, water solubility, chemical functional groups, configuration or charge distribution of both the organic compound and solid (Doucette 2000; Warren *et al.* 2003). For example, the clay composition of sediment particles has a greater influence on the interaction of cationic organic compounds compared with unionised compounds, where organic carbon content is of important consequence for sorption (Kookana *et al.* 1998; Doucette 2000; Gevao *et al.* 2000; Li *et al.* 2000; Warren *et al.* 2003; Gao and Pedersen 2005).

Sorption, which includes processes such as surface adsorption, partitioning into hydrophobic domains and incorporation within the physical matrix of solids, can range from being a completely reversible to completely irreversible process (Gevao *et al.* 2000). The extent of reversibility is dependent on the interactions that lead to the binding. For example, an irreversible process, such as sequestration, involves the integration of the

organic compound within the structural matrix of the solid (Pignatello and Xing 1996; Warren *et al.* 2003). As diffusion into the matrix can take from periods of days up to months, measured strength and irreversibility of sorption can increase over time (Pignatello and Xing 1996; Xing and Pignatello 1996; Lesan and Bhandari 2003). Irreversibility of the sorption process is defined by desorption studies, where contaminant-free solution is added to a matrix that already contains the compound of interest. Irreversibility of sorption can have a number of consequences, such as reducing the bioavailability and, therefore, toxicity of a bound organic contaminant (Cornelissen *et al.* 1997; Gevao *et al.* 2000). However, uptake by organisms is still dependent on a number of organism specific processes, such as digestion of sediment or food and exposure to pore water (James and Kleinow 1994; Leppanen and Kukkonen 2000; Sijm *et al.* 2000; Eggleton and Thomas 2004). Therefore, the bioavailability of a sorbed compound should also be considered in terms of the solid acting as a reservoir.

Since sorption to solids is important in determining the environmental fate and effects of organic contaminants, recent guidelines for the ecological risk assessment of human pharmaceuticals have recommended assessment of sorption (EMEA-CPMP 2005). A common approach to determine the extent of sorption is undertaken using batch sorption methods. Batch sorption assessment of organic compounds usually involves shaking a solution containing a compound of interest with a solid phase within an experimental vessel for a given period (usually within 24 h). The concentration of the organic compound within solution (and sometimes within the solid phase) is then determined to assess the affinity of the compound to the solid phase. To assess whether this process is

concentration dependent, the organic compound is spiked in solution as a range of concentrations and the relative affinity at these concentrations is then determined. This concentration dependent relationship is known as the Freundlich isotherm and is defined by the following relationship:

$$C_s = K_f C_{aq}^n \qquad (4.1)$$

where C_s is the concentration of the compound sorbed, C_{aq} is the concentration of compound remaining within solution, K_f is the Freundlich distribution coefficient and n indicates the linearity of the relationship. The Freundlich isotherm is often converted to a logarithmic relationship for convenience as follows:

$$\log C_s = n \log C_{aq} + \log K_f \qquad (4.2)$$

When the relationship is independent of the concentration of organic compound spiked into the system, n=1 and the following relationship is formed:

$$C_s = K_d C_{aq} \qquad (4.3)$$

where K_d is the distribution coefficient. The K_d value, which gives an indication of the affinity that an organic compound has for the solid phase, encompasses a range of sorption processes. As the nature of the solid phase is an important factor for the K_d value, standardised soils can be used to encompass a number of variables that can affect

sorption (USEPA 1998; OECD 2000). Standardised solutions are usually composed of 10 mM $CaCl_2$, although it is has been demonstrated that ionic strength of solution has an influence on the extent of sorption (Figueroa *et al.* 2004; Zhou *et al.* 2004; ter Laak *et al.* 2006a).

Assessment of desorption is undertaken following sorption assessment, where quantification of desorption is the same as sorption; that is, a Freundlich isotherm of the desorption process is generated. When a desorption isotherm is found to have a greater K_f value compared with the sorption isotherm, irreversibility of sorption is implied. There are a number of methods to determine desorption from sediments, including serial, parallel or dilution approaches, each with its own advantages and disadvantages (Bowman and Sans 1985; OECD 2000; Della Site 2001).

This study therefore aims to identify the extent of sorption and desorption of a number of human pharmaceuticals to a freshwater sediment. The pharmaceuticals selected (*see* Chapter 3; Table 3.1) had a variety of physicochemical properties and this was anticipated to give a range of K_d values for the test pharmaceuticals.

4.2 Materials and methods

4.2.1 Test pharmaceuticals

The following pharmaceuticals were used for the generation of sorption and desorption isotherms:

Atenolol (ATL), caffeine (CAF), carbamazepine (CBZ), cimetidine (CIM), diclofenac (DCF), diltiazem (DIL), desipramine (DMI), 17α-ethynylestradiol (EE2), fluoxetine (FLX), ibuprofen (IBU), imipramine (IMI), paracetamol (PAC) and propranolol (PRL). Stock solutions, prepared as 1 gL^{-1} standards in methanol and stored at -18°C, were diluted for use for all working solutions (*see* Chapter 3).

4.2.2 Test sediment

The sediment used for the batch sorption experiments was collected from Mackreath Creek, Scott Creek, South Australia in March, 2005. This sediment has been previously used as a reference test for ecotoxicological assessments and is considered to be free of contaminants due to its position within the catchment area of the Adelaide Hills.

The top 50 mm of sediment was removed using a shovel and transported back to the laboratory in plastic buckets. Sediments were stored at +4°C for 24 h, before being placed

in aluminium foil trays and dried in ovens at 30°C. Total drying time was 4-5 days. After attaining constant weight, samples were sieved through a mesh size of <2 mm. Sediment was then placed in plastic bags and stored in the dark at +4°C until required for use.

Prior to placing in plastic bags, several sub-samples were taken and were used as replicates for assessment of physicochemical properties (Table 4.1). All physicochemical properties were determined on four replicate sediment sub-samples. Sediment pH and electrical conductivity were determined by shaking with 100 mM KCl solution and pH and electrical conductivity of the extract was measured using a Thermo Orion pH/EC meter. Organic carbon content was determined using the Walkley-Black chromic acid digestion (Allison 1965). The cation exchange capacity was determined using ammonium acetate (Chapman 1965) and particle size analysis was undertaken using a hydrometer, with sodium hexametaphosphate used as a dispersing agent (Day 1965).

Solid state ^{13}C NMR of the sediment samples was also undertaken to make further assessments of the sediment characteristics. NMR spectra were obtained at a ^{13}C frequency of 50.3 MHz on a Varian Unity200 spectrometer, using methods described in Smernik and Oades (2000).

4.2.3 Sorption validation experiments

To assess the length of time for each drug to reach sorption equilibrium in the batch sorption system, batch sorption experiments were conducted for respective

pharmaceuticals. The concentration of drug in solution was measured at 0, 1, 2, 5, 8, 16 and 24 h. A one-way analysis of variance (ANOVA) was used to determine whether there was a significant difference between the K_d values for each for each compound following variation of pH or $CaCl_2$ concentration of solution. A Tukey's test ($\alpha = 0.05$) was used as a multiple comparison test if a treatment was found to be significantly different. Toxstat (Version 3.4; Western EcoSystems Technology, Inc; WY, USA) was used to perform ANOVA and Tukey test.

Sorption of the compounds was assessed for the pharmaceuticals on an individual basis and as a mixture to assess whether competitive sorption was apparent. Spiking solutions were prepared, with methanol maintained at a volume of 0.01% of solution for both treatments. The shaking period was 24 h.

To assess whether degradation occurred over the 24 h shaking period, sediment was sterilised by autoclaving in a sealed Schott bottle for 0.5 h. All other materials for the batch sorption analysis, such as containers and solutions, were also sterilised by autoclaving. Stock solutions of drugs (1 gL^{-1} in methanol) were spiked into sterilised 10 mM $CaCl_2$ solutions and batch sorption experiments were performed as in section 4.3.2. All preparation was undertaken in a laminar flow cupboard, using appropriate aseptic techniques.

To assess the effect of sediment particle size, the < 2 mm sieved sediments were further sieved through a < 0.425 mm sieve size. Also, 10 g of sediment was added to another

treatment to test whether the sediment : solution ratio would affect the sorption of the drugs, under the test conditions.

For validation experiments, nominal spiking concentrations were 100 μgL^{-1} for ATL, CIM, CAF, CBZ, PAC and 500 μgL^{-1} for CIM, DIL, IMI, DMI, IBU and 1000 μgL^{-1}.for DCF and FLX.

4.2.4 Isotherm sorption test procedure

As a number of test pharmaceuticals were used with a large variation in their physicochemical properties (*see* Chapter 3; Table 3.1), the sediment to solution ratio for the batch sorption experiments was 1:100. Test pharmaceutical stock solutions were spiked into a volumetric flask and dried with N_2. Methanol was then added to re-solubilise the pharmaceuticals, such that the final volume of methanol was 0.01%. $CaCl_2$ was added to 18.2 $M\Omega cm^{-1}$ water and spiking solutions were made up to volume, with 10 mM $CaCl_2$ used as the reference solution.

Test containers were 100 mL volume Schott bottles, with polytetrafluoroethylene (PTFE) inserts placed into the lids. Schott bottles were wrapped in aluminium foil to ensure solutions were always protected from light. Sediment (1 g) was weighed and placed into pre-weighed Schott bottles. Sediment then had 10 mL of the appropriate molarity $CaCl_2$ added to it and shaken for 24 h. Spiking solution, also weighed, was placed into the Schott bottles to give a total volume of 100 mL. Spiking solutions for the sorption

isotherms were 50, 100, 250, 500, 1000 and 2000 μgL^{-1} (*ca.* 0.2-10 μM) for each drug, with triplicate solutions used for each concentration. The 500 μgL^{-1} spike was not able to be used for analysis.

Schott bottles were placed into a rotating, end-over-end shaker for an appropriate time period. For each batch sorption experiment, ambient temperature was measured on a regular basis over the period of shaking. Schott bottles were then removed from the shaker and centrifuged at 2000 *g* for 10 min and a 10 mL aliquot was removed. This aliquot was filtered through a 0.45 μm nylon syringe filter (Biolab, Australia) prior to HPLC analysis.

The concentration of the test pharmaceuticals within solution were determined by HPLC-PDA (*see* Chapter 3; section 3.3) analysis. The concentration of test pharmaceuticals within spiking solutions was also measured and the difference in initial and final solution concentrations was used to indirectly determine the concentration of drugs within the sediment. The remaining solution had pH and electrical conductivity of solution measured with a Thermo Orion pH/EC meter. The pH probe was calibrated daily, using pH 4, 7 and 10 solutions, while the electrical conductivity meter was calibrated using a 1071 μScm^{-1} solution for a given room temperature.

K_f values were determined using from Equation 4.2, using log transformed data. Log transformation of sediment and solution concentrations were undertaken due to the broad

range of spiking values within the isotherm. K_d values were determined using Equation 4.3 for each batch sorption system.

4.2.5 Desorption isotherm test procedure

Two desorption methods were employed to assess the extent of desorption of pharmaceuticals after sorption equilibrium had been reached. In both cases, the highest spiking concentration in the sorption series was used for desorption. Parallel desorption was undertaken as outlined in Organisation for Economic Cooperation and Development (OECD) guidelines for batch sorption (OECD 2000). Following decanting of sorption solutions, 100 mL of 10 mM $CaCl_2$ (without pharmaceuticals) was added to the sediment and aqueous concentrations were measured after defined 2, 4, 15, 24 and 28 h. This method was used to assess when desorption equilibrium occurred.

Dilution desorption was also undertaken through respectively adding 10, 50, 100, 150 and 200 mL of 10 mM $CaCl_2$ following decanting of sorption solutions of the same spiking concentration. Dilution solutions were removed for analysis after 24 h. This method was undertaken to mimic sequential volumes of drug free solution added to the sediment (Bowman and Sans 1985).

The highest spiking concentration used in the sorption isotherm experiments was used to assess desorption. Care was taken to minimise the loss of sediment with solution. Schott bottles were weighed prior to addition of 10 mM $CaCl_2$ to estimate the contribution of

pharmaceuticals in the solution remaining following the sorption experiments. The extent of hysteresis was determined using the hysteresis index (H):

$$H = \frac{n_f}{n_{fd}} \qquad (4.4)$$

where n_f is the Freundlich constant (Equation 4.1) for the sorption isotherm and n_{fd} is the Freundlich constant for the desorption isotherm (Celis and Koskinen 1999).

4.2.6 Quality assurance / quality control (QA/QC)

Control samples containing sediment and solution, without test pharmaceuticals, were used to assess the potential for interfering peaks for HPLC-PDA analysis. Control samples containing only solution spiked with test pharmaceuticals (containing no sediment) were used to account for losses, such as degradation or sorption to surfaces of the test containers.

During HPLC-PDA analysis, an external standard was run every 5-10 samples to assess reproducibility of peak area.

4.3 Results and discussion

4.3.1 Sediment characterisation

The sediment quality parameters for the sediment used in the experiments are shown in Table 4.1. The measured properties of the test sediment were found to be similar to Soil 7, outlined in OECD Guideline for Testing of Chemicals 106 (2000). The organic carbon content of the sediment was found to be reasonably high, relative to other standardised soil types (OECD 2000). The particle size analysis found a high proportion of the sediment to be sand and silt, with only a moderate proportion consisting of clay. The sediment was also found to be acidic when extracted with 0.1 M KCl, while extraction with 0.01 M $CaCl_2$ was found to give a lower pH (*see* section 4.3.2).

The NMR spectra of the sediment indicated the majority of carbon within the sediment was composed of O-alkyl carbon, with the peak at ~74 ppm due to carbohydrates (Dickens *et al.* 2006; Smernik 2006). The resonance at ~31 ppm is due to unsubstituted hydrocarbons (alkyl), which are commonly found within sediments due to lipids or biopolymers from plants (Dickens *et al.* 2006; Smernik 2006). The prevalence of these lipids in sediments is often attributed to sediments having a stronger sorption capacity than soils, even when normalised to organic carbon content (Doucette 2000). Also, a significant peak was found at ~173 ppm, which can be due to carbonyl groups associated with carboxylic acids, esters or amides (Smernik and Oades 2000). The low pH of the

sediment and of the batch sorption experiments would also indicate the presence of organic acids in this sediment.

Table 4.1: Selected physicochemical properties of the sediment used for sorption experiments. Values shown are mean ± standard deviation of 4 replicates.

pH [a]	EC [b] (μScm^{-1})	% OC [c]	CEC [d] ($cmol_{+}kg^{-1}$)	Particle size analysis		
				%Sand	%Silt	%Clay
6.04±0.07	260±3	5.08±0.05	20.3±0.2	49.7±2.7	34.8±1.7	15.5±1.5

[a] 0.1 M KCl
[b] Electrical conductivity
[c] % organic carbon
[d] Cation exchange capacity

Table 4.2: Integral regions for replicate solid-state ^{13}C NMR analysis for sediment replicates A-D

Replicate	% Carbonyl [a] (190-160 ppm)	% Aryl (160-110 ppm)	% O-alkyl (110-45 ppm)	% Alkyl (45-0 ppm)
A	8.6	23.2	43.8	24.5
B	8.7	23.3	43.5	24.5
C	8.2	22.8	44.3	24.7
D	9.3	24.5	42.6	23.6
Average	**8.7**	**23.5**	**43.5**	**24.5**
Std Dev.	**0.5**	**0.7**	**0.7**	**0.5**

[a] percentage of signal within the carbonyl range

4.3.2 Sorption protocol validation

All pharmaceuticals were found to reach apparent sorption equilibrium within the batch sorption system within 24 h (Figure 4.2). The average solution pH (±standard error) for the kinetic series of treatments reduced from 5.36±0.51 at t = 0 h to 4.75±0.09 for all other times. For most of the drugs, a constant solution concentration within the water / sediment system was reached in less than an hour. Exceptions included although

for the highly water soluble ATL this process took nearly 10 h. This apparent equilibrium represents the rapid phase of sorption, since true sorption equilibrium can take months to occur (Pignatello and Xing 1996).

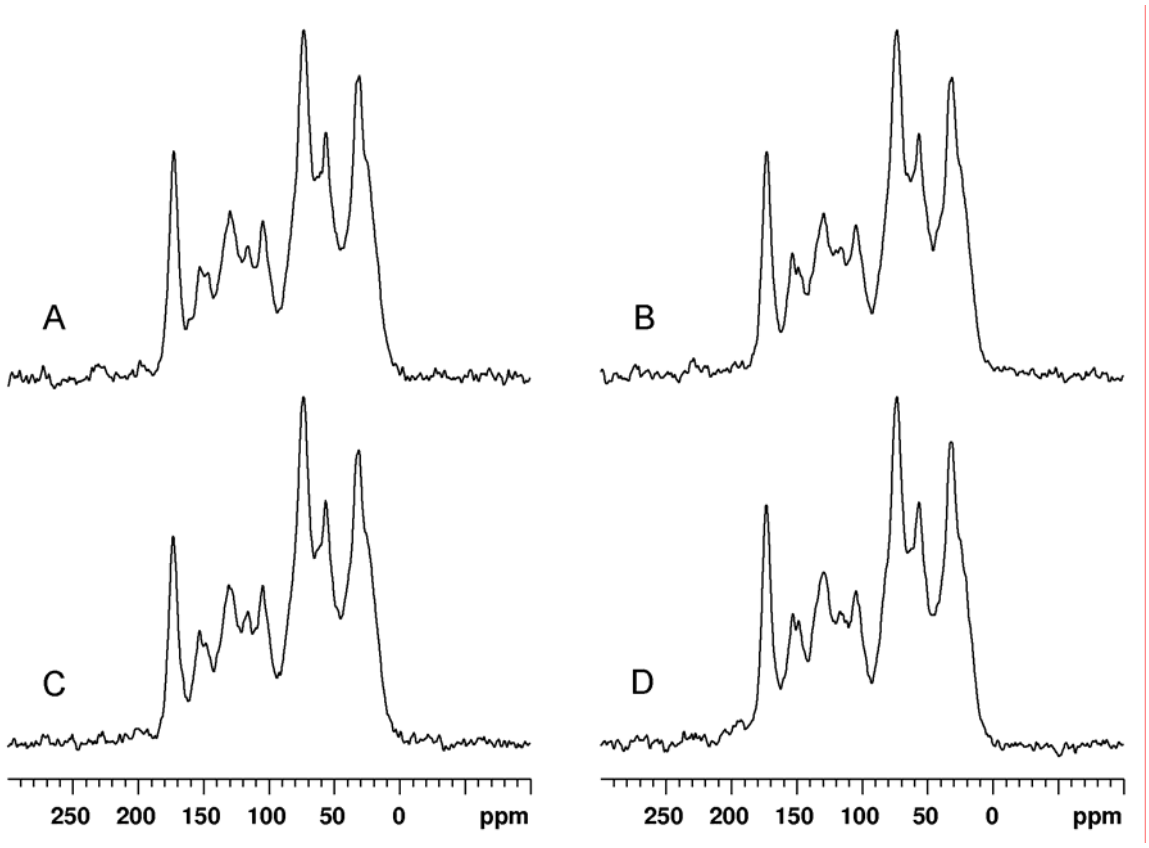


Figure 4.1: Replicate NMR spectra of sediment used for batch sorption experiments. NMR spectra A-D represent 4 sediment replicates.

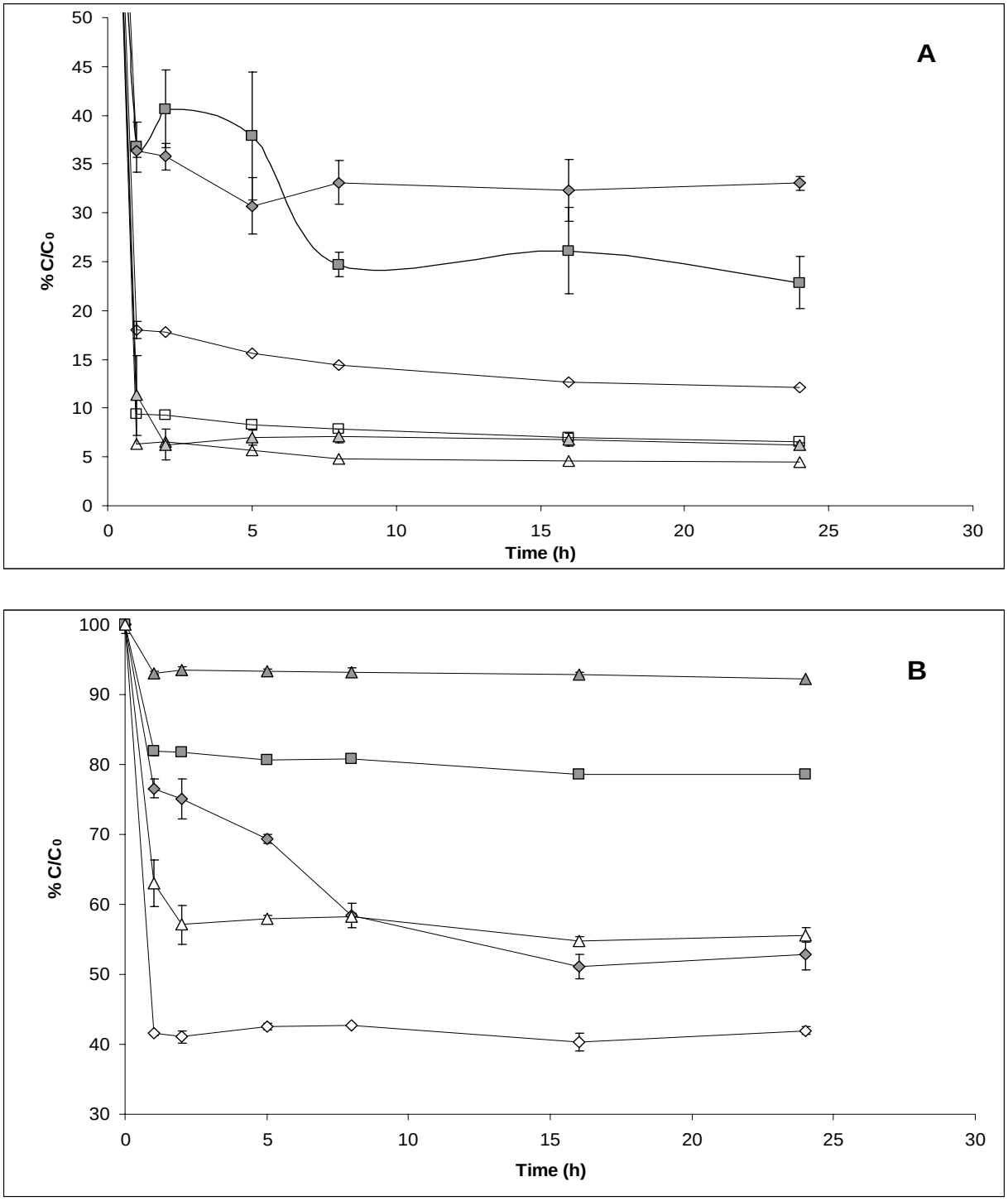


Figure 4.2: Percentage of spiking concentration of test pharmaceuticals in solution, compared with initial spiking concentration (C_0), after various batch sorption shaking times for A: CIM (■), DCF (▲), DIL (◆), DMI (◇), FLX (△), and IMI (□); B: ATL (◆), CAF (◇), CBZ (■), IBU (△) and PAC (▲). Error bars are the standard error of triplicate samples. In all cases, $\%C/C_0$ is 100 at t = 0 h.

However, for practical purposes, such as minimising the potential for degradation, the use of the apparent equilibrium to determine the distribution coefficient was used in this study. Apparent equilibrium was not found to change after it was attained for all test pharmaceuticals within 24 h. This indicates that it was unlikely that degradation of the test compounds occurred within the 24 h shaking period. If degradation was occurring then a slow decrease over the 24 h would be expected. However, an apparent equilibrium was attained rapidly within the 24 h period, without solution concentrations further reducing after an apparent equilibrium was reached (Figure 4.2). A summary of the statistical analyses of the apparent equilibrium concentration obtained after 24 h is given in Appendix F.

All drugs, except PAC and CBZ, had 40 % or more of the spiked amount sorbed to the sediment at the selected spiking values after 24 h. Adjusting the sediment to solution ratio to 1 : 10 was not found to change the extent of sorption for both PAC and CBZ, although, surprisingly, it was found to reduce the K_d values of both DIL and PRL (Figure 4.4). This may be attributed to “noisier” HPLC chromatograms where these drugs eluted, leading to overestimation of peak areas. Another explanation may be related to the overestimation through interaction of these drugs with non-filterable colloidal material, which would have been at relatively higher concentrations with the higher sediment ratio. It was decided to maintain the sediment : solution ratio at 1 : 100.

While the sterilised system seemed to have an effect on the K_d value for CAF, CIM, IBU and PAC (Figure 4.4), it should be noted that the average solution pH (±standard error) was found to be 5.05 ±0.02 in the sterilised treatments, while it was 4.36±0.04 for the sediment ratio and particle size treatments. This would suggest that autoclaving affected the physicochemical properties of the sediment. For ionisable compounds, such as CIM

and IBU, the pH of solution is important in determining the extent of interaction with the solid phase (Doucette 2000; Della Site 2001; ter Laak *et al.* 2006a). This is particularly important when there is a pH change within 2 pH units of the pK_a value of a compound, which was the case for both CIM and IBU (*see* Chapter 3; Table 3.1). Furthermore, it might be expected that a pH change would influence the sorption capacity of this sediment, considering the likelihood of the sediment containing ionisable organic acids (*see* section 4.3.1). While modification of sediment and drug physicochemical properties might have occurred between these treatments, degradation still cannot be discounted. For example, both CAF (Buerge *et al.* 2003) and IBU (Roberts and Thomas 2006) have been shown to be almost completely removed from WWTPs, although it has not been established if this is due to biodegradation.

4.3.3 Sorption isotherms

For the 24 h shaking period of the isotherm experiment, the room temperature was 22±2.5 °C. The final pH (±standard error) of the isotherm solutions was 4.60±0.03. The K_f and mean K_d values for each drug are depicted in Table 4.3, with the log transformed sorption/desorption isotherms depicted in Figure 4.5. The K_f and K_d values indicated the extent of sorption to the sediment was highly variable between drugs (Table 4.3).

4.3.3.1 Sorption isotherm parameters

For many of the pharmaceuticals there was a marked degree of non-linearity for this sediment, as shown for *n* values. An *n* value < 1 implies that as the aqueous concentration of the drug increases the associated sorption to the solid phase decreases, with the inverse

occurring when $n > 1$. For this experiment where n was less than 1, mean K_d values were overestimated by K_f values, while the inverse was apparent where $n > 1$. Where n approached 1, there was good agreement between K_f and mean K_d. Non-linearity of sorption isotherms can occur due to the nature of the adsorbed compound or the sediment. The case of $n < 1$ can occur with polar or ionic species that have a greater tendency to remain in the aqueous phase (Doucette 2000).

This was especially apparent for the drugs with comparatively high water solubilities, such as ATL, PAC, CIM and CAF (Table 4.3). Compared with these water soluble drugs, DCF, EE2 and FLX have substantially lower water solubilities, although these compounds all had $n < 1$ (Table 4.3). Higher variability for aqueous concentrations was apparent for DCF, EE2 and FLX, indicating water solubility may have become an issue at these higher spiking concentrations. Since the linearity of sorption isotherms is dependent on aqueous phase solubility of an organic compound the higher spiking levels for EE2 and DCF may have influenced the linearity of the isotherms (Doucette 2000). Most of the isotherms had $r^2 > 0.92$, except for IBU and PAC (Table 4.3). When the highest spiking concentrations were removed from the PAC isotherm, the linear relationship and linearity of the curve were considerably enhanced, although linearity of the isotherm was still low ($r^2 = 0.94$, $n = 0.55$).

It is possible that the sediment : solution ratio selected for the batch sorption experiments was not ideal for PAC, as more than 80 % of drug remained in solution following the sorption experiments. When the solution concentration is used as an indirect measure of K_d values, small differences in solution concentration can often cause greater experimental error (Chiou 1989). However, the selected ratio was appropriate for the

remainder of the drugs and, furthermore, the K_d for each individual batch sorption system remained reasonably constant for PAC (Table 4.3). Other studies, which suggest a low affinity of PAC to solids, suggest that the K_f value overestimated the extent of PAC sorption (Furlong *et al.* 2004; Jones *et al.* 2006; Lorphensri *et al.* 2006).

With respect to IBU, there was a large degree of variability associated with the isotherm (Figure 4.5), although this was not apparent for the individual K_d values for each batch sorption system (Table 4.3). In this instance, K_f underestimated the mean K_d value for IBU.

Both FLX and IMI had isotherms with a smaller concentration range than the other drugs, since the aqueous concentrations at the lower spiking levels were not detectable. At the higher spiking level, IMI had a high degree of linearity ($n = 0.96$), while the linearity of the FLX isotherm was comparatively poor ($n = 0.41$). Since the concentration range for FLX was less than intended, the higher weighting of the higher concentrations (due to a greater number of samples) may have caused the low linearity that was apparent.

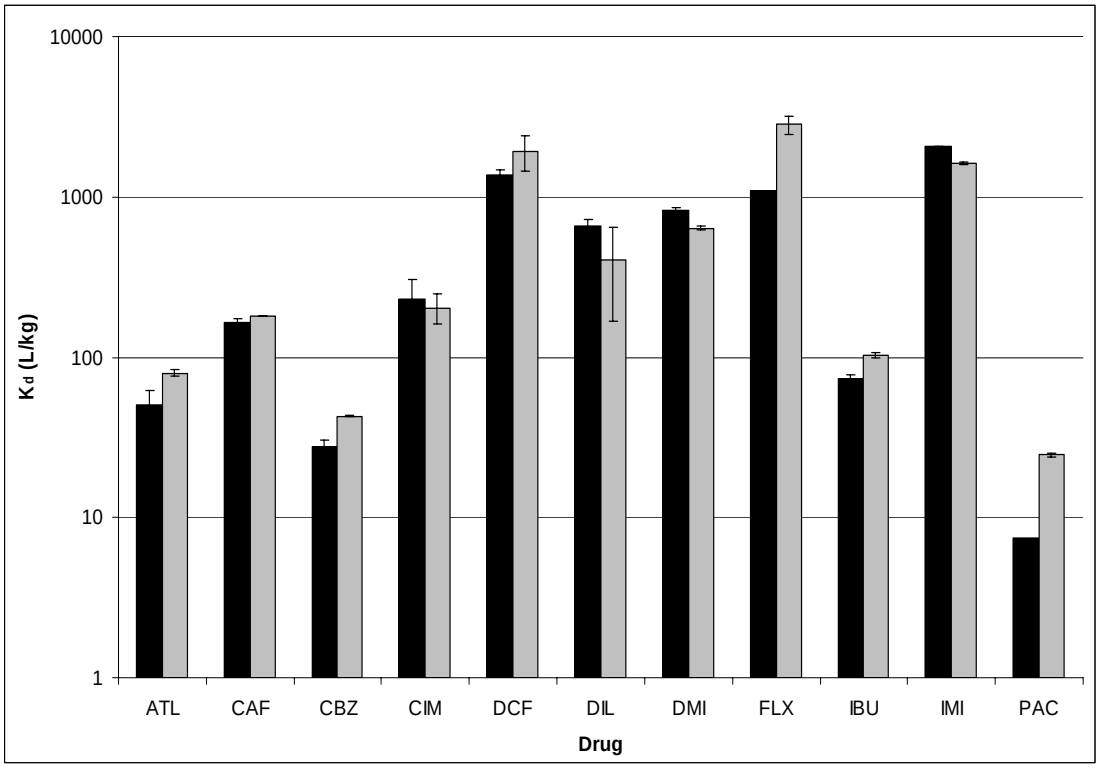


Figure 4.3: Effect of spiking pharmaceuticals as a mixture (■) in batch sorption system, compared with spiking each drug individually (□) into the batch sorption system. Error bars are the standard error of triplicate samples.

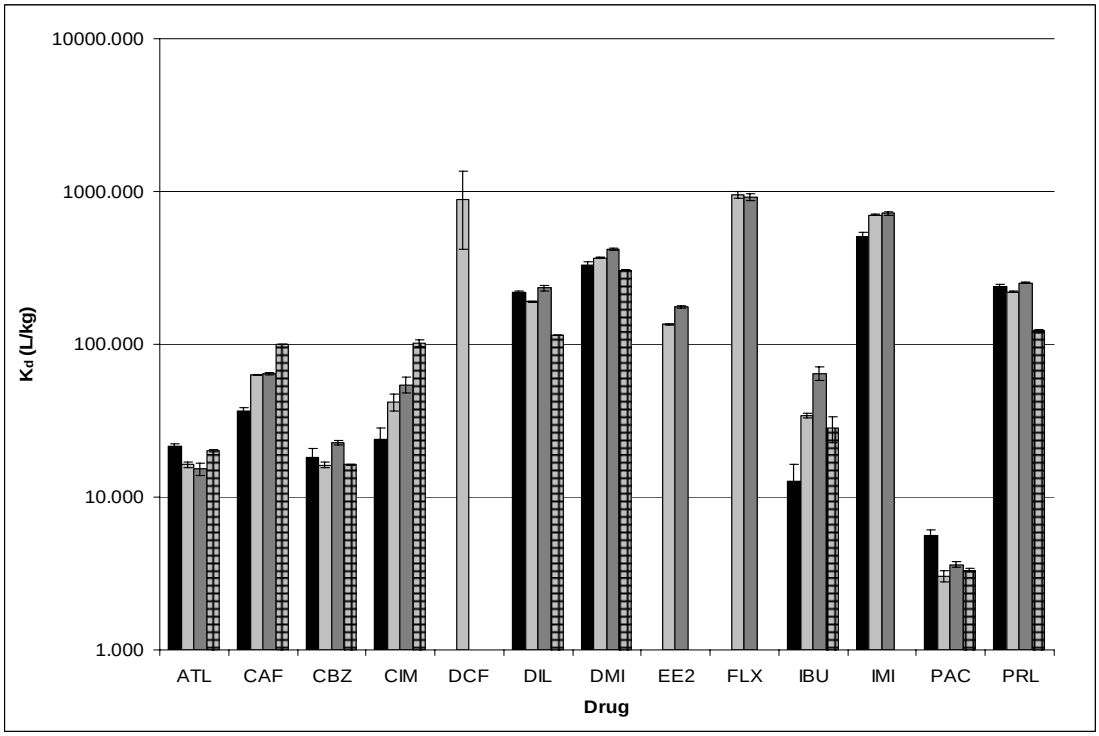


Figure 4.4: The effect of sterilisation (■), particle size (0.425 mm (□) and 2 mm (▩)) and sediment : solution ratio (1 : 10 (▦), compared with 1 : 100 for all other treatments) on distribution coefficient (K_d) of test pharmaceuticals. For the sterile and 1/10 sediment : solution treatments DCF, EE2 and FLX were not detected. DCF was also not detected for the 2 mm sediment treatment. Error bars are the standard error of triplicate samples.

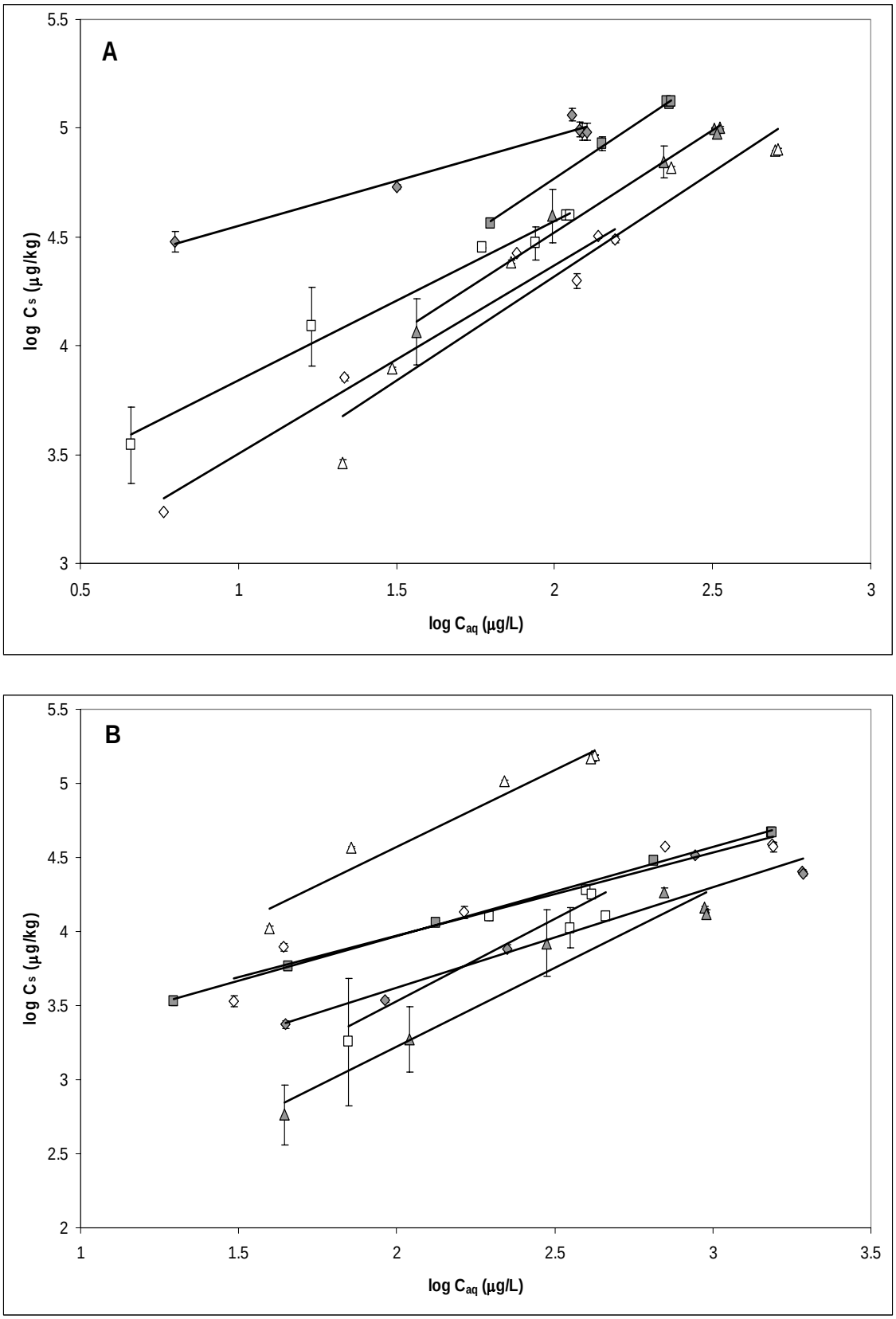


Figure 4.5: Log-transformed sorption isotherms for (A): DCF (◇), DMI (▲),EE2 (□), FLX (◆), IMI (■) and PRL (△); and (B): ATL (◆), CAF (■), CBZ (▲), CIM (◇), DIL (△) and IBU (□). Data is log transformed for aqueous concentrations (C_{aq}) and apparent sediment concentrations (C_s). Error bars are standard error of triplicate samples.

Table 4.3: Sorption parameters for sorption isotherm experiment and literature comparisons. Additional information relating to solid phases from literature values are given in Appendix

Drug	K_f (L/kg)	*n*	r^2	K_d ± s.e.[a] (L/kg)	K_{OC}[b] (L/kg)	Literature K_d (L/kg)	Literature K_{OC}[c] (L/kg)
ATL	186	0.68	0.93	30.6±5.9	602	-[d]	-
CAF	571	0.61	0.99	80.6±22.5	1586	26 (Furlong *et al.* 2004)	-
CBZ	12.4	1.06	0.92	19.8±3.5	389	0.5-37 (Drillia *et al.* 2005) 0.2-5.3 (Scheytt *et al.* 2005) 0.1 (Scheytt *et al.* 2006) 1.2 (Ternes *et al.* 2004) 1.3 (Loffler *et al.* 2005) 69 (Furlong *et al.* 2004)	132-521 161-2650 101 3.5 83 -
CIM	712	0.54	0.92	79.8±25.2	1570	22 (Furlong *et al.* 2004)	-
DMI	407	0.97	0.97	321±25.5	6311	-	-
DCF	460	0.85	0.94	269±30.6	5295	0.5-165 (Drillia *et al.* 2005) 0.6-4.7 (Scheytt *et al.* 2005) 16-459 (Ternes *et al.* 2004)	121-2310 423-2330 47-1310
DIL	331	1.02	0.94	399±41.5	7846	53 (Furlong *et al.* 2004)	-
EE2	1333	0.72	0.92	482±81.2	9484	584 (Andersen *et al.* 2005) 692 (Clara *et al.* 2004b) 2.3-23.4 (Lee *et al.* 2003) 278-349 (Ternes *et al.* 2004) 24.2 (Ying *et al.* 2003)	2089 2042 813-1096 794-860 4840
FLX	13877	0.41	0.96	1618±340	31860	-	-
IBU	10.6	1.2	0.77	44±7.8	866	0.2-1.7 (Scheytt *et al.* 2005) 0.1 (Scheytt *et al.* 2006)	138-1308 238
IMI	702	0.96	0.98	584±8.8	11495	-	-
PAC	208	0.41	0.74	10.6±4.3	209	4.5 (Furlong *et al.* 2004)	-
PRL	279	0.93	0.93	218±34	4299	16-199 (Drillia *et al.* 2005)	2803-4405

[a] Standard error of K_d (n = 16-22)
[b] K_d corrected for organic carbon content of 5.03 %
[c] K_{OC} values derived where values given in literature or where organic carbon content of sediment stated
[d] Data not available

4.3.3.2 Sorption of pharmaceuticals – comparisons with other studies

There is comparatively little data for the sorption of human pharmaceuticals to soils or sediments. Sorption studies that have been undertaken tend to focus on relatively few drugs, such as CBZ, NSAIDS (e.g. DCF and IBU), steroidal estrogens and antibiotics

(Lee *et al.* 2003; Ying *et al.* 2003; Clara *et al.* 2004b; Furlong *et al.* 2004; Ternes *et al.* 2004; Andersen *et al.* 2005; Drillia *et al.* 2005; Loffler *et al.* 2005; Scheytt *et al.* 2005; Scheytt *et al.* 2006) and, therefore, there were few studies to make comparisons with. Furthermore, the experimental procedures, where sorption has been estimated for pharmaceuticals, have not all been batch sorption experiments. As diverse solid matrices such as sediments, soils and sewage sludge have been used in other sorption experiments, K_d values adjusted for organic carbon content (K_{OC}) have also been included to standardise the comparison (Table 4.3). K_{OC} has been commonly used for comparing sorption of non-ionic organic compounds and this assumes sorption is highly dependent on the compound interacting with the solid phase organic carbon. However, this assumption may not be appropriate for ionisable compounds that can interact with the solid phase through other mechanisms (Doucette 2000).

From this series of sorption experiments, EE2, FLX and IMI had the highest sorption values, both in terms of K_f and K_d, while CBZ, IBU and PAC had the lowest sorption values. CBZ, a commonly studied and detected environmental contaminant, is usually found to have a low affinity to the sediment in a variety of solid phases in batch sorption or column experiments and environmental surveys (Furlong *et al.* 2004; Ternes *et al.* 2004; Drillia *et al.* 2005; Hari *et al.* 2005; Loffler *et al.* 2005; Scheytt *et al.* 2005; Scheytt *et al.* 2006) (Table 4.3). While the physicochemical properties of PAC (*see* Chapter 3; Table 3.1), along with a number of studies, have suggested that PAC has low affinity with the solid phase (Furlong *et al.* 2004; Jones *et al.* 2006; Lorphensri *et al.* 2006), Loffler and co-workers (2005) found a substantial fraction of ^{14}C-labelled PAC migrated to sediments, in a sediment-water system. However, they concluded this was likely to be

principally caused by metabolites of PAC that were formed within the sediment, as opposed to sorption.

DCF and IBU are two other pharmaceuticals that have been used in a number of sorption studies. There was good agreement between the K_{OC} values from this experiment (Table 4.3) and those found for DCF and IBU in other studies (Ternes *et al.* 2004; Drillia *et al.* 2005; Scheytt *et al.* 2005; Scheytt *et al.* 2006). Jones *et al.* (2006) also found that just under 20 % of spiked IBU was sorbed in batch experiments.

An environmental survey by Furlong and co-workers (2004) determined concentrations of a number of human pharmaceuticals in water and sediments (n = 44). From this data, the mean K_d values of CBZ, DIL, CAF, CIM and PAC were estimated. In comparison with the drugs used in this work, there was a reasonable agreement with these K_d values, although the DIL K_d value from this study was nearly an order of magnitude higher (Table 4.3). While there is likely to be greater variability associated with field-collected samples, it is worth noting that such sediment could represent the pharmaceuticals in an "aged' state, where lengthy interaction with the sediment gives a more appropriate estimate of true equilibrium concentration (assuming inputs are more or less constant) (Pignatello and Xing 1996; Lesan and Bhandari 2003).

The sorption values obtained in this study for PRL were in good agreement with Drillia and co-workers (2005), where a soil with high organic carbon content and low pH was also used. On the other hand, Jones *et al.* (2006) found at the same solid : solution ratio (1 : 100) that less than 10 % of PRL was bound to the solid phase after 5 h of mixing. The hydrophobic nature of PRL, even in its ionised state, would suggest a reasonable extent

of PRL should bind to solids. Sorption of organic contaminants has often been related to the octanol-water partition coefficient (K_{OW}) (Doucette 2000). There was poor agreement with the K_f values and K_{OW} ($r^2 = 0.01$, $p = 0.75$), which was likely to be related to the degree of non-linearity associated with the K_f values. However, there was a significant relationship between K_d and K_{OW} ($r^2 = 0.46$, $p = 0.01$), which was probably related to K_d taking into account the non-linearity of sorption.

The concentrations of drugs spiked into the batch sorption systems are at least an order of magnitude higher than aqueous concentrations found in environmental surveys (Kolpin *et al.* 2002; Andreozzi *et al.* 2003; Ashton *et al.* 2004; Ferrari *et al.* 2004). Where sorbent concentrations are dilute, it is often assumed that sorption will be linear (Pignatello 1989). It has been shown for EE2 that sorption to sewage sludge is linear over a concentration range of orders of magnitude, even when its water solubility is approached (Lee *et al.* 2003; Clara *et al.* 2004b; Andersen *et al.* 2005). Drillia and co-workers (2005) also found linear sorption for PRL, CBZ and DCF in the low mgL^{-1} range, where the water solubility of CBZ and DCF (at least for the pH 4.3 treatment) was approached. However, a number of pharmaceuticals were seen to have non-linearity associated with their isotherms in this work. While the properties of the sediment may be a contributing factor in the non-linearity, a number of other factors may have contributed. As previously mentioned, these factors could have been the high water solubility of a number of the drugs, while, for the less water soluble drugs, variability of measured solutions at the highest spikes was likely to have led to observed non-linearity (Figure 4.5). Also, this type of sorption isotherm, known as an “L-curve”, could indicate the sorption capacity of the sediment decreases as binding sites become less available at higher contaminant concentrations (Giles *et al.* 1960). Visual assessment of the isotherms and reproducible

mean K_d values for all drugs indicated concentration dependence of sorption was not likely to occur over the range of spiking concentrations used.

4.3.4 Desorption isotherms

4.3.4.1 Desorption of test pharmaceuticals

For the time series desorption experiments, the concentration of drug in solution for all drugs, except FLX, varied little. The percent coefficient of variation (%CV) of the extent of desorption ranged from 1.4 % for CAF and DMI to 9.8 % for IBU (Table 4.4). However, the %CV for FLX was 25 %, where there was no difference between the concentrations at 2 and 4 h or 15, 24 and 28 h (where concentrations were 3 times higher than the earlier time period). This indicates that there was some time dependence of desorption within the 28 h period for FLX only.

The dilution series showed that after 24 h desorption could be simulated using varying sizes of reservoirs of drug-free solution (Figure 4.6). For some drugs, such as EE2 and FLX, the measured solution concentrations did not vary greatly from those seen for the adsorption series (Figure 4.6 and Table 4.4). This would suggest that while a large fraction of the drug bound to the sediment could be removed through desorption, a substantial fraction remained within the sediment. In contrast, the desorption isotherms of CAF and CBZ tended to fall on the sorption isotherm (Figure 4.6). This indicates that there was little desorption hysteresis evident for these compounds. The final desorption point of CBZ seems to indicate a degree of hysteresis occurred (Figure 4.6). However, considering the relatively weak sorption found for CBZ, it is likely that this point was an anomaly.

In general, the pharmaceuticals had either the majority of drug remaining sorbed to sediment after 28 h (DMI, DCF, DIL, EE2, FLX and PRL) or the majority being desorbed into solution (Table 4.4). While the extent of desorption seemed to be correlated with the water solubility (*see* Chapter 3; Table 3.1) and extent of adsorption of the drugs (Table 4.3), there were two notable exceptions. IBU was observed to have more than 50 % of its original sediment load desorbed in contrast with the similarly hydrophobic, acidic DCF. This contrasting behaviour was also noticed for the K_f / K_d values. Also, CBZ has a limited water solubility, although its low K_f and K_d values indicate a comparatively low affinity for the sediment. Based on the moderately high K_{OW} and low water solubility of CBZ and IBU (*see* Chapter 3; Table 3.1), it might be expected that they would have a stronger affinity with the sediment. An alternative explanation could be that CBZ and IBU associate with the non-filterable colloidal fraction of the sediment, which would lead to lower observed adsorption and desorption values (Huang *et al.* 1998). Such an artefact may be the cause of the anomalous point seen for the desorption isotherm of CBZ (Figure 4.6).

4.3.4.2 Desorption hysteresis

Non-singularity, or hysteresis, of sorption isotherms is indicated where the slope of the desorption isotherm is greater than the adsorption isotherm (Pignatello 1989). Desorption hysteresis is due to the test compound being retained within “resistant” and irreversible fractions of the sorbent (Di Toro and Horzempa 1982; Chiou 1989; Pignatello 1989). Resistant fractions represent domains of the sediment from which the sorbate will be released beyond the completion of the desorption experiment (Pignatello 1989). This is in

contrast with irreversible fractions that essentially capture, or absorb, the sorbate within their domain, where desorption is extremely limited.

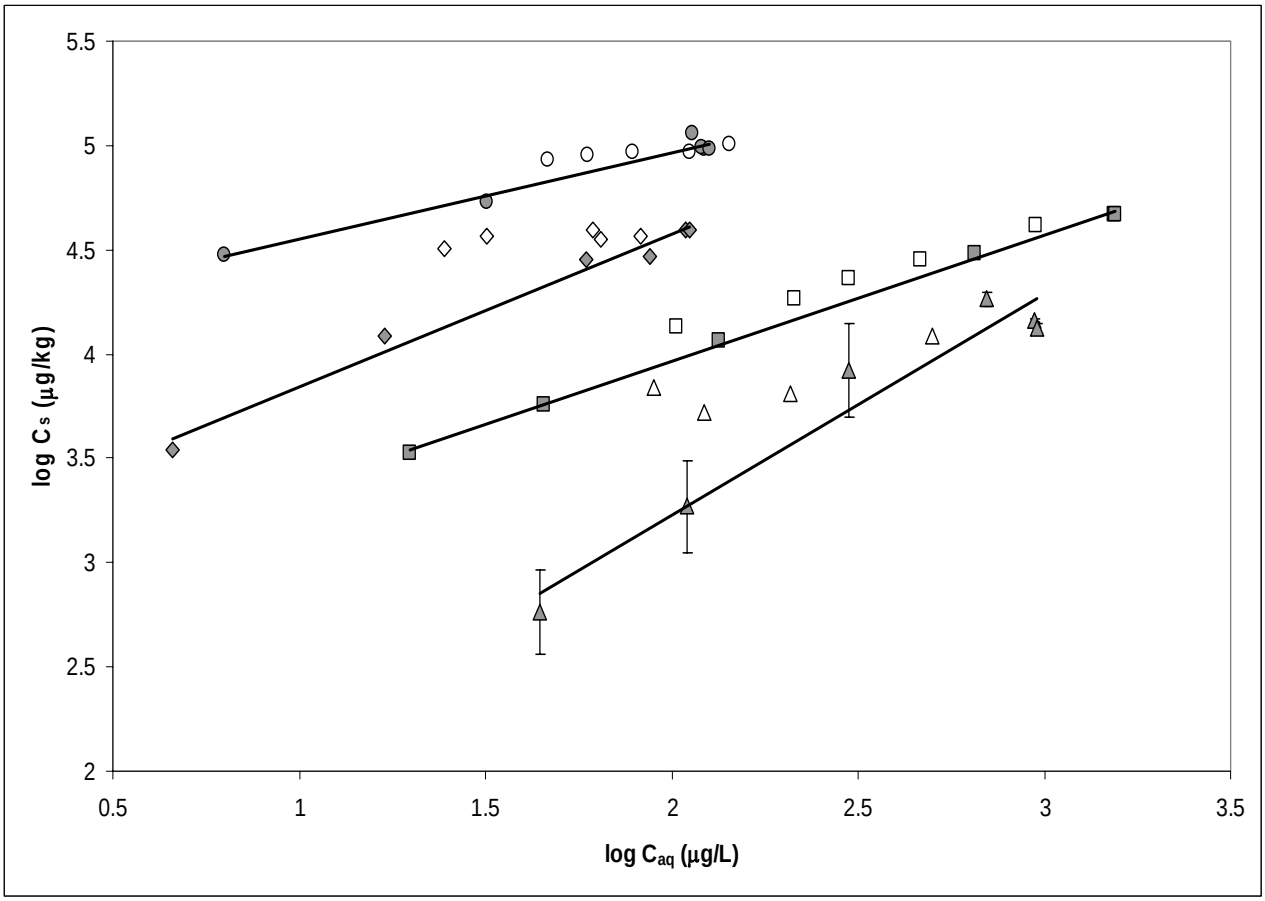


Figure 4.6: Log-transformed sorption and desorption isotherms for selected drugs; CAF (sorption (■) and desorption (□)), CBZ ((▲) and (△)), EE2 ((◆) and (◇)) and FLX ((●) and (○)). Regression lines are for sorption isotherm only. Desorption isotherms were derived from dilution method. Error bars are standard error of triplicate samples.

Hysteresis can also occur due to a number of other factors, such as artefacts specific to the experimental procedure undertaken, degradation of the test compound, competitive sorption from materials residing within the sorbent, loss of sorbed compound from the system or sorption equilibrium not being attained (Bowman and Sans 1985; Pignatello 1989).

Table 4.4: Isotherm parameters for desorption isotherm experiment

Drug	K_f (L/kg) [a]	*n*	r^2	H [b]	% drug desorbed (%CV) [c]
ATL	762	0.46	0.99	0.68	69 (4.6)
CAF	1633	0.46	0.99	0.75	54.8 (1.4)
CBZ	1015	0.38	0.87	0.36	61.5 (4.7)
CIM	2187	0.38	0.85	0.70	68.9 (7.2)
DMI	22367	0.25	0.76	0.26	19.6 (1.6)
DCF	8873	0.24	0.59	0.28	19.3 (4.6)
DIL	3824	0.61	0.92	0.60	17.5 (7.1)
EE2	24808	0.1	0.51	0.14	9.8 (7.6)
FLX	53641	0.13	0.7	0.32	5.9 (25.5)
IBU	422	0.61	0.8	0.79	50.5 (10.7)
IMI	5253	0.59	0.72	0.61	13.8 (3.7)
PAC	6123	0.06	0.22	0.15	53.8 (5.8)
PRL	18117	0.24	0.89	0.26	26 (2)

[a] From dilution series (*see* Figure 4.2)
[b] Hysteresis index from Equation 4.4, based on dilution series for desorption
[c] % of drug originally bound to sediment desorbed after 28 h based on desorption time series, with % coefficient of variation (n=5)

Adsorption to the colloidal matter may also lead to hysteresis, as removal of colloidal material through decanting will reduce their effect during the desorption step (Pignatello 1989; Della Site 2001). That is, there will be less drug associated with the colloidal fraction during the desorption step, leading to lower measured aqueous concentrations of the drug. If there was significant binding to the colloidal fraction for a number of the drugs, then this could explain the deviation seen in the desorption series, particularly for the higher dilution levels (for example, CBZ in Figure 4.6). As higher dilutions are used (and, therefore, lower aqueous concentrations of drug), the relative effect of removal of the colloid-associated drug would have been more apparent. The dilution method has been suggested to reduce the occurrence of desorption hysteresis, as it does not require centrifugation, which can lead to an experimental hysteresis (Bowman and Sans 1985; Huang *et al.* 1998).

The desorption experiment using the dilution method was undertaken within a relatively short time frame, in order to reduce the potential for degradation to occur. The low %CV of desorption measured for the majority of drugs within solution for the desorption time series suggests that degradation during desorption was unlikely within 28 h (Table 4.4). Also, the use of the dilution method required only one decanting step, which minimised the loss of drug bound to sediment or colloids following the sorption period. An adsorption / desorption experiment looking at a range of human pharmaceuticals was undertaken by Drillia and co-workers (2005). The desorption isotherms obtained by Drillia *et al.* (2005) showed hysteresis, although not to the same degree as that seen in this study (Table 4.4). While the desorption methods employed were different, making comparisons between degrees of hysteresis difficult, desorption hysteresis was apparent in both studies.

4.3.5 Limitations and implications of batch sorption

There are a number of limitations associated with the batch sorption assessment undertaken in this study. For example, the interaction is of a short-term nature and, therefore, the apparent equilibrium achieved is not likely to represent a true equilibrium state (Pignatello and Xing 1996). The non-attainment of true equilibrium would also have an influence on the extent of desorption of a drug from the sediment (Doucette 2000; Lesan and Bhandari 2003). The sediment that was used for this study was from a highly specific source; any differences in the physicochemical properties determined for this sediment would be expected to have an influence on the extent of sorption determined for each drug (Table 4.3) Due to the diverse physicochemical properties of the test

pharmaceuticals, the extent of sorption found within other systems would be expected to be variable and unpredictable. That is, a number of interacting factors of the system and drug would be likely to influence the extent of sorption, making direct comparisons with other system more difficult (de Jonge and de Jonge 1999; Fabrega *et al.* 2001; Drillia *et al.* 2005; ter Laak *et al.* 2006b). Finally, the concentrations of test pharmaceuticals in solution were, for practical purposes, much higher than what would be considered environmentally relevant from current surveys of waterways.

Despite a number of limitations existing with the methodology used in this study, a number of important generalisations regarding the sorption of pharmaceuticals within an aquatic system can be made. When organic contaminants associate with sediments, they can take on the transport characteristics of these sediments (Warren *et al.* 2003). For example, suspended sediment can accumulate in areas further downstream from discharge points, leading to localised areas of high concentrations of associated organic contaminants, such as estuaries (Warren *et al.* 2003). In general, association with solids and desorption hysteresis can reduce the biodegradability of organic contaminants (Pignatello and Xing 1996; Huang *et al.* 2003) and bioavailability to sediment feeding organisms (Lamoureux and Brownawell 1999). However, hysteresis has been linked with bioavailability (Lawrence *et al.* 2000) and poorly understood feeding and digestive regimes of aquatic organisms would have an important influence on bioavailability of sediment bound contaminants (James and Kleinow 1994; Pignatello and Xing 1996; Standley 1997).

4.4 Conclusions

Rapid equilibrium processes occurred within the first 24 h of the batch experiments for all the test pharmaceuticals, with the extent of sorption to the sediment found to be highly variable. Also, varying degrees of desorption hysteresis implied a fraction of the drugs that adsorbed to the sediment were unable to be removed following addition of drug-free water over an additional 24 h period. Pharmaceuticals are often found to be continually released into the aquatic environment, which is conducive to slow sorption processes occurring. If the isotherm non-singularity observed in this work exists for other sediment / solution systems, there could be a number of important implications for transport within aquatic systems and bioavailability to aquatic organisms. This degree of hysteresis, particularly for drugs with higher affinities to the sediment, would also be expected to be more pronounced, if the length of the sorption experiment was substantially longer than 24 h.

To exclude the possibility of artefacts causing the apparent hysteresis, more work is required to assess the sorption / desorption potential for human pharmaceuticals. Another important factor requiring further investigation, is the influence of specific factors existing within the sorption system (including both the sediment and solution) and relating to the respective drugs that influence the extent of their sorption. This will give an insight into how release into various aquatic systems can influence the fate of pharmaceuticals and the potential implications this has for their risk assessment in aquatic ecosystems. This seems especially prudent considering the lack of information relating to the sorption of human pharmaceuticals in aquatic systems.

Chapter 5. The influence of water quality parameters (pH and Ca^{2+} ionic strength) on the sorption of pharmaceuticals

5.1 Introduction

Current environmental risk assessment procedures for human pharmaceuticals recommend undertaking studies to assess the potential for pharmaceuticals to remain within sediment, in both short- and long-term assessments (OECD 2002; EMEA-CPMP 2005). The results from these studies should indicate whether sediment ecotoxicity testing is necessary. However, the sediments used for these studies are field collected and only need to vary in organic carbon content and texture (OECD 2002).

Many factors related to the physicochemical properties of solution or of the solid phase can influence the extent of sorption of organic compounds. This can occur through altering the sorption capacity of the solid phase or the compound or both. The Organisation for Economic Cooperation and Development (OECD) guidelines recommend using a number of standardised soils to account for the factors that can influence the extent sorption of an organic compound, in batch sorption assessments (OECD 2000). These factors include the organic carbon content, clay content and soil texture and pH. However, the extent of sorption, often described by the partition coefficient (K_d), only needs to be normalised to the organic carbon content of the solid phase (K_{OC}) in the OECD guidelines. The use of K_{OC} has been found to be a useful means of predicting the extent of sorption of an organic compound when soils with diverse organic carbon contents are compared (Doucette 2000; Della Site 2001; OECD 2001a).

While this is especially useful for organic compounds that are not able to undergo ionisation, pharmaceuticals are often found to have pH-dependent functional groups that can be ionised. Ionised organic compounds will have different physicochemical properties of the unionised compound which will modify their affinity for the solid phase (de Jonge and de Jonge 1999; Lorphensri *et al.* 2006; ter Laak *et al.* 2006b; ter Laak *et al.* 2006a). The solid phase can also have its physicochemical properties affected by changes in solution pH and ionic strength (Murphy *et al.* 1994; Doucette 2000). Changes in the properties of the solid phase can then influence the affinity of an organic compound to the solid phase. Variation in the ionic strength of an environmental solution has also been shown to alter the K_d value of organic compounds (Brownawell *et al.* 1990; de Jonge and de Jonge 1999; Oste *et al.* 2002; Zhou *et al.* 2004; ter Laak *et al.* 2006a).

The aim of this study was to assess the role of pH and ionic strength (in terms of concentration of Ca^{2+}) of solution in influencing the distribution of the test pharmaceuticals, in a batch sorption system. Furthermore, the species-specific contribution to distribution was estimated at different solution pH values. Therefore, a number of pharmaceuticals were assessed that have a range of ionisation potentials (i.e. acidic, basic and neutral) over a range of pH values. The data from these experiments were then discussed in terms of the importance of distribution, and the factors that influence distribution, of human pharmaceuticals when evaluating the potential for their fate.

5.2 Methods and materials

5.2.1 Test pharmaceuticals

For the batch sorption experiments, 11 human pharmaceuticals were selected; they were atenolol (ATL), caffeine (CAF), carbamazepine (CBZ), cimetidine (CIM), desipramine (DMI), diltiazem (DIL), diclofenac (DCF), fluoxetine (FLX), ibuprofen (IBU), imipramine (IMI) and paracetamol (PAC) (*see* Chapter 3; Table 3.1). Both 17α-ethynylestradiol (EE2) and propranolol (PRL) were not available for this series of experiments. All pharmaceuticals were obtained from SigmaAldrich Pty Ltd. (NSW, Australia) and were prepared as 1 gL^{-1} stock solutions in methanol (Malinckrodt, Germany). A working solution of 1000 μgL^{-1} was prepared by mixing an appropriate volume of the respective stock solutions, evaporating the methanol under a gentle stream of N_2 and making to volume with 18.2 $M\Omega cm^{-1}$ water. Pharmaceuticals were prepared as spiking solutions from the working solution. Initial spiking concentrations were 100 μgL^{-1} for ATL, CAF, CBZ, CIM and PAC, 500 μgL^{-1} for DMI, DIL, IBU and IMI and 1000 μgL^{-1} for DCF and FLX.

5.2.2 Batch sorption

The batch sorption sorption / desorption isotherms produced in Chapter 4 were all seen to have a degree of non-linearity associated with them (*see* Chapter 4; Table 4.3). Non-linearity of the isotherms seemed to be related to the K_d values associated with the higher spiking levels. Either the highest spiking concentration was seen to have a high degree of

concentration dependence, compared with the rest of the isotherm (e.g. ATL, PAC, CAF, CIM), or there was a high level of variability associated with highest spiking concentration replicates (e.g. DCF, EE2, FLX, IBU). Therefore, the spiking level for each drug was at the lower range of the isotherms, although still high enough to enable acceptable analysis. At the lower concentrations, the effect of non-linearity on sorption isotherms is progressively smaller. The extent of sorption was therefore defined by the following relationship:

$$K_d = \frac{C_s}{C_{aq}} \qquad (5.1)$$

5.2.3 Adjustment of pH and ionic strength

Solutions used for the batch sorption experiments had their pH and [Ca^{2+}] manipulated to assess their influence on the K_d values of the pharmaceuticals. A 10 mM $CaCl_2$ solution had its pH varied through the addition of 1 M KOH, where target final solution pH values were 4.5, 5.5, 6.5 and 9.5. This broad range of pH values included all of the pK_a values of the represented pharmaceuticals. The drugs with ionisable functional groups, therefore, had varying levels of ionisation for at least one of the pH treatments. 1 M KCl was added to solutions to ensure the ionic strength of the solutions were the same for all treatments.

Ionic strength of solution was altered by preparing solutions of $CaCl_2$ with concentrations of 1, 5, 10, 50 and 100 mM. The presence of Ca^{2+} in solution can influence the configuration of humic acids and surface binding sites of sediment particles (Zhou *et al.* 2004). This includes aggregation of sediment particles, which can occur for [Ca^{2+}] = 3 mM at pH 4 (Oste *et al.* 2002). Standard batch sorption experiments are

conducted at $[Ca^{2+}] = 10$ mM to minimise cation exchange processes and give some degree of coagulation of the solids (OECD 2000). Changing the concentration of $CaCl_2$ was therefore undertaken to demonstrate how this variable environmental parameter could influence the sorption of pharmaceuticals.

A mixture of salts was also used for the solution to assess how this would influence the sorption of the pharmaceuticals. Moderately hard water (MHW), commonly used for ecotoxicological assays, was used as a solution for this purpose. MHW is composed of 0.35 mM $CaSO_4.2H_2O$, 0.5 mM $MgSO_4$, 1 mM $NaHCO_3$ and 0.054 mM KCl (USEPA 2002). The MHW used in this experiment used 0.41 mM $CaCl_2.2H_2O$ in place of $CaSO_4.2H_2O$, along with the other salts required to prepare MHW, for this series of experiments. For another treatment, MHW was prepared with 1 mM $CaCl_2$ to make a comparison with the 1 mM $CaCl_2$ treatments. The ionic strength (*I*) of each solution was determined based on the following relationship:

$$I = 0.5(v_C^2[C] + v_A^2[A]) \qquad (5.2)$$

where v_C and v_A are the valencies of the cation and anion of the salt, respectively, and *[C]* and *[A]* are the respective concentrations of the cation and anion of the salt.

Sediment used for the batch sorption experiments (*see* Chapter 4; Table 4.1) was field collected, sieved to < 2 mm particle size, air dried (30±1 °C) to a constant weight and stored at + 4 °C, prior to use in the experiments. For more details, refer to Chapter 4, section 4.2.2.

A one-way analysis of variance (ANOVA) was used to determine whether there was a significant difference between the K_d values for each for each compound following variation in pH or $CaCl_2$ concentration of solution. A Tukey's test ($\alpha = 0.05$) was used as a multiple comparison test if a treatment was found to be significantly different. Toxstat (Version 3.4; Western EcoSystems Technology, Inc; WY, USA) was used to perform ANOVA and Tukey test.

5.2.4 Test procedure

The test procedure for batch sorption and subsequent preparation and analysis by HPLC was undertaken as in Chapter 4 (*see* Chapter 4, section 4.2).

5.2.5 QA/QC

Controls containing spiked solution without sediment for each treatment accounted for any loss of pharmaceuticals not related to sediment sorption. Also, controls containing sediment with unspiked solutions showed no interference of HPLC analysis occurred. Limits of detection and quantitation for the HPLC analysis were determined using data from Chapter 3 (*see* Chapter 3; Table 3.2).

5.3 Results and discussion

5.3.1 Variation of sorption as a function of pH

The final pH of the test solutions were 4.48±0.02, 5.53±0.13, 6.46±0.05 and 9.53±0.03, while ambient temperature was 22.0-24.0 °C. Figure 5.1 gives an overview of the changes in ionisation of an acidic or basic functional group for the pharmaceuticals dependent on the pH of solution. Among the test pharmaceuticals that were ionisable over the tested pH range, only one ionisable functional group was present per compound. The pK_a values for each drug are given in Chapter 3, Table 3.1.

For the basic pharmaceuticals, no definite trend could be found for the K_d values over this range of pH values (Figure 5.2). The K_d values of IMI and DMI were found to increase as pH increased, whereas the K_d values for FLX, another cationic drug with a high log K_{ow}, decreased with increasing solution pH. The other cationic pharmaceuticals showed a decreasing trend in K_d values with increasing pH (Figure 5.2). Also, K_d values for neutral CBZ, structurally related to IMI and DMI, decreased as solution pH increased.

A summary of the statistical analyses of the K_d values obtained after adjustment of solution pH is given in Appendix G.

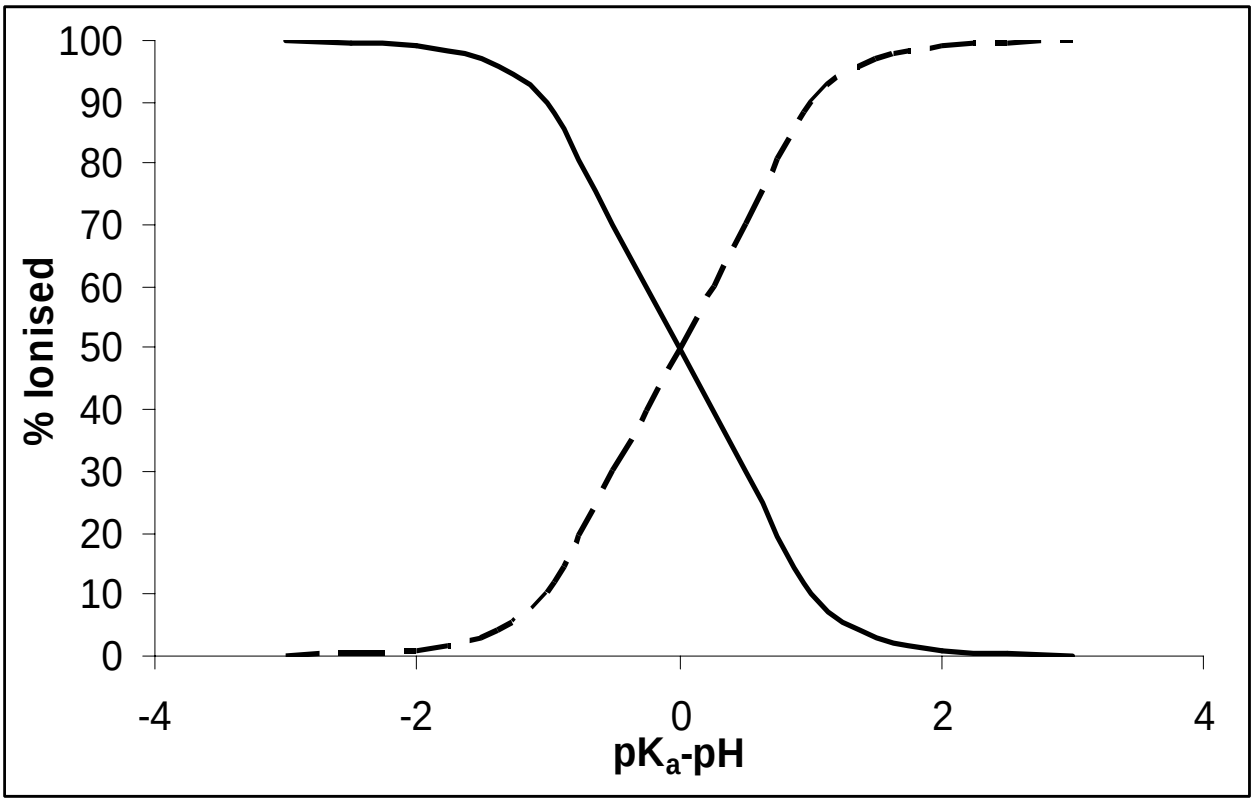


Figure 5.1: The effect of pH on the extent of ionisation of a drug with a mono-acidic (solid line) or mono-basic (dashed line) functional group. The x-axis shows the difference between the pK_a value of the drug and the pH of solution it is dissolved in.

5.3.1.1 Neutral and acidic pharmaceuticals

The K_d values of IBU were found to substantially decrease as pH increased. A decrease was evident even at pH 5.5, less than one pH unit above the pK_a of IBU (Figure 5.2). The K_d value of PAC, which would have been partially ionised at pH 9.5 being a weak acid, increased only at this pH. For CAF and CBZ, which remained unionised over the experimental pH range, increasing solution pH led to a decreasing trend in their respective K_d values.

Hari *et al*. (2005), with reported K_d values substantially lower than this study, found that the K_d of CBZ and PAC were not affected by changing solution pH. As there was no influence of pH on the physicochemical properties of PAC or CBZ, varying pH was likely to have had an affect on the binding capacity of the sediment. The binding capacity of the sediment would be affected by the pH-dependent change in the extent of ionisation of functional groups, particularly of anionic carboxyl and phenolic hydroxyl groups, within the sediment (Gevao *et al.* 2000). For example, at an equivalent ionic strength, a solution of higher pH will reduce the aggregation of organic acids within sediment as repulsion between charged functional groups increases (Murphy *et al.* 1994). Reducing the aggregation of organic acids would have implications for access to the physical structure and surface area of the sediment.

Where the change of solution pH led to the ionisation of a pharmaceutical, the effect of the charged species, as well as the sediment, needs to be considered for the sorption process. For acidic IBU, the decrease in sorption as pH increased could be explained by both the increasing ionisation of IBU and sediment functional groups. As a greater proportion of anionic carboxylate groups formed, the water solubility of IBU would have increased, giving IBU a greater tendency to remain within the aqueous phase. Furthermore, the sediment would have increased its overall negative charge as more anionic groups formed, leading to electrostatic repulsion between the sediment and IBU. This effect has been seen with acidic antibiotics, where sorption greatly decreases as pH increases beyond their respective pK_a values (Hari *et al.* 2005; ter Laak *et al.* 2006a).

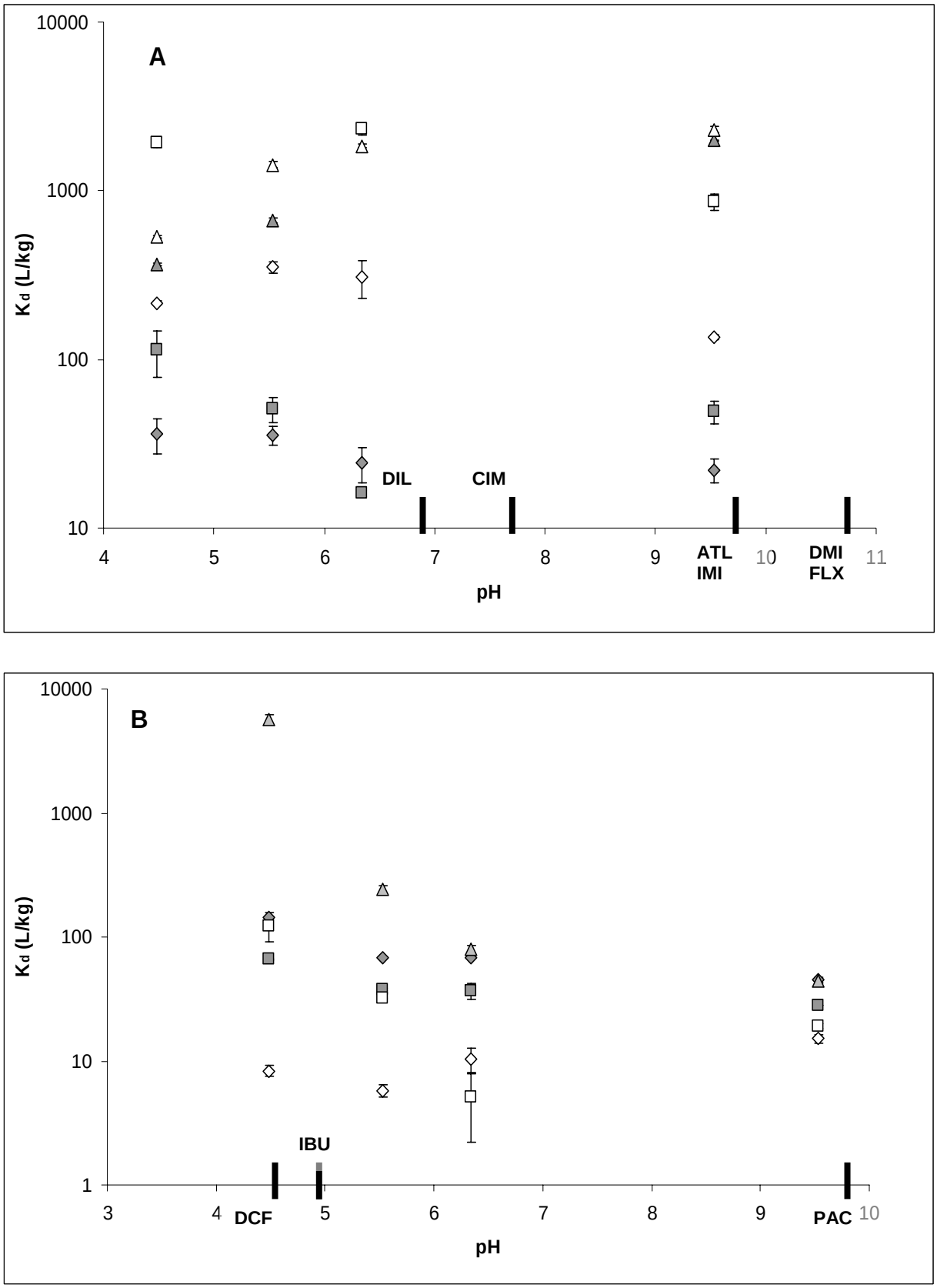


Figure 5.2: Distribution coefficient (K_d) plotted against solution pH for drugs that were (A) basic; ATL (◆), CIM (■), DMI (▲), DIL (◇), FLX (□), IMI (△) or (B) neutral and acidic; CAF (◆), CBZ (■), DCF (△), IBU (□), PAC (◇) within the measured pH range. The pK_a values for each drug are marked on the x-axis with solid, black lines. Error bars are standard error of the mean K_d value.

5.3.1.2 Basic pharmaceuticals

The less predictable pH dependence of sorption for the cationic pharmaceuticals is likely to be related to the diversity of test pharmaceuticals within this category. The weaker bases CIM and DIL, while both experiencing similar extents of ionisation over the pH range, had distinctly different sorption behaviour (Figure 5.2). The same is also apparent for the drugs with stronger basic groups (higher pK_a values), FLX, IMI, DMI and ATL (Figure 5.2). Part of this may be explained by other physicochemical properties of the drugs, such as the water solubilities of the unionised form of the drug. A number of sorption processes, such as ion exchange and hydrophobic interactions, can occur simultaneously (Gevao *et al.* 2000; Tolls 2001). For example, it has been reported that the highest extent of sorption for triazine pesticides is close to their pK_a value, where ionic and hydrophobic sorption processes would occur (Gevao *et al.* 2000). Also, the sorption of dodecylpyridinium was found to slightly decrease with decreasing pH, which was attributed to alteration of the surface charge of the sorbent or ion exchange between H^+ and the dodecylpyridinium cation (Brownawell *et al.* 1990). If the water solubility of a drug is such that hydrophobic interactions are less important than ionic interactions, then the cationic species would play a more important role in binding.

5.3.2 pH-dependent species contribution to sorption

Further analysis of the role of speciation of the drugs was assessed by determining species-specific contribution to the K_d value. Recently, ter Laak and co-workers (2006b) determined the species-specific contribution to K_d of 3 ionisable antimicrobials, tylosin, oxtetracycline and sulfachloropyridazine. Using batch sorption experiments with

artificially altered pH values, the contribution of each species to the K_d value was found from the relationship:

$$K_d' = K_{d1}.\alpha + K_{d2}.(1-\alpha) \qquad (5.3)$$

where K_d` is the apparent K_d value of both ionised and unionised species, K_{d1} is the sorption coefficient for the species at pH lower than the pK_a value of the drug, K_{d2} is the sorption coefficient for the species at pH greater than their pK_a value and α is $1/(1+10^{(pH-pKa)})$ at a particular system pH value. This relationship is derived from the Henderson-Hasselbach equation (*see* Appendix A for derivation of Equation 3).

Using this approach, the species-specific K_d values for each pharmaceutical in this study was assessed. From rearranging Equation 3 (ter Laak *et al.* 2006b):

$$K_d' = K_{d2} + \alpha(K_{d1} - K_{d2}) \qquad (5.4)$$

K_{d2} can be estimated from the intercept of the regression between K_d` and α, while K_{d1} is the difference of the slope and intercept.

A similar analysis for most of the pharmaceuticals in this study was not appropriate since regression analysis was not representative of the entire range of ionisable moieties possible. This is because many of the basic pharmaceuticals have pK_a values greater than 9.5, so even at the pH 9.5 treatment more than half of the representative species would have been ionised. Even for the acidic drug DCF, a substantial fraction of the drug species would have been ionised at the lowest pH treatment.

Only the weakly basic CIM and acidic IBU had α-values spanning greater than 0.5. Respective plots of K_d against α indicate that, while pH is an important factor in determining K_d for this set of pharmaceuticals, it is likely that a number of other factors within the system are influencing K_d. For example, as pH increased K_d was seen to decrease, while IBU was completely ionised ($\alpha << 0.1$) (Figure 5.3). In the case of CIM, there seems to be a sorption minimum around its pK_a (Figure 5.3). The opposite of this has been observed, with the maximum K_d being found around the pK_a (Gao *et al.* 1998; Gevao *et al.* 2000). The authors concluded a combination of sorption factors were possible at the pK_a, such as ionic and hydrophobic interactions, which led to maximum sorption.

Analysis using Equation 5.4 assumes that only the physicochemical properties of the compounds are influenced by the change in solution pH. In the case of the work done by ter Laak *et al.* (2006a; 2006b) this may be a reasonable assumption considering the limited pH range used. However, this assumption cannot be expected to hold for the solution pH range (4.5-9.5) that was used in this study. As previously stated, the binding capacity of the sediment may have been altered by the wide pH range of solution.

While it may have been useful to consider *a priori* an analysis using a smaller range of pH values within the pK_a values of individual drugs, this would have had implications for environmental realism; 1.5 pH units below 4.5 and above 10.5 would be required to encompass all the representative pK_a values of the test pharmaceuticals.

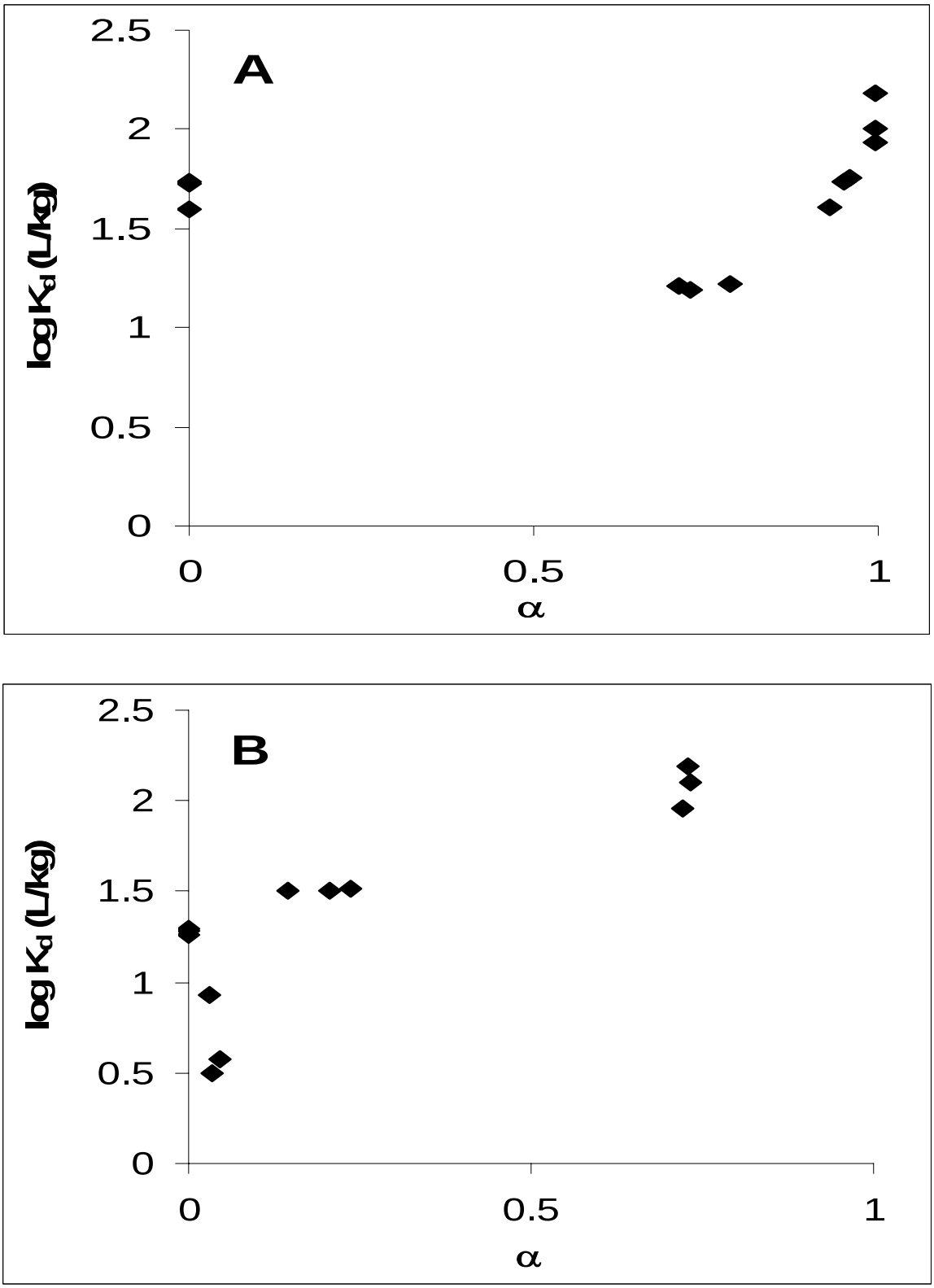


Figure 5.3: Log distribution coefficient (log K_d) plotted against α for (A) CIM and (B) IBU. At $\alpha = 0$, basic CIM is in its unionised form, while acidic IBU is in its ionised form. The ionisation state then changes until $\alpha = 1$, where CIM is ionised and IBU is unionised.

5.3.3 Variation of sorption as a function of [Ca^{2+}]

Addition of $CaCl_2$ to the solution was found to decrease the final pH of the batch sorption solutions, where the pH ranged from 4.74±0.01 at 1 mM $CaCl_2$ to 4.30±0.03, for 100 mM $CaCl_2$, while the temperature ranged from 22.9-23.8 °C. This decrease in pH is related to the displacement of protons from the surface of the sediment by Ca^{2+} (de Jonge and de Jonge 1999). For the neutral and basic pharmaceuticals, this decrease in pH would not have greatly affected the extent of ionisation of the test pharmaceuticals. A summary of the statistical analyses of the K_d values obtained following adjustment of the $CaCl_2$ concentration in solution is given in Appendix G.

5.3.3.1 Acidic pharmaceuticals

The decrease in solution pH with increasing $CaCl_2$ concentrations may have been a more important factor for the acidic pharmaceuticals IBU and DCF due to their pK_a values (*see* Chapter 3; Table 3.1) being close to the experimental pH range. However, increasing solution $CaCl_2$ concentration did not affect the sorption of IBU, except at 100 mM $CaCl_2$, where the K_d value was seen to be slightly elevated (Figure 5.4). According to the Henderson-Hasselbach equation:

$$\frac{A^-}{HA} = 10^{(pH - pK_a)} \qquad (5.5)$$

the ratio of ionised (A^-) to unionised (HA) for IBU in this pH range would be around 0.25 at pH 4.3 to 0.67 at pH 4.74. Therefore, the influence of a higher ratio of unionised IBU

would have caused the higher K_d at the lower pH value. Also, increased amounts of the Ca^{2+} ion bound to the sediment could facilitate bridging between anionic IBU and the sediment surface (Spark and Swift 2002).

The other acidic drug, DCF, showed an apparent increase in K_d as the concentration of $CaCl_2$ increased to 50 mM, while it was below the limit of quantitation for the 100 mM Ca^{2+} treatment. This led to a very high series of K_d values for DCF (Figure 5.5). It is also interesting to note that the K_d value of DCF for the 10 mM Ca^{2+} treatment was more than 10 times greater than those found in the sorption / desorption study (*see* Chapter 4; Table 4.3). The pH for 10 mM $CaCl_2$ in the Ca series was 4.48±0.01, compared with 4.60±0.03 in the sorption / desorption work (*see* Chapter 4; section 4.4.3). The Henderson-Hasselbach equation (Equation 5.5) indicates that slightly more unionised DCF, compared with ionised DCF, would have existed for the former (ratio = 0.95) and the reverse for the latter (ratio = 1.26). Whether this was enough to lead to such a dramatic difference in K_d values is difficult to determine without a bigger dataset. However, the fact that the 1 mM $CaCl_2$ treatment had a pH of 4.74 and a similar K_d value to the 10 mM $CaCl_2$ treatment (from the Ca series) would indicate that there may have been another experimental artefact that dramatically altered the K_d value.

Controls containing no sediment for the Ca series indicated that recovery of DCF was around 46 % compared with 70 % for the isotherm series, despite being in solutions of identical $CaCl_2$ molarity. The pH of $CaCl_2$ solutions, without the addition of sediment, was found to be 5.32±0.08. The water solubility of unionised DCF is 2.37 mgL^{-1} (*see* Chapter 3; Table 3.1), although it would be substantially higher when the ionised species is present. However, the spiking level of DCF for this series of experiments (1 mgL^{-1})

may still have presented problems, in terms of DCF water solubility. Furthermore, the addition of Ca^{2+} to solution may have exacerbated this problem by causing a "salting out" effect, leading to variability in K_d values (Rogers 1993; Brunk *et al.* 1997; Zhou and Liu 2000). DCF has been spiked in other studies at concentrations of up to 12 mgL^{-1} in 10 mM $CaCl_2$ solutions with a soil of pH 4.3, although the K_d value determined (165 Lkg^{-1}) and isotherm linearity (n = 0.92) did not indicate solubility issues (Drillia *et al.* 2005). In another study, indirect measurement of K_d (measurement of solution concentrations only) of DCF did not yield any data, when DCF was spiked at 0.86 mgL^{-1} in 10 mM $CaCl_2$ with a soil of pH 4.8 (Scheytt *et al.* 2005). However, in the same study a direct measurement (measurement of solution and sediment concentrations) revealed that DCF had a low K_d (0.55 Lkg^{-1}). From the present study, and others at pH values around the pK_a of DCF, it is difficult to ascertain whether DCF is affected by water solubility, although it does suggest more careful analysis for DCF is required (such as including more pH values for treatments) when working at pH values close to its pK_a value.

5.3.3.2 Basic pharmaceuticals

The K_d values of the basic pharmaceuticals were found to decrease with an increasing molarity of $CaCl_2$ (Figure 5.4). This type of result has been found for a number of other cationic compounds and is likely to be related to competitive binding of Ca^{2+} with negatively charged sites on the sediment surface (Brownawell *et al.* 1990; Kookana *et al.* 1998; Della Site 2001; ter Laak *et al.* 2006a). A study by ter Laak and co-workers (2006a) showed that increasing the concentration of $CaCl_2$ from 0.01 to 500 mM led to a concurrent decrease in the sorption of the weakly basic antibiotic tylosin. A similar effect was demonstrated for aniline and α-naphthylamine in soil / water systems, particularly

when the concentration of $CaCl_2$ increased from 5 to 50 mM in acidic soil treatments (Fabrega *et al.* 2001). Brownawell and co-workers (1990) found that the sorption of the dodecylpyridinium cation was found to decrease as the concentration of inorganic cations, in particular Ca^{2+}, increased in solution.

5.3.3.3 Neutral pharmaceuticals

There was no consistent effect of increasing the concentration of $CaCl_2$ on the neutral pharmaceuticals. The K_d value of CBZ increased at 5 and 10 mM $CaCl_2$, before decreasing at 100 mM $CaCl_2$ (Figure 5.4). A similar effect has been demonstrated for the neutral compound phenanthrene, with its sorption greater at 50 mM $CaCl_2$ compared with 5 and 500 mM $CaCl_2$ (Zhou *et al.* 2004). It was postulated that the intermediate concentrations were a balance between Ca^{2+} facilitating uptake and sediment aggregation excluding phenanthrene. In the same study, using a different soil, increasing the concentration of $CaCl_2$ led to a decreasing trend in phenanthrene sorption. A similar trend was seen in this work for CAF, unionised at the pH of the $CaCl_2$ experiments, while the K_d values of PAC were not affected over the range of solution [Ca^{2+}] (Figure 5.4).

5.3.3.4 Sorption to colloids – implications for K_d

One aspect of the experimental design which needs to be considered is the use of 0.45 μm filters. A solution of low ionic strength is found to be associated with a greater degree of dispersion of sediment, leading to higher concentration of colloids within the water column (de Jonge and de Jonge 1999). Following filtration, colloid-associated drugs would have been classified as part of the C_{aq} fraction, effectively reducing the K_d value

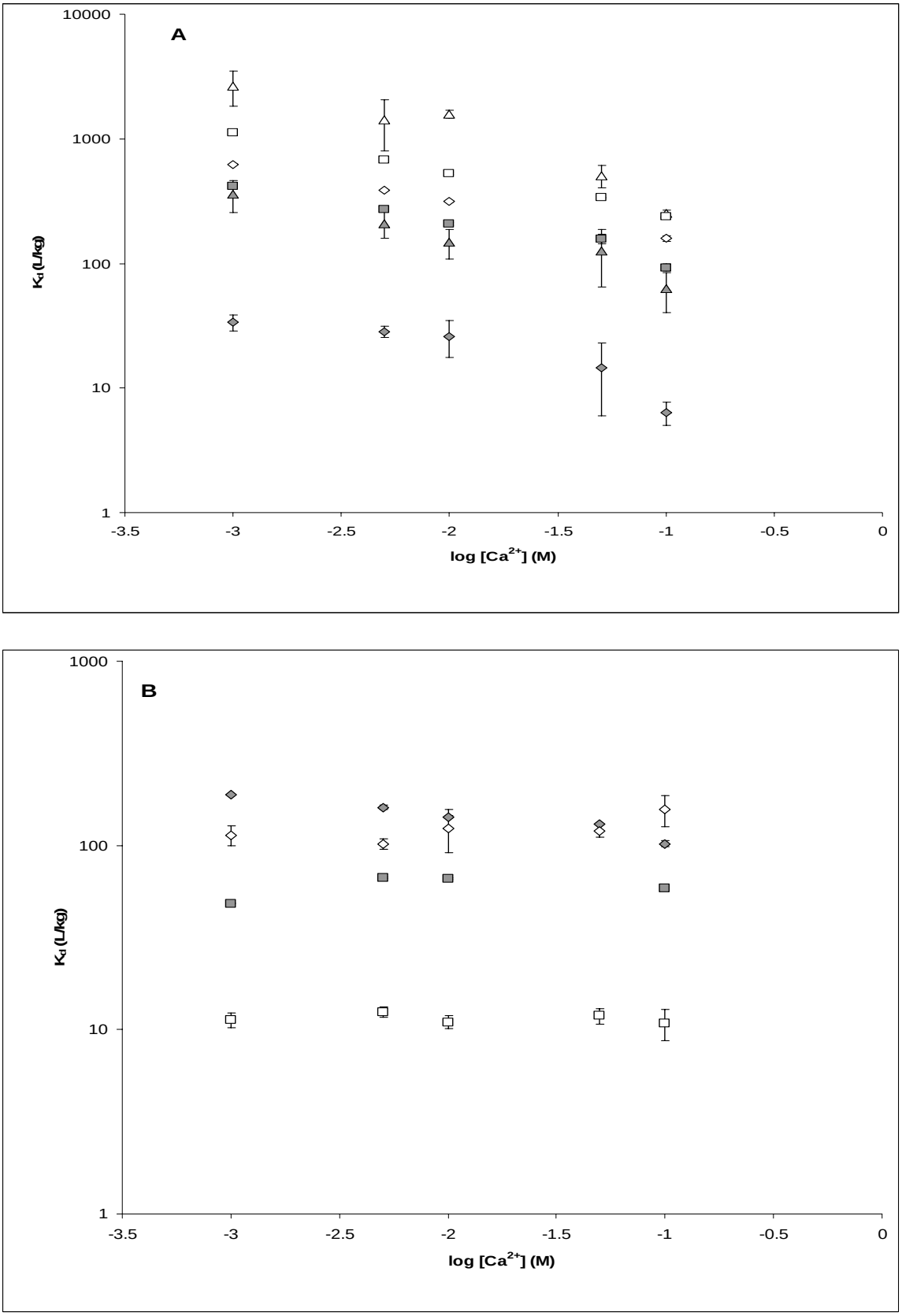


Figure 5.4: The effect of Ca^{2+} (expressed as molarity) on the K_d value of pharmaceuticals with (A) basic; ATL (◆), CIM (▲), DIL (■), DMI (◇), FLX (△), IMI (□) and (B) acidic or neutral; CAF (◆), CBZ (■), IBU (◇), PAC (□) functional groups. Error bars are standard error of triplicate K_d values.

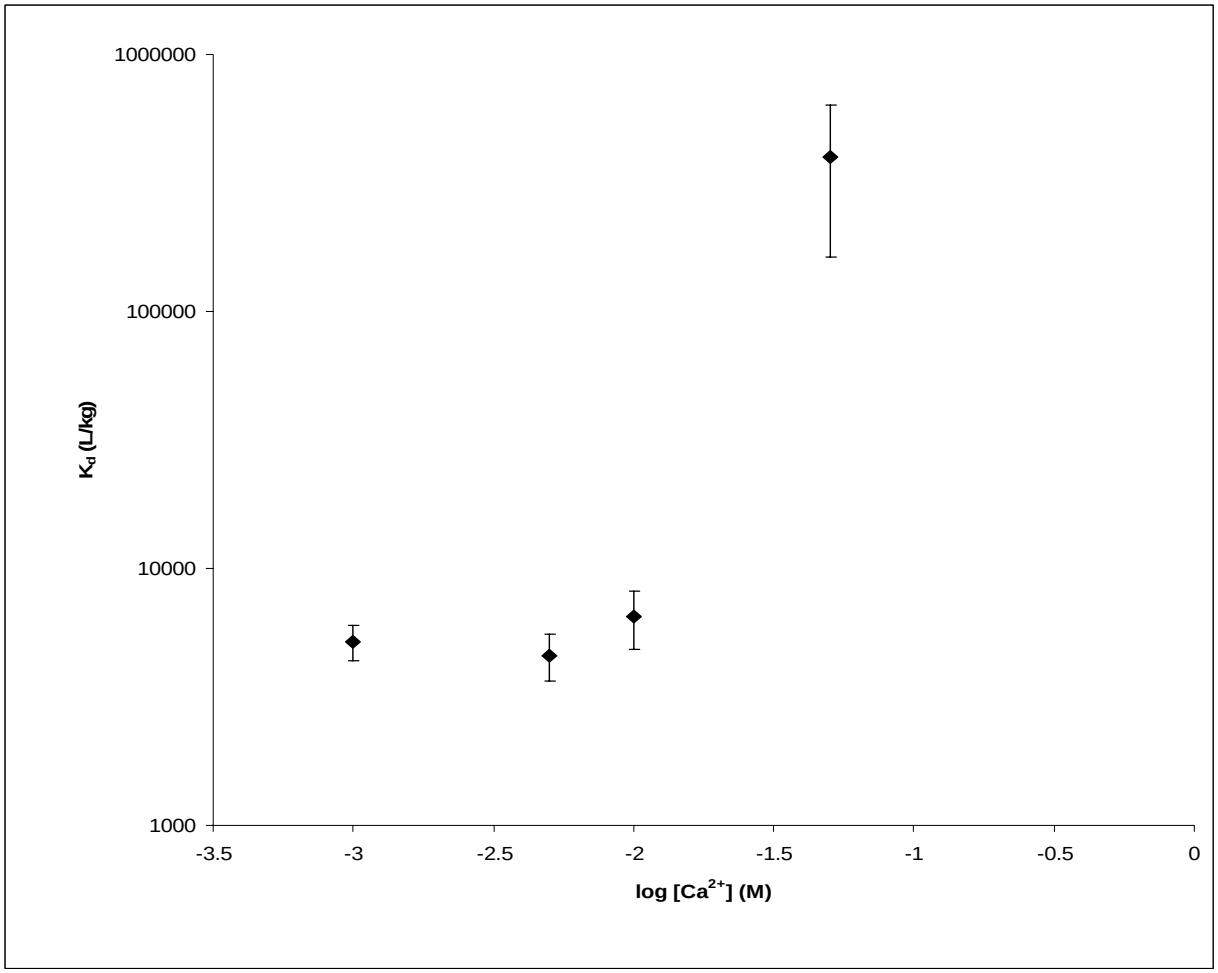


Figure 5.5: The K_d value of DCF based on the solution concentration of Ca^{2+}, expressed as molarity

(Equation 5.1). However, the opposite effect was seen for CAF and the cationic drugs, suggesting sorption to colloids was not as important as sorption to the bulk sediment.

Increasing the concentration of Ca^{2+} would have affected the sorption potential of the sediment, particularly when it facilitates bridging between ionised sites on the sediments (Murphy *et al.* 1994; Spark and Swift 2002) At higher concentrations of Ca^{2+}, the overall charge of sediment would become more positive and reduce the affinity of the solid phase for cationic species (Zhou *et al.* 2004; ter Laak *et al.* 2006a). The further complication of variation of solution pH would also influence the extent that this bridging can occur, as

sediment anionic groups vary their charge and, therefore, extent of interaction with cations.

5.3.4 Variation of sorption as a function of solution salts and pH; the case of moderately hard water (MHW)

Addition of MHW led to a solution pH of 6.51±0.02, while amendment with 1 mM $CaCl_2$ led to a reduction of pH to 6.07±0.03. The temperature of the batch sorption systems over 24 h was 22.0-24.0 °C. Based on Equation 5.2 the ionic strength of the MHW solution was 0.0043, the 1 mM $CaCl_2$ 0.003 and the MHW with 1 mM $CaCl_2$ 0.006. Therefore, while the absolute ionic strengths of the solution were low, the ionic strength of the solution containing MHW combined with 1 mM $CaCl_2$ was twice that of the 1 mM $CaCl_2$ solution, with the MHW solution an intermediate value.

The difference in pH between the two systems would have been important for CIM and, to a lesser extent, DIL and IBU, which have pK_a values close to the pH range. The lower pH solution (MHW amended with 1 mM $CaCl_2$) showed a decrease in K_d for CIM. While Figure 5.3 suggests this is where CIM has a minimum sorption value for pH, all other basic drugs also exhibited a decrease in K_d values when MHW had 1 mM $CaCl_2$ (Figure 5.6). Although pH may have had an effect on the K_d of CIM it was also likely that increased molarity of $CaCl_2$ also reduced the K_d value of CIM (and other basic drugs), as occurred in the other $CaCl_2$ series of experiments (Figure 5.4).

For IBU, there was no difference between K_d values of the MHW solutions, while the K_d value substantially increased for the 1 mM $CaCl_2$ solution (Figure 5.6). A similar pattern was apparent for DCF. Considering the difference in pH between solutions, this would

further indicate the importance of solution pH, compared with Ca^{2+} concentrations, for sorption of these acidic pharmaceuticals.

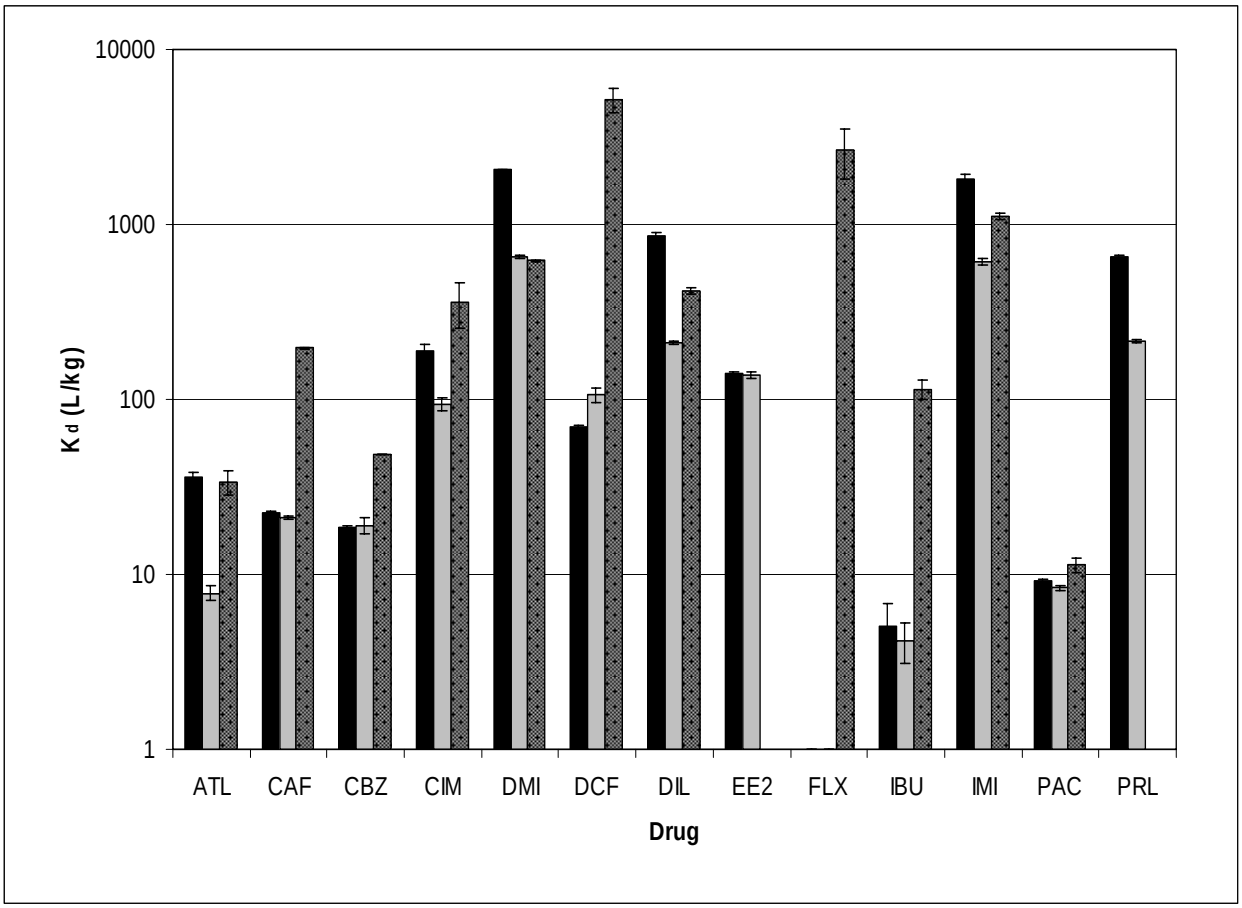


Figure 5.6: The K_d value of pharmaceuticals in moderately hard water (■), moderately hard water with 1 mM $CaCl_2$ (□) and 1 mM $CaCl_2$ (▩). Error bars are standard error of triplicate $K_d$values.

It is worth noting for the drugs that were not ionised over the experimental pH range (i.e. CAF, CBZ, EE2 and PAC), amending MHW with 1 mM $CaCl_2$ did not affect the K_d values relative to MHW. However, for the same molarity of $CaCl_2$, the K_d values from the $CaCl_2$ experiments were considerably higher. For these drugs with no change in ionisation, variation in K_d values may be related to another pH-dependent process, such as the effect on structure and dispersion of the sediment.

FLX was below the limit of detection for both the MHW treatments (Figure 5.6). Considering all the cationic pharmaceuticals showed an increase in sorption as less Ca^{2+} was present in the system (Figures 5.4 and 5.6) it is possible that FLX was bound to the sediment such that aqueous concentrations were below the limit of its detection (*see* Chapter 3, Table 3.2).

5.3.5 Implications for sorption in the aquatic environment

This study further indicates the important role that the solid phase plays when assessing the environmental fate of human pharmaceuticals. All the test pharmaceuticals were found to interact with the sediment to a varying degree. Furthermore, within the pH range and $CaCl_2$ concentrations used for this study, the test pharmaceuticals were observed to exhibit differences in affinity to sediments within the batch sorption system. This would suggest that distribution among solid and solution phases in various aquatic receiving systems, with variable ionic strength or pH, is likely to vary considerably. Recognition of pH and Ca^{2+}, among many other variables, as factors influencing the phase partitioning of pharmaceuticals is an important step in improving risk assessments of the aquatic environment. For example, release of acidic pharmaceuticals into aquatic systems 1 pH unit or more above their pK_a values will likely result in their presence within the water column as ionised species; which would be particularly relevant where sediment systems are dominated by high levels of ionisable organic acids. The common detection of acidic pharmaceuticals (such as NSAIDs) in aquatic environmental surveys might be partly explained by their likely extent of ionisation (alongside their volume of use).

Conversely, the increase of Ca^{2+} hardly affected the neutral or acidic drugs (with the notable exception of DCF), although increasing salinity has been found to increase the sorption of organic contaminants of low water solubility (Rogers 1993; Brunk *et al.* 1997; Zhou and Liu 2000). In the case of cationic pharmaceuticals, it might be expected that the extent of sorption would decrease as salinity increased, which would have implications if bound pharmaceuticals were transported along a salinity gradient, as would occur in estuarine or brackish environments.

In the current risk assessment process required by the EMEA for new drug products relevant physicochemical properties of the compound in question would already be well defined (EMEA-CPMP 2005). However, properties of the receiving system would not necessarily need to be as well defined but, rather, different scenarios of the receiving system should be taken into account. For example, sewage effluent outflows entering marine environments will be subject to a predictable pH range and substantially higher concentrations of Ca^{2+} (among other inorganic cations) compared with freshwater systems. In a freshwater system, significantly lower concentrations of Ca^{2+} (and other cationic species) would suggest the relative sorption of cationic pharmaceuticals is likely to be greater than anionic compounds. This effect would be exacerbated by the negative or neutral charge of ionic sediment moieties and mineral surfaces at typical pH values found in freshwater systems (Warren *et al.* 2003). An assessment of interaction with sediments must also be taken within the context of other factors that could influence sorption of human pharmaceuticals, such as temperature, concentration of dissolved organic carbon and suspended solids, or quality of organic carbon (Della Site 2001; Warren *et al.* 2003; Golding *et al.* 2005; Jones *et al.* 2006). Due to the limited amount of

work relating to human pharmaceuticals, work from existing research on other polar or ionic organic contaminants (e.g. pesticides) should also be utilised.

While such considerations would further complicate EMEA risk assessment strategies, interaction with sediments is likely to have an impact on the bioavailability and, therefore, ecotoxicity of human pharmaceuticals (Warren *et al.* 2003; Eggleton and Thomas 2004). EMEA guidelines recommend sediment ecotoxicological assessments of a new drug product if an extensive testing regime indicates long-term residence in test sediment (EMEA-CPMP 2005). Modifying estimates of the potential for sorption, such as taking into account the likely physicochemical properties of the receiving environment known to modify K_d, can therefore enable an improved risk assessment process. This can be done prior to undertaking extensive experimental procedures and would give a stronger basis for deciding how such procedures should be undertaken.

5.4 Conclusions

From this series of experiments, it can be seen that solution pH and concentration of $CaCl_2$ can have an important influence on the extent of sorption of a number of human pharmaceuticals. An increase of solution pH led to a decrease in sorption of acidic pharmaceuticals, although this effect was less predictable for the cationic pharmaceuticals, including the weak bases CIM and DIL. The pH of solution also influenced the K_d value of the neutral compounds, indicating that sediment physicochemical properties and, therefore, its sorption potential were pH-dependent.

Increasing $CaCl_2$ in solution was also found to reduce the K_d value for the cationic pharmaceuticals and CAF, although a marked, opposite effect was seen for DCF. Competitive sorption of the Ca^{2+} ion for anionic sites on the sediment with the basic pharmaceuticals was a probable factor. Also, the influence of Ca^{2+} on the sediment structure may have been important for the neutral drug CAF. Water solubility of DCF may have been decreased by the increasing ionic strength of solution.

The dependence of K_d on the solution concentration of $CaCl_2$ and pH is likely to be related to their interaction with both the pharmaceuticals and the sediment. Therefore, estimating the sorption of ionisable pharmaceuticals based on organic carbon normalised K_d (K_{OC}) is unlikely to give a suitable comparison of sorption between systems. Further investigation into the mechanistic processes relating to the pH and Ca^{2+}-dependent sorption of pharmaceuticals is thus required. Furthermore, an approach that encompasses the parameters of the environment, including the solution and sediment, and the pharmaceuticals in estimating their potential sorption in an aquatic system is desirable. The following chapter describes an approach where the known pharmacological parameters of a drug could be used in assessing its relative extent of sorption. This approach could be used in place of defining a number of physicochemical properties of the system that are likely to influence the extent of sorption.

Chapter 6. Can the aquatic distribution of human pharmaceuticals be related to pharmacological data?

6.1 Statement of contributions

Chapter 6 has been published (*see* Appendix C) and has been slightly modified to maintain consistency between chapters within this thesis (Williams *et al.* 2006). The abstract has been removed but the introduction, while repeating some information from previous chapters, has been maintained as it contains other information relevant to the manuscript.

As primary author, I was responsible for experimental design, experimental undertaking, sample analysis, data collection, collation and analysis and the primary preparation of the manuscript. The role of D.B. Williams, C.L.A. Saison and R.S. Kookana in this publication was for revision of drafts of the manuscript prior to publication.

M. Williams C.L.A. Saison D.B. Williams R.S. Kookana

6.2 Introduction

Post-therapeutic fate and effects of human pharmaceuticals have only recently become an issue of environmental interest, especially since more powerful analytical techniques have allowed their detection and quantification from environmental surveys (Daughton 2002; Heberer 2002; Barcelo 2003). Human pharmaceuticals have been detected in diverse environmental compartments following therapeutic use; from sewage treatment plants, to sewage sludge applied onto the land, to surface waters such as rivers, lakes, estuaries, the open ocean and water destined for human consumption (Buser *et al.* 1998; Daughton and Ternes 1999; Heberer *et al.* 2002; Kolpin *et al.* 2002; Stackelberg *et al.* 2004). Ecotoxicity testing has also been undertaken, although the number of pharmaceuticals detected in environmental surveys, which represent only a fraction of those in the marketplace, means such tests are unable to assess every pharmaceutical for every representative species. Therefore, despite the increasing awareness of the presence of pharmaceuticals, little is still known about their behaviour, in terms of fate and effects, after their entry into the environment (Jones *et al.* 2004). Ecological risk assessments for pharmaceuticals have been formulated in the United States and European Union, by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA), as part of the approval process for new drug applications (FDA-CDER 1998; EMEA-CPMP 2005). This is to account for the uncertainty surrounding the fate and effects of pharmaceuticals once they enter the environment, following their intended therapeutic use. The discussion paper released by the EMEA has recommended assessing the potential for interaction with sediments during the risk assessment process (EMEA-CPMP 2005). Interaction with sediment can modify the potential toxicity for organisms

living within aquatic ecosystems (Brooks *et al.* 2003b; Oetken *et al.* 2005). There has been comparatively little work assessing interactions of pharmaceuticals with the solid phases within aquatic ecosystems or what this means in terms of the potential for ecotoxicity of pharmaceuticals. Of the few studies that have been undertaken, a relatively small number of pharmaceuticals that are currently on the market were assessed (Ternes *et al.* 2004; Drillia *et al.* 2005; Loffler *et al.* 2005; Scheytt *et al.* 2005). Pharmaceuticals, as environmental contaminants, are in a unique position of potentially having existing data that can be used to predict their environmental behaviour. Due to the large number of pharmaceuticals on the market and the number of new drugs being developed, it is worth considering the information already available for these compounds. In this paper, we propose the use of pharmacological data as a means of predicting the distribution of pharmaceuticals within an aquatic system.

6.2.1 Pharmacological principles

The description of fate processes in pharmacology is covered by the field of pharmacokinetics. Pharmacokinetics is a function of the interaction between the body and a pharmaceutical compound and gives information on the absorption, distribution, metabolism and elimination (ADME) processes over time. Clinical pharmacokinetics is based upon measuring the amount of drug in a reference fluid (such as blood plasma) over time. The volume of distribution (V_D), an essential pharmacokinetic parameter, relates the concentration of drug (C) in a reference fluid with the known amount of drug (A) originally dosed. That is, it can be related to the measured concentration in plasma as follows:

$$V_D = \frac{A}{C} \qquad (6.1)$$

V_D is experimentally determined by plotting the blood concentration of a drug against time after a single dose of a drug and back extrapolating to time zero to yield an estimate of concentration. From knowing the amount of drug originally administered allows the calculation of V_D. Both single and multiple drug dosing can be used to determine V_D. It is essential to quantify V_D, since it can (along with other pharmacokinetic parameters) predict where and how long a drug will remain within the body. Pharmacokinetics thus forms the basis of a rational design for a drug dosing regimen, so the levels of the drug in the body remain between those that are effective and those that produce toxic effects (Mehta 1987; Wilkinson 2001). Any pharmaceutical product designed for use as a therapeutic agent must be registered with a governing body, such as the FDA, EMEA or Therapeutic Goods Administration (TGA; Australia). The methodology of pharmacokinetics, in deriving terms to characterise the fate of a drug in the body, is well defined and essential in the process of registering drugs for marketing. As pharmacokinetic information is required for every pharmaceutical product on the market, which thereby represents a potential environmental contaminant, it seems highly pertinent to try to adapt pharmacokinetic information for assessment of their environmental fate.

Other studies have suggested using pharmacological data as a means of predicting the potential ecotoxicological effects of pharmaceuticals (Lange and Dietrich 2002; Seiler 2002; Huggett *et al.* 2003a) or the total amount of pharmaceuticals that can enter the aquatic environment (Miao and Metcalfe 2003; Heberer and Feldmann 2005). However, we propose that pharmacokinetic data could also be used to prioritise pharmaceuticals in terms of their environmental fate. The contribution to the risk assessment process would

enable prediction of the likely extent of partitioning of a pharmaceutical relative to others. Estimating the relative extent of partitioning would also provide guidance for direction of ecotoxicological assessment. For example, if a pharmaceutical compound is considered likely to partition principally to sediment, sediment-based toxicity assessments would be more appropriate compared with water-only testing.

6.2.3 Comparison between volume of distribution (V_D) and the partition coefficient (K_d)

The proposed functional relationship between the environmental partition coefficient (K_d) and V_D is based on the premise that when a drug is introduced to the body, not only the physicochemical properties of the drug affect its pharmacokinetic profile but also the parameters of the system. Both V_D and K_d are related to the extent of distribution of an organic compound between an aqueous and organic phase and are also dependent on the interplay between the properties of the compound and those of the system (Mehta 1987; Doucette 2000; Wilkinson 2001; Warren *et al.* 2003) (Table 6.1). Due to the numerous interactions between a drug and the system it is in, both V_D and K_d values can be highly variable, depending on the specific mitigating factors within the body or the receiving environmental system. Despite the variability of K_d and V_D, they are highly useful in both absolute and relative terms in guiding therapeutic dosing of pharmaceuticals (Thummel and Shen 2001) or understanding their environmental distribution (Doucette 2000). This is especially so when a number of known system parameters can be rationalised as having influence on the values of V_D or K_d (Table 6.1). For example, total lipid content or charged proteins circulating in the bloodstream can influence the value of V_D through interacting with the drug (Wilkinson 2001; Casati and Putzu 2005), while organic carbon and charged mineral surfaces are important factors in influencing the sorption of a neutral

or ionized organic compound (Doucette 2000). Also, both V_D and K_d are strongly influenced by the lipophilicity and ionisability of the drug/organic compound (Doucette 2000; Wilkinson 2001). If a drug is ionisable, the pK_a of the drug and pH of the system is important to consider. K_d and V_D are, therefore, bulk parameters integrating a number of various drug and system-specific processes. As both parameters are a measure of the tendency for a drug to move from the aqueous to a more organic-rich phase and dependent on system or compound-specific parameters, a good correlation between the two may be expected. The determination of K_d values can be undertaken using standardised methods, such as OECD Guideline for Testing Chemicals 106 (OECD 2000). However, variation in environmental parameters influences the extent of sorption of organic contaminants (Warren *et al.* 2003). Therefore, we used field-collected soil and sediment to test whether variable and arbitrary physicochemical properties would affect the Kd values of the test pharmaceuticals. We subsequently assessed whether altering the Kd value of the selected pharmaceuticals would affect the relationship between K_d and V_D.

Table 6.1: Comparison of the volume of distribution (V_D) and partition coefficient (K_d) processes and the system and compound-specific parameters that influence their respective values in the human body or aquatic ecosystem

	Volume of Distribution (Human)	**Partition Coefficient (Aquatic system)**
System specific parameters	• Blood proteins (α_1-acid glycoprotein and albumin) which can bind drugs (basic and acidic) • Reservoirs for lipid soluble drugs i.e. lipid content of body • Highly specialised membrane types e.g. blood-brain barrier • pH differences across membrane surfaces (for dissociable compounds) • Health/age/gender of individual	• Concentration of suspended solids/colloids/mineral matter which can bind drugs in the water column • Solid phase characteristics e.g. % organic carbon, cation exchange capacity, amount and structure of binding surface • Bulk solution characteristics e.g. pH, hardness/alkalinity/electrical conductivity (competing ions) • Type and content of organic/mineral matter and organisms in a steady-state system
Compound specific parameters	• pK_a of drug affects plasma binding (e.g. only basic drugs bind to α_1-glycoprotein) and area of uptake (e.g. intestine vs. stomach) • pH-dependent log K_{ow} of drug influences uptake through lipophilic membranes (i.e. distribution from systemic circulation) • Molecular weight (affects ability to cross membranes) • Relative properties of other drugs present (i.e. drug interactions can modify process)	• pK_a of drug affects charge of compound i.e. water solubility and lipophilicity are modified • pH-dependent log K_{ow} affects partitioning/bioavailability • Molecular weight (affects extent of sorption) • Interactions with competing species for the solid phase

The objectives of this work were therefore to assess the relative importance of sorption of pharmaceuticals in three batch sorption systems. Each batch sorption system had a different combination of solid and aqueous phases, to assess how this would affect the K_d values of each test pharmaceutical. From these K_d values, the functional relationship between the K_d and V_D values of each pharmaceutical was tested for three different systems. That is, if a reasonable and consistent relationship is determined, this would suggest available pharmacological data could be used to enhance the risk assessment required by the EMEA.

6.3 Materials and methods

6.3.1 Test pharmaceuticals

Thirteen human pharmaceuticals were selected to ensure there was a broad range of V_D values. A number of these pharmaceuticals have been used previously in sorption experiments or have data available that can be used to estimate K_d values (Furlong *et al.* 2004; Ternes *et al.* 2004; Drillia *et al.* 2005; Scheytt *et al.* 2005). The pharmaceuticals used (Table 6.3) were atenolol (ATL), caffeine (CAF), carbamazepine (CBZ), cimetidine (CIM), desipramine HCl (DMI), diclofenac Na (DCF), diltiazem HCl (DIL), 17α-ethynylestradiol (EE2), ibuprofen (IBU), imipramine HCl (IMI), fluoxetine HCl (FLX), paracetamol (PAC) and propranolol HCl (PRL). All pharmaceuticals were obtained from SigmaAldrich Pty Ltd. (NSW, Australia).

6.3.2 Test procedure

Batch sorption experiments were used to determine K_d values. Air-dried sediment or soil (1 g, 0.125 mm sieved) was added to 100 mL Schott bottles, with Teflon lined lids, along with 100 mL of solution spiked with the test pharmaceuticals. Schott bottles were wrapped in aluminium foil to minimise photodegradation. The solution added to the solids included 10 mM $CaCl_2$ and moderately hard synthetic freshwater (MHW) (USEPA 2002). MHW was composed of 1 mM $NaHCO_3$, 0.35 mM $CaSO_4.2H_20$, 0.5 mM $MgSO_4$ and 0.05 mM KCl and was used to assess whether a complex mixture of salts influenced K_d values. The solid phases used included sediment and a soil and were collected from uncontaminated reference sites for an unrelated ecotoxicity study.

Table 6.2: Physicochemical properties of solid phases used for the batch sorption experiments

System	pH	EC[a] (μScm^{-1})	% OC[b]	CEC[c] ($cmol_+kg^{-1}$)	Particle size analysis		
					%Sand	%Silt	%Clay
Sediment	6.04±0.07	260±3	5.08±0.05	20.3±0.2	49.7±2.7	34.8±1.7	15.5±1.5
Soil	9.06±0.02	474±3	2.17±0.13	23.0±0.1	22.3±1.5	45.9±1.6	31.9±0.1

Values shown are mean±s.d. of triplicate samples
[a] electrical conductivity
[b] % organic carbon
[c] cation exchange capacity

Controls containing drug-spiked solution without sediment were used to account for degradation or removal of the drugs during the shaking and sample preparation. Both soil and sediment were collected in the field, air-dried and sieved through a 0.125 mm pore size sieve. The three batch sorption systems were composed of sediment and 10 mM $CaCl_2$, sediment and MHW and soil and 10 mM $CaCl_2$ (Table 6.2). Spiking concentrations ranged from 1–2 μM, depending on the affinity of the test pharmaceuticals for the solid phase. Sorption isotherms were determined for the drugs in the 10 mM $CaCl_2$ / sediment system and

concentration dependence for all drugs was not observed. Schott bottles containing solid and aqueous phases were placed on a vertically rotating shaker for 24 h.

Table 6.3: Literature dissociation constants (pK_a), octanol-water partition coefficient (K_{OW}) and V_D values of selected pharmaceuticals

Compound	pK_a[a]	Log K_{OW}[b]	V_D (L)[c]
Atenolol (ATL)	9.6 (b)	0.16	66.5±10.5
Caffeine (CAF)	0.6 (b)	-0.07	42.7±1.4
Carbamazepine (CBZ)	- (n)	2.45	98±35
Cimetidine (CIM)	6.8 (b)	0.4	70±14
Desipramine (DMI)	10.4 (b)	4.9	1400±210
Diclofenac (DCF)	4.5 (a)	4.4	11.9±7.7
Diltiazem (DIL)	7.7 (b)	2.7	231±84
17α-ethynylestradiol (EE2)	- (n)	3.67	265±56 [d]
Fluoxetine (FLX)	10.5 (b)	4.05	2450±1470
Ibuprofen (IBU)	4.9 (a)	3.97	10.5±1.4
Imipramine (IMI)	9.5 (b)	4.8	1260±140
Paracetamol (PAC)	9.3 (a)	0.51	66.5±8.4
Propranolol (PRL)	9.5 (b)	3.56	301±4.2

[a] Acid dissociation constant (SRC 2004) and function of charged group: acid (a), base (b) or neutral (n);
[b] log octanol-water partition coefficient (SRC 2004)
[c] Volume of distribution based on a 70 kg person (Thummel and Shen 2001) where values are mean±s.d.;
[d] V_D value for EE2 from Back *et al.* (1979)

A kinetic batch sorption experiment over 24 h showed that the concentration of drugs in solution reached a steady-state, indicating an apparent sorption equilibrium had been reached (*see* Chapter 4, Figure 4.2). Following shaking, Schott bottles were centrifuged at 2000·*g* for 10 min. Supernatant solutions were filtered through 0.45 μm nylon syringe filters (Biolab, Australia) prior to HPLC analysis.

The concentrations of the pharmaceuticals in the sediment were determined indirectly by difference from the amount spiked to the system and final concentration of water. The K_d values were then determined from the average value of four replicates using the relationship:

$$K_d = \frac{C_s}{C_{aq}} \qquad (6.2)$$

The K_d value depicts the ratio between the concentration of compound in sediment (C_s) and the aqueous phase (C_{aq}), at the time of determined sorption equilibrium (OECD 2000). The K_d values obtained were then compared with the V_D values (Table 6.3) of each drug by regression analysis (Figure 6.1).

6.3.3 Chemical analysis

HPLC-UV analysis was undertaken with an Agilent 1100 HPLC, with a polydiode array (PDA) detector. The column used for separation was an Apollo C_{18} (250x 4.6 mm, 5 μm) (Alltech), with an Alltima C_{18} guard column (7.5x 4.6 mm; 5 μm) (Alltech); 100 μL of sample was injected onto the column. The mobile phases used were; mobile phase A: 0.05 M sodium formate/formic acid (pH 3); mobile phase B: acetonitrile, with the percentage of mobile phase A used for gradient analysis as follows; 0–4 min 90 %, 6–11 min 65 %, 12–15 min 45 %, 16–19 min 30 %, 22–25 min 90 %. Detection wavelengths for each pharmaceutical were as follows: IBU 215 nm; CIM 230 nm; FLX, PAC, IMI 254 nm; ATL, CAF, DIL, DMI, CBZ, EE2, DCF 280 nm; PRL 290 nm. Calibration was undertaken using an 8 point curve, while external standards and Milli-Q water blanks were run every 5–8

samples, to account for instrumental performance and sample overlap. Calibration standards were prepared in Milli-Q water as there was no significant effect found on detection response from the various matrices.

6.4 Results and discussion

6.4.1 Functional relationship between K_d and V_D

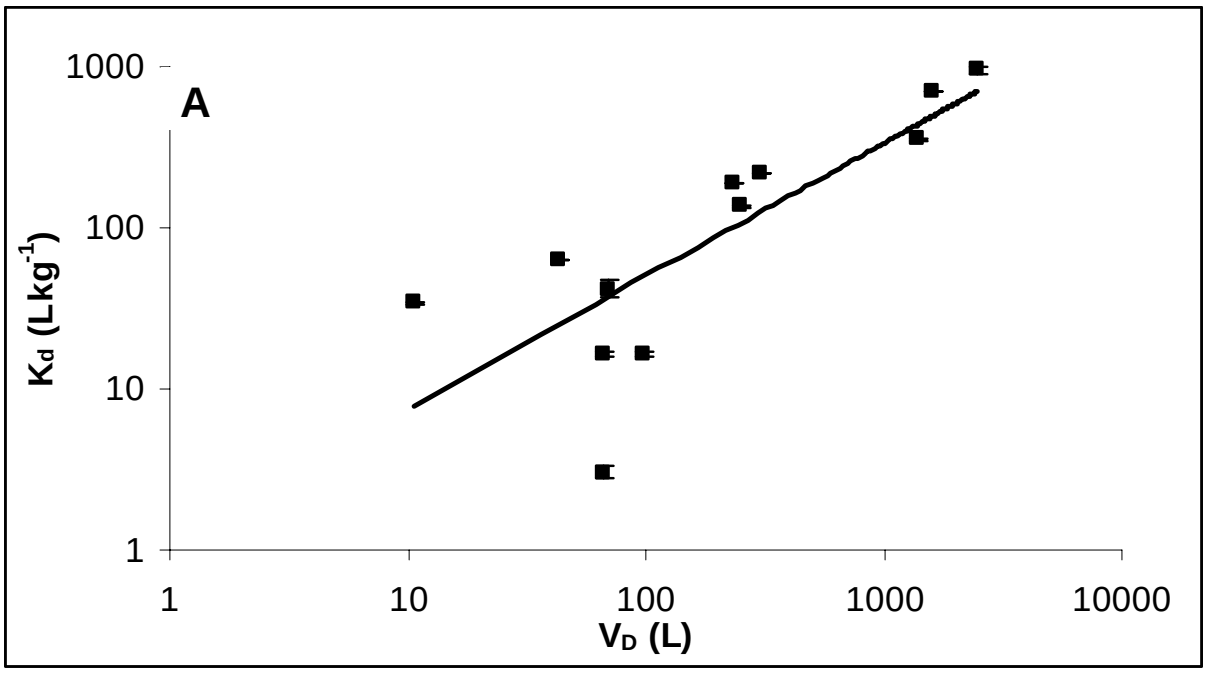
Each batch sorption system was found to have varying physicochemical properties (Table 6.2) which caused variable K_d values for each pharmaceutical compound (Figure 6.1). Also, each batch sorption system showed that the pharmaceuticals with higher V_D values tended to have comparatively higher K_d values (Figure 6.1). That is, while there were physicochemical variations between the systems, an increasing V_D value corresponded with a relatively higher K_d value (Figure 6.1). Log transformation of the K_d and V_D data was done due to the orders of magnitude difference of values within both the K_d and V_D datasets, respectively. This avoided any undue leverage by the highest V_D or K_d values in regression analysis of untransformed data. When 10 mM $CaCl_2$ was used for both sediment and soil, the regression was highly significant for both the sediment ($r^2 = 0.62$, $p = 0.002$) and soil ($r^2 = 0.72$, $p < 0.001$). When MHW was combined with sediment, the regression was also found to be highly significant ($r^2 = 0.69$, $p < 0.001$). Furthermore, regression coefficients for the three batch sorption systems indicated a reasonable amount of variation in K_d could be consistently explained by V_D.

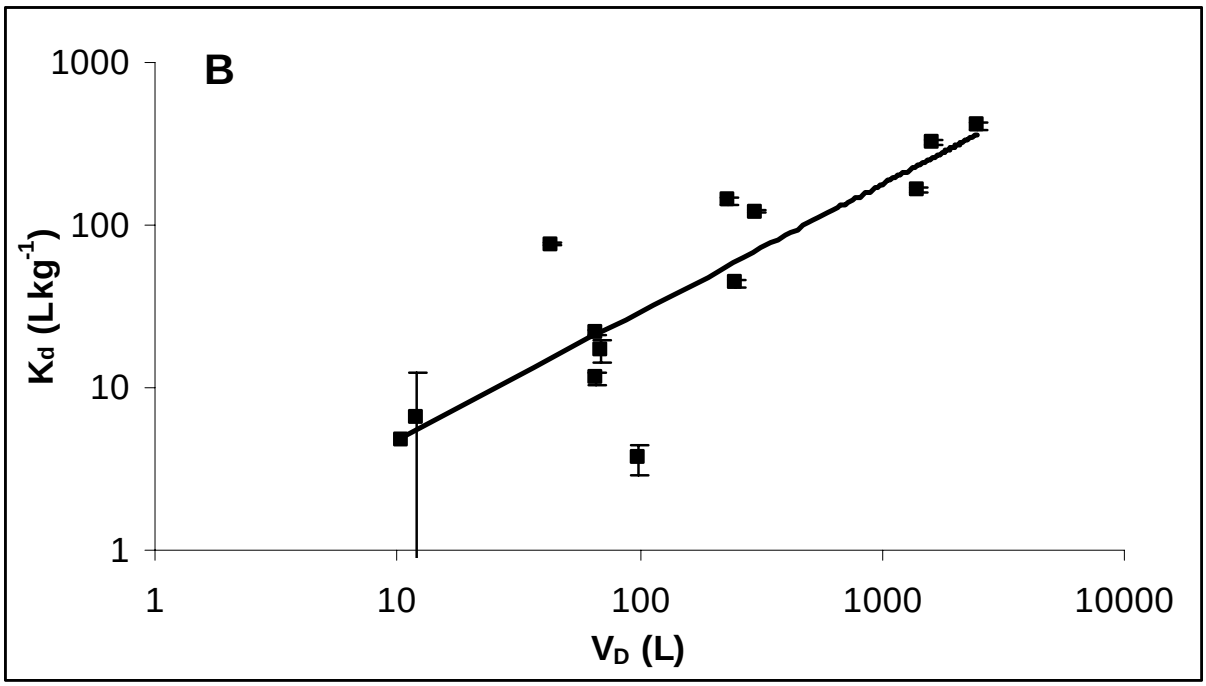
6.4.2 Comparisons with other studies

Other work consisting of batch sorption studies, or where data was available to estimate K_d values, were compared with our work. These studies had systems where the solid and aqueous phases used were distinct from our study. Drillia *et al.* (2005) used solid phases with both high and low organic carbon content and varying pH values. Based on the V_D values of these drugs it would be expected that the sorption coefficient order would be PRL > CBZ > DCF ≈ clofibric acid, where clofibric acid has a V_D value of 9.8 L (Thummel and Shen 2001). While the K_d value of PRL was consistently highest, DCF (0.45 Lkg^{-1}) had a Kd value similar to CBZ (0.49 Lkg^{-1}) for the low organic carbon, neutral pH soil (% organic carbon = 0.4, pH = 6.8). However, DCF (165 Lkg^{-1}) had a K_d similar to PRL (199 Lkg^{-1}) for the high organic carbon content, low pH soil (% organic carbon = 7.1, pH = 4.3). Scheytt *et al.* (2005) also found DCF (1.9 Lkg^{-1}) and CBZ (3.9 Lkg^{-1}) to have similar K_d values at around pH 7, while Ternes *et al.* (2004) found DCF (453 Lkg^{-1}) to have a substantially higher K_d value than CBZ (1.2 Lkg^{-1}) in a sewage sludge around neutral pH. Furthermore, Loffler *et al.* (2005) found that diazepam had a K_d value double that of CBZ (3 and 1.3 Lkg^{-1}, respectively), although the V_D of diazepam (77 L) is less than CBZ (Thummel and Shen 2001). While in this case, the octanol–water partition coefficient (K_{OW}) may give a better indication of sorption behaviour, it is not the only factor that can predict K_d, particularly when considering a complex system containing ionisable compounds (Doucette 2000).

We compared the K_{OW} values of the pharmaceuticals (Table 6.3), with the experimentally obtained K_d values from each system to assess whether it formed a better basis for estimation of K_d. The regression coefficient between K_d and K_{ow} for the 10 mM $CaCl_2$ / sediment system was significant (r^2 = 0.52, p = 0.01), although still less than the regression coefficient between K_d and V_D. The regression coefficients between K_d and K_{OW} for both the

MHW / sediment and 10 mM $CaCl_2$ / soil systems were both weak and insignificant ($r^2 < 0.25$, $p > 0.1$). We also made a comparison between the K_{OW} values of 88 pharmaceuticals (*data from* SRC 2004) and their V_D values (*data from* Thummel and Shen 2001) but no relationship was apparent (data not shown) between the two parameters.





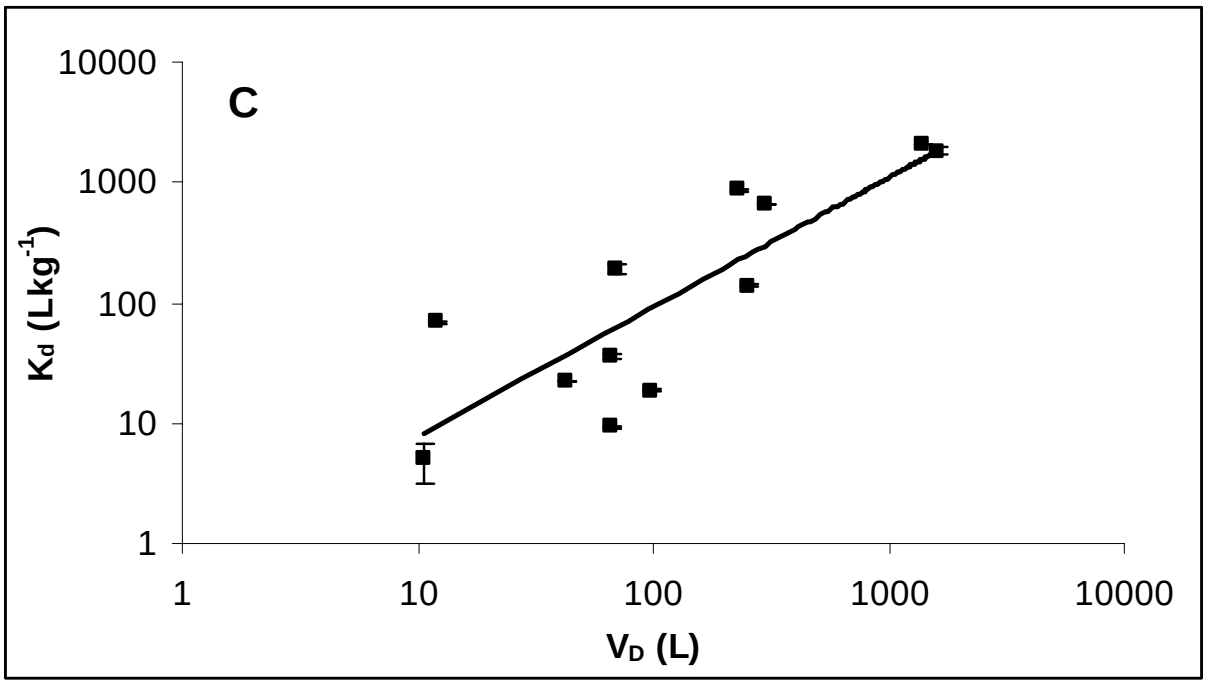


Figure 6.1: The relationship between K_d and V_D for (A) the 10 mM $CaCl_2$/sediment system, (B) the 10 mM $CaCl_2$/soil system and (C) the MHW/sediment system. The values are mean ± s.e. for K_d while error bars are not shown for V_D values.

Therefore, K_{OW} is unlikely to serve as the sole predictor of K_d in our experimental systems or for V_D in the human body. This further indicates the dependence of V_D and K_d on other system and compound-specific parameters. In an environmental survey by Furlong *et al.* (2004) concentrations of a number of pharmaceuticals, including CBZ (K_d = 69 Lkg^{-1}), DIL (53 Lkg^{-1}), CAF (26 Lkg^{-1}), CIM (22 Lkg^{-1}) and PAC (5 Lkg^{-1}) were measured in both field collected sediment and water. Relative comparison with our work indicates this dataset of five pharmaceuticals does not follow the same pattern of sorption. In our three batch sorption experiments, DIL had the highest K_d values (190 Lkg^{-1} for 10 mM Ca^{2+} and sediment / 140 Lkg^{-1} for 10 mM Ca^{2+} and soil / 869 Lkg^{-1} for MHW and sediment), followed by either CAF (63 / 76 / 22 Lkg^{-1}) or CIM (142 / 17 / 188 Lkg^{-1}), while CBZ (16 / 4 / 19 Lkg^{-1}) or PAC (3 / 5 / 4 Lkg^{-1}) had the lowest K_d values of these five pharmaceuticals. However, the thirteen

pharmaceuticals used in our study indicated a general trend can exist between K_d and V_D, despite anomalies between individual K_d and V_D values of pharmaceuticals.

6.4.3 Influence of system parameters on K_d values

Both K_d and V_D values are a consequence of a number of system and compound-specific parameters and this was demonstrated to affect the K_d values of pharmaceuticals between systems. Altering the physicochemical properties of the aqueous phase by using either 10 mM $CaCl_2$ or MHW would have altered the physicochemical properties of the pharmaceuticals. This would particularly relate to pharmaceuticals with pH-dependent functional groups. For example, increasing pH of solution would increase the extent of ionised acidic carboxyl groups on DCF and IBU, in turn increasing their water solubility and decreasing their lipophilicity. In our study, control samples (Schott bottles with no added sediment) from the 10 mM $CaCl_2$ / sediment treatment indicated DCF had poor water solubility at pH 4.5. Based on the pK_a value of DCF, half of the molecules would be un-ionised at this pH, meaning its water solubility would be substantially less than the treatments of higher pH. No data for DCF was therefore available for comparison with the other studies. Increasing solution pH would increase the extent of ionisable acidic groups in the sediment or soil as well, influencing their interaction with charged functional groups of the pharmaceuticals. Changing the concentration of Ca^{2+} would also have affected conformational structure of the solid phase (Zhou *et al.* 2004) or led to an increase in competitive binding with cationic functional groups, found in basic pharmaceuticals (Doucette 2000). Furthermore, the organic carbon content of the respective solid phases had an influence on the absolute K_d values of the pharmaceuticals. The soil, with a lower organic carbon content, had comparatively lower K_d values for respective pharmaceuticals when

compared with the systems containing sediment. Following the 24 h equilibration period, the pH of solution was found to be similar for both the sediment / MHW and soil / 10 mM $CaCl_2$ systems (6.51±0.02 and 6.49±0.03, respectively), while the sediment / 10 mM $CaCl_2$ system had a significantly lower pH (4.31±0.01). The relationship between K_d and V_D was also found to be weaker in the sediment / 10 mM $CaCl_2$ system, which may indicate pH plays an important role for both parameters. Furthermore, it could also suggest when the value of the batch sorption system moves further away from the physiological pH of human blood (pH 7.4), the relationship between K_d and V_D becomes weaker. Despite the variation in physicochemical properties of the batch sorption systems, the relative relationship between K_d and V_D remained constant. As this relationship was found for a dataset of thirteen diverse pharmaceuticals in three different batch sorption systems, there seems to be a case for further exploring the use of pharmacological data for ecological risk assessment of pharmaceuticals. There is an obvious need to expand the number of pharmaceuticals to test this relationship. This would ensure adequate representation of the thousands of pharmaceuticals currently in use.

6.4.4 Considerations for ecological risk assessment of pharmaceuticals

While V_D does not give an indication of the absolute K_d value of a pharmaceutical, it could still be useful in gaining a better appreciation of the relative extent of partitioning in a system. Sorption studies for pharmaceuticals have been limited to a small proportion of registered pharmaceuticals under limited variations of conditions. Even if the tiered risk characterisation required by the EMEA leads to only a relatively small number of pharmaceuticals requiring sorption assessment, the number of sorption assessments could still be substantial. Expanding the current database of test pharmaceuticals from this study to

test the relationship between K_d and V_D might be a more prudent use of resources to assess the potential for distribution of pharmaceuticals in a receiving environmental system. As the relationship between K_d and V_D remained reasonably constant in these experiments, pharmaceuticals could be ranked in terms of their relative sorption capacity based on their V_D values. This has the advantage of drawing on data already available in pharmacological literature. Also, better estimation of sorption capacity would enable appropriate ecotoxicological assays for higher tier risk assessment, if required by the EMEA. For example, sediment toxicity testing might not be useful for IBU, especially if the pH of a system was around neutral. Alternatively, sediment toxicity testing for FLX would be more appropriate than a water-only exposure assessment.

6.5 Conclusions

The variations in physicochemical properties of three batch sorption systems used in this study led to K_d values of thirteen diverse pharmaceuticals to vary as well. Despite the variability of K_d values, the relationship between K_d and V_D values remained constant and significant (r^2 = 0.62–0.72, p < 0.001–0.002). This gives an encouraging insight into how pharmacokinetic parameters could be exploited for gaining understanding into the fate processes of pharmaceuticals. The use of existing data in such a way could be a useful means of contributing to the ecological risk assessment, required by regulatory agencies. This is particularly important considering the paucity of data relating to the fate and effects of this diverse class of environmental contaminant.

Chapter 7: Isotopic dilution of carbamazepine as a measure of its exchangeable fraction

7.1 Introduction

Desorption hysteresis often indicates that an organic contaminant at sorption equilibrium has accessed domains of the solid phase that slow the rate of release of the contaminant into the aqueous phase. The assessment of desorption is commonly undertaken using sorption / desorption batch equilibration methods, although the extent of desorption using this experimental method is often overestimated due to a number of experimental artefacts (Bowman and Sans 1985; Pignatello 1989; Della Site 2001). Assessment of the extent of a bound organic contaminant that can be released back into the aqueous phase is important in terms of understanding fate processes, such as potential for transport pathways, degradation and bioavailability. For example, the potential for desorption (or "lability") has been suggested as a means of approximating the bioavailability of sediment-bound organic contaminants (Standley 1997; Lamoureux and Brownawell 1999; Northcott and Jones 2000; Huang *et al.* 2003).

Artefacts leading to apparent desorption hysteresis can be reduced by ensuring experimental approaches avoid factors that cause desorption hysteresis, such as degradation, transformation, insufficient equilibration time or loss of sorbate. Isotopic techniques have been proposed as a means of avoiding the introduction of some of the artefacts that can lead to hysteresis of organic contaminants (Celis and Koskinen 1999a, 1999b; Sander and Pignatello 2005). Two techniques that have been employed for assessment of desorption hysteresis of organic compounds are the isotopic tracer and isotopic dilution techniques.

Isotopic tracer experiments involve the addition of a labelled isotope of a compound along with the unlabelled compound into the experimental vessel (Sander and Pignatello 2005). On the other hand, isotopic dilution involves the addition of an isotopically-labelled test compound to an aqueous phase / solid phase system, where the unlabelled compound is already at equilibrium between the two phases (Celis and Koskinen 1999a, 1999b). In this case, the labelled compound can exchange with the unlabelled compound that is weakly bound to the sediment. Such an exchange then provides an estimate of the fraction easily exchanged between the aqueous and solid phases and could therefore indicate the potentially bioavailable fraction of the test compound.

7.1.1 Exchangeable (E) value of contaminants

Isotopic techniques have been more commonly employed in studies of inorganic species, such as trace metals or phosphorous (Fardeau 1996; Hamon *et al.* 2002). Such techniques have been used in agricultural sciences to estimate the pool of the analyte of interest that is available for uptake by plants, whether from a nutritive or toxicological perspective. The fraction of an analyte associated with the solid phase that is rapidly re-released into the solution phase is considered to be the exchangeable pool of the analyte (Figure 7.1). When the analyte accesses domains of the solid phase from which rapid desorption is hindered, it is said to be within the non-exchangeable pool. If solids containing a non-exchangeable pool are found within the aqueous phase, then the analyte can exist within solution as colloids, without exchanging with the solution phase (Figure 7.1). While the terms “exchangeable” and “labile” have been applied to the pool of analyte that undergoes rapid desorption, exchangeable will be used here to avoid potential confusion.

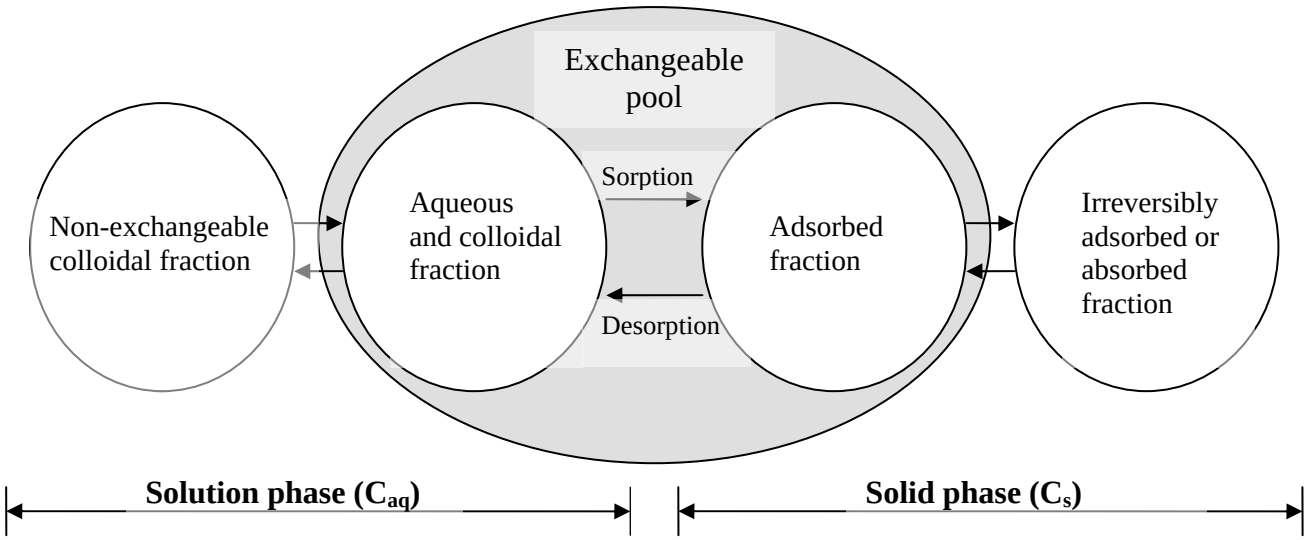


Figure 7.1: Labile and non-labile pool of an organic contaminant in a water sediment system. The exchangeable pool is where the radiolabelled isotope added in solution can exchange with the sediment-bound non-labelled analyte. Aqueous concentration (C_{aq}) and solids-bound concentration (C_s) are used to define the partition coefficient (K_d) of the analyte

Isotopic dilution techniques can be used to estimate the exchangeable pool of analyte within a system consisting of an aqueous and solid phase (Hamon *et al.* 2002; Nolan *et al.* 2003). The exchangeable (E) value of an analyte indicates the fraction that exists within the exchangeable pool. When the E-value of an analyte is compared against the total amount of analyte introduced into the system, an estimate of the percentage of analyte originally added to the system within the exchangeable pool can be attained.

The aim of this chapter was to determine the E-value of carbamazepine (CBZ) within a batch sorption system. CBZ was used as a model compound to assess whether the hysteresis observed for it using a more common desorption method (*see* Chapter 4, Figure 4.6) was apparent using isotopic dilution. Sorption equilibrium was apparently reached for CBZ within 24 h for the experiments undertaken in Chapter 4 (*see* Chapter 4; Figure 4.2) although this may only represent rapid sorption processes. Since the extent of desorption has been found to decrease as slow sorption processes become more important (Pignatello and Xing

1996), the effect of the length of shaking time on the E-value of CBZ was assessed. This evaluation was undertaken at a single spiking concentration. However, the extent of desorption of organic contaminants has been found to be dependent on their equilibrium concentration (Celis and Koskinen 1999b; Della Site 2001; Sander and Pignatello 2005). Therefore, the influence of a range of CBZ spiking concentrations on its E-value was also assessed.

CBZ was chosen as a model compound since it has been found to be persistent in a number of environmental surveys and laboratory experiments (Ternes 1998; Drewes *et al.* 2002; Heberer 2002; Tixier *et al.* 2003; Clara *et al.* 2004a; Joss *et al.* 2005). Therefore, its inclusion was less likely to lead to experimental artefacts from degradation. However, previous batch sorption experiments indicated that CBZ does not sorb strongly to the test sediment (*see* Chapter 4). Additional treatments were included, where sediment was amended with char to increase the probability of irreversible sorption of CBZ occurring. The presence of char within soils or sediments is often associated with both an enhanced sorption capacity and a decrease in the extent of desorption (Braida *et al.* 2003; Cornelissen *et al.* 2005; Koelmans *et al.* 2006). Decreasing the extent of desorption, while using the same test sediment, was considered to be an important measure to further test the applicability of the isotopic exchange technique.

7.2 Methods and materials

7.2.1 Principles for determining the E-value

There are a number of assumptions for the isotopic dilution experiment (Hamon *et al.* 2002; Nolan *et al.* 2003):

(1) The ^{14}C-CBZ added to the system containing sediment and solution does not alter the sorption equilibrium of ^{12}C-CBZ

(2) The ^{12}C-CBZ within the system is exchangeable with the added ^{14}C-CBZ

(3) The ^{14}C-CBZ added to the system will physically mix with the entire exchangeable pool of ^{12}C-CBZ

The following derivation of the E-value has been adapted from Hamon *et al.* (2002). The total CBZ within the system can be given by:

$$CBZ_{total} = CBZ_{aq} + CBZ_{e} + CBZ_{n} \qquad (7.1)$$

Where CBZ_{total} is the total amount of CBZ within the system, CBZ_{aq} is the amount of CBZ within the aqueous phase (not irreversibly associated with colloids), CBZ_{e} is the amount of adsorbed CBZ within the exchangeable pool (Figure 7.1) and CBZ_{n} is the amount of adsorbed CBZ that is not within the exchangeable pool. When a quantity of radiolabelled CBZ (R) is added to the system, it will equilibrate amongst the aqueous phase and exchangeable pool of the sediment, such that:

$$\frac{r_{aq}}{r_e} = \frac{CBZ_{aq}}{CBZ_e} \qquad (7.2)$$

where r_{aq} and r_e represent the fractions of the specific activity of the added R in solution and in the exchangeable sediment pool, respectively. In other words:

$$r_e = R - r_{aq} \qquad (7.3)$$

From combining Equations 7.2 and 7.3:

$$CBZ_e = CBZ_{aq} \frac{(R - r_{aq})}{r_{aq}} \qquad (7.4)$$

where rearrangement of Equation 7.4 leads to:

$$(CBZ_e + CBZ_{aq}) = CBZ_{aq} \frac{R}{r_{aq}} \qquad (7.5)$$

In Equation 7.5, (CBZ_e+CBZ_{aq}) represents CBZ that exists within the exchangeable pool (both within the sediment and solution) or, in other words, the E-value. Therefore, to determine the E-value of a system, the ratio of specific activity of added radiolabelled CBZ and radiolabelled CBZ within solution after equilibrium needs to be quantified. Also, the amount of non-labelled CBZ within solution needs to be quantified.

In Equation 7.5 the term r_{aq} is often referred to as r_t due to its dependence on time (Hamon *et al.* 2002). However, since an apparent sorption equilibrium for CBZ was previously found to occur within 24 h (*see* Chapter 4; Figure 4.2) shaking of radiolabelled CBZ with the sediment was undertaken for 24 h. Any increase in sorption of the unlabelled CBZ after this period would therefore be assumed to be related to slow sorption processes, which could be expected to influence the E-value of CBZ.

7.2.2 Test pharmaceutical

Carbamazepine (CBZ) was obtained from Sigma-Aldrich (NSW, Australia) and had a chemical purity of >99%. Radiolabelled CBZ (carbonyl ^{14}C) was obtained from Sigma-Aldrich (NSW, Australia) and had a specific activity of 22.6 μCi $mmol^{-1}$. Unlabelled CBZ (^{12}C-CBZ) was prepared as a 1 gL^{-1} stock solution in methanol, from which working solutions were prepared. For the radiolabelled CBZ (^{14}C-CBZ) stock solution, 3700 kBq was added to 1 mL of methanol, from which a 2000 $BqmL^{-1}$ working solution was prepared in 18.2 $M\Omega cm^{-1}$ water. Stock solutions were stored at -18 °C.

7.2.3 Batch sorption procedure

The sediment used for the experiments was the same as that used for the batch sorption experiments (*see* Chapter 4; Table 4.1) and was prepared in the same way. The sediment / solution ratio was adjusted to 0.5 g sediment to 5 mL solution (1 : 10), as this was not found to have an effect on the partition coefficient (K_d) of CBZ (*see* Chapter 4; Figure 4.4). The volume of solution used was reduced to enable a smaller amount of ^{14}C-CBZ to be spiked to the solution, without affecting its detection. Batch sorption vessels were 10 mL

glass tubes, with polytetrafluoroethylene (PTFE) lined lids. The stock solution of ^{12}C-CBZ was spiked into a volumetric flask and dried with N_2. Methanol was then added to re-solubilise ^{12}C-CBZ, such that the final volume of methanol was 0.01%. A solution of 10 mM $CaCl_2$ (in 18.2 MΩcm^{-1} water) was added to a volumetric flask and the spiking solution was made up to volume. Sediment was weighed and placed into pre-weighed glass tubes. Sediment had 1 mL of the 10 mM $CaCl_2$ solution added to it and was pre-equilibrated for 24 h. After pre-equilibration, 0.5 mL of ^{12}C-CBZ spiking solution and 3.5 mL 10 mM $CaCl_2$ solution were added to the glass tubes to give a total volume of 5 mL.

Glass tubes were wrapped in aluminium foil and placed into a rotating, end-over-end shaker for an appropriate time period. Following this, the glass tubes were removed from the shaker and had 0.105 mL of 2 kBqmL^{-1} ^{14}C-CBZ added to them and were placed on the shakers for an additional 24 h period. Glass tubes were then removed from the shaker and centrifuged at 2000 *g* for 10 min. Aliquots of the supernatant were used for analysis of ^{12}C-CBZ and ^{14}C-CBZ.

The remaining solution was used for pH measurement using a pH probe (Thermo Orion). The pH probe was calibrated daily, using pH 4, 7 and 10 solutions (LabChem NSW, Australia).

7.2.4 Concentration dependence of E-value

The concentration dependence of the E-value of CBZ was tested by spiking a range of concentrations of ^{12}C-CBZ to a batch sorption system. The amount of ^{12}C-CBZ spiked to the respective systems was 0.5, 1, 5, 10 and 50 μg, which was achieved by altering the volume of the 10 mgL^{-1} ^{12}C-CBZ spiking solution and 10 mM $CaCl_2$ added to the sediment. All

treatments were conducted in triplicate. The batch sorption experiments were undertaken for 24 h, prior to the addition of ^{14}C-CBZ. Equal volumes of ^{14}C-CBZ were spiked to solutions and shaken for a further 24 h, as per the time dependence study. A Freundlich isotherm was determined from the equation:

$$\log C_s = n \log C_{aq} + \log K_f \qquad (7.7)$$

where n is the exponent indicative of concentration dependence of sorption (linearity of the relationship) and K_f is the Freundlich sorption coefficient.

7.2.5 Time dependence of E-value

Spiking solutions of 10 mgL^{-1} ^{12}C-CBZ were added to the glass tubes containing pre-equilibrated sediment, such that 5 μg ^{12}C-CBZ was added to each system. To test the effect of the shaking period on the E-value of CBZ, shaking periods of 0, 1, 3, 7, 14, 21 and 28 d were used, prior to the addition of ^{14}C-CBZ. All treatments were conducted in triplicate. To assess whether ^{12}C-CBZ associated with colloidal material would influence the size of the exchangeable pool, supernatants collected from 0, 1, 14 and 28 d were also filtered through 0.45 and 0.2 μm nylon syringe filters (Biolab, Australia) prior to chemical analysis.

Values of the distribution coefficient (K_d) for ^{12}C-CBZ and ^{14}C-CBZ were determined from the relationship:

$$K_d = \frac{C_s}{C_{aq}} \qquad (7.8)$$

where C_s is the concentration of CBZ adsorbed and C_{aq} is the concentration of CBZ in solution. The value of C_s was obtained from the difference of the amount of CBZ spiked and of that measured in solution.

7.2.6 Sediment amendment with char

Additional treatments at 1, 3, 7 and 28 d had their sediment amended with 10 mg Red Gum (*Eucalyptus camaldulensis*) wood char. The char was prepared at CSIRO Land and Water laboratories by combusting Red Gum chips at 850 °C for 1 h and grinding them into a fine powder. Analysis of the micropore content (pores < 2 nm size) and N_2-BET specific surface area showed a micropore content of around 90 %, while the N_2-BET specific surface area was 605 m^2g^{-1} (Bornemann *et al.* 2007).

7.2.7 Analytical methods

Liquid scintillation counting (LSC) was used to measure the activity of ^{14}C-CBZ within solution, based on the number of radioactive disintegrations per minute (dpm) of the ^{14}C nucleus, as follows:

$$^{14}_{6}C \rightarrow ^{14}_{7}N + \beta^{-} \qquad (7.9)$$

The instrument used for LSC was a Wallac WinSpectral α/β 1414 Liquid Scintillation Counter. The software used was WinSpectral 1.10.00 (Wallac, 1994).

Clear glass LSC vials (Biolab, Australia) were used for analysis. Into each vial, 1 mL of sample was added and 10 mL of Optiphase HiSafe (Perkin Elmer, USA) liquid scintillant was added. This mixture was allowed to equilibrate for a minimum 6 h prior to counting. A count time of 10 min was undertaken for all samples to give an average count per minute (cpm) value, which was adjusted to dpm based on counting efficiency. The counting efficiency was determined from an external colour quench calibration.

7.2.7.1 Quench curve

The efficiency of the light measured by the scintillation counter is specific to the radioisotope being investigated and it can be reduced further by quenching. Quenching can either occur through coloured solutions and chemical groups, such as Cl^-, which reduce the amount of light reaching the scintillation counters.

Solutions used to mimic sample matrices were prepared from a humic acid stock solution. A 20 gL^{-1} stock solution of humic acid (sodium salt) (Aldrich, Australia) was prepared and various volumes were added to 18.2 $M\Omega cm^{-1}$ water. Solutions of the sample matrix were spiked with a 42 μL aliquot of 2000 $BqmL^{-1}$ ^{14}C-CBZ to give a total volume of 1 mL and a final solution activity of 83 Bq or 5000 dpm. Typical counting efficiencies of the ^{14}C-CBZ were 80%.

To account for background levels of radiation, duplicate samples of 1 mL solution matrix, without any added ^{14}C-CBZ, were counted. The limit of quantitation (LOQ) was then set at 10 times this dpm value for respective analyses. The LOQ was determined to be 420 dpm from preliminary experiments.

7.2.8 QA/QC considerations

As with previous batch sorption experiments (*see* Chapter 4), control samples containing sediment not spiked with CBZ and CBZ-spiked solution without sediment were used to assess analytical interferences and loss due to the experimental procedure, respectively. For the time series, such controls were used at 1, 14 and 28 d. Furthermore, systems spiked only with ^{12}C-CBZ or ^{14}C-CBZ were used to assess the effect of interaction between the isotopes on their respective K_d values. This control was used to test the assumption that spiking the radiolabelled CBZ would not disturb the sorption equilibrium of the unlabelled CBZ. To assess whether ^{12}C-CBZ and ^{14}C-CBZ are equally exchangeable, they were spiked at the same time (t = 0 d treatment) and shaken for 24 h. If the two isotopes were equally exchangeable, the E-value should be 100 % for the t = 0 d treatment, as the isotopes would equilibrate at the same rate.

7.3 Results and discussion

7.3.1 Concentration dependence of E-value

The pH of the equilibrated solutions in the concentration treatments was 6.08±0.05. The Freundlich parameters over the tested concentration range of ^{12}C-CBZ were K_f = 28.8 Lkg^{-1}, n = 0.88 and r^2 = 0.999 (Figure 7.3), compared with the previous isotherm experiments where K_f = 12.4 Lkg^{-1}, n = 1.06 and r^2 = 0.92 (*see* Chapter 4; Table 4.3). However, the mean K_d value in the previous sorption experiments was 19.8±3.5 Lkg^{-1} (*see* Chapter 4; Table 4.3), which was similar to those obtained for both the time and concentration treatments in this study (Figures 7.4 and 7.6). The value of n indicated that, for this concentration range, there

was an extent of concentration dependence for K_d, where the K_d values decreased as the spiking concentration of CBZ increased (Figure 7.4) However, the use of Equation 7.8 to approximate K_d values was still considered to be appropriate.

Since the E-values varied according to the spiking concentrations of CBZ, only the percentage of spiked CBZ that remained within the exchangeable pool was shown (Figure 7.2). A slight but non-significant increase in the E-value was apparent with increasing concentration of CBZ, although the lowest E-value (for the 0.1 mgL^{-1} spiking concentration) was still above 90 %.

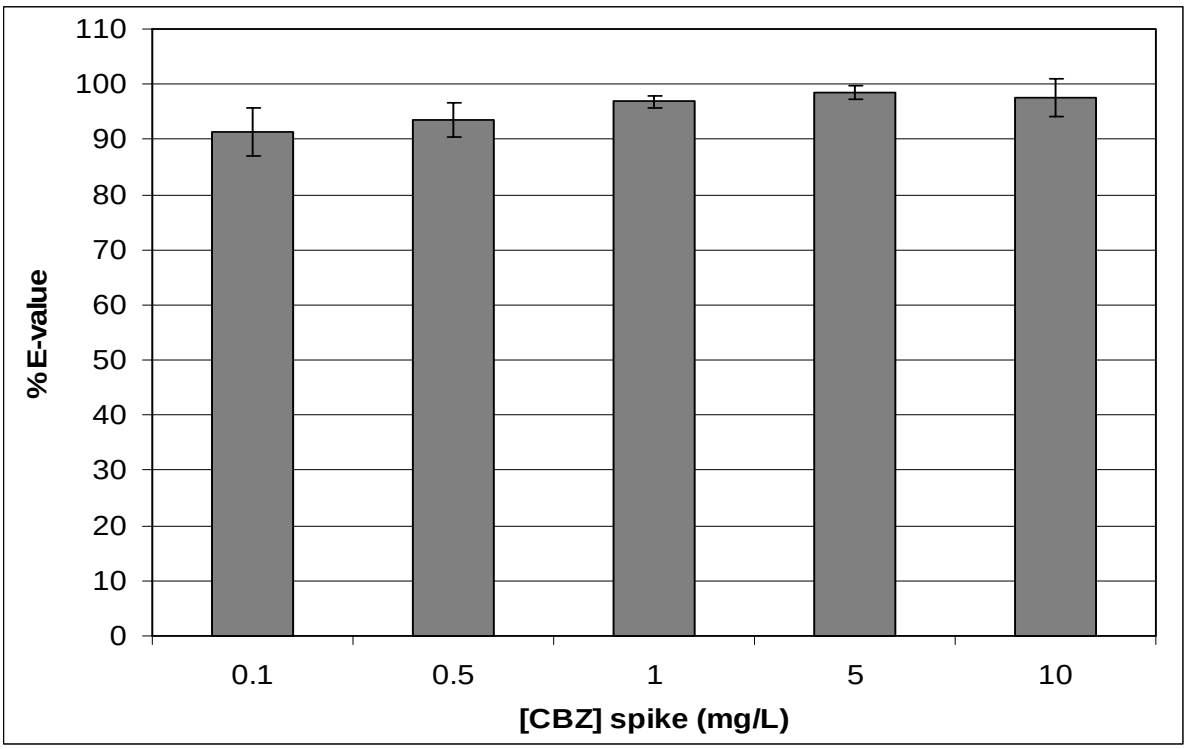


Figure 7.2: Effect of concentration of carbamazepine ([CBZ]) on the percentage of originally spiked CBZ remaining within the exchangeable pool. Error bars shown are standard error of triplicate samples.

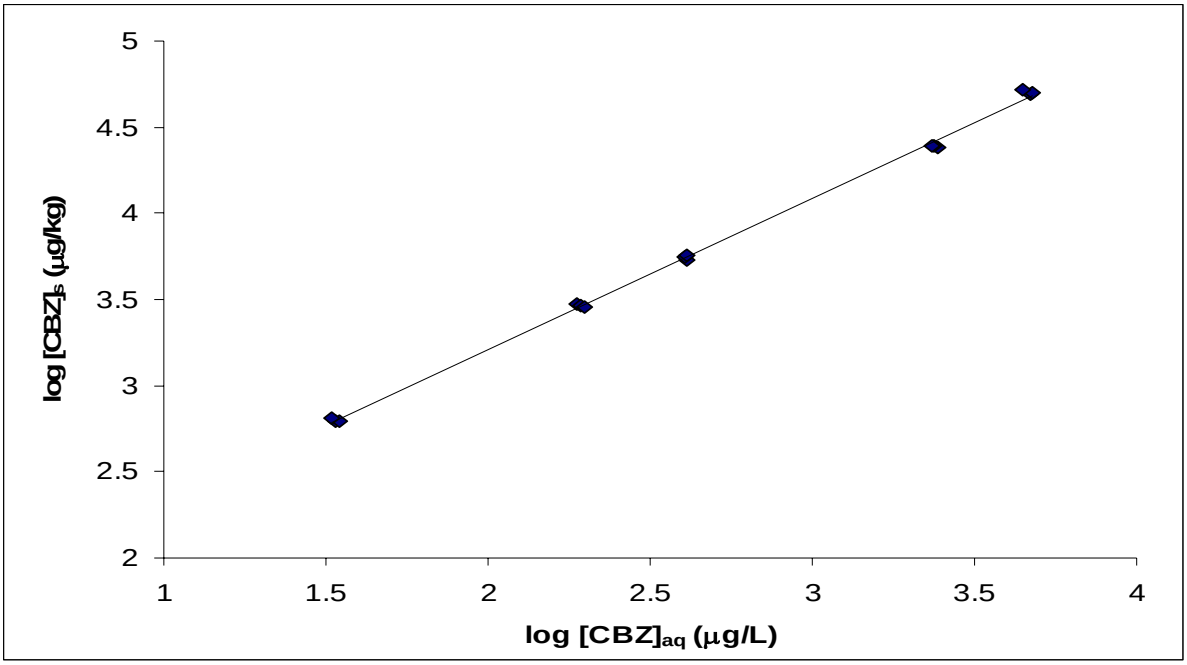


Figure 7.3: Sorption isotherm for carbamazepine from batch sorption experiments for triplicate samples

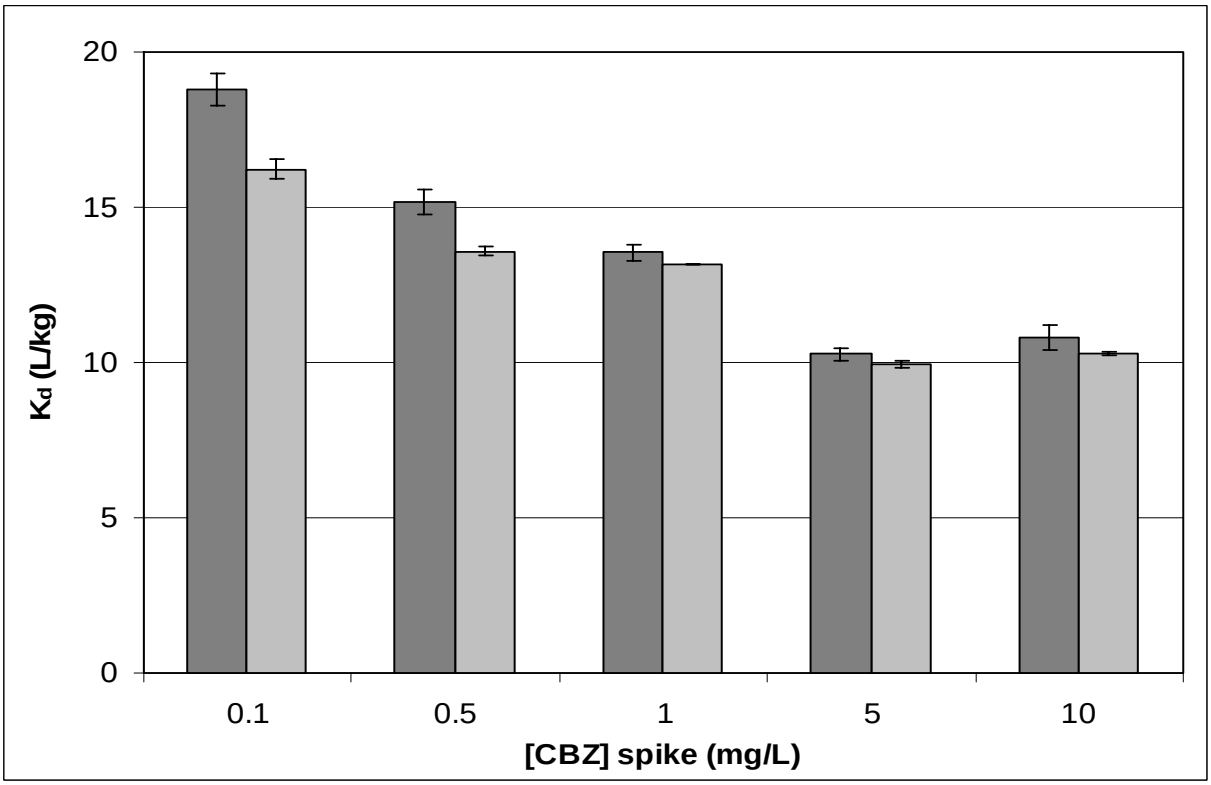


Figure 7.4: Effect of concentration of carbamazepine ([CBZ]) on the distribution coefficient (K_d) value of unlabelled carbamazepine (^{12}C-CBZ; ■) and radiolabelled carbamazepine (^{14}C-CBZ; ■)

7.3.2 Time dependence of E-value

The pH of the system was not affected by time, with the pH of solutions for all time treatments 6.04±0.15. However, the solution pH is substantially higher than that determined for previous batch sorption experiments using the same sediment, where 10 mM $CaCl_2$ and a 1 : 10 solution : sediment ratio was used (*see* Chapter 4; Section 4.3.2). The K_d values for this series of experiments ranged from 14 to 21 Lkg^{-1} for the 0.45 μm treatments (Figure 7.6). This is compared with a K_d value of 16 Lkg^{-1} for the 1 : 10 solution : sediment ratio treatment in Chapter 4 (*see* Chapter 4; Figure 4.4). Therefore, in this case pH was not found to influence the K_d value of CBZ.

For both the filtered and unfiltered treatments of non-amended sediments, there was a decrease in the amount of CBZ in the exchangeable pool as the time of shaking increased. This is depicted as the percentage of CBZ that was found in the exchangeable pool, relative to the theoretical amount that would be found if CBZ was 100 % exchangeable for each treatment (Figure 7.5). After day 7, the E-value for the unfiltered samples did not significantly decrease, while the same could be seen for the filtered treatments after day 14 (Figure 7.5). While the 0.2 μm filtered treatment had a considerable degree of variability associated with it, it followed the same pattern as the other treatments.

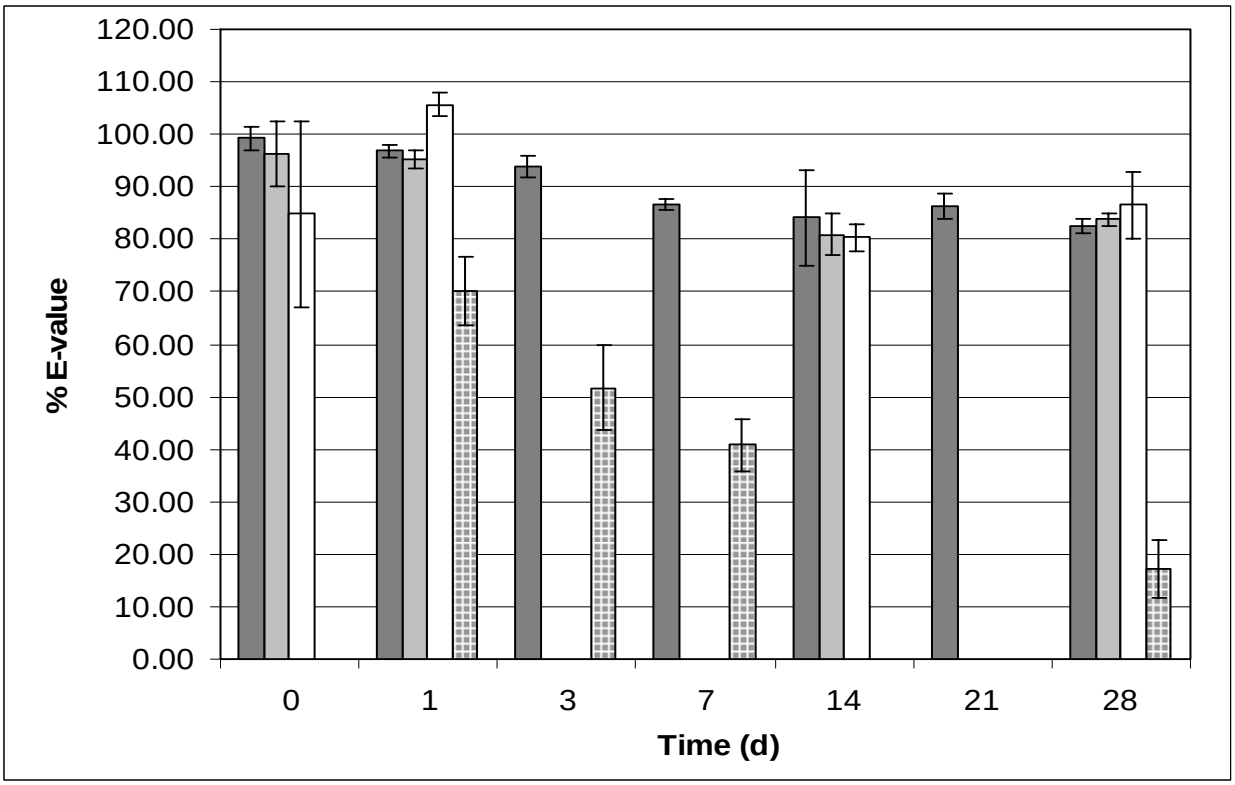


Figure 7.5: Effect of time of batch sorption experiments on the percentage of originally spiked carbamazepine remaining within the exchangeable pool for unfiltered treatment (■), 0.45 µm filtered treatment (■), 0.2 µm filtered treatment (□) and char-amended sediment treatment (▦). Error bars shown are standard error of triplicate samples.

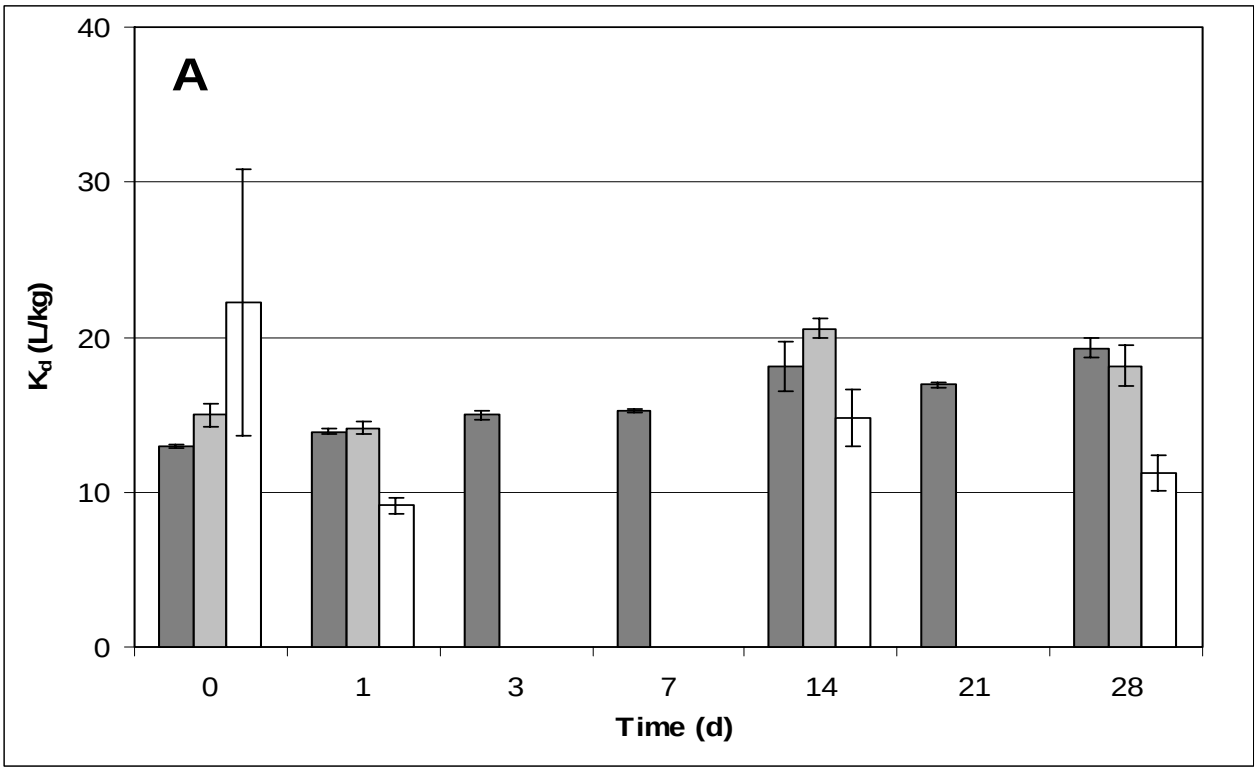
The E-value of CBZ spiked to the char-amended sediment was seen to decrease until the final treatment at day 28 (Figure 7.5). Previous work with CBZ, in a static sediment / solution system, found that it took nearly 60 d to reach a steady-state between the sediment and solution (Loffler *et al.* 2005). The relatively shorter 7-14 d equilibration time found in this work for non-amended sediment may have been attributable to a number of factors. One important factor may have been due to the active mixing of the batch sorption technique. Also, since the char treatment is not likely to have attained equilibrium after 28 d, the quality of sediment is a probable influence on the time to sorption equilibrium. However, both the work undertaken by Loffler and co-workers (2005) and the char and non-char amended treatments in this study highlight the importance of considering slow sorption

processes in sediment binding, especially where sorbents of a microporous nature, such as char, are present.

The K_d values of the ^{14}C-CBZ were constant with time for the non-amended sediment treatments (Figure 7.6), while they decreased slightly (27 to 23 Lkg^{-1}) for the char-amended treatments (Figure 7.5). Furthermore, the K_d values of ^{14}C-CBZ in the char-amended sediment were higher than the K_d values in the non-amended char. A number of points can be made about these observations of the K_d values. Along with the significantly higher K_d values for ^{12}C-CBZ in the char-amended sediment compared with the non-amended char treatments, the K_d values of the ^{14}C-CBZ indicated that the char enhanced the affinity of CBZ to the sediment.

Also, as the length of shaking time increased, the amount of ^{12}C-CBZ exchanging with ^{14}C-CBZ decreased. Since the K_d of the ^{14}C-CBZ was effectively constant over time for both the char-amended and non-amended sediment treatments, it seems likely that the exchangeable domains within the sediment were not affected by time of shaking.

Conversely, the increase in K_d for ^{12}C-CBZ in both sediment treatments indicates that the increasing sorption is likely to be associated with greater access to the non-exchangeable pool of the sediment. Char has been found to enhance the sorption of organic compounds to the extent where non-linearity of the sorption isotherm occurs (James *et al.* 2005; Koelmans *et al.* 2006). Also, the potential for non-exchangeable sorption is greatly enhanced in char due to its structure and quality of organic carbon (Braida *et al.* 2003; Cornelissen *et al.* 2005; Golding *et al.* 2005).



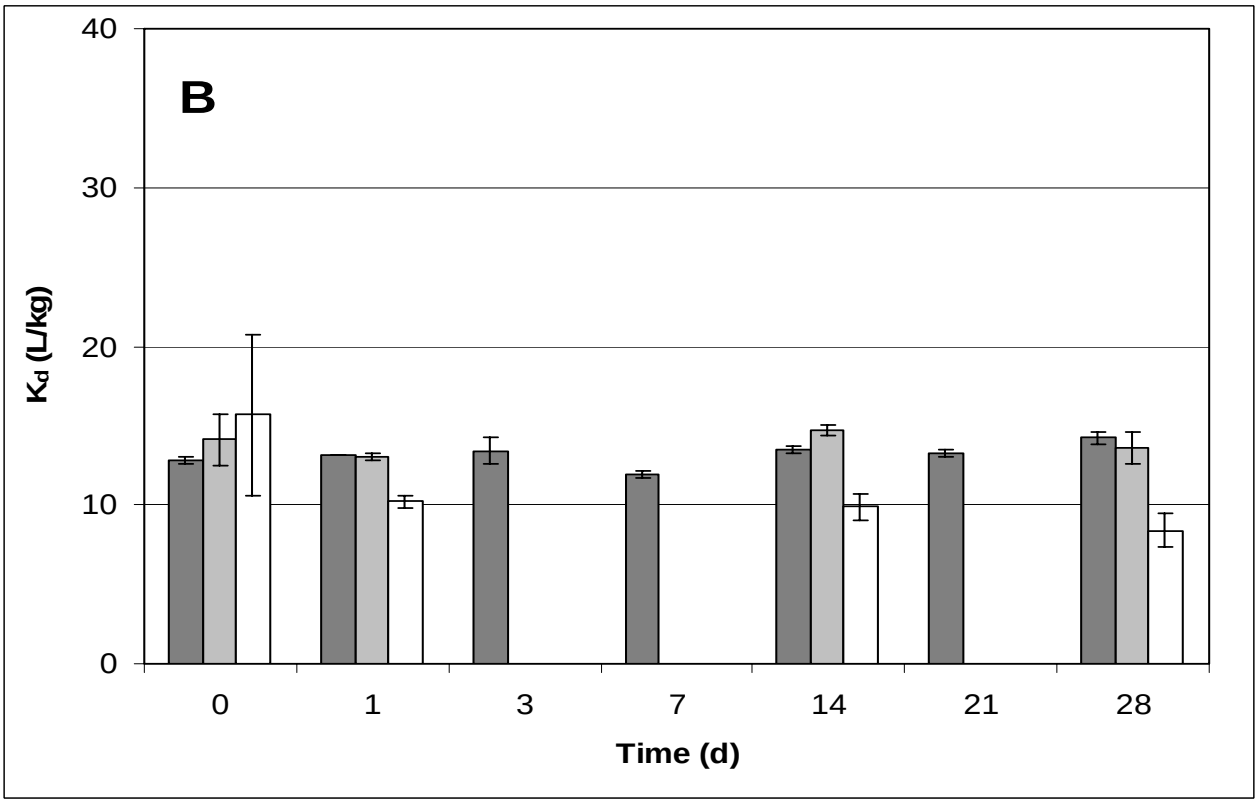


Figure 7.6: Effect of time of batch sorption experiments on the distribution coefficient (K_d) value of (A) unlabelled carbamazepine (^{12}C-CBZ) and (B) radiolabelled carbamazepine (^{14}C-CBZ) for unfiltered treatment (■), 0.45 μm filtered treatment (■) and 0.2 μm filtered treatment (□). Error bars shown are standard error of triplicate samples.

While this has been found for hydrophobic organic contaminants, a strong influence on the potential for desorption also seems to be occurring in the char-amended sediments. Since the K_d values in this study are determined indirectly (from solution concentrations only) it is possible that biodegradation is enhancing the K_d value and, therefore, causing an apparent decrease in the E-value of CBZ. However, CBZ has consistently found to be highly resistant to degradation in both natural and laboratory aquatic systems (Metcalfe *et al.* 2003a; Lam *et al.* 2004; Loffler *et al.* 2005). For example, after 100 d in a water / sediment system, a mass-balance of CBZ showed more than 80 % of the initially spiked CBZ remained, corresponding with a disappearance half-life of 328 d (Loffler *et al.* 2005). Also, association of organic compounds to char has been found to reduce the extent of their biodegradation (Cornelissen *et al.* 2005). The reduction in the E-value of CBZ, particularly where no limit was reached in the char-amended treatments, is most likely to be associated with increased association with a non-exchangeable pool compared with degradation over time. Degradation and losses due to non-sediment processes was found to be negligible by the sediment free controls.

The K_d value of the ^{12}C-CBZ control, spiked without ^{14}C-CBZ, was 14.02±0.3 Lkg^{-1}, while the ^{14}C-CBZ control, spiked without ^{12}C-CBZ, was 15.4±0.1 Lkg^{-1}. This indicates that the sorption capacity of the sediment was slightly reduced for ^{14}C-CBZ in the presence of ^{12}C-CBZ (K_d = 13.2±0.2 Lkg^{-1}, for all treatments), while addition of ^{14}C-CBZ did not affect the sorption capacity of ^{12}C-CBZ (K_d = 14.4±0.3 Lkg^{-1}, for all treatments). The assumption that addition of the radiolabelled CBZ did not affect the sorption equilibrium of the unlabelled CBZ was therefore appropriate.

7.3.4 Use of the E-value and implications for sorption of carbamazepine (CBZ)

7.3.4.1 Comparison with other desorption methods

The use of the E-value to estimate the extent of desorption of CBZ was a different method than that employed in Chapter 4 (*see* Chapter 4; Section 4.2.5). For example, slightly different parameters to assess batch sorption were undertaken in the two studies, while another analytical method was required to measure concentrations of ^{14}C-CBZ. These factors make it difficult to make a direct comparison between the extent of hysteresis seen in Chapter 4 and in this chapter. However, qualitative analysis of the two methods is worth considering. In Chapter 4, the comparison between the sorption and desorption isotherms of CBZ suggest that a large degree of hysteresis occurred (*see* Chapter 4; Tables 4.3 and 4.4). However, the final point on the desorption isotherm plot can be seen to contribute greatly to the K_f of desorption, with the other points falling on sorption isotherm, suggesting little desorption hysteresis actually occurred (*see* Chapter 4; Figure 4.6). In the present chapter, a slight reduction in the exchangeable fraction of CBZ was found after 24 h of shaking, for both time and the majority of concentration treatments. However, the extent was not found to be substantial, where more than 90 % of CBZ was found to still remain within the exchangeable pool (Figures 7.2 and 7.5). It would therefore seem that a qualitative assessment between the different methods indicates that the isotopic dilution method experiences less experimental artefacts that can indicate desorption hysteresis, or that it suffers them to a lesser extent. The time of the sorption experiments were of the same and degradation of CBZ was unlikely to differ between the methods. Therefore, if experimental artefacts were a cause of the apparently lower level of desorption in the dilution method (*see* Chapter 4), it was most likely to be linked with the methodology. While an attempt was made

to account for potential loss during the decanting process, the potential for loss of analyte still existed in the dilution method (*see* Chapter 4, Section 4.2.4). The loss of analyte was much less likely to occur using the isotopic dilution approach since no solution was removed between the commencement of the batch sorption experiment and removal of sample for analysis. Also, any loss of CBZ during sample preparation would be expected to be identical for the two CBZ isotopes, which would not influence the ratio between the two used to determine the E-value (Equation 7.5). Other studies have found that isotopic dilution can determine hysteresis while reducing the extent of experimental artefacts (Celis and Koskinen 1999b; Sander and Pignatello 2005). However, due to the relatively few studies that have used the isotopic exchange method for organic contaminants, substantially more work needs to be undertaken to prove its utility in this respect.

7.3.4.2 Presence of char in sediment

Amending the sediment with char was found to lead to a substantial increase in the K_d value of ^{12}C-CBZ, with a concurrent decrease in the E-value for CBZ. The presence of char in soils or sediments has been previously found to increase the extent of sorption, as well as desorption hysteresis (Braida *et al.* 2003; Sander and Pignatello 2005; Yu *et al.* 2006). However, while this association was found between the K_d of ^{12}C-CBZ and the E-value, it is not necessarily a case of the value of K_d influencing the E-value. As Figure 7.1 indicates, the K_d value is a measure of the concentration of an analyte associated with the sediment, while the E-value is only a measure of the concentration of analyte associated with the sediment that can rapidly exchange with the analyte in the aqueous phase.

As previously mentioned, sediment was amended with char to provide a case where sorption was significant for otherwise weakly sorbed CBZ and to assess the exchangeable fraction of CBZ from a sorbent of strong affinity. Addition of the char led to not only a significant increase in sorption but significant reduction in the exchangeability of CBZ. Numerous other studies have found that the quality of organic carbon plays an important role in the sorption of organic compounds (Huang *et al.* 2003; Golding *et al.* 2005; James *et al.* 2005). Char, along with other black carbon material, has been identified as being a domain associated with having a high affinity with organic compounds (Huang *et al.* 2003; Cornelissen *et al.* 2005; Koelmans *et al.* 2006). The highly aromatic nature of black carbon plays an important role in its structure and affinity for organic compounds (Cornelissen *et al.* 2005). For example, a quantitative assessment for the sorption of phenanthrene to a number of sediments was found to be due to the quantity of aromatic domains within the sediments, while the type of aromatic carbon was important for the extent of sorption (Golding *et al.* 2005). Also, the sorption of the pesticides phosalone and carbaryl were found to be strongly correlated with the content of aromatic carbon in a number of soils (Ahmad *et al.* 2001). The surface area and presence of micropores (pores width < 2 nm) within char has also been found to have an important influence on the affinity and desorption hysteresis of organic compounds. For example, Yu *et al.* (2006) noted a strong correlation between microporosity of char and the hysteresis index of a non-ionic pesticide, showing an increase in desorption hysteresis with increasing microporosity. The char used in this work was found to have a specific surface area of around 570 000 m^2g^{-1} and contained a micropore volume of 0.28 cm^3g^{-1} (Yu *et al.* 2006). Micropores play an important role in increasing desorption through their deformation (Braida *et al.* 2003; Sander and Pignatello 2005; Yu *et al.* 2006). This suggests that the decrease in the E-value was indicative of CBZ actually entering the non-exchangeable pool to the significant level found in the char-amended treatments.

The sediment treatment that did not have char added to it was found to have an appreciable quantity of aromatic carbon (around 25 %), based on ^{13}C-NMR analysis (*see* Chapter 4, Table 4.2). While the quality of the aromatic carbon was not assessed, this fraction of aromatic carbon may have contributed to the slow sorption process, where the E-value of CBZ reduced to around 80 % of spiked values after 28 d (Figure 7.5). This E-value is substantially higher than was found in the char-amended sediments, which suggests that the aromatic carbon found in the sediment was not of the same quality as that in the char. That is, the addition of char increased the percentage of carbon in the sediment from around 5 % to 7 %, which is unlikely to have led to the disparity seen for the respective E-values (Figure 7.5).

7.3.4.3 Implications for the exchangeability of CBZ

Since pharmaceuticals are released into the environment at consistent rates year round (Heberer *et al.* 2002), a steady-state within a receiving aquatic environment is likely to exist. If a steady-state existed, this would allow slow sorption processes to occur. These processes would increase the likelihood of a certain fraction of a drug like CBZ to remain absorbed. While there is a disparity in the extent of desorption that occurred between the methods in Chapter 4 and in this chapter, both conclude that a fraction of CBZ is likely to enter the non-exchangeable phase. Integration of organic compounds within the slowly desorbing pool of the solid phase is often associated with a reduction in the extent of biodegradation (Howard 2000; Della Site 2001; Bondarenko and Gan 2004; Cornelissen *et al.* 2005) and bioavailability to aquatic organisms (Kaag *et al.* 1998; Lamoureux and Brownawell 1999; Sijm *et al.* 2000; Koelmans *et al.* 2006).

These assumptions should also be taken in the context of the experimental conditions under which they were created. As previously noted, the batch sorption technique has a number of limitations that could restrict its interpretation for environmental systems.

Also, the use of the isotopic exchange technique has not been thoroughly investigated as a means of quantifying the exchangeability of organic compounds associated with solids. However, this study has given important insights into the behaviour of CBZ within a batch sorption system and should flag a number of potential avenues for future investigations. For example, the marked decrease in the E-value of CBZ in the amended char, consistent with the microporous nature of the char, indicated that the isotopic dilution method is probable a useful approach to measuring the labile fraction of sorbed CBZ. The use of the E-value as a means of assessing the strength of association for CBZ and other pharmaceuticals to sediments needs to be further developed. Since the E-value gives an indication of the extent of the interactions between the organic compounds and a solid phase, assessment of the E-value in a number of different sediments and for a number of different pharmaceuticals should be undertaken. The implications of access to sediment drugs has important implications for biodegradation and bioavailability and, therefore, in the overall approach to risk assessment. Therefore, assessment of the biodegradation in terms of the amount of a drug within the exchangeable pool is worth considering.

7.4 Conclusions

An isotopic dilution method was used to determine the E-value of CBZ, within a batch sorption system containing sediment and 10 mM $CaCl_2$ solution. The E-value was found to decrease to around 80 % of the spiked CBZ over a 28 d period, although the E-value did not

change after 14 d. This highlights the importance of considering slow sorption process in the aquatic fate of CBZ.

Amendment of the sediment with char led to a substantial decrease in E-value, with no minimum apparently reached after 28 d. This expected result, due to the microporous nature of the char, demonstrates isotopic dilution could be an important tool for assessing the desorption potential of other pharmaceuticals. This should be the subject of future investigations.

Desorption hysteresis was not observed after 24 h contact with sediment, which seemed to be confirmed from previous batch desorption work. Whether isotopic dilution suffers from experimental artefacts to a lesser extent is another opportunity for future work, with a number of other studies already suggesting that the use of isotopic dilution may eliminate artefacts altogether (Celis and Koskinen 1999a, 1999b; Sander and Pignatello 2005). If isotopic dilution was found to reduce experimental artefacts then this may present a powerful technique for understanding sorption processes and their subsequent influence on the fate of pharmaceuticals and other organic contaminants in sediments.

The exchangeable sorption to sediments could have important consequences with respect to the fate of CBZ within aquatic systems, in terms of biodegradation and bioavailability. Along with this knowledge, the exposure to aquatic organisms within an aquatic system containing sediment would be an important part of determining whether sediment could play an important role in the toxicological profile of a drug.

Chapter 8. Ecotoxicology of carbamazepine in sediments; the case of the freshwater midge, *Chironomus tepperi*

8.1 Introduction

The major concern with the presence of pharmaceuticals present in the aquatic environment is that they are a class of compounds designed to specifically bind to receptors to exert biological effects. Human pharmaceuticals as a class of environmental contaminants alone represent a vast diversity of compounds in terms of their physicochemical properties and biological effects. This raises the concern that exposure to a mixture of pharmaceuticals at once may lead to effects at concentrations that are lower than expected from exposure to a single drug (Cleuvers 2003). Despite the relatively low levels at which pharmaceuticals are found, constant exposure of aquatic organisms over their life-cycle may lead to effects that may have profound consequences on development, fitness or reproduction. Exposure over a number of generations may lead to subtle changes that would be falsely attributed to natural processes (Daughton and Ternes 1999).

8.1.1 Ecotoxicology of pharmaceuticals

The conservation of receptors in aquatic organisms may also suggest that pharmaceuticals interacting with receptors might be predictable based on the targeted receptors within humans (Huggett *et al.* 2004). However, although interaction with receptors might be predictable, the effect of this interaction in aquatic organisms may lead to profoundly different physiological effects. For example, the selective-serotonin reuptake inhibitors (SSRIs) paroxetine and fluvoxamine were found to induce spawning in the fingernail clam, *Sphaerium striatinum* at concentrations as low as 10 nM (Fong *et al.* 1998). Serotonin (5-hydoxytriptamine or 5-HT)

has important effects on reproduction in bivalve molluscs, while in humans 5-HT regulates a number of functions, including sleep, appetite, sensory perception and mood (Fong *et al.* 1998; Sanders-Bush and Mayer 2001). Therefore, while it is important to characterise the likely receptors pharmaceuticals will have an effect on in aquatic organisms, it is equally important to define what effects such interactions may have on the functioning of the organism. In ecotoxicology, the use of biological markers of effect, or biomarkers, can be used to define whether exposure and / or sub-physiological effects have occurred (Schlenk 1999; Eason and O'Halloran 2002). It follows that biomarkers do not necessarily indicate an effect is likely but, rather, that uptake and/or alteration of some function within the organism has occurred following exposure to a compound not found within that organism, also known as a “xenobiotic”.

There has been substantially less work undertaken on ecotoxicological assays of human pharmaceuticals, compared with environmental surveys. Therefore, it is important to put into context the concentrations of pharmaceuticals that have been detected in aquatic systems. While ecotoxicological assays are only recommended by FDA and EMEA guidelines after threshold concentrations of pharmaceuticals are breached (FDA-CDER 1998; EMEA-CPMP 2005), concentrations below the threshold level may also be of importance. For example, entry of pharmaceuticals into the aquatic environment would be expected to occur along with other pharmaceuticals and anthropogenic contaminants. Therefore, additive or even synergistic (greater than additive) effects of individual compounds may contribute to effectively lower the threshold concentration (Cleuvers 2003, 2004). Also, the constant release of pharmaceuticals into the environment, consistent with their therapeutic use, implies chronic exposure of aquatic organisms to pharmaceuticals is likely (Daughton and Ternes 1999). In this case, the use of acute toxicity assays for risk assessment is less likely to provide an accurate reflection of the ecotoxicological impacts based on the concentrations detected in the environment. This is because exposure at concentrations considered sub-lethal

from acute toxicity assays could still lead to effects over longer exposure periods (Nash *et al.* 2004; Fent *et al.* 2006). For example, the ecotoxicological effect, or endpoint, of an acute assay is usually associated with mortality, while chronic assays can elucidate long-term endpoints, such as developmental or reproductive success. Chronic toxicity assays can therefore elucidate endpoints that have an impact on the life-cycle of an organism or even the success of a population.

Most data that currently exist on the potential for ecotoxicity of human pharmaceuticals are based on acute toxicity data (Webb 2004; Fent *et al.* 2006). However, the few ecotoxicity assays that have involved chronic exposure tend to show effects at lower concentrations compared with acute exposures (Wollenberger *et al.* 2000; Webb 2004; Flaherty and Dodson 2005; Hoeger *et al.* 2005; Oetken *et al.* 2005). Furthermore, the majority of these tests are related to aqueous exposures only. This is despite the moderate level of interaction that pharmaceuticals would be expected to undergo with sediments once released within an aquatic system. Sorption of organic contaminants has been found to influence their extent of bioavailability and, therefore, their potential ecotoxicity (Lamoureux and Brownawell 1999; Reid *et al.* 2000; Sijm *et al.* 2000). This influence usually leads to a reduction of the bioavailable fraction and subsequent toxicological impacts, although there is still a poor understanding of processes related to the bioavailability of organic contaminants from sediments (Reid *et al.* 2000; Eggleton and Thomas 2004).

Therefore, one aim of this work was to try to elucidate what role sorption of CBZ to the sediments, previously found to be relatively weak, might have on the potential for its uptake in an aquatic organism. In other words, sorption could act as a protective mechanism for aquatic organisms by reducing bioavailability (a “sink”) or, conversely, providing an alternative route of uptake for bound contaminants that are readily desorbed (a “source”).

8.1.2 Ecotoxicology of carbamazepine (CBZ)

Following the focus on the exchangeability of carbamazepine (CBZ) in Chapter 7, the ecotoxicological profile of CBZ was examined in this chapter. CBZ has been commonly detected at high part per trillion (ngL^{-1}) to low part per billion (μgL^{-1}) concentrations in environmental surveys (Heberer *et al.* 2002; Metcalfe *et al.* 2003b; Ferrari *et al.* 2004) and has also been used in a number of ecotoxicological assays (Cleuvers 2003; Ferrari *et al.* 2003; Jos *et al.* 2003; Oetken *et al.* 2005). To assess the potential ecotoxicity of sediment bound CBZ, Oetken *et al.* (2005) found a significant effect on the development of the freshwater midge, *Chironomus riparius*, at sediment concentrations as low as 0.16 $mgkg^{-1}$ in a chronic exposure assay.

In humans, CBZ is used primarily to treat generalised and partial epileptic seizures (McNamara 2001). Its mode of action is related to binding to receptors that would activate voltage-sensitive, membrane-bound sodium (Na^+) channels (Willow *et al.* 1984) and presynaptic inhibition of synaptic transmission (Smyth *et al.* 2002). Therefore, CBZ regulates the flow of Na^+ across cellular membranes by prolonging the inactivation of Na^+ channels (McNamara 2001).

Sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) is an enzyme that facilitates the energy-dependent transfer of Na^+ across cellular membranes (Emery *et al.* 1998; Hoffman and Taylor 2001). The activity of this enzyme has been used as an ecotoxicological endpoint for a number of aquatic organisms, both vertebrates and invertebrates, as the flow of Na^+ across cellular membranes is a critical process for the functioning of an organism (Emery *et al.* 1998; Lionetto *et al.* 2000; Cotou *et al.* 2001; Webb *et al.* 2001).

8.1.3 The use of Chironomus tepperi *(freshwater midge) for ecotoxicity testing*

Larvae of the freshwater midge (*Chironomus* spp.) have been commonly used in ecotoxicological assays as they are relatively easy to culture, their larvae interact with both the sediment and water column and a number of ecotoxicological endpoints can be used (OECD 2001b). *Chironomus* are amenable to acute toxicity testing in aqueous solutions or in sediment-based chronic toxicity testing that encompasses their developmental stages. After hatching from eggs, *Chironomus* larvae burrow into sediment and feed on organic matter within the sediment, after which the larvae commence pupation from which they emerge as an adult midge. The period of this lifecycle is dependent on the species of *Chironomus*, and can take from 20-28 days for *C. riparius* to 28-65 days for *C. tentans* (OECD 2001b). *Chironomus tepperi* was used in this ecotoxicological assay, as another species of the freshwater midge, *C. riparius*, has previously been found to be sensitive to CBZ (Oetken *et al.* 2005). This species of midge has been used for previous ecotoxicological assays, with the length of the lifecycle similar to that of *C. riparius*.

In this chapter, *C. tepperi* were exposed to CBZ using both 48 h, water-only assays (acute testing) and 14-21 d sediment-based assays (chronic testing). An assay for the activity of Na^+/K^+-ATPase within *C. tepperi* cell homogenates was used as an endpoint in both the acute and chronic assays, as well as for an *in vitro* assay. This was to determine whether CBZ affected the flow of Na^+ across cell membranes in *C. tepperi* and, therefore, whether the activity of Na^+/K^+-ATPase would be a useful biomarker of exposure or as a sub-organism level effect. Therefore, the expected biological effect in *C. tepperi* was tested, based on the known effect within humans. Other endpoints assessed were mortality (acute assay), wet weight of larvae (acute and chronic assays) and the length of time and number of larvae that emerged as adults, following chronic exposure. These assays were used to determine whether CBZ had an effect on the organism level functioning of *C. tepperi*, following acute and

chronic exposure periods through exposure to both sediment and water-only concentrations of CBZ.

8.2 Methods and materials

8.2.1 Test chemicals

Carbamazepine (CBZ) was purchased from Sigma-Aldrich (NSW, Australia). Stock solutions of 1 gL^{-1} CBZ were prepared in Malinckrodt methanol (Biolab; Victoria, Australia) and stored at -18°C until required. Working solutions were prepared from this stock solution, with the methanol evaporated under N_2 prior to addition to test systems and made up to solution using 18.2 $M\Omega cm^{-1}$ water. If necessary, working solutions were placed in an ultrasonic bath to solubilise CBZ.

8.2.2 Test sediment and solutions

The test solution used for all the experiments was moderately hard water (MHW), prepared following USEPA guidelines (USEPA 2002). Prior to use in the tests, the MHW was aerated for a minimum of 24 h and tested for pH, electrical conductivity and dissolved oxygen content.

The sediment used in the tests was the same as that collected for the sorption experiments in Chapter 4 with physicochemical parameters listed in Table 4.1. Sediment was filtered through a 0.425 mm sieve prior to use in the tests. During toxicity testing, sediment was added to 2 L test beakers at a ratio of 50 g (dry weight) : 350 mL water to give a volumetric ratio within the test beaker of 1 : 4.

8.2.3 Chironomus tepperi *culturing*

The organism used for the test was the aquatic midge, *Chironomus tepperi*, which were originally sourced from Dr Mark Stevens (NSW Agriculture). Culture tanks for *C. tepperi* were 2 L glass beakers containing ca. 1.8 L MHW and ca. 10 mm depth of tissue paper. Since the tissue paper was used as a substrate for the egg sacs and hatched larvae to reside, tissue paper needed to be suitably prepared to avoid any adverse effects of contaminants within it. Tissue paper was shredded and placed in 2 L glass beakers, immersed in 95 % ethanol and boiled for approximately 1 h. The ethanol was then decanted and the tissue paper was immersed in 18.2 $M\Omega cm^{-1}$ water, which was also decanted. This rinsing was repeated 5 times before the remaining solution was squeezed out of the tissue by hand and the clean tissue paper was kept at +4 °C for a maximum of 2 weeks. The *C. tepperi* were fed with Nutramin® tropical fish flakes flakes, ground with a mortar and pestle and a prepared as a suspension of 1 g/25 mL 18 $M\Omega cm^{-1}$ water. Approximately 2 mL (80 mg of Nutramin®) of this suspension was added to each beaker per day. Feeding was conducted *ad libitum* depending on the developmental stage of the larvae; cultures were monitored daily to ensure under- or overfeeding was not occurring. Culture tanks were kept in an incubator at 24 °C with a 16 : 8 h light : dark cycle maintained.

For testing, egg sacs were produced by placing adult *C. tepperi* into a fish tank containing still MHW at a depth of *ca.* 100 mm. All egg sacs produced within a 24 h period were collected and placed inside culture tanks. Aeration was ceased prior to their addition and recommenced once the egg sacs attached to the tissue paper. Larvae used for acute and chronic toxicity assays were collected at the 1st instar larval stage, collected 3 d after hatching or 10 d after the egg sacs were initially collected. At this stage larvae were *ca*. 2-3 mm long. Larvae used for the *in vitro* assay were collected at the final instar (prior to pupating), when they had taken on a reddish hue and were *ca*. 10 mm long.

8.2.4 Degradation of CBZ within solution

To assess whether CBZ was likely to be susceptible to degradation during the ecotoxicity assay, photolytic, hydrolytic and biological stability were determined in solution. Both sterile and non-sterile solutions of MHW, previously mixed with the test sediment, and sterile 18.2 MΩcm^{-1} water were spiked with CBZ and subjected to either light or dark conditions, respectively. The concentration of CBZ in solution was then assessed over time.

Sediment was mixed with MHW in a 2 L Schott bottle at a ratio of 1 : 10 and mixed on a rotating shaker for 24 h. The Schott bottle was allowed to stand for *ca*. 2 h before the solution was filtered through a 0.45 μm nylon syringe filter (Biolab; Victoria, Australia). 10 mL of the solution was set aside, while the remainder of the solution was collected in a 2 L Schott bottle and autoclaved, using a Siltex HC2 autoclave, for 20 min at 121°C and 220 kPa. Half of the autoclaved solution was then placed in a separate Schott bottle and re-inoculated with the 10 mL of solution previously set aside. A 10 gL^{-1} stock solution of CBZ in methanol was filtered through a 0.2μm PTFE syringe filter and 50 μL of this was spiked to 500 mL of the respective MHW or sterile 18.2 MΩcm^{-1} water solutions to give a test concentration of 1 mgL^{-1}. 10 mL of the solutions were then added to previously autoclaved glass culture tubes with PTFE-lined lids.

Culture tubes were wrapped in aluminium foil for the dark treatment solutions. All tubes were laid flat in a tray to maximise exposure to light. The light source was a 400 W Philips Son-T-Agro light placed 400 mm above the culture tubes. Light exposure was continuous, with a total exposure time of 350 h. The light intensity was measured daily using a LiCor LI-185A light meter (Adelab, Australia). The experimental set-up was in a temperature controlled room, set at 22 °C. A fan was set-up near the tray to disperse heat from the light

source and independent thermometers were placed around the tray to ensure that the temperature around the tubes was not different from the ambient temperature.

Triplicate samples from each treatment were removed at time 0, 24, 48, 96, 168, 264 and 350 h. A 1 mL aliquot was removed (using autoclaved pipette tips) for HPLC analysis. A further 1 mL aliquot was taken from the tubes and pooled for triplicate treatments for assessment of the sterility of solution. The remainder of solution was used for assessment of pH, electrical conductivity and dissolved organic carbon.

Plate counting techniques were used to assess the sterility of the samples. Difco™ nutrient broth (BD and Co.; MD, USA) was prepared in a 2 L Schott bottle and autoclaved for 20 min at 121 °C at 220 kPa. While still liquid, the agar was poured into sterile, plastic petri dishes (Techno-Plas; S.A., Australia) and allowed to cool. Pooled aliquots of experimental solution were applied to the cool agar using a sterilised wire loop. Agar plates were then incubated at 25 °C in the dark for 24 h. The presence of colony forming units (CFUs) indicated the presence of viable microorganisms.

8.2.5 Na^+/K^+-ATPase assay

The activity of the Na^+/K^+-ATPase enzyme within homogenised midge tissue was adapted from a number of methods (Schin and Kroeger 1980; Lionetto *et al.* 2000; Cotou *et al.* 2001; Webb *et al.* 2001). The general approach is to spike homogenised cells with adenosine triphosphate (ATP) and measure the amount of inorganic phosphorus (P_i) liberated as ATP is hydrolysed to adenosine diphosphate (ADP) over a period of time. Hydrolysis of ATP occurs through the ATPase enzyme using the energy for transport of ions into the cells. The ATPase activity is the sum of Na^+/K^+-ATPase and Mg^{2+}-ATPase activity within cells. Ouabain (OUB) is a cardiac glycoside drug that specifically inhibits the activity of Na^+/K^+-ATPase.

Therefore, a comparison of the P_i released in the presence and absence of OUB gives the specific activity of Na^+/K^+-ATPase. This activity is then normalised to the content of protein within the cell homogenate to give an activity of moles of P_i released per gram of protein per unit of time.

C. tepperi collected for assessment of Na^+/K^+-ATPase activity were placed in 2 mL polypropylene microcentrifuge tubes (Degersheim, Switzerland). An SEI buffer containing 300 mM sucrose, 20 mM ethylenediaminetetracetate disodium (Na_2EDTA) and 115 mM imidazole (all chemicals from Sigma-Aldrich; NSW, Australia) was adjusted to pH 7.2 with 0.6 % v/v concentrated hydrochloric acid (HCl). The SEI buffer was added at a ratio of 10 μL per 1 mg of *C. tepperi* tissue. The activity of Na^+/K^+-ATPase has been found to be dependent on pH, where the pH of the SEI buffer is within the optimal range (Schin and Kroeger 1980). Samples were then immediately placed at -80 °C until required.

For the Na^+/K^+-ATPase assay, the frozen midges were thawed on ice and homogenised using a PTFE pestle attached to an electric drill. The pestle was rinsed with a volume of 18 $M\Omega cm^{-1}$ water (chilled to +4 °C) equivalent to the added SEI buffer. Eppendorf tubes were kept on ice during homogenisation. Homogenised midges were then centrifuged at 1000 *g* for 4 min at +4 °C. The supernatant of the centrifuged samples was collected and used to assay the Na^+/K^+-ATPase activity. The assay was undertaken by adding 50 μL of homogenate supernatant to 350 μL of 115 mM imidazole, 155 mM sodium chloride (NaCl), 75 mM potassium chloride (KCl), 23 mM magnesium chloride ($MgCl_2$) and 5 mM OUB (as $OUB.8H_2O$) in a 2.5 mL plastic cup. Also, 50 μL of homogenate was added to an identical solution without OUB. The assay was initiated by spiking the solutions with 25 μL of disodium adenosine triphosphate (Na_2ATP) (Sigma-Aldrich: NSW, Australia) and placing in

an incubator at 30 °C for 1 h. To stop the reaction, 750 μL of 0.8 M sulphuric acid (H_2SO_4) was added to each cup.

The amount of P_i in solution was measured using on-line colorimetry using the molybdate reactive P_i following methodology developed by Hamon and McLaughlin (2002). Due to the sensitivity of this technique to pH, solutions were neutralised immediately prior to analysis by the addition of 825 μL 0.5 M sodium hydroxide (NaOH). The colour reagent was prepared using 40 mgL^{-1} ammonium molybdate, 0.14 gL^{-1} potassium antimony tartrate, 9 gL^{-1} ascorbic acid and 0.2 gL^{-1} sodium lauryl sulphate in 540 mM sulphuric acid (H_2SO_4). The reagent was prepared immediately prior to analysis. Solutions were pumped through a Burkard Scientific SFA-2 segmented flow analyser, with the flow tube length adjusted to allow the sample and colour reagent to react for 3.5 min at room temperature. The solution was then directed to a spectrophotometer cell and the absorbance measured at 880 nm. Standards were prepared by adding 50 μL of sodium orthophosphate (Na_2PO_4), in place of cell homogenate, to obtain final P_i concentrations of ranging from 0 to 1 mgL^{-1}.To assess whether Na_2ATP may have affected the amount of P_i measured in solution, 0, 5, 10, 25 and 50 μL of the 25 mM Na_2ATP solution were added to the reaction vessel, while keeping the final volume of the reaction mixture the same.

The P_i released was normalised to the amount of protein in the homogenate by using the Lowry technique for measuring protein (Lowry *et al.* 1951). Reagents for the Lowry technique were 200 mM sodium carbonate (Na_2CO_3) and 500 mM NaOH (solution A), 90 mM potassium sodium tartrate (solution B) and 40 mM copper sulphate ($CuSO_4.5H_2O$). Protein standards were prepared from bovine serum albumin (BSA) (Sigma-Aldrich; NSW, Australia), from a 1 gL^{-1} BSA standard prepared in 0.5 M NaOH. BSA standards from 5 to 1000 mgL^{-1} were prepared by diluting 1 gL^{-1} BSA in 0.5 M NaOH to make a final volume of 1 mL Analysis of protein in the homogenate was undertaken by adding 50 μL of centrifuged

homogenate supernatant to 950 μL 0.5 M NaOH in glass culture tubes. 5 mL of a 100 : 1 : 1 ratio of solutions A : B : C was immediately added to both standards and samples. The samples were then vortexed and incubated at room temperature for 10 min. To prepare the colouring reagent, Folin Ciocalteau's reagent (Sigma-Aldrich; NSW, Australia) was mixed at a 1:1 ratio with 18.2 MΩcm^{-1} water. Each sample had 500 μL of colouring reagent added to it, vortexed and allowed to stand for 30 min at room temperature. An aliquot of the samples was then added to 2 mL plastic cuvettes (Crown Scientific; NSW, Australia). The absorbance of the solutions was measured on a Shimadzu UV-1061 UV-Visible spectrophotometer at 720 nm.

8.2.6 In vitro *assay*

For an *in vitro* assessment on the effect of CBZ on the function of Na^+/K^+-ATPase of *C. tepperi*, larvae were collected from culture tanks *ca.* 8 d after hatching. This was done to approximate the lifestage of *C. tepperi* during the sediment toxicity assay. At this stage, *C. tepperi* larvae had changed from colourless to a red-brown colour. *C. tepperi* were pooled and processed for the Na^+/K^+-ATPase assay as per section 8.2.5. Spiking solutions of CBZ were prepared in 18.2 MΩcm^{-1} water and 100 μL of this solution was added to 350 μL of the enzyme reaction mixture in 2 mL plastic cups to give final exposure concentrations of 0, 0.32, 1.6, 8, 40, 200 and 1000 μgL^{-1}. To this mixture, 50 μL of *C. tepperi* homogenate was added, with 4 replicates used for each spiking treatment. The reaction was initiated by adding 25 μL of 25 mM Na_2ATP and incubation of the reaction mixture was for 1 h at 30 °C in the dark.

8.2.7 Aqueous exposure

The exposure of *C. tepperi* to CBZ in water was used to assess the importance of aqueous uptake. 20 mL glass beakers were used for the assay, where 10 mL of solution was added to them. CBZ solutions of 0, 0.01, 0.1, 1 and 10 mgL^{-1} were prepared in MHW, previously aerated for a minimum of 24 h. First instar *C. tepperi* larvae were collected and 10 larvae added per beaker. The pH, electrical conductivity and dissolved oxygen were measured using a TPS 90-FL meter (Analytical Equipment Co., Australia) prior to the addition of larvae and following termination of the test. A copper (Cu) reference test was set-up alongside the CBZ exposure test. This reference test is used for all aquatic toxicity tests in the Ecotoxicology Unit at CSIRO Land and Water, to ensure that the larvae collected from each culture is of a sufficient and consistent robustness. For the reference test, copper sulphate ($CuSO_4$) was prepared at concentrations of 0, 31, 62, 125, 250 and 500 μgL^{-1} in the MHW used for the parallel test. The ecotoxicological effects (or endpoints) that were assessed for the CBZ test were mortality, wet weight of larvae and Na^+/K^+-ATPase activity in the homogenised cells. Duplicate beakers were set-up for all concentrations, while 3 *C. tepperi* were pooled from each beaker to measure Na^+/K^+-ATPase activity.

8.2.8 Sediment exposure

Exposure of *C. tepperi* to CBZ through sediment was adapted from OECD chemical testing guideline 218 (OECD 2001b). CBZ was prepared at spiking concentrations of 0.16, 0.8, 4, 20 and 100 mgL^{-1} by adding 8, 40, 200, 1000 and 5000 μL of 1 gL^{-1} CBZ (in methanol) to 50 mL volumetric flasks and blowing to dryness under N_2. The volumetric flasks were made up to volume with 18.2 $M\Omega cm^{-1}$ water and the flasks were placed in an ultrasonic bath for 30 min. For the 100 mgL^{-1} treatment, complete dissolution did not occur and a suspension of the drug was formed. To 2 L glass beakers, 50 g (dry weight) of sediment was added

followed by the 50 mL of spiking solution, forming a slurry. Addition of *ca.* 10 mL MHW was necessary to quantitatively transfer CBZ from the volumetric flask for the 100 mgL^{-1} treatment to the sediment. A solution containing 0.6 mg of Tetramin per midge per day was added to the sediment slurry. The sediment slurries were allowed to sit for 24 h prior to the addition of 300 mL MHW. MHW was added to the beakers by pouring onto a plastic disc held just above the sediment slurry to minimise the extent of suspension. The beakers were then placed into a temperature controlled room (22 °C), where the toxicity assay was to take place, and solutions were aerated using glass pipette tips connected to a aquarium air pump (Hailea, Japan) with polyethylene tubing. Aeration was at a rate of *ca*. 5 bubbles per second and did not lead to resuspension of the sediment. The light source used for the test were 360 W cool white fluorescent tubes (Osram, Australia) and were set on a 16 h : 8 h light:dark cycle.

Prior to the sediment assays, triplicate beakers were set-up without *C. tepperi* and the concentration of CBZ in solution was assessed after 1, 7 and 14 d, to estimate the time for equilibration. The system was allowed to equilibrate in this manner for 7 d before the addition of 1st instar larvae. Prior to the addition of *C. tepperi* larvae, aeration was stopped. 20 larvae were added to each beaker and were allowed to burrow into the sediment for 24 h before aeration was recommenced. The pH, electrical conductivity and dissolved oxygen of solution were measured every 7 d, room temperature was monitored every 24 h, while the redox potential of the sediments were measured at the end of the tests.

All treatments were undertaken in duplicate, with two tests run in parallel. One set of duplicates were stopped after 7 d (when larvae were found to have maximal growth) to measure the wet weight and the Na^+/K^+-ATPase activity of the larvae. *C. tepperi* larvae that were collected were rinsed with 18 $M\Omega cm^{-1}$ water, blotted dry on paper towelling and pooled in Eppendorf tubes sitting in ice. Triplicate samples were prepared for Na^+/K^+-

ATPase analysis as previously outlined. The parallel test was allowed to continue to measure the length of time and number of larvae that emerged to become adult *C. tepperi*. The emergence test was considered completed once 80 % of the control *C. tepperi* emerged as adults.

8.2.9 Statistical analysis

For the *in vitro*, aqueous exposure and sediment exposure assays, a one-way analysis of variance (ANOVA) was used to assess whether there was any difference between the concentration treatments for mortality, wet weight of larvae and Na^+/K^+-ATPase activity of the homogenates. The ANOVA tests whether the mean values for each group are the same (the null hypothesis) by comparing the ratio of the variance within the treatments and the variance between the treatments, to give an F-value. This is compared with a critical F-value ($F_{critical}$) which is dependent on the level of significance required, the number of treatments and the number of samples within the treatments (Zar 1984).

For the emergence assay, a one-way ANOVA was used to determine whether there was a significant difference between concentration treatments on the day that 80 % of *C. tepperi* emerged as adults. Also, a one-way ANOVA was conducted within each concentration treatment to assess whether there was any significant difference of the number emerged, compared with day 7 (0 % emergence). Tukey's test ($\alpha = 0.05$) was used as a multiple comparison test if a treatment was found to be different.

Toxstat (Version 3.4; Western EcoSystems Technology, Inc; WY, USA) was used to perform ANOVA and Tukey test. The Trimmed Spearman-Karber method (Hamilton *et al.* 1977; Hamilton *et al.* 1978) was used to perform the LC50 for the Cu reference test.

8.3 Results and Discussion

8.3.1 Aqueous degradation of pharmaceuticals

The room temperature range measured was 22.4-24.2 °C. The daily light exposure was found to be 0.3 x 10^5 Lux. The pH of 18.2 MΩcm^{-1} water, sterile MHW and re-inoculated MHW were found to be 7.04±0.13, 7.39±0.06 and 7.45±0.06, repectively, over the exposure period, while the electrical conductivity was found to be 15.1±3.5, 376.2±7.7 and 376.1±6.7 μScm^{-1}, respectively.

For the sterile and re-inoculated MHW solutions the dissolved organic carbon content was 62±3.6 and 63±3.6 mgL^{-1}.Following 350 h, no degradation of CBZ was apparent for all of the treatments (Figure 8.1). A number of other test pharmaceuticals, such as CAF and ATL, also did not degrade under the test conditions. However, a number of other drugs, such as DCF and FLX, were found to be sensitive to light exposure under all exposure conditions (Appendix B). Also, a number of drugs, such as IMI and CIM, were only sensitive to light when there was organic carbon present (Appendix B). All drugs were found to be stable in solution after 350 h when protected from light.

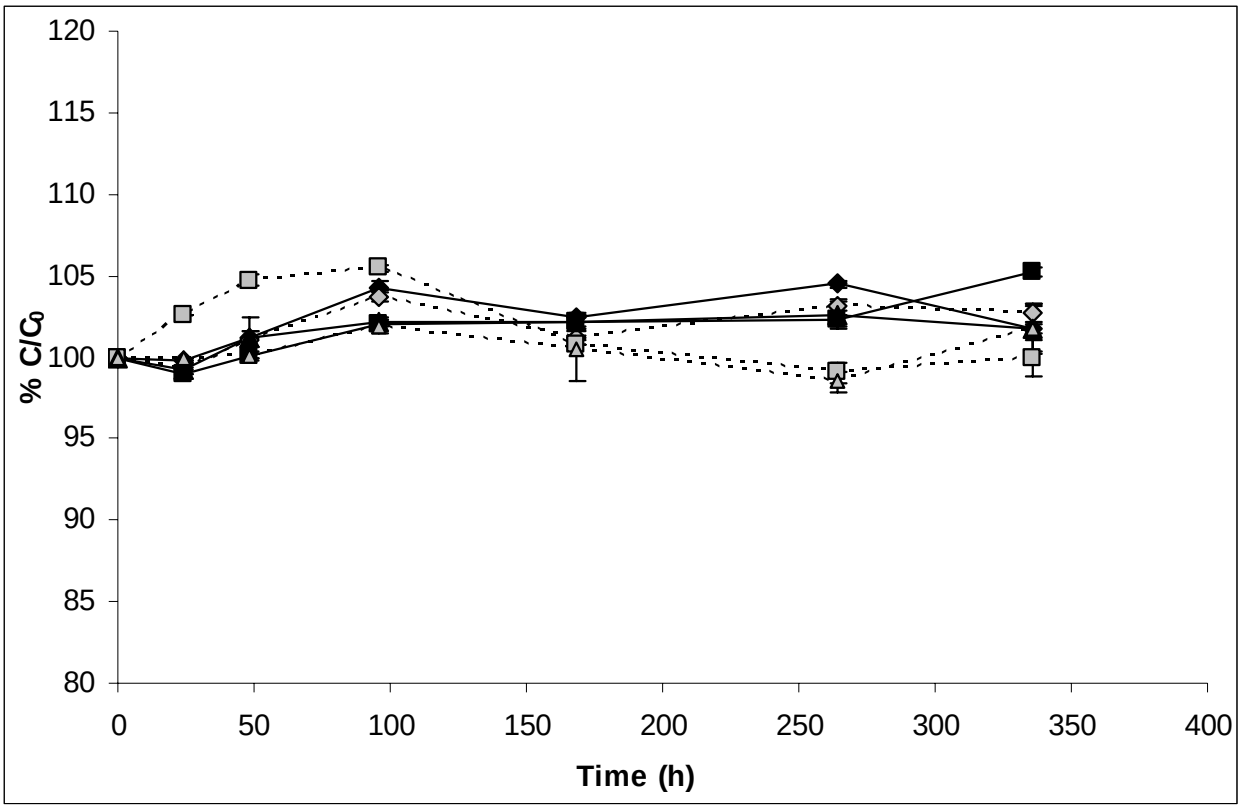


Figure 8.1: Concentration of carbamazepine in solution (C) compared with initial spiking concentration (C_0) following exposure to light and colloidal material; treatments were 18.2 MΩcm^{-1} water (—◆—), moderately hard water (MHW) mixed with sediment and sterilised (—■—) and MHW mixed with sediment and re-inoculated (—▲—); controls were protected from light for 18.2 MΩcm^{-1} water (- - -◆- -), MHW mixed with sediment and sterilised (- - -□- -) and MHW mixed with sediment and re-inoculated (- - -△- -). Error bars are standard errors of the mean of triplicate samples.

CBZ was spiked into the sediment, allowed to equilibrate for 7 d followed by the addition of *C. tepperi* for 14 days for the emergence test. Since a 16 : 8 light : dark cycle was used, CBZ would have been exposed to light for a total of *ca*. 340 h. Within the 350 h of exposure in the degradation experiment, under stronger exposure conditions, CBZ was not found to degrade. Therefore, it is unlikely that the amount of CBZ within the system would have reduced during the toxicological assay due to photolysis, hydrolysis or biodegradation due to the presence of sediment.

8.3.2 Na^+/K^+-ATPase assay validation

Addition of Na_2ATP was found to affect the amount of inorganic phosphorus (P_i) released during the assay (Figure 8.2). The number of moles of ATP used for the assay was 1.4 mM (25 μL of 25 mM Na_2ATP in 425 μL). This compares with a previous validation study of Na^+/K^+-ATPase activity in *Chironomus thummi*, where the P_i released in the assay reached a maximum at 2 mM ATP (Schin and Kroeger 1980). At this molarity of ATP, the Na^+/K^+-ATPase activity was found to be around 2.5 mmol P_i/g protein/h (Schin and Kroeger 1980). However, with the present assays it was found that increasing the molarity of ATP within the standards led to a decrease in the linearity of the calibration curve. Therefore, a volume of 25 μL 25 mM Na_2ATP was chosen as a compromise.

The volume of OUB added to the reaction mixture also seemed to influence the amount of P_i measured, with a maximum response found when 100 μL of 5 mM OUB was added to the reaction mixture (Figure 8.2). However, all of the spiking values led to a molarity greater than 0.1 mM OUB, which Schin and Kroeger (1980) found caused complete inhibition of Na^+/K^+-ATPase activity.

The length of reaction time on the assay was shown to lead to a slight decrease of P_i released up until 60 min, followed by large increase after 90 min (Figure 8.2). As the amount of P_i released remained relatively constant until 60 min, it was thought that the increase after 90 min may have been related to degradation of Na_2ATP. Therefore, the maximum reaction time where there was no change in P_i released (60 min) was used for the assay. A steady increase in the amount of P_i released was found in the study by Schin and Kroeger (1980), as time increased.

Based on the difference between the control and OUB-exposed homogenate, the Na^+/K^+-ATPase activity during the validation experiments ranged from 0.133-0.451 mmol P_i/g protein/h. This is compared with maximal values ranging from 0.5-1 mM P_i/g protein/h (Schin and Kroeger 1980).

8.3.3 In vitro *exposure*

Mean values for the Na^+/K^+-ATPase activity ranged from 0.137-0.538 mmol P_i/g protein/h. Although there was an apparent decrease in the Na^+/K^+-ATPase activity for the 0.034 μM (*ca.* 8 mgL^{-1}) CBZ treatment, Na^+/K^+-ATPase activity was not found to be significantly different between all treatments. This was likely to be related to the large degree of variability between replicates. The summary of statistical analyses is given in Table 8.1.

While the equivalent concentrations of CBZ used for the *in vitro* assessment was similar to the exposures used for the acute toxicity assay, an effect on the wet weight of *C. tepperi* was found following aquatic exposure (Figure 8.4). Direct application of CBZ to the cell homogenate may be reasonably expected to elucidate a response at concentrations lower than an external exposure, as in the acute assay, due to the variable nature of uptake from an external source. Based on this reasoning, it may be concluded that CBZ has no effect on the Na^+/K^+-ATPase activity in *C. tepperi*. However, *in vitro* exposure is often found to be considerably less sensitive, compared with *in vivo* exposure models, where equivalent concentrations of up to 2 orders of magnitude higher have been found to elicit a response for *in vitro* systems (Castano *et al.* 1996; Magwood and George 1996; Kilemade *et al.* 2002; Gulden and Seibert 2005). This discrepancy can be attributed to a reduction in bioavailability of organic compounds spiked *in vitro*, through binding to serum proteins and partitioning into lipids (Gulden and Seibert 2005). The variability between *in vitro* and *in vivo* exposure is also found to be greater when the toxicity of the test compound is greater (Gulden and

Seibert 2005). Until now there has been limited attention on comparing *in vitro* and *in vivo* exposure, although the need to address this issue is highly relevant, especially when considering the use of *in vitro* assessment as a screening tool in risk assessments (Kilemade *et al.* 2002; Gulden and Seibert 2005; Caminada *et al.* 2006). While fish *in vitro* assays have been found to correlate with *in vivo* acute testing assays, it is often more difficult to do so with invertebrates due to limited knowledge of their physiology and, in particular, the functioning of cellular receptors (Hutchinson 2002). Therefore, it is worthwhile further exploring *in vitro* assays that can be used for estimating potential effects in aquatic organisms, especially for invertebrates used in risk assessments for pharmaceuticals.

8.3.4 Aqueous exposure

The pH of solution was found to be 7.8±0.03, electrical conductivity was 246±0.4 and percentage dissolved oxygen was 79.7±0.6. The ambient temperature measured over the 48 h exposure period ranged from 21.7-21.9 °C. Following exposure to aqueous solutions of CBZ, there was no apparent effect on the Na^+/K^+-ATPase activity of the larvae. There was similarly no effect on the survival of the larvae, with the survival in the treatments being greater than the controls. This was due to one *C tepperi* in each replicate being observed to be eaten by another. The test was still deemed to be valid, since the minimum survival of controls is required to be 90 % (USEPA 2002). The Cu reference toxicity test had an LC_{50} of 92.8 μgL^{-1} (95 % confidence interval 46.3-185.9 μgL^{-1}), compared with the mean LC50 of 112 μgL^{-1} (95 % confidence interval range 87-144 μgL^{-1}) for $CuSO_4$ for the Ecotoxciology Unit. While the 95 % confidence interval is reasonably broad, the LC_{50} data was consistent with that of the mean LC_{50} data for *C. tepperi* used previously. This added to the confidence of the robustness of the cultures selected for analysis.

There appeared to be a decrease in the mean wet weight of *C. tepperi* for the 10 mgL^{-1} CBZ exposure, which was found to be significant for the 1 and 10 mgL^{-1} CBZ treatments (Table 8.1). Oetken *et al.* (2005) found no effect of CBZ on *Chironomus riparius* at exposure concentrations up to 4 mgL^{-1}, although the end-point used was lethality. In the same study, no toxicity through exposure to CBZ in solution was apparent for the oligochaete *Lumbriculus variegatus* at the same exposure concentrations. Also, freshwater snails exposed to aqueous concentrations of CBZ up to 250 μgL^{-1} were not adversely affected after a 28 d exposure period (Oetken *et al.* 2005). EC_{50} values (the concentration where an effect is expected to occur in 50 % of exposed organisms) for the cladocerans *Ceriodaphnia dubia* and *Daphnia magna* have been found in the range of 77.7 mgL^{-1} to greater than 100 mgL^{-1} for a 48 h test period (Cleuvers 2003; Ferrari *et al.* 2003). This concentration of effect was reduced (implying a greater potential for ecotoxicity) for tests of greater duration or when CBZ was present with other drugs, although this was still at mgL^{-1} levels (Cleuvers 2003; Ferrari *et al.* 2003; Ferrari *et al.* 2004). A study assessing exposure of the algae *Selenastrum capricornutum* indicated no adverse effects on growth up to a concentration of 20 mgL^{-1} CBZ (Andreozzi *et al.* 2002). These ecotoxicological assays, including the present study, would therefore suggest that ecotoxicological impacts of aqueous exposure to CBZ would be unlikely at environmentally relevant concentrations. However, previous studies that found the potential toxicity of CBZ increases when another pharmaceutical is present raise the issue of the likely exposure of organisms to a mixture of pharmaceuticals in aquatic ecosystems (Cleuvers 2003).

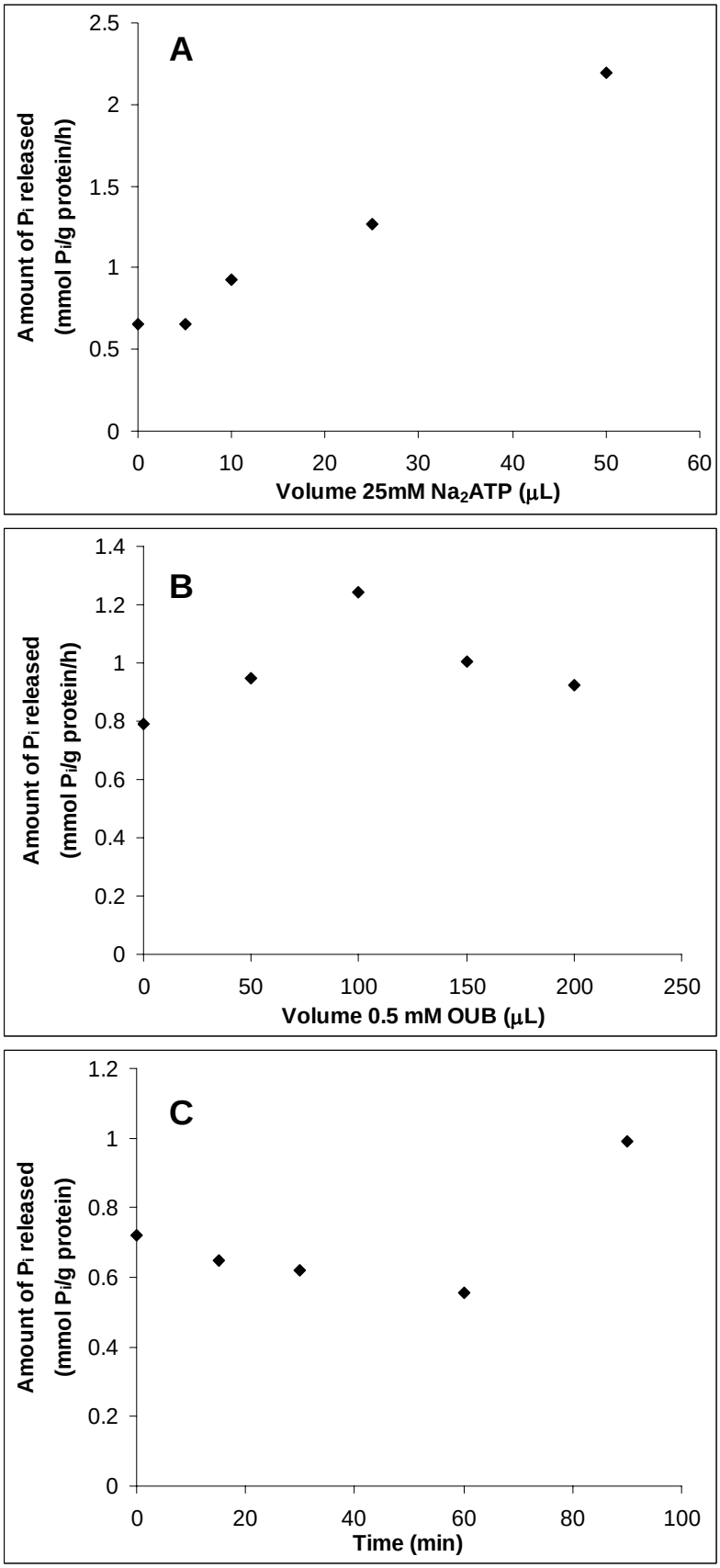


Figure 8.2: Amount of P released during Na^+/K^+-ATPase assay depending on (A) the volume of 25 mM Na_2ATP, (B) the volume of 0.5 mM ouabain (OUB) added to the reaction solution and (C) the length of time the assay was allowed to proceed. Values are means of duplicate samples

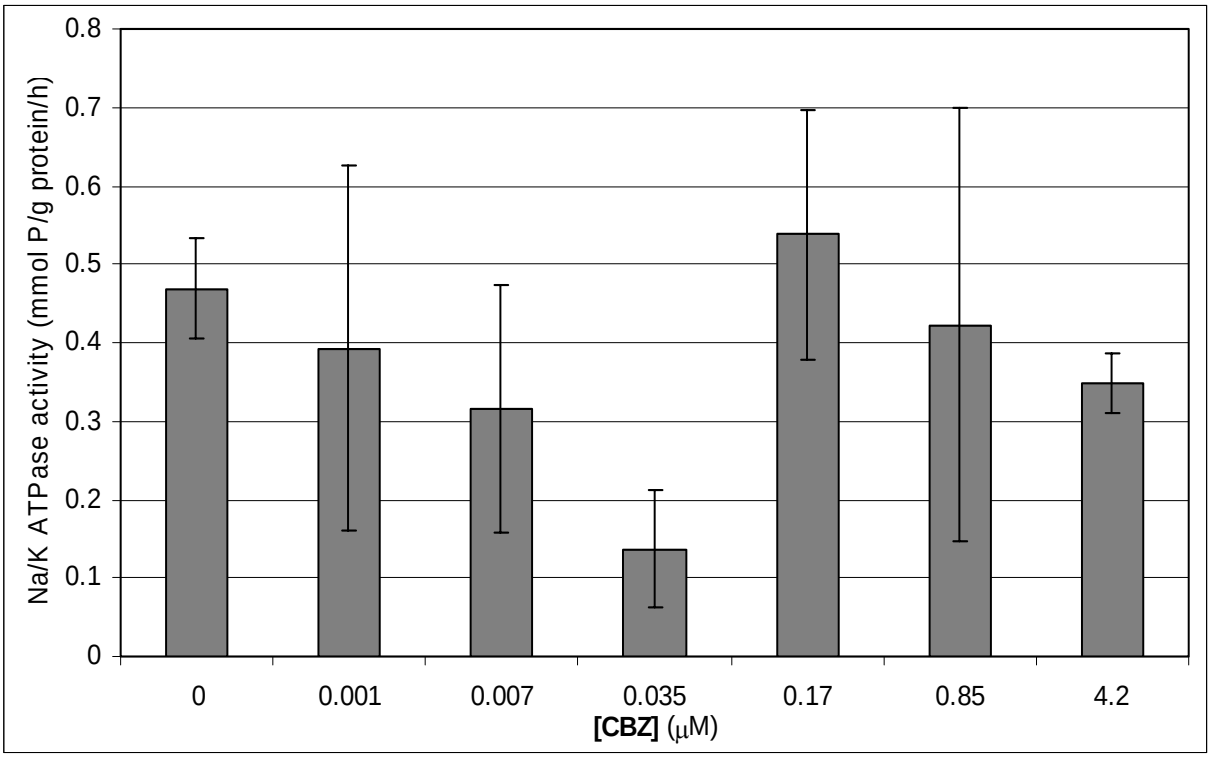
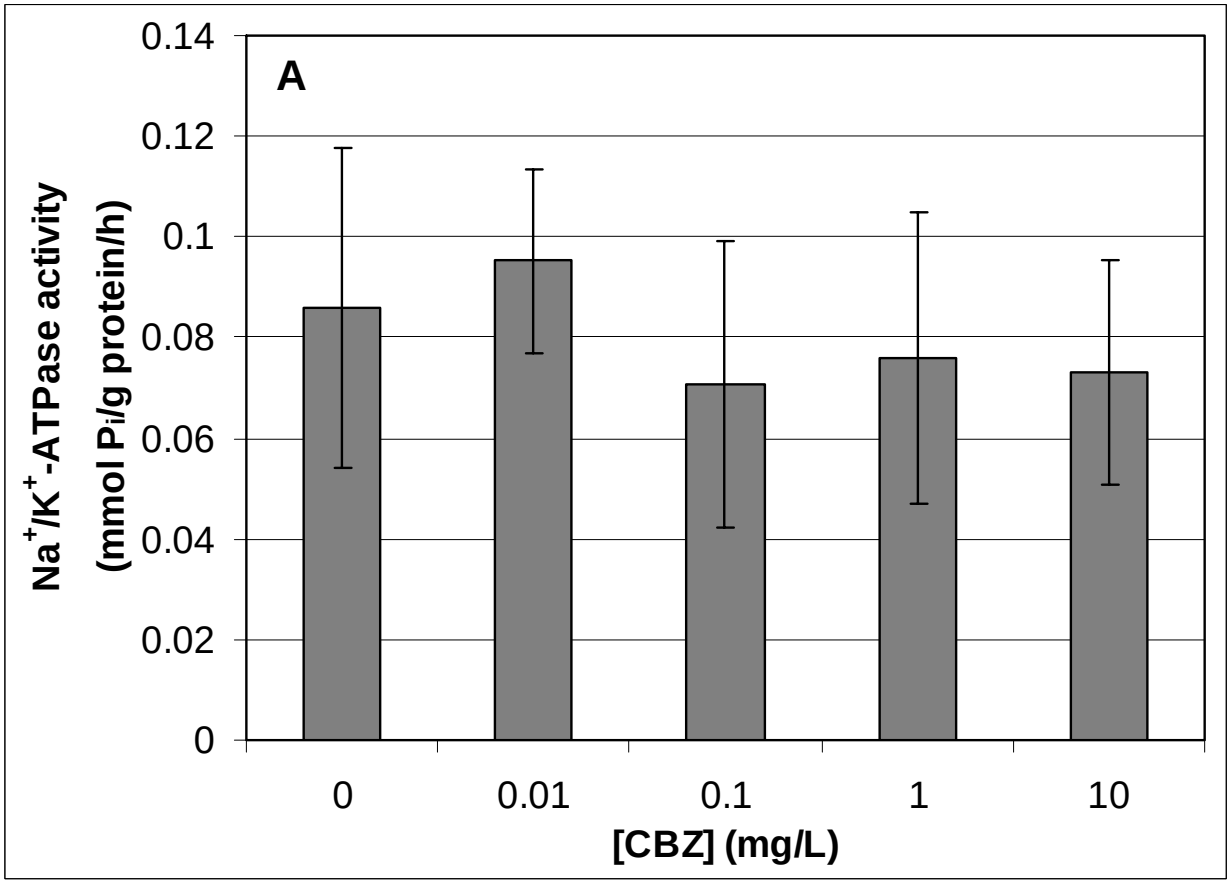
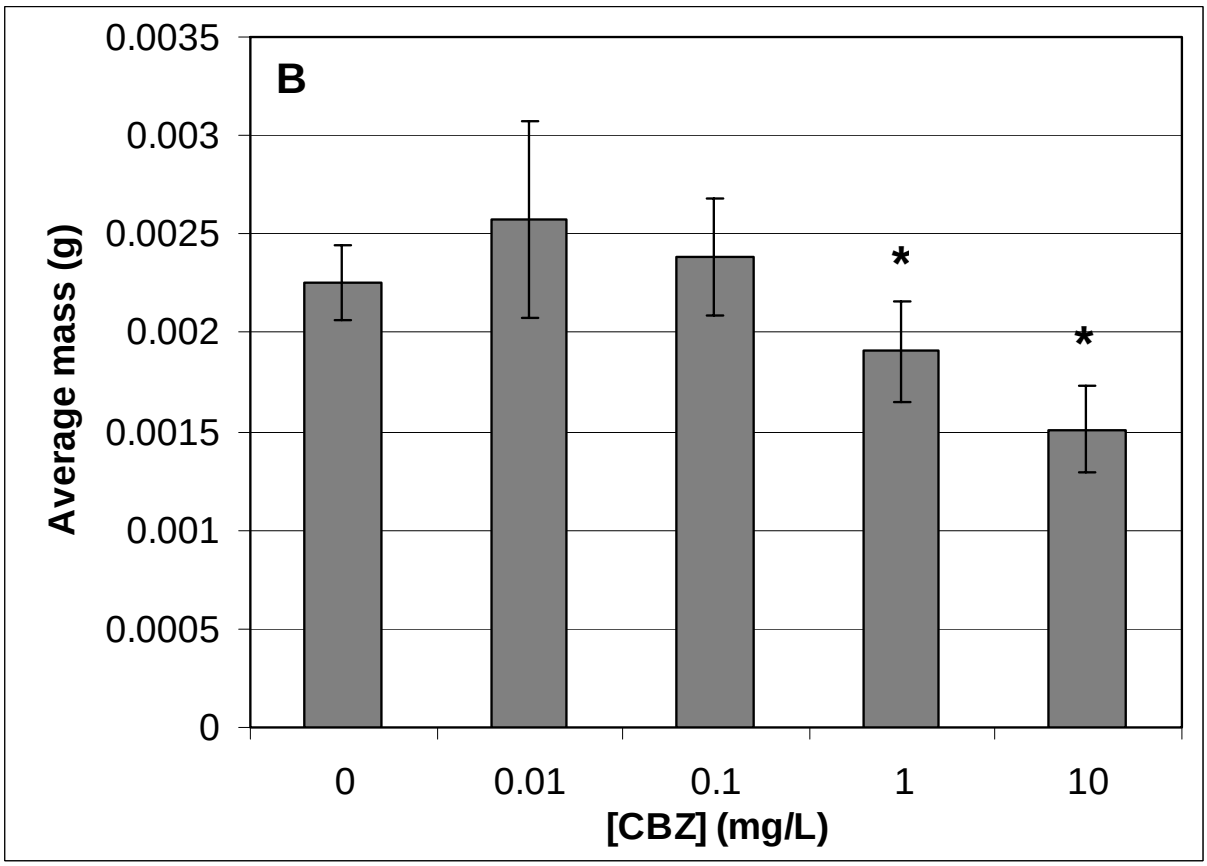


Figure 8.3: Na^+/K^+-ATPase activity in *C. tepperi* cell homogenate exposed in vitro to varying concentrations of carbamazepine (CBZ). Error bars are the standard error of four replicates. The molarity of CBZ is relative to the volume of cell homogenate.





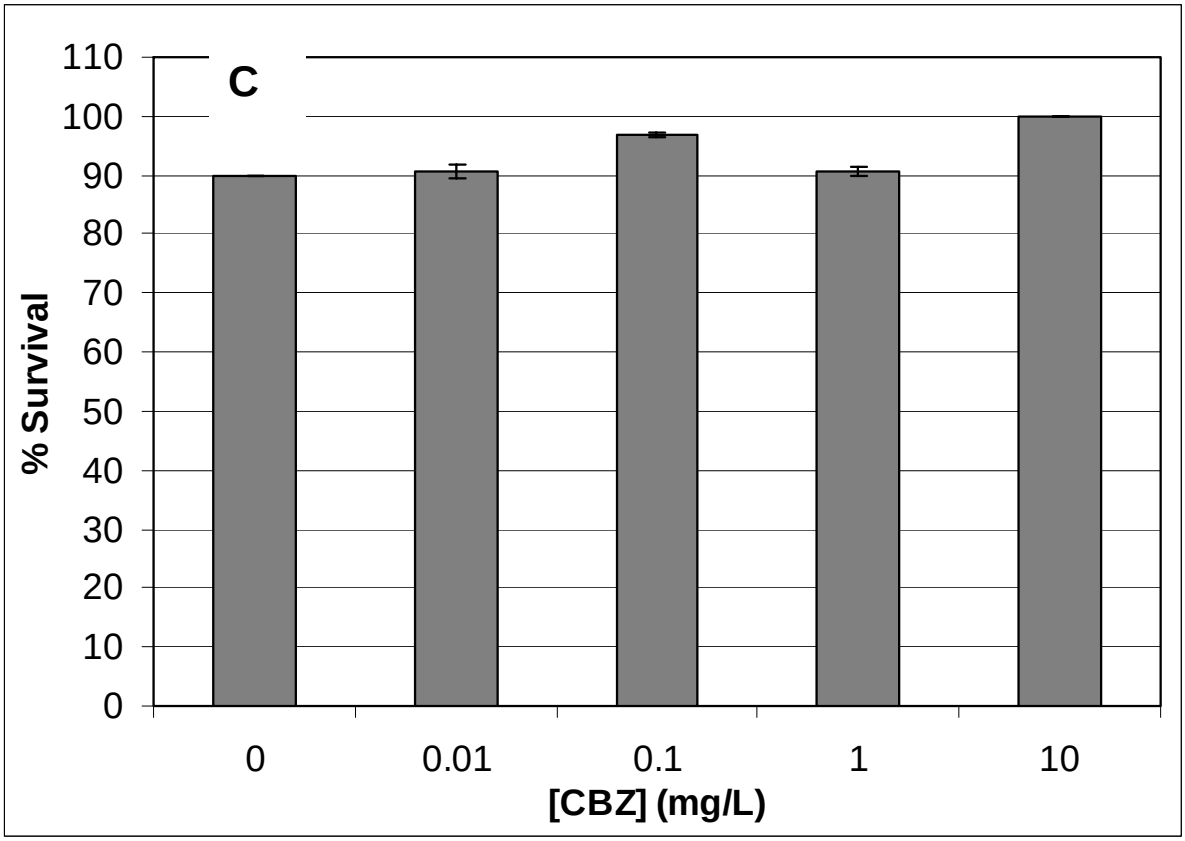


Figure 8.4: (A) The Na^+/K^+-ATPase activity of *C. tepperi* cell homogenates, (B) the mean wet weight of larvae and (C) the percentage survival of added *C. tepperi* following a 48 h exposure to aqueous concentrations of carbamazepine (CBZ). * indicates significantly different values using ANOVA followed by Tukey's multiple comparison test. Error bars are standard errors of the mean of four replicates

8.3.5 Sediment exposure

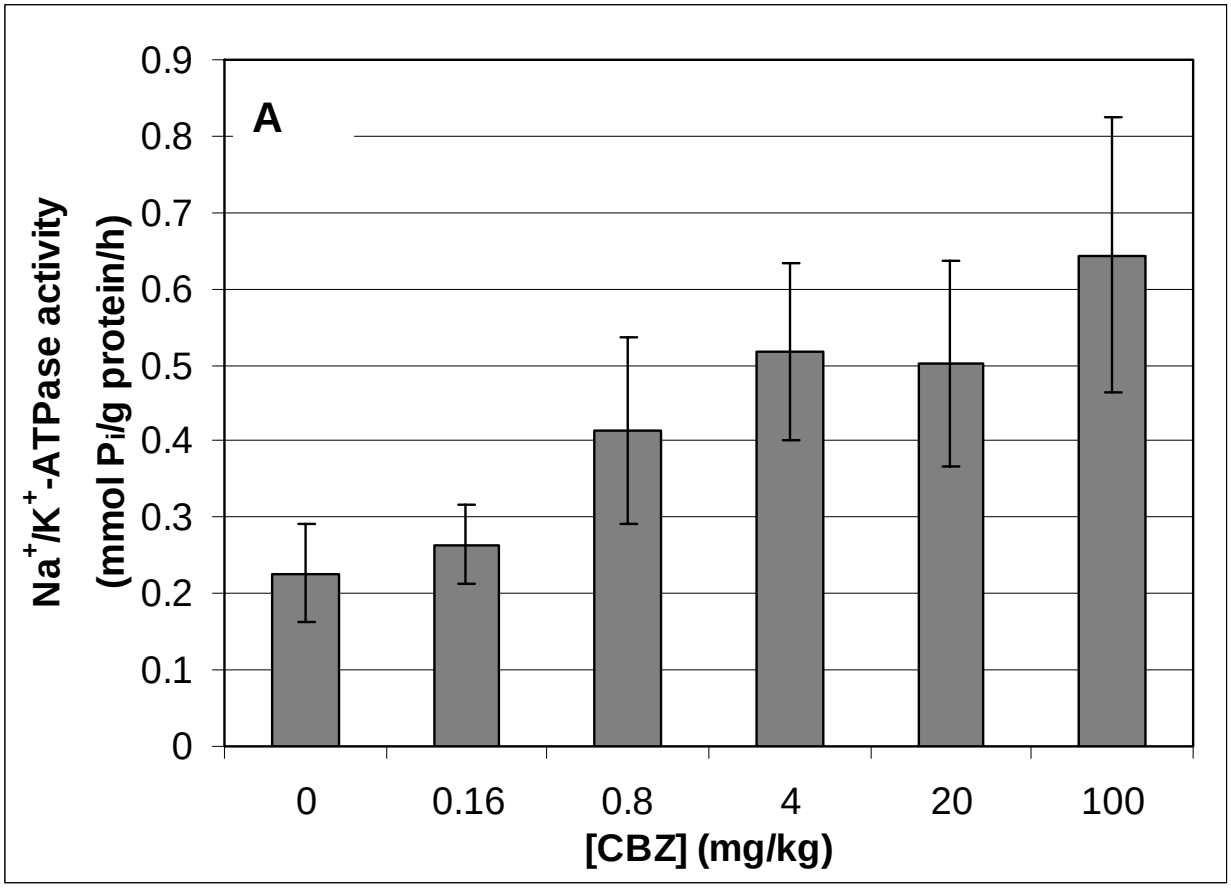
For the equilibration period, after 1 h, 24 h, 7 d and 14 d the concentration of CBZ in solution was 141±47, 394±87, 477±57 and 422 μgL^{-1}, respectively, after sediment was spiked with 40 $mgkg^{-1}$ CBZ. The solution parameters were measured as follows: pH 7.88±0.03, electrical conductivity 333±4.4 μScm^{-1}, percentage dissolved oxygen 79±1 %, while the redox potential of the sediment was 52.9±6.9 mV. The ambient temperature range measured over the exposure period was from 21.7-22.4 °C.

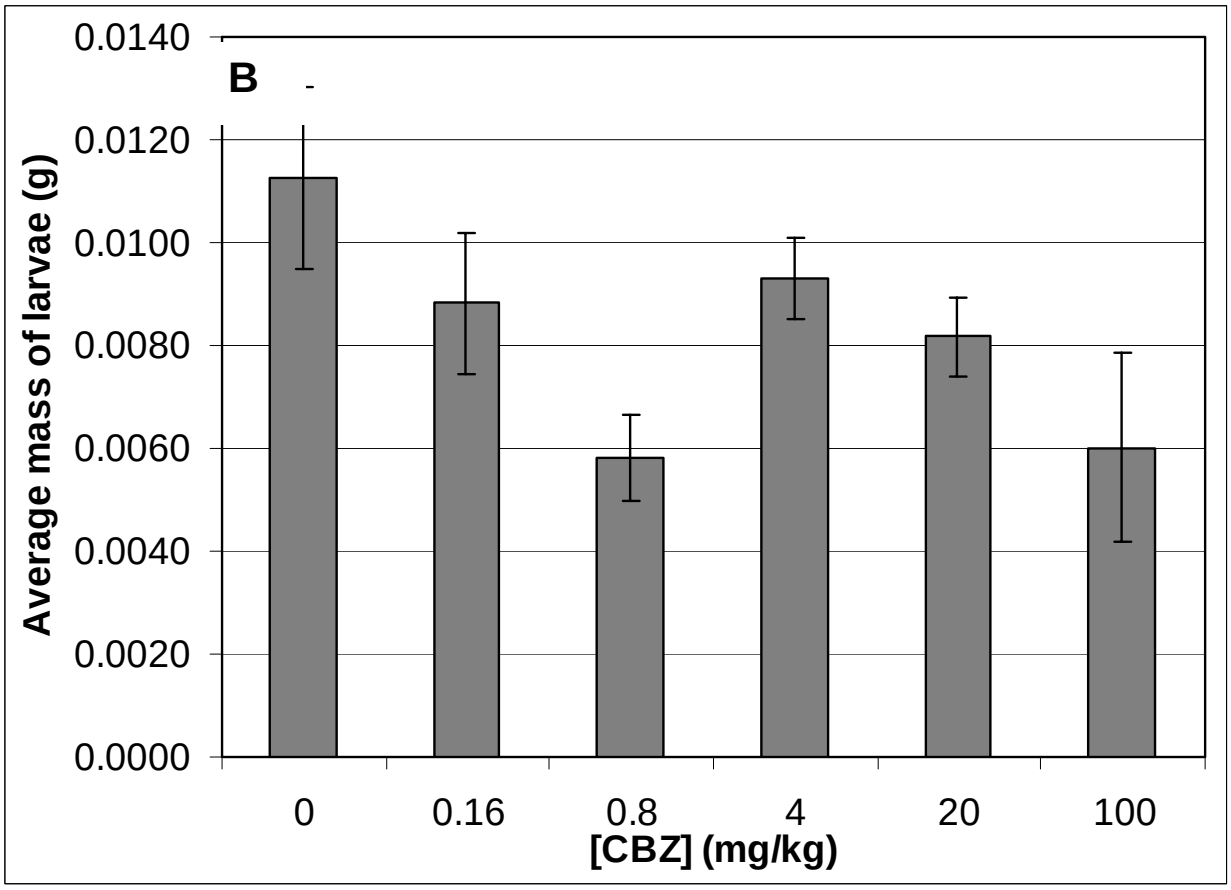
Validation of the assay requires 70 % survival of larvae in the control and, therefore, the test was deemed to be valid (OECD 2001b).

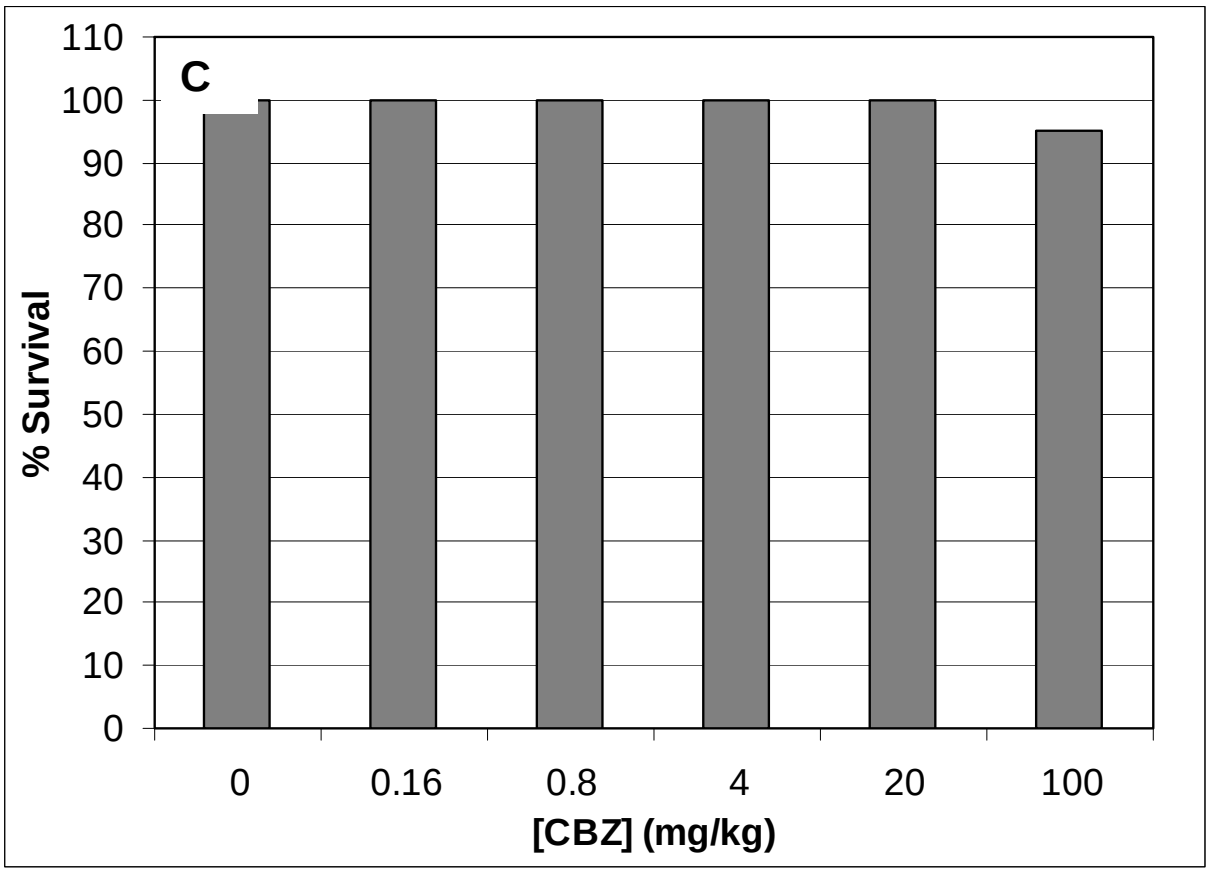
The activity of Na^+/K^+-ATPase in the cell homogenates was found to be higher than the cell homogenates from the aqueous exposure, although they were of a similar range to the *in vitro* exposed homogenates (Figures 8.3, 8.4 and 8.5). This may indicate that the activity of this enzyme is dependent on the developmental stage of *C. tepperi*, although there is no example of this to be found in the literature. The Na^+/K^+-ATPase activity in the cell homogenates seemed to increase over time, although no significant difference was found between the treatments. Since Na^+/K^+-ATPase is used to maintain an ionic balance in cells, the blocking of Na^+ channels would be expected to reduce this requirement. Therefore, it was expected an increasing exposure to CBZ might reduce the Na^+/K^+-ATPase activity within the cell homogenates. However, statistical analysis was unable to determine whether CBZ had an effect on the OUB-sensitive Na^+/K^+-ATPase activity of *C. tepperi*. This would indicate that the Na^+/K^+-ATPase activity may not be an appropriate indicator of exposure to CBZ in *C. tepperi*.

Analysis of emergence seemed to indicate that the development of larvae into adults was inhibited at the higher sediment concentrations of CBZ (Figure 8.5). However, no significant difference between the control and all concentrations was found where 80 % of the control had emerged at day 11 (Figure 8.5). However, when a one-way ANOVA was conducted on each individual treatment, no significant difference was found in number of adults emerging for the 100 $mgkg^{-1}$ CBZ treatment. For the remainder of treatments, a significant difference in emergence from the control treatment only occurred after day 10 of exposure (Figure 8.5). Therefore, although the data seems to indicate effects were apparent during emergence, statistical analysis could only determine an effect for the highest spiking concentration. Observation of *C. tepperi* collected for the Na^+/K^+-ATPase analysis indicated that the larvae were smaller than those collected for the other treatments. However, the observation of relative size was not supported by analysis of the average mass of collected larvae (Figure 8.5). The colour of collected larvae in the 100 $mgkg^{-1}$ CBZ treatment were also noticeably paler than the other treatments; prior to pupation, larvae become a deep red colour but the larvae from the highest treatment were still colourless. While there was no difference in mortality during the collection of larvae for the 7 d sediment test, there was an effect on the ability of *C. tepperi* to pupate at 100 $mgkg^{-1}$ CBZ.

Oetken *et al.* (2005) found that pupation of *C. riparius* was also inhibited by CBZ, with an EC50 as low as 0.16 $mgkg^{-1}$ determined. The sediment used in this study was an artificial sediment, with an organic carbon content < 1 %, compared with the substantially higher value of the sediment in this work (*see* Chapter 4; Table 4.1). Based on the direct measurement of CBZ in both water and sediment, the K_d of CBZ in their assay was around 0.8 Lkg^{-1} (Oetken *et al.* 2005).


A
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Na+/K+-ATPase activity
(mmol Pi/g protein/h)
0
0.16
0.8
4
20
100
[CBZ] (mg/kg)


B
0.0140
0.0120
0.0100
0.0080
0.0060
0.0040
0.0020
0.0000
Average mass of larvae (g)
0
0.16
0.8
4
20
100
[CBZ] (mg/kg)



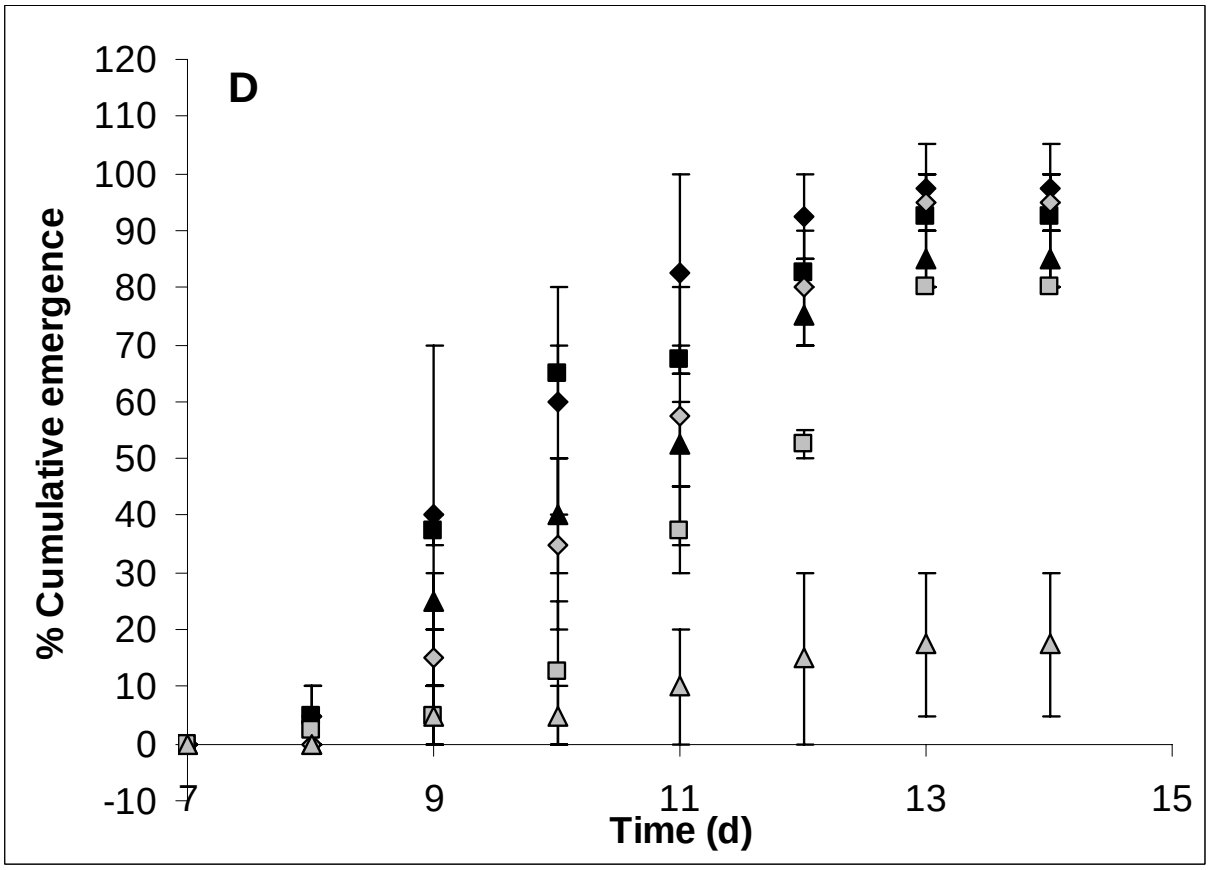


Figure 8.5: (A) The Na^+/K^+-ATPase activity of *C. tepperi* cell homogenates, (B) the mean wet weight of larvae and (C) percentage survival of added *C. tepperi* following a exposure to carbamazepine (CBZ) spiked to sediment. The time taken for *C. tepperi* to emerge as adults (D) is shown for a sediment spiking concentration of 0 (◆), 0.16 (■), 0.8 (▲), 4 (◇), 20 (□) and 100 (△) $mgkg^{-1}$. Error bars are standard errors of the mean of two to four replicates

In comparison, indirect measurement of CBZ in solution indicated a K_d of CBZ after 14 d equilibration within the present study to be around 88 Lkg^{-1} (based on a final sediment concentration of 37 $mgkg^{-1}$ and solution concentration of 422 μgL^{-1}). This may give an indication that the sediment used in the present study may have provided some measure of protection to the *C. tepperi*, possibly through reducing the bioavailability of CBZ. The E-values of CBZ suggest a slight decrease in bioavailability over time (*see* Chapter 7; Figure 7.5). Whether the extent of decrease of E-values over time would contribute to the discrepancy between the studies requires more work in elucidating the role sorption to sediments plays in the overall bioavailability of organic contaminants. A number of other studies suggest sorption to sediments can decrease the toxicity of xenobiotics for aquatic organisms (Kaag *et al.* 1998; Lamoureux and Brownawell 1999; Sijm *et al.* 2000), including the pesticides molinate and atrazine in *C. tepperi* (Phyu *et al.* 2005).

Alternatively, the difference in the levels of toxicity found between this study and by Oetken *et al.* (2005) may have been related to how *Chironomus* spp. are exposed to contaminants. For example, while *Chironomus* larvae spend the majority of their feeding on and moving within sediments, uptake of CBZ from interstitial water (water found between the sediment pores) may have been an important pathway. That is, the effective concentrations where effects were apparent between the studies were similar, when taking into account the apparent K_d values of the two studies. For a K_d value of 88 Lkg^{-1}, the solution concentration of CBZ would have been around 1 mgL^{-1} following equilibration of the 100 $mgkg^{-1}$ CBZ treatment. A similar inhibition of emergence (around 10 %) was found at ca. 0.25-0.5 $mgkg^{-1}$ in the study by Oetken *et al.* (2005); assuming a K_d of 0.8 Lkg^{-1} this corresponds with a solution concentration similar to the present study. It would thus seem to indicate that the solution concentration of CBZ may be a more useful indicator of potential ecotoxicity to the sediment-dwelling *Chironomus* spp. larvae, compared with the concentration of CBZ in

sediment. This would suggest that sorption of CBZ to sediments may provide protection to *Chironomus tepperi*, since uptake may be principally from solution.

The persistence of CBZ within aquatic ecosystems has been repeatedly demonstrated (Lam *et al.* 2004; Bendz *et al.* 2005; Joss *et al.* 2005) and its stability in sediment over a prolonged period has also been demonstrated (Loffler *et al.* 2005). It is therefore worth considering the potential for association with sediments to decrease the biodegradation of CBZ as has been found for other trace organic contaminants (Warren *et al.* 2003). Futhermore, the E-value decreasing slightly over time for CBZ (*see* Chapter 7; Figure 7.5) would point to the need for more effort to be directed towards the effects of "ageing", or the effect of interaction time on the extent of sorption and decrease in bioavailability. For example, resuspension of sediments could lead to the re-equilibration of sediment-bound CBZ within the aqueous phase. While there has been limited research conducted in this area, bioavailability of organic contaminants may be enhanced through resuspension of sediments (Eggleton and Thomas 2004).

Table 8.1: Summary of the F-values determined for the various assays by ANOVA. The $F_{critical}$ values are the critical F-values based on the number of degrees of freedom and desired confidence level for the anlaysis. The null hypothesis (H_0) is that there is no difference between the mean values determined at each treatment.

Exposure	Endpoint	F-value	$F_{critical}$	Accept H_0?[a]
In vitro	Na^+/K^+-ATPase	0.603	3.87 (0.05,6,7)[b]	Yes
Aqueous	Na^+/K^+-ATPase	0.894	3.06 (0.05,4,15)	Yes
	Wet weight	6.858	3.06 (0.05,4,15)	No
Sediment	Na^+/K^+-ATPase	0.49	2.53 (0.05,5,30)	Yes
	Wet weight	0.923	2.45 (0.05,5,40)	Yes
	Emergence (t=11d)	2.039	3.2 (0.05,5,11)	Yes
	Emergence (control)	7.662	3.5 (0.05,7,8)	No
	Emergence (0.16 $mgkg^{-1}$)	28.944	3.5 (0.05,7,8)	No
	Emergence (0.8 $mgkg^{-1}$)	38.491	3.5 (0.05,7,8)	No
	Emergence (4 $mgkg^{-1}$)	14.367	3.5 (0.05,7,8)	No
	Emergence (20 $mgkg^{-1}$)	36.743	3.5 (0.05,7,8)	No
	Emergence (100 $mgkg^{-1}$)	0.623	3.5 (0.05,7,8)	Yes

[a] Null hypothesis (H_0) is accepted if $F_{critical}$ > F-value
[b] The $F_{critical}$ value is 3.87 at the 5% level (=0.05) for total number of groups minus 1 (=6) and for the total number of subjects in the experiment minus the total number of groups (=7)

Table 8.2: Summary of results from Tukey's multiple comparison test for the effect of time on emergence following sediment (chronic) exposure. Treatments with the same letter denotes there was no significant difference found between the means.

	Control	**0.16 mgkg^{-1} CBZ**	**0.8 mgkg^{-1} CBZ**	**4 mgkg^{-1} CBZ**	**20 mgkg^{-1} CBZ**
Day 7	a	a	a	a	a
Day 8	a,b	a	a	a	a
Day 9	a,b,c	a,b	a,b	a	a
Day 10	a,b,c	b,c	b	a,b	a,b
Day 11	b	b,c	b,c	a,b,c	b,c
Day 12	c	c	c,d	b,c	c,d
Day 13	c	c	d	c	d
Day 14	c	c	d	c	d

8.3.6 Effect of exposure period

While the only apparent effect of CBZ on *C. tepperi* could be found at the highest spiking concentration of CBZ for the emergence assay, there are important implications for the length of exposure on the assessment of ecotoxicity of contaminants. Most of the ecotoxicological assays conducted for human pharmaceuticals have been undertaken as short-term exposures, thereby focussing on a relatively short period of the life-cycle of the organism. However, a comparison between short-term and long-term exposure to a xenobiotic will show more relevant responses in the long-term exposures occurring at lower concentrations (Cleuvers 2005). Where such comparisons have been undertaken with human pharmaceuticals, greater sensitivity, in terms of concentrations where endpoints can be measured, are seen for the chronic exposures (Webb 2004; Hoeger *et al.* 2005; Oetken *et al.* 2005; Fent *et al.* 2006).

This demonstrates the importance of considering the effects of exposure over the life-cycle of an organism or, at least, during their more sensitive developmental stages. Early life stages of aquatic invertebrates and fish tend to be more sensitive than adults, while being of a similar

sensitivity to entire life-cycle assays (Hutchinson *et al.* 1998). While this implies early life stage tests can be used to as a substitute for entire life-cycle assays, early life stage assays may not take into account other critical developmental periods, such as reproduction. For example, the effects of 2 endocrine disrupting chemicals (EDCs) were only apparent in the second generation of exposed *Chironomus riparius*, while no effect was seen for any life stage of the first generation (Segner *et al.* 2003). Chronic exposure to pharmaceuticals in ecotoxicological assays is particularly relevant for aquatic organisms, considering their consistent release into aquatic environments. Therefore, the use of acute toxicity testing is limited in that no observable effects following acute testing does not imply that exposure to the test compound poses no risk.

8.3.7 The use of biomarkers for ecotoxicological screening

The concept of using common biomarkers of humans and other organisms has been previously proposed and demonstrated as a potential tool for screening pharmaceuticals for their likely ecotoxicological effects (Fong *et al.* 1998; Huggett *et al.* 2003a). In this case the biomarker proposed for CBZ, the Na^+/K^+-ATPase enzyme activity, did not show any observable disruption to its function. The function of this enzyme has been widely used to not only measure a specific interaction with the enzyme but as a more general indicator of the general physiological functioning of the organism (Emery *et al.* 1998; Lionetto *et al.* 2000; Cotou *et al.* 2001; Webb *et al.* 2001). From this series of tests it seems that the Na^+/K^+-ATPase enzyme function of *C. tepperi* is not a sensitive endpoint for the tested CBZ exposure levels.

Along with the previous comments on the comparison of *in vitro* and *in vivo* assays, it might also be the case that the Na^+/K^+-ATPase enzyme might not be a good indicator of the general physiological function of *C. tepperi*. This assumption is based on the fact that while

emergence was delayed for the highest CBZ sediment treatment (Figure 8.5), there was no apparent influence on the activity of the Na^+/K^+-ATPase enzyme for the same level of exposure (Figure 8.5). Since the amount of Na_2ATP spiked to the system influenced the response of the Na^+/K^+-ATPase colorimetric assay, other assays should be considered in case this led to a decreased sensitivity of the assay.

An advantage of the use of targeted biomarkers is that it can provide an economical screening tool that, when properly developed, does not require extensive test procedures with organisms (Schlenk 1999; Fent *et al.* 2006). While changes in the function of a biomarker do not necessarily indicate a physiological response, it can give an indication of exposure and potential for impairment of physiological functions. However, the use of biomarkers might lead to a number of short-comings. For example, the change of a biomarker response does not take into account the potential for other effects. In this study, there was no influence on the expected biomarker through exposure to CBZ, even though an effect on emergence was apparent. Also, developing embryos of the Japanese Medaka (*Oryzias latipes*) exposed to sub-μgL^{-1} concentrations of the selective-serotonin reuptake inhibitor fluoxetine showed a 5-fold increase in the prevalence of deformities (Foran *et al.* 2004). However, there was no concentration-dependence found on the levels of plasma estradiol and testosterone, both regulated by serotonin in fish. In another study, Hoeger *et al.* (2005) assessed the effect of diclofenac on the *in vitro* synthesis of prostaglandin in the brown trout, *Salmo trutta*. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) that inhibits the synthesis of prostaglandin in humans. The synthesis of prostaglandin in *Salmo trutta* kidney macrophages was reduced following exposure to diclofenac, although this was not significantly different from control samples. However, physiological assessment of the kidney showed potentially adverse effects following chronic exposure of the whole fish to diclofenac.

Therefore, while defining biomarkers of effect is an important initial step in defining potential ecotoxicological impacts, physiological endpoints following chronic exposure to pharmaceuticals could be just as enlightening. Despite the conservation of receptors and enzyme systems amongst organisms, the selection of appropriate biomarkers in aquatic organisms may not be as easily defined because of the distinct physiological function between mammals, aquatic vertebrates and aquatic invertebrates (Huggett *et al.* 2004). However, the use of generalised biomarkers, as a comparison with physiological responses following chronic exposure, should still be employed, at least as a screening tool.

The Na^+/K^+-ATPase activity was selected as a biomarker of effect, where an effect on its function could be considered a potential prelude to effects apparent at the organism level. Another approach could be to screen *C. tepperi* for a biomarker indicating exposure to the compound of interest. For example, CBZ is known to increase its rate of removal in humans by activating the cytochrome-P450 enzyme activity (McNamara 2001). *Chironomus* spp. are known to have a mixed-function oxidase (MFO) enzyme system, including cytochrome-P450, which gives it the ability to metabolise xenobiotics (Miota *et al.* 2000; Simkiss *et al.* 2001; Verrengia Guerrero *et al.* 2002; Fisher *et al.* 2003). Exposure to xenobiotics, including the pesticides permethrin and atrazine, has been found to induce cytochrome-P450 activity in *Chironomus* spp. (Miota *et al.* 2000; Fisher *et al.* 2003). If exposure to CBZ was found to enhance cytochrome-P450 activity in *Chironomus*, then this endpoint could be used as an indicator of uptake from the environment. While this does not necessarily suggest physiological effects would occur, it would give an indication of bioavailability to the organism; if there is no uptake then there is no possibility of toxicological effects. Alternatively, an aquatic organism that exhibits little biotransformation of xenobiotics could be used as an indicator of bioavailability, if the xenobiotic can be chemically extracted from it. For example, the freshwater oligochaete *Lumbriculus variegatus* has been suggested for such a purpose (Verrengia Guerrero *et al.* 2002). Bioavailability is an important step in

defining the relevance of detecting pharmaceuticals within aquatic systems (including sediments) from environmental surveys.

8.4 Conclusions

It should be noted that the ecotoxicological assays used in this study were at a screening level, where large concentration ranges were used. However, a number of important conclusions can still be made from this work.

CBZ was found to reduce the rate of developing *C. tepperi*, where the mass of larvae following 48 h of exposure to solutions containing 1 and 10 mgL^{-1} CBZ was found to be reduced. Comparisons with environmental surveys would suggest these concentrations are 2 to 3 orders of magnitude greater than environmentally relevant concentrations. A significant decrease in the ability for larvae of a freshwater midge, *Chironomus tepperi*, to emerge as adults was found at a sediment spiking concentration of 100 $mgkg^{-1}$ CBZ. This was estimated to correspond with a solution concentration of CBZ of around 1 mgL^{-1}. Based on similar results from another test using freshwater midge larvae (Oetken *et al.* 2005) may suggest that the solution concentration of CBZ is a useful indicator for its potential toxicity. Furthermore, based on the comparative amount of CBZ spiked to the sediment between these two studies could also suggest that sediment can reduce the potential for uptake of CBZ in the sediment-feeding *Chironomus*.

The solution concentration where an effect was found in both acute and chronic exposures was around 1 mgL^{-1} CBZ. While this suggests the acute test could be used to give an indication of chronic level toxicity, the presence of sediment needs to be considered in terms of its mitigating effect. The concentration of solution CBZ was an indirect estimate based on the sorption of CBZ and, therefore, an assessment of the extent of effect on bioavailability

that sorption can have needs to be addressed. Included in such an assessment should be the role sediment can play in acting as a reservoir for pharmaceuticals, particularly with environmentally recalcitrant compounds such as CBZ. When environmental conditions, such as resuspension of sediments, lead to re-equilibration between sediment-bound and solution CBZ, the role of sediment acting as a reservoir may become more important.

An attempt was made to rationalise a likely receptor-level effect of CBZ, although no effect on the function of the Na^+/K^+-ATPase activity was apparent following *in vitro*, acute and chronic testing regimes. Therefore, the use of this biomarker did not correspond with the effects seen in emergence assay. The use of a biomarker within a tiered risk assessment has the important function of flagging the potential, through an effect on their function prior to physiological changes, for higher-order physiological or community impacts (Schlenk 1999). Pharmaceuticals represent a unique environmental contaminant, in that their physiological pathways are well-defined in mammalian systems (Huggett *et al.* 2003a). The challenge that exists for defining potential ecotoxicological impacts in aquatic systems is to determine whether analogous receptor systems within aquatic species are similarly affected through exposure to pharmaceuticals. Once this can be adequately established within a number of organisms, representing a range of trophic levels, a more rigorous risk assessment can be used without necessarily increasing the complexity of the process.

Chapter 9. Conclusions and recommendations

Pharmaceuticals have been found in the aquatic environment following a number of environmental surveys at relatively low concentrations. However, the inherent potency of these compounds has raised a number of concerns regarding the potential ecotoxicological consequence of their presence in the environment. While a considerable amount of research has been undertaken over the past decade, particularly over the last 3 years, a consensus on the implications of their environmental presence has yet to be reached. With the number of pharmaceuticals currently registered for therapeutic use, a targeted approach has been undertaken by most researchers in an attempt to identify drugs that are likely to present the greatest risk. This approach has centred on the recommendations formulated by the EMEA for the ecological risk assessment of human pharmaceuticals (EMEA-CPMP 2005). These recommendations attempt to define the likely levels of pharmaceuticals in the aquatic environment and what implications these levels may have on aquatic organisms. Most work to date has focussed on the aquatic concentrations of pharmaceuticals, with relatively few studies seeking to define the consequences of interaction with sediments.

9.1 Sorption of human pharmaceuticals

The development of a multi-residue method for HPLC analysis enabled the assessment of their sorption to sediments (Chapter 4). The selected pharmaceuticals had a diverse range of physicochemical properties and this was reflected in their extent of sorption to the test sediment. However, the extent of sorption was not predictable based on the physicochemical properties of the test compounds. Environmental variables, such as the pH and ionic strength of solution, were also shown to have an influence on the extent of sorption to the sediment (Chapter 5). The relationship between pH and sorption seemed to be reasonably

straightforward for the acidic pharmaceuticals. In this instance, an increase in their water solubility, through increasing their level of ionisation, led to a decrease in sorption. However, an increase in ionisation and, therefore, water solubility of the cationic pharmaceuticals did not necessarily lead to a lesser degree of sorption. Furthermore, the pH was found to have an effect on the sorption on drugs that were not ionisable at the experimental pH values. It was likely that altering a parameter such as solution pH will not only affect the properties of the drug and, therefore, its sorption potential, but also that of the sediment. The sorption of ionisable, organic contaminants, such as pharmaceuticals, is therefore difficult to predict based solely on the physicochemical properties of the drug itself.

9.2 The use of pharmacological data to estimate the potential sorption of pharmaceuticals

The use of human pharmacological data, used to predict the fate of pharmaceuticals within the human body, was used to assess whether this may enable the prediction of the extent of sorption pharmaceuticals once they enter an aquatic ecosystem. The rationale behind this approach was that these empirically determined values are dependent on not only the properties of the drug but also those of the body. In a number of different systems there was a reasonable relationship between the extent of sorption to sediment and distribution throughout the body (Chapter 6). A number of factors relating to the nature of the system would have had an influence on the relationship between partitioning coefficient (K_d) and volume of distribution (V_D). For example, the body is a closely regulated system, in terms of pH and ionic strength, compared with aquatic ecosystems. Therefore, to ensure that the tested relationship is indeed robust, a number of different aqueous systems should be tested with respect to pH and ionic strength. Also, potential interactions with other drugs should also be considered as is commonly reported in pharmacological studies. While the drugs chosen to test the relationship between K_d and V_D were used based on their range of physicochemical

properties and V_D values, a greater number of drugs would be desirable to further test this relationship. From this preliminary work, the next step would be to consider all of these factors and determining whether deviation from human physiological parameters for these factors influences the relationship. If the relationship between K_d and V_D was found to be a useful indicator in estimating the environmental fate of pharmaceuticals, then this would have important implications in the use of existing pharmacological data to contribute to the ecological risk assessment process.

While sorption was found to be a variable process, dependent on the characteristics of the drugs and the system, it is likely to play an important role in the environmental fate of these compounds. Transport, degradation and bioavailability are all influenced by the extent of interaction with sediments. A number of drugs found in environmental surveys, such as the acidic NSAIDs, are likely to have a relatively low affinity with sediments due to their level of ionisation. However, a number of other drugs found in environmental surveys are likely to interact with sediments to such an extent that their fate is intrinsically linked with this process. Sorption and desorption isotherms indicate desorption hysteresis was likely to have occurred for a number of the drugs (Chapter 4).

9.3 Exchangeability of carbamazepine (CBZ) within a water / sediment system

To test whether this hysteresis may have been related to experimental artefacts, an isotope exchange approach was used for CBZ (Chapter 7). This indicated that there was a slight decrease in the exchangeability of CBZ with the solution phase, particularly as time and organic carbon content increased. The quality of the organic carbon was also likely to be an important factor in determining the extent of observed exchangeability. Even though the amount of exchangeable CBZ remained above 80 % for the unamended sediment after 28 d, the non-exchangeable fraction would still modify the fate of CBZ in an aquatic system. This

has a number of potentially important consequences, when considering how pharmaceuticals enter the aquatic environment. The level of a drug's association with sediments within an aquatic ecosystem would be dependent on the concentration of the drug being discharged into the system. If the concentration was to vary markedly, the steady-state conditions would be altered and the ratio of drug within solution and within the sediment would also be expected to vary. While drugs may be expected to enter the environment consistently over the year, a number of factors, such as flooding, drought, temperature or seasonal dependence on the use of therapeutic goods, would disrupt a system at an apparent steady-state (Warren *et al.* 2003). Where sorption decreases the extent of degradation (Pignatello and Xing 1996), or in the case of drugs recalcitrant to degradation (Loffler *et al.* 2005), the role of sediment acting as a reservoir is an important consideration. The use of standardised, long-term sediment degradation experiments would be an important step to characterise environmental fate of human pharmaceuticals (EMEA-CPMP 2005; Loffler *et al.* 2005).

The use of the isotope exchange technique has yet to be proved as an effective alternative to the traditional sorption / desorption isotherms. Also, the use of radioisotopes is more expensive, due to the need for specialised laboratories and the highly regulated nature of its use and disposal. However, if such a technique is found to be less prone to experimental artefacts that make the occurrence of hysteresis ambiguous, then the associated costs would be traded off with clear indications of fate in sediments (Celis and Koskinen 1999a; Sander and Pignatello 2005).

Sorption to sediment is often found to reduce the bioavailable fraction of organic contaminants (Lamoureux and Brownawell 1999; Sijm *et al.* 2000; Phyu *et al.* 2005; Koelmans *et al.* 2006), which would be expected to reduce the potential for ecotoxicological impacts. For organisms that feed on sediment, bioavailability would also be associated with specific factors such as the extent of digestion of sediment particles (Sijm *et al.* 2000;

Simkiss *et al.* 2001). Therefore, the ease with which organic contaminants can be released from sediments is often related to bioavailability, where the extent of desorption hysteresis has been associated with the extent of reduction in the bioavailability of contaminants (Lamoureux and Brownawell 1999; Leppanen and Kukkonen 2000). If the exchangeable fraction of a drug can be related to its bioavailability within an aquatic ecosystem, then this method could prove to be a useful tool in the assessment of potential ecotoxicological impacts. The E-value has been used in agricultural sciences to assess the fraction of total trace metals in soils that can desorb into soil solution (Hamon *et al.* 2002). This concept has been taken further by comparing the E-value with the labile (L) value of plants growing within the soil (Nolan *et al.* 2003; Massoura *et al.* 2004). The L-value is analogous to the E-value, in that it measures the isotopic ratio in plants. When the L-value and E-value are compared using this method within a soil plant system and the values are equal, this suggests that the plant is only taking up contaminants from the exchangeable pool within the soil. If the L-value is greater than the E-value, then this suggests that the plant is not only taking the contaminant from the exchangeable pool but also from the non-exchangeable pool (which can be facilitated by soil microorganisms). The advantage of using this technique is that information can be obtained with respect to where the plant is taking a contaminant up from. If an analogous situation was tested in an aquatic ecosystem, then it may also be possible to determine whether the non-exchangeable pool is bioavailable for a sediment bound contaminant. This would contribute further to understanding whether sediment is acting as a protective sink for contaminants or as a source. Unfortunately, due to time constraints, this was not possible for this project.

9.4 Ecotoxicological impacts of carbamazepine (CBZ) on Chironomus tepperi

CBZ spiked to sediments did not seem to increase the ecotoxicological impact on the sediment-feeding *Chironomus tepperi*, compared with short-term exposure in solution only.

This finding was based on the estimated concentration of CBZ within solution between systems being the same where significant effects were apparent (Chapter 8). This would indicate that sediment can reduce the bioavailability of CBZ to *Chironomus tepperi*, even though they are feeding on the sediment. It would therefore be desirable to further assess the role that sorption to sediment can have on the fate of human pharmaceuticals, in the context of the potential for modifying bioavailability to aquatic organisms. Understanding this process would be important in defining the risk that human pharmaceuticals present in the aquatic environment.

The effect on the development of *Chironomus tepperi* larvae, based on the concentration of CBZ in solution, would suggest that environmental concentrations of CBZ would not present a risk to *Chironomus tepperi*. However, exposure to a mixture of compounds may indicate that the potential for additive or synergistic effects within *Chironomus* spp. cannot be ruled out. Effects from exposure to mixtures of pharmaceuticals are particularly relevant considering the origin of pharmaceuticals. Other environmental stressors related to WWTP effluent, such as elevated levels of phosphorus or ammonia, might be exacerbated if a number of pharmaceuticals are also present in effluent. This issue might be more relevant for water regulatory authorities, as opposed to the regulatory groups concerned with pharmaceuticals. Safety factors within guidelines for pharmaceuticals may already account for the possibility of mixture or co-stressor effects (EMEA-CPMP 2005).

The use of a biomarker to assess whether effects at the cellular level could indicate whether *Chironomus tepperi* was exposed to CBZ and whether changes in the functioning of the biomarker occurred at concentrations below the whole organism proved to be unsuccessful. That is, the use of Na^+/K^+-ATPase as a biomarker of exposure and effect for CBZ in *Chironomus tepperi* did not seem to be appropriate (Chapter 8). Although the physiological effects in humans might suggest that exposure to CBZ would influence the activity of

Na^+/K^+-ATPase, this might not be the case for invertebrates. While pharmaceuticals are designed to interact with specific receptors within mammals, the effect may not be as predictable for lower organisms such as invertebrates (Huggett *et al.* 2003a). However, it is apparent that CBZ does have an influence on the development of *Chironomus* spp. based on this study (Chapter 8) and that of Oetken *et al.* (2005). Therefore, it might be useful to focus on other biomarkers; for example, the activity of cytochrome P450 in *Chironomus* spp., based on the presence of these enzymes in *Chironomus* spp. and the effect CBZ has on cytochrome P450 in humans, may be a useful biomarker of exposure. Determining the actual body burden of pharmaceuticals in aquatic organisms is probably the most effective measure of bioavailability (Kaag *et al.* 1998). Compared with whole body extractions and powerful analytical methodologies for trace organic analysis, however, the use of biomakers represents a more cost-effective approach to assessing bioavailability and would represent a more attractive option for regulatory agencies (Schlenk 1999). It is therefore desirable to investigate the presence of appropriate biomarkers for pharmaceuticals in aquatic organisms. The extensive pharmacological data available for pharmaceuticals represents a unique opportunity to use similar endpoints in ecotoxicological assays, especially considering the limited work done in this area (Seiler 2002; Huggett *et al.* 2003a).

9.5 Application of results to the regulatory approach to ecological risk assessment (ERA)

The current draft guidelines recommended by the EMEA have become more detailed since their initial publication (EMEA-CPMP 2001; EMEA-CPMP 2005). The general premise of newly developed pharmaceuticals requiring extra assessment if their predicted environmental concentration is greater than 10 ngL^{-1} remains unchanged. The information required for this prediction has become more sophisticated, incorporating factors such as a market penetration

factor (F_{pen}). However, the potential for interaction with sediments might be useful during this initial assessment.

While this value represents an arguably low concentration for organic contaminants, using precedents from pharmaceuticals currently in use may be appropriate. For example, CBZ has been found in the environment at more than 10 times the concentration of FLX or PRL (Kolpin *et al.* 2002; Metcalfe *et al.* 2003b; Ashton *et al.* 2004). However, the sorption of FLX, PRL and CBZ determined during this study (Chapters 4, 5 and 6) would suggest that the relative sorption of FLX or PRL would be substantially higher than CBZ. This relatively higher sorption potential might therefore indicate that, while at lower concentrations in solution, the sediment pool of FLX or PRL could be relatively larger than CBZ. This would be of importance, particularly if FLX was similarly as recalcitrant to degradation as CBZ. Therefore, potential sorption of a drug would be a useful consideration in the Phase I assessment of EMEA guidelines, particularly when predicted environmental concentrations (PECs) are close to 10 ngL^{-1}.

If a Phase II assessment is required, a number of tests are recommended, including chronic toxicity assays for a number of trophic levels (sludge microorganisms, algae, invertebrates and fish), assessment of sorption / desorption using batch equilibrium studies and the assessment of degradation within a water / sediment system (EMEA-CPMP 2005). The use of chronic toxicity assays is a significant improvement, based on the nature of exposure aquatic organisms have to pharmaceuticals and the different endpoints that are apparent, as occurred in this study (Chapter 8). However, it has been suggested that the use of effective concentration (EC) values would be more robust measures of assessment in ecotoxicological assays than statistically-based no observed or lowest observed effect concentration (NOEC or LOEC) (Chapman *et al.* 1996; Dhaliwal *et al.* 1997). The use of sorption and sediment degradation assessments is highly appropriate, based on the findings of this work. However,

the outcomes of the guidelines for these assessments are to determine whether terrestrial toxicity should be considered (for highly adsorbed drugs) or whether sediment ecotoxicity assays are necessary for sediment bound drugs (EMEA-CPMP 2005). Once again, the implication of sorption of pharmaceuticals, in terms of the subsequent modification of fate, ecotoxicological effects and whether sediment is acting as a sink or source may be important considerations in the risk assessment process. Further work on pharmaceuticals currently on the market, in the context of the recommended EMEA guidelines, will provide information with respect to these considerations. This information will then allow a clearer indication of the potential environmental risk of new drug products, as well as currently available drugs, and how our use patterns could mitigate these potential risks. Such measures are crucial measures to consider, prior to the development and implementation of effective wastewater treatment technologies.

9.6 Summary

A number of important points have been raised by this work, with respect to the risk assessment of human pharmaceuticals:

- Human pharmaceuticals found in the aquatic environment are likely to be associated with sediments. Sorption would be a particularly important process for cationic and neutral compounds.
- For both neutral and ionisable pharmaceuticals, solution pH and concentration of calcium ions are important modifying factors of the sorption process.
- The use of distribution data within humans may be an important tool for estimating the relative extent of sorption of human pharmaceuticals within an aquatic environment.
- The exchangeable pool of CBZ was found to decrease slightly over time and decrease markedly when the quality and quantity of organic carbon in the sediment was modified.

- The exposure of *Chironomus tepperi* to CBZ from solution may have been more important than from CBZ adsorbed to the sediment. This may indicate that sorption has a protective effect, although the potential for sediment to act as a reservoir needs to be further considered.
- CBZ was found to affect the development of *Chironomus tepperi*, although the concentration of effect was higher than concentrations of CBZ found in environmental surveys.

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Appendix A - Equation 5.3 derivation

Derivation of Equation 5.3 (Chapter 5) from the Henderson-Hasselbach equation, based on the case of a drug as a weak base.

In water, the conjugate acid of a weak base can dissociate according to the equilibrium:

$$BH^+ \rightleftharpoons B + H^+$$

The acid dissociation constant for this equilibrium is:

$$K_a = \frac{[H^+][B]}{[BH^+]} \qquad (1)$$

where [B] is the concentration of free base, $[BH^+]$ is the concentration of the conjugate acid of the weak base and K_a is the acid dissociate constant for the conjugate acid.

The distribution of the conjugate acid of the weak base between sediment and water can be expressed as:

$$K_{d1} = \frac{[BH^+]_s}{[BH^+]_w} \qquad (2)$$

Similarly, the distribution of the weak base between sediment and water can be expressed as:

$$K_{d2} = \frac{[B]_s}{[B]_w} \qquad (3)$$

The distribution of total substance between water and sediment can be represented by:

$$K_D = \frac{[BH^+]_s + [B]_s}{[BH^+]_w + [B]_w} \qquad (4)$$

which can be expressed as:

$$K_D = \frac{[BH^+]_s}{[BH^+]_w + [B]_w} + \frac{[B]_s}{[BH^+]_w + [B]_w} \qquad (5)$$

Rearrangement of (1) and substitution into (5) yields:

$$K_D = \frac{[BH^+]_s}{[BH^+]_w + \frac{[BH^+]_w K_a}{[H^+]}} + \frac{[B]_s}{\frac{[B]_w [H^+]_w}{K_a} + [B]_w} \qquad (6)$$

Collecting like terms:

$$K_D = \frac{[BH^+]_s}{[BH^+]_w\left(1+\frac{K_a}{[H^+]}\right)} + \frac{[B]_s}{[B]_w\left(1+\frac{[H^+]}{K_a}\right)} \qquad (7)$$

The distribution constants for the conjugate acid and the unionised weak base can be inserted using the relationships defined in (2) and (3) and rearranging:

$$K_D = \frac{K_{d1}[H^+]}{[H^+]+K_a} + \frac{K_{d2}K_a}{[H^+]+K_a} \qquad (8)$$

$\frac{[H^+]}{[H^+]+K_a}$ = α is the fraction of the weak base in its conjugate acid form and $\frac{K_a}{[H^+]+K_a}$ = 1 - α is the fraction of the weak base in its unionised state. Therefore the distribution between sediment and aqueous phase can be written:

$$K_D = K_{d1}.\ \alpha + K_{d2}.(1 - \alpha) \qquad (9)$$

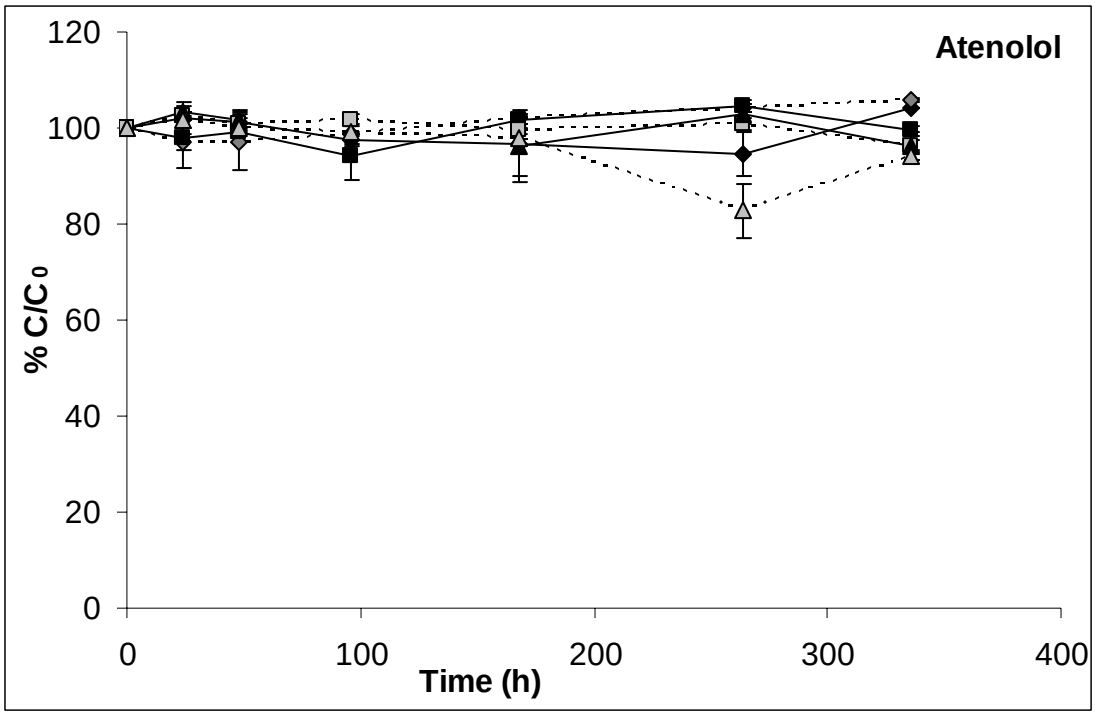
Various linear transforms can be employed to determine the values for K_{d1} and K_{d2}, eg:

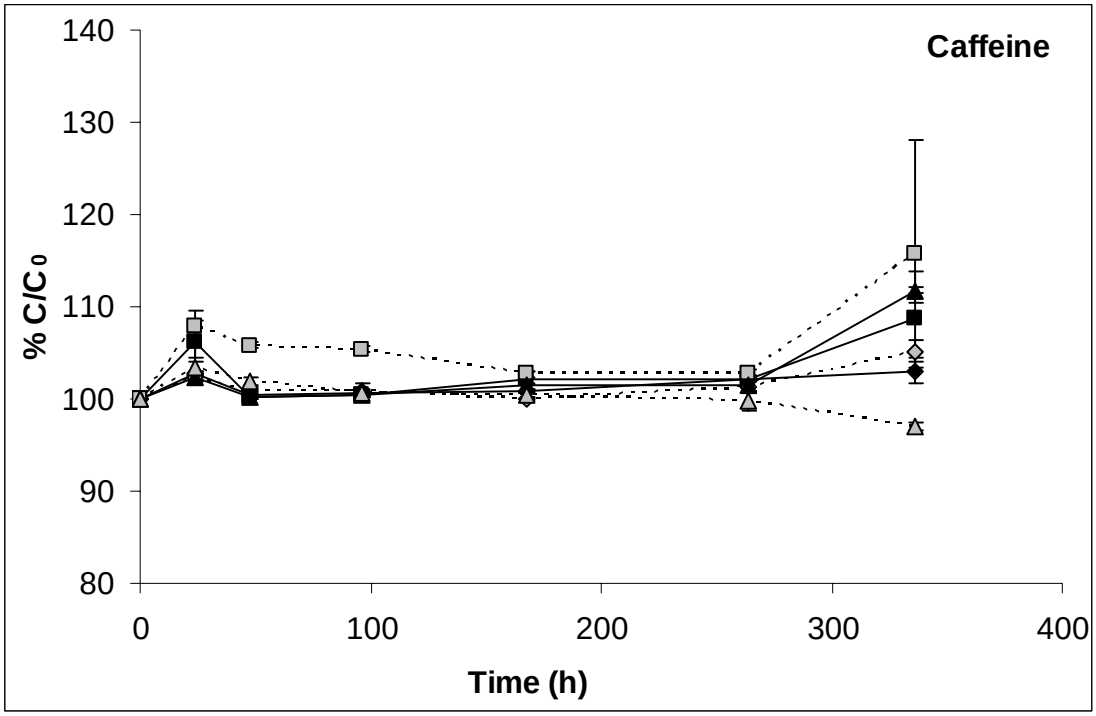
$$K_D/\alpha = K_{d1} + K_{d2}.(1 - \alpha)/\alpha \qquad (10)$$

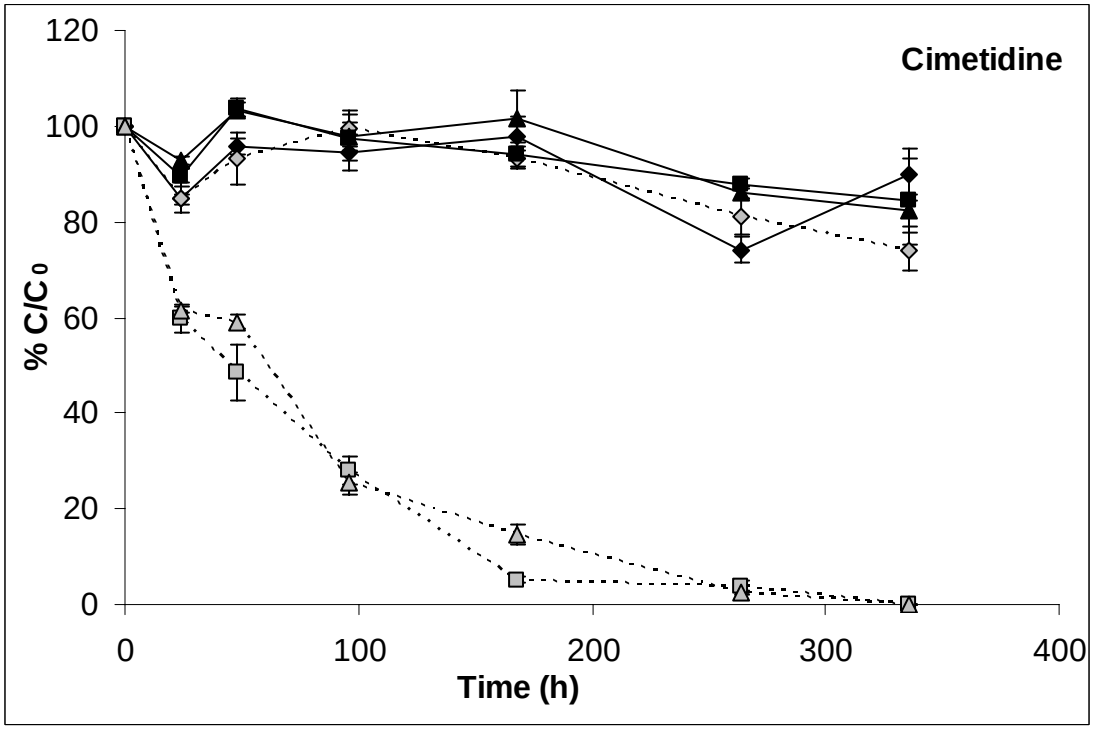
In this case, a plot of K_D/α *vs* $(1 - \alpha)/\alpha$ will yield an intercept of K_{d1} and a slope of K_{d2}.

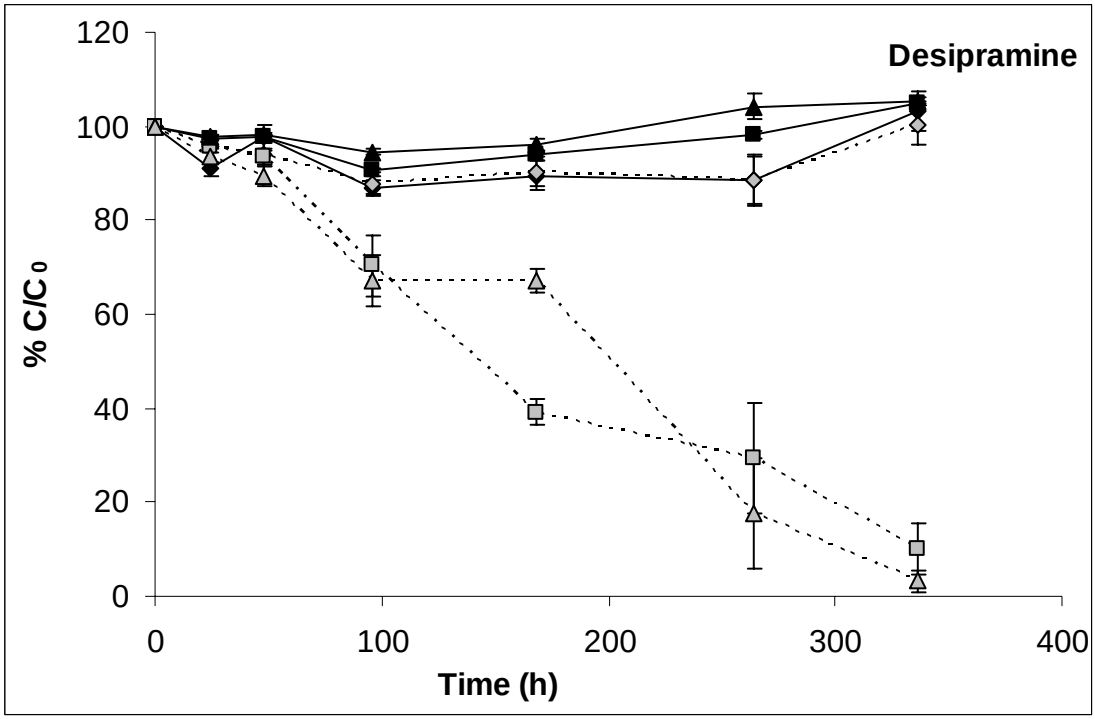
Appendix B – Aqueous degradation

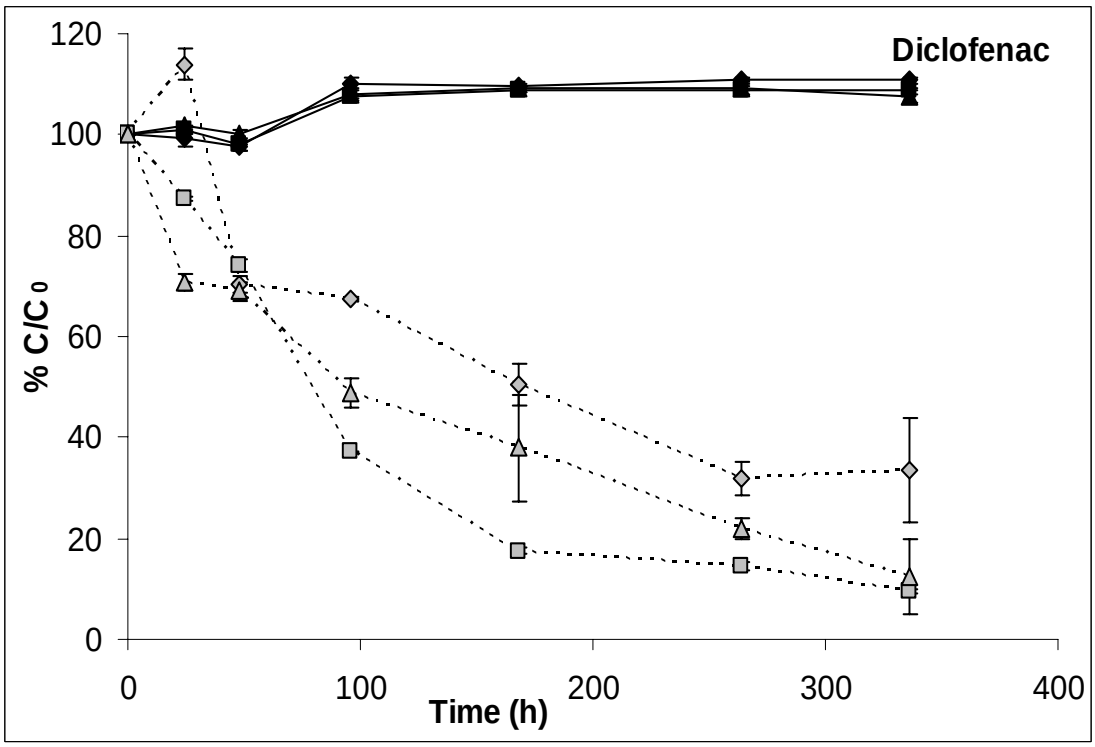
Figures representing the degradation of pharmaceuticals in solution under the same conditions as those for carbamazepine (*see* Chapter 8, section 8.2.4). Figures show the concentration of drug in solution (C) compared with initial spiking concentration (C_0) following exposure to light and colloidal material; treatments were 18.2 MΩcm^{-1} (Milli-Q) water (—◆—), moderately hard water (MHW) mixed with sediment and sterilised (—■—) and MHW mixed with sediment and re-inoculated (—▲—); controls were protected from light for Milli-Q water (- - -◆- -), MHW mixed with sediment and sterilised (- - -□- -) and MHW mixed with sediment and re-inoculated (- - -△- -). Error bars are standard errors of the mean of triplicate samples.

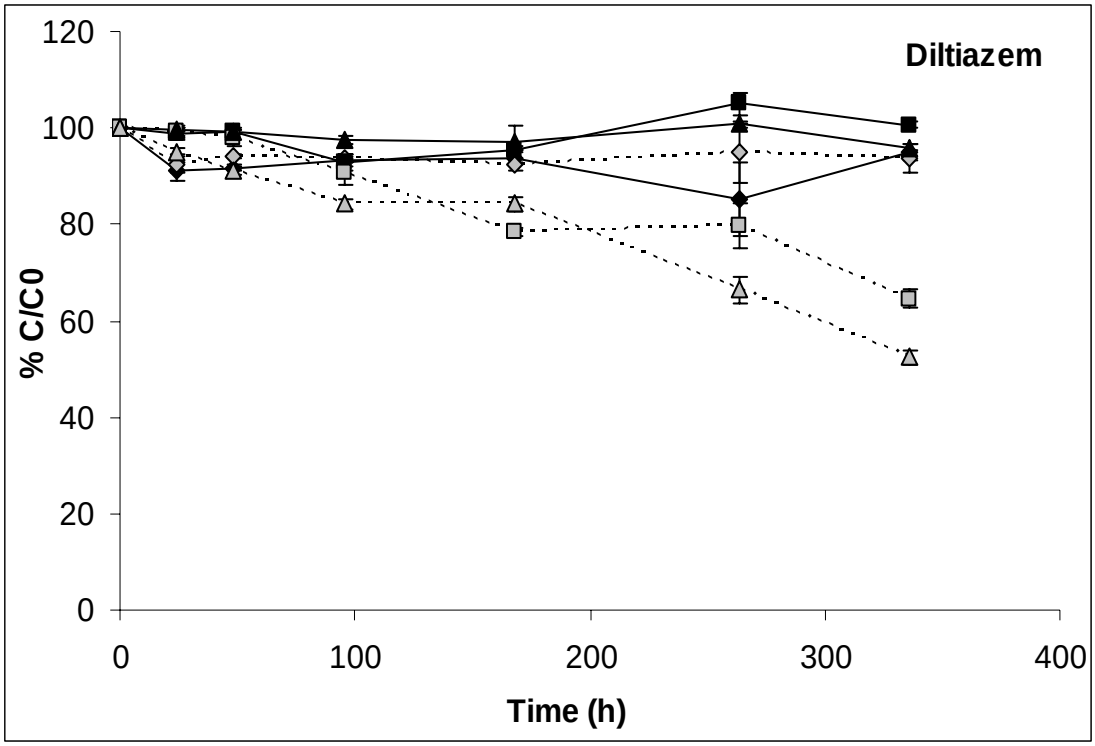


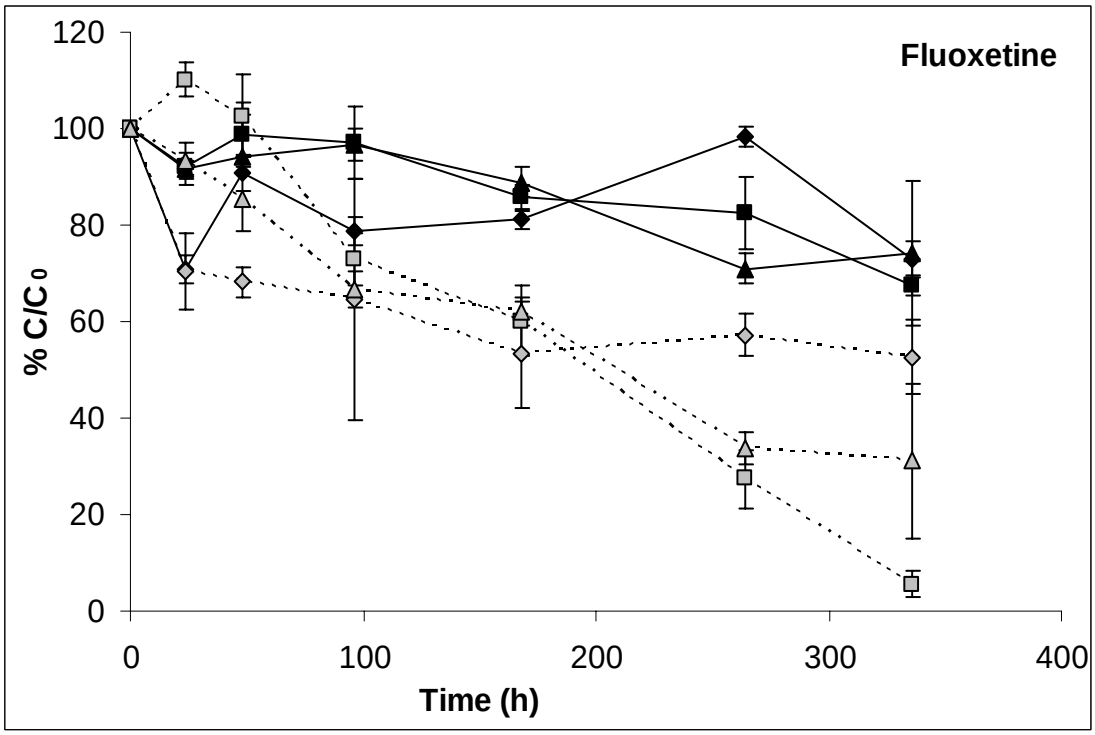

Caffeine
140
130
120
110
100
90
80
% C/C0
0
100
200
300
400
Time (h)

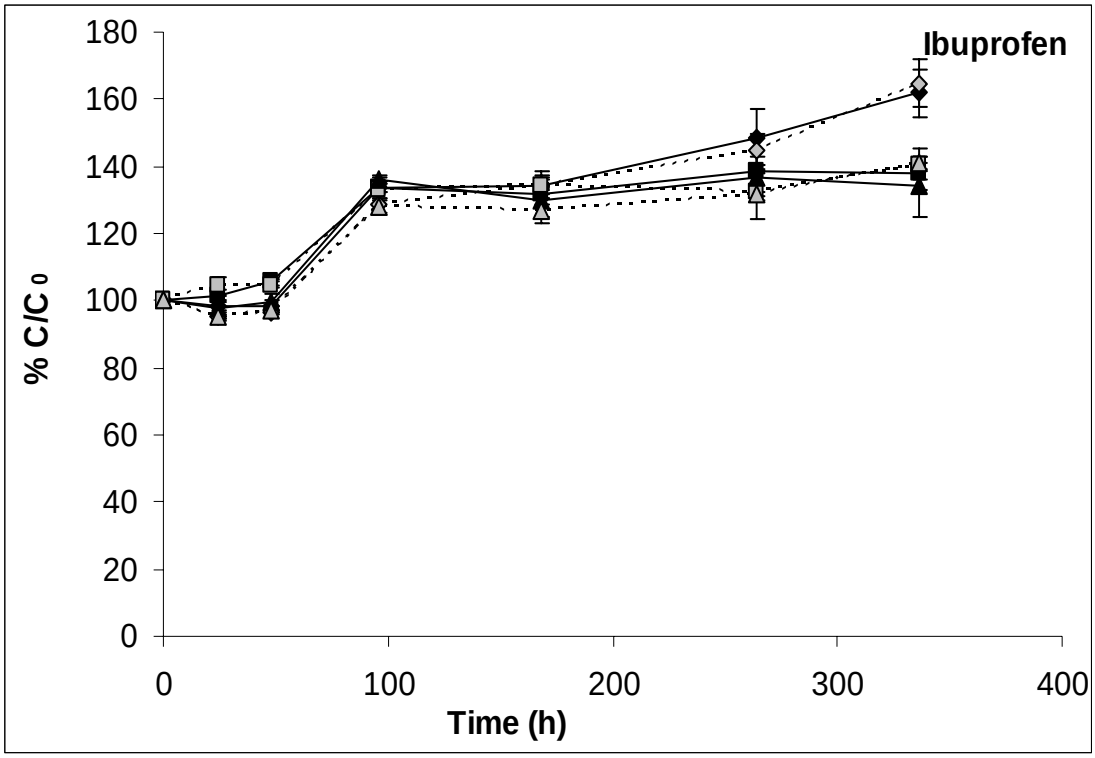

Cimetidine
120
100
80
60
40
20
0
% C/C0
0
100
200
300
400
Time (h)

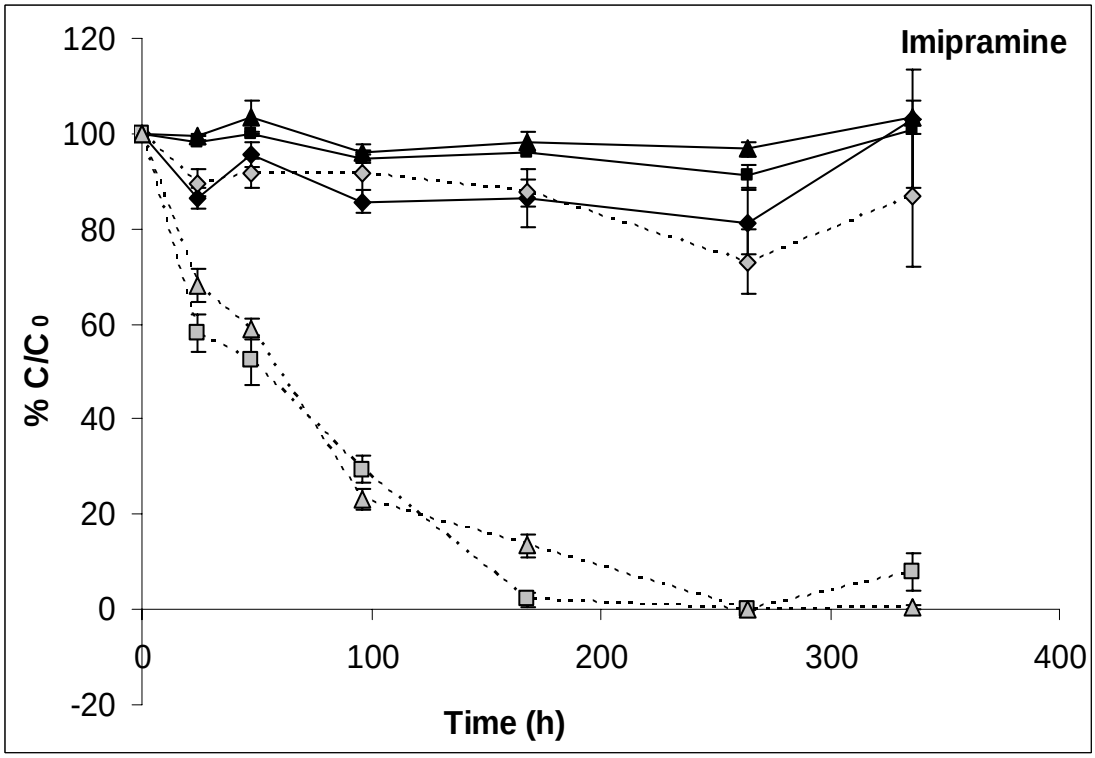

Desipramine
% C/C0
Time (h)
0
20
40
60
80
100
120
0
100
200
300
400

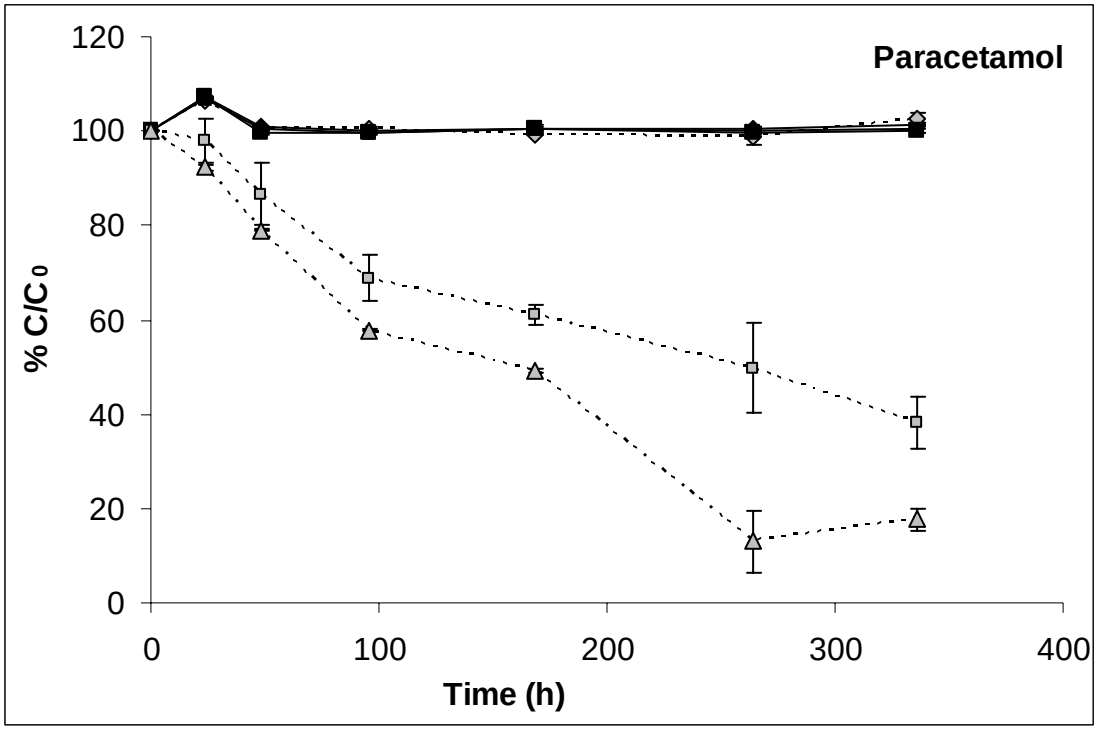

Diclofenac
% C/C0
Time (h)
0
20
40
60
80
100
120
0
100
200
300
400


Diltiazem
% C/C0
Time (h)
0
20
40
60
80
100
120
0
100
200
300
400


Fluoxetine
% C/C0
Time (h)
0
20
40
60
80
100
120
0
100
200
300
400


Ibuprofen
% C/C0
Time (h)
180
160
140
120
100
80
60
40
20
0
0
100
200
300
400


Imipramine
% C/C0
Time (h)
120
100
80
60
40
20
0
-20
0
100
200
300
400


Paracetamol
120
100
80
60
40
20
0
% C/C0
0
100
200
300
400
Time (h)

Appendix C – Publication

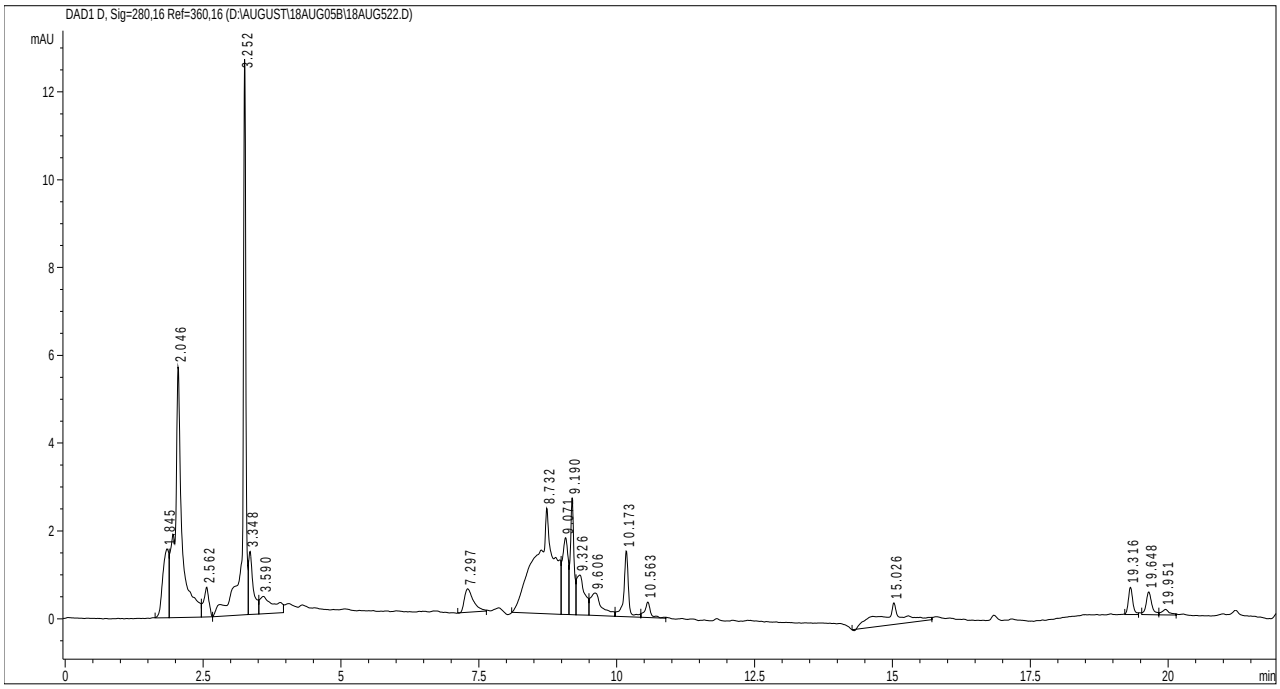
Williams, M., Saison, C. L. A., Williams, D. B. and Kookana, R.S. (2006). Can aquatic distribution of human pharmaceuticals be related to pharmacological data? *Chemosphere* **65** (11): 2253-2259

Williams, M., Saison, C.L.A., Williams, D.B. and Kookana, R.S. (2006) Can aquatic distribution of human pharmaceuticals be related to pharmacological data? *Chemosphere v.65 (11) pp. 2253-2259, December 2006*

Appendix D – Sample chromatograms from batch sorption

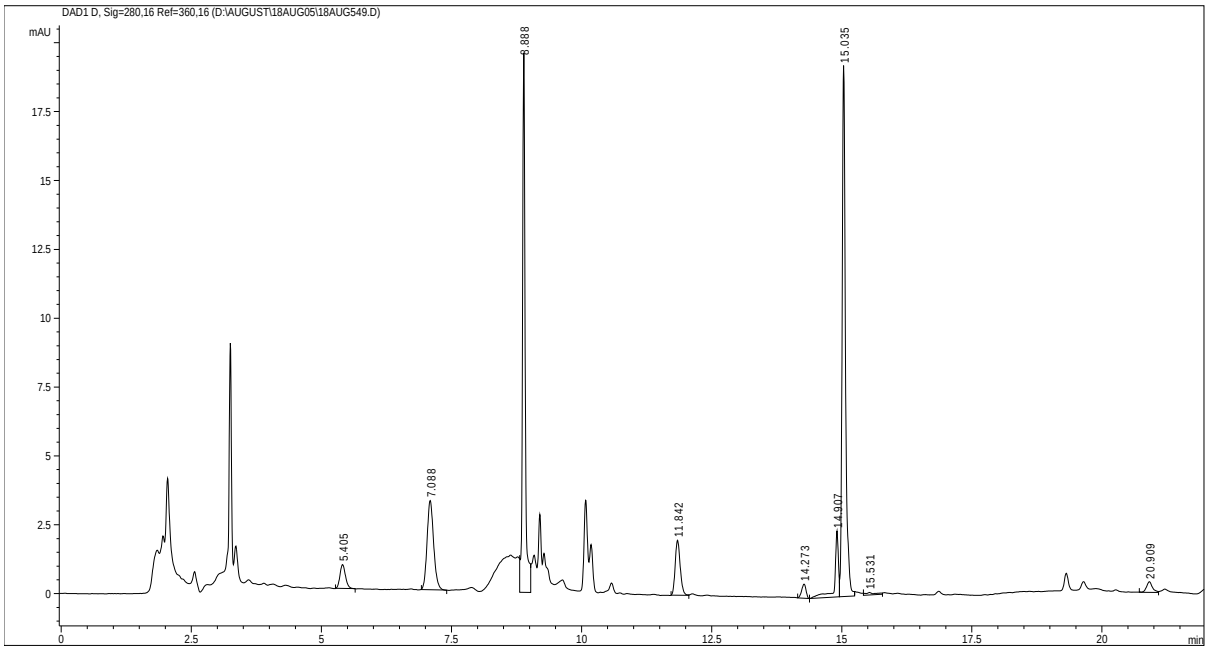
The following chromatograms show an example of the controls used for batch sorption experiments. The first two HPLC chromatograms (Figure and Table D1 and D2) compare a typical sediment blank (no compounds added) with a spiked sample after 24 h shaking with sediment. In the case where peaks for compounds aligned with peaks in the sediment blank (for example, at 15 min where CBZ elutes) the peak areas of the sediment blank were subtracted from the peak areas in the samples only if this was likely to interfere the sample. For example, the peak area and height at 15.03 min in the blank sample would not likely have interfered with the elution of CBZ and was not adjusted accordingly.

The final two chromatograms (Figure and Tables D3 and D4) compare a spiking solution of the compounds with a no sediment control (spiked with compounds) at pH 6.5 and shaken for 24 h. In this case there was little evidence of degradation, although in some cases (such as lower pH spiking solutions with higher spiking concentrations) some compounds, such as DCF, were found to be substantially reduced in the no sediment control. In most cases, this was accounted for by adjusting for the extent of difference between the spiking solution and the no sediment control (following duplicate analysis). This was considered to be appropriate since most of the loss of analyte was expected to be due to filtration losses (*see* Chapter 4, section 4.3.2).



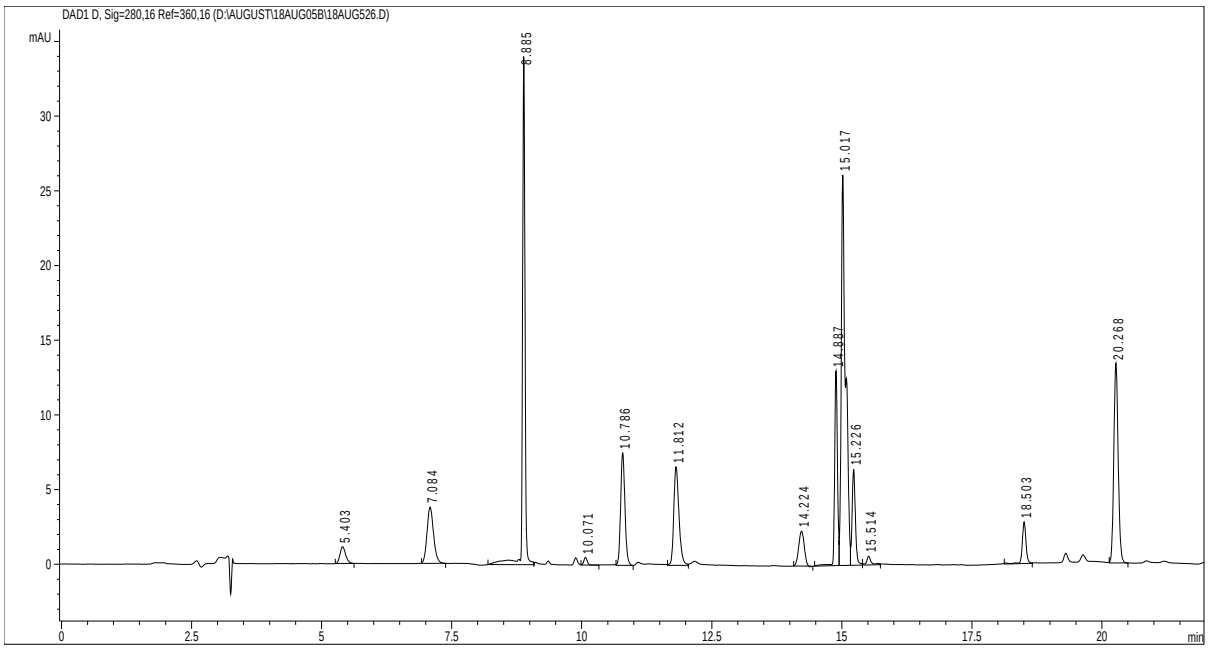
#	Time	Area	Height	Width	Area%	Symmetry
1	1.845	12.2	1.6	0.1263	5.367	2.437
2	2.046	41.4	5.7	0.0984	18.267	0.431
3	2.562	4.5	6.8E-1	0.0915	1.993	1.344
4	3.252	51.2	12.7	0.0611	22.609	1.82
5	3.349	8.5	1.4	0.0851	3.761	0.424
6	3.591	5.7	3.9E-1	0.1984	2.524	0.501
7	3.896	3	2.3E-1	0.1719	1.309	0.403
8	7.296	5.8	5.3E-1	0.1628	2.539	0.56
9	8.732	19.2	2.4	0.1056	8.466	0.521
10	9.071	12.7	1.8	0.105	5.592	1.146
11	9.191	12.1	2.7	0.0664	5.349	0.891
12	9.329	9.8	9.4E-1	0.1514	4.308	0.561
13	9.606	8.6	5.4E-1	0.2208	3.798	0.516
14	10.173	8.7	1.5	0.0826	3.830	1.047
15	10.563	2.8	3.6E-1	0.1084	1.246	0.923
16	15.026	13.3	4.9E-1	0.3333	5.853	1.775
17	19.316	3.5	6.2E-1	0.0854	1.540	0.828
18	19.648	3.7	5.2E-1	0.1078	1.648	0.796

Figure and Table D1: Example of an HPLC chromatogram and chromatogram specifications of a sediment blank used as a control in batch sorption experiments



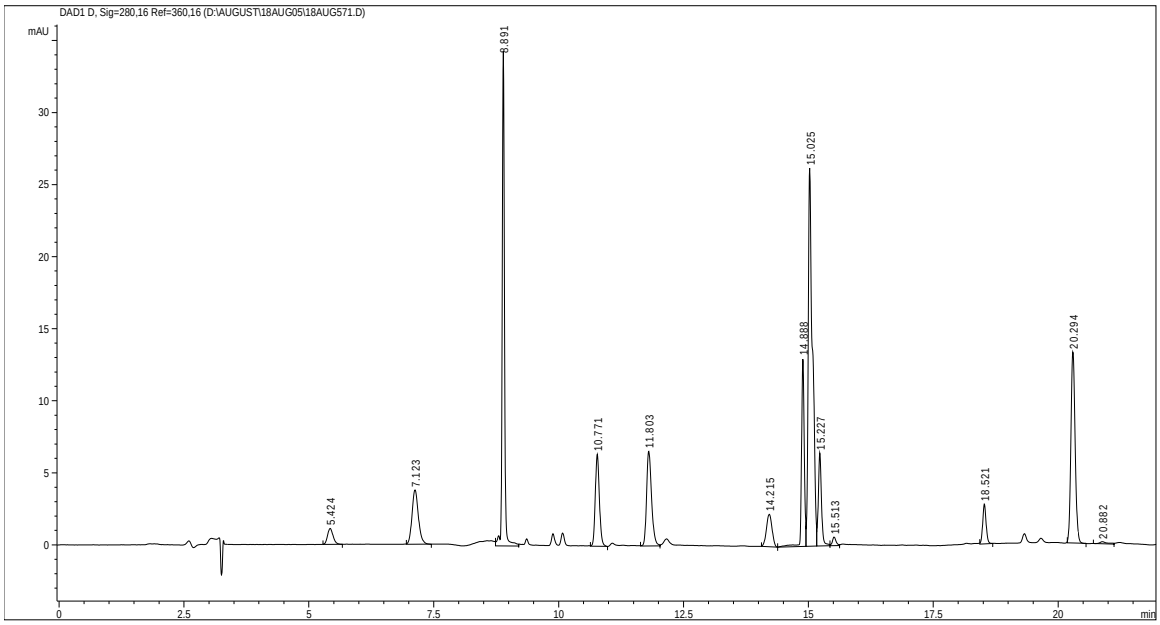
#	Time	Area	Height	Width	Area%	Symmetry
1	5.405	6.5	8.7E-1	0.1148	2.889	0.819
2	7.088	28.5	3.2	0.1365	12.698	0.843
3	8.888	70.2	19.6	0.0558	31.290	0.809
4	11.842	13.5	2	0.1045	6.004	0.804
5	14.273	3	5.1E-1	0.0939	1.331	1.158
6	14.907	11.7	2.4	0.0732	5.232	1.934
7	15.035	86.6	19.3	0.0704	38.593	0.75
8	15.531	1.3	9.3E-2	0.1803	0.578	0.587
9	20.909	3.1	3.9E-1	0.1195	1.387	0.845

Figure and Table D2: Example of an HPLC chromatogram and chromatogram specifications of a sample in batch sorption experiments



#	Time	Area	Height	Width	Area%	Symmetry
1	5.403	8.7	1.1	0.1195	1.480	0.801
2	7.084	33.1	3.8	0.1363	5.638	0.847
3	8.885	110	34.2	0.0515	18.749	0.89
4	10.786	43.6	7.6	0.0913	7.429	0.872
5	11.812	46.1	6.6	0.1053	7.853	0.742
6	14.224	17.8	2.4	0.1197	3.034	0.998
7	14.887	49.8	13.1	0.0603	8.481	1
8	15.017	152.3	26.2	0.0839	25.957	0.48
9	15.226	28.4	6.5	0.0673	4.838	0.815
10	15.514	3.4	6.1E-1	0.0834	0.585	0.895
11	18.503	13.7	2.8	0.0754	2.333	0.949
12	20.268	78.4	13.4	0.0923	13.361	0.88
13	20.856	1.5	1.7E-1	0.1345	0.263	0.572

Figure and Table D3: Example of an HPLC chromatogram and chromatogram specifications of a spiking solution used as a control in batch sorption experiments



#	Time	Area	Height	Width	Area%	Symmetry
1	5.424	8.6	1.1	0.1201	1.470	0.823
2	7.123	33.3	3.8	0.1352	5.713	0.844
3	8.891	112.3	34.4	0.0521	19.289	0.891
4	10.771	36.8	6.4	0.0892	6.321	0.867
5	11.803	45.8	6.6	0.1071	7.864	0.735
6	14.215	17.7	2.3	0.1231	3.034	0.988
7	14.888	50.1	13.1	0.0607	8.602	0.99
8	15.025	153.1	26.3	0.0841	26.302	0.494
9	15.227	28.7	6.5	0.0676	4.927	0.797
10	15.513	3.2	6.1E-1	0.0794	0.550	0.814
11	18.521	13.2	2.8	0.0755	2.261	0.875
12	20.294	77.9	13.3	0.0924	13.385	0.867
13	20.882	1.6	1.5E-1	0.1556	0.281	0.465

Figure and Table D4: Example of an HPLC chromatogram and chromatogram specifications of a no sediment sample used as a control in batch sorption experiments

Appendix E – Parameters of solid phase used from literature in Table 4.3

Reference	Compound	K_d (L/kg)	pH	% OC	Solid phase
Lee *et al.* 2003	EE2	2.3 23.4	7.3 [a] 7.2 [a]	0.22 2.91	Soil
Ying *et al.* 2003	EE2	24.2	8.9	0.5	Sediment
Clara *et al.* 2004	EE2	692	NR [d]	34	Sewage sludge
Furlong *et al.* 2004	CAF CBZ CIM DIL PAC	26 69 22 53 4.5	NR	NR	Sediment
Ternes *et al.* 2004	CBZ DCF EE2	NR [d] 1.2 16 459 278 349	6.6 7.5 6.6 7.5 6.6 7.5	35 41 35 41 35 41	Sewage sludge
Andersen *et al.* 2005	EE2	584	7.1 [c]	28	Sewage sludge
Drillia *et al.* 2005	CBZ DCF PRL	0.5 37 0.5 165 16 199	6.8 [b] 4.3 [b] 6.8 4.3 6.8 4.3	0.4 7.1 0.4 7.1 0.4 7.1	Soil
Loffler *et al.* 2005	CBZ	1.3	7.7	1.4	Sediment
Scheytt *et al.* 2005	CBZ DCF IBU	0.2 5.3 0.6 4.7 0.2 1.7	4.8 [b] 6.7 [b] 4.8 6.7 4.8 6.7	1.3 2 1.3 2 1.3 2	Sediment
Scheytt *et al.* 2006	CBZ IBU	0.1 0.1	4.8 [b]	1.3	Sediment

[a] pH from H_2O
[b] pH from $CaCl_2$
[c] pH of added solution
[d] Not reported

Appendix F: Statistical analysis of solution concentrations obtained after apparent sorption equilibrium

Table F1: Summary of statistical analyses of solution concentrations after various shaking periods within the batch sorption system. A one-way ANOVA was used to determine whether any significant differences existed between times for a particular compound, with Tukey's test used to assess which treatments were different. Treatments that share a common letter were not found to be significantly different.

Compound	**Shaking time (h)**					
	1	**2**	**5**	**8**	**16**	**24**
ATL	a	a	a	b	b	b
CAF	a	a	a	a	a	a
CBZ	a	a	a,b	a,b	b	b
CIM	a,b	a	a,b	a,b	a,b	b
DCF	a	a	a	a	a	a
DIL	a	a	a	a	a	a
DMI	a	a	a	a,b,c	b	c
DPH	a	a	a	a	a	a
FLX	a	a	a,b	b	b	b
IBU	a	a	a	a	a	a
IMI	a	a	a	a,b	b,c	c
PAC	a	a	a	a	a	a

Appendix G: Statistical analysis of K_d values obtained after altering solution pH and $CaCl_2$ concentrations

Table G1: Summary of statistical analyses of K_d values for each compound after altering pH of solution within the batch sorption system. A one-way ANOVA was used to determine whether any significant differences existed between treatments for a particular compound, with Tukey's test used to assess which treatments were different. Treatments that share a common letter were not found to be significantly different.

Compound	Treatment (pH of solution)			
	pH 4.5	pH 5.5	pH 6.5	pH 9.5
ATL	a	a	a,b	b
CAF	a	b	b	b
CBZ	a	b	b	b
CIM	a	b	b	b
DCF	a	b	b	b
DIL	a,c	b	c	a,c
DMI	a,c	b,c	c,d	d
FLX	a	b	c	a
IBU	a	b	b	b
IMI	a	b	c	d
PAC	a,b	a	b	c

Table G2: Summary of statistical analyses of K_d values for each compound after altering the concentration of $CaCl_2$ in solution within the batch sorption system. A one-way ANOVA was used to determine whether any significant differences existed between treatments for a particular compound, with Tukey's test used to assess which treatments were different. Treatments that share a common letter were not found to be significantly different.

Compound	**Treatment (solution concentration of $CaCl_2$)**				
	1 mM	**5 mM**	**10 mM**	**50 mM**	**100 mM**
ATL	a	a	a	b	b
CAF	a	b	c	d	e
CBZ	a	b	b	c	d
CIM	a,c	c	b,c	b,c	b
DCF	a	a	a	a	a
DIL	a	b	c	d	e
DMI	a	b	c	-	d
FLX	a	b	b	a,b	a,b
IBU	a	a	a	a	a
IMI	a	b	c	d	d
PAC	a,b	a	a,b	b	a,b

- not available