Occurrence and effects of pharmaceuticals in freshwater ecosystems

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Over the last 10-15 years pharmaceuticals have been identified as a widespread pollutant in freshwater systems having entered the environment predominantly via the domestic sewage system where removal by treatment systems is often poor. This thesis provides detailed reviews and meta-analyses of existing data regarding pharmaceutical pollutants, examines the occurrence of five pharmaceuticals in semi-rural and urban catchments of West Yorkshire and their effects on freshwater ecosystems using both laboratory and field experiments.

A critical review and meta-analysis of 155 published pharmaceutical papers found 204 pharmaceuticals were present in rivers across large parts of Europe, North America and Asia. However, spatial and methodological gaps were identified in the research body with almost no research evident in Africa, South America, the Middle East and large parts of Asia. Furthermore, research effort was focused around a small number of commonly studied compounds often employing poorly representative grab sampling techniques.

Treated and untreated effluent of sewage treatment plants, combined sewer overflows and their receiving freshwaters were monitored for five pharmaceuticals (diclofenac, erythromycin, ibuprofen, mefenamic acid and propranolol). All compounds were detected at very high frequencies across all samples confirming them as a ubiquitous and widespread pollutant in freshwaters. Data showed pronounced seasonal (winter maxima) and diurnal (late morning and late evening) peaks in concentrations. Periods of high flow were characterised by reduced concentrations, possibly due to dilution within receiving waters. No appreciable attenuation of pharmaceuticals was observed across an intensively sampled 5 km study reach of the River Aire in Leeds suggesting the pollution burden placed on rivers by pharmaceuticals extends well downstream of individual waste water point sources.

Laboratory experiments revealed significant increases in mortality of the freshwater shrimp *Gammarus pulex* were observed during extended exposures to environmental levels of erythromycin and when combined in mixture with propranolol. A first application of ^{1}H NMR environmental metabolomics to the study of pharmaceutical effects on freshwater biota was coupled with this experiment highlighting its potential use in ecotoxicological research. Sublethal, metabolic changes associated with energy storage and metabolism were observed with potential future applications for biomarker development centred on the osmolyte TMAO.

The pharmaceuticals studied here were found to pose no detectable risk to leaf litter decomposition in streams although a further experiment demonstrated a reduction in organic matter processing of freshwater sediments, coupled with some complex stimulatory and inhibitory effects on respiration and nutrient cycling at environmentally

relevant concentrations. Taken as a whole this work has added substantial knowledge to this growing research area and has allowed the construction of a conceptual framework that links measured environmental concentrations with effects at the sublethal and individual organism level mediated to the ecosystem and functional level via complex interactions between macroinvertebrates and microbial communities.

Overall, this body of research has demonstrated that pharmaceuticals should be treated as a widespread pollutant of on-going major concern capable of eliciting significant effects on freshwater ecosystems. Therefore, they require substantial further research and scrutiny from regulators and policy makers if the negative consequences of their presence in rivers are to be avoided or mitigated.

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List of Definitions

CA – concentration addition

CSO – combined sewer overflow

DDD – defined daily dose

EA – Environment Agency

EC_{50} – median effective concentration

EMEA – European Medicines Agency

EQS – Environmental Quality Standard

K_{OW} – octanol-water partition coefficient

GC-MS – gas chromatography with mass spectroscopy

HPLC-MS/MS – high-performance liquid chromatography with tandem mass spectroscopy

IA – independent action

LC_{50} – median lethal concentration

LOD – limit of detection

LOEC – lowest observed effect concentration

LOQ – limit of quantification

MOA – mechanism of action

MEC – measured environmental concentration

NOEC – no observed effect concentration

NSAID – non-steroidal anti-inflammatory drug

OTC – over-the-counter

PCPs – personal care products

PEC – predicted environmental concentration

PPCPs – pharmaceuticals and personal care products

Q-TOF – quadrupole time-of-flight

RQ – risk quotient

SSRI – selective serotonin reuptake inhibitor

STP – sewage treatment plant

WFD – Water Framework Directive (2000/60/EC)

1 INTRODUCTION AND CONTEXT

Human society relies on rivers for myriad functions and services including: drinking water, irrigation, navigation, transport, recreation and waste disposal. Despite accounting for just 0.4 % of the Earth's surface and 0.006 % of the Earth's freshwater they are estimated to contain 6 % of all described species and provide 5.1 % of global ecosystem services worth an estimated $1.7 trillion per year (Costanza *et al.*, 1997; Giller & Malmqvist, 1998; Dudgeon *et al.*, 2006). Despite their importance, human interventions are threatening the biodiversity of over 65 % of global rivers (Vorosmarty *et al.*, 2010) which in turn impacts upon their ability to provide the vital services upon which human civilisation relies. Over abstraction for drinking water and irrigation, flow and channel modification, invasive species, pollution and climate change are all playing a part in drastically altering the physical, chemical and biological makeup of rivers (Giller & Malmqvist, 1998). The level of threat to freshwater biodiversity is strongly related to the presence of large human populations and degradation increases downstream associated with large urban settlements (Abell *et al.*, 2008). Even such alarming assessments of global river systems are likely to be an underestimate of the true situation owing to insufficient information on a range of stressors such as mining, interbasin transfers and pollution by synthetic compounds such as pharmaceuticals (Vorosmarty *et al*., 2010).

A recent survey of EU citizens asked participants to list their main environmental concerns; 47 % of respondents listed water pollution as their second highest concern (behind climate change) and a further 35 % chose the potential health impacts of chemicals used in everyday life as their 6th (EC, 2008a). Research on the occurrence, fate and effects of emerging contaminants in the environment has, thus, grown rapidly in recent years and has focussed mainly on pharmaceuticals. Pharmaceuticals are comprised of a vast array of compounds used to treat, prevent and cure diseases and include: antibiotics, analgesics, anti-inflammatories, anti-depressants, cancer treatments and x-ray diagnostics amongst many others. This broad group of substances certainly falls within the classification of 'everyday chemicals'. The consumption of pharmaceuticals occurs worldwide where people have access to medical care and continues at all times regardless of season (Daughton, 2001). Individual households contribute insignificant amounts when considered in isolation but the cumulative contributions of thousands and even millions of households, particularly within urbanised areas, means that environmental contamination by pharmaceuticals has become an increasingly important scientific concern in recent years (Kümmerer, 2008a). This has been aided by the substantial refinement in analytical techniques such as high-performance liquid chromatography (HPLC), enabling detection of these previously cryptic pollutants. The study of pharmaceuticals in the environment represents a

departure from the more ‘conventional’ pollutants which tend to be highly delineated with high volume inputs from industry or diffuse inputs from agriculture. Unlike conventional pollutants, very little is known about the environmental fate and effects of pharmaceuticals and there are no current statutory water quality limits in the European Union (UK Parliament, 2012).

The dispersed consumption of pharmaceuticals takes places predominantly in domestic households with varying but significant amounts entering the sewage system either from direct disposal or post-consumption excretion. As such, effluent from sewage treatment plants (STPs) is widely considered to be the major source of pharmaceuticals to surface waters. This is because of generally poor removal rates during conventional sewage treatment (primary and secondary) due to the non-volatile, polar nature of such compounds (Kümmerer, 2009a). There is, thus concern that pharmaceuticals present at ultra-trace concentrations may be adversely affecting freshwater ecosystems, particularly those adjacent to sewage outfalls. Of most concern is the potential for long-term, multigenerational exposure of aquatic organisms to low levels with even easily degraded compounds exhibiting ‘pseudo-persistence’ due to continual replenishment (Daughton, 2001).

Over the last thirty or so years, European and UK legislation has been responsible for a steady improvement in general water quality triggered largely by greater investment in sewage treatment facilities during the 1980s and 1990s (Thompson, 2006; EA, 2012; Vaughan & Ormerod, 2012). However, this improvement has slowed in recent years and only 26 % of English and Welsh rivers satisfy ‘good ecological status’ requirements of the Water Framework Directive (2000/60/EC; (EC, 2000)). This has been blamed partly on the need for water quality management to broaden from the existing narrow focus on point source pollution and a small number of prescribed pollutant lists (EA, 2012). More subtle environmental effects such as endocrine disruption in rivers demonstrate that pollutants are still eliciting negative consequences in freshwater ecosystems (Luoma, 1999; Pawlowski *et al.*, 2004).

The majority of current ecotoxicological pharmaceutical research has focused on short-term, usually acute exposures at concentrations unlikely to occur in the natural environment (Fent, 2008; Santos *et al*., 2010). However, the main concern in environmental exposures is the potential for chronic or long-term toxicity at low-level concentrations, the effects of pharmaceutical mixtures and impacts on populations, communities and ecosystem functioning (Hughes *et al*., 2013). Also, relatively little data on the occurrence of pharmaceuticals has been gathered with existing research heavily biased towards western Europe and north America (Hughes *et al*. 2013). There is a clear need for further research into both the occurrence and effects of pharmaceutical compounds in river ecosystems. In depth understanding of the spatial and temporal

variability of pharmaceutical pollution will help to inform future management and policy decisions. Further knowledge on the effects of environmental exposure to pharmaceuticals on individual aquatic organisms and whole ecosystem structure and function will aid in prioritising the highest risk compounds for future interventions. Thus, this thesis will aim to add substantially to current knowledge on the spatial and temporal aspects of pharmaceutical pollution in rivers and the effects of such pollution on ecosystem structure and function.

Chapter 2 presents a comprehensive review of the literature covering existing research on the consumption, environmental occurrence and effects of human use pharmaceuticals in freshwater ecosystems. Chapter 3 outlines a systematic review of existing pharmaceutical occurrence research across the globe and highlights spatial and methodological research gaps. Chapter 4 presents results from several monitoring campaigns in two UK semi-rural and urban catchments examining spatial and temporal variation in pharmaceutical concentrations in rivers. Chapter 5 presents the first application of NMR environmental metabolomics techniques alongside an extended exposure of the freshwater amphipod *Gammarus pulex* to two pharmaceutical compounds in mixture. Chapter 6 outlines the results of two experiments examining the effect of pharmaceutical exposure on the microbial and macroinvertebrate mediated decomposition of leaf litter in freshwaters. Chapter 7 examines the effects of exposure to pharmaceuticals on respiration in freshwater sediments and the effects on nutrient exchange with overlying waters. Finally, Chapter 8 presents a thesis outline and research synthesis and also highlights limitations, research gaps and opportunities for continued research in this area.

2 REVIEW OF PHARMACEUTICAL POLLUTION RESEARCH

2.1 Chapter overview

During the course of this study, a thorough review of existing literature on pharmaceuticals and their consumption, environmental occurrence, fate and potential impacts in aquatic ecosystems was conducted. This helped to define clear aims and objectives and progress research with particular reference to known gaps in data and knowledge. The first section of this chapter gives an introduction to emerging environmental contaminants and how PPCPs and particularly pharmaceuticals fit into this category. After this is a consideration of pharmaceutical consumption data and patterns across Europe and the globe but with particular emphasis on the UK. The third section summarises current knowledge on the known and potential sources of pharmaceuticals into a number of environmental components (including soils, surface waters and groundwater) but with a particular focus on rivers and the fate of such compounds in freshwater ecosystems. The fourth section details existing knowledge on the presence of pharmaceuticals in the environment, again with a focus on human-use compounds in rivers. Following this is a summary of the existing research and data gaps on the potential ecotoxicological and environmental impacts of pharmaceuticals in rivers. The last section focuses on the legislative and policy drivers in the EU and UK and concludes with a short summary of research needs in the broad area of pharmaceutical pollution of rivers.

2.2 A brief history of emerging contaminants

Much has been achieved in the latter part of the 20th century onwards in improving the quality of surface waters in the developed world and rivers such as the Thames have come a long way from their days as 'open sewers' in the 19th century. However, significant threats to water quality from human activity remain (Tinsley, 1998). Environmental legislation such as the Environmental Protection Act (1990), the Urban Waste Water Treatment Directive (UWWT; 91/271/EEC) and the Drinking Water Directive (98/83/EC) focused on reducing the environmental levels of organic pollution and a number of prescribed pollutants in order to improve water quality. These pieces of legislation, particularly the UWWT Directive, triggered further investment by water companies into sewage treatment facilities which has largely been responsible for a steady improvement in general river water quality in England and Wales since the 1990s (EA, 2012; Vaughan & Ormerod, 2012). Similar legislation was enacted in the USA with the Federal Water Pollution Control Amendments of 1972 (often abbreviated as the Clean Water Act) which introduced controls on the discharge of toxic substances to surface waters and provided substantial federal grants for the construction of sewage

treatment plants. Initially, sewage treatment efforts focused on the removal of microbial contamination and organic pollution with a more recent shift towards nutrient (nitrogen and phosphorous) stripping (Thompson, 2006).

The Water Framework Directive (EC, 2000) requires member states of the European Union to achieve 'good ecological status' for all inland and coastal waters. This has been classified using both chemical and biological indicators thereby integrating the effects of all polluting substances present in the aquatic environment and not just those 'conventional' pollutants currently monitored. The Environment Agency estimates that only 26 % of English and Welsh rivers currently meet these requirements showing a clear need to broaden the scope of water quality management from the existing narrow focus on point sources and prescribed lists (EA, 2012). Daughton (2004a) stated that conventional pollutants represent only a tiny proportion of the total number of pollutants present in the aquatic environment and that rivers and their ecosystems are being exposed to a wide variety of pollutants that are either unregulated or unidentified. Indeed, Luoma (1999) points out that although legislation and technology have been successful in reducing the number of overt pollution events (e.g. fish kills) the discovery of more subtle environmental effects, such as endocrine disruption in fish (Pawlowski *et al.*, 2004), means that pollutants are still exerting a significant influence on river ecosystems.

Recent developments in analytical techniques have highlighted the presence of a number of 'emerging contaminants' present at trace or ultra-trace concentrations in the environment. Research and regulation for emerging contaminants represents a significant departure from conventional pollutants as many of these substances are produced industrially but subsequently enter the environment through domestic, commercial and agricultural use via both point and diffuse sources (USGS, 2009). The concept of an emerging contaminant is an interesting one as there are a number of ways in which a substance can be considered to be 'emerging' (Daughton, 2004a):

- Previously recognised or naturally occurring substances with a newly identified environmental impact;
- Synthesis and subsequent release of a newly derived substance into the environment;
- Advances in analytical techniques allowing the detection of very low environmental concentrations of a substance that is likely to have had a long-term environmental presence;
- Changes in usage or disposal methods for an existing substance representing a new environmental input.

The broad class of emerging contaminants includes myriad anthropogenic and naturally-occurring substances including brominated flame retardants, disinfection by-products, hormones, endocrine disrupting compounds, PPCPs and surfactants (Petrovic, 2008). These substances have been linked to a range of acute and chronic effects such as endocrine disruption, teratogenicity, interference with reproduction, immune system depression and behavioural changes (see Smital, 2008 for a review). Despite these documented impacts and the potential for human health effects the majority of emerging contaminants are not routinely monitored nor are they generally subject to legislative or regulatory scrutiny (Daughton, 2007; Farré *et al.*, 2008). A report by the Royal Commission on Environmental Pollution (2003) described the lack of knowledge, risk assessment and regulation of so many chemicals as a key area for concern.

The broad group of PPCPs fits neatly into the class of emerging contaminants as the vast majority of these compounds are not subject to regulatory scrutiny and it is only in recent years that a significant amount of research has been conducted into their environmental presence and potential impacts (Daughton & Ternes, 1999; Daughton, 2001). Furthermore, due to their generally non-volatile nature, these compounds have the capacity to readily enter the environment, particularly surface waters. However, Daughton (2004a) suggested that PPCPs be considered as an 'emerging concern' as most of these compounds are likely to have had a stable environmental presence since their initial commercial release which in some cases can be over 50 years ago. For example aspirin has been marketed and sold worldwide since 1899 (Jeffreys, 2005). It is only recently that analytical techniques have become sufficiently refined to detect the very low environmental concentrations of PPCPs which are generally within the low ng L^{-1} to low µg L^{-1} range (Kümmerer, 2009a).

In view of these concerns, stakeholders from government, industry and academia ranked PPCPs in a list of the top five surface and groundwater contaminants of concern in the USA and Europe (Doerr-MacEwen & Haight, 2006). However, Williams (2005) questioned the scientific basis for grouping pharmaceuticals and personal care products under the same banner based purely on their emerging status. Indeed, pharmaceuticals and PCPs are subject to quite different regulatory oversight and consumption patterns; furthermore the inherent bioactivity of pharmaceuticals may merit a specific framework for risk assessment that distinguishes them from PCPs (Tarazona, 2005). Based on these suggestions, this thesis will focus solely on pharmaceuticals and PCPs will only be considered to provide a wider context from the literature. Furthermore, for similar reasons as above, this thesis will focus only on human-use pharmaceutical compounds and will not specifically consider those compounds used solely or largely in veterinary medicine.

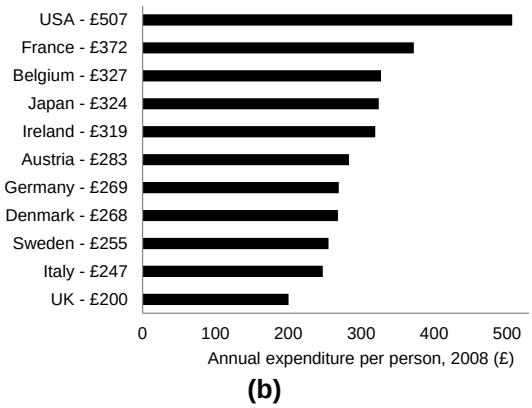
2.3 Pharmaceutical consumption

2.3.1 Consumption and disposal of pharmaceuticals across the globe

Consumption can vary significantly between individual countries for a wide variety of reasons; within Europe, the United Kingdom, France and Germany account for 46 % of the pharmaceuticals market followed by Russia, Spain and Italy (EEA, 2010). Cohen *et al*. (2007) found that the rate and speed of the drug approval processes in individual countries were major factors affecting consumption; the USA approved 15-18 % more drugs than the other countries. The cost per prescription and evenness of availability within the individual countries also played a role in overall pharmaceutical consumption. For example, access to the vast majority of approved pharmaceuticals is guaranteed in the UK but this is not so in the USA where access is dependent on an individual's insurance arrangements. The NHS guarantees access to all approved pharmaceuticals for all UK citizens either free at the point of need or for a fixed flat-rate prescription cost and the UK government claim that as many as 89 % of prescription items are dispensed free of charge through various exemptions (BBC, 2009a).

Pharmaceutical sales vary considerably both internationally and within the EU (Figure 2-1a). These data show the clear dominance of the USA in the world market which is singly greater than the rest of the world combined. The UK is similarly dominant within the EU accounting for almost half of the total sales in 2007. However, this is not the case when the average annual spend per capita is considered (Figure 2-1b) as the UK spends considerably less than the USA and several other European countries. This may be due to differences in healthcare provision between nations and also the Pharmaceutical Price Regulation Scheme which operates in the UK (DoH, 2009). Disposal methods can also vary significantly between countries particularly within the EU where a number of countries classify pharmaceuticals as 'special waste' that must be returned to a pharmacy; return rates range from 0.19 tonnes million capita^{-1} for Croatia to 237 tonnes million capita^{-1} in Switzerland (EEA, 2010).

Country	Global sales share (%)
USA	54
UK	19
Switzerland	11
Japan	6
France	5
Germany	3
Belgium	<1
Denmark	<1
Israel	<1



(b)

Figure 2-1: (a) Sales share of the world's top 100 prescription medicines in 2007 and (b) Average annual per capita medicines expenditure in 2008 (adapted from ABPI (2010))

2.3.2 Spatial and temporal variation in pharmaceutical consumption

The spatial variation in consumption of pharmaceuticals within an individual country can depend on a number of factors (Bush & Rabin, 1976; Davey *et al*., 2002; Schafheutle *et al*., 2002; Porteous *et al*., 2005; Cohen *et al*., 2007; Davey *et al*., 2008; Daughton, 2009a):

- Local population demographics;
- Level of education;
- Self-reported or perceived health status;
- Prescription exemption status or prescription charge (varies across the UK);
- Current prescriptions;
- Advertising;
- Local prescribing practices or cultural practices;
- Perceptions of short or long-term risks associated with drugs;
- Disposable income and patient willingness to spend money;
- Local NHS trust budgets or funding decisions.

Porteous *et al*. (2005) confirmed existing evidence that women, more highly educated, younger (<60 years old) people and those with a self-perceived fair to poor health status were significantly more likely to regularly use OTC analgesics. They found that 55 % of respondents in Scotland had recently used OTC analgesics compared to 68 % consulted

in a survey of English university students (French & James, 2008). The age of a particular population can influence the prevalence of illness and pharmaceutical consumption; for example only 17 % of 15-34 year olds in Finland are prescribed NSAIDs compared to 34 % of those aged 75 and over (Ahonen, 1992). Johnson *et al*. (2007) suggested that an average age in a small rural catchment that exceeded the national average by 3.8 years may have contributed to slightly higher than expected modelled concentrations of pharmaceuticals in a receiving river.

The consumption of individual pharmaceuticals can also show marked seasonal variation. Goossens *et al*. (2005) found peaks in the consumption of certain antibiotics during winter in Europe due to an increase in respiratory tract infections during the colder months. However, this variation was not prevalent for all antibiotics; for example ciprofloxacin demonstrates little effectiveness against the streptococci causing respiratory tract infections (Hooper, 2002) and as such shows no appreciable winter peaks. Seasonal allergic rhinitis (hay-fever) is a common condition during the summer months when higher levels of antiallergic drugs or specific immunotherapy injections are consumed due to elevated pollen counts (Smith *et al*., 2004). Seasonal influenza has long been considered a self-limiting, controllable disease despite its high morbidity and mortality rate. However, with increasingly virulent seasonal and pandemic flu strains such as avian flu (H5N1) and more recently swine flu (H1N1) more attention has been given to public health measures such as vaccination programmes and the use of antiviral drugs (ACP, 2006; Sahni & Mossad, 2009). Clearly, the consumption of vaccines, antivirals (e.g. Tamiflu) and other pharmaceuticals to treat seasonal diseases will be strongly related to disease prevalence or particular outbreaks such as a flu pandemic (Straub, 2009).

This spatial and temporal variation in consumption of pharmaceuticals means that annual consumption rates do not give an accurate indication of the use (and subsequent environmental release) of certain compounds at fixed points in time or at specific locations. Thompson (2006) highlighted the potential for the ageing UK population to cause a long-term shift in the amounts and types of drugs being prescribed; this concern was echoed in a recent workshop on pharmaceuticals in the environment (EEA, 2010). Other potential drivers for increasing consumption include: expanding populations, increases in direct advertising/marketing, patent expirations causing a shift to less expensive generic drugs and new uses for existing drugs (Daughton, 2003). However, consumption data are still highly useful for providing a national overview of widely used compounds and in helping to identify those compounds likely to have the greatest environmental footprint.

2.3.3 Pharmaceutical consumption in the UK

Information on total consumption of pharmaceuticals in the UK or elsewhere is not readily available (Thompson, 2006) but is clearly very important when prioritising compounds for occurrence or effects studies in the environment. Ayscough *et al*. (2000) obtained data from the Department of Health stating that approximately 3000 pharmaceutically active substances are approved for human use in the UK although not all of these are necessarily available on the market at any given time. Pascoe *et al*. (2003) discovered that there were 19 835 Marketing Authorisations for medicinal products covering 5091 active ingredients which target around 500 distinct biochemical receptors. All of these figures are likely to be higher today as new products are frequently being developed and brought to market. Ayscough *et al*. (2000) highlighted the difficulty of obtaining an overall picture of total consumption due to a number of different agencies holding data on various aspects of consumption including community dispensing, hospital usage and over-the-counter (OTC) purchases.

The NHS Prescription Pricing Authority (PPA) began publishing data on the total number of prescriptions issued in England from 2000 onwards (Thompson, 2006), split into a number of prescribing categories (Table 2-1). In the 2008/09 financial year, the NHS prescribed a total of 841 000 000 items at a total cost of almost £ 8.2 billion (NHS, 2008a; NHS, 2009a). It must be noted that these data do not include hospital usage, OTC drugs or prescriptions made in Wales, Scotland or Northern Ireland. It is estimated that as much as 20 % of pharmaceutical prescriptions are accounted for by a relatively small number of patients in hospitals (Sebastine & Wakeman, 2003). Therefore, the data presented below provide a useful summary and indication of the likely high risk classes in terms of usage but they must not be considered comprehensive.

Table 2-1: Summary of English prescribing data in England 2007-2009

Prescribing Category	Volume (no. prescriptions, millions)		Cost (£, millions)	
	2007/08	2008/09	2007/08	2008/09
Cardiovascular	253.70	268.38	1736.12	1618.83
Central nervous	136.75	144.72	1670.21	1667.76
Endocrine	68.23	73.18	898.95	910.35
Gastro-intestinal	60.37	64.83	466.13	449.19
Respiratory	53.42	56.05	899.04	960.86
Infection	40.30	41.24	199.73	186.09
All others	180.35	193.46	2227.03	2365.60
Total	793.12	841.86	8097.21	8158.68

Sources: (NHS, 2008a; NHS, 2009a)

A much more detailed dataset is the NHS Prescription Costs Analysis (NHS, 2009b) which collects data on all NHS drug usage, including that in hospitals across the whole of the UK. The total cost of drugs in 2008 was estimated at £ 11.6 billion with hospital consumption accounting for 28.7 % of overall costs. These data are useful because they present the number of prescriptions for individual pharmaceutical compounds and preparations prescriptions. Consumption data show a number of specific pharmaceuticals that are consistently prescribed in very high numbers; these include bendroflumethiazide, levothyroxine sodium and aspirin which showed steady increases over a five year period (Table 2-2). In addition there are a number of drugs that have risen notably in rank from 1997 onwards. The most obvious of these is simvastatin (a blood lipid modifier) whose prescriptions have increased by 1400 % since 1997 making it the most widely prescribed drug in 2008. This was despite simvastatin only being approved for UK use in the mid-1990s (Thompson, 2006) and low doses being made available OTC in 2004 (Nash & Nash, 2004).

Table 2-2: Estimated prescription volumes and rankings for the top 10 prescribed drugs in the UK in 2008

Compound	Therapeutic class	DDD (mg day^{-1})	Prescriptions (thousands) and rank in brackets							
			1997	1999	2000 England only	2004	2005	2006	2007	2008
Simvastatin	Lipid modifier	30.0	2400.0 (47)	-	-	12680.9 (6)	16536.9 (4)	22556.1 (2)	29353.5 (2)	33853.5 (1)
Aspirin	NSAID	4000.0	11634.6 (3)	13900.0 (2)	16769.0 (1)	23641.7 (1)	25883.3 (1)	27971.7 (1)	30210.4 (1)	32682.0 (2)
Levothyroxine Sodium	Thyroid hormones	0.2	-	-	-	14237.8 (5)	15607.7 (6)	17095.7 (4)	18736.4 (4)	20426.4 (3)
Bendroflumethiazide	Diuretic	2.5	7591.5 (8)	9600.0 (7)	-	18839.8 (2)	19791.1 (2)	19667.7 (3)	19304.1 (3)	19151.5 (4)
Salbutamol	Inhalant	10.0	16167.8 (2)	16700.0 (1)	-	16182.4 (4)	16368.6 (5)	16613.1 (5)	17046.7 (5)	17887.8 (5)
Ramipril	ACE inhibitor (pain)	2.5	-	-	-	8978.7 (13)	10258.9 (13)	12074.2 (11)	15005.1 (7)	17417.3 (6)
Paracetamol	Analgesic	3000.0	9569.5 (6)	10400.0 (4)	10636.0 (4)	12360.7 (8)	14281.1 (7)	15113.3 (7)	16062.4 (6)	17398.4 (7)
Omeprazole	Proton pump inhibitor	20.0	-	-	-	5597.2 (22)	8324.0 (17)	10961.4 (13)	13074.0 (12)	15634.3 (8)
Amlodipine	Selective calcium channel blocker	5.0	-	-	-	8191.9 (15)	9291.1 (15)	11238.5 (12)	13324.0 (11)	14961.1 (9)
Atenolol	Beta-blocker	75.0	8133.0 (7)	10000.0 (5)	11554.0 (3)	17349.0 (3)	17589.2 (3)	16605.4 (6)	14961.6 (8)	14319.4 (10)

DDD: defined daily dose (http://www.whocc.no/atc_ddd_index/)
Sources: (Ayscough *et al.*, 2000; Jones *et al.*, 2001; Jones *et al.*, 2002; NHS, 2005; NHS, 2006; Thompson, 2006; NHS, 2007; NHS, 2008b; NHS, 2009b)

Whilst the data sets based on prescription numbers are the most widely available, such ranking of compounds does not necessarily hold true if total consumption mass is considered. For example, Thompson (2006) found that although cardiovascular drugs exceeded NSAIDS in terms of prescription numbers, NSAIDs far exceeded cardiovascular drugs in mass terms due to greater dosages. The technique of using Defined Daily Doses (DDD) and prescription numbers has been employed to estimate mass consumption (for example see Sebastine and Wakeman (2003)). However, this methodology does have limitations as dosages for individual patients, compounds or mixtures may differ from the DDD and no correction is made for the reduced doses for children (Goossens *et al.*, 2005). Furthermore, not all patients complete the full course of medication and this may remain unused for an extended period of time prior to disposal (Bound *et al.*, 2006).

When such data are compiled they reveal differing trends between therapeutic classes (Table 2-3). Certain NSAIDs and analgesics (paracetamol and aspirin) which are prescribed widely for osteoarthritis and back pain show consistently high consumption (>50 tonnes year^{-1}). Consumption of amoxicillin, an antibiotic, remains consistently high although this does appear to be steadily dropping from 1997 onwards. Other antibiotics such as flucloxacillin, erythromycin and furosemide which were widely used in 1997 and the early 2000s have seen a marked decline in recent years. This may be due to general practitioners following prescribing guidelines more closely or a more general fall in the number of respiratory infections (Thompson, 2006). Goossens *et al*. (2005) identified a general increase in broad-spectrum antibiotics at the expense of narrow-spectrum compounds although these older antibiotics are still widely prescribed, particularly in northern Europe. Cardiovascular drugs such as simvastatin have seen significant increases in consumption in recent years (Table 2-2; Table 2-3). These data outline well the importance of using up-to-date consumption statistics as prescribing trends are shown to vary from year to year.

Data on pharmaceutical consumption volumes highlight marked variation between different years and studies for the same pharmaceuticals. For example, Webb (2001a) estimated 2000 tonnes of paracetamol were consumed in 1995 but Sebastine & Wakeman (2003) estimated only 403 tonnes and the 2004-2008 PCA estimates range from 37 to 52 tonnes. The source and coverage of the data may create discrepancies between years that cannot be explained by actual changes in consumption. For example, the 1995 estimates (Webb, 2001a) were obtained directly from Intercontinental Medical Statistics but it is not clear whether these data include OTC use or amalgamates the use of paracetamol in other end-products (e.g. Co-codamol) although such a high estimate would suggest they do. Furthermore, the Sebastine and Wakeman (2003) data for 2000 do not include OTC or hospital consumption but all of these estimates are

considerably larger than those derived directly from the 2004-2008 NHS PCA data which do include hospital prescriptions. It is likely that consumption figures based solely on PCA data are significantly underestimating total consumption as they do not include OTC purchases or the inclusion of single APIs in multiple products. For example, Thompson (2006) used data from Huschek *et al*. (2004) to estimate that UK prescription data for paracetamol represented only 20.6 % of total usage; if this was extrapolated using the 2008 PCA estimate from Table 2-3 then the total annual consumption would be 253.4 tonnes.

Overall, there is a broad trend of increasing consumption of prescription pharmaceuticals in the UK with modest changes in some classes of antibiotics, steady and sustained increases in the consumption of painkillers (particularly aspirin) and NSAIDs and the rapid rise of blood lipid regulators and other cardiovascular drugs used to prevent or treat long-term conditions. Clearly, such an increase is of major concern as a source of potential freshwater pollution by pharmaceutical compounds. The better collation of consumption data in a form accessible to environmental scientists should be seen as a priority in order to inform and prioritise future research into the occurrence and effects of pharmaceuticals in the environment.

Table 2-3: Estimated consumption of 26 widely prescribed pharmaceuticals in the UK using data from the NHS PCA and previous studies

Pharmaceutical	Estimated annual consumption[1] (tonnes)									
	1995	1999	1997	2000	2004	2005	2005	2006	2007	2008
Simvastatin	-	-	-	2.67	0.38	0.22	0.50	0.68	0.88	1.02
Aspirin	770.00	55.60	46.54	78.57	94.57	-	103.53	111.89	120.84	130.73
Levothyroxine Sodium	-	-	-	-	0.01	-	0.01	0.01	0.01	0.01
Bendroflumethiazide	-	0.02	0.02	-	0.05	0.05	0.05	0.05	0.05	0.05
Salbutamol	-	0.17	0.16	-	0.16	0.19	0.16	0.17	0.17	0.18
Ramipril	-	-	-	-	0.02	0.02	0.03	0.03	0.04	0.04
Paracetamol	200.00	31.20	28.71	403.11	37.08	97.20	42.84	45.34	48.19	52.20
Omeprazole	-	-	-	3.44	0.01	-	0.14	0.22	0.26	0.31
Amlodipine	-	-	-	-	0.04	-	0.02	0.06	0.67	0.08
Atenolol	-	0.75	0.61	28.98	1.30	1.35	1.32	1.25	1.12	1.07
Co-codamol (paracetamol/codeine)	-	-	29.85	444.34	40.53	-	47.63	51.11	54.24	57.46
Amoxicillin	-	-	16.59	71.47	12.51	41.60	12.97	12.55	13.53	13.27
Atorvastatin	-	-	-	-	0.23	0.12	0.26	0.26	0.22	0.21
Ibuprofen	-	-	-	162.91	5.63	5.28	5.80	5.49	5.38	7.39
Flucloxacillin	-	-	-	23.38	6.03	25.60	6.02	6.33	6.40	6.56
Propranolol Hydrochloride	-	-	-	8.16	0.40	-	0.41	0.41	0.42	0.44
Carbamazepine	-	-	-	40.35	2.36	-	2.34	2.34	2.37	2.40
Erythromycin	67.70	-	-	26.48	2.16	12.80	2.15	2.09	2.17	2.05
Metoprolol Tartrate	-	-	-	2.63	0.12	-	0.13	0.14	0.14	0.14
Metformin	106.10	-	-	205.79	14.83	15.20	16.67	18.43	20.48	23.23
Co-proxamol (dextropropoxyphene/paracetamol)	-	-	32.51	336.21	23.07	-	9.39	4.45	3.06	1.22
Co-Trimoxazole (trimethoprim/sulfamethoxazole)	-	-	-	-	0.10	-	0.11	0.11	0.12	0.13
Furosemide	-	-	-	-	0.40	0.42	0.41	0.42	0.43	0.45
Naproxen	-	-	-	35.14	0.51	-	0.57	0.58	0.65	0.90
Diclofenac	-	-	-	26.11	0.78	0.72	0.85	0.85	0.89	0.84
Oxytetracycline	33.70	-		27.20	1.01	4.00	0.97	0.91	0.91	0.89

Notes:

1. Total annual consumption estimated by multiplying the prescription number by the Defined Daily Dose as at http://www.whocc.no/atc_ddd_index/;

Sources: (Ayscough *et al.*, 2000; Jones *et al.*, 2001; Webb, 2001; Jones *et al.*, 2002; Sebastine & Wakeman, 2003; NHS, 2005; NHS, 2006; Thompson, 2006; NHS, 2007; NHS, 2008b; NHS, 2009b)

2.3.4 Over-the-counter (OTC) consumption in the UK

Current UK government policy encourages the self-care of minor, self-limiting illnesses and as such a number of previously prescription drugs are now available OTC (Porteous *et al.*, 2005). In 2008 the total value of the OTC market was £ 2.3 billion with consumers purchasing 977 million packs of over 1000 commercially available products (PAGB, 2010). Clearly, the OTC market rivals the prescription sector both in terms of financial and consumption volume; therefore it is vital that any assessment of total pharmaceutical consumption takes the OTC sector into account. Data are collected on the total spent on a range of OTC therapeutic areas but, unlike prescription data, statistics for individual compounds are not freely available. However, these overall data are still useful in highlighting trends (Table 2-4) (PAGB, 2010). Furthermore, a 1997 study (PAGB, 1997) found that almost 88 % of OTC drugs purchased were used for two days or less, suggesting that a significant majority of purchased remedies may be only partly used with the remainder being stored for a later date or disposed of. There is a clear trend in increasing sales revenue for OTC medicines with an overall 38 % increase from 2000-2008. However, without specific statistics relating to sales volumes it is difficult to attribute how much of this growth in market value is due to increased consumer demand rather than cost increases or inflation. Unfortunately such information is commercially sensitive and is not readily available.

As with prescription pharmaceuticals, the available data indicate a strong increase in consumption with particularly strong growth for skin treatments, eye care, sleeping aids and smoking cessation products (Table 2-4). This growth in sales represents an increase in the potential source of pollution by such compounds in freshwaters. This is of particular concern given that existing pharmaceutical pollution research tends to focus on a small number of widely used prescription compounds and OTC pain relief. For example, little or no monitoring has been conducted for smoking cessation products (Hughes *et al*., 2013) and as such these represent an unknown risk in terms of environmental pollution. Better data on actual consumption volumes/masses would enable research into such pollution to prioritise the highest risk compounds.

Table 2-4: Summary of OTC pharmaceutical sales in the UK from 2000-2008

Therapeutic class	Total sales (£ millions)									
	2000	2001	2002	2003	2004	2005	2006	2007	2008	% inc. (2000-2008)
Pain relief	396	407	416	465	460	471	500	527	539	36
Skin treatments	253	276	299	353	377	386	416	423	420	66
Cough/cold/sore throat	341	351	339	376	357	369	390	437	453	33
Gastro-intestinal	187	192	210	236	253	257	264	262	262	40
Medicated mouthwash	16	17	20	23	25	26	27	26	28	76
Hay fever remedies	54	64	68	79	76	79	88	79	85	56
Eye care	32	34	38	39	40	41	43	46	48	51
Sleeping aids	12	13	24	28	30	31	32	35	37	198
Smoking cessation	58	65	72	778	84	89	92	108	101	72
Vitamins and minerals	307	293	277	296	321	326	333	336	322	5
TOTAL	1658	1711	1762	1973	2023	2074	2184	2282	2294	38

Source: (PAGB, 2009)

2.4 Environmental sources and fate of pharmaceuticals

Pharmaceuticals comprise a vast array of compounds that are produced, consumed and disposed of in a variety of settings including agriculture, veterinary medicine, hospitals, domestic households and commercial settings. As such, there are numerous pathways of entry into the environment including point, diffuse, continuous and intermittent sources some of which receive some form of biological or chemical treatment or dilution whereas others may pass directly into the environment (Figure 2-2). Therefore, this section will briefly outline these sources with a particular focus on those discharging to the aquatic environment.

2.4.1 Pharmaceutical manufacturing facilities (PMFs)

Losses of pharmaceuticals to the environment during their manufacture are generally considered to be negligible given the high economic value of the products and strict good practice regulations imposed on manufacturers, particularly in Europe and North America (Velagaleti & Gill, 2001; Kümmerer, 2009a). The use of degradation techniques and technology such as scrubbers and filters to minimise waste streams are also widely adopted by manufacturers (USEPA, 1997). However, there is some evidence for detectable levels in manufacturing effluents. Larsson *et al*. (2007) detected the antibiotic ciprofloxacin at levels 1000x greater than the toxic threshold for some bacteria in effluent from a large Indian manufacturer, and ecotoxicological studies on the same effluent demonstrated adverse effects on amphibians and fish (Carlsson *et al.*, 2009). High levels in manufacturing effluent have also been detected in Norway with a total loading of 2000 kg year^{-1} of the same antibiotic (Thomas & Langford, 2008). Phillips *et al*. (2010) surveyed a number of STPs in New York, USA that receive waste water from both PMFs and pharmaceutical formulation facilities (PFFs). These effluent concentrations were generally below 1 µg L^{-1} but several compounds did exceed this level including

oxycodone (analgesic; 1,700 µg L^{-1}) and metaxolone (muscle relaxant; 3800 µg L^{-1}). Indeed Li *et al*. (2008) found very high concentrations of up to 800 mg L^{-1} and 2 mg L^{-1} in effluent and receiving waters respectively from a STP serving an antibiotic manufacturing facility in China.

Further monitoring is required to either challenge or support the assumption that manufacturing inputs are negligible although it must be noted that the effects are likely to be highly localised and restricted to areas with a large manufacturing presence (Daughton, 2007).

2.4.2 Veterinary pharmaceuticals

This section provides a brief overview of veterinary pharmaceuticals in order to provide context for human-use compounds and in order to compare and contrast the different environmental pathways and risks. A wide range of veterinary pharmaceuticals are used to prevent disease and promote growth in livestock production and aquaculture (Boxall *et al.*, 2002). However, the use of antibiotics as growth promoters for livestock was banned by the EU in 2006 (Kemper, 2008; Kools, 2008). The major classes of veterinary pharmaceuticals include anti-parasitic agents and antibiotics. This range of compounds includes those specifically designed for veterinary use and a number that are also used by humans (Jørgensen & Halling-Sørensen, 2000).

There are a number of review articles available on the use and exposure pathways of veterinary pharmaceuticals in the environment (Boxall *et al.*, 2002; Sarmah *et al.*, 2006) and as such these will only be briefly summarised here. The major potential exposure routes include:

- Consumption of large quantities of pharmaceuticals in combined animal feeding operations (CAFOs);
    - Agricultural land application of manure or slurry from CAFOs as fertiliser has the potential to contaminate soils, groundwater and surface waters via overland flow, subsurface flow and drain flow (Boxall *et al.*, 2002);
    - Airborne transport of contaminated dust via the ventilation systems in CAFOs (Zahn *et al.*, 2001);
    - Leakage from manure/slurry storage systems and waste lagoons (USEPA, 2000; Sarmah *et al.*, 2006).
- Direct deposition of dung from treated livestock (Wall & Strong, 1987);
- Direct contamination of sediment beneath aquaculture facilities with treated feed and via contaminated faeces (Samuelsen *et al.*, 1992);

- Treatment of companion animals; this is not likely to be a significant source as these animals are not treated in large numbers in intensively reared conditions (Boxall *et al.*, 2002);

- Washing off of topically applied treatments directly into rivers; this is likely to be a sporadic and highly localised source (Daughton & Ternes, 1999);

- Disposal of unwanted or expired pharmaceutical products.

The vast majority of research into veterinary pharmaceuticals has focused on the application of manure/slurry from CAFOs as fertiliser. A study by the US EPA (2000) estimated that as much as 80 % of administered antibiotics could enter waste lagoons. The ultimate fate of veterinary (and human) pharmaceuticals is dependent on a number of factors (Tolls, 2001; Kay *et al.*, 2004; Kay *et al.*, 2005a; Kay *et al.*, 2005b; Blackwell *et al.*, 2007; Blackwell *et al.*, 2009; Gielen *et al.*, 2009; Monteiro & Boxall, 2009):

- The physical-chemical nature of the compound which will dictate the extent of degradation in both the manure and the soil;

- The affinity of the compound for solid phases;

- The length of time that manure or slurry is stored prior to application;

- Soil type (pH and clay content) and moisture content play a major role in dictating loss to the aqueous phase;

- Prevailing climatic and weather conditions;

- The application regime of the manure or slurry, particularly with regards to total loads, frequency and timing;

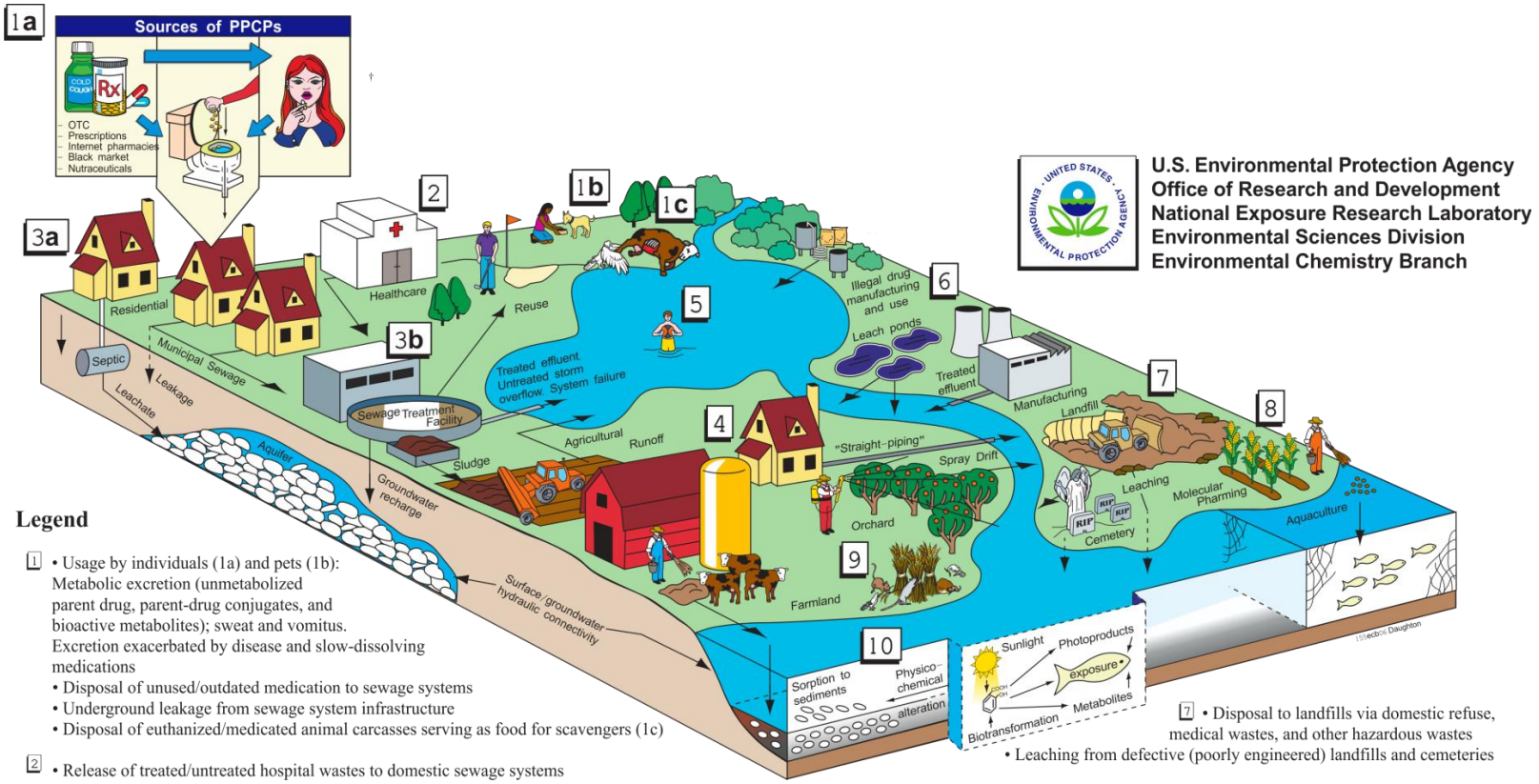
- The drainage regime of the agricultural land and the presence of piped drainage networks;

- The presence of other contaminants within a mixture.

The major source of veterinary pharmaceuticals is via indirect application of manure/slurry after excretion. In addition, some inactive metabolites or conjugates have the potential to be re-transformed into parent compounds after excretion meaning a significant proportion of administered pharmaceuticals can enter the environment (Warman & Thomas, 1981).

Jones-Lepp & Stevens (2007) presented a review of the potential for agricultural application of human sewage sludge to be a source of pharmaceuticals to the

environment. Their review found concentrations ranging from low µg kg^{-1} to low g kg^{-1}; these concentrations depended on the type of compound and the degree of sludge pre-treatment prior to application with synthetic musks generally found at the highest levels. However, the agricultural application of sewage sludge in the EU is in decline due to public concern over pathogens and pollution despite a 1986 European Directive (86/278/EEC) to regulate the practice (Jones-Lepp & Stevens, 2007). Studies have also found pharmaceuticals to be present in soil irrigated with reclaimed wastewater generally within the ng g^{-1} range (Kinney *et al.*, 2006).



U.S. Environmental Protection Agency
Office of Research and Development
National Exposure Research Laboratory
Environmental Sciences Division
Environmental Chemistry Branch

Legend

1 • Usage by individuals (1a) and pets (1b):
Metabolic excretion (unmetabolized parent drug, parent-drug conjugates, and bioactive metabolites); sweat and vomitus.
Excretion exacerbated by disease and slow-dissolving medications
• Disposal of unused/outdated medication to sewage systems
• Underground leakage from sewage system infrastructure
• Disposal of euthanized/medicated animal carcasses serving as food for scavengers (1c)

2 • Release of treated/untreated hospital wastes to domestic sewage systems (weighted toward acutely toxic drugs and diagnostic agents, as opposed to long-term medications); also disposal by pharmacies, physicians, humanitarian drug surplus

3 • Release to private septic/leach fields (3a)
• Treated effluent from domestic sewage treatment plants discharged to surface waters, re-injected into aquifers (recharge), recycled/reused (irrigation or domestic uses) (3b)
• Overflow of untreated sewage from storm events and system failures directly to surface waters (3b)

4 • Transfer of sewage solids ("biosolids") to land (e.g., soil amendment/fertilization)
• "Straight-piping" from homes (untreated sewage discharged directly to surface waters)
• Release from agriculture: spray drift from tree crops (e.g., antibiotics)
• Dung from medicated domestic animals (e.g., feed) - CAFOs (confined animal feeding operations)

5 • Direct release to open waters via washing/bathing/swimming

6 • Discharge of regulated/controlled industrial manufacturing waste streams
• Disposal/release from clandestine drug labs and illicit drug usage

7 • Disposal to landfills via domestic refuse, medical wastes, and other hazardous wastes
• Leaching from defective (poorly engineered) landfills and cemeteries

8 • Release to open waters from aquaculture (medicated feed and resulting excreta)
• Future potential for release from molecular pharming (production of therapeutics in crops)

9 • Release of drugs that serve double duty as pest control agents:
examples: 4-aminopyridine, experimental multiple sclerosis drug → used as avicide; warfarin, anticoagulant → rat poison; azacholesterol, antilipidemics → avian/rodent reproductive inhibitors; certain antibiotics → used for orchard pathogens; acetaminophen, analgesic → brown tree snake control; caffeine, stimulant → *coqui* frog control

10 Ultimate environmental transport/fate:
• most PPCPs eventually transported from terrestrial domain to aqueous domain
• phototransformation (both direct and indirect reactions via UV light)
• physicochemical alteration, degradation, and ultimate mineralization
• volatilization (mainly certain anesthetics, fragrances)
• some uptake by plants
• respirable particulates containing sorbed drugs (e.g., medicated-feed dusts)

Figure 2-2: Environmental sources and fate of PPCPs (USEPA, 2010)

2.4.3 Human-use pharmaceuticals

This section highlights the main pathways by which human-use pharmaceutical compounds enter the environment, particularly freshwater ecosystems. The actual occurrence of pharmaceuticals in the environment will be summarised elsewhere (Section 2-5; Chapters 3 and 4). The main environmental sources of pharmaceuticals are listed below and each will be considered in more detail (Figure 2-2):

- Disposal of pharmaceuticals via solid waste and subsequent landfill or incineration;
- Disposal of pharmaceuticals down the drain into the domestic sewage system;
- Domestic consumption and subsequent excretion to the sewage system which passes through a sewage treatment plant (STP) prior to discharge to surface waters;
- Washing off of topically applied treatments into the domestic sewage system (Daughton & Ruhoy, 2009b);
- Consumption and subsequent excretion in hospitals and long-term care facilities. The effluent generally undergoes the same treatment processes as domestic effluent (Kümmerer, 2001a);
- Discharge of raw or partially treated sewage from combined sewer overflows (CSOs) during a flood or significant rainfall event;
- Leakage from aging or poorly maintained infrastructure and illegal 'straight-piping' of sewage direct to surface waters (Daughton, 2007);
- Application of treated sewage sludge from STPs to agricultural land as fertiliser or the reuse of treated wastewater for irrigation (Daughton, 2004b; Daughton, 2004c);
- Leachate from landfills or buried waste storage systems (e.g. cess tanks, see Halling-Sorensen *et al*. (1998)).

2.4.3.1 Domestic disposal to solid and liquid waste streams

A survey by Kuspis & Krenzelok (1996) found that disposal of pharmaceuticals either into solid waste or down the drain was common in the USA despite instructions on packaging regarding proper disposal. Bound *et al*. (2006) conducted a similar survey in the UK and found that 63 % of unused pharmaceuticals disposal was to solid waste and

22 % down the drain. Clearly, this represents a potentially significant source of pharmaceuticals to the environment, particularly as they are passing unmodified into the waste streams and demonstrates a clear case for more appropriate disposal (Bound *et al.*, 2006). Niquille & Bugnon (2008) suggest that community pharmacies and practitioners should be more involved in encouraging the return of unused pharmaceuticals but also to proactively reduce potential disposal through better prescription practices and encouraging patient adherence. Washing of dermal residues during bathing is generally considered to be a minor or inconsequential sources but recent reviews by Daughton & Ruhoy (2009a; 2009b) suggest that more research emphasis be placed on these routes particularly as the potential for pollution prevention will be greater when compared to post-consumption excretion. Although direct disposal does carry an environmental risk this should be weighed against the risk of inappropriate use or inadvertent poisonings (particularly to infants and the elderly) when large amounts of unused or unwanted drugs are stored long-term (Daughton, 2007).

Studies have shown that leachate from landfill and underground waste storage systems (e.g. cess tanks) can contain concentrations of pharmaceuticals which then contaminate ground water and possibly aquifers used for drinking water abstraction (Halling-Sørensen, 1998). Up to the 1970s it was common practice to dispose of pharmaceutical industry waste to landfills with no leachate collection systems. The resultant leachate plumes contained detectable concentrations of three pharmaceuticals decades after they were first disposed of (Holm *et al.*, 1995).

2.4.3.2 Hospitals and long-term care facilities

Hospitals and long-term care facilities represent concentrated points of consumption for pharmaceuticals and other compounds used for medicinal purposes such as anaesthetics, diagnostic (e.g. x-ray) contrast media and disinfectants. The usage patterns and compounds used within hospitals often vary significantly from those in households due to different prescribing practices between hospital staff and general practitioners (GPs) (Kümmerer, 2001a). In addition, certain drug types are prescribed much more widely in hospitals; Sebastine & Wakeman (2003) estimate that as much as 80 % of antibiotics are prescribed in hospitals. These hospital-specific drugs are generally weighted towards those with high acute toxicity and genotoxicity and which are used for short-term therapy or diagnostics (Daughton, 2007). However, the basic pathways of entry to the waste streams are similar to the domestic setting and include: excretion of parent or metabolised compounds, washing off of topically applied substances and washing of residues from equipment or clothing into the sewage system. Furthermore, there is the potential for outpatients to excrete substances taken during a hospital visit into the domestic sewage system after they return home; this is particularly

the case for anti-cancer drugs where much of the treatment is now handled in outpatient departments (Rowney *et al.*, 2009). Larger, more specialised hospitals represent significant concentrated point sources for particular drugs (Moldovan, 2006). Studies have shown that concentrations of pharmaceuticals in hospital effluent are higher than those detected in STP effluent (Kümmerer, 2001a; Kümmerer, 2009b; Kümmerer, 2009c). However, the total volume of effluent leaving hospitals is much lower than from domestic households and the dilution by domestic wastewater can be by more than a factor of 100. However, there is the potential for hospitals to make a significant contribution, particularly for smaller STPs that serve a relatively small 'sewer catchment' but which may have a large hospital within them (Jørgensen & Halling-Sørensen, 2000).

2.4.3.3 Domestic consumption and excretion

Domestic consumption and excretion follow similar pathways to hospitals usage as effluent from both is generally conveyed within the same combined sewer system prior to treatment. The major difference between hospital and domestic effluent will be in the types and quantities of pharmaceuticals present (Kümmerer, 2001a). The main routes of pharmaceutical uptake in humans are oral intake and injection after which they are metabolised to varying degrees depending on individual compound properties (Sebastine & Wakeman, 2003). After ingestion, pharmaceuticals undergo partial or complete Phase I metabolism (oxidation, reduction or hydrolysis) before Phase II metabolism (conjugation with glucornic, sulphate, acetic or amino acids). This produces polar metabolites which are then excreted in the urine and do not usually exhibit pharmacological activity (Ayscough *et al*., 2000). Pharmaceuticals are then excreted in the urine and/or faeces prior to discharge to the domestic sewage system. The urine and faeces contain a mixture of parent compounds, metabolites and inactive conjugated substances (Sebastine & Wakeman, 2003). The degree to which a parent compound is metabolised is strongly influenced by individual factors including: genetics, age, gender, health/disease status and individual metabolic idiosyncrasies as well as the pharmacokinetic characteristics of the compound and its delivery form (Daughton, 2007). In addition to this inactive conjugated metabolites have the potential to be re-transformed into active metabolites or parent compounds after excretion (Warman, 1981).

Lienert *et al*. (2007) conducted a thorough review of the metabolism and excretion of 212 APIs which highlighted large variability both within and between different therapeutic classes and even for individual APIs. X-ray contrast media were the most readily excreted via urine with 94% unchanged parent compound lost via this route. The extent of metabolised products lost via urine varies between groups from an average of 6 % for antidepressants to 100 % for blood lipid regulators. However, it must be noted that variation between compounds in the same group also ranged from 0 to 100 % and

certain individual compounds can skew the average of an entire group. Also, individual compounds can demonstrate variation ranging from 0 to 100 % which is strongly dependent on the factors mentioned above (Figure 2-3) (Daughton, 2007). Such a high proportion of excretion via urine amongst certain therapeutic use classes may mean that urine source separation techniques could play a role in reducing the input of pharmaceuticals into the sewage system (Lienert *et al.*, 2007a; Lienert *et al.*, 2007b).

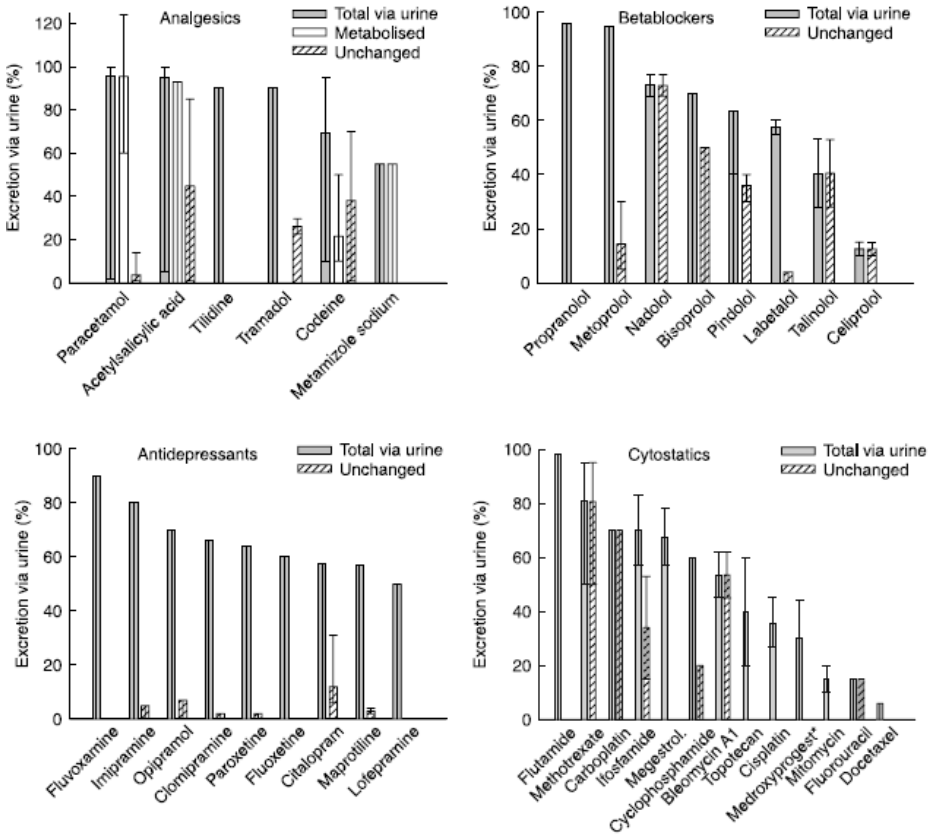


Figure 2-3: Excretion of analgesics, beta-blockers, antidepressants and cytostatics via urine
[Bars indicate average for each group and error bars indicate min and max values if available (Lienert *et al.*, 2007a)]

2.4.3.4 Sewage treatment plants (STPs)

In the UK, the majority of effluent from domestic households and hospitals is conveyed through a combined sewer system to a sewage treatment plant where it undergoes treatment prior to being discharged to rivers or the sea. O'Brien & Dietrich (2004) estimate that 80 % of European wastewater passes through a STP although the proportion in England and Wales is much higher with 96 % entering a STP and 80 % receiving at least secondary level treatment. 80 % of the 11 000 billion litres of sewage effluent produced daily in England and Wales is of domestic origin (Mason, 2002). However, each STP varies in both amount and composition of the effluent it receives and the treatment technologies it employs. The Urban Waste Water Treatment Directive (91/271/EEC) requires the treatment of effluent from all settlements of >2000 population equivalent (p.e.). This includes the reduction of biochemical oxygen demand (BOD),

chemical oxygen demand (COD) and total suspended solids by primary (e.g. settling) and secondary (e.g. biological) treatment. Apart from reducing organic pollution some STPs discharging to sensitive areas (drinking or eutrophic waters) are also required to reduce the levels of nitrate and phosphate in effluent discharge. Furthermore Directive 2008/105/EC (EC, 2008b) lists 33 priority substances and 8 other pollutants that must be monitored for and maintained within strict Environmental Quality Standards (EQS) and in some cases discharges or emissions phased out within 20 years. Many of these compounds are likely to be detectable within STP effluent. This Priority List covers a range of pollutants including heavy metals, industrial chemicals and a small number of pesticides and biocides (e.g. alachlor, DDT, diuron, endosulfan and isoproturon). A proposed amendment to this Directive (EC, 2012) would add 15 additional substances to this list including two pharmaceuticals (the NSAID, diclofenac and the synthetic oestrogen, EE2). Initial proposals recommended an EQS of 10 ng L^{-1} for diclofenac although this was later removed and now both diclofenac and EE2 are merely named on the list without an EQS. The reason for removal was an estimated £ 27 billion additional cost over 20 years in the UK alone for advanced waste water treatment deemed necessary to meet the proposed EQS targets (UK Parliament, 2012). Furthermore, the pharmaceutical industry stated that the evidence of negative environmental effects was flawed and that much more information was required for policy development (EC, 2012).

O'Brien & Dietrich (2004) raised the issue of an increasing burden on existing STPs due to urban expansion thus reducing treatment efficiencies. In recent years, much research has been conducted on the presence of pharmaceuticals in STP influent, effluent and adjacent surface waters (Hughes *et al.*, 2013) aided by developments in analytical techniques such as GC-MS and HPLC-MS (Kümmerer, 2009a). STP effluent is generally considered to be the major source of pharmaceuticals into the environment, particularly surface waters (Heberer, 2002; Daughton, 2004c; Jones *et al.*, 2005; Tambosi *et al.*, 2010). These point sources are particularly important as they discharge continuously into surface waters which allow even those pharmaceuticals with very low environmental half-lives to demonstrate 'pseudo-persistence' as any compound lost to degradation is continually replenished (Daughton, 2001). This has potentially important implications for aquatic organisms as their exposure will generally be low-level (chronic) and life-long or even multi-generational with the potential for toxic effects to manifest slowly which may then go undetected or be attributed to natural change (Daughton, 2004a; Daughton, 2004c).

Once a pharmaceutical compound has reached the aquatic phase degradation can occur through biotransformation but it is thought that abiotic processes play a much more important role. Hydrolysis is likely to be negligible for pharmaceuticals but direct and indirect photolysis can play a major role in removal, particularly close to the water surface

(Farré *et al.*, 2008). Photolysis has been shown to be the major removal process for diclofenac (Buser *et al.*, 1998) but removal is strongly dependent on substance properties, solar irradiation (and hence on latitude and season) and the presence of photosensitiser constituents in the water column (Fent *et al.*, 2006). Some sorption to sediment and suspended particles does occur but this is generally considered to be low, particularly for those compounds present in ionic forms at ambient pH (Scheytt *et al.*, 2005).

2.4.3.5 Pharmaceutical removal during sewage treatment

The degree of removal of a pharmaceutical depends on a number of factors but is largely dictated by the treatment technologies in place and the physico-chemical properties of the individual compound (Santos *et al.*, 2010). Pharmaceuticals have a wide variety of structures and functional groups but unsaturated aliphatic compounds, esters, nitriles and aromatic alcohols are generally more biodegradable whereas compounds with long, highly branched side chains, iodide, nitro and azo functional groups are generally more recalcitrant (Tunkel *et al.*, 2000; Jones *et al.*, 2005). An early study by Richardson & Bowron (1985) examined a number of highly used substances (including clofibrate, aspirin, ibuprofen, paracetamol and a range of antibiotics) for their biodegradability during sewage treatment. They found that many of these substances (apart from paracetamol, ibuprofen and aspirin) were either non-biodegradable or only partially bio-degraded during treatment. Adsorption to the solid (sludge) phase and biodegradation are the key elimination processes in STPS. Adsorption depends on hydrophobic and electrostatic interactions with pH playing a key role in dictating the ionic state of compounds and whether or not they remain in the soluble phase.

Generally acidic pharmaceuticals such as ibuprofen are poorly adsorbed during treatment as they occur as hydrophilic ions at neutral pH. Biodegradation (aerobic and/or anaerobic) is therefore the key elimination process for dissolved pharmaceuticals and also for re-transformation of conjugated metabolites to parent compounds (Fent *et al.*, 2006). Biotransformation (or biodegradation) can range from partial transformation to complete mineralisation of a pharmaceutical by microorganisms (bacteria) using these compounds as an energy source. A fundamental factor governing the degree of biodegradation is the structure of the bacterial community within the STP. This community can be comprised of fast growing generalists that can degrade a wide variety of chemicals and more specialised organisms that occupy narrower niches and may have generation times of several days. The structure of the bacterial community within STPs depends upon pH, temperature, redox potential and also the substrate (i.e. pharmaceutical) concentrations (Kümmerer *et al*., 2005). High substrate concentrations often favour generalists whereas specialists tend to thrive in low concentration, nutrient

poor conditions. Furthermore, very high concentrations of certain compounds can be directly toxic to all or parts of the microbial community within a STP which can have a significant impact on treatment efficiency (Kümmerer *et al.*, 2005). A STP with a high microbial density and species richness will be able to degrade the largest range of pharmaceuticals under fluctuating conditions.

Removal rates in STPs are highly variable and range from 0 to 100 % depending on site and compound specific factors (Table 2-5). Numerous reviews are available on this subject (Fent *et al.*, 2006; Onesios *et al.*, 2009). Some compounds demonstrate consistently low removal rates throughout various studies; for example carbamazepine never showed removal rates of greater than 30 % whereas paracetamol was consistently removed by more than 90 % (Onesios *et al.*, 2009). Generally, the secondary treatment phase is much more effective at removal than the primary phase. The variation in removal rates is not surprising given the diverse nature of pharmaceuticals and also local factors such as treatment technology, wastewater volumes, temperature and weather. Even individual compounds can show different elimination rates, for example diclofenac is eliminated between 17 % and 100 % at different STPs (Fent *et al.*, 2006). This may in part be due to the chiral nature of many pharmaceuticals that are often administered as racemic mixtures. Different enantiomers of the same compound are known to behave differently in the environment and this may have implications for removal during treatment and the subsequent fate and transport in the aquatic environment (Kümmerer *et al.*, 2005).

Some studies have compared the removal efficiency of a laboratory STP with that of a full-scale treatment works; Letzel *et al*. (2010) showed relatively good agreement between laboratory removal of the pyschostimulant Ritalin (13 %) and an activated sludge STP (23 %). However, these studies often only consider the gross difference in parent compounds prior to treatment (i.e. raw sewage influent) and post-treatment (i.e. treated effluent) with often little or no attempt to understand the mechanisms of removal (i.e. biodegradation, mineralisation and metabolism) and the potential for compounds to be re-transformed to the parent state (Jones *et al.*, 2005). Also, the OECD guidelines for testing biodegradability (e.g. the 'closed bottle test' OECD 301D) stipulate very high initial concentrations (mg L^{-1}) which are not representative of average influent concentrations at STPs (ng L^{-1} to µg L^{-1}). Therefore the results from such experiments should be used with caution and further research on removal efficiencies is required (Jones *et al.*, 2005).

Table 2-5: Summary of sewage treatment plant removal efficiencies for 16 widely used pharmaceuticals

Compound	Treatment stage removal efficiency (%)		
	Primary	Secondary[1]	Tertiary[2]
Aspirin		81-100	
Atenolol			>90
Carbamazepine	0	0-45	0-81
Clofibric acid		51-91	
Diclofenac		0-80	25-54
Erythromycin		38-100	0-89
Fluoxetine	10	85	
Ibuprofen	14-44	0-100	0-99
Ketoprofen			>90
Mefenamic acid		87-97	0-100
Metoprolol			>90
Oxytetracycline		0	
Paracetamol		86-100	
Propranolol		0-96	83
Salbutamol		95	
Sulfamethoxazole		18-100	27-87

Notes:
1. Includes both fixed film and suspended technologies *i.e.* trickling biological filters and activated sludge
2. Includes a range of technologies such as sand filtration, ozonation and biological nutrient removal
Sources: (Ternes, 1998; Stumpf *et al.*, 1999; Kanda, 2003; Paxeus, 2004; Versteeg *et al.*, 2005; Jones *et al.*, 2007; Maurer *et al.*, 2007; Nakada *et al.*, 2007; Coetsier *et al.*, 2009; Lundström *et al.*, 2010; Tambosi *et al.*, 2010)
Blank cells mean no data available

Clearly, STPs were designed and constructed to reduce the levels of organic and nutrient pollution in surface waters and there are currently no treatment technologies available that are specifically designed to remove or reduce pharmaceutical concentrations in sewage effluent. However, some existing technologies such as membrane filtration and photo-oxidation have shown promising removal rates for pharmaceuticals but these have additional capital and energy burdens (Heberer, 2008; Martins *et al.*, 2008). One study (Lundström *et al.*, 2010) compared the removal efficiencies of conventional treatment with sand filtration, membrane bio-reactors, moving-bed biofilm reactors and ozonation as additional treatments. The study found that generally ozonation was the most effective at removal but that the resulting effluent was slightly more toxic compared to other treatments; this was most likely due to the formation of toxic transformation by-products. Zheng *et al*. (2010) demonstrated very efficient removal (96 %) of the antibiotic oxytetracycline with ozonation at a dose of 657 mg O_3 L^{-1}.

Simpler, process orientated measures such as increasing the hydraulic retention time (HRT) within a STP and using mature activated sludge can also improve the biodegradation of some compounds during treatment (Fent *et al.*, 2006). Indeed, one study (Onesios *et al.*, 2009) found that many pharmaceuticals have a critical solids retention time (SRT) and HRT below which there is very little removal. They also found that removal rates were generally higher during summer, perhaps due to lower influent flow rates, increases in retention times and elevated temperatures. Kanda *et al*. (2003) also found that increased retention times can improve removal and placed treatment

technologies in the following order based on pharmaceutical removal efficiency: filter bed < non nitrifying activated sludge < nitrifying activated sludge < oxidation ditch. This same study also showed that final effluent concentrations can vary throughout the day (likely due to a combination of fluctuating influent concentrations, flow rates and removal efficiencies; Figure 2-4). Models are also available which attempt to predict the removal of pharmaceuticals during various stages of the sewage treatment process. For example, Jones *et al*. (2002) used the STPWIN model to predict the fate of 25 pharmaceuticals and found that total removal for most compounds was predicted as <5 % except ibuprofen which had a predicted removal of 29 %.

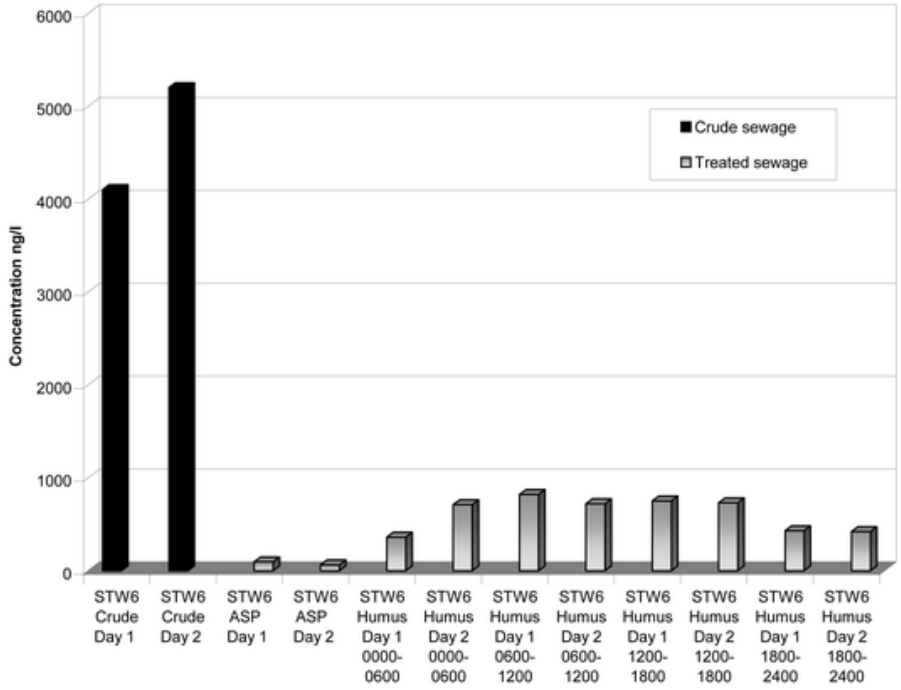


Figure 2-4: Daily variation in ibuprofen effluent concentrations at an activated sludge STW
(Kanda, 2003)

2.4.3.6 Combined sewer overflows (CSOs)

CSOs are designed to divert excess storm water into surface waters during a high rainfall or flood event to prevent flooding in urban areas. They remove the need for large, high capacity sewer systems and the provision of expensive additional capacity in STPs that would be unused during normal flow conditions (Mulliss *et al.*, 1997). Furthermore, they allow the discharge of storm water effluent at controlled and managed locations which are owned and operated by the water companies and regulated by the Environment Agency (in England and Wales). The EA maintain a register of CSO discharge consents which stipulate the operational limits for each CSO and requires that they only discharge during heavy rainfall or snowmelt events (i.e. that they do not discharge routinely during normal, dry conditions).

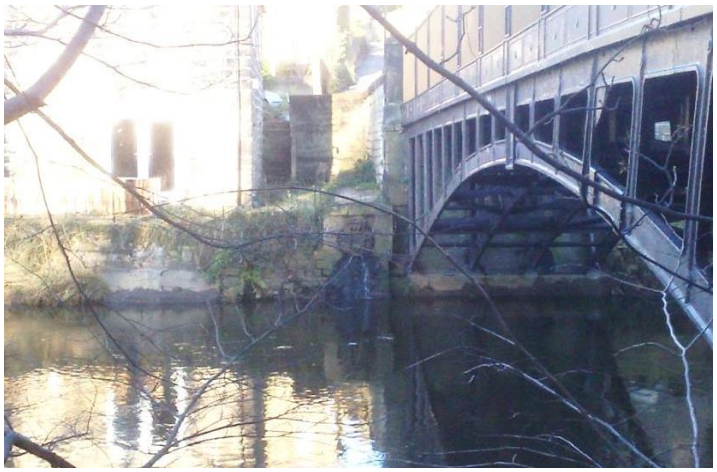
Generally speaking, premises (both domestic and commercial) constructed since the 1960s will discharge to a non-combined sewerage system but this means a significant proportion of the UK sewage system is combined (i.e. urban runoff is mixed with domestic sewage) resulting in dilute but untreated effluent being discharged directly into receiving waters (both rivers and the sea) (Water UK, 2009). During the early 1990s it was estimated that there were 25 000 CSOs in the UK with as many as one third of these being classed as unsatisfactory under the terms of the UWWT Directive (Thompson Rpm, 2006). These discharges generally occur when rainfall and river levels are high allowing greater dilution of the CSO effluent. Besides pharmaceuticals, CSO effluent can contain a wide variety of pollutants including anoxic bacterial scums from roadside catch pits, suspended material and heavy metals from road surfaces, dog faeces and seasonally high levels of chloride from road-salting operations (Mason, 2002).

Unlike STP effluent, CSOs are designed only to discharge on a sporadic basis and studies have found that the frequency, duration and volume of overflows can be used as an indicator of deterioration in water quality (Lau *et al.*, 2002). However, recent findings by the popular media (BBC, 2009) highlighted a number of CSOs in England and Wales that were discharging more frequently and in some cases hundreds of times per year. The UK water industry economic regulator Ofwat (http://www.ofwat.gov.uk/) included as part of the latest 5 year Periodic Review (PR09 for the period 2010-2015) a requirement for the water industry as a whole to invest £ 985 million in capital expenditure to reduce unsatisfactory intermittent discharges from CSOs and other emergency overflows which is a requirement of the UWWT Directive (Ofwat, 2009). Furthermore, the Flood and Water Management Act which became law in April 2010 (HMSO, 2010) requires the adoption of sustainable urban drainage systems (SUDS) in some types of new development which it is hoped will go some way in reducing the volume of surface runoff during heavy rainfall and help to reduce the frequency of CSO discharges (Water UK, 2009).

The significant degradation of chemical, physical and biological quality of receiving waters due to CSO discharges is well documented (Saul, 1989). CSOs have the potential to release significant amounts of pharmaceuticals into receiving waters without undergoing any form of treatment and could be a major source into the environment, particularly for those compounds that would otherwise be well removed during the treatment process. Boyd *et al*. (2004) surveyed storm water channels in New Orleans and found that pharmaceutical concentrations increased with rainfall suggesting a ‘first flush’ response where heavily contaminated sediment in the pipes is re-suspended during the initial stages of a storm event. Mulliss *et al*. (1997) discovered a similar flushing response for nitrate and phosphate and Old *et al*. (2003) for suspended

sediment concentrations during CSO discharges to UK rivers. Ellis (2006) suggests that the presence of pharmaceuticals in urban waters far from STP discharges are most likely a result of sporadic releases from CSOs and other cross-connections or leakages in ageing sewer systems. Clearly, understanding the contribution of these non-STP point sources to overall pharmaceutical levels in receiving waters requires more research particularly as the relationship between flow and contamination is strongly catchment and site specific for individual CSOs (Old *et al.*, 2003).

To date there has been no published research focusing specifically on pharmaceuticals in CSO discharges in the UK despite their potential to represent significant sources of pharmaceutical pollution. The degree of risk will be strongly dependent on the individual CSO, its contributing sewer 'catchment' and the frequency of overflow events. A further concern is that many CSO outfalls are 'flapped' in order to prevent flow backup from high levels in the receiving channel (Figure 2-5) but this may lead to CSOs discharging when river levels have receded and the subsequent dilution of effluent is much lower. Ellis (1979) recorded high suspended sediment concentrations in the falling limbs of storm hydrographs believed to be due to release from CSOs that were drowned and hydraulically locked during the flood peak. However, Kolpin *et al*. (2004) have demonstrated the opposite phenomenon where concentrations due to storm water effluent decreased with increasing stream flow and the subsequent dilution. Therefore, analysis of CSO effluent and receiving waters should be a research priority in an attempt to quantify this currently overlooked source.



(a)

(b)

Figure 2-5: Examples of a 'flapped' (a) combined sewer overflow outfall [centre of image] and (b) sewage treatment plant overflow

2.4.4 Pharmaceutical sources to the aquatic environment: a hierarchy of importance

It is generally agreed that STP effluent is the predominant source of human pharmaceuticals to the aquatic environment (Halling-Sørensen, 1998; Heberer, 2002; Santos *et al.*, 2010) as this integrates discharges from domestic households, commercial properties and hospitals into a single point source for each STP which discharges continuously to its receiving waters. However, CSOs have been subject to very little research, particularly for emerging contaminants and as such these should be prioritised due to their capacity to discharge untreated effluent albeit on a generally sporadic basis. The remaining potential sources (indirectly via agricultural application) are likely to be less important as any pharmaceuticals will be subject to degradation and transformation in the terrestrial environment (see Section 2.4.2).

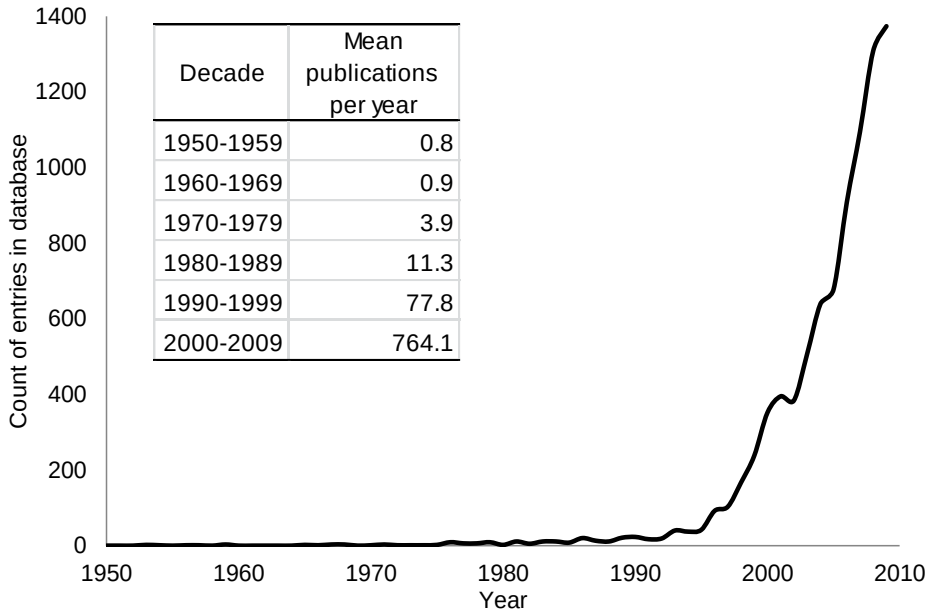
2.5 Occurrence of pharmaceuticals in the environment

This section will summarise existing research on the occurrence of pharmaceuticals in a range of environmental compartments, including soils, the atmosphere and groundwater. The main focus of this section will be on the occurrence and fate of human use pharmaceuticals in STP effluent and receiving freshwater systems.

2.5.1 Expansion of research

Apart from a few pioneering studies which detected drugs in the aquatic environment in the 1970s and 1980s (Hignite & Azarnoff, 1977; Aherne *et al.*, 1985; Richardson, 1985; Aherne *et al.*, 1990) the majority of studies into the environmental occurrence of pharmaceuticals have been conducted from the late 1990s onwards due to the advent of analytical techniques such as high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) (Daughton, 2001). This is despite concerns about the potential environmental effects of pharmaceuticals being raised by the UK government as early as 1969 (Swann *et al.*, 1969). Williams (2005) conducted a review of pharmaceutical occurrence research from the 1970s to 2004 and found that an average of one paper per year was written from 1975 to 1995 which rose to over 50 in 2004. The majority of these studies were conducted in Germany (56 %) with only 5 % in the UK. However the 'detection' rate of pharmaceuticals varied significantly from 14 % in the USA, 40 % in the UK and 70 % in Switzerland highlighting the importance of locally relevant occurrence data. A review of the publically available citations database maintained by the US EPA (USEPA, 2010) showed a similar increase in publications from 1950 onwards (Figure 2-6). The data show a relatively small number of studies from the 1950s to the 1980s and a seemingly exponential increase in the publication rate from the mid-1990s onwards with a current mean publication rate of over 764 articles per year. It must be noted that this database includes literature covering both pharmaceuticals and personal care products and contains journal articles, books, web pages, reports and other grey literature (USEPA, 2010).

Hughes *et al*. (2013) conducted a systematic review of pharmaceutical occurrence research using the Web of Knowledge publications database. They identified 236 studies from 13 journals, which had specifically identified pharmaceutical compounds in STP effluent or receiving waters. The analyses showed a clear upward trend in publications from the late 1990s onwards (the earliest included paper was from 1998) and the majority of studies (over 80 %) were published between 2005 and 2010 (Figure 2-7). It was hypothesised that this increase was driven by advancements in analytical techniques, evidenced by the relatively high proportion (22 %) of studies published in journals with a specific analytical focus such as *Journal of Chromatography A, Analytical and Bioanalyitcal Chemistry* and *Talanta* (Hughes *et al*., 2013).



Decade	Mean publications per year
1950-1959	0.8
1960-1969	0.9
1970-1979	3.9
1980-1989	11.3
1990-1999	77.8
2000-2009	764.1

Figure 2-6: Summary of PPCP publications from 1950-2009 (USEPA, 2010)

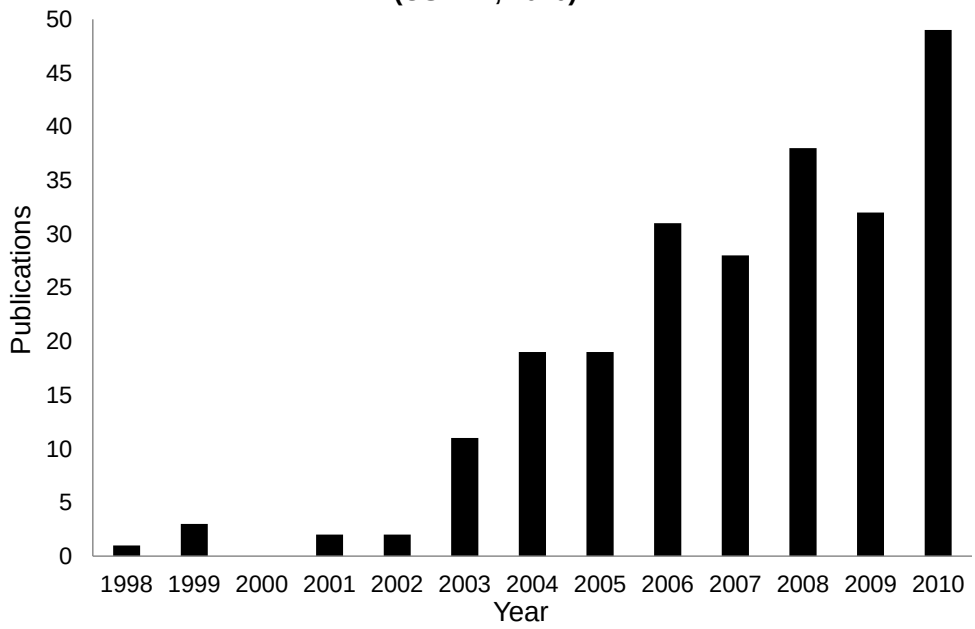


Figure 2-7: Number of publications identifying pharmaceutical compounds in sewage treatment plant effluent or receiving waters from a systematic review of the Web of Knowledge publications database (Hughes *et al*., 2013)

A number of review articles (Halling-Sørensen, 1998; Heberer, 2002; Fent *et al.*, 2006; Calisto & Esteves, 2009; Santos *et al.*, 2010; Hughes *et al.*, 2013) and three editions of a textbook (Kümmerer, 2001b; 2004a; 2008a) have been published focusing solely on PPCPs in the environment. Furthermore, special volumes of the journals *Chemosphere* (April 2000: Volume 40 Issue 7), *Environmental Toxicology and Chemistry* (December 2009: Volume 28 Issue 12) and *Science of the Total Environment* (Virtual Special Issue: http://tinyurl.com/muz64ck) have been published which dealt solely with this issue. The tones of these review articles and particularly the textbook edited by Klaus Kümmerer (2001b; 2004; 2008a) shifted from the early 2000s to the present. The 1st edition of

Kummerer's textbook (2001b) had a majority of chapters devoted to summarising occurrence data whereas the latest edition (2008a) has much more emphasis on understanding the effects of pharmaceuticals and gaps in current knowledge. The books and review articles present useful summaries of existing data from across the world so they will not be reproduced here. However, there is no published article that presents the UK occurrence data in one single place and so these values will be presented later in this chapter as they are particularly relevant to this study.

Kasprzyk-Hordern *et al*. (2007) estimated that between 80 and 150 PPCPs have been explicitly studied for their occurrence in various environmental compartments. Hughes *et al.* (2013) synthesised global pharmaceutical research via a systematic review of research and found a total of 203 pharmaceutical compounds identified in rivers across 41 countries. Despite the relatively large number of compounds monitored, the majority of research effort has so far focused on just 14 widely used substances. Furthermore, research is spatially biased towards North America and Europe with perhaps the most research conducted in Germany. The greater emphasis on pharmaceutical occurrence research in Europe is perhaps due to higher concentration of sewage outfalls, lower per-capita water usage and lower stream flows (resulting in less dilution) when compared to North America (Daughton, 2001). Based on the findings of Hughes *et al*. (2013) very little or no research has been conducted in Africa, South America, the Middle East and large parts of Asia. However, as the previous section on pharmaceutical consumption has demonstrated, the transfer of measured environmental concentrations is not appropriate between countries due to variations in consumption and also differing levels of sewage treatment.

2.5.2 Occurrence in non-aquatic environmental compartments

Pharmaceuticals have been widely detected in a range of environmental compartments including: surface water, ground water, soils, landfill leachate, sewage sludge, manure and in marine waters and sediment (Daughton & Ruhoy, 2009b). Active pharmaceutical ingredients (APIs) have even been detected in drinking water and in the Arctic environment (Stan et al., 1994; Kallenborn et al., 2008). This section will give a very brief overview of the occurrence data for non-aquatic compartments in order to provide context. A small number of studies have shown veterinary antibiotics may enter the airborne phase within and outside CAFOs (Zahn *et al.*, 2001; Chapin *et al.*, 2005). However, the generally non-volatile nature of pharmaceuticals means that airborne exposure is not considered to be a major concern, except perhaps for communities living downwind of large CAFOS (Wing & Wolf, 2000).

Pharmaceuticals can enter soils by direct deposition from cattle, the application of manure/slurry from animal rearing or the application of human sewage sludge (see

Section 2.4.2). Furthermore, the use of reclaimed wastewater for irrigation and aquifer recharge can act as an indirect source (Xu *et al.*, 2009). Ternes *et al*. (2007) analysed soil water samples for 52 pharmaceuticals and two musk fragrances from a German field that had been treated with sewage sludge for 45 years. Only four of the compounds were detected in soil water (up to several µg L^{-1}) despite being consistently present in the associated STP effluent. A number of European studies (Winckler, 2000; Hamscher *et al.*, 2002; Hamscher *et al.*, 2005) have detected antimicrobials in agricultural soils ranging from 4.5 µg kg^{-1} to over 900 µg kg^{-1}. It should be noted that a number of the detections were well above the detailed risk assessment trigger value for soil concentrations of 100 µg kg^{-1} (EMEA, 2006). The concentrations detected also vary with soil depth (Hamscher *et al.*, 2002) and with soil pH, microbial communities and redox conditions (Sarmah *et al.*, 2006). River and marine sediments directly adjacent to fish farms have been studied for the presence of pharmaceuticals, particularly antibiotics. These compounds are administered to the fish as a feed additive which can enter the sediment as food falls directly through the farm cages or in fish faeces; concentrations ranging from 100 to 4900 µg kg^{-1} have been detected (Jacobsen & Berglind, 1988; Capone *et al.*, 1996).

The disposal of unwanted or unused pharmaceuticals into solid waste accounts for the majority of household disposal in the UK (Bound, 2006). All or some of this solid waste may then be sent to a landfill site where pharmaceuticals can collect and contaminate ground and surface waters. This is of particular concern for landfills where there are no leachate collection and treatment technologies in place (Kümmerer, 2009a). A study at a Florida landfill found a total of 22 APIs (equivalent to 22 910 mg) in 2800 kg of solid waste, representing an average of 8.1 mg kg^{-1} (Musson & Townsend, 2009). Holm *et al*. (1995) conducted a study on a landfill site which received solid waste from the pharmaceutical industry from the 1940s to the 1970s; they found very high concentrations of sulphonamides (up to 5 mg L^{-1}) and the analgesic propylphenazone (up to 4 mg L^{-1}) adjacent to the landfill. However, concentrations decreased rapidly within 50 metres downstream of the groundwater plume and none of the compounds exceeded the detection limit after 150 metres. This was due to anaerobic degradation, dilution and sorption (Holm *et al.*, 1995). The presence of pharmaceuticals in groundwater is a concern as it is often a source of drinking water and the persistence of compounds may be greater due to reduced microbial activity and the absence of photochemical breakdown processes (Jones *et al.*, 2001). Indeed, Stan *et al*. (1994) detected the blood lipid regulator clofibrate in German drinking water at concentrations ranging from 10 to 165 ng L^{-1} indicating this compound is readily persistent in the environment, this hypothesis is further supported by its regular detection in European surface waters (Halling-Sørensen, 1998).

2.5.3 Occurrence in STP effluent and receiving waters

The vast majority of occurrence studies have focused on effluent from STPs discharging to surface river water as these are considered to be the major source of pharmaceuticals into the environment (Ellis, 2006). Pharmaceutical occurrence studies have been conducted in at least 41 countries although research is extremely limited or absent in most of South America, central and southern Asia and Africa (Hughes *et al.*, 2013). Perhaps the most comprehensive single study to date is that of Kolpin *et al*. (2002) who monitored for the presence of 95 micro-pollutants (including a range of prescription and non-prescription drugs, antibiotics, disinfectants and fragrances) in 139 polluted streams across the USA. In some streams as many as 38 of the target compounds were detected. Review articles are available which summarise much of this global occurrence data so these will not be reproduced here (Halling-Sørensen, 1998; Ayscough *et al.*, 2000; Jones *et al.*, 2001; Heberer, 2002; Daughton, 2004a; Enick & Moore, 2007; Caliman & Gavrilescu, 2009; Kümmerer, 2009a; Kümmerer, 2009b; Kümmerer, 2009c; Pal *et al.*, 2010; Brausch & Rand, 2011; Hughes *et al.*, 2013). However, a detailed breakdown of pharmaceutical occurrence in UK rivers is given to provide the research background for this thesis (Table 2-6). A detailed global synthesis and critical evaluation of research into the occurrence of over 200 pharmaceutical compounds detected in rivers across 41 countries is given in Chapter 3.

A total of 69 pharmaceutical compounds have been identified in UK rivers or STP effluent. Overall, median pharmaceutical concentrations in receiving waters are in the low tens of ng L^{-1} range for all but the most widely used compounds (i.e. painkillers and antibiotics). Receiving water maxima frequently exceed 100 ng L^{-1} for most compounds and several exceeding 1000 ng L^{-1} (predominantly antibiotics and painkillers such as erythromycin and ibuprofen). The mean receiving water concentration across all compounds was 134.6 ng L^{-1} with the highest recorded concentration of 7731 ng L^{-1} for the painkiller tramadol. Effluent concentrations are generally greater than respective receiving waters with a median concentration across all compounds of 1547 ng L^{-1}. Here painkillers, antibiotics, antiepileptics and cardiovascular drugs demonstrated the highest concentrations. The highest measured effluent concentration was again for the painkiller tramadol (97 616 ng L^{-1}). Where compounds were identified in both STP effluent and receiving waters, median concentrations were greater in effluent for all but five compounds out of a total of 38 (clofibric acid, clotrimazole dextropropoxyphene, salbutamol, sulfamethoxazole). Effluent concentrations were generally 19 x greater than the respective receiving water values although such comparisons should be treated with caution given differences in sampling and analyses between studies (Table 2-6; 2-7). This supports the prevailing evidence that STP effluent is the dominant pharmaceutical source to rivers.

Table 2-6: Summary of pharmaceutical occurrence in UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
Amitriptyline	Antidepressants	2.0	17.0	43.8	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Amoxicillin	Antibiotics	117	622.0	29.5	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008)
Amphetamine	Illicit drugs	5.5	9.0	53.4	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008)
Aspirin	Painkillers	31.0	36.0	46.8	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Atenolol	Other cardiovascular drugs	124.3	560.0	86.4	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Bendroflumethiazide	Other cardiovascular drugs	1.0	15.0	2.1	Taff and Ely	Kasprzyk-Hordern et al. (2008))
Benzoylecgonine	Painkillers	13.0	17.0	47.3	Taff and Ely	Kasprzyk-Hordern et al. (2008); Zuccato et al. (2008)
Bezafibrate	Blood lipid regulators	30.4	76.0	49.4	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Loos et al. (2009)
Bleomycin	Cancer treatments		17.0		SE England	Aherne et al. (1990)
Carbamazepine	Antiepileptics	95.5	684.0	92.3	Sussex Ouse, Taff, Ely, Fleetwood, Lancaster, Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford and Naburn Lock	Zhang et al. (2008); Zhang & Zhou (2007); Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Loos et al. (2009); Zhou et al. (2009))
Chloramphenicol	Antibiotics	4.0	40.0	3.9	Taff and Ely	Kasprzyk-Hordern et al. (2008)
Cimetidine	Gastro-intestinal drugs	35.5	220.0	75.1	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Clofibrate	Blood lipid regulators	40.0			Thames	Richardson & Bowron (1985)
Clofibric acid	Blood lipid regulators	14.8	164.0	31.5	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Clotrimazole	Others	20.8	34.0	100.0	Tyne	Roberts & Thomas (2006)
Cocaine	Illicit drugs	4.0	6.0	10.6	Taff and Ely	Kasprzyk-Hordern et al. (2008); Zuccato et al. (2008)
Codeine	Painkillers	165.0	815.0	84.0	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Dextropropoxyphene	Painkillers	285.0	682.0	35.3	Thames and Tyne	Richardson & Bowron (1985); Ashton et al. (2004); Roberts & Thomas (2006)
Diazepam	Others	10.0			Thames	Richardson & Bowron (1985)

Table 2-6: Summary of pharmaceutical occurrence in UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
Diclofenac	Painkillers	13.9	568.0	70.9	Sussex Ouse, Lea, Thames, Fleetwood, Lancaster, Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford and Naburn Lock	Zhang et al. (2008); Zhang & Zhou (2007); Ashton et al. (2004); Ellis (2006); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Loos et al. (2009); Zhou et al. (2009)
Diltiazem	Other cardiovascular drugs	12.0	65.0	56.3	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Erythromycin	Antibiotics	185.2	1022.0	58.0	Thames, Taff and Ely	Richardson & Bowron (1985); Ashton et al. (2004); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Furosemide	Other cardiovascular drugs	31.0	630.0	63.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Gabapentin	Antiepileptics	434.5	1887.0	94.2	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Gemfibrozil	Blood lipid regulators		2.0	35.0	Fleetwood, Lancaster, Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford and Naburn Lock	Loos et al. (2009)
Ibuprofen	Painkillers	447.7	5044.0	73.5	Thames, Lea, Taff, Ely, Tyne, Fleetwood, Lancaster, Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford and Naburn Lock	Ashton et al. (2004); Bound & Voulvoulis (2006); Ellis (2006); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Loos et al. (2009); Roberts & Thomas (2006)
Indomethacin	Painkillers	1.1	3.0	100.0	Sussex Ouse, Taff, Ely	Zhang et al. (2008); Zhang & Zhou (2007); Zhou et al. (2009)
Ketoprofen	Painkillers	1.5	14.0	74.1	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
MDA	Illicit drugs	3.0	4.0			Zuccato et al. (2008)

Table 2-6: Summary of pharmaceutical occurrence in UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
MDMA (ecstasy)	Illicit drugs	4.0	6.0			Zuccato et al. (2008)
Mefenamic acid	Painkillers	47.0	366.0	60.9	Thames, Taff and Ely	Ashton et al. (2004); Bound & Voulvoulis (2006); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Metoprolol	Other cardiovascular drugs	7.5	12.0	94.4	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Metronidazole	Antibiotics	3.5	24.0	66.1	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Miconazole	Others	8.0	9.0	100.0	Thames	Roberts & Bersuder (2006)
Morphine	Painkillers	8.0	42.0			Zuccato et al. (2008)
Naproxen	Painkillers	22.5	146.0	82.4	Taff, Ely, Fleetwood, Lancaster, Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford and Naburn Lock	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Loos et al. (2009)
Paracetamol	Painkillers	194.1	2382.0	89.3	Thames, Lea, Taff, Ely, Tyne, Fleetwood, Lancaster, Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford, Naburn Lock	Bound & Voulvoulis (2006); Ellis (2006); Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Propranolol	Other cardiovascular drugs	38.2	215.0	76.2	Sussex Ouse, Taff, Ely, Thames, Lea and Tyne	Ashton et al. (2004); Ellis (2006); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006); Zhang & Zhou (2007); Zhang et al. (2008); Zhou et al. (2009)
Ranitidine	Gastro-intestinal drugs	7.0	73.0	22.7	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Salbutamol	Respiratory drugs	69.0	471.0	41.3	Taff and Ely	Bound & Voulvoulis (2006); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Sulfamethoxazole	Antibiotics		95.8	73.7	Sussex Ouse, Thames, Taff, Ely, Fleetwood, Lancaster,	Zhang & Zhou (2007); Richardson & Bowron (1985); Kasprzyk-Hordern et al. (2008); Loos et al. (2009); Zhou et al. (2009)

Table 2-6: Summary of pharmaceutical occurrence in UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
					Glasgow, Hull, Middlesbrough, Runcorn, Edinburgh, Stafford and Naburn Lock	
Sulfapyridine	Antibiotics	17.5	142.0	55.3	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Sulfasalazine	Antibiotics	26.8	168.0	81.2	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Tamoxifen	Cancer treatments	78.0	212.0	100.0	Tyne	Roberts & Thomas (2006)
Tetracycline	Antibiotics	1000.0			Thames	Richardson & Bowron (1985)
Theophylline	Respiratory drugs	1000.0				Richardson & Bowron (1985)
Tramadol	Painkillers	1771.0	7731.0	80.5	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Trimethoprim	Antibiotics	23.3	569.0	62.2	Taff, Ely and Tyne	Ashton et al. (2004); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006)
Valsartan	Other cardiovascular drugs	16.0	144.0	94.1	Taff and Ely	Kasprzyk-Hordern et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)

Notes:
Median value of all concentrations from combined studies
Maximum value of all concentrations from combined studies
Mean detection frequency of all samples from combined studies (not all studies stated detection frequencies)
Blank cells indicate data were unavailable

Table 2-7: Summary of pharmaceutical occurrence in treated STP effluent discharging to UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
Amitriptyline	Antidepressants	141.0	357.0	85.5	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Amphetamine	Illicit drugs	3.0	11.0		Taff, Ely and Thames	Boles & Wells (2010)
Aspirin (acetylsalicylic acid)	Painkiller	349.0	85.0	71.0	Taff, Ely and Thames	Richardson & Bowron (1985); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Atenolol	Other cardiovascular drugs	2496.5	7602.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Bendroflumethiazide	Other cardiovascular drugs	11.0	58.0	19.0	Taff and Ely	Aherne et al. (1990)
Bezafibrate	Blood lipid regulators	204.0	667.0	92.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Bleomycin	Cancer treatments	15.8	19.0		SE England	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Carbamazepine	Antiepileptics	1066.8	4596.0	100.0	Taff, Ely, Sussex Ouse	Zhang et al. (2008); Zhang & Zhou (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Zhou et al. (2009)
Chloramphenicol	Antibiotics	21.0	69.0	25.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Chlorpromazine	Other CNS drugs	10.0	11.0	33.3	Thames	Roberts & Bersuder (2006)
Cimetidine	Gastro-intestinal drugs	1533.5	9395.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Clofibric acid	Blood lipid regulators	10.5	75.0	43.5	Taff and Ely	Kasprzyk-Hordern et al. (2009; 2008); Roberts & Thomas (2006)
Clotrimazole	Others	14.0	27.0		Tyne	Roberts & Thomas (2006)
Codeine	Painkillers	3993.5	15593.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Dextropropoxyphene	Painkillers	160.3	585.0	74.0	Tyne	Ashton et al. (2004); Hilton & Thomas (2003); Roberts & Thomas (2006)
Diclofenac	Painkillers	192.1	2349.0	93.0	Taff, Ely, Lea, Thames, Sussex Ouse, Tyne	Zhang et al. (2008); Zhang & Zhou (2007); Ashton et al. (2004); Ellis (2006); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006); Zhou et al. (2009)
Diltiazem	Other cardiovascular drugs	312.0	1156.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Ephedrine	Illicit drugs	12.6	14.8		Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)

Table 2-7: Summary of pharmaceutical occurrence in treated STP effluent discharging to UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
Erythromycin	Antibiotics	761.0	2841.0	72.0	Taff, Ely, Tyne	Ashton et al. (2004); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006)
Fluoxetine	Antidepressants	33.5	34.0	33.3	Thames	Roberts & Bersuder (2006)
Furosemide	Other cardiovascular drugs	895.0	1956.0	96.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Gabapentin	Antiepileptics	9169.5	42611.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Ibuprofen	Painkillers	1437.7	27256.0	92.0	Tyne	Kanda et al. (2003); Ashton et al. (2004); Ellis (2006); Hilton & Thomas (2003); Jones et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006)
Indomethacin	Painkillers	16.2			Sussex Ouse	Zhang & Zhou (2007); Zhou et al. (2009)
Ketoprofen	Painkillers	17.0	37.0	72.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
MDMA (ecstasy)	Illicit drugs	5.0	10.0		Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Mefenamic acid	Painkillers	274.6	1440.0	84.3	Tyne	Ashton et al. (2004); Hilton & Thomas (2003); Jones et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006)
Methotrexate	Cancer treatments	1000.0			SE England	Aherne et al. (1985)
Metoprolol	Other cardiovascular drugs	55.0	130.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Metronidazole	Antibiotics	309.0	561.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Miconazole	Others	9.0		100.0	Thames	Roberts & Bersuder (2006)
Naproxen	Painkillers	270.0	703.0	96.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Paracetamol	Painkillers	4038.7	24525.0	93.0	Taff and Ely	Ellis (2006); Jones et al. (2007); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Propranolol	Other cardiovascular drugs	151.8	523.0	100.0	Taff, Ely, Sussex Ouse, Lea Thames, Tyne	Zhang et al. (2008); Zhang & Zhou (2007); Ashton et al. (2004); Ellis (2006); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006); Zhou et al. (2009)
Pseudoephedrine	Illicit drugs	26.7	27.7		Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Ranitidine	Gastro-intestinal drugs	324.5	783.0	87.5	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Salbutamol	Respiratory drugs	36.5	234.0	87.0	Taff and Ely	Jones et al. (2007); Kasprzyk-Hordern et al. (2009; 2008)

Table 2-7: Summary of pharmaceutical occurrence in treated STP effluent discharging to UK rivers

Compound	Therapeutic class	Concentration (ng L^{-1})		Mean detection freq (%)[3]	Catchment(s) or location(s)	References
		Med[1]	Max[2]			
Simvastatin	Blood lipid regulators	5.0	20.0	19.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Sulfamethoxazole	Antibiotics	23.6	132.0	50.0	Taff, Ely, Sussex Ouse	Zhang et al. (2008); Zhang & Zhou (2007); Ashton et al. (2004); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Zhou et al. (2009)
Sulfapyridine	Antibiotics	366.0	1112.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Sulfasalazine	Antibiotics	242.3	2185.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Tamoxifen	Cancer treatments	199.0	369.0	4.0	Tyne	Ashton et al. (2004); Roberts & Thomas (2006)
Tramadol	Painkillers	35980.0	97616.0	100.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Trimethoprim	Antibiotics	501.8	3052.0	82.5	Taff, Ely, Tyne	Ashton et al. (2004); Hilton & Thomas (2003); Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009); Roberts & Thomas (2006)
Valsartan	Other cardiovascular drugs	173.0	711.0	96.0	Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)
Venlafaxine	Antidepressants	130.0	248.3		Taff and Ely	Kasprzyk-Hordern et al. (2008); Kasprzyk-Hordern et al. (2009)

Notes:
Median value of all concentrations from combined studies
Maximum value of all concentrations from combined studies
Mean detection frequency of all samples from combined studies (not all studies stated detection frequencies)
Blank cells indicate data were unavailable

These data are in agreement with occurrence data from other countries which generally lie within the range of a few ng L^{-1} up to tens of µg L^{-1} (Hughes *et al.*, 2013). Williams (2005) showed the average detection concentration in surface waters for human pharmaceuticals was 43 ng L^{-1} (compared to 18 ng L^{-1} for drinking water, 159 ng L^{-1} for ground water and 360 ng L^{-1} for treated STP effluent). These data show substantial variation between minimum and maximum concentrations ranging up to a factor of >1300 for paracetamol within the same study (Kasprzyk-Hordern *et al.*, 2007). There are a number of possible factors for such wide variation which may include: climate, weather and flow conditions, position within the catchment, STP specific factors (treatment technologies, flow rates, retention times, removal efficiencies and overflows etc.) and nature of the channel sediment which can affect both the input of pharmaceuticals associated with sewage effluent and the degradation in receiving waters (Kümmerer *et al.*, 2005). For example, the biodegradation rate of synthetic oestrogens in UK rivers has been shown to vary by a factor of 10 due to season and catchment location (Jürgens *et al.*, 2002). The degree of dilution of sewage effluent in receiving waters is also a critical factor. Waiser *et al*. (2010) found a range of pharmaceuticals at relatively high concentrations (µg L^{-1}) in a glacier fed river in Canada; certain reaches of this river comprised almost 100 % sewage effluent during periods of the year when glacial melt water is absent resulting in a dilution factor of 0.

2.5.3.1 Non-steroidal anti-inflammatory drugs (NSAIDs) and painkillers

The widely consumed NSAIDs (such as ibuprofen, paracetamol and diclofenac) were readily detected in these studies and at some of the highest concentrations in both STP effluent (up to 27 000 ng L^{-1} for ibuprofen) and receiving waters (5044 ng L^{-1} for ibuprofen). Perhaps surprisingly, aspirin which was the second most widely prescribed drug in 2008 (130.7 tonnes, see Table 2-2) and is also a popular generic OTC pain reliever, was only detected in relatively low river concentrations with a median of 4 ng L^{-1} and a maximum of just 36 ng L^{-1}. This is likely due to the fact that approximately 80 % of the active ingredient in aspirin (acetylsalicylic acid) is readily metabolised (to salicylic acid) in the human liver (Levy, 1972). However, these metabolites have been shown to have toxic effects on *Daphnia magna* (Marques *et al.*, 2004). Ibuprofen, by contrast is excreted 70-80 % in active forms which is a possible reason for its high detected concentrations (Pounds *et al.*, 2008). The analgesic dextropropoxyphene was detected in generally lower concentrations (median river concentration 285 ng L^{-1}) than other NSAIDs. Perhaps this is due to the relatively small DDD of 200 mg even though it was prescribed 380 000 times (ranked 287th) in 2008 as co-proxamol in combination with paracetamol (see Table 2-3). The total prescribed amount of dextropropoxyphene in 2008 was an estimated 0.08 tonnes compared to 1.22 tonnes of co-proxamol

demonstrating that paracetamol accounts for the large majority of active pharmaceutical ingredients in this particular treatment. Richardson and Bowron (1985) detected dextropropoxyphene in UK surface waters in the 1980s and estimated an environmental half-life of >1 year.

2.5.3.2 Antibiotics

After the NSAIDs, antibiotics were generally detected in the highest concentrations in effluent and receiving waters. The macrolide antibiotic erythromycin and the β-lactam antibiotic amoxicillin were detected at the highest receiving water concentrations at maximums of 1022 and 622 ng L^{-1} respectively. It is worth noting that the prescription volume of these two antibiotics has reduced within recent years with amoxicillin falling from rank 1 in 2005 to rank 13 in 2008 and erythromycin falling from 7 to 85 in the same period (NHS 2006; 2009b). Therefore, the peak concentrations measured for erythromycin by Ashton *et al*. (2004) may not be reached if measured today. Also, the peak concentration reported by Kasprzyk-Hordern *et al*. (2008) for amoxicillin may be considerably less than in 2005 when this compound was prescribed much more widely. These examples highlight the importance of up to date information on both consumption and environmental occurrence. The sulphonamide antibiotic sulfamethoxazole is often combined with trimethoprim to form co-trimoxazole which was prescribed 65 500 times in 2008 resulting in a mass consumption of just 0.13 tonnes (see Table 2-3) which is reflected in the relatively low receiving water concentrations (maximum 37 ng L^{-1}). However, the major metabolite acetyl-sulfamethoxazole was detected at slightly higher levels of up to 2235 ng L^{-1} and 240 ng L^{-1} in effluent and receiving waters respectively. As little as 15% of sulfamethoxazole is excreted as the parent compound and up to 43% can be excreted as the acetyl-sulfamethoxazole metabolite (van der Ven et al., 1994). Furthermore Radke *et al*. (2009) found that these metabolites can retransform into the parent compounds under environmental conditions.

2.5.3.3 Blood lipid regulators and cardiovascular drugs

Clofibric acid is the major active metabolite of the blood lipid regulator clofibrate and has been readily detected in surface waters in the UK and other countries; approximately 96 % of the parent compound is excreted as active clofibric acid or its conjugates (Winkler *et al*., 2001). Clofibrate has now been withdrawn from usage in much of Western Europe (it was not present in the 2008 PCA dataset) and replaced with other cholesterol lowering drugs after evidence of increased mortality rates amongst clofibrate treated patients (WHO, 1984). Despite this, clofibric acid continues to be detected in the environment and is known to be highly persistent and mobile in the aquatic phase (see Heberer, 2002). It is also known to be very resistant to both sewage (5-51% removal; Ternes, 1998; Zweiner & Frimmel, 2003) and drinking water treatment (Ternes *et al*., 2002).

Clofibric acid has been detected in Berlin tap water at concentrations up to 165 ng L^{-1} (see Heberer *et al*., 1998). Simvastatin is a drug used to regulate cholesterol levels in the blood (similar to clofibrate) and prevent cardiovascular disease and has risen from rank 47th in prescription numbers in 1997 to rank 1st in 2008 (see Table 2-2) but the relatively low DDD of 30 mg day^{-1} meant that only 1.02 tonnes were prescribed in 2008. This is reflected in the relatively low frequency of detection in the UK (when compared to NSAIDs and antibiotics) and the low concentrations in receiving waters (<50 ng L^{-1}). However, blood lipid regulators alongside other drug types such as antiepileptics and contraceptives tend to be prescribed to patients on a long-term or permanent basis so the inputs of these drugs may represent a continuous source into the aquatic environment with much less seasonal variation (see Bound *et al*., 2006).

Beta-blockers are a class of pharmaceuticals used primarily for the treatment of cardiac arrhythmia (abnormal electrical activity in the heart), hypertension (high blood pressure) and as protection following myocardial infarction (heart attack). Of these compounds, atenolol was the most widely prescribed in 2008 followed by propranolol and metoprolol and this order is reflected in the occurrence data in (see Tables 2-3 and 2-4) with maximum receiving water concentrations of 560, 215 and 12 ng L^{-1} respectively. Propranolol was the most frequently detected beta-blocker in UK surface waters with Ashton *et al*. (2004) finding it to be ubiquitous in all samples taken. However, it must be noted that propranolol was frequently analysed in all of the studies shown in Table 2-5 whereas atenolol and metoprolol were only analysed for in three of the studies. Another reason for the possible variation in detected concentrations is the percentage excretion of unchanged parent compound which varies from <0.5, 10-30 and 50 % for propranolol, metoprolol and atenolol respectively (Kasprzyk-Hordern *et al*., 2007). The metabolites of metoprolol are deactivated whereas approximately 20 % of the propranolol metabolites are in the form of glucuronide conjugates which have the potential to be re-transformed into the parent compound.

2.5.3.4 Other widely used drugs

Salbutamol is a bronchodilator used in the treatment of bronchospasm for sufferers of asthma or other similar obstructive pulmonary disease. It was the fifth most widely prescribed compound in 2008 but its low DDD of 10 mg day^{-1} and its predominant inhaled administration route (Moore et al., 1994) may be the reason for infrequent detection and generally low measured concentrations in UK surface waters (average 76 ng L^{-1}). Also, only an estimated 30 % of salbutamol is excreted unmetabolised by humans (see Kasprzyk-Hordern *et al*., 2007). Carbamazepine is an anticonvulsant and mood stabiliser used in the treatment of epilepsy and other disorders such as ADHD (attention-deficit hyperactivity disorder). It was the 77th most widely prescribed drug in 2008 (see Tables

2-3 and 2-4) but the relatively high DDD of 1000 mg day^{-1} results in a total estimated consumption of 2.4 tonnes. Table 2-5 shows that carbamazepine was quite widely detected at relatively high concentrations up to a maximum of 684 ng L^{-1} in the River Ouse (Zhou *et al*., 2009). Carbamazepine was found to be ubiquitously present in samples taken by Kasprzyk-Hordern *et al*. (2008) in South Wales although they found another antiepileptic (gabapentin) to also be ubiquitous and at higher concentrations (maximum 1887 ng L^{-1}). This difference can be explained by the excretion ratios which is approximately 100 % unchanged for gabapentin but only 3 % unchanged for carbamazepine. Both substances were found to be persistent in river water and demonstrated poor removal during sewage treatment (Kasprzyk-Hordern *et al*., 2008). Much higher concentrations of carbamazepine have been detected in Berlin (up to 1075 ng L^{-1}; Heberer (2002) and the River Elbe (up to 7100 ng L^{-1}; Wiegel *et al*. 2004). Studies into the occurrence of metabolites and transformation products are much less common; a recent review by Mompelat *et al*. (2009) found that over 160 pharmaceuticals had been explicitly studied but only 30 by-products. This is surprising as some studies indicate that the environmental concentrations of metabolites and transformation can match or even exceed those of the parent compound (Celiz *et al*., 2009).

This summary of the UK occurrence data for pharmaceuticals demonstrates how river water and effluent concentrations can vary between regions and even within the same river across multiple years. Therefore, it is important that any study into the potential impacts of pharmaceuticals on river ecosystems is accompanied with relevant, local and up-to-date occurrence data so that laboratory and field studies can be informed by realistic environmental concentrations for that particular river. In turn, these occurrence studies and compound selection should be designed using the most relevant and recent consumption data available. A long-term routine monitoring study for a number of compounds (of differing use classes) at a range of STPs with different treatment technologies will provide valuable data on seasonal variations in effluent and receiving water concentrations. Higher temporal resolution monitoring will also provide much needed data on how these concentrations vary over shorter time steps particularly in response to heavy rainfall events. Finally, sampling a longer stretch of river away from the immediate influence of STP effluent discharges will further understanding of the fate and transport of pharmaceuticals in freshwater ecosystems.

2.5.4 Predicting the occurrence of pharmaceuticals in rivers

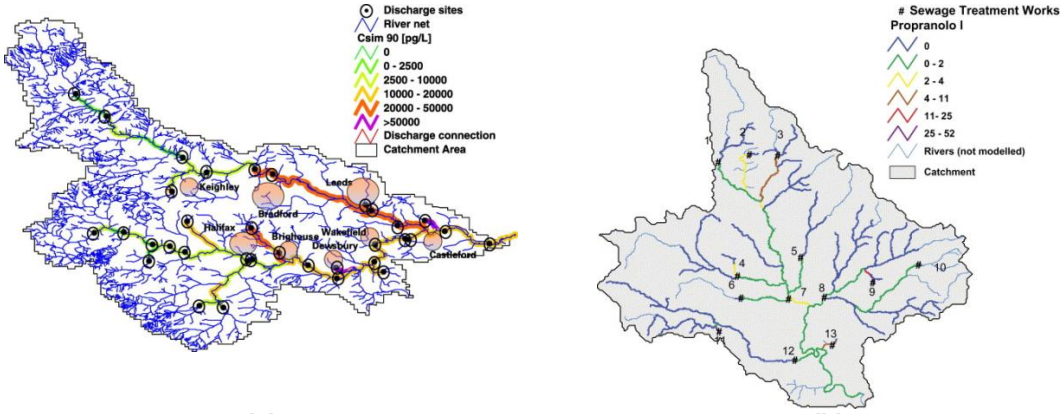
The data presented in section 2.5.3 and elsewhere (e.g. Hughes *et al*., 2013) demonstrate that there is a relative paucity of studies with an explicit focus on the presence of pharmaceuticals in surface waters (Tables 2-6 and 2-7). Using the UK as an example, most studies have been conducted in southern and southeast England and

Wales. Clearly, the geographic spread of these studies is biased to densely populated urban areas and the relevant research institutions but as the previous section (Section 2.2.3) demonstrates, these occurrence data may not represent surface water concentrations in other parts of the UK. In response to this some efforts have been made to predict potential surface water concentrations using models that combine water quality and hydrological flow models in a GIS environment. The most frequently used model in Europe is GREAT-ER (Geography Referenced Regional Exposure Assessment Tool for European Rivers; see http://www.great-er.org/pages/home.cfm for details and technical documentation). Other models used in north America include LF2000-WQX (Low Flows 200-Water Quality modelling eXtension; Keller & Young (2004; 2006)) and PhATE™ (Pharmaceutical Assessment and Transport Evaluation; Anderson *et al*., (2004)), a mass balance model developed for use in US surface waters.

Two of these models have been applied explicitly to pharmaceuticals in the UK; Johnson *et al*. (2007) used LF2000-WQX to predict diclofenac and propranolol concentrations in a small rural catchment in Devon (England) and Johnson *et al*. (2008) used GREAT-ER to predict river concentrations of the anti-cancer drug 5-fluorouracil in the Aire and Calder catchments in West Yorkshire (England). Both of these studies presented their final results for the 90th percentile flow; a low flow that is exceeded 90 % of the time and so can be assumed to be worst-case due to minimal levels of dilution. The rural study found concentrations were generally low (<1 ng L^{-1}) except immediately downstream of some STPs where concentrations were >25 ng L^{-1}. Much higher concentrations were predicted for the more densely populated and urbanised Aire and Calder catchments (983 people km^{-2} c.f. 15 people km^{-2} in the Devon study) where concentrations ranging from 5 to >50 ng L^{-1} were predicted for long stretches of the catchment. Schowanek & Webb (2002) also used GREAT-ER to predict concentrations of five pharmaceuticals and the synthetic oestrogen EE2 in the Aire catchment. The final predicted environmental concentrations (PECs) ranged from 20 ng L^{-1} for dextropropoxyphene to 6800 ng L^{-1} for paracetamol. These models can also produce geographically referenced graphical outputs to give users an idea of how concentrations vary across a catchment (e.g. Figure 2-8).

Modelling studies predict relatively low concentrations in the upper reaches which increase as the burden of STP discharge increases downstream and the catchments become gradually more influenced by urban areas; this is particularly evident in the Aire and Calder. Direct comparisons between these two modelled catchments are difficult as the authors chose to model different pharmaceuticals in each study and neither of these models was directly validated through analysis of the compounds measured in water samples. However, validation of these models was undertaken using chloride and orthophosphate for the Devon catchment (Johnson *et al.*, 2007) and boron and a

detergent for the Aire and Calder (Neal *et al.*, 1998; Fox *et al.*, 2000). Both models were able to predict these concentrations reasonably well but it is questionable whether the same can be assumed for the much more varied and biologically active class of pharmaceuticals. Schowanek & Webb (2002) attempted to validate their results with measured environmental concentrations (MECs) from previous studies and found that GREAT-ER over predicted for paracetamol and under predicted for dextropropoxyphene. They attributed these errors to variations in consumption patterns between the modelled and MEC catchments, simplistic or worst-case assumptions about treatment removal and the poor representation of some in-stream degradation processes such as photolysis.



(a) **(b)**

Figure 2-8: (a) Predicted 5-fluorouracil concentrations in the Aire and Calder Catchments in Yorkshire under low flow conditions using GREAT-ER, (b) Predicted propranolol concentrations (ng L^{-1}) in the Tamar catchment in Devon under low flow conditions using LF2000-WQX
(Johnson *et al.*, 2007; Johnson *et al.*, 2008)

Modelling may be useful in directing and prioritising compounds and catchments for more detailed occurrence studies without the need for preliminary fieldwork. Versteeg *et al.* (2005) conducted basic comparisons between predicted and measured environmental concentrations for dextropropoxyphene and paracetamol in the Aire catchment (Table 2-8).

Table 2-8: Comparison between predicted and measured concentrations in the River Aire catchment, UK (data adapted from Versteeg et al. 2005)

API	Human loss (%)	STP removal (%)	In-stream decay (day^{-1})	Basic $PEC_{surfacewater}$ ($ng\ L^{-1}$)[1]	GREAT-ER $PEC_{surfacewater}$ ($ng\ L^{-1}$)[2]	$MEC_{surfacewater}$ ($ng\ L^{-1}$)[3]
Dextropropoxyphene	99	0	0	7.5	20	58
Paracetamol	10	98	0.047	630	6800	<50

Notes:
Basic predicted environmental concentration calculated as part of the environmental risk assessment process: assumes a mean wastewater flow of 259L $person^{-1}$ day^{-1} and a dilution factor of 10
This is the 90th percentile PEC under mean flow conditions
Measured environmental concentration; data taken from Hilton *et al*. (2003)

These data show that the basic PEC is 3 to 10 times lower than the GREAT-ER predictions suggesting the dilution factor of 10 may be overestimating actual dilution in the Aire. The MEC for dextropropoxyphene was approximately 3 and 10 times greater than the GREAT-ER and basic estimates respectively whilst the PECs for paracetamol were more than ten times greater than the MECs. Possible reasons for these discrepancies include varying flow magnitudes in the Aire during water sampling (compared with the mean flow assumed during PEC calculation) and a lack of more reliable consumption, metabolism and treatment removal data for these pharmaceuticals (Versteeg *et al.*, 2005). Thompson (2006) conducted comparisons between MECs and PECs for a total of 10 pharmaceuticals and found that PECs gave generally good agreements (within one order of magnitude) but were over-estimates for all but one compound (diazepam). Aspirin was the most notable anomaly in these comparisons as it was grossly overestimated by the PEC calculation (by a factor > 10 000). This was likely due to the high metabolism and removal of aspirin during treatment which is assumed to be 0 % in the basic PEC calculation. It also must be noted that these two comparison studies used different consumption and MEC data.

A comparison between monitoring and GREAT-ER predictions was also conducted by Küster *et al*. (2010) for the beta-blocker propranolol; these results indicated an agreement consistently within a factor of 2 for three European catchments (Germany and Switzerland). However, these agreements were not as strong for another beta-blocker atenolol which is much more readily biodegradable and this factor was not accounted for in the model. The research summarised above indicate the potential applications of models such as GREAT-ER to screen a large number of pharmaceutical compounds for potential environmental risk and to use the results to better inform monitoring campaigns of STP effluent and rivers.

2.5.5 The fate of pharmaceutical compounds in surface waters

Consideration must be given to the transport and fate of pharmaceutical compounds once they enter river systems as research into their occurrence and effects rely on such

knowledge to highlight key areas of risk. In their most basic forms, the risk assessment models mentioned earlier (such as GREAT-ER; Schowanek *et al*., 2001) make the simple assumption of first-order decay along a river reach once a compound has entered via a STP outfall. However, this is oversimplified especially given the huge array of different pharmaceutical compounds with widely varying physico-chemical properties that enter rivers (Table 2-9). The ultimate transport and fate of pharmaceuticals in rivers are subject to a number of key processes including (Farré *et al*., 2008; USEPA, 2010):

- Photo transformation (both direct and indirect reactions via UV radiation);
- Physical-chemical alteration, degradation and ultimately mineralisation;
- Volatilisation;
- Dispersion and dilution;
- Biological uptake and alteration.

The relative importance of the above processes has been shown to vary between specific compounds with different properties and depend on site-specific factors (Table 2-9). Pharmaceuticals demonstrate a wide range of solubilities (see Table 2-9) which will have a distinct impact on their transport and fate within aqueous systems; it is expected that more soluble compounds will readily pass through STPs. The acid dissociation constant (pK_a) gives an indication of the degree to which a specific compound will dissociate into ionic forms. The majority of pharmaceutical compounds listed below (Table 2-9) have positive pK_a values indicating they are moderate or weak acids and will remain largely in their parent (i.e. active, non-dissociated form) at environmental pH and temperature ranges (Harrison & deMora, 1996). Log K_{OW} is a the ratio of concentrations between the aqueous phase and the model hydrophobic phase (octanol) and can be seen as a measure of how hydrophilic/hydrophobic a chemical substance is (Leo *et al.*, 1971). The guidelines for environmental risk assessment of pharmaceuticals in the EU suggest that any compounds with log K_{OW} greater than 4 should be considered persistent, bioaccumulative and toxic (PBT; EMEA, 2006). Most pharmaceuticals demonstrate lower values but some compounds do approach the PBT threshold indicating varying degrees of hydrophobicity. Sorption to the solid phase (K_d) or to organic carbon (K_{OC}) is also highly variable between compounds; although substances containing amine functional groups (e.g. propranolol) appear to sorb more readily than neutral or carboxylic acid pharmaceuticals such as paracetamol and ibuprofen (Yamamoto *et al.*, 2009). Brown *et al*. (2007) identified NSAIDs as a key group as they demonstrate sufficient solubility to avoid sorption during passage through STPs but are sufficiently lipophilic to bioconcentrate in aquatic organisms. Furthermore,

sorption to organic carbon is dependent on the amount of carbon available either suspended in freshwaters or in sediments and is therefore likely to be highly variable across temporal and spatial scales in complex river systems (Giller & Malmqvist, 1998). All of these parameters are in turn dependent on temperature and pH both of which vary in freshwater systems (Harrison & deMora, 1996); this highlights the need for a combination of lab and field experiments if the fate of pharmaceuticals is to be better understood.

Natural attenuation in the environment plays a key role in decontamination with associated implications for water quality and the ecological status of freshwaters (Farré *et al*., 2008). However, estimates of environmental fate depend strongly on experimental conditions (lab vs. field studies) and particularly the testing system used (e.g. water only or a combined sediment/water system and the presence/absence of biota). Identical compounds can demonstrate varying attenuation between different studies (attenuation of diclofenac ranged from 0 to 69 % across reaches of several kilometres in three studies by the same research group; see Table 2-9). This is most likely due to specific local and experimental factors.

Despite these complications, the available data suggest that direct or indirect photo-degradation is the dominant removal mechanism for most pharmaceutical compounds in surface waters (Mompleat *et al*. 2009). Local factors play a large role in determining ultimate fate and transport of pharmaceutical compounds. For example, latitude, cloud cover, rainfall, riparian and aquatic vegetation, channel depth and turbidity can act as strong controls on photo-degradation in rivers and such factors have been used to explain low, variable attenuation of photo labile compounds such as diclofenac (Packer *et al*., 2003). Flow rates and velocities have been shown to have a significant effect on downstream transport and attenuation with greater removal generally observed during low flow events due to longer travel times and reduced turbidity (Kolpin *et al*., 2004; Radke *et al*. 2010). Research into the fate and transport of pharmaceuticals in freshwaters is relatively sparse and there are only a small number of laboratory and field studies. However, the available data show variability within and between compounds, testing regimes and field sites (Table 2-9). Much more research is necessary if the fate of these compounds is to be understood. It is clear that the assumption of first-order decay in rivers used by risk assessment models (e.g. Schowanek *et al*. 2001) is unsafe and wherever possible reliable experimental data, preferably combining laboratory and field techniques, should be used to inform this aspect of the risk assessment process.

Table 2-9: Summary of environmental fate and transport data for selected pharmaceutical compounds in rivers

Compound	Physicochemical properties	Dominant attenuation mechanism (lab or field)	Transport and attenuation	Environmental half-life (days)	Reference
Atenolol	Kd = 1.3-110 K_{OC} = 310 - 5000 log K_{OW} = 0.16 Solubility = 300 mg L^{-1} pKa = 9.6	Lab: water/sediment system		2.3-3.0 (water) 2.3-3.0 (water/sediment)	Ramil *et al*. (2009)
Acebutolol	log K_{OW} = 1.71 Solubility = 259 mg L^{-1}	Lab: water/sediment system		2.0-5.9 (water) 10.9-27.7 (water/sediment)	Ramil *et al*. (2009)
Bezafibrate	log K_{OW} = 4.25 Solubility = 0.335 mg L^{-1}	Lab: biodegradation	50% removal along 87km and 113km under slow and fast water-sediment exchange	4.3-8.4 (biodegradation)	Kunkel & Radke (2008)
		Field: none	0% removal along 16km stretch of small river		Kunkel & Radke (2011)
Bisoprolol	log K_{OW} = 1.87 Solubility = 2240 mg L^{-1}	Lab: water/sediment system		3.9-8.4 (water) 8.6-27.7 (water/sediment)	Ramil *et al*. (2009)
Carbamazepine	Kd = 0.08 – 1.8 K_{OC} = 0.01 – 0.06 log K_{OW} = 2.45 Solubility = 17.7 mg L^{-1} pKa = 13.9	Field: unknown	0% removal along 11km stretch of shallow river		Gross *et al*. (2004)
		Field: unknown		63.0	Tixier *et al*. (2003)
		Lab: water/sediment system		328.0	Löffler *et al*. (2005)
Celiprolol	log K_{OW} = 1.92 Solubility = 93.9 mg L^{-1}	Lab: water/sediment system		3.0-13.1 (water) 23.9-67.0 (water/sediment)	Ramil *et al*. (2009)
Ciprofloxacin	log K_{OW} = 0.28 Solubility = 0.0003 mg L^{-1} pKa = 6.1	Lab: biodegradation		>29.0 (biodegradation)	Girardi *et al*. (2011)
Clofibric acid	log K_{OW} = 2.57 Solubility = 583 mg L^{-1}	Lab: biodegradation			Kunkel & Radke (2008)
		Field: biofilm	35% removal along 16km stretch of small river	2.5	Kunkel & Radke (2011)
		Field: unknown		>63.0	Tixier *et al*. (2003)
		Lab: water/sediment system		119.0	Löffler *et al*. (2005)
Diclofenac	log K_{OW} = 4.02	Field: photolysis and sediment biotransformation	69% and 41% removal along a 12km river during sunny and rainy conditions		Kunkel & Radke (2012)

Table 2-9: Summary of environmental fate and transport data for selected pharmaceutical compounds in rivers

Compound	Physicochemical properties	Dominant attenuation mechanism (lab or field)	Transport and attenuation	Environmental half-life (days)	Reference
	Solubility = 2430 mg L^{-1}	Lab: biodegradation	50% removal along 193km and 144km under slow and fast water-sediment exchange	5.5-18.6 (biodegradation)	Kunkel & Radke (2008)
	pKa = 4.0				
		Field: photolysis		8	Tixier *et al*. (2003)
		Lab: photolysis		<0.1 (rapid photolysis)	Buser *et al*. (1998)
		Field: none	0% removal along 16km stretch of small river		Kunkel & Radke (2011)
Gemfibrozil		Field: photolysis	64-72% removal along 12km stretch depending on time of day	0.6 (photolysis)	Lin *et al*., (2006)
	log K_{OW} = 4.77	Lab :biodegradation	50% removal along 278km under fast water-sediment exchange	2.5 (biodegradation)	Kunkel & Radke (2008)
	Solubility = 10.9 mg L^{-1}	Field: unknown	58% to 67% removal along 11km stretch of shallow river		Gross *et al*. (2004)
Ibuprofen		Field: none	Full transport along 12km		Kunkel & Radke (2012)
		Field: photolysis	87% removal along 12km stretch depending on time of day	0.1-0.6 (photolysis)	Lin *et al*., (2006)
		Lab: biodegradation	50% removal along 53km and 66km under slow and fast water-sediment exchange	2.5-5.1 (biodegradation)	Kunkel & Radke (2008)
	Kd = 0.09 – 0.9				
	K_{OC} = 0.01 – 9.4	Lab: biodegradation		>37.0 (photolysis)	Buser *et al*. (1999)
	log K_{OW} = 3.79			20.0 (biodegradation)	
	Solubility = 21 mg L^{-1}	Field: unknown	47% to 83% removal along 11km stretch of shallow river		Gross *et al*. (2004)
	pKa = 4.91				
		Field: biofilm	100% removal along 16km stretch of small river	0.4	Kunkel & Radke (2011)
		Field: sorption		32	Tixier *et al*. (2003)
		Lab: water/sediment system		<6.0	Löffler *et al*. (2005)
Ivermectin		Lab: water/sediment system		15.2	Löffler *et al*. (2005)
Ketoprofen	log K_{OW} = 3.12	Field: unknown	100% removal along 11km stretch of shallow river		Gross *et al*. (2004)
	Solubility = 51 mg L^{-1}	Field: photolysis and biotransformation			Tixier *et al*. (2003)
	pKa = 4.45				
Metoprolol		Field: none	0% removal along 16km stretch of small river		Kunkel & Radke (2011)
		Lab: water/sediment system		4.1-8.7 (water) 17.8-28.9 (water/sediment)	Ramil *et al*. (2009)

Table 2-9: Summary of environmental fate and transport data for selected pharmaceutical compounds in rivers

Compound	Physicochemical properties	Dominant attenuation mechanism (lab or field)	Transport and attenuation	Environmental half-life (days)	Reference
Nadolol	log K_{OW} = 0.81 Solubility = 8330 mg L^{-1}	Lab: water/sediment system		3.1-3.7 (water) 4.0-9.0 (water/sediment)	Ramil *et al*. (2009)
Naproxen		Field: sediment biotransformation	50% and 43% attenuation along 12km river during sunny and rainy conditions		Kunkel & Radke (2012)
		Field: photolysis	75-100% removal along 12km stretch depending on time of day	0.3-0.6 (photolysis)	Lin *et al*., (2006)
		Field: unknown	100% removal along 11km stretch of shallow river		Gross *et al*. (2004)
	log K_{OW} = 3.18 Solubility = 15.9 mg L^{-1} pKa = 4.15	Lab: biodegradation	50% removal along 144km and 272km under slow and fast water-sediment exchange	10.3-13.9 (biodegradation)	Kunkel & Radke (2008)
		Field: photolysis and biotransformation		14	Tixier *et al*. (2003)
		Field: none	0% removal along 16km stretch of small river		Kunkel & Radke (2011)
Oxazepam	log K_{OW} = 2.24 Solubility = 179 mg L^{-1}	Lab: water/sediment system		54.0	Löffler *et al*. (2005)
Paracetamol	Kd = 1.0 – 7.7 K_{OC} = 170 - 13000 log K_{OW} = 0.46 Solubility = 0.0004 mg L^{-1} pKa = 9.38	Lab: water/sediment system		3.1	Löffler *et al*. (2005)
Pindolol	log K_{OW} = 1.75 Solubility = 7880 mg L^{-1}	Lab: water/sediment system		0.1-0.5 (water) 0.1-0.5 (water/sediment)	Ramil *et al*. (2009)
Propranolol	log K_{OW} = 2.60	Field: sediment biotransformation	70% and 42% attenuation along 12km river during sunny and rainy conditions		Kunkel & Radke (2012)
	Solubility = 31.7 mg L^{-1} pKa = 9.4	Lab: water/sediment system		0.4-1.8 (water) 9.9-33.0 (water/sediment)	Ramil *et al*. (2009)
Sotalol	log K_{OW} = 0.24	Field: photolysis and sediment biotransformation	42% and 36% attenuation along 12km river during sunny and rainy conditions		Kunkel & Radke (2012)
	Solubility = 5510 mg L^{-1}	Lab: water/sediment system		7.6-8.2 (water) 11.4-30.0 (water/sediment)	Ramil *et al*. (2009)
Sulfamethoxazole	log K_{OW} = 0.89	Field: sediment biotransformation	26% and 25% attenuation along 12km river during sunny and rainy conditions		Kunkel & Radke (2012)
	Solubility = 610 mg L^{-1}	Lab: water/sediment system		25.6 (biodegradation in sediment)	Radke *et al*. (2009)

2.6 Consequences of pharmaceuticals in the environment

2.6.1 Introduction to current ecotoxicological methods and concerns for pharmaceuticals

Definitions for the science of ecotoxicology range from a focus on individual organisms like that of Jørgensen (1990) towards more focus on higher levels of biological organisation like that of Walker *et al*. (2001) who emphasised the study of harmful effects on ecosystems. In fact, ecotoxicological effects can be demonstrated from biomolecular and cellular scales, through to single organisms, populations and ecosystems and potentially up to landscape and biosphere scales (Newman & Unger, 2003). Processes at one level draw mechanisms from the level below and exhibit consequences at the level above (Caswell, 1996). Therefore, ecotoxicologists should consider all levels of ecological organisation across a range of temporal scales if they are to understand, predict, prevent and mitigate the impacts of toxic substances in the environment (Newman & Unger, 2003).

Recent research has attempted to understand their potential environmental impacts (Daughton, 2007). The widely reported 95 % decline in vulture (*Gyps benegalensis*) populations in India and Pakistan has been directly linked to the anti-inflammatory drug diclofenac which caused renal failure, visceral gout and uric acid accumulation eventually leading to death (Oaks *et al.*, 2004). Vultures consumed livestock carcasses that had been treated with diclofenac and subsequent lethal effects for vultures were observed at just 10 % of the recommended mammalian dose. This cause was only discovered after exhausting more 'traditional' causes of death (heavy metal poisoning, viruses, bacteria etc.) and as a result veterinary application of diclofenac is now banned in India (Dorne *et al.*, 2007). This is an excellent example of the unintentional release of a pharmaceutical into the environment and a subsequent devastating impact on a non-target organism manifesting at population level. Unintended environmental consequences have been experienced for other species, such as an increase in food availability for the feral dog (*Canis familiaris*) and rat (*Rattus* spp.); this also brings associated public health and social concerns (Dorne *et al.*, 2007). It must be noted that traditional acute toxicity tests conducted in the laboratory did not, and could not anticipate the effects on *Gyps* vultures because of their focus on short-term effects on single species (Oaks *et al.*, 2004).

Another widely studied and acknowledged impact on non-target organisms is that of the synthetic oestrogen ethinylestradiol (EE2, used in the human contraceptive pill) on fish and other aquatic organisms. Concentrations as low as 0.1 to 5.0 ng L^{-1} have been linked with reductions in fecundity, suppression of male secondary sexual characteristics, intersex and population failure due to reduced fertility in a number of fish

species (Nash *et al.*, 2004; Pawlowski *et al.*, 2004; Colman *et al.*, 2009). Other chemical compounds produced and consumed in domestic and industrial settings have been widely acknowledged to disrupt the endocrine system in fish and to mimic the action of natural oestrogens (Mittwoch *et al.*, 1993; Ashby *et al.*, 1997). Compounds such as detergents, pesticides and surfactants have been shown to elicit such effects with high concentrations capable of feminising male fish, particularly those present in rivers which receive high STP effluent loads (Jobling & Sumpter, 1993; Purdom *et al.*, 1994; Sumpter, 1995; Tyler *et al.*, 1998). The above examples highlight the potential for unforeseen consequences for organisms at all trophic levels and environmentally relevant concentrations. This section of the literature review will summarise current knowledge and data gaps on the ecotoxicological impacts of pharmaceuticals with a particular focus on aquatic organisms.

2.6.1.1 Standardised toxicity testing procedures

Pharmaceuticals are subject to very rigorous pharmacological, mammalian toxicology and clinical testing prior to approval for human use but Pascoe *et al*. (2003) highlighted a paucity of data examining their effects on aquatic fauna, particularly important freshwater taxa such as invertebrates. Sanderson *et al*. (2003) estimate that ecotoxicological data is publicly available for just 1 % of approved human pharmaceuticals. Despite the need for ecotoxicological studies to consider a range of ecological levels and temporal scales, the vast majority of data that do exist have been derived from short-term acute toxicological studies using a range of standard organisms in idealised laboratory experiments (Santos *et al*., 2010). These acute toxic effects are generally manifested through non-specific mechanisms of action (MOAs) usually disrupting cellular membranes (known as narcosis) resulting in cell death (cytotoxicity) or oxidative stress that results in cellular damage (Fent, 2008). Generally, these tests are conducted in line with established guidance such as the Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals Section 2 and include a range of organisms, toxicological endpoints and life stages:

- OECD Report 201 (OECD, 2011) – Alga, Growth Inhibition Test – assesses the effect of a substance on the growth rate of unicellular algal species (e.g. *Selenastrum capricornutum*) – 72 hour duration;

- OECD Report 202 (OECD, 2004) – *Daphnia sp.*, Part I Acute Immobilisation Test and Part II Reproduction Test – assesses the effects on swimming as well as the timing and rate of reproduction of the planktonic crustacean, *Daphnia magna* – 48 hours for Part I and up to four weeks for Part II which should include approximately nine batches of offspring;

- OECD Report 210 (OECD, 1992) – Fish, Early Life Stage Toxicity Test – assesses lethal and sub-lethal effects on fertilised eggs. The report includes a list of recommended fish species such as Rainbow Trout (*Oncorhynchus mykissi*) and Zebrafish (*Brachydanio rerio*) – continues until all hatched fish are free-feeding which can be > 1 month;

- OECD Report 221 (OECD, 2006) – *Lemna* sp. Growth Inhibition Test – 7-day test to assess the impact on growth of the free-floating aquatic macrophyte genus *Lemna* (usually *Lemna gibba or L. minor* – 7 days).

Other tests include the effects on soil microorganisms, honeybees, sediment dwelling *Chironomids* and respiration of activated sludge; the guidelines for these are available freely on the OECD website (OECD, 2012). Data from these experiments are fitted to a dose-response model from which either the LC_{50} (median lethal concentration), EC_{50} (median effective concentration), lowest-observed-effect-concentration (LOEC) and no-observed-effect concentration (NOEC) can be calculated (Newman & Unger, 2003). The majority of these tests are concerned with acute toxicity which will have an effect on the organism in question within 96 hours (Sprague, 1969) although some such as OECD Reports 202 and 204 (OECD, 1984; OECD, 2004) look at prolonged toxicity for up to four weeks. However, even 14 day tests of fish toxicity do not cover a sufficient proportion of their life cycle to be classed as chronic studies (Suter, 1993).

2.6.2 Summary of short-term toxicity data

There are a number of reviews of the toxicity of pharmaceuticals to aquatic organisms available in Crane *et al*. (2006), Enick & Moore (2007), Fent *et al*. (2006), Fent (2008), Halling-Sørensen *et al*. (1998), Hughes *et al*. (2013), Santos *et al*. (2010) and Webb (2001). As such, these data will not be replicated here but rather an overview of general acute toxic effects is presented.

The review articles show that short-term toxicity can vary markedly for the same compound across trophic levels and even between similar species. For example, *D. magna* is much more tolerant of diclofenac when compared to the macrophyte *Lemna minor* and the beta-blocker metoprolol when compared to another crustacean *Ceriodaphnia dubia* (Cleuvers, 2003; Ferrari *et al.*, 2003). Data also show that acute toxicity varies with exposure time and toxicity endpoint and that final LC_{50} or EC_{50} values can vary due to differing susceptibility of sourced test organisms and actual exposure concentrations (when nominal concentrations are used) (Fent, 2008). These data also show that generally very high concentrations (>1 to 10 mg L^{-1}) are required to elicit an acute toxic effect over short time scales. These concentrations are generally well above those encountered in the aquatic environment, often exceeding them by several orders

of magnitude (10^3 to 10^7; (Fent *et al.*, 2006)). Webb (2001) compared the acute toxicity responses in various trophic levels and found that the antidepressant, antibiotic and antipsychotic compounds were generally the most toxic and that algae were generally the most sensitive organisms. A similar comparison by Jones *et al*. (2002) identified the same high risk compound classes (Table 2-10). Hughes *et al*. (2013) compared the measured environmental concentrations of major classes of pharmaceuticals and their potential risk of chronic toxicity to fish, invertebrates, bacteria, algae and plants in freshwater ecosystems. At particular risk were invertebrates and fish due to exposure to relatively high concentrations of antibiotics and cardiovascular drugs (particularly in Asia); antidepressants and painkillers pose a similar risk in North America. Contrast media and tranquilisers were also identified as posing a significant global risk to fish and invertebrates. Despite this such compounds remain relatively poorly studied both in terms of their environmental occurrence and ecotoxicology.

There are some notable exceptions to the rule of low acute toxicity. For example the antidepressants fluoxetine and fluvoxamine demonstrate acute toxicity to green algae (EC_{50} = 31 000 ng L^{-1}) and *Sphaerium* clam species (4 hour LOEC = 3000 ng L^{-1}) at much lower concentrations (Webb, 2001; Brooks *et al.*, 2003). Also, carbamazepine demonstrates acute lethality to zebrafish at 43 000 ng L^{-1} (Santos *et al.*, 2010). However, the maximum measured environmental concentration of fluoxetine was 596 ng L^{-1} taken from estuarine samples in New York, USA (Benotti & Brownawell, 2007) and the global median concentration was 17.8 ng L^{-1} (Hughes *et al.*, 2013). Fluvoxamine has only been studied twice in the environment with median and maximum concentrations of 0.7 and 4.6 ng L^{-1} respectively (Vasskog *et al.*, 2008; Schultz *et al.*, 2010). Carbamazepine is much more widely studied in the environment with at least 50 studies detecting it (Hughes *et al.*, 2013; Loos *et al.*, 2009) at median and maximum concentrations of 176 and 11 561 ng L^{-1} respectively.

These data demonstrate that for most compounds the margin of safety for acute toxicity is high but there are exceptions to this rule. This may be of particular concern in areas immediately adjacent to large or poorly performing STPs, CSOs or pharmaceutical manufacturing facilities (Hughes *et al.*, 2013). The widely held opinion that the risk of acute toxicity is low (Fent, 2008) does therefore hold for most compound classes but further research is required.

Table 2-10: Summary of toxicity of pharmaceuticals to aquatic organisms (adapted from Jones *et al*., 2002)

Substances	Extremely toxic EC_{50} <0.1 mg L^{-1}	Very toxic EC_{50} 0.1-1 mg L^{-1}	Toxic EC_{50} 1-10 mg L^{-1}	Harmful EC_{50} 10-100 mg L^{-1}	Non-toxic EC_{50} >100 mg L^{-1}
Analgesics			D	D, E	
Antibiotics	A	B			
Antidepressants		D			
Anti-epileptics			C		D, E
Cardiovascular		D			
Cytostatics		A		D, E	
X-ray contrast media					A, B, D, E

Most sensitive taxonomic groups: A (micro-organisms), B (algae), C (cnidarians), D (crustaceans), E (fish).

2.6.3 Summary of long-term and chronic toxicity data

Fent (2008) states that 38 % of tested compounds showed acute toxicity above 100 mg L^{-1}, classifying them as "*not harmful to aquatic organisms*" according to EU Directive 2001/83/EC. Given this and the generally low risk of widespread, frequent acute toxicity the focus of toxicological testing should address the potential for long-term and chronic effects at environmentally realistic concentrations (Hughes *et al.*, 2013). This is of particular relevance to aquatic organisms adjacent to STP outfalls for which there is potential for life-long and multigenerational exposure through the 'pseudo-persistence' of continually discharging effluent (Daughton, 2001). However, there is a relative lack of chronic data perhaps due to the increased costs, logistics and analytical requirements of longer exposure times or the assessment of sub-lethal endpoints. A chronic toxicity study is defined by convention to be at least 10 % of the species' life span (Suter, 1993) but this is often not achieved and much shorter tests are incorrectly classed as chronic (Newman & Unger, 2003). More investigations into specific receptors, effects, life stages and life cycle studies are almost completely lacking for the vast majority of pharmaceuticals (Fent *et al.*, 2006).

Reviews of chronic ecotoxicological data are available elsewhere (see references in Section 2.6.2 and Hughes *et al*., 2013) which provide useful indicators of likely sub-lethal impacts resulting from longer-term exposure to low level concentrations. This continuous, chronic exposure is of particular concern as any negative effects may accumulate and manifest so slowly that they will be attributed to natural, ecological succession and by the time they have been identified the effects may be irreversible (Daughton & Ternes, 1999). These review data show that the majority of chronic toxicity values lie within the range of 10 µg L^{-1} to >10 mg L^{-1} which are generally at least 1 to 2 orders of magnitude greater than the concentrations measured in sewage effluent; this gap is greater when dilution in receiving waters is taken into account (Hughes *et al.*, 2013). This demonstrates that the margin of safety between measured and toxic concentrations is much narrower for chronic toxicity.

Indeed, there are some compounds which demonstrate chronic toxicity at or close to concentrations measured in the environment. For example, ibuprofen has been shown to affect the polyp structure of *Hydra vulgaris* at 10 000 ng L^{-1} (Pascoe *et al.*, 2003) and has been detected in Turkish rivers at concentrations up to 31 323 ng L^{-1} (Loos *et al.*, 2009) with a global median of 517 ng L^{-1} (Hughes *et al.*, 2013). Propranolol has also been shown to reduce fecundity and recruitment in Japanese medaka (*Oryzias latipes*) at 500 ng L^{-1} (Huggett *et al.*, 2002) which corresponds with the maximum environmental concentration of 590 ng L^{-1} measured in a German river (Ternes, 1998); although the global median concentration was just 19 ng L^{-1} (Hughes *et al.*, 2013). Fluoxetine demonstrates chronic toxicity at some of the lowest concentrations; for example fathead minnow (*Pimephales promelas*) escape velocity was reduced at a concentration of just 25 ng L^{-1} which in turn, may have secondary impacts on predator avoidance (Painter *et al.*, 2009). This concentration is very close to the global median river concentration of 18 ng L^{-1} indicating a strong likelihood of chronically toxic effects in fish (Hughes *et al.*, 2013). However, no information is available on the length of time at which rivers were at the 'maximum' exposure to these measured concentrations and as such direct comparisons between laboratory exposures of several hours to several weeks and instantaneous samples of river concentrations should be treated with some caution.

There are also examples of positive (or at least non-adverse) effects at relatively low concentrations. For example, fluoxetine caused an increase in brood size of *Daphnia* at 36 000 ng L^{-1} which may be attributed to a toxicological phenomenon known as hormesis (Flaherty & Dodson, 2005). This bi-phasic response may be an adaptive change to low levels of stress resulting in increased fitness for a finite period of time, often at the expense of growth (Figure 2-9; (Calabrese & Baldwin, 2002)).

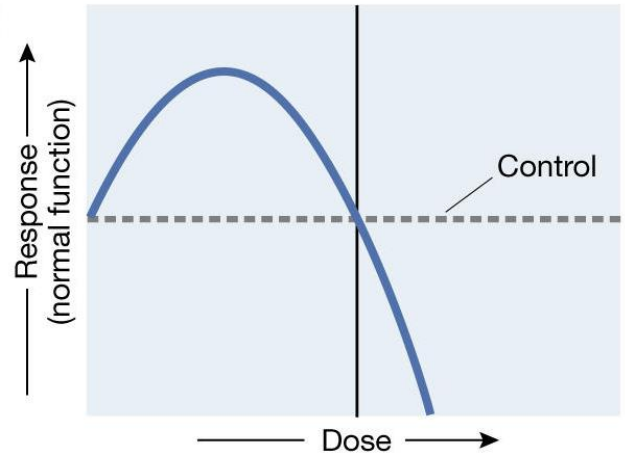


Figure 2-9: Graphical example of a U-shaped hormetic dose response (Calabrese, 2004)

Apart from a few exceptions (Oetken *et al.*, 2005; Triebskorn *et al.*, 2007; Nentwig, 2008; Painter, 2009), most toxicological studies focus on non-specific endpoints of growth,

survival, reproduction and immobilisation to the individual with much less emphasis on species-specific endpoints (e.g. polyp structure test of Pascoe *et al*. (2003)). There is even less evidence of research addressing behavioural, morphological or physiological responses and a clear need for much more research into effects at the population, community or ecosystem level (Figure 2-10; Santos *et al*. (2010)). Ankley et al. (2005) suggest adopting other species into standard ecotoxicological testing to represent taxa that are currently overlooked. For example *Chironomus* are sediment-dwelling aquatic insects that may be particularly useful for testing for a number of reasons: insects are a critical group of aquatic organisms, they have a number of distinct life stages which may vary in sensitivity and unlike most test invertebrates (e.g. cladocerans) they reproduce sexually, a process which may be sensitive to pharmaceuticals.

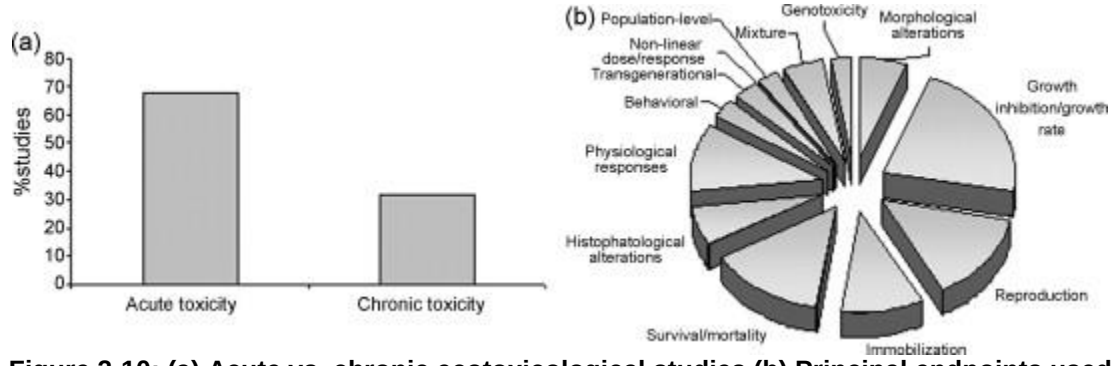


Figure 2-10: (a) Acute vs. chronic ecotoxicological studies (b) Principal endpoints used in ecotoxicological studies (relative %)
(data collected from 94 articles published between 1996 and 2009; Santos *et al*. (2010))

2.6.4 The effects of pharmaceutical mixtures

Single compound studies can only go so far in predicting the total toxic burden or 'exposure totality' of aquatic organisms in the environment; Daughton (2003) describes this as the '4Ts' of toxicant-totality-tolerance-trajectory which accounts for an organism's complete exposure timeline. Environmental parameters, particularly pH, may have an influence on speciation which can influence the hydrophobicity and subsequently the bioavailability of pharmaceuticals but this has only rarely been investigated (Fent *et al.*, 2006). As well as the dynamic physico-chemical nature of the aquatic environment, pharmaceuticals are present not as individual compounds but as a multi-component mixture including a vast array of compounds, their transformation products and metabolites alongside other organic and inorganic pollutants (Fent, 2008). Furthermore, it is likely that the relative proportions of individual compounds in this mixture will fluctuate over time as the concentrations of pharmaceuticals in sewage effluent are known to be highly variable (Kanda *et al.*, 2003). Despite this, only a limited number of studies have been conducted looking at the effects of pharmaceutical mixtures and their toxicity to aquatic organisms.

Cleuvers (2003; 2004) evaluated the mixture toxicity of NSAIDs and other pharmaceuticals on *Daphnia magna*, algae and *L. minor*. These studies found that drugs

acting in combination follow the concept of concentration addition (CA) although the combination effect was slightly stronger for *Daphnia*. This is a very important conclusion as compounds present in the environment below their individual NOECs can still exert a toxic effect when acting in combination. Dietrich *et al*. (2010) tested the effects of a mixture of four diverse pharmaceuticals (carbamazepine, diclofenac, metoprolol and 17α-ethinylestradiol) on the moulting and reproduction of the freshwater amphipod *Gammarus fossarum*. The study found no significant effects on reproduction but some disruption to moulting and growth in control individuals.

Pharmaceuticals with similar mechanisms of action (MOAs) tend to follow concentration addition but those with dissimilar MOAs have been found to follow independent action (IA), where only those compounds with an effect at that particular concentration will contribute (Escher *et al*., (2005). A study by Dyer *et al*. (2000) suggests that cumulative baseline toxicity (narcosis) of mixtures with a large number of compounds may dominate over the combined effect of specific MOAs, particularly if the mixture components are below individual effect thresholds. Baseline toxicity is a non-specific disturbance of cellular membranes due to the solid-phase partitioning of hydrophobic pollutants and this constitutes the minimal toxicity of every chemical (Newman & Unger, 2003). Christensen *et al.* (2006; 2007) concluded that concentration addition was the dominant mixture model for antibiotics and antidepressants although some synergistic effects were present for combinations of erythromycin and oxytetracycline used in aquaculture. Overall, Cleuvers (2003) recommended that CA be assumed unless evidence indicates otherwise. Even mixture toxicity tests have a number of limitations for use in representing actual environmental conditions (Backhaus, 2008):

- Different degradation kinetics between compounds means the relative concentrations in the mixture will vary over time;
- Inability to infer the effects of individual compounds from the mixture;
- The mixtures are often relatively simple with <10 component compounds;
- The mixture is static with no compounds being added or lost whereas environmental mixtures are dynamic in time;
- Toxicity can only be inferred for the specific mixture(s) tested for and the results are not applicable for different mixtures.

Indeed, O'Brien & Dietrich (2004) suggest that the situation of pharmaceutical mixture toxicity is so complex that the most economically viable solution would be to simply

upgrade all European STPs; although this opinion has not been widely discussed amongst the scientific community or water industry.

2.6.5 Toxicity of pharmaceutical metabolites and transformation products

There have been relatively few studies focusing specifically on the environmental toxicity of pharmaceutical transformation products and metabolites to aquatic organisms. This is despite a recommendation in the EMEA (2006) guidelines on risk assessment to explicitly consider any metabolite that may be present at a concentration >10 % of the parent compound (Celiz *et al*., 2009). Perhaps the most widely studied is clofibric acid, an active metabolite of the blood lipid regulator clofibrate which is known to persist in the environment and has been the subject of a number of ecotoxicological studies (Halling-Sørsensen *et al*., 1998). Miao & Metcalfe (2003) monitored the occurrence of carbamazepine in surface waters; this drug is considered to be particularly problematic with approximately 30 associated metabolites. Just 3 % of the parent compound is excreted in its active form and this study revealed the occurrence of one metabolite at three times the concentration of carbamazepine. La Farré *et al*. (2008) conducted a review of ecotoxicological data with a particular focus on transformation products and suggested that more research effort be spent on these as some have been found to be more toxic or present in higher environmental concentrations than their parent compounds. For example, transformation products of paracetamol after sewage treatment were found to be up to 58x more toxic to mice when compared with the parent compound (Bedner & MacCrehan, 2005).

It is generally considered that transformation products or metabolites are more polar and hence less bioavailable, for example the phototransformation products of propranolol were found to be less toxic to algae and rotifers (Liu *et al*., 2009). However, the same authors recommend that risk assessment of a parent compound that is mostly or completely metabolised in the human body is not representative and that the risk assessment procedures should be more flexible allowing assessors to specifically target transformation products in such cases. Lienert *et al*. (2007b) conducted a modelling study using quantitative structure activity relationships to predict the mixture toxicity of parent compounds and metabolites for 30 pharmaceuticals. This review concluded that human metabolism generally decreases overall mixture toxicity for most pharmaceuticals but certain APIs have specifically acting metabolites which may serve to increase toxicity. Therefore, they recommended that metabolites be considered as an integral part of the risk assessment process.

2.6.6 Multiple stressors

Pharmaceuticals not only interact within mixtures of other pharmaceutical compounds but also the very large number of other chemicals in everyday use; this is estimated to

be between 30 000 and 100 000 in developed nations (Mihaich *et al.*, 2005). In addition to these myriad chemicals, aquatic organisms are exposed to other natural or man-made stressors which include:

- pollution (in its many forms);
- habitat degradation;
- reduced food availability;
- exotic or invasive species;
- disease;
- pH, temperature, DO, salinity, turbidity etc.;
- artificial flow regimes (increased flooding or excessive low flows).

Prioritising the ecological impact of pharmaceuticals relative to these other stressors is a difficult task but one that must be addressed in order that research and regulatory scrutiny can be focused best on those stressors having the greatest impact. Furthermore, it must be realised that different stressors can interact and influence each other. For example, changes in pH can alter the ionic state of pharmaceutical compounds which can in turn affect their bioavailability (Mihaich *et al.*, 2005).

2.6.7 Environmental relevance of toxicity studies

The assumption behind standardised laboratory tests is that through testing the most 'sensitive' species and applying safety factors the structural and functional components of the whole aquatic ecosystem are protected (Graney *et al.*, 1994). However, idealised single species tests cannot predict potential indirect effects in complex ecosystems (Brooks *et al.*, 2003). There are a wide variety of factors which can influence an organism's response during toxicological testing (Figure 2-11; Mason, 2002). The majority of these factors are not considered during standardised testing with only age, sex, water quality and life cycle being routinely accounted for. Environmental factors such as temperature and pH can also be added to this list which can strongly influence the speciation and uptake of toxic substances (Mason, 2002).

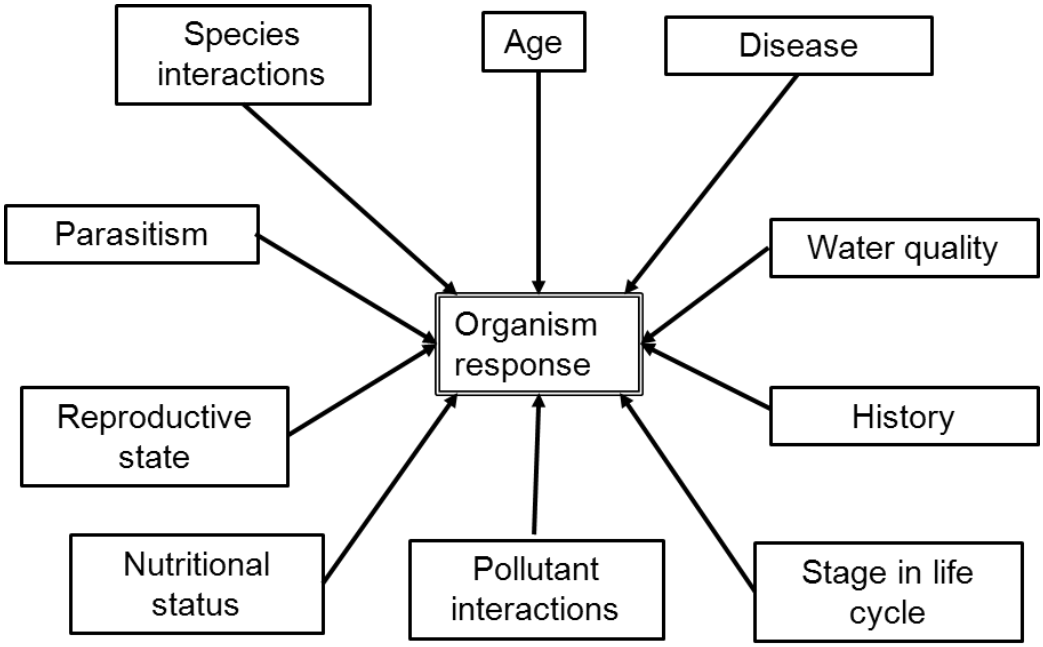


Figure 2-11: Factors influencing an organism's response during standard toxicity testing (adapted from Mason (2002))

Attempts have been made to simulate the environmental effects of pharmaceutical mixtures in model or artificial ecosystems known as microcosms and mesocosms. These can be either closed or open to the environment and may be placed in the field or laboratory. Mesocosms are generally larger than microcosms, with field installations which allow a part of the natural environment to be brought under experimental control. Mesocosms have the distinct advantage of being much more representative of natural conditions than studies in laboratory aquariums and hence are more ecologically relevant. This comes at the price of reduced replicability within the system, thus reducing the probability of detecting changes due to treatment (Rowe & Dunson, 1994). Despite these issues, mesocosms represent a vital part of the experimental continuum between simplified laboratory tests and field studies (Wilbur, 1989).

Mesocosms can also allow the inclusion of a representative community including bacteria, diatoms, algae, macroinvertebrates and possibly fish to adequately represent a natural system (Mihaich *et al.*, 2005). Sanderson et al. (2009) stated that the high cost per study makes sufficient replication difficult. This is in agreement with Kraufvelin (1998) who highlighted the natural variability in ecosystem variables and the often impractically high number of replicates needed to draw statistically robust conclusions from mesocosm studies. 'Aquarium individuality' is a phenomenon where two otherwise identical systems (mesocosms) are the same at the outset but diverge from one another over time due to small undetectable differences at the beginning of the study (Kraufvelin, 1998). Clearly, micro and mesocosms are extremely useful because they provide conditions of the natural environment whilst having capacity for replication and control

over experimental parameters but their limitations must be acknowledged (Graney *et al.*, 1994). Field studies have also been used to assess the effects of emerging contaminants on aquatic organisms. For example fish can be placed in cages downstream of STPs to test for the effects of oestrogenic chemicals (Jobling & Sumpter, 1993; Purdom *et al.*, 1994; Sumpter, 1995; Tyler *et al.*, 1998; Mihaich *et al.*, 2005).

2.6.8 Microcosm and mesocosm studies of pharmaceutical effects

Despite their obvious advantages, studies employing artificial ecosystems to examine the effects of pharmaceuticals on freshwaters are relatively sparse. Brain *et al*. (2004a; 2004b) conducted still water mesocosm studies using 12 000 L containers in which a semi-natural ecosystem containing algae, zooplankton, macrophytes and fish species was created. Twenty five individual compounds and an eight pharmaceutical mixture were tested on two aquatic macrophyte species (*Lemna gibba* and *Myriophyllum sibiricum*) over a 35-day period which showed that somatic endpoints were the most sensitive. Effects on growth were observed at high but environmentally relevant concentrations of >37 µg L^{-1} with antibiotics (chlortetracycline, lomefloxacin, levofloxacin and sulfamethoxazole) and the blood lipid regulator, atorvastatin, proving the most toxic. However, the observed effects were not above the biologically significant population threshold of 20 % mortality (Christman *et al.*, 1994). A further study by Laird *et al*. (2007) used the same microcosms to assess the toxicity of a mixture of three antidepressants on zooplankton and found that such mixtures did not pose a risk at environmental concentrations. This is contrary to the observed high acute toxicity of these compounds in isolation (see Sections 2.5.2 and 2.5.3). A further study by Richards *et al*. (2004) found that a three pharmaceutical mixture (ibuprofen, fluoxetine and ciprofloxacin) had diverse effects at environmentally realistic concentrations (≥6000 ng L^{-1}) across different trophic levels. The effects included: increased fish and macrophyte mortality and abundance, reduced diversity and community-level effects on plankton. Finally, Sanderson *et al*. (2007) used the same facilities to assess the impact of the veterinary drug ivermectin and concluded that 30 to 1000 ng L^{-1} caused significant reductions in zooplankton species richness but no corresponding decrease in total abundance. This was due to a dominance shift in *Cladocerans* from sediment-dwelling (*Chydorus* sp.) to more littoral and pelagic species; this is perhaps due to the high sorption potential of ivermectin in sediments (Sanderson *et al*., 2007). Wilson *et al*. (2003) used natural algal assemblages sampled from up and downstream of an STP outfall to examine the effects of ciprofloxacin and triclosan (an antimicrobial agent often used in cleaning personal care products) on algal community structure. Environmentally relevant concentrations (120 to 1500 ng L^{-1}) caused significant changes in both total algal biomass and community structure; the changes were less pronounced in the downstream samples where

continuous exposure to effluent may have increased the adaptation of algal communities. Furthermore, there was evidence of a dose-response relationship whereby increasing concentrations of these compounds were associated with a decrease in algal species richness. Brodin *et al*. (2013) used laboratory microcosms to expose perch (*Perca fluviatilis*) to environmentally relevant concentrations of the anti-anxiety drug oxazepam for seven days. Fish exposed at 1800 ng L^{-1} fed at a greater rate and were more active but less social. Fish exposed at 910 000 ng L^{-1} displayed increased boldness with potential secondary consequences for predation risk.

These examples highlight the value of experimental studies in identifying effects ranging from the individual to ecosystem level that would not be possible using standardised laboratory procedures. One of the main advantages of mesocosm and field studies is their potential to identify impacts which are mediated through complex intra and inter-specific interactions. A whole lake study conducted in Ontario, Canada found that additions of low levels of EE2 (5 to 6 ng L^{-1}) caused fathead minnow populations to collapse due to vastly reduced recruitment. This continued for three years after the addition of EE2 ceased (Kidd, 2007). Such a drastic effect on one species may have knock-on effects through the rest of the ecosystem. For example, McMahon *et al*. (2012) demonstrated the effects of fungicides (≥164 µg L^{-1} over four weeks) on a range of species, community and ecosystem level responses in a relatively complex mesocosm. They found that mortality was increased for algae, invertebrates, macrophytes and amphibians as well as reductions in leaf litter decomposition and net primary productivity. Applications of similar tests to the effects of pharmaceuticals at environmentally relevant concentrations are necessary if such complex, indirect effects are to be understood.

2.6.9 Functional indicators of stress response

Although, the micro and mesocosm studies mentioned above are more representative of environmental conditions when compared to simplified laboratory studies, the endpoints assessed are often the same with a focus on individual growth and mortality with often little consideration of ecosystem functioning. Functional measures have a number of key advantages over structural ones and furthermore it is possible for changes in structure to occur with no corresponding change in functioning and vice versa. Despite this, bioassessment is overwhelmingly based on structural measures alone (Matthews *et al.*, 1982; Gessner & Chauvet, 2002).

An often cited concern over the use of functional indicators for monitoring stressor impacts is their inherent environmental variability and reduced replicability (Hill *et al.*, 2000). However, Niemi *et al*. (1993) found that functional measures were generally more sensitive in detecting stressor impact and recovery. Despite this, most effects studies

continue to focus on single-species examinations of toxicity (Rosi-Marshall & Royer, 2012).

2.6.9.1 Leaf litter decomposition in freshwater ecosystems

This section will examine the importance of the decomposition of leaf litter in freshwater ecosystems and how this functional process has been adopted in the effects testing of pharmaceuticals. Inputs of allochthonous carbon to streams in the form of coarse particulate organic matter (CPOM) can be a major energy source in freshwater ecosystems. Input rates depend on latitude, season, riparian vegetation cover and rainfall (Giller & Malmqvist, 1998), varying from 7.5 g C m^{-2} y^{-1} for high order streams to 1394 g C m^{-2} $year^{-1}$ for low order streams (Conners & Naiman, 1984; Webster *et al.*, 1995). These inputs have been shown to be limiting in some streams (Richardson, 1991). The decomposition of leaf litter is influenced by: leaf species (more 'woody' plants take longer to decompose), water temperature, pH, water chemistry, flow and the community composition of macroinvertebrates, fungi and microorganisms (Giller & Malmqvist, 1998).

Decaying leaf litter from terrestrial inputs contains cellulose, lignin and other resistant carbohydrate compounds, which are not readily available as a food source to most aquatic animals. Therefore, the carbon within is released through three physical and biological processes, which can all act simultaneously (Webster & Benfield, 1986):

- Rapid leaching of dissolved organic carbon (DOC) and soluble inorganic constituents accounting for up to 25 % mass loss;

- Microbial and fungal colonisation and decomposition – these penetrate the leaf surface with hydrolysing enzymes capable of degrading cellulose which can convert up to 75 % of leaf mass into fine particulate organic matter (FPOM). Furthermore, these processes aid breakdown by macroinvertebrates through ‘softening’ the leaf tissue and increasing palatability;

- Biological and mechanical fragmentation – the physical action of flow can breakdown leaf tissue into FPOM but, more importantly, invertebrate shredders bite and feed on leaves releasing material as FPOM. The contribution of invertebrates to total decomposition can be >40 % where they are abundant. CPOM breakdown rates are higher when macroinvertebrates are present (Webster & Benfield, 1986). In turn, the activities of shredding macroinvertebrates serves to increase the surface area available for microbial colonisation (Giller & Malmqvist, 1998).

These processes aid the conversion of CPOM to FPOM and in turn to DOC which is then available as an energy source to other aquatic organisms. Without these conversions, the carbon would be unavailable at higher trophic levels and downstream transport would be reduced (Giller & Malmqvist, 1998). Disruption of detrital processing in low-order streams has been shown to significantly reduce the downstream export of organic material which can in turn affect downstream macroinvertebrate assemblages (particularly collectors and filterers). This has been demonstrated experimentally by Cummins *et al*. (1973) and Cuffney *et al*. (1990). Indeed experiments have shown that carbon storage, processing and downstream transport occurs predominantly in low-order streams whereas the majority of carbon consumption occurs in higher-order streams, highlighting the importance of this upstream-downstream link (Naiman *et al.*, 1987; Cuffney *et al.*, 1990). Therefore, the decomposition of leaf litter is a key ecosystem function with important implications for energy flow and nutrient cycling (Giller & Malmqvist, 1998). For example, Wallace *et al*. (1997) showed that removing leaf litter inputs leads to a 40 % reduction in secondary production and abundance drops for shredders, collectors and predators. This clearly demonstrates the potential for changes in leaf litter breakdown to act as a bottom-up control cascading to higher trophic levels.

The second of the three phases of Webster & Benfield's (1986) conceptualised decomposition regime includes direct decomposition by fungal and microbial communities as well as the softening of leaf tissue for macroinvertebrate shredders. Irons *et al*. (1994) showed that as temperature decreased (with increasing latitude and altitude) the relative importance of microbial/fungal processing decreased when measured as a proportion of total decomposition rates from up to 70 % at 35 ° latitude to approximately 10 % at 65 ° latitude. However, even when the fungal and microbial stage is less important in terms of total mass of decomposition there are still significant implications for food quality and selection by invertebrates (Graça *et al.*, 2001). Studies have shown that macroinvertebrate shredders may actually derive a small but significant proportion of their dietary energy requirements from the fungal and microbial communities which have colonised decaying leaf tissue rather than from the leaves themselves (Merritt *et al.*, 1984). Furthermore, the shredder *Gammarus* has been shown to actively select leaf tissue that has been pre-conditioned by fungal/microbial communities both in the laboratory and in field studies (Graça, 1993; Graça *et al.*, 2001). There are a number of possible reasons why *Gammarus* actively choose pre-conditioned leaves (Giller & Malmqvist, 1998) which include: 'softening' of leaf tissue rendering it more amenable to shredding, fungal and microbial communities lending a more palatable 'flavour' to the leaf tissue, and the increased energy contained within the fungal/microbial colonisers.

The relative abundance of fungal and bacterial communities present on colonised leaves may also influence overall decomposition rates. Studies have shown that fungi dominate the microbial biomass colonising leaf litter (fungi accounted for up to 99.8 % of biomass) and that increasing nutrient concentrations stimulated leaf litter decomposition (Gulis & Suberkropp, 2003a; Gulis & Suberkropp, 2003b). However, when fungi were excluded (and hence only bacteria were present) the decomposition rate was reduced, particularly at high nutrient concentrations. This suggests that fungi are overwhelmingly dominant in terms of colonisation (biomass) and microbial decomposition and that bacteria are subject to strong competition for the available resources of carbon in leaf litter.

2.6.9.2 The effects of pharmaceuticals on leaf litter decomposition

There are very few examples of studies that assess the effects of pharmaceuticals on ecosystem structure and functioning which can in turn impact on important ecological factors such as resilience, redundancy, nutrient cycling and energy availability. One exception is the study of Bundschuh *et al*. (2009) which tested the effects of a mixture of five antibiotics on the food selection and decomposition of leaf litter by *Gammarus*. This study found that breakdown of antibiotic-treated leaf disks was greater than control disks, possibly due to increased conditioning of the leaves by fungal communities triggered by competitive release due to a reduction in the colonising bacterial communities. It is also possible that the bacterial communities developed resistance to the antibiotic compounds during the treatment period (as demonstrated for soil bacteria exposed to a sulphonamide antibiotic by Schmitt *et al*. (2005) but the initial competitive advantage gained by colonising fungi during the early stages was sufficient for them to maintain dominance. It is also reasonable to hypothesise that reduced nutritional quality of leaf material may lead to an increase in processing by detritivores to maintain necessary dietary intake (Maul *et al.*, 2006a). Hahn & Schulz (2007) found the opposite effect from a mixture of two different antibiotics (oxytetracycline and sulfadiazine) whereby *Gammarus pulex* preferred leaves conditioned in the absence of antibiotics but this caused no change in the total decomposition rate. Notably, neither of these studies conducted parallel exposures of individual compounds in isolation so it is not possible to attribute changes to specific antibiotics or to evaluate any additive or antagonistic effects of these compounds in mixture. Furthermore, no studies are currently available which examine the effects of non-antibiotic pharmaceuticals on the breakdown of leaf litter in freshwaters.

2.6.9.3 Sediment respiration, carbon and nutrient cycling

Early studies of DOC in stream sediments showed that concentrations were generally higher in groundwater and sediment pore waters when compared to the overlying waters

(Moss, 1988). This shows that stream sediments are biologically active zones which modify the chemical nature of water passing through them and act as both sinks and sources for carbon and nutrients from both upwelling groundwater and down welling stream water (Rutherford & Hynes, 1987). The majority of metabolic activity in small streams occurs within bed sediments, either at the sediment-water interface or in the deeper hyporheic zone (Sobczak & Findlay, 2002). The microbial processes within sediments play a major role in carbon and nutrient cycling in rivers. This is of particular importance as the downstream fluxes of DOC are frequently the largest components of stream organic matter budgets (Rutherford & Hynes, 1987).

Respiration occurring in streams, even with relatively small hyporheic zones has been shown to account for more than 40 % of whole ecosystem respiration. This process can increase overall ecosystem efficiency by allowing more rapid recycling of carbon and nutrients that would otherwise be lost to downstream transport (Battin, 1999; Battin *et al.*, 2003). The degree to which the hyporheic zone can influence whole river ecosystem functioning depends on a number of factors including: physical dimensions, biogeochemistry, temperature, oxygen, nutrient supply and the proportion of total flow rate passing via the hyporheic zone. Many important ecosystem functions (primary productivity, respiration and nutrient cycling) occur either at the sediment-water interface or within the top few centimetres of the hyporheic zone (Battin, 1999; Battin *et al*., 2003).

There are two general mechanisms of carbon supply to stream sediments; these are the burial of POC during episodic disturbances and the direct transport of suspended POC and DOC via intrusions from river water or groundwater (Findlay, 1995). Coarse and fine POC can be directly utilised by invertebrates but this is not often the case for DOC where some form of microbial transformation into a particulate form is necessary. The relatively slow velocity of pore waters (when compared to the overlying stream water) can allow ample time for the microbial metabolism of bioavailable solutes including DOC and nutrients. Sobczak & Findlay (2002) have shown that DOC losses along hyporheic flowpaths can be as high as 50 % and this depends predominantly on microbial metabolism and less so on other parameters such as dilution, residence time and initial DOC concentrations.

2.6.9.4 The role of microbial communities in decomposition and nutrient cycling

Rosenfeld & Roff (1991) have shown that respiration by benthic microbial communities (particularly bacteria) can exceed whole stream primary production in forested streams. These communities consist of autotrophic filamentous algae, cyanobacteria, diatoms, fungi and heterotrophic bacteria. The major energy source for bacterial communities is DOC leached from detritus, excreted by consumers or released by their own enzymatic degradation of detritus (Miller, 1987). These communities are responsible for significant

energy fluxes and nutrient cycling both within the hyporheic zone and in exchange with overlying waters (Giller & Malmqvist, 1998). Therefore, any change in benthic respiration by microorganisms has the potential to significantly alter the stream P/R ratio (e.g. Wilson *et al*. (2004)).

Smith (1973) estimated that bacteria were responsible for the majority of sediment respiration (30 to 60 %) whereas macrofaunal respiration accounted for just 5 to 26 %. The major energy source for bacteria is DOC and thus the transfer of energy and nutrients through this 'microbial loop' is particularly important as only a few animal species are capable of directly assimilating DOC (Giller & Malmqvist, 1998). The disruption of microbial cycling in sediments can reduce ecosystem productivity and cause the sediment to become a carbon sink, rendering organic carbon and nutrients unavailable for secondary productivity which will have knock-on effects via food web interactions (Burton, 1991). Microbial communities present within pore waters play a major role in both the respiration of sediment and DOC cycling required making it available to other trophic levels. Therefore, pharmaceuticals (particularly those designed to target microorganisms such as antibiotics and antifungals) have the potential to alter or disrupt these sediment dwelling organisms which in turn may impact on the important ecosystem functions they perform.

2.6.9.5 The effect of stressors on sediment respiration and nutrient cycling

Community respiration (CR) in stream sediments is a widely measured attribute of ecosystem functioning that has been shown to be a sensitive indicator of ecosystem stress (Hill et al., 1998). Studies comparing CR to other functional attributes in stream ecosystems have shown it is the most sensitive to perturbations from a range of stressors (Matthews *et al*., 1982). For example, Crossey *et al*. (1988) showed CR was significantly increased (by of 2-3x) in a Montana stream experiencing chronic heavy metal pollution but structural indices of the periphyton community did not show any change. Bunn *et al*. (1999) found that both gross primary production (GPP) and CR were highly sensitive to changes in catchment land use. Reductions in riparian vegetation cover lead to increases in both processes and crucially a shift in affected ecosystems from being net carbon consumers (P<R) to net producers (P>R).

Bunch & Burnot (2011) examined the effects of ibuprofen and paracetamol at environmentally realistic concentrations (100-1000 ng L^{-1} and 55-10000 ng L^{-1}, respectively) on sediment respiration and nitrate uptake using both *in vitro* and *in situ* techniques. *In vitro* exposures demonstrated increased respiration rates at low levels of both pharmaceuticals and a slight inhibition of respiration at the highest exposure of paracetamol. Furthermore, lower levels of paracetamol appeared to enhance nitrate uptake. None of the above differences were evident in the more variable *in situ*

experiments although this may be due to the long exposure history to pharmaceuticals at the site yielding greater adaptation and tolerance in the microbial community. The picture is further complicated as different pharmaceutical compounds may be stimulatory (due to the uptake of pharmaceuticals as a nutritive source) or inhibitory (due to direct toxic effects) although this is dependent on specific compound properties (Lawrence *et al.*, 2005). Certain antibiotics (amoxicillin, clarithromycin and erythromycin) have been shown to reduce the denitrification rates of specialised sediment-dwelling bacteria although this effect was only seen at relatively high concentrations (1 mg L^{-1}; Costanzo *et al*. (2005)). Similar effects are widely known to occur within nitrogen cycling processes in STPs (Gomez *et al.*, 1996; Campos *et al.*, 2001). Despite the inherent complexities in such processes there is ample evidence that low levels of pharmaceutical compounds can have a significant impact on stream microbial activity and the uptake of nitrogen which may have further implications for autotrophic production and algal blooms (Dodds & Welch, 2000).

2.6.9.6 The effects of pharmaceuticals on freshwater microbial communities and processes

Antibiotic compounds are known to cause effects on microbial community structure in both the soil and water environment as well as changes in associated ecosystem functions such as nitrogen cycling, methanogenesis and sulphate reduction (Ding & He, 2010). Maul *et al*. (2006a) found that environmentally relevant concentrations of the antibiotic ciprofloxacin (100 000 ng L^{-1}) caused a significant shift in microbial community composition and a decrease in microbial respiration. However, this change in microbial communities did not translate to any significant change in leaf litter decomposition or growth of two detritivore species (*Gammarus* spp. and a caddisfly species, *Lepidostoma liba*). There were three possible reasons for this:

- The complex matrix of water, sediment and leaf material used in the experiment may have reduced the bioavailability of ciprofloxacin;

- Functional redundancy within the microbial communities (i.e. fungi) may have persisted and provided the necessary enzymes for leaf breakdown and the additional nutritional source for detritivores (Wohl *et al.*, 2004);

- Some detritivores (e.g. *Gammarus*) may be able to endogenously produce the necessary enzymes to digest leaf material or to rely on endosymbionts within their gut (Monk, 1977; McGrath & Matthews, 2000)

All of these studies demonstrate the potential for pharmaceuticals (and antibiotics in particular) to have a direct impact on leaf litter breakdown which is widely considered to

be an important source of organic matter and energy in streams (Cummins *et al.*, 1973; Petersen & Cummins, 1974; Cummins & Klug, 1979). However, there are further areas that require research, namely using more realistic exposure concentrations at environmentally measured levels. For example, both Bundschuh *et al*. (2009) and Hahn & Schulz (2007) experiments used 20 000 to 200 000 ng L^{-1} exposures whereas median environmental concentrations are generally <1000 ng L^{-1}. However, there are examples of highly polluted rivers receiving effluent from pharmaceutical manufacturing facilities where concentrations well exceed the LOECs observed in the laboratory (Li *et al.*, 2008; Fick *et al.*, 2009; Lin & Tsai, 2009; Hughes *et al.*, 2013). Even for less polluted rivers, the margin of safety is just one or two orders of magnitude and more research is clearly needed. Furthermore, no consideration has been given to other pharmaceutical classes (e.g. NSAIDs or beta-blockers). Finally, the studies mentioned above were conducted entirely in the laboratory under controlled conditions whereas actual rates *in situ* may be impacted differently under dynamic environmental conditions. One particular question is how pharmaceutical exposure during the leaf pre-conditioning process will affect colonisation by whole macroinvertebrate communities and not just isolated species of detritivores in the laboratory (e.g. Arsuffi & Suberkropp, 1989).

Antibiotics have also been shown to impact on other aspects of ecosystem functioning; for example, Wilson *et al*. (2004) showed that a mixture of four tetracycline antibiotics increased CR in a microcosm study at low concentrations. However, gross primary productivity (GPP) was less affected resulting in a reduced community metabolism (P/R ratio). Also the net daily metabolism (P-R) resulted in an oxygen deficit which increased with concentration. Such changes in the balance between production and respiration are common in stressed ecosystems and indicate an inefficient state as more oxygen is consumed than is produced by autotrophs (Odum, 1985).

Another study by Näslund *et al*. (2008) indicated that the antibiotic ciprofloxacin had a negative impact on the bacterial diversity in marine sediments which in turn reduced the degradation rate of an organic contaminant (pyrene). Although the relationship between bacterial community structure and function is complex (Bell *et al.*, 2005) the degradation of pollutants is an important ecosystem service with obvious socioeconomic value (Costanza *et al.*, 1997). As above, no studies have been conducted into how non-antibiotic pharmaceuticals affect community respiration and potential knock-on effects. For example, the microbial cycling of dissolved organic carbon and nutrients within sediment pore waters and subsequent exchange with overlying stream water (Sobczak & Findlay, 2002).

Pharmaceuticals may also alter inter-specific interactions within aquatic ecosystems. For example, Morley *et al*. (2009) conducted a review of literature on the impacts of pharmaceuticals on aquatic host-parasite relationships. This review

concluded that some pharmaceuticals may alter host physiology and immunology rendering them more susceptible to parasites and that some parasites may be developing resistance to certain compounds. Painter *et al*. (2009) conducted a study which used high speed video recordings to examine the changes in predator avoidance speed of fish exposed to mixtures of antidepressants at ng L^{-1} concentrations. This study found that certain individual compounds (e.g. fluoxetine) did not slow predator avoidance but slowing was consistent when the antidepressants were mixed; such a toxic effect may have important indirect effects on survival and reproduction that have yet to be determined.

Pharmaceuticals clearly have the potential to impose subtle changes on individual behaviour, survival and reproduction, which may in turn manifest at the population level and be further mediated via food-web interactions to yield an effect at the ecosystem level. Parallel examples and lessons can be drawn from trophic interaction studies for non-pharmaceutical compounds. Maul *et al*. (2006b) demonstrated the trophic transfer and subsequent bioaccumulation of PCBs (polychlorinated biphenyls) via sediment-dwelling aquatic insects to tree swallows (*Tachycineta bicolor*). This transfer route may also be active for pharmaceuticals present in rivers and aquatic sediments, particularly those compounds with high log K_{OW} (i.e. more lipophilic compounds). However, elucidating these extremely complex interactions will be difficult and much further research is required although it is vital that experiments should combine organism-level effects with structural and functional indicators and food-web interactions in order to provide a more holistic and relevant understanding of real world processes (Matthews *et al.*, 1982; Brown *et al.*, 2011).

2.6.10 Limitations of current ecotoxicological testing procedures

The field of ecotoxicology draws many of its principles from human and mammalian toxicology which rightly focus on the protection of individuals from toxic effects. This is less relevant from an ecological perspective where greater emphasis should be placed on impacts at the population, community and ecosystem level (Calow, 1998). A possible exception to this would be the protection of critically endangered species in a particular area (Dorne *et al*. 2007). However, very few attempts have been made to directly relate the toxic effects of pharmaceuticals from individuals up to population level and beyond. This could be achieved using life-history models, such as the Euler-Lotka equation, to predict population multiplication rates (Calow, 1997). In the absence of reliable population-level effects data, two approaches are available to extrapolate individual toxicity data to that at a ‘systems’ level but these are based on a number of key assumptions:

- The Assessment Factor (AF) approach simply divides the lowest available single-species toxicity (*i.e.* LOEC) by an arbitrary amount considered safe for the ecosystem. A similar approach is adopted during risk assessment to extrapolate acute to chronic ratio (ACR) data. The value of the AF or ACR depends on available experimental data and is arbitrarily defined with no explicit ecological basis (Dorne et al., 2007). Ankley *et al*. (2005) call for a high quality database of AFs and (ACRs) for a range of species, MOAs and endpoints;

- The Species Sensitivity Distribution (SSD) approach fits a statistical distribution over all available toxicity data. The usual assumption is that that protection of 95 % of individuals will maintain a viable population. This assumes that the SSD calculated from known data is representative of all species, particularly the most sensitive ones (Posthuma, 2002).

However, the species used in standard toxicity tests are typically selected more for their amenability to laboratory culture rather than any direct ecological relevance (Fent, 2008). A test species should be representative of the aquatic ecosystem in question and its sensitivity should be high to ensure other (less sensitive) species are protected (Newman & Unger, 2003). A modelling study conducted by Stark *et al*. (2004) concluded that *Daphnia* spp. were the least sensitive amongst six other arthropod species; this suggests levels deemed non-toxic to *Daphnia* individuals or populations in the laboratory may not be protective of other species in the aquatic environment. Henschel *et al*. (1997) tested the relative sensitivity of standard (i.e. acute) and non-standard toxicity tests to four pharmaceuticals and found that for three compounds it was the non-standard methods that were most sensitive. These studies indicate that the standard battery of toxicity tests may be underestimating the potential for toxic effects of pharmaceuticals in the environment (Länge & Dietrich, 2002; Kümmerer *et al.*, 2004b).

Attention must also be drawn to the concept of NOECs etc. which are actually just indicators of a statistical difference between treatments within an experiment (which in turn are strongly influenced by experimental design) and do not have any direct mechanistic, ecological or toxicological relevance (Newman & Unger, 2003). Jager (2012) calls the widespread use of NOECs an "*outdated...bad habit*" that should be replaced with more widespread use of mechanistic models. Furthermore, the concept of a NOEC implies there is a threshold concentration below which no adverse effect will occur; known as the 'threshold model' (Newman & Unger, 2003). Some toxic stressors (such as radiation) are believed to have no threshold as cancerous tumours can be initiated from damage to the DNA in a single cell resulting from a single exposure event (Newman & Unger, 2003). The same may be true for anti-cancer drugs (antineoplastics or cytotoxic drugs) which are known to have fetotoxic, genotoxic and teratogenic

properties (Kümmerer, 2001a). This would mean that a single exposure to an extremely low dose would have a probability (albeit very low) of eliciting a toxic effect to an individual.

2.6.11 How to improve the ecotoxicological testing of pharmaceuticals

The above review of ecotoxicological data has identified a distinct bias towards idealised laboratory experiments focusing on short-term, acute endpoints on a small number of individual, standard species. These tests are relatively easy and cheap to conduct and are useful for inter-comparisons but they do not adequately capture the diverse range of toxicological effects that pharmaceuticals can cause. Fent *et al*. (2006) suggest that ecotoxicological studies be much more targeted based on existing information that is readily available from human and mammalian studies during the pharmaceutical development process, this includes:

- Known receptors or target molecules;
- Known side effects;
- Known drug-drug interactions as a possible indicator of mixture toxicity;
- Pharmacokinetic properties (Adsorption-Distribution-Metabolism-Excretion: ADME) i.e. what the body does to the drug;
- Pharmacodynamic properties (drug-receptor interactions and the relationship between dose and effect) i.e. what the drug does to the body;
- Any known specific MOAs.

Targeting studies based on MOAs and receptor sites is particularly promising as many of these are known to be strongly evolutionarily conserved and can be present in lower animal forms such as fish and invertebrates (see Fent *et al*., 2006). For example, NSAIDs work by inhibiting one or both of the two isoforms of the cyclooxygenase enzyme (COX-1 and COX-2) which regulate the synthesis of prostaglandins which in turn play a role in pain and inflammatory response. COX enzymes occur in fish but their role in prostaglandin synthesis is not fully understood in lower invertebrates. This may mean that NSAIDs inhibit prostaglandins in the same way but the resultant effect is different (Fent *et al*., 2006). Triebskorn *et al*. (2007) conducted a more targeted study looking at the structural changes caused by pharmaceuticals in the liver, kidney and gills of fish; they discovered that diclofenac caused a particularly strong reaction and eventually induced cell death (necrosis). The LOECs derived from this study were up to three orders of magnitude lower than those derived from the more traditional tests suggesting

that standardised tests may be missing toxic effects or quite dramatically underestimating their toxic potential in surface waters (Kümmerer *et al*., 2004b). Certain classes of pharmaceuticals have also been shown to inhibit the cytochome-P450 (CYP) enzymes in fish; this is a widely preserved metabolic pathway for the removal of xenobiotics and is thought to be the major oxidative system for most pharmaceuticals (see Dorne *et al*., 2007). For example, antidepressants were shown to inhibit more than 90 % of a particular CYP isoform having potential implications for metabolism and accumulation of toxins within the organism.

Despite the usefulness of targeted studies the lack of a similar receptor sites in aquatic organisms does not necessarily mean the compound in question will not cause adverse effects. We know from human toxicology that these can be caused by previously unrecognised interactions and unknown receptor sites which are poorly characterised in aquatic organisms; this means that the transfer of data from human and mammalian toxicology should be done with caution (Cleuvers, 2005). Finally, Owen *et al*. (2010) highlight the importance of repeating studies prior to drawing important conclusions about potential environmental effects. This study repeated a 28 day growth test of Rainbow Trout (*Oncorhynchus mykiss*) exposed to clofibric acid; the initial experiment found a concentration-dependent reduction in growth at exposures greater than 100 ng L^{-1}. However, a larger repeat study found no such effect on growth. This highlights the importance of considering data in detail, particularly when no repeats are available or when comparing what would otherwise appear to be similar studies.

2.6.12 Complex and unexpected effects of pharmaceuticals in the environment

Even with knowledge of MOAs and presence of appropriate receptors in non-target organisms, there is still the potential for pharmaceuticals to elicit unexpected effects due to receptor systems performing different functions in these organisms. The class of antidepressants known as SSRIs (selective serotonin reuptake inhibitors) are an excellent example of this (Fong, 2001). Serotonin is a neurotransmitter involved in regulating hormonal and neuronal mechanisms but is also important for feeding and reproduction (Berger *et al.*, 2009). SSRIs are used as antidepressants in humans but serotonin is well conserved even amongst lower invertebrates and can affect such diverse functions as immune response, appetite, behaviour and sexual function (Fong, 1998a; Fong *et al.*, 1998b). However, some SSRIs are known to strongly affect the reproductive behaviour of shellfish whereas others have little or no effect (Fong, 2001). Brodin *et al*. (2013) found that low concentrations of the anti-anxiety drug oxazepam increased the boldness but reduced the social activity of European perch (*Perca fluviatilis*) with implications for predation and feeding rates. These complex, adverse and unexpected effects in non-target organisms are likely to increase as newly developed

pharmaceuticals begin to target much more fundamental receptors including individual proteins or mechanisms involved with signal transduction or cell division all of which are very well conserved in evolution (Seiler, 2002).

Indirect effects may also be propagated through different trophic levels via inter-specific or predator-prey interactions. De Lange *et al*. (2006) found that fluoxetine and ibuprofen reduced activity of *G. pulex* at 10-100 ng L^{-1} whilst increasing activity at higher concentrations 1000 to 1 000 000 ng L^{-1}) due to stress or flight response (i.e. movement away from a stressor). Reduced activity could have subsequent effects on feeding and energy uptake and on predator avoidance without being directly lethal to *Gammarus* individuals. Initially, this may be beneficial to predators due to increased prey consumption but would then become detrimental due to overexploitation of the prey resource. Studies have also shown that pharmaceuticals can have indirect effects on survival and reproduction mediated through processes such as host-parasite relations and predator avoidance (Morley *et al*., 2009; Painter *et al*., 2009).

2.6.12.1 The need to consider biological uptake, bioavailability and bioaccumulation

Current aquatic ecotoxicological methods quote the final toxicity of a compound in terms of its aqueous concentration in the surrounding water (e.g. LOEC = 1000 ng L^{-1}). Such concentration values give no direct indication of uptake and accumulation within the test organism's tissue (and/or blood and plasma) and assume that the concentration in the biological compartment is at steady state with the surrounding water. However, many factors can affect the uptake and potential bioconcentration/bioaccumulation of toxic compounds (Mace, 2002) including:

- bioavailability of the compound (influenced by temperature, pH, solid phase partitioning, log K_{OW} etc.);
- temperature and the subsequent impacts on organism physiology;
- other organism and species specific factors such as size, age, sex, diet and lipid content;
- internal fate and therefore the internal active dose at the receptor site, this may be dependent on the plasma half-life and the degree and rate of metabolism (Mihaich *et al*., 2005). Huggett *et al*. (2005) suggest using the plasma active dose derived during mammalian and human screening as an indicator of potential activity thresholds in aquatic organisms.

There is a requirement to test uptake into fish from surrounding water when a drug's log K_{OW} exceeds 3 (EMEA, 2006). However, this requirement is waived if the substance is rapidly degraded in the environment (Koschorreck & Hickmann, 2008) but even readily

degraded compounds that are 'pseudo-persistent' may still have the potential to bioconcentrate. Such standardised tests cannot account for the natural variations in uptake to tissue (for the myriad reasons listed above). In particular they do not account for important environmental parameters (such as pH) which can strongly affect speciation and subsequent bioavailability of acidic or basic pharmaceuticals (Fent, 2008).

Due to these myriad factors, uptake and bioconcentration of pharmaceuticals vary dramatically between compounds and species. For example Schwaiger *et al*. (2004) found the bioconcentration factor (BCF) of diclofenac varied between 3 and 2732 in brown trout. The final BCF was dependent on the surrounding water concentration and on the tissue type analysed with liver tissue demonstrating the highest BCF**;** this is despite diclofenac having a low log K_{OW}. An additional study by Brown *et al*. (2007) used the Fish Plasma Model (FPM) to predict uptake into *O. mykiss* and compare these with laboratory and field tissue samples. The NSAIDs (and the blood lipid regulator gemfibrozil) were selected as their log K_{OW} range meant they were sufficiently soluble to avoid sorption to sludge during sewage treatment but lipophilic enough to bioconcentrate. This study found marked differences in BCF between drugs and field sites sampled with the lowest BCF of 5 measured for diclofenac ranging to a BCF of 18 667 for ibuprofen at the same site. These values were considerably higher than the BCFs derived in the laboratory which ranged from 0.1 to 63.0. Environmental conditions (temperature, pH), pharmaceutical mixtures and the presence of colloidal material are likely to be the reasons for such striking differences (Brown *et al*., 2007). The FPM model generally underestimated BCFs due to it being developed for non-ionic, lipophilic pollutants. Underestimation in such a model may render it unsuitable for risk assessment of pharmaceuticals where it is often best to overestimate risk particularly during early screening stages. Dussault *et al*. (2009) found that the BCF of EE2 ranged between 0.8 and 142 depending on species (*Chironomus* or *Hyalella*) and whether the exposure route was water-only or via sediment. These studies indicate the complex nature of uptake and bioconcentration of pharmaceuticals in complex environmental systems.

2.6.12.2 The development and use of biomarkers

The use of biomarkers for EDCs is relatively well established in fish and invertebrates. The most widely used is vitellogenin, a precursor to the egg-yolk proteins (vitellins; see Matozzo *et al*. (2008) for a review). However, such a specific biomarker is not available for pharmaceuticals given their diverse nature. An attempt at identifying biomarkers of exposure in mussels to three pharmaceuticals (carbamazepine, caffeine and methotrexate) and an extract of STP effluent was made by Martin-Diaz *et al*. (2009). This study used enzymes associated with phase I and II metabolism, oxidative stress

and DNA damage as indicators of exposure and found that varying effects were induced depending on the compound and exposure concentration. However, such biomarkers would not necessarily be solely indicative of pharmaceutical exposure in the natural environment given the complex nature of STP effluent which contains myriad other xenobiotics capable of inducing oxidative stress and xenobiotic metabolism pathways.

One possible answer to this question is the emerging field of metabolomics; the study of chemical metabolic fingerprints left behind by cellular processes which can detect minute but important disturbances that other techniques may miss (Daviss, 2005). Rapid advancements in analytical techniques such as chromatography and nuclear magnetic resonance (NMR) spectroscopy mean that reliable quantification of a large number of metabolites (i.e. the metabolic profile or 'metabolome') is now possible (Fernie *et al.*, 2004). A metabolite is defined as a small molecule (< 2.5 kDa in size) that is either an intermediate or the product of metabolism (Preti, 2005). Metabolic profiling can be used to quantify both an organisms 'normal metabolic operating range' (Viant, 2007) and to detect the physiological and metabolic changes caused by specific stressors such as temperature, pH or the toxic action of a specific compound or mixture (Robertson, 2005).

The application of these techniques to characterise an organism's response to natural and anthropogenic stressors is known as environmental metabolomics. When an organism becomes stressed or is exposed to a toxicant this triggers specific molecular changes, altering its phenotype, which can in theory be measured and quantified. Environmental metabolomics is the newest of the suite of so called 'omics' techniques alongside genomics, transcriptomics and proteomics (Viant, 2007). Omics offer a key advantage over more traditional biochemical methods in that they allow rapid and simultaneous assessments of tens, hundreds and even thousands of endpoints (metabolites) which, coupled with statistical methods such as principal component analysis (PCA), make it a powerful tool in discovering biomarker profiles of toxicant exposure, disease and stressor response (Viant *et al.*, 2008). However, it is estimated that even the best analytical techniques can currently only detect around 1000 of the existing metabolites (Viant *et al.*, 2003a) and practitioners should stay well acquainted with this rapidly developing field. Environmental metabolomics studies tend to focus more on questions rather than hypotheses which allows for the discovery of unexpected relationships and responses which in turn can generate hypotheses about the underlying biochemical causes (Bundy *et al.*, 2009).

2.6.12.3 NMR spectroscopy

There are a range of analytical techniques available to metabolomics researchers including the hybrid technologies: gas chromatography or liquid chromatography coupled with mass spectrometry (GC-MS and LC-MS), nuclear magnetic resonance (NMR)

spectroscopy, capillary electrophoresis, Fourier transform infrared spectroscopy and direct infusion mass spectrometry (Koek *et al.*, 2011). The two most widely adopted analytical methods are NMR and LC-MS both of which have key but differing advantages over other techniques. NMR is considered to be a high throughput, highly reproducible, platform requiring minimal sample preparation; however compared to LC-MS it is less advantageous in terms of sensitivity and the number of resolvable metabolites (Robertson, 2005; Aliferis & Chrysayi-Tokousbalides, 2011). Scalbert *et al*. (2009) found that 62 % of metabolomics studies in human nutrition used proton (^{1}H) NMR despite this technique only being able to identify around 60 specific metabolites compared to >1000 from MS-based techniques (Lindon & Nicholson, 2008a; Lindon & Nicholson, 2008b). A further key advantage of NMR spectroscopy is the ability to derive absolute concentrations of metabolites by adding an internal standard of known concentration; this is not as straightforward with MS due to variable ionisation and ion suppression effects (Lindon & Nicholson, 2008b).

2.6.12.4 Why environmental metabolomics?

The most important characteristic which sets environmental metabolomics apart from the other omics techniques, which makes it particularly suited to characterising organism stressor response, is the degree to which the metabolome varies under normal and stressed conditions (Viant, 2007). It is the first to respond to stressors often when no changes in the transcriptome or proteome occur. The metabolome therefore can act as an indicator of organism's energetic, oxidative and reproductive state. Genes and transcripts do not manifest functional changes at the organismal level and are much more suited to characterising population structure and genealogy. This dynamism and responsiveness is a key advantage but also one the major obstacles when it comes to producing useable data. This is due to high levels of metabolic 'noise' as metabolite concentrations vary due to differences in individual genetics, fluctuating environmental and stressor conditions, diet, age and sex (Viant, 2007). Separating differences between stressed and healthy individuals from this noise is the biggest challenge to using metabolomics as an indicator of toxicity. Controlling for such traits as strain, sex and age (known as genotypic or phenotypic anchoring) may play a part in addressing this (Viant, 2007). For example, studies with marine mussels have shown that variability in the metabolome was more pronounced in species transferred from the laboratory and that gender was the principal source of variation (Hines *et al.*, 2007). They recommended the use of directly field-sampled individuals to reduce metabolic variability for toxicological testing.

Despite such 'noise' metabolomics has been applied to a range of organisms and has demonstrated considerable success in identifying aspects of biological, chemical

and temperature stress (Viant *et al.*, 2003; Viant, 2007). Viant *et al.* (2003a) were the first to apply metabolomics techniques (^{1}H NMR) to aquatic organisms which was successful in distinguishing between healthy and diseased groups of the marine gastropod red abalone (*Haliotis rufescens*). Williams *et al.* (2009) exposed a freshwater fish to low levels (50 000 ng L^{-1}) of the polycyclic aromatic hydrocarbon dibenzanthracene for four days and were able to detect very small (6-24 %) but significant changes in four metabolites (taurine, malonate, glutamate and alanine). Similar techniques have been successful in identifying toxic effects of pesticides and industrial solvents on freshwater fish (Viant, 2005; Viant *et al.*, 2006; Viant *et al.*, 2006). Numerous studies have also demonstrated significant effects of low-level exposure to EE2 (Samuelsson *et al.*, 2006; Ekman *et al.*, 2008). Short-term exposure of fathead minnow (*Pimephales promelas*) to 10 ng L^{-1} caused perturbations in energy metabolism and liver toxicity (Ekman *et al.*, 2008). A study by Bundy *et al*. (2007) sampled earthworms from a range of UK sites and found that NMR metabolomics could resolve individual sites and that certain specific metabolites correlated with zinc contamination of soils across all the sites. This conclusion lends support to the use of environmental metabolomics as a diagnostic tool in ecotoxicology.

Viant *et al*. (2008) conducted an inter-comparison exercise between NMR laboratories in the USA, Canada, UK and Australia and concluded that NMR metabolomics produces sufficiently reproducible results to justify its continued use in environmental science and regulation. Despite the progress in recent years, environmental metabolomics is still a growing field and researchers are still a long way from being able to routinely infer underlying biochemistry from metabolic profiles. This linking of metabolomics to clear physiological endpoints should be seen as a priority for future work in this area (Bundy *et al.*, 2007).

Biomarker profiles from metabolomics would be able to directly distinguish metabolic effects of pharmaceutical compounds or mixtures from other confounding factors in waters receiving complex STP effluents. However, these techniques have not yet been widely applied to the problem of pharmaceutical pollution in freshwaters. Their application to aquatic invertebrates (even widely used species such as *D. magna*) is almost entirely absent (Bundy *et al.*, 2009). Only two studies have addressed this issue with the effects of the antibiotic oxytetracycline on red abalone (Viant *et al.*, 2003a) and propranolol on *D. magna* (Taylor *et al*., (2010). However, neither of these studies includes exposure concentrations that are representative of the very low concentrations present in freshwater ecosystems nor did they consider the effects of pharmaceutical mixtures.

2.6.13 Potential human health impacts of pharmaceuticals in rivers

This thesis is concerned with the occurrence and toxicological effects of pharmaceuticals in freshwater ecosystems but this brief section will provide a wider context by outlining some of their known and potential impacts on human health.

2.6.13.1 Drinking water

Low levels of some active pharmaceutical ingredients or their metabolites have been found in drinking water supplies (Huerta-Fontela & Ventura, 2008). For example, Stackelberg *et al*. (2004) detected a number of prescription and OTC drugs, cosmetics and fragrances in finished drinking water from a US treatment plant with carbamazepine present at a maximum concentration of 258 ng L^{-1}. Heberer & Stan (1997) detected clofibric acid in Berlin tap water at concentrations up to 270 ng L^{-1} but the study by Stackelberg *et al*. (2004) suggests that drinking water treatment reduces the levels of most pharmaceuticals below the current limits of detection. The drinking water treatment process itself has been linked with the creation of potentially harmful transformation products. For example the chlorination of the antibacterial triclosan (used in many hand soaps and disinfectants) has been found to form chloroform and other harmful by-products (Farré *et al.*, 2008) which can increase a person's annual chloroform exposure by 40 % over background levels. At the concentrations of carbamazepine measured in US finished drinking water, Stackelberg *et al*. (2004) estimated that a lifetime intake (assuming 2 litres of water per day for 70 years) would be just 13 mg compared an average DDD of up to 100 mg. Despite such low concentrations the presence of pharmaceuticals in drinking water does raise potential human health concerns, particularly related to low-level chronic exposure, transformation products and mixture toxicity. These potential effects are poorly understood and more research is required (Guo *et al.*, 2009). Rowney *et al*. (2009) estimated the potential human health consequences of cytotoxic (anti-cancer) drugs in UK drinking water to be of low risk to healthy adults but that certain vulnerable subgroups (e.g. pregnant or breast-feeding women, infants and the elderly) may have cause for concern.

2.6.13.2 Antibiotic resistance

The fostering of antibiotic resistance (ABR) amongst bacteria is considered to be one of the major current public health concerns as many antibiotics are becoming less effective and there is a paucity of new antibiotics being developed (APUA, 2010). Harrison & Lederber (1998) describe it as a worsening phenomenon with our tools to combat it decreasing in power and number. Indeed, a Royal Commission was established in 1969 which looked at the potential for environmental ABR from pharmaceuticals (Swann *et al*., 1969). Perhaps the most prominent recent example of a resistant strain is methicillin-resistant *Staphylococcus aureus* (MRSA) but many other resistant strains have been

widely found in the aquatic environment and in drinking water (Kümmerer *et al.*, 2004b; Kümmerer, 2008b). Resistance is fostered amongst bacterial communities that are exposed to sub-inhibitory concentrations, particularly in areas of high bacterial density such as biofilms (Kümmerer, 2008b). There are two possible mechanisms of resistance in the environment: the *in-situ* fostering of resistance due to the environmental release of antibiotics or the transport of already resistant bacteria via the sewage network from domestic households and hospitals. The second mechanism is generally considered to be the most important as there is a strong correlation between resistant bacteria and the input of urban waste water (Edge & Hill, 2005). A study by Akiyama & Savin (2010) in the United States demonstrated significantly elevated levels of resistance to the antibiotics sulfamethoxazole and trimethoprim downstream of an advanced residential STP. However, this relationship was more variable for two other antibiotics (ofloxacin and tetracycline) and resistance to all four compounds began decreasing further than 640 metres downstream of the STP input.

Antibiotic resistant strains of bacteria are proliferating globally and are increasingly being encountered outside of hospitals and long-term care facilities (Taubes, 2008). Densely populated areas of China have a particularly high prevalence of resistance perhaps driven by the linking of doctor's salaries with prescription and sale rates of antibiotics (Hvistendahl, 2012). Bacteria demonstrating up to 58 % resistance to multiple antibiotics have been detected downstream of a freshwater aquaculture effluent discharge (Lim *et al*., 2013). River biofilms, sediment and 'floc' have been shown to be key reservoirs of concentrated ABR (Drudge *et al*., 2012; Winkworth, 2013). Increased resistance to most classes of antibiotic have been demonstrated in rivers including ampicillins, quinolones, tetracyclines, beta-lactams (Ash *et al*., 2002; Goñi-Urriza *et al*., 2000). Crucially, levels of ABR have in some cases been shown to increase markedly downstream as rivers become more influenced by STP effluent (Lupo *et al*., 2012).

The acquisition and exchange of ABR in the environment is likely a natural phenomenon but water pollution may be a key factor in forcing the speed of its evolution (Lupo *et al*., 2012). This proliferation is of particular concern considering only five of the major global pharmaceutical companies now have significant antibiotic development research programmes underway. The lack of research is perhaps due to lower success rates of antibiotic compound discovery and reduced rates of financial return when compared with other classes such as antidepressants or cardiovascular drugs. There is likely no easy solution to this problem but newly discovered antibiotics need to be combined with more intelligent and prudent use of existing drugs alongside more rigorous infection control procedures within hospitals (Taubes, 2008).

2.7 Legislative context and future research needs

2.7.1 The Water Framework Directive and other relevant legislation

The primary piece of legislation now driving catchment management and water quality improvements in European rivers is the Water Framework Directive (2000/60/EC). This requires member states to achieve good status in the chemical and biological quality of surface waters by 2015. UK rivers saw a steady improvement during the 1990s in chemical and biological quality but recent years have seen a slowing in this, particularly in biological quality (EA, 2012; Vaughan & Ormerod, 2012). It is likely that pharmaceuticals and the myriad other domestic chemicals and emerging contaminants, alongside channel modifications and nutrient enrichment are partly responsible.

In response to this concern, the European Union is currently in the process of adding diclofenac and EE2 to its list of Priority Substances (EC, 2012) which list substances that member states must maintain mean and maximum concentrations below a defined Environmental Quality Standard (EQS) (2008/105/EC). The Annual Average EQS was proposed at 10 ng L^{-1} for diclofenac and 0.4 ng L^{-1} for EE2 although these were subject to significant opposition raised on cost grounds by the pharmaceutical and water industries (UK Parliament, 2012) with the UK leading successful opposition to the EQS at the European Commission (SCHER, 2013). The estimated cost to the UK water industry for additional advanced waste water treatment in order to meet the EQS was £ 27 billion although the evidence base for these costs was not presented to the UK Parliament and is therefore not publicly available (UK Parliament, 2012). Despite the removal of the mandatory EQS it is refreshing to see emerging contaminants coming onto the policy agenda and regulators, researchers and other stakeholders should see this as an opportunity to expand understanding beyond diclofenac to the myriad other pharmaceuticals and chemicals that routinely enter rivers via STPs.

2.7.2 Environmental Risk Assessment (ERA) of pharmaceuticals

EC Directives 2001/82/EC and 2001/83/EC amended earlier EEC directives (81/851/EEC and 93/39/EEC) and set out the legal requirement for environmental risk assessments of new veterinary and human use pharmaceuticals seeking market approval. These were in turn amended by EC Directives 2004/27/EC and 2004/28/EC to include extensions of existing approvals in 2004. In addition, there are guidance documents available to aid applicants in the ERA procedure and although these are non-legally binding, they are widely adopted across the industry (Koschorreck & Hickmann, 2008). These documents are published by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products (VICH, 2000; VICH, 2004) for veterinary drugs and the European Agency for Evaluation of

Medicinal Products (EMEA, 2006) for human drugs. The EC Directives and guidance documents help the applicant to demonstrate that use of their product is safe for the environment and that any potential adverse effects will be mitigated or managed. There are numerous summaries and examples of the ERA procedure (Carlsson *et al.*, 2006) for human pharmaceuticals and only a brief outline flow chart is presented here (Figure 2-12).

There is one fundamental distinction between the human and veterinary ERA procedures. Potentially serious environmental risks can prevent the authorisation of a veterinary drug if no mitigation is possible but this cannot happen for human pharmaceuticals where legislation can stipulate risk reducing measures only (Koschorreck & Hickmann, 2008). This distinction between veterinary and human pharmaceuticals is purely a political and legislative one and there is no scientific justification for a differing approach between the two particularly as some compounds are used in both veterinary and human applications (e.g. amoxicillin). However, the environmental risks of pharmaceuticals should be rightly weighed against their significant health benefits to humans (Jones *et al.*, 2002).

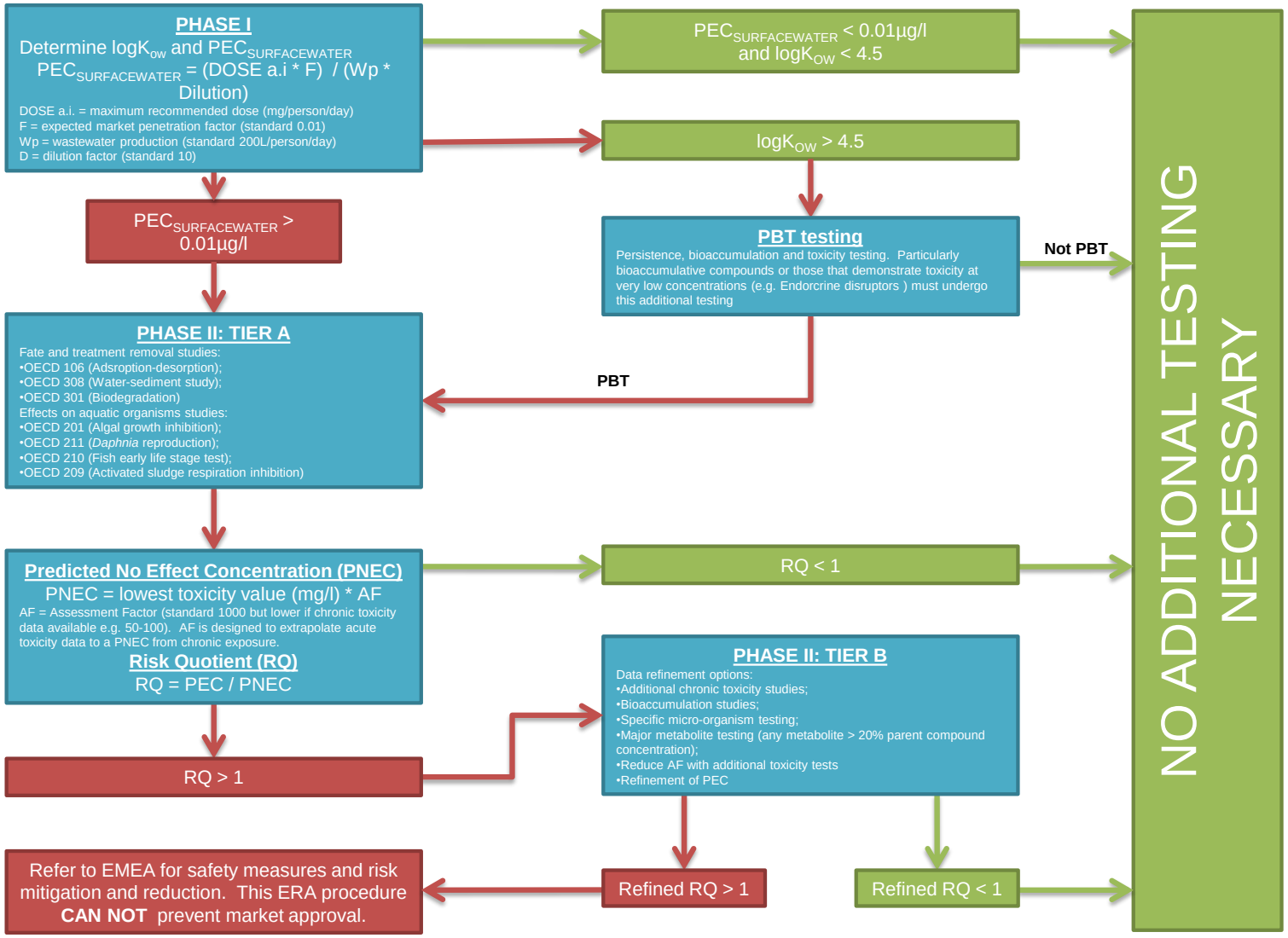


Figure 2-12: Flow chart summary of the environmental risk assessment procedure for human-use pharmaceuticals (EMEA, 2006)

2.7.2.1 Limitations of environmental risk assessment

Perhaps the major limitation of current procedures is that ERAs are only required for new substances or those seeking a variation of an existing approval. This means that the thousands of pharmaceuticals already approved for use will not be subject to a formal ERA. Cooper *et al*. (2008) used a variety of data combinations (including prescriptions, toxicity, solubility and persistence) to rank over 300 APIs for environmental risk and place their findings in a publicly accessible database (available at http://www.chbr.noaa.gov/peiar/default.aspx). This study found that central nervous system (CNS) drugs (which includes NSAIDs) were most frequently high ranking followed by cardiovascular drugs and then antibiotics. Sanderson *et al*. (2003) used QSAR models (Quantitative Structure Activity Relationships), log K_{OW} and treatment removal data to predict hazards towards algae, *Daphnia* and fish. The study concluded that cardiovascular and gastrointestinal drugs were the most hazardous. Other studies (Jones *et al*., 2002) have used prescription volumes to prioritise compounds for ERA; they found that four compounds (paracetamol, amoxicillin, oxytetracycline and mefenamic acid) have the potential to illicit acute toxic effects at environmental concentrations. A cause for concern was that all of the assessed compounds exceeded the 10 ng L^{-1} threshold requiring Phase II assessment (Figure 2-12). These examples demonstrate that priority and ranking of pharmaceuticals based on risk is dependent on the type and quality of data used. Koschorreck & Hickmann (2008) estimate that at the current rate of progress it will take approximately 100 years to evaluate all currently approved compounds. Hemminger (2005) highlights the problem of who should conduct retrospective testing as many of the drugs originators are no longer the main manufacturers. Furthermore, Carlsson *et al*. (2006) suggest that ERA and effects data be reviewed and updated in the light of new information during the life cycle of an API after it has achieved market approval.

There are other limitations with this risk assessment methodology, particularly for the initial PEC calculation which is based on broad assumptions of market penetration, metabolism, dilution and treatment removal. The assumption of 1 % market penetration may be too low for very popular drugs. For example, the rapid rise of simvastatin from the mid-1990s to being the most widely prescribed drug in the UK in 2008 (Table 2-2). Also, as mentioned in the previous section, there may be no lower toxic threshold for some compounds (e.g. anti-cancer drugs) and the 10 ng L^{-1} for Phase II analysis would be inappropriate. There are counter arguments from the pharmaceutical industry that the PEC values are overly conservative whereas some scientists have suggested they may be too low as the assumed 1:10 dilution factor may not be achieved in some areas particularly during low flows (Hemminger, 2005) or in ‘effluent dominated streams’

(Waiser *et al*., 2010). However, Schmitt *et al*. (2010) took a critical approach to the 10 ng L^{-1} threshold for effects testing and they recommend that such tests be mandatory for all compounds (regardless of PEC) under certain circumstances. For example, where drugs target biological functions known to be strongly conserved across species, high potency drugs or those structurally similar to other drugs known to have an effect and particularly for drugs in new therapeutic classes. Furthermore, the threshold may become increasingly inappropriate as drug development progresses yielding much more potent drugs requiring lower doses (and hence lower PECs) which may have effects at much lower aquatic concentrations (Schmitt *et al*., 2010). Kostich & Lazorchak (2008) suggest using the minimum daily dose of a new drug as a measure of its potency and this approach has already been adopted in prioritisation studies by regulators (EA, 2009b). Another major area for concern is the treatment of compounds in isolation with no requirement to consider mixture toxicity. Lienert *et al*. (2007b) suggest that the risk assessment process should also include more in depth testing of metabolites as high metabolism rates can both reduce or increase overall toxicity. Considering mixtures and metabolites in more detail may prevent overly conservative toxicity estimations for some drugs and highlight those with higher toxic potential.

Von der Ohe *et al*. (2011) tested a new form of risk assessment methodology using decision trees to classify 500 emerging contaminants in terms of the availability of analytical methodologies and effects and occurrence data. Comparisons were made against maximum measured concentrations and LOECs from chronic effects testing. These results were then ranked based on both the probability of exceedance of effects concentrations and the extent of exceedance. The majority of high risk compounds identified during this process were pesticides and some industrial products (von der Ohe *et al*. 2011). Such a risk assessment methodology which relies first and foremost on measured effects and occurrence values may prove useful for pharmaceuticals in the future as increased research efforts begins to expand the amount of data available. Despite the above requirements, the majority of pharmaceuticals assessed in the USA do not pass beyond Tier A although more are expected to enter this stage of testing in the EU due to lower threshold values (Hemminger, 2005). The toxicological testing procedures recommended in the EMEA guidelines have also come under criticism for being inappropriate, poorly replicated and insufficient to detect very low level effects (Enick & Moore, 2007). Also, the continued use of single species laboratory tests will mean that the ERA procedure will be unable to predict the potential for indirect effects manifesting at community or ecosystem level. These authors also suggest that the conservative burden of proof required by the scientific community (i.e. 95 % statistical significance) is increasing the number of false negatives and that substances are deemed to pose no risk when in fact they do. Indeed, the over reliance on significance

thresholds at the expense of effects size and R^2 in ecology has long been acknowledged (Yoccoz, 1991). False negatives arising from inappropriate testing has been blamed for the diclofenac induced reduction in Indian vulture populations (Oaks *et al*., 2004). This requirement for a heavy burden of proof can be reversed using the Precautionary Principle when there is plausible but limited evidence of an environmental risk so that evidence is required to indicate the absence of risk rather than its presence. For example, this stance has been adopted by the EU in imposing the 2006 ban on the use of antibiotics as growth promoters in livestock (Enick & Moore, 2007; Kemper, 2008).

2.7.3 Strategies for reducing pharmaceutical inputs to the aquatic environment

When considering the environmental risks associated with pharmaceuticals the human health benefits for which they were derived must take precedence; this means that banning or restricting usage is not a viable option unless an alternative compound is readily available and alternative strategies for reducing environmental input must be developed (Jones *et al*., 2002). Kümmerer (2008c) splits proposed strategies for reducing the input of pharmaceuticals into the aquatic environment into three broad categories:

- Short-term: advanced effluent treatment and end-of-pipe technologies;
- Medium-term: altering disposal, consumption and prescription practices and the collection of detailed consumption and disposal data;
- Long-term: adoption of ‘green pharmacy’ principles that all drugs should achieve their therapeutic purpose and then readily degrade and other developments such as improved targeting of MOAs or improved delivery mechanisms to reduce dosage levels (Daughton, 2003).

Short-term measures could include source control (e.g. additional treatment of highly contaminated hospital effluent) or source separation (urine separation) in households which would yield a waste source that is much more amenable to treatment, particularly as the majority of metabolites are in a water-soluble form (Larsen *et al.*, 2004; Lienert *et al.*, 2007a; Lienert *et al.*, 2007b). End-of-pipe technologies such as ozone treatment and granular activated carbon have demonstrated increased removal rates at STPs but these do not work for all compounds and carry additional capital and energy costs (Larsen *et al*., 2004). Increased HRT of sewage within a STP can improve removal rates by increasing biodegradation and sorption to the sludge phase but the ultimate fate of sorbed compounds depends on the disposal route of the sludge i.e. whether it is incinerated or applied to agricultural land (Boxall *et al*., 2002). Williams (2005) estimated that the cost to completely remove oestrogenic substances from UK STP effluent would

be £ 20 million per plant, a cost that would easily run into billions of pounds for all STPs in the UK. However, it may only be necessary to apply these additional treatment steps to a small number of particularly affected plants whilst others would be satisfactorily controlled by more traditional technologies.

Daughton & Ruhoy (2009) suggest the concept of 'personalised medicine' where each patient (human or animal) would receive exactly the type, degree and duration of treatment they require thus reducing residues from both waste and excretion. The environmental benefits from such a perfectly working healthcare system could have additional health benefits for humans, for example by reducing the fostering of ABR in the environment. The longer-term aim of 'benign by design' drugs will require the co-operation of the pharmaceutical industry and the scientific community. Such co-operation in response to legislative, ethical and socio-economic concerns has already produced positive results. For example the increased biodegradability of many household washing powders (Larsen *et al*., 2004). The principles of 'green chemistry' (Anastas & Warner, 1998) have been successfully applied to the pharmaceutical sildenafil citrate (Viagra) in reducing the manufacturing process waste from 1300 L kg^{-1} in 1990 to just 7 L kg^{-1} in 2004 (Williams, 2005). However, green chemistry is a relatively recent strategy in the pharmaceutical industry which has understandably focused much of its efforts on achieving therapeutic benefits in humans with only secondary consideration of environmental consequences. A recent EEA workshop (2010) suggested benign by design drugs be incentivised by extending their patent duration therefore encouraging pharmaceutical companies to consider environmental impacts at the earliest stages of drug development. One other possibility is the consideration of environmental factors when prescribing or recommending drugs for use; this approach has been piloted with some success in Sweden (Daughton & Ruhoy, 2009a; Roig, 2010). Pharmaceutical take-back schemes for unwanted or unused medicines may also play a role in reducing the flow of drugs to the environment via disposal to solid or liquid waste streams (Roig, 2010).

2.7.4 Research needs and data gaps

A recent review of global threats to human water security and biodiversity (Vorosmarty *et al.*, 2010) highlighted the lack of knowledge on occurrence, fate and effects of emerging contaminants such as pharmaceuticals as a major limitation to fully understanding global threats to freshwater resources. The above review of literature has highlighted three key areas for future research required to understand and mitigate the consequences of pharmaceuticals in the aquatic environment:

1. Continued research into analytical techniques and sampling of surface water and STP effluent to better quantify the presence of pharmaceuticals in the

environment. Further sampling will assist in understanding the spatial and temporal variation in surface water concentrations, the relationship between PECs and MECs during risk assessment and the occurrence of particular mixtures and metabolites of pharmaceuticals in surface waters. Additional work to reliably validate models such as GREAT-ER for pharmaceuticals may help to prioritise rivers for more detailed sampling and analysis;

2. Continued research into treatment removal efficiencies and the development of new treatment technologies to prevent or reduce the input of pharmaceuticals to surface waters;

3. A shift in focus of ecotoxicological testing to more ecologically relevant endpoints which focus on higher levels of organisation and assess the effects of pharmaceuticals on ecosystem structure and functioning and attach more importance to mixture toxicity. Bound & Voulvoulis (2004) and Länge & Dietrich (2002) suggest adopting a more flexible toxicity screening process during risk assessment to allow the use of bespoke tests tailored to known MOAs or toxic endpoints. However, it is important to continue some standardised testing to maintain reproducibility and allow comparisons between different drugs to be made.

This thesis will focus on numbers 1 and 3. Detailed sampling in catchment areas ranging from the highly urbanised to semi-rural will be undertaken. Urban catchments are likely to represent a worst-case scenario due to high population density and large STPs although smaller works (i.e. those serving small semi-rural communities) may be problematic as they are less regulated under the UWWT Directive and may not have such comprehensive treatment systems. It will add substantially to the existing small dataset of pharmaceutical occurrence in England and will widen the geographical spread of such data beyond the current focus in SE England and Wales. The work will also be of global relevance given the similar patterns of consumption and environmental occurrence observed in other countries.

The review of literature has highlighted the current weaknesses in the ecotoxicological testing of pharmaceuticals where the focus is on acutely toxic effects to individuals at concentrations unlikely to be encountered in the environment. Focusing on higher levels of ecological organisation (population, community and ecosystem level), considering aspects of ecosystem functioning and mixture toxicity will help to make conclusions about pharmaceutical toxicity much more relevant to actual environmental conditions. The use of *in situ* field techniques will also help to bridge the relevance gap between laboratory studies and environmental conditions. These studies are particularly

important as toxic effects to individuals seen in the laboratory may not manifest at population level or above due to phenomena such as functional redundancy, stress response (hormesis), adaptation, migration and availability of alternative resources (Moss, 1988; Giller & Malmqvist, 1998; Hauer, 2006). Furthermore, using ecosystem service or functional endpoints for ecotoxicological testing may provide a better basis and justification for the decisions of environmental managers, policy makers and regulators as these can have obvious socioeconomic values.

2.8 Thesis aims and objectives

2.8.1 Aims

The review of literature highlighted numerous knowledge gaps both in terms of the occurrence of human-use pharmaceutical compounds in STP effluent and receiving waters and in their effects on freshwater ecosystems. Thus, the broad aim of this thesis is to examine the occurrence of pharmaceuticals in typical UK catchments and consider the effects of such pharmaceuticals on important freshwater taxa and aspects of ecosystem structure and functioning. This aim can be separated into the following four sub-questions:

1. What is the current spatial and compound coverage of existing pharmaceutical pollution research and how reliable/representative are data obtained from these studies?

2. How do pharmaceutical concentrations in STP and CSO effluent and receiving waters vary over diurnal, seasonal, reach and catchment scales?

3. What lethal and sublethal impacts does extended exposure to environmentally relevant concentrations of pharmaceuticals have on important freshwater taxa?

4. What impact does exposure to pharmaceuticals have on important aspects of ecosystem functioning?

2.8.2 Objectives

The research gaps identified in the literature review and the research questions outlined above can be divided into five key objectives which this thesis will address:

1. Synthesise and critically evaluate current pharmaceutical pollution research by means of a systematic review examining spatial and compound coverage, sampling strategies and comparisons between measured environmental concentrations and ecological effect thresholds;

2. Quantify the temporal and spatial dynamics of the occurrence of five human-use pharmaceutical compounds in STP effluent, CSO effluent and receiving waters across two densely populated catchments over an 18 month period;

3. Examine the effects of extended exposure to erythromycin, propranolol and their mixture on the functionally important freshwater amphipod *Gammarus pulex* through assessment of growth, feeding, mortality and NMR environmental metabolomics;

4. Assess the impact of extended exposure to low and high levels of pharmaceuticals on leaf litter decomposition in the laboratory and *in situ* and how exposure to pharmaceuticals affects colonisation of leaf litter by freshwater macroinvertebrates;

5. Examine the effect of pharmaceutical exposure in the laboratory on sediment metabolism and nutrient exchange with overlying waters.

It is anticipated that this thesis will add substantially to the relatively small body of data examining the occurrence of pharmaceuticals in freshwater systems and provide novel information on the effects of extended exposure to environmentally relevant concentrations of pharmaceuticals on important freshwater taxa and aspects of ecosystem structure and function.

3 GLOBAL SYNTHESIS AND CRITICAL EVALUATION OF PHARMACEUTICAL DATASETS COLLECTED FROM RIVER SYSTEMS

This chapter is comprised entirely of material included in the below paper. It provides a global-scale critical review of research into the pharmaceutical pollution of rivers. The aim of this chapter was to highlight spatial, temporal and methodological gaps in current research in order to inform the monitoring and experimental chapters presented later in this thesis.

Hughes, S. R., P. Kay and L. E. Brown (2013). "Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems." *Environmental Science & Technology* **47**(2): 661-677.

3.1 Introduction

Pharmaceuticals have been used by humans for centuries with commercialisation beginning in the late 19th Century. Aside from pioneering studies in the 1970s and 1980s (Hignite & Azarnoff, 1977; Aherne *et al.*, 1985; Richardson, 1985) pharmaceuticals have only emerged as a major group of environmental contaminants over the last 15 years (Wall & Strong, 1987; Oaks *et al.*, 2004; Schwarzenbach *et al.*, 2006; Kidd, 2007). Their presence in numerous environmental compartments including surface and ground waters, soils and biota is now well established (Daughton & Ruhoy, 2009b) and the predominant pathway of entry to the environment is considered to be post-consumption excretion to the sewer network and subsequent passage to rivers via straight piping, STPs (Tambosi *et al.*, 2010) or sewer overflows (Heberer, 2002; Daughton, 2004c; Jones *et al.*, 2005). Pesticide research in the 1990s identified clofibric acid as a widespread aquatic contaminant (Heberer *et al.*, 1995), which in turn sparked an expansion of method development and pharmaceutical research in subsequent years (Stan, 1992; Petrovic *et al.*, 2010). These studies have vastly improved the reliability, availability and precision of pharmaceutical detection methods (Petrovic *et al.*, 2010).

The shift from gas to high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) has been a key driver in improving knowledge in recent years (Kümmerer, 2009a). Despite the availability of these methods, analysis and monitoring of pharmaceuticals in freshwaters remains far from routine and research is often sporadic and isolated. This is despite an increased awareness of the potential effects of pharmaceuticals on ecosystems and the services they provide (Fent *et al.*, 2006; Kümmerer, 2008a; Kümmerer, 2009a; Kümmerer, 2009b; Santos *et al.*, 2010). Existing research indicates that pharmaceuticals are generally present in freshwaters within the ng L^{-1} range and, at these sub-therapeutic levels, the risk of acute toxicity is thought to be negligible (Enick & Moore, 2007). However, there are substantial knowledge gaps in terms of chronic, long-term exposure of non-target aquatic organisms

and the effects on ecosystem functioning (Kümmerer, 2009a). Data are available to suggest that some compounds may display chronic effects at or close to the levels detected in the environment (Fent *et al.*, 2006; Santos *et al.*, 2010). Moreover, the development of antibiotic resistant bacteria is a major public health concern; the prudent use of pharmaceuticals in the future, is seen as key to reducing risks to public health and the environment (Kümmerer, 2009b; Kümmerer, 2009c).

It is likely that pharmaceutical consumption will increase in coming years, particularly in developing countries and those with ageing human demographics (Daughton, 2003; EEA, 2010). Nevertheless, pharmaceutical compounds currently receive minimal consideration by regulators, policy makers and managers (Farré *et al.*, 2008), perhaps because there have been few attempts to amalgamate research findings from disparate spatial and temporal studies. However, the status quo is unlikely to remain in future, and the European Union has already started the process of adding the anti-inflammatory drug diclofenac to its list of Priority Substances (EC, 2012). This change would mean member states must maintain concentrations below a defined Environmental Quality Standard in an attempt to meet the requirements of good ecological status under the Water Framework Directive (EC, 2000).

This chapter synthesises the disparate research on pharmaceutical occurrence in freshwaters at national, regional and global scales but goes much further than a review by providing a critique of current research effort by compound class and individual substance. A critical review of sampling strategies and methods adopted by researchers in this field is also provided, a crucial factor in considering how reliable and representative data are. Moreover, a brief summary of the environmental effects of pharmaceuticals in freshwater ecosystems is given in order to highlight potentially high risk compound classes. The substantial assembled database is provided as a tool to better inform future research on the occurrence and effects of pharmaceuticals in freshwaters, and to identify key areas where future research should be focused. A number of regulators and researchers have already accessed the database to inform their own work. Finally, the benefits of meta-analyses such as this is discussed in support of policy development to target the highest risk and most widespread compounds.

3.1.1 Aims and objectives

The aim of this chapter was to synthesise and critically evaluate current research into pharmaceutical pollution of receiving freshwaters. Specific objectives were:

1. Through the use of systematic review techniques, evaluate spatial coverage of current monitoring research at global, national and provincial levels and identify areas where research is sparse or absent;

2. Quantify the number of compounds currently detected in receiving waters and synthesise data to provide median and maximum measured concentrations at global, national and provincial level;

3. Critique the sampling strategies adopted by researchers in this field and use this to inform future research;

4. Compare synthesised receiving water concentrations with known ecological effects data in order to provide an indication of environmental risk posed by pharmaceuticals in freshwaters.

3.2 Methodology

A systematic review was conducted via a search of the Web of Knowledge (WoK) publications database (http://apps.isiknowledge.com/) on 6th March 2011. The search term below was applied to the title, abstract and keywords of articles:

(((((pharmaceutical OR API* OR drug* OR PPCP* OR PhAC*) AND (aquatic* OR river* OR stream* OR "surface water*" OR freshwater* OR effluent* OR wastewater* OR "waste water*")))))*
Refined by: Document Types=(ARTICLE OR REVIEW OR ABSTRACT) AND Research Domains=(SCIENCE & TECHNOLOGY) AND Languages=(ENGLISH) AND Subject Areas=(ENVIRONMENTAL SCIENCES & ECOLOGY OR PUBLIC, ENVIRONMENTAL & OCCUPATIONAL HEALTH OR MARINE & FRESHWATER BIOLOGY OR WATER RESOURCES OR BIODIVERSITY & CONSERVATION)

This was intended to identify all studies that analysed for pharmaceuticals in either STP effluent or receiving waters; there was no restriction on time span for this query. Research conducted by governmental departments or reported in the 'grey' literature is also available; for example the US Environmental Protection Agency http://www.epa.gov/ppcp/but such work was not included here. The acronyms API (active pharmaceutical ingredient), PPCP (pharmaceuticals and personal care products) and PhAC (pharmaceutically active compound) are the most widely used by researchers in this field. This initial search yielded 57 289 results which were sorted by their relevance to the search term (using the in-built WoK algorithm) and refined using the criteria above.

The refined search criteria yielded 18 245 results and consequently the study was constrained further to the 28 most common journals returned by the search; which accounted for ~50 % of results (9072 records) and included most major environmental and analytical chemistry journals. The remaining records were sorted by relevance and

the first 1000 results were examined individually. The cut-off of 1000 and top 28 journals results was deemed necessary due to the time taken to assess each individual data source for the meta-analyses, versus the 'success rate' for inclusion. The criterion for inclusion in the database was that a study explicitly analysed for, detected and quantified at least one human-use pharmaceutical compound in either STP effluent or receiving waters. From these 1000 studies, only 236 met the inclusion criterion (Table 3-1). While a more exhaustive search may have improved coverage, the assembled database represents the broadest review of the most detailed research studies to date.

Table 3-1: Summary of journal sources for the 236 studies included in the full database

Journal	Record Count	% total (236)
Journal of Chromatography A	42	17.80
Environmental Science & Technology	34	14.41
Water Research	34	14.41
Science of the Total Environment	33	13.98
Chemosphere	27	11.44
Environmental Toxicology and Chemistry	23	9.75
Analytical and Bioanalyitcal Chemistry	11	4.66
Environmental Pollution	9	3.81
Journal of Hazardous Materials	9	3.81
Water Science and Technology	8	3.39
Aquatic Toxicology	1	0.42
Environmental Science and Pollution Research	1	0.42
Environmental Health Perspectives	1	0.42
Talanta	1	0.42
Archives of Environmental Contamination and Toxicology,	1	0.42
Ecotoxicology and Environmental Safety	1	0.42

Analysis of publication dates of the 236 studies showed a clear upward trend from the late 1990s onwards (Figure 3-1). The majority of studies included (>80%) were published between 2005 and 2010, a trend most likely driven by the advancement of analytical techniques such as HPLC-MS/MS and increased interest in pharmaceutical pollution (Kümmerer, 2009a). This is reflected in the relatively high number of studies published in analytical chemistry journals such as *Journal of Chromatography A* and *Analytical and Bioanalytical Chemistry* (Table 3-1) despite neither of these journals having a specific environmental focus.

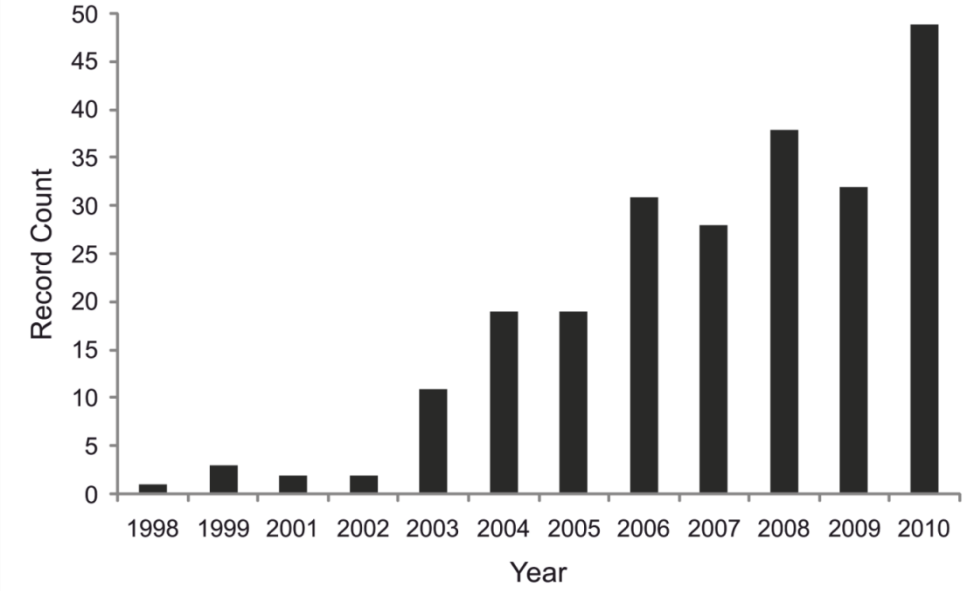


Figure 3-1: Number of publications per year for 236 studies included in the database

For each of the 236 studies the following data were extracted from the main report body or supplementary material: compound(s) detected; country; median and maximum concentration(s); sampling technique(s) adopted; sampling period; analytical methodologies; number of samples; number of detections; number of no detections; frequency of detection; referencing information and any pertinent information (e.g. raw drinking water or estuarine sampling). Median and maximum concentrations were recorded when explicitly stated by authors or in some cases were calculated using data tables and in a small number of cases, concentrations had to be derived from graphical representations such as boxplots. Other concentrations stated in the studies were not recorded as it was felt that median levels were appropriately representative of 'normal' conditions and maxima represent peaks in concentrations indicative of a worst-case scenario in terms of ecological effects.

The ratio of maximum: median concentration was also calculated and recorded and available for 74 % of the studies. For the purposes of this review only data concerning receiving waters (and not treated effluent) were subjected to meta-analysis because these were the most relevant indicators of potential effects in freshwater ecosystems. Effluent concentrations are inherently variable (Kanda *et al.*, 2003) and are less reliable as indicators of effects given the broad range of dilution factors and chemical transformations to which they are subjected when entering receiving waters. When published studies reporting only treated effluent concentrations were excluded, a total of 156 studies were retained. Fifty studies (26.8 %) contained samples of a non-riverine source (marine, estuarine, groundwater, raw drinking water or sediment samples); from which lentic and raw drinking water samples were included as these were deemed relevant. Five studies contained samples extracted from freshwater sediments and

although these are relevant, the small number of studies was deemed insufficient to merit inclusion. The full database containing all receiving water records is freely available upon request from http://www.wateratleeds.org (further details are given in Appendix A).

All concentrations were recorded in the database as nanograms per litre (ng L^{-1}) to enable direct comparison. Individual compounds were then grouped into the following classes: antibiotics; antidepressants; antiepileptics; antivirals; blood lipid regulators; cancer treatments; contrast media; endocrine drugs; gastro-intestinal drugs; illicit drugs; other cardiovascular drugs; other CNS (central nervous system) drugs; others; painkillers and respiratory drugs.

3.3 Results

The full database containing pharmaceutical occurrence records from the 156 published studies with data for 203 compounds across 41 countries is available in Appendix A. Median concentrations ranged from 6.2 ng L^{-1} for the antibiotic sulfathiazole to 163 673 ng L^{-1} for the antibiotic ciprofloxacin which also had the highest maximum concentration of the entire database at 6 500 000 ng L^{-1}. Of the 61 (=50th) most frequently detected compounds, 39 % were antibiotics, 21 % painkillers, 20 % cardiovascular drugs or blood lipid regulators and 3 % antidepressants.

3.3.1 Spatial coverage of research

Pharmaceutical compounds were identified in receiving waters across 41 countries on all continents except Africa and Antarctica (Figure 3-2). The database included 1417 records representing 67 903 analyses and median/maximum concentrations from >14 155 samples (nb. 27 studies did not state sampling numbers) and >24 989 positive detections of pharmaceuticals; equating to an overall detection frequency of 37 %. The results illustrated a heavy bias towards research in Europe and North America which accounted for 80 % of studies, with a further 16 % in Asia (predominantly China). Only three studies reported from South America and just one from the Middle East (Israel). The USA had the most studies (34) and Spain, China, Germany, Canada and the UK were the only other countries to have >10 studies. Seventy one percent of nations in the database had three or fewer studies conducted, including very large or populous nations such as Japan, Brazil, Mexico, Pakistan and Australia.

When examining data at the 'continental' scale strong biases are again evident (Figure 3-3). For example, just 6/176 provinces in Asia accounted for >50 % of published studies in that region, with the most populous province in China (Guangdong) having the highest number (six). In Europe most studies have been undertaken in Germany, Spain and Switzerland (in the Elbe, Rhine, Ebro and Llobregat basins). The UK has a relatively good spread of research, covering most countries except South West England and Northern Ireland. However, studies are generally isolated, with the only repeated work

undertaken in Wales, Greater London and the North East. A similar story is evident in North America with nine Canadian provinces (69 %) and 28 US states (55 %) having one or no studies.

Globally, painkillers were the most frequently detected compounds accounting for 31 % of records with a median concentration of 230 ng L^{-1} followed by antibiotics (21 %, 8128 ng L^{-1}; Figure 3-4 a). The remaining compound classes each demonstrated medians of <100 ng L^{-1} except for Others (830 ng L^{-1} incl. antiepileptics and contrast media). The picture changes when data are examined regionally (Figure 3-4 b-d). For example, the most commonly encountered pharmaceuticals were painkillers in Europe (34 %, median concentration 261 ng L^{-1}) and antibiotics in North America (38 %, 71 ng L^{-1}) and Asia (42 %, 33 446 ng L^{-1}). Contrast media and respiratory drugs had particularly high median concentrations in Asian waters of 1257 and 50 023 ng L^{-1} respectively, although these were from a small number of studies contaminated by effluent from pharmaceutical manufacturing facilities (Li *et al.*, 2008; Fick *et al.*, 2009). Additionally, antidepressants are more frequently detected in North America (9 %, 25 ng L^{-1}) compared to Europe and Asia where they account for <1 % of records.

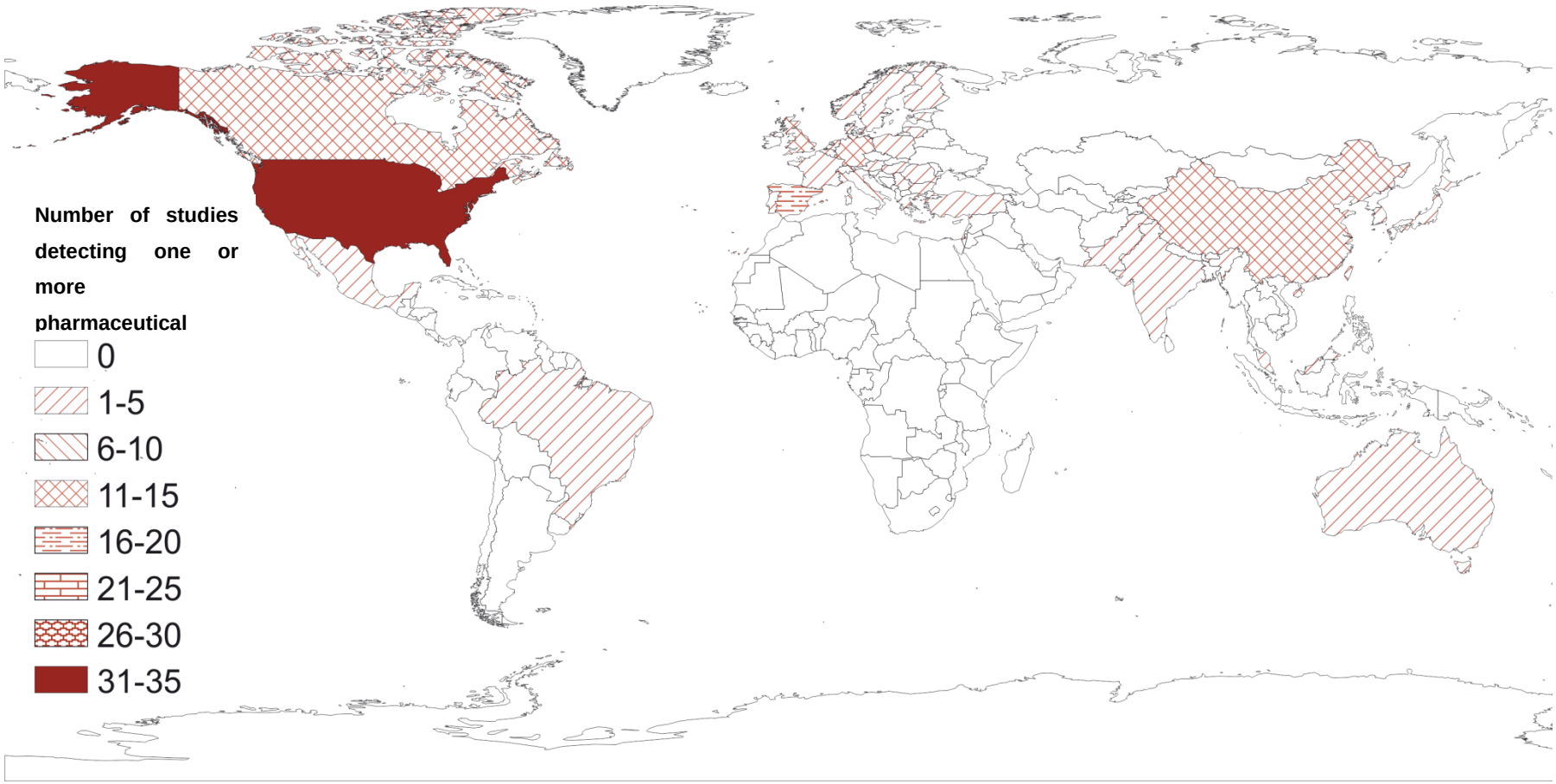


Figure 3-2: Global-scale distribution of the number of published studies identifying pharmaceuticals in inland surface waters

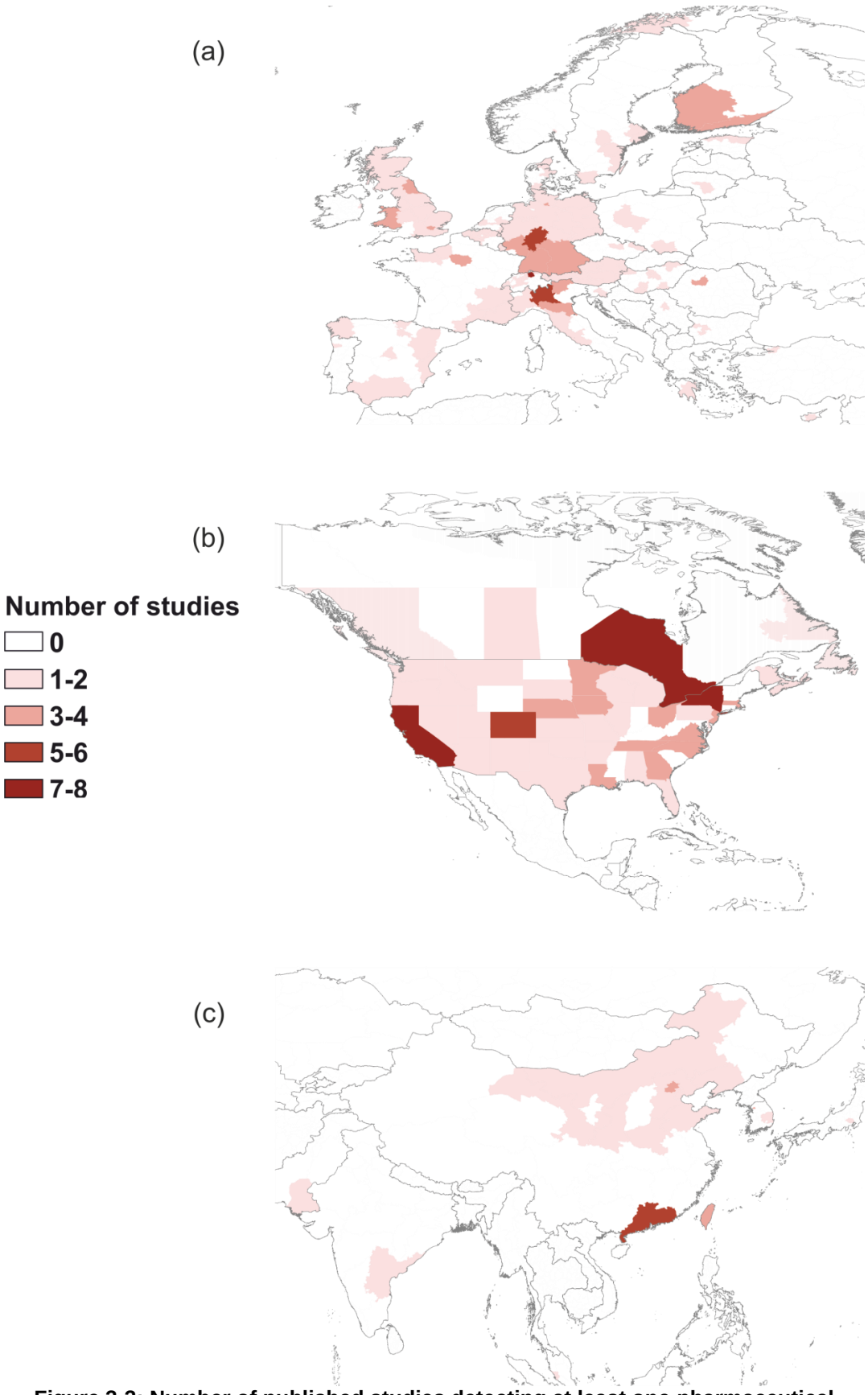


Figure 3-3: Number of published studies detecting at least one pharmaceutical compound in (a) European regions (b), North American states and (c) south Asian provinces

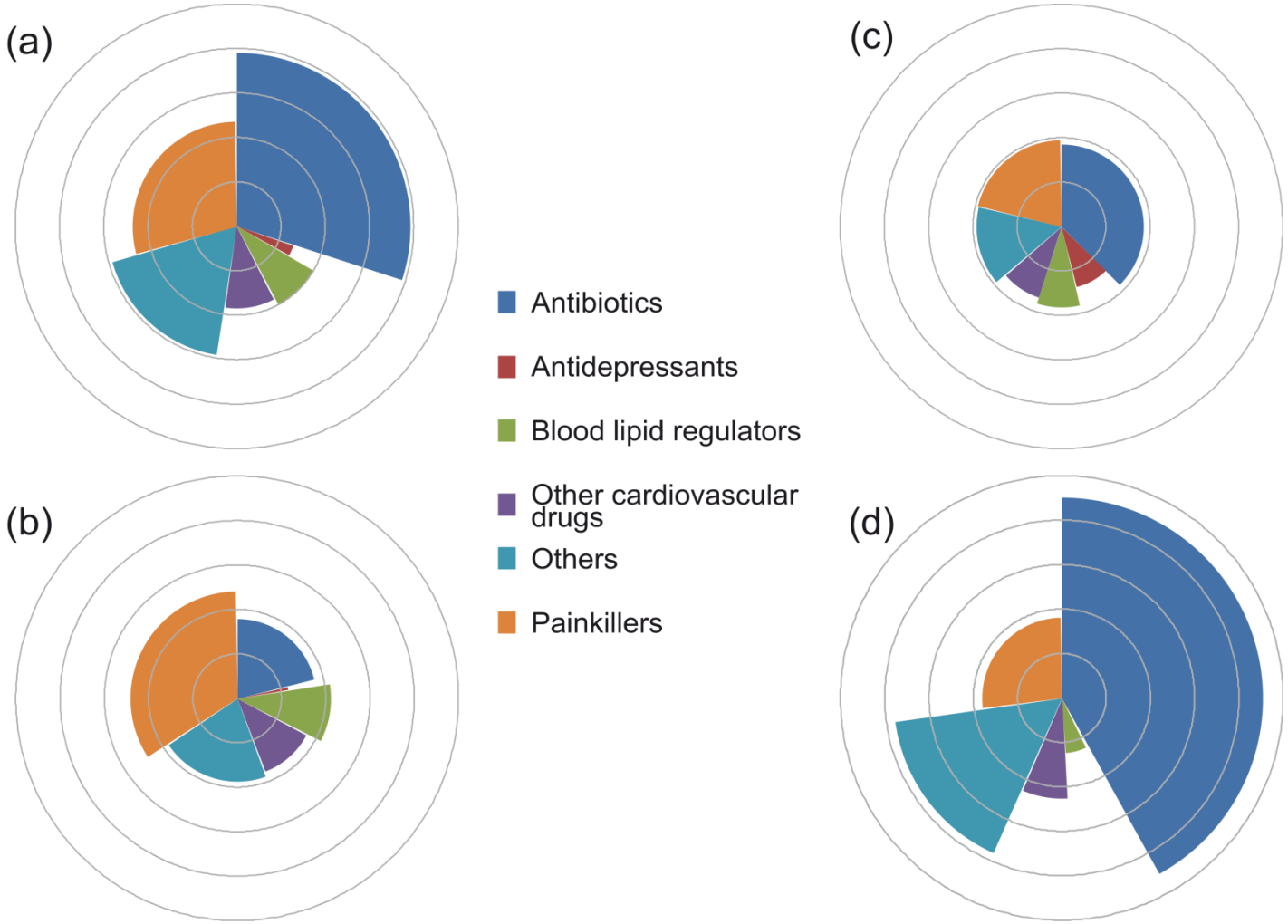


Figure 3-4: Relative frequency of detection, and median concentration, of pharmaceuticals in receiving waters: (a) global, (b) Europe, (c) North America and (d) Asia.
[The circumference of each fan is scaled by the relative proportion of detections. Each point outwards on the radial axis represents 10^y of the median concentration in ng L^{-1}. For example, the innermost circle represents 10^1 ng L^{-1}; the second represents 10^2 ng L^{-1}, etc.

3.3.2 Concentrations of pharmaceuticals in receiving waters

A striking result from mean concentrations in the top 10 most studied countries was that all except China demonstrated very low proportions of the global mean (<2 %) for antibiotics (Figure 3-5); this was due to the very high concentrations detected in a small number of Asian studies. Interestingly, China displayed values well below the global means for all other compound classes ranging from 2 to 25 %. Spain was above the global mean (171 to 441 %) for all classes except Antibiotics and Others compared to Italy, Switzerland, Japan and the UK which were below the global mean for all classes. No single country in the top 10 demonstrated mean concentrations above 40 % of the global mean for Others. This is due to there being only seven countries within this group which are heavily skewed by one study in India detecting very high concentrations (15 000 ng L^{-1}) of the antifungal terbinafine (Fick *et al.*, 2009).

The top 20 most frequently studied compounds in Europe included eight painkillers, six cardiovascular drugs and four antibiotics with median concentrations for all compounds below 100 ng L^{-1} except aspirin and paracetamol (median 190 ng L^{-1}; Figure 3-6). A similar picture was evident in North America and Asia with mostly antibiotics, cardiovascular drugs and painkillers present in the top 20 compounds with median concentrations generally <100 ng L^{-1}. A small number of compounds (six in Europe and Asia and two in North America) had maxima >10 000 ng L^{-1}. Of particular note was carbamazepine which was the most frequently studied and detected compound in both North America and Europe and third in Asia. The minimum detection frequency for all compounds studied (Figure 3-6) was 9 % (diclofenac in North America) but average detections frequencies were 70 % (Europe), 27 % (North America) and 65 % (Asia).

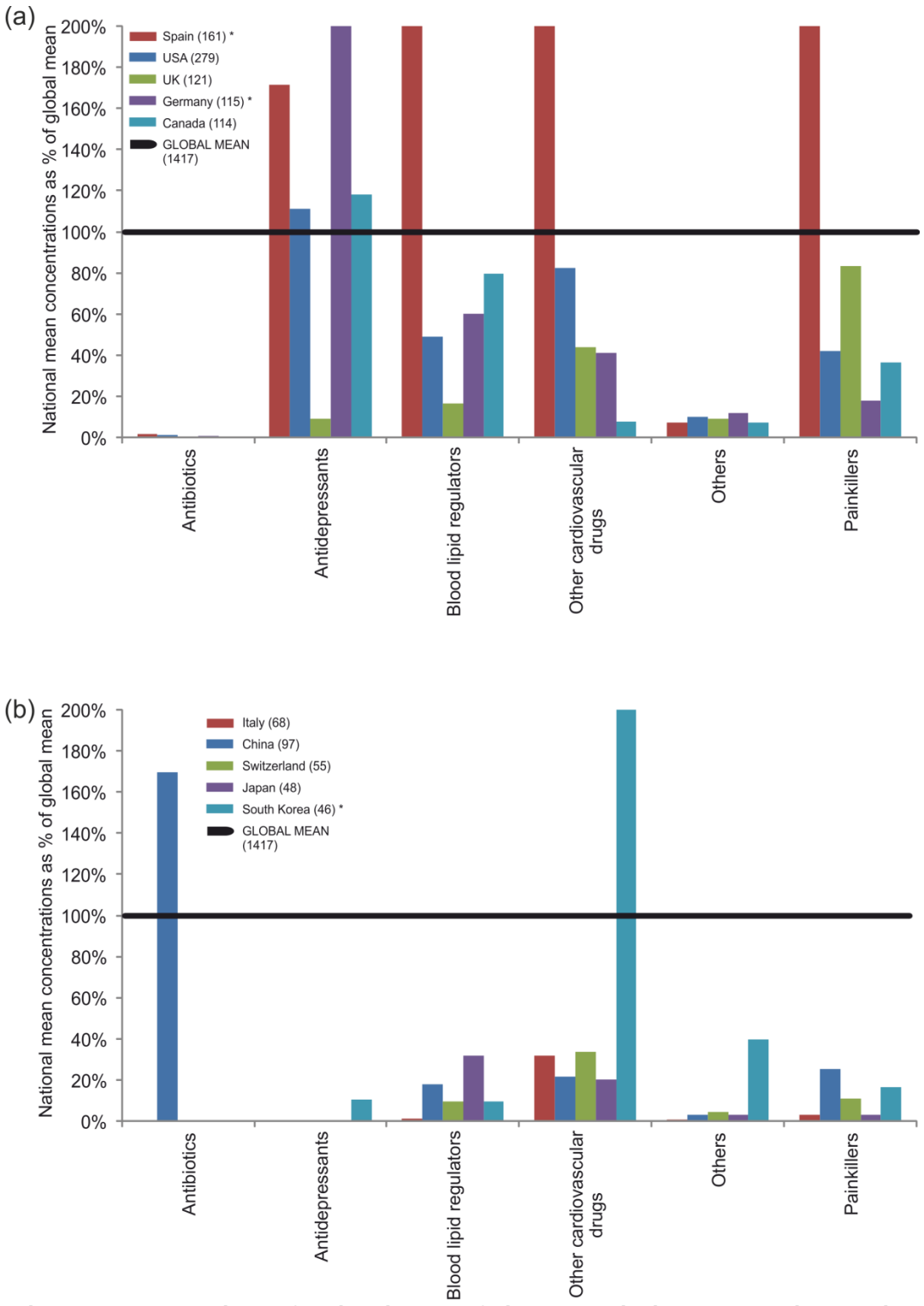


Figure 3-5: Comparison of national mean of pharmaceuticals concentrations to the global mean for (a) the 1st to 5th and (b) the 6th to 10th top countries [as determined by number of entries in the database; represented by the number in brackets. Global mean is the mean concentration for all records of that compound group. Values exceeding 200% of global mean: Spain (blood lipid regulators 441%, other cardiovascular drugs 213%, and painkillers 209%), Germany (antidepressants 246%), and South Korea (other cardiovascular drugs 404%)]

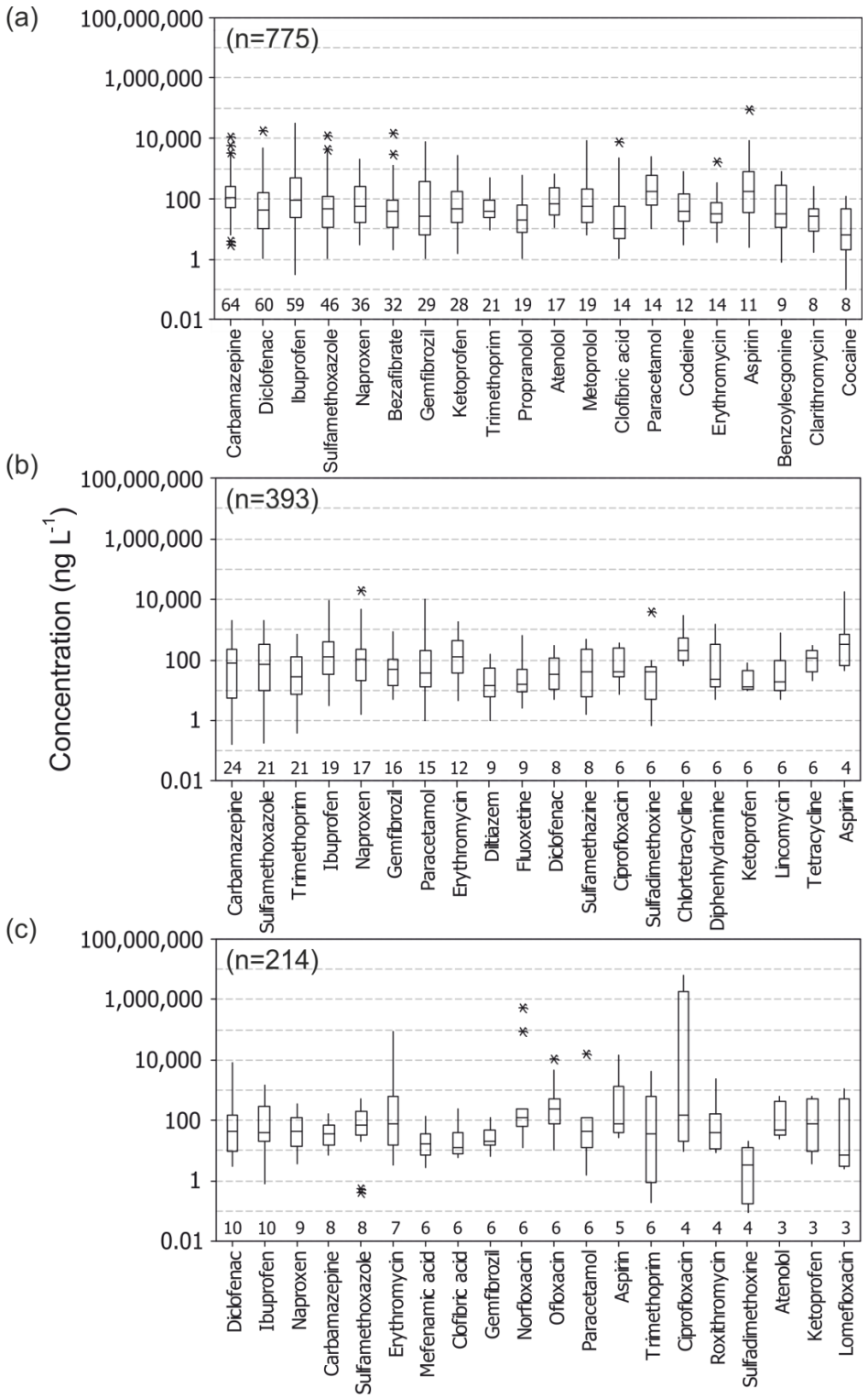


Figure 3-6: Boxplots of the 20 most commonly encountered pharmaceuticals in (a) European, (b) North American, and (c) Asian receiving waters. [n values represent total number of records for the respective region and values above the x axes represent records for each of the 20 specific compounds. Boxes represent interquartile ranges with median concentration represented by the horizontal line. Whiskers show the range of data and asterisks represent outliers].

3.3.3 Sampling strategies adopted in pharmaceutical monitoring studies

A major finding from this analysis was that 45 % of European and Asian and 23 % of North America studies failed to provide any details of the sampling regime and techniques adopted (Figure 3-7). Isolated and non-repeated grab sampling was by far

the most common technique and was adopted in 31-34 % of studies. Slightly more representative repeated grab sampling over periods ranging from days to years was undertaken in just 12 % of studies. Additional techniques are available including composite sampling and the use of passive samplers (e.g. polar organic contaminant integrative sampling, POCIS) which give an integrated sample over a period of days to weeks. However, these were adopted by just 11 % of European and 9 % of North American studies and not at all elsewhere.

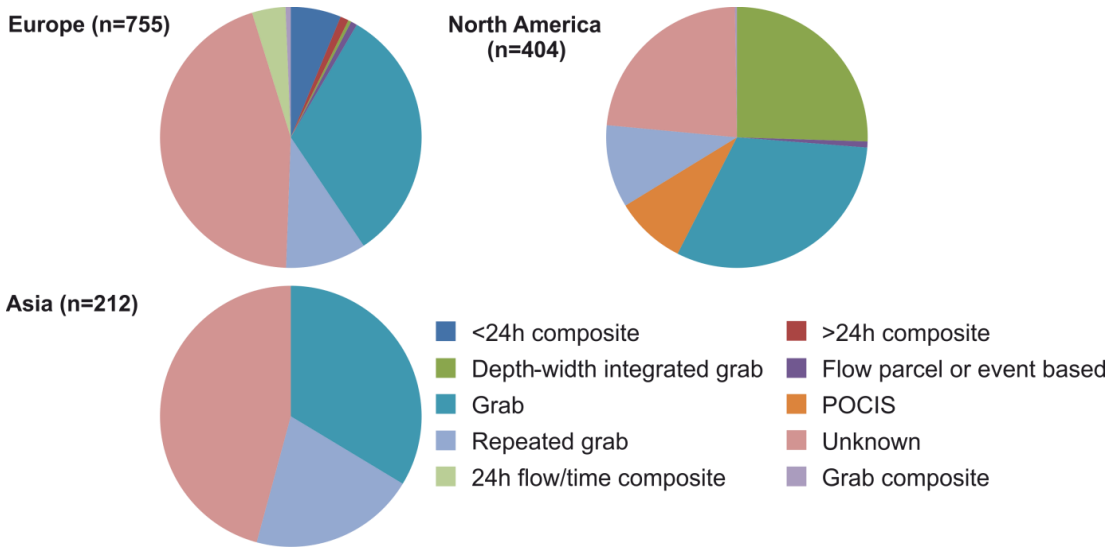


Figure 3-7: Summary of sampling methodologies employed in detection of pharmaceutical compound(s) in rivers.
[n = number of records in the database; POCIS = polar organic contaminant integrative sampler]

3.3.4 The effects of long-term exposure to pharmaceuticals in receiving waters

Chronic effects data for the six main compound classes were collated from four major reviews (Crane *et al.*, 2006; Fent *et al.*, 2006; Enick & Moore, 2007; Santos *et al.*, 2010) and any duplicate results were removed manually. Chronic (i.e. long-term) data (rather than short-term, acute studies) were chosen as these were deemed to be more representative of actual low-level exposure of aquatic organisms (Newman & Unger, 2003). The endpoints assessed included lethal and sub-lethal indices (e.g. feeding, growth, behaviour and reproduction). The data included here are not intended as a direct assessment of particular compound toxicity but rather to highlight where overlaps between measured environmental concentrations and chronic toxicity indicate potential environmental risk.

The ranges of long-term toxicity vary markedly across compound types and taxa (Figure 3-8). From these data antidepressants appear to pose a particular risk to all taxa except bacteria with effective concentrations ranging from low µg to mg L^{-1}. Invertebrates and fish show chronic toxic effects at sub mg L^{-1} levels for cardiovascular drugs and Others. Fish also appear susceptible to painkillers with effects manifesting at 40 000 ng L^{-1}. This summary highlights where research is lacking; in particular there were no

studies examining the effects of antibiotics on fish, antidepressants on aquatic plants or cardiovascular drugs on bacteria. Overall, bacteria and aquatic plants appear to be the least well studied whereas research on chronic effects in aquatic invertebrates is relatively abundant.

3.4 Discussion

3.4.1 Spatial distribution of research

This study shows that knowledge of pharmaceutical occurrence is poor or absent for large parts of the globe, particularly in developing countries. The research that has been undertaken displays a heavy bias towards North America, Europe and the more populous parts of China. Given that the consumption of pharmaceuticals is ubiquitous across the globe (Daughton, 2001), there is clearly a pressing need to expand research into their occurrence particularly in Russia, southern Asia, Africa, the Middle East, South America and Eastern Europe. As more data become available, the better able researchers will be able to highlight the scale of the problem and inform management decisions. Moreover, even where studies have been relatively numerous knowledge is still minimal compared to that for other stressors such as nutrients (Smith & Schindler, 2009; Woodward *et al.*, 2012), acidification (Evans, 2001), river regulation (Nilsson *et al.*, 2005) and sedimentation (Viers *et al.*, 2009).

When looking at the spread of research it is apparent that the spatial biases manifest at multiple scales. In Europe, an obvious cluster of research is evident around central Europe particularly and to a lesser extent in the UK and Spain. Research effort appears to be clustered around the high population areas of (London, Paris, Hamburg, Frankfurt, California and the North American eastern seaboard and Beijing and Guangdong province in China). This is understandable given that pharmaceutical pollution can be expected to be at its greatest in densely populated areas and a risk based approach (Borja *et al.*, 2006) should target these receiving waters first. However, this leaves substantial proportions of Europe and North America with insufficient research. For example, in the UK no research has been conducted in the major urban areas of the West Midlands, Greater Manchester or West Yorkshire which have a combined population of 6 million people (Pointer, 2005). Researchers should expand monitoring efforts to previously unstudied catchments whilst at the same time increasing the length/breadth and temporal resolution of monitoring campaigns if the dynamics of pharmaceutical pollution are to be fully understood. Research in less densely populated and rural areas should also be a priority as it is probable that STPs in such locations are smaller and less advanced when compared to their larger counterparts serving urban areas and septic tanks may also represent a risk (Neal *et al.*, 2005).

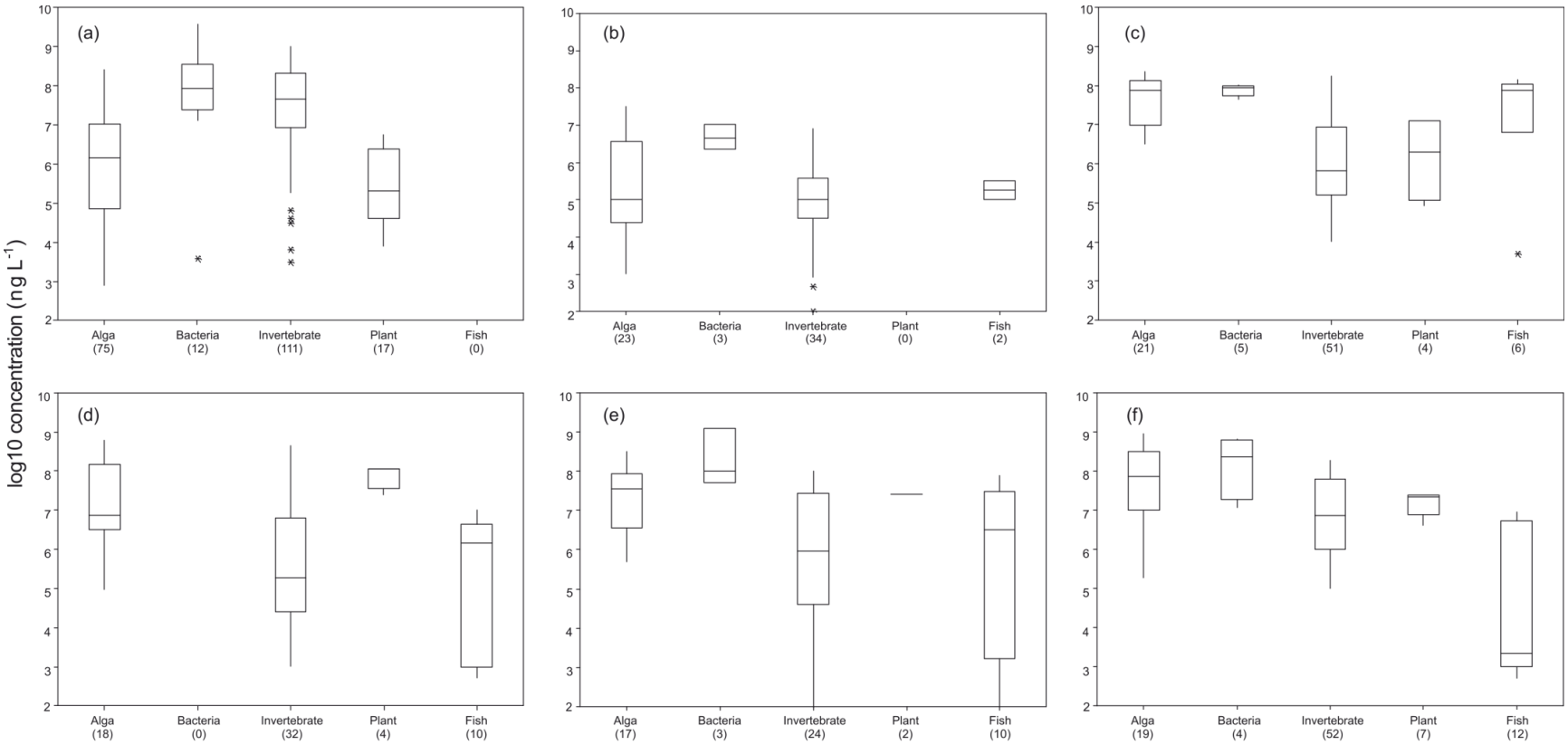


Figure 3-8: Summary of chronic ecotoxicological data for pharmaceuticals and freshwater organisms across (a) antibiotics, (b) antidepressants, (c) blood lipid regulators, (d) other cardiovascular drugs, (e) others and (f) painkillers data summarised from (Crane *et al.*, 2006; Fent *et al.*, 2006; Enick & Moore, 2007; Santos *et al.*, 2010)

3.4.2 Coverage of compound classes

When viewed globally, antibiotics, antiepileptics, cardiovascular drugs (including blood lipid regulators) and painkillers accounted for 86 % of all database records with carbamazepine being the single most commonly identified compound. A likely reason for this bias is that these drug types are the most widely prescribed and purchased over-the-counter (NHS, 2009b; Gu, 2010). For example, cardiovascular drugs and painkillers were amongst the top three compound types prescribed to adults in the USA during 2007 (Gu, 2010) and as such could be expected to enter the environment in the highest concentrations. However, this leaves major groups of potentially toxic pharmaceuticals being poorly studied. For example, antidepressants, antivirals, cancer treatments, tranquilisers and antifungals combined accounted for just 6.3 % of records. A reason for this imbalance in detections could be that researchers have not been analysing these compounds in environmental samples, rather than them being genuinely scarce. It may also be possible that less frequently detected compounds are less refractory and are more efficiently removed during sewage treatment (Tambosi *et al.*, 2010). Further studies on these 'rarer' compounds are particularly necessary given documented effects on microalgal communities even at very low concentrations (Porsbring *et al.*, 2009). Interestingly, antidepressants were the most widely prescribed compounds amongst young American adults (Gu, 2010), and this is reflected in the relatively high number of North American studies analysing these compounds (9 %) when compared to Asia and Europe (1 %).

The analysis confirmed that knowledge of some pharmaceutical groups is almost completely lacking with >50 % of entries in the database represented by just 14 compounds (all of which are antibiotics, antiepileptics, cardiovascular drugs or painkillers). The entire database represents only a snapshot (203) of all pharmaceuticals approved for use, which is estimated to be well over 5000 in Europe alone (Pascoe *et al.*, 2003). This gives a conservative estimate that fewer than 4 % of pharmaceuticals have been analysed for and detected in freshwaters. It demonstrates a clear need for future research to be expanded across the less well studied compounds, particularly those identified as posing environmental risk.

3.4.3 Pharmaceutical concentrations in freshwaters

One of the most striking results from the data was the relatively high concentrations for antibiotics and Others (comprising just the respiratory drug ceterizine and the contrast medium ioprimide in Asia) compared to other classes. However, these very high concentrations were skewed by a small number of studies which analysed waters receiving pharmaceutical manufacturing effluent (Li *et al.*, 2008; Fick *et al.*, 2009). Up until recently pharmaceutical manufacturing facilities (PMFs) were considered a low

priority given the high value of pharmaceutical products and the adoption of 'good management practice' by the industry intended to reduce wastage and loss to the environment (USEPA, 1997). However, PMFs can be a source of pharmaceuticals to receiving waters both within developed (Phillips *et al.*, 2010) and developing countries (Larsson *et al.*, 2007; Li *et al.*, 2008); these should be considered a priority for future research effort given the potential for strong localised effects.

National means were compared to global means to evaluate the appropriateness of local studies as indicators of concentrations at larger spatial scales. The differences shown in these comparisons highlight the importance of local studies to inform stakeholders and regulators, and emphasise a need for caution when scaling up to larger spatial scales, or vice-versa. Given the relative paucity of data it is possible for a small number of studies at particularly polluted locations to have a disproportionate influence on national and even global mean concentrations. The very high concentrations for antibiotics in Asia and globally are an example of this problem The global median antibiotic concentration of 8135 ng L^{-1} reduces to 58 ng L^{-1} if just two Chinese and Indian studies are excluded (Li *et al.*, 2008; Fick *et al.*, 2009).

3.4.4 Frequently studied compounds

Ibuprofen and sulfamethoxazole were consistently within the top five most studied compounds across all regions and had similar concentration ranges (1 to 10 000 ng L^{-1}). Notably though, very high concentrations (up to a maximum of 10 000 000 ng L^{-1}) of ciprofloxacin, erythromycin, norfloxacin and ofloxacin were evident in Asia; this is of concern because these are well within the range known to cause acute and chronic toxic effects to aquatic organisms (Santos *et al.*, 2010). Also of note was the presence of the antidepressant fluoxetine in the top 20 North American compounds; this type of antidepressant causes chronic effects at the low ng L^{-1} range (Flaherty & Dodson, 2005; Painter *et al.*, 2009), well below the median and maximum concentrations detected in the environment. Carbamazepine is the single most widely studied and detected compound in Europe and North America but is not generally considered to pose a risk of acute or chronic toxicity to freshwater organisms (Quinn *et al.*, 2008). However, its refractory nature and generally ubiquitous presence in STP effluent may make carbamazepine a useful source-specific tracer of domestic wastewater contamination (Gasser *et al.*, 2011).

3.4.5 Sampling strategies

Perhaps the most crucial step in reliably quantifying the presence of pharmaceuticals in freshwaters is the collection of a representative sample using appropriate strategies and methodologies (Ort *et al.*, 2010a; 2010b). Sampling uncertainty can often exceed that during the analytical procedure (Ramsey & Thompson, 2007). Large sample numbers

and sophisticated analytical techniques, although important, should not be seen as a substitute for the collection of a representative sample (Ort *et al.*, 2010a) and poor sampling regime can be the dominant source of error in water quality data (Martin *et al.*, 1992). Some exponents of sampling theory have gone so far as to argue that "*nothing good (certainly nothing representative) has ever come from grab sampling*" (Petersen *et al.*, 2005) due to the fact that it provides a mere snapshot in time. Grab sampling does have a part to play in furthering our understanding of pharmaceutical occurrence particularly for extensive spatial or long-term studies where the equipment and set-up costs of more representative techniques may be prohibitive. However, efforts should be made to adopt more representative techniques wherever possible. For pharmaceuticals in particular, non-repeated grab sampling is unlikely to be representative as concentrations have been shown to vary considerably over time (Kanda *et al.*, 2003).

A possible explanation for the lack of robust sampling is the relatively high proportion of studies published in journals with a focus on analytical chemistry (namely *Journal of Chromatography A, Analytical and Bioanalytical Chemistry* and *Talanta*). The main thrust of these journals is method development rather than the collection of large, representative datasets and this is borne out by the fact that 64 % of these papers did not state the sampling methodologies adopted (c.f. 44 % across all journals) and only 11 % of studies adopted repeated grab or composite sampling strategies (c.f. 21-23 % across all journals). This propensity of analytical studies to collect fewer samples for shorter periods is probably due to analytical chemists needing to validate methods on 'real' samples which requires only minimal numbers to be collected. The contribution of these papers should not be understated, as they provide reliable and sophisticated analytical methods that can be adopted as part of more representative sampling studies. The occurrence data alone provided by these studies covers 94 compounds in 34 countries, so minor modifications to sampling strategies and subsequent reporting by these scientists could benefit the field substantially.

In addition to the techniques adopted, appropriate sampling numbers, duration and frequency are crucial to obtain a reliable quantification of variable water quality parameters. Most studies collected a relatively small number of samples (1-50) with only 13 % of studies collecting for >12 months. This indicates a dearth of long-term monitoring which may be crucial in understanding seasonal and annual dynamics. Conversely, sampling at an appropriately high temporal resolution over the short-term is equally important if we are to fully quantify variability over the periods of minutes to days (Kanda *et al.*, 2003; Ort *et al.*, 2010a; Ort *et al.*, 2010b). Studies on other aquatic contaminants have shown the importance of a robust sampling strategy; Rabiet *et al.* (2010) demonstrated that fixed time interval grab sampling underestimated pesticide fluxes from a small catchment by as much as a factor of five. One study (Robertson,

2003) recommended tailoring sampling strategies to the variable of interest and the duration of the study when monitoring in small streams. An interesting research task would be the systematic comparison of measured concentrations using grab sampling and continuous or composite techniques; the database assembled here may prove useful in such a study.

These data clearly highlight the importance of adopting flexible, appropriate sampling strategies in order to supply reliable and representative data. Furthermore, researchers should explicitly state the techniques employed in addition to sample numbers, frequencies and locations so that other researchers can fairly evaluate the results presented and use them to inform their own research.

3.4.6 Effects of pharmaceuticals on freshwater ecosystems

Despite the widespread presence of many pharmaceuticals in freshwaters, some risk assessments have suggested that they pose little risk of acute or chronic toxicity at environmentally relevant concentrations (Jones *et al.*, 2002; Fent *et al.*, 2006). The data summaries presented here and elsewhere indicate potential overlap between chronic effects levels and the concentrations detected within freshwaters however. In particular, potential risks are evident for invertebrates and fish from antibiotics and cardiovascular drugs in Asia and from the contrast media, tranquilisers and antiepileptics (carbamazepine) across all regions. Additionally, invertebrates and fish are potentially at risk (i.e. low margin of safety) from antidepressants and painkillers in North America. While this may suggest that future research should shift towards less well studied but more 'toxic' compounds, particular care needs to be taken with these conclusions because many effects studies on aquatic 'ecosystems' have been undertaken using ecologically unrealistic (i.e. single species) laboratory experiments. Additionally, such studies tend to test at concentrations that are orders of magnitude above those detected in the environment and be run over short time-scales, and as such do not adequately address the issue of low-level, chronic exposure. Studies which have examined the effects of low-level pharmaceutical exposure on aquatic ecosystem structure and functioning are rare but have indicated that pharmaceuticals can display significant effects on important ecosystem services (Wilson *et al.*, 2004; Näslund *et al.*, 2008; Bundschuh *et al.*, 2009; Painter, 2009).

The data presented here are a useful indicator of particular compound classes exhibiting some kind of toxic effect at the levels stated. As such, the combined occurrence and effects datasets can serve as a guide for future ecotoxicological research. However, they should be treated with caution as they integrate a variety of toxicological tests which employ a range of exposures and endpoints across a variety of species. The exposure regime of any testing should attempt to cover both the median

as a measure of frequently encountered concentrations, and the maximum concentration as an indicator of worst-case exposure. Furthermore, standard assessments of toxicity, whilst useful in producing comparable results, suffer from low bio complexity and environmental relevance and so researchers need to adopt a more flexible, knowledge based approach tailored to specific risks (Länge & Dietrich, 2002). A problem with undertaking research in 'natural' freshwaters is that there will probably be myriad other compounds in addition to the one of interest, meaning a high potential for confounding effects. More realistic controlled experiments need to be designed and undertaken (Brown *et al.*, 2011) before researchers can make better predictions about ecosystem response to pharmaceutical pollution. Researchers and regulators must therefore be wary of dismissing particular pharmaceuticals as 'low risk' on the basis of isolated and overly simplified laboratory studies.

3.5 Conclusions

This study is the first to assemble a comprehensive database of widely distributed information on pharmaceutical concentrations at global, continental, national and provincial scales. In doing so it has highlighted large parts of the globe where knowledge of pharmaceutical occurrence is minimal or non-existent. In particular, large parts of Asia, Africa, South America and Australia should be seen as a priority for future research. Additionally, the majority of countries where research is sparse or absent are developing nations, and as such it is possible that the pharmaceutical consumption (and hence pollution) profile may differ markedly from developed nations. It could be the case that in developing countries water resource management has not yet progressed to the stage where environmental monitoring and regulation of pollutants typically start to be taken seriously (Wescoat & White, 2003).

Even within the developed nations of Europe and North America there is a pressing need for better spatial coverage across all provinces and states. Future research should learn lessons from other fields and use appropriate, representative sampling strategies to give much more reliable estimates of the pollution problem. The current proposal for statutory monitoring of diclofenac in European waters (EC, 2012) will provide a substantial boost to our knowledge but researchers and regulators should also see this as an opportunity to increase our understanding of the thousands of other pharmaceuticals and domestic chemicals that routinely enter freshwaters via STPs.

The most striking results of this study were the very high concentrations reported (particularly antibiotics, painkillers and antidepressants) that are within the range known to cause acute or chronic toxicity in aquatic systems. This challenges the assumption that pharmaceuticals in rivers generally pose little risk (Kanda *et al.*, 2003) and highlights the need for an expansion of robust monitoring and the adoption of more realistic

ecotoxicological experiments. Expanding research effort to previously understudied compounds (some antibiotics, antidepressants, respiratory drugs and contrast media) and areas of the globe where research coverage is poor should be a priority. This is particularly important as in coming decades it is anticipated that pharmaceutical use will increase substantially, partly due to the ageing population structure in developed countries, and with ongoing increases in the standard of living in developing countries. These improvements in quality of life are accompanied by an increase in meat consumption in developing countries (Stokstad, 2010) which in turn may boost the consumption of veterinary pharmaceuticals, posing additional environmental risk.

The database assembled in this study should serve as a useful tool for industry professionals, academics, regulators and water managers in prioritising future work on both pharmaceutical occurrence and the effects of such compounds on aquatic ecosystems. Ultimately, the results indicate that despite almost two decades of research effort, our knowledge of pharmaceutical occurrence and effects in the environment is still substantially lacking when compared to other aquatic pollutants.

4 PHARMACEUTICALS IN SEWAGE EFFLUENT, COMBINED SEWER OVERFLOWS AND RECEIVING WATERS

4.1 Introduction

An increasing global population is placing great strain on over 65 % of the Earth's rivers with chemical pollution one of the main causes of degradation and biodiversity loss in aquatic ecosystems (Vorosmarty *et al*., 2010). In chemical pollution research there has been an increasing focus on emerging contaminants over recent decades (Daughton & Ternes, 1999) which enter the aquatic environment following excretion to the sewer system and passage through STPs (Kolpin *et al.*, 2002). The technologies employed in most STPs are designed to reduce organic pollution and excess nutrients but they are often poor at removing pharmaceutical compounds (Halling-Sørensen, 1998). Removal rates are highly variable between compounds and individual STPs but it is now widely considered that STP effluent is the dominant source of pharmaceuticals entering the aquatic environment (Heberer, 2002; Daughton, 2004c; Jones *et al.*, 2005; Tambosi *et al.*, 2010). The presence of pharmaceuticals in continually discharged effluent is a major concern due to 'pseudo-persistence' resulting in continuous, low-level and multigenerational exposure of aquatic organisms (Daughton, 2001). The ultra-trace concentrations of pharmaceuticals in rivers mean that the risk of acute toxicity and human health impacts are low but there is increasing evidence of chronic, longer-term effects on aquatic biota and important ecosystem processes (Fent *et al.*, 2006; Kümmerer, 2009a; Santos *et al.*, 2010). The potential for effects at very dilute concentrations and the effects of mixture toxicity must also be considered (Cleuvers, 2003; Cleuvers, 2004; Caliman & Gavrilescu, 2009).

Research into pharmaceutical pollution is expanding largely due to increased concern over potential adverse effects and advancements in the analytical techniques necessary to detect such compounds at extremely low concentrations (Daughton, 2001; WIlliams, 2005). Research to date has been overwhelmingly biased towards certain European countries (Germany, Italy, Spain, Sweden, Switzerland and the UK), the USA, Canada and China (Petrovic *et al.*, 2004; Caliman & Gavrilescu, 2009). Very little research has been conducted in large parts of Africa, Asia, the Middle East and South America (Hughes *et al*. 2013). Even within those countries with a relatively high level of research, studies are spatially biased around a small number of densely populated urban areas or around key research institutions. For example, UK pharmaceutical pollution research is heavily clustered around south east England and parts of north Wales with very few studies in central, western and northern England or Scotland and where such studies are present they are often single, non-repeated studies (Hughes *et al*. 2013).

When compared to other aquatic stressors such as nutrients, acidification and sedimentation (Evans, 2001; Smith & Schindler, 2009; Viers *et al.*, 2009; Woodward *et al.*, 2012) relatively little is known about pharmaceutical pollution and there remains a pressing need for more widespread and reliable monitoring studies (Hughes *et al*., 2013). Furthermore, existing studies have typically provided very few details on the adopted sampling regime, making it difficult to draw conclusions about the reliability or representativeness of the data presented. Hughes *et al*. (2013) showed that 45 % of European studies provided no details of sampling techniques, numbers, frequency or duration. Of those studies which did provide such data, isolated and non-repeated grab sampling was by far the most common technique adopted with more representative composite techniques used in just 11 % of studies.

Combined sewer overflows (CSOs) and storm water discharges have been identified as potential sources of pharmaceutical pollution under differing flow conditions which can potentially discharge untreated sewage effluent to receiving waters. Despite this, there are very few studies which attempt to examine the contribution they make to overall pharmaceutical loads in rivers (Boyd *et al*., 2004; Kolpin *et al*., 2004). This is of concern as it has been hypothesised that such non-STP point sources may be the key contributor of high pharmaceutical concentrations in reaches far from STP effluent outfalls (Ellis, 2006).

Examinations of the longitudinal (up-downstream) profile of pharmaceutical pollution in rivers generally present the same view of relatively uncontaminated upper reaches with increasing concentrations moving downstream through more urbanised areas (Glassmeyer *et al.*, 2005; Kasprzyk-Hordern *et al.*, 2008; Tamtam *et al.*, 2008; Schultz *et al.*, 2010; Waiser *et al.*, 2010). In some cases, residual low levels of pharmaceuticals have been detected tens or hundreds of kilometres downstream of STP outfalls (Waiser *et al*., 2010) demonstrating the potential for much wider, catchment-level impacts. However, only 16 % of studies included in a recent review collected samples more than 1 km downstream of STP outfalls indicating a tendency of research to focus on the effluent dominated reaches immediately downstream (see Appendix A; Hughes *et al*., 2013). This is understandable given the likelihood that these areas are most affected by pharmaceutical pollution but this leaves long reaches of even well studied catchments with little or no monitoring data available.

The degree of attenuation of pharmaceuticals over reaches of several kilometres is also highly variable and dependent on local and compound specific factors (Packer *et al*., 2003; Radke *et al*., 2010). Furthermore, it is likely that in highly urbanised reaches with many STP and CSO outfalls that at least some pharmaceutical attenuation over such distances is replaced by additional inputs (Ellis, 2006). However, the number of field studies examining the longitudinal profiles across the reach scale is small (Radke

et al., 2010) and this is a pressing research question given the potentially unsafe assumption in many risk assessment models of first-order in-stream decay of pharmaceuticals (e.g. GREAT-ER; Schowanek *et al*., 2001).

Research examining temporal variation in pharmaceutical concentrations over the short-term (daily) is also relatively rare. This is despite evidence demonstrating high degrees of variation in STP effluent pharmaceutical concentrations over hourly and daily periods (Kanda *et al*., 2003). Given the tendency towards non-repeated grab sampling it is unlikely that such variation has so far been adequately captured in existing monitoring datasets (Ort, 2006; Ort *et al.*, 2010a; Ort *et al.*, 2010b). Monitoring over longer, seasonal timescales is well represented with 50 studies in a recent review having collected samples for a period of greater than six months (Hughes *et al*., 2013). There are no general seasonal trends in pharmaceutical concentrations in either effluent or receiving waters (Kasprzyk-Hordern *et al.*, 2008) as these are dependent on a number of factors including seasonal consumption trends, flow/dilution, temperature and site specific factors (Goossens *et al.*, 2005; Ort *et al.*, 2010b). However, Conkle *et al*. (2008) suggest that due to catchment retention effects and the tendency of many drugs to be prescribed for longer-term (monthly) usage, the majority of variation in pharmaceutical concentrations will be at the seasonal scale. For example, Lorraine & Pettigrove (2006) showed markedly higher emerging contaminant concentrations during the dry season in southern California, USA. However, this was largely caused by higher summer consumption of certain compounds and the import of water from adjacent effluent-dominated catchments during the summer months. This highlights the importance of local monitoring data and demonstrates the pitfalls of using even nearby proxy sites as an indicator of the likely pollution extent in a particular catchment. Therefore, there is a pressing need to further expand the temporal and spatial coverage of pharmaceutical monitoring to previously unstudied catchments.

In the UK, to date 19 published studies have analysed for pharmaceuticals in UK STP effluent or receiving waters, and in total 73 different compounds have been monitored. Chapter 3 showed that although there was a good spread of research across the UK, most of the studies were isolated with repeated work only conducted in small clusters throughout the country (Figure 4-1). The Thames is by the far the most studied catchment with eight different published papers. Substantial parts of the UK have little or no existing data on pharmaceutical pollution in rivers. For example, in England no published research has been conducted in the major urban areas of the West Midlands, Greater Manchester or West Yorkshire which have a combined population of 6 million people (Pointer, 2005). Research is also lacking in much of Wales, south west England, Scotland and Northern Ireland. The region of Yorkshire has been subject to only one published study (Loos *et al*., 2009) despite being home to a population of 5.3 million

people and the fifth most populous region outside of London (ONS, 2012). There is a need to expand pharmaceutical pollution research in the UK beyond London and the South East to densely populated areas where the risk of pollution is also high.

The contribution of CSOs to reductions in water quality are well acknowledged (Muliss *et al*., 1997; Old *et al*., 2003; Saul, 1989) and some recent research has highlighted their capacity to contribute significant loads of pharmaceuticals to rivers under differing flow conditions in the USA (Boyd *et al*., 2004). Despite this no such research has been conducted in the UK and CSOs represent an unquantified but potentially significant source of pharmaceutical pollution. The above studies indicate that emerging contaminants are present and potentially eliciting negative effects on the freshwater ecosystems in the UK. However, the relative paucity of data for large parts of the UK means a substantial monitoring study is required to broaden the spatial coverage of research and add to the global knowledge base on temporal and spatial variation, in-stream degradation and the contribution of CSOs to pharmaceutical pollution.

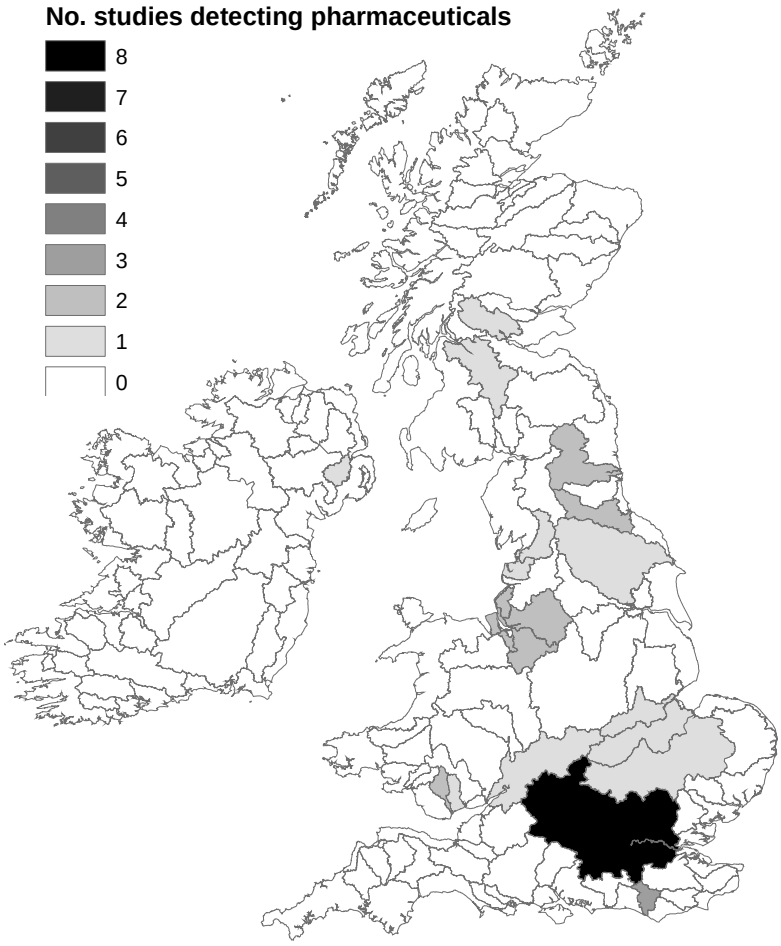


Figure 4-1: Pharmaceutical pollution research in the UK from Hughes *et al*. (2013) Boundaries are major UK river catchments, and the total no. studies reporting sampling locations = 14)

4.1.1 Aims and objectives

The aim of this study was to build on existing pharmaceutical occurrence research by improving spatial coverage to previously unstudied catchments, considering a substantial range of sites from semi-rural to urban and including both nested temporal monitoring (diurnal-seasonal). Compounds posing a high risk to the environment were selected for monitoring and pollution from combined sewer overflows was also considered. Specific objectives were to:

1. Carry out repeated monthly grab sampling of effluent and receiving waters downstream of several STPs to examine seasonal trends in pharmaceutical concentrations;

2. Quantify short-term diurnal variations in receiving water pharmaceutical concentrations by carrying out intensive sampling of the receiving waters from two STPs;

3. Use the seasonal dataset to compare concentrations between different STPs of differing treatment technologies and populations served throughout the catchments;

4. Sample along a 5 km stretch of the River Aire downstream of a large STP to examine longitudinal concentration profiles;

5. Collect samples from combined sewer overflows to examine the contribution these sources make to overall pharmaceutical pollution of rivers.

The following specific hypotheses were tested: (**H_1**) that pharmaceuticals will be consistently present in STP effluent at concentrations greater than receiving waters and that concentrations would show significant temporal variation across both seasonal and diurnal timescales (**H_2**). It was also hypothesised that differences in concentrations would be present between different STP sites based on the treatment technologies employed within (**H_3**) and that significant attenuation along the 5 km study reach of the River Aire would take place (**H_4**). Finally, it was hypothesised that pharmaceuticals would be present in CSO effluent at reduced concentrations and frequencies when compared to STP effluent (**H_5**).

4.2 Methods

4.2.1 Study area

The Aire and Calder catchments are ideal for studying the occurrence of pharmaceuticals in rivers given the 105 STPs that discharge effluent into them (Yorkshire Water, pers. comm.). Within Yorkshire the major conurbation known as the West Yorkshire Urban Area (WYUA) comprises the cities of Leeds, Bradford, Wakefield and Huddersfield alongside the adjacent large towns of Castleford and Halifax, with a combined population of 2.2 million. Two major rivers, the Aire and Calder, flow from west to east through the WYUA and they have a long history of receiving runoff affected by textiles manufacturing, heavy industry and coal mining, as well as agricultural production. The River Aire rises at Malham Tarn in North Yorkshire whilst the Calder rises at Heald Moor close to Todmorden, West Yorkshire. The upper reaches of these rivers are dominated by Carboniferous Limestone with some overlying Millstone Grit in the south progressing through Coal Measures around Leeds, Wakefield and Huddersfield into Magnesian Limestone in the lower reaches, which are subject to groundwater abstraction in parts of the catchment (Aldrick, 1978). Soil cover ranges from blanket peat bog and acid loams in the upper reaches in the southern Pennines through more freely draining acid loams and clays in the lower reaches of both catchments. Freely draining, lime-rich loams dominate the lower extremes of the catchments east of Leeds and Wakefield

(Soilscapes, 2013). Average air temperatures across the catchments vary from 3.1 °C in winter to 15.2 °C in July although these are generally much lower in the Pennine headwaters. Average rainfall can exceed 1500 mm in parts of the Pennines and drops to approximately 660 mm in the lower eastern parts. Rainfall is distributed relatively evenly across the year although there is a seasonal trend of dry springs and wet autumn/winter period which is much more prevalent in upland areas (Met Office, 2008).

The Aire flows for 114 km prior to joining the Yorkshire Ouse at Airmyn in the East Riding of Yorkshire with the River Worth (a major tributary) joining at Keighley. The Calder flows for approximately 72 km prior to joining the Aire east of Castleford with its major tributaries being the Rivers Hebble and Colne which join at Halifax and Brighouse, respectively. Both rivers are canalised along parts of their length forming part of the Aire & Calder and Calder & Hebble Navigations and the Calder in particular is heavily reservoired for drinking water supply (Jarvie *et al.*, 1997).

Generally speaking the water quality in these two catchments has improved over recent years due largely to declines in heavy industry and developments in sewage treatment. However, the improvement has been much slower in reaches downstream of the major population centres of Bradford, Wakefield and Leeds (EA, 2009a). In particular, biological water quality was much slower to improve when compared to chemical indices and the UK Environment Agency have identified toxic discharges from storm systems and STPs as the main reasons for this lack of improvement (EA, 2009a). These problems may be exacerbated by the abundance of small and medium-sized STPs discharging to smaller tributaries where dilution factors of effluent can be as low as 2:1 (Fox *et al.*, 2000). In addition to the 105 operational STPs currently discharging in the Aire and Calder catchments, there are estimated to be at least 70 CSOs spread across the entire catchment (EA, 2010) representing significant potential point sources of pharmaceutical pollution during heavy rainfall events (Figure 4-2).

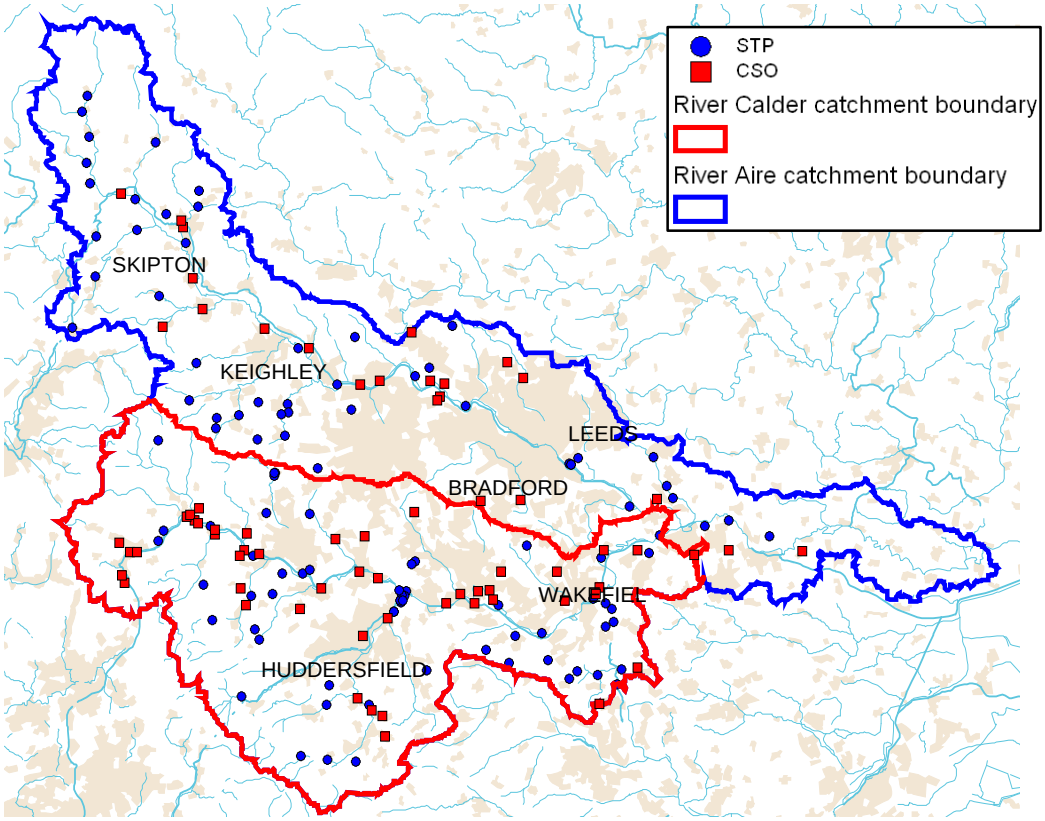


Figure 4-2: Map of combined sewer overflow (CSO) and sewage treatment plant (STP) locations in the Aire and Calder catchments
[Based on data supplied by the Environment Agency and Yorkshire Water]

4.2.2 Monitoring sites

Seven STP sites were identified for monthly monitoring over an eighteen month period (September 2010 to March 2012) which comprised four in the Aire and three in the Calder catchments (Table 4-1; Figure 4-3). Sites were chosen based on access via public rights of way and where it was possible to sample both the effluent and receiving waters directly. These STPs represented a range of sizes and treatment technologies and they also discharged to receiving waters of various sizes ranging from the relatively small Sheffield Beck to the main channels of the Aire and Calder themselves. Furthermore, they were distributed throughout the catchments from the semi-rural upper reaches down to the urbanised lower reaches.

In addition, five CSO monitoring sites were selected in the Aire catchment (Figure 4-3) where it was possible to directly sample the effluent. Furthermore, it was necessary for sites to be in relatively close proximity to the University of Leeds to allow responsive grab sampling during rainfall events. CSOs were grab sampled during periods of intensive rainfall from March 2010 to August 2012 and sites were chosen based on access, safety and ease of sampling.

4.2.2.1 River Aire reach sampling

The stretch of the River Aire between two large STPs at Knostrop and Oulton/Lemonroyd (Figure 4-4; Table 4-1) was chosen for monitoring of a river reach based on the absence of any additional known point sources of pharmaceutical pollution. During the period from February to July 2011 (seven sampling occasions) receiving waters were collected at five sites between the two STP outfalls. Sampling across winter, spring and summer periods was conducted to capture a range of flow conditions in the River Aire. The presence of a large and reliable flow gauging structure (Fleet Weir; EA Station Number 8079) approximately 30 m upstream of sampling point 5 meant it was possible to obtain accurate flow information for the whole sampling campaign. Although not a fully synoptic survey, this sampling campaign still gave an informative indication of the longitudinal concentration profiles of the study compounds across a range of flow conditions.

Table 4-1: Summary of pharmaceutical sampling sites

Location *	Channel, catchment and catchment area (km^2)	Sample type	Treatment type, population (design dry weather flow, $m^3 d^{-1}$)	OS NGR
Garforth Owlwood [1] [A]	Sheffield Beck (Aire) 15.6	STP effluent and receiving water	Secondary BF 36 868 (10 204)	SE 41683 28738
Heaton Lodge [B]	River Calder 393.4	STP effluent and receiving water	Secondary BF Unknown	SE 17652 20677
Horbury Junction [C]	River Calder 779.5	STP effluent and receiving water	Secondary BF 15 842 (3974)	SE 29992 17362
Knostrop [1] [D]	River Aire 847.5	STP effluent and receiving water	Unknown	SE 34177 30982
Oulton/Lemonroyd [E]	River Aire 890.5	STP effluent and receiving water	Tertiary A1 33 262 (6178)	SE 37869 27952
Oxenhope No. 2 [F]	Bridgehouse Beck (Aire) 14.9	STP effluent and receiving water	Secondary AS 2204 (1632)	SE 03496 35565
Ripponden [G]	River Rybrun (Calder) 37.9	STP effluent and receiving water	Secondary BF 4995 (1150)	SE 04460 20558
Canal Road [V]	River Aire 684.5	CSO effluent	n/a	SE 27685 34037
Commercial Road [W]	River Aire 678.5	CSO effluent	n/a	SE 26497 35079
Cotton Mill Beck [X]	Cotton Mill Beck (Aire) 3.3	CSO effluent	n/a	SE 28233 28655
Oulton Park [Y]	Oulton Beck (Aire) 22.48	CSO effluent	n/a	SE 34700 28307
Newlay Lane [Z]	River Aire 655.5	CSO effluent	n/a	SE 23852 36963
Fishpond Lock weir	River Aire 854.5	Receiving water	n/a	SE 35409 30090
Woodlesford Lock weir	River Aire 857.3	Receiving water	n/a	SE 36695 29519
Fleet weir	River Aire 861.4	Receiving water	n/a	SE 38021 28539

* For site locations see Figure 4-3 and 4-4

Effluent samples collected from an effluent channel downstream of the STP outfall. Approximate downstream distance of 15m at Garforth and 600m at Knostrop.

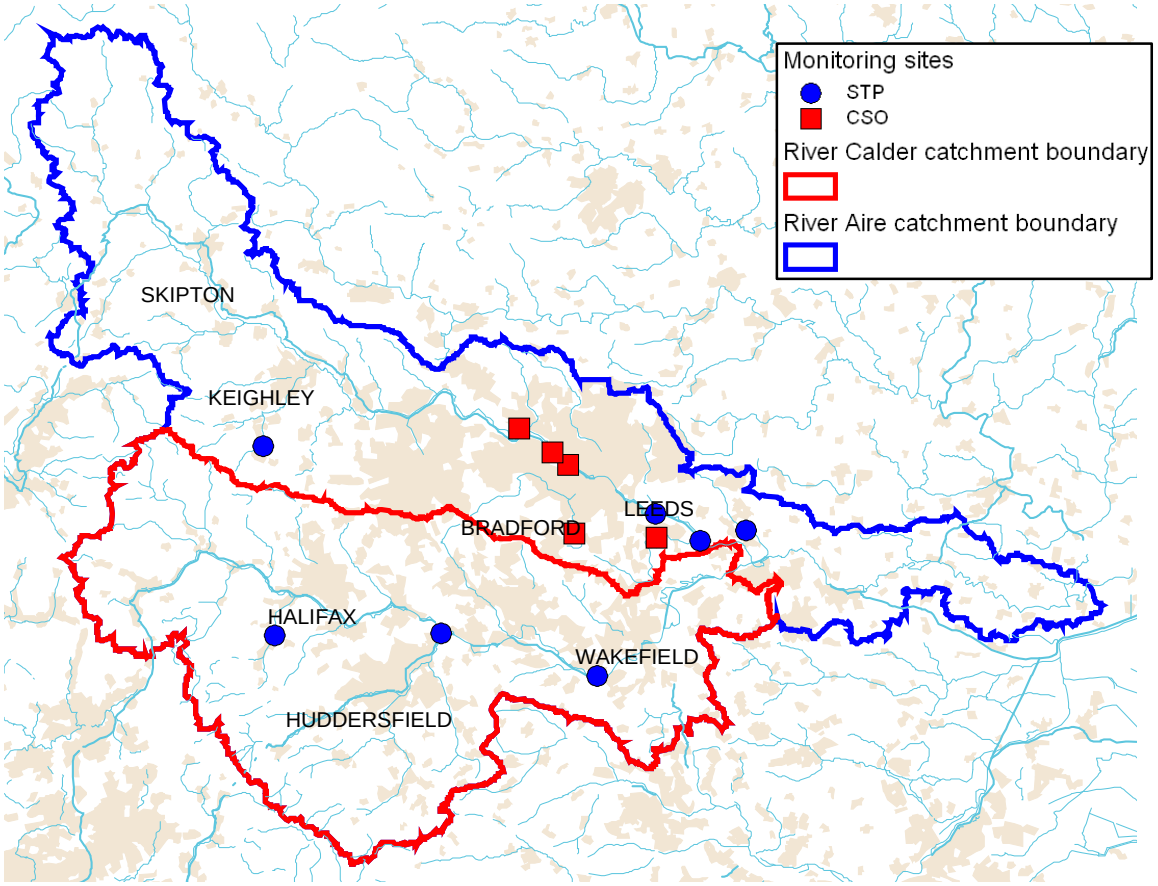


Figure 4-3: Map of sewage treatment plant (STP) and combined sewer overflow (CSO) sampling sites monitored during the period September 2010 to March 2012
For site details see Table 4-2

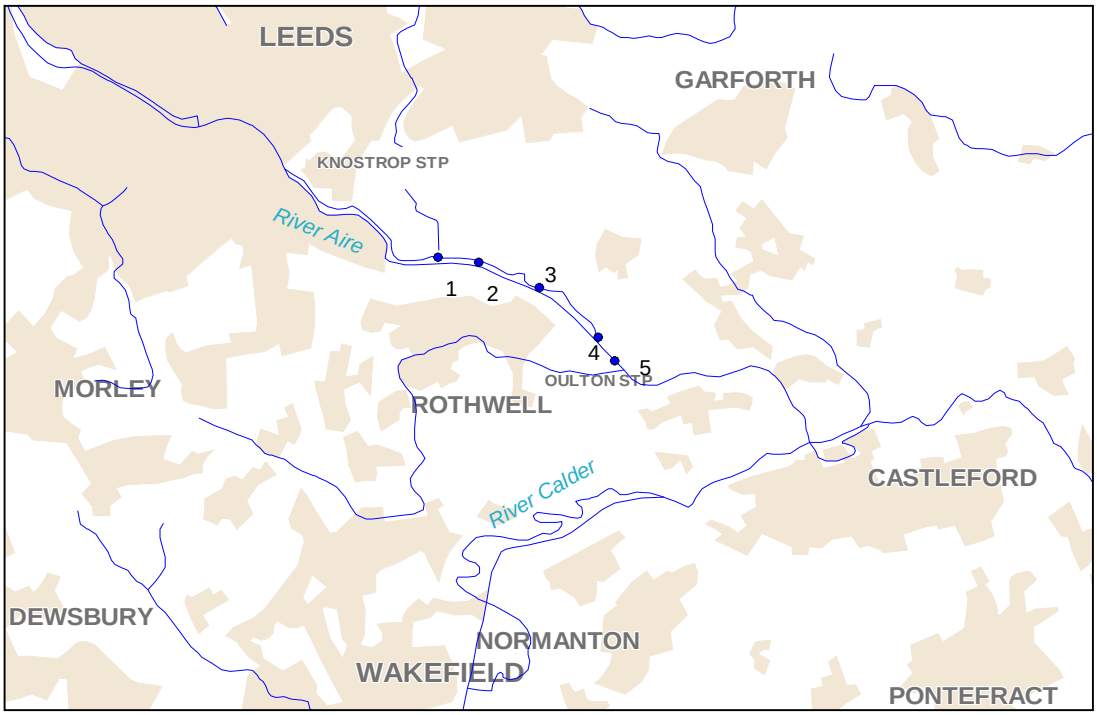


Figure 4-4: Locations of the River Aire reach sampling sites monitored during the period February to November 2011
1. Knostrop outfall, 2. Fishpond Lock weir, 3. Woodlesford Lock weir, 4. Fleet weir, 5. Upstream of Oulton/Lemonroyd outfall

4.2.2.2 Intensive diurnal sampling

Garforth Owlwood and Oulton/Lemonroyd were selected for intensive 24 h sampling given their close proximity to one another and relative ease of sample collection which allowed for the collection of samples from both sites within one hour. Furthermore, the differing treatment technologies in place at these two STPs provided a useful comparison.

4.2.3 Sample collection procedure

Samples of treated effluent and receiving channel water (0.8 L) for all field surveys were collected in 1 L amber glass bottles with Teflon® lined caps (Fisher Scientific, Leicestershire, UK) and kept in the dark on ice during transit. Samples were collected from the centre of the stream at 50 % depth in line with established guidelines (USGS, 2006) where possible although the size of the channel and bank topography meant it was necessary at some sites to collect samples at bankside (Heaton Lodge, Horbury Junction, Oulton/Lemonroyd, and Knostrop). Once returned to the laboratory, samples were stored in the dark at 4 °C before extraction within 48 hours. Solid phase extraction recoveries indicated no appreciable change in pharmaceutical concentrations when 48 h stored samples were compared to those extracted immediately. Other studies have also shown that extended periods of storage (up to 6 months) even at 4 °C caused no appreciable change in spiked concentrations. All apparatus and glassware used during sample collection and preparation were thoroughly washed with 100 % methanol (1 x)

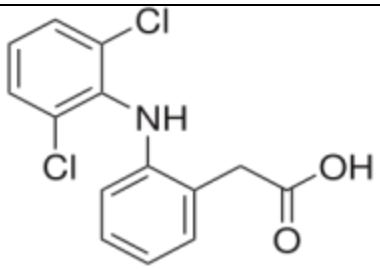
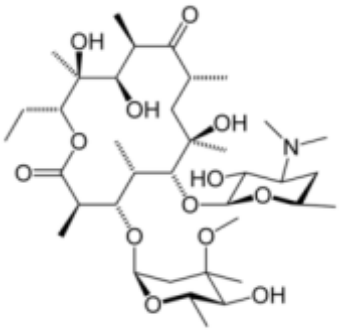
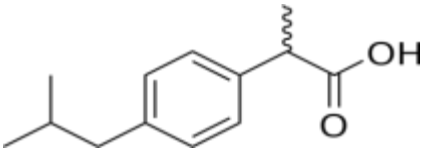
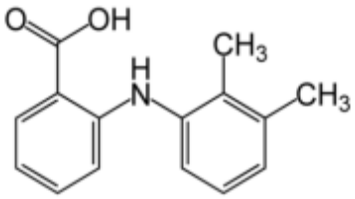
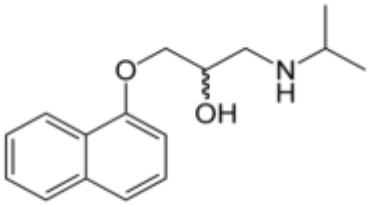
and de-ionised water (3 xs) prior to each use to remove potential contamination. For all samples collected, field measurements of pH, dissolved oxygen and electrical conductivity were recorded using an Intellical LDO probe and HACH HQ30d multi-meter (HACH Company, CO, USA).

Routine STP samples were collected each month at the same time of day to minimise errors associated with diurnal fluctuations in pharmaceutical concentrations (Kanda *et al.*, 2003). At Garforth Owlwood and Knostrop access to the immediate STP outfall was not possible and at these two sites it was necessary to sample from undiluted effluent channels 15 m and 600 m downstream, respectively. Receiving water samples were collected at a point of five times the stream width downstream of the effluent outfall to allow for mixing (Morris, 2013). Grab samples of CSO effluents were collected directly from the outfall.

4.2.4 Study compounds

An initial set of five study compounds was chosen based on potential environmental risk in previously published studies (Table 4-2). Environmental risk was assessed using published estimated risk quotients (RQ) (which is the ratio PEC: PNEC or MEC: PNEC). A RQ≥1 indicates the potential for impacts on aquatic organisms so this was used as the basis for selection. In addition only compounds with a published analytical method and which had previously been detected in UK rivers were selected for monitoring (Table 4-2).

Table 4-2: Structure and properties of the five study compounds

Compound	Therapeutic use	Structure	Physico-chemical properties and risk quotients
Diclofenac	Non-steroidal anti-inflammatory drug (NSAID)		MW = 296.15 Solubility (mg/L) = 2430 pK_a = 4.0 log K_{OW} = 4.02 Excretion rate = 15% parent, <1% conjugates RQ_{min} = 0.01 RQ_{max} = 1.13
Erythromycin	Macrolide antibiotic		MW = 733.95 Solubility (mg/L) = 1.44 pK_a = 8.9 log K_{OW} = 2.48 Excretion rate = 5% parent RQ_{min} = 0.01 RQ_{max} = 1.25
Ibuprofen	NSAID		MW = 206.29 Solubility (mg/L) = 21.00 pK_a = 4.91 Excretion rate = 1% parent log K_{OW} = 3.79 RQ_{min} = 0.55 RQ_{max} = 4.20
Mefenamic acid	NSAID		MW = 241.29 Solubility (mg/L) = 20.00 pK_a = 4.2 Excretion rate = 52% conjugates log K_{OW} = 5.12 RQ_{min} = 1.03 RQ_{max} = 8.31
Propranolol	Non-selective beta-blocker		MW = 259.35 Solubility (mg/L) = 31.7 pK_a = 9.4 log K_{OW} = 2.60 Excretion rate = <0.5% parent, 20% active conjugates RQ=0.21

Notes:
MW= molecular weight; pK_a = dissociation constant; log K_{OW} = octanol: water partition coefficient
RQ data from: (Jones *et al.*, 2002; Thompson, 2006; Yamamoto *et al.*, 2009)
Physicochemical data from: (Ternes, 1998; Kasprzyk-Hordern *et al.*, 2007), ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/), DrugBank (Knox *et al.*, 2010) and ECOSAR v1.00a (http://www.epa.gov/oppt/newchems/tools/21ecosar.htm)

There was some variation between RQs calculated by Thompson (2006) and Jones *et al*. (2002), the major reason for which is the assumed dilution factor; Jones assumed 1:10 dilution whereas Thompson assumed none. Thompson's estimates are

conservative but 1:10 dilution factor may not be achieved in some rivers, particularly during low summer flows (Hemminger, 2005). It is likely that actual RQs will fall somewhere between the two estimates.

4.2.5 Collation of flow data

Flow data (15 min) from the closest relevant river gauge to each sampling location were obtained from the Environment Agency (see Appendix B1) to compare receiving water concentrations with flow. Flow or stage duration curves were therefore available at or close to all but one sampling site (Garforth Owlwood).

4.2.6 Analysis of receiving water and effluent samples

4.2.6.1 Chemicals and materials

Pharmaceutical standards were used to create working and stock solutions in dilution series for calibration of analytical instruments. All pharmaceuticals were supplied by Sigma-Aldrich Company Ltd. (Dorset, UK) and were of the highest purity available (>99 %). Individual stock standard solutions were prepared on a weight basis in 100% methanol and stored in the dark at -20 °C until used. A fresh working mixture solution of all pharmaceuticals was prepared by appropriate dilution of the individual stocks in methanol-water (20:80, v/v) immediately before each analytical run and used as working standard solutions. Working standards were also stored in the dark at -20°C between uses. HPLC-grade methanol was supplied by Fisher Scientific UK Limited (Loughborough, UK). Deionised water was supplied by a Purite Select HP160/BP/IT deioniser.

4.2.6.2 Solid phase extraction (SPE)

The SPE procedure and subsequent analysis by HPLC and Q-TOF MS/MS used during this study followed that of Petrovic *et al*. (2006). A total of 800 mL of unfiltered sample was accurately measured for SPE. For the SPE, a 20-position Waters vacuum extraction manifold was used in conjunction with Oasis HLB SPE cartridges (6 cc, 150 mg; Waters Corporation, Milford, MA, USA). First the SPE cartridges were conditioned with 5 mL 100% methanol followed by 5 mL de-ionised water at a flow rate of 1 mL min^{-1}. The 800 mL river and effluent samples were then loaded onto the SPE cartridge at a flow rate of 10 mL min^{-1} during which care was taken not to let the sorbent material dry out. The cartridges were then rinsed with 5 mL de-ionised water at a flow rate of 1 mL min^{-1} prior to being thoroughly air dried under vacuum for 15 minutes to remove excess water. Elution of the cartridges was then performed with 2 * 4 mL of 100 % methanol at a flow rate of 1 mL min^{-1} directly into glass centrifuge tubes. The eluent was evaporated to dryness under vacuum centrifugation and reconstituted in 0.5 mL of methanol: water

(20:80, v/v). Sample extracts were stored in the dark at -20 °C prior to analysis. Data reported are for extractions from unfiltered samples and as such will potentially include pharmaceuticals sorbed to suspended particulate material. However, previous studies have shown that this fraction only represents a small proportion of the total load (Miao & Metcalfe, 2007) and observations during SPE showed very little suspended particulate material was present in the samples. Sample extracts were stored for periods up to six months as tests have shown that storage of samples at frozen (-20 °C) and chilled temperatures (up to 4 °C) caused no significant change in sample concentration (Radke *et al.*, 2010).

4.2.6.3 HPLC-Q-TOF-MS/MS

Liquid chromatography was performed using an Ultimate 3000 nanoLC system (Dionex) on a 2.1 * 100mm Acclaim RSLC 120 C18 2.2 µm particle silica column. In positive ion (PI) mode analysis a solvent system of 5 mM ammonium acetate/acetic acid (pH 2.4; buffer A) and methanol-acetonitrile (2:1, v/v, buffer B) was used at a flow rate of 0.3 mL min^{-1} and the column held at constant temperature (30 °C). In negative ion (NI) mode analysis a solvent system of 2 mM ammonium acetate (buffer A) and 2mM ammonium acetate in methanol-water (95:5, v/v, buffer B) was used at a flow rate of 0.3 mL min^{-1} and the column held at constant temperature (30°C). After injection the gradient was held at 5 % B for 5 minutes followed by 5-80 % B over 20 minutes. A 15 minute column wash at 95 % B and an equilibration of 5 min at 5 % B was performed between each sample injection.

The LC eluent was directly infused into the Z-spray electrospray source of a quadrupole-ion mobility-orthogonal time of flight (Q-TOF) mass spectrometer (Synapt HDMS, Waters UK). Electrospray desolvation temperature was 150 °C and desolvation gas was 500 L h^{-1}, capillary and cone voltages were 3.2 kV and 30 V respectively. Backing pressure was 2.1 bar, the argon pressure in the trap and transfer region of the IMS cell was 2.17 mbar. MS/MS analysis was performed only on the target pharmaceutical compounds when their intensity was greater than 10 counts per second and their retention time was within ±1 min of that of the corresponding standards. Trap collision energy used for each compound was optimised individually between 15 and 30 V. The lockspray frequency was set to one 1 s scan every 10 s. For PI mode a 5 µM solution of Glu fibrinopeptide in methanol/0.1 % formic acid (50:50, v/v) was used as the lock mass, in NI mode the *m/z* 276 peak of a 2 ng μL^{-1} of sodium iodide in methanol water (50:50, v/v) was used as the lock mass.

4.2.7 Data analysis and method validation

All data analysis was performed in MassLynx™ (v4.1, Waters Ltd.). Positive identification of the target pharmaceuticals was based on: accurate mass measurement

of the parent ion within an error of ±20 ppm, accurate mass of at least one product ion also within an error of ±20 ppm and LC retention time of the target analyte with reference to that of a standard (±5 %). Quantitation was performed by constructing extracted ion chromatograms (XICs) of the protonated (PI mode) and deprotonated (NI mode) ion using a 20 mDa window centred on the theoretical *m/z* of the compound and taking the area of the resulting peak using MassLynx's inbuilt integration algorithms.

Reproducibility of the method was tested with repeated injections of a standard solution both sequentially and day-to-day. Instrumental detection limits (IDLs) were estimated using a standard solution in dilution series until reaching a concentration yielding a signal: noise (S: N) ratio of three. Linear dynamic ranges were determined for each compound by injecting a dilution series of the working standard mixture solutions across a wide concentration range (2-1000 ng L^{-1}). Calibration curves of peak area (from XICs) vs. concentration were created using linear regression analysis and the linear range that gave good fit (r^2>0.95) was established for each compound.

4.2.8 Matrix effects and signal suppression

Ion suppression (and occasionally enhancement) due to matrix effects is a phenomenon in LC-MS whereby components of a complex matrix sample co-elute from the LC column with the analyte(s) of interest and influence the ionisation process at the beginning of the MS stage. This can lead to a reduced (or enhanced) analyte signal and reduce reproducibility. The mechanisms are not fully understood but it is believed that co-eluted compounds compete with the analytes for space and charge on the surface of the small droplets formed during electrospray ionisation (ESI) (Jessome, 2006). The source of the interfering components may be endogenous within the sample (e.g. a complex river or effluent sample) or may be due to contamination during sample preparation. A common method to attempt to separate the analyte(s) of interest from interfering matrix components is to use sample preparation techniques such as solid phase extraction (SPE).

SPE recovery and signal suppression due to matrix effects were evaluated by spiking known concentrations of all five pharmaceuticals into replicates of deionised water and a sample matrix, in this case a sample of 'clean' river water from Silsden Beck immediately downstream of Silsden Reservoir (OSNGR SE 0446147504). This location is upstream of all STP inputs. Three replicates each of both deionised and river water were spiked at the following concentrations: 20, 100 and 200 ng L^{-1} for a total of 18 samples. The pharmaceutical standards (in the form of working solutions dissolved in 100 % methanol) were added prior to SPE and handled in the same way in order to account for losses during the sample preparation and extraction and that due to matrix assisted signal suppression (Kasprzyk-Hordern *et al.*, 2007).

4.2.9 Data analysis

Detection frequencies were calculated by dividing the total number of samples by the number of samples containing a detectable pharmaceutical concentration. All pharmaceutical concentration values were tested for normality using the Anderson-Darling normality test and were found to be generally non-normal; therefore non-parametric techniques were used throughout.

Flows at sampling dates were classified based on the daily mean flow from individual site flow duration curves as follows: $<Q_{25}$ high flow, Q_{25} to Q_{75} normal flow, $>Q_{75}$ low flow. Comparisons of receiving water concentrations between flow categories were tested using the Kruskal-Wallis H test. Pairwise comparisons between CSO and STP effluent were performed using the non-parametric Mann-Whitney U test.

Differences between concentration based on sampling site and date were tested using a General Linear Model (GLM) which also tested for interaction between site and date. Any subsequent pairwise comparisons between categories were performed with a Tukey's family error rate of 0.05 to correct for multiple comparisons.

4.3 Results

4.3.1 Total sample numbers and frequency of detection

A total of 317 samples were collected across all campaigns representing 121 STP effluent, 185 receiving water and 11 CSO effluent samples (Table 4-3). Detection frequencies were high in STP effluent and receiving waters (≥51 % overall) although detection frequencies in CSO effluent were usually lower (Table 4-4). Diclofenac (≥67 %), erythromycin (≥78 %) and ibuprofen (≥83 %) were detected at particularly high frequencies. Notably, several compounds were detected at 100 % frequency in certain sampling campaigns, particularly during the intensive diurnal sampling (Table 4-4). Furthermore, detection frequencies were comparable between channel and effluent samples.

Table 4-3: Summary of sample numbers collected from September 2010 to March 2012

Campaign	No. effluent samples	No. receiving water samples	No. CSO samples	TOTAL
Monthly STP and receiving water	121	125	-	246
Intensive diurnal	-	28	-	28
River Aire reach	-	32	-	32
CSO	-	-	11	11
TOTAL	121	185	11	317
Aire	71	131	11	
Calder	50	54	-	

Table 4-4: Summary of detection frequency (%) of pharmaceutical compounds in samples from the Aire and Calder catchments

	Erythromycin	Propranolol	Mefenamic acid	Ibuprofen	Diclofenac
Monthly sampling					
Secondary AS Channel	77.8	44.4	27.8	83.3	66.7
Secondary AS Effluent	88.9	77.8	16.7	83.3	88.9
Secondary BF Channel	95.5	71.9	68.5	94.4	96.6
Secondary BF Effluent	89.4	75.3	52.9	89.4	94.1
Tertiary Channel	94.4	77.8	94.4	94.4	100.0
Tertiary Effluent	100.0	88.9	77.8	100.0	100.0
Intensive diurnal sampling					
Garforth Owlwood	100.0	100.0	100.0	100.0	100.0
Oulton/Lemonroyd	100.0	92.9	100.0	100.0	100.0
River Aire reach samples					
	93.8	84.4	96.9	96.9	100.0
CSO effluent samples					
	64.3	35.7	21.4	100.0	85.7
TOTAL CHANNEL	94.6	76.2	76.8	94.6	95.1
TOTAL EFFLUENT	90.9	77.7	51.2	90.9	94.2

Notes: AS = activated sludge, BF = biological filter

4.3.2 Method performance

Overall the performance of the HPLC-MS/MS method was very good (Table 4-5) with a detection limit of 5 ng L^{-1} for all study compounds except ibuprofen (25 ng L^{-1}). Precursor ion mass accuracies were within acceptable ranges for all five compounds although product ion mass accuracies fell outside the 20 ppm range for three compounds (EC,

2002). Overall, SPE recovery in both deionised water and matrix samples compared favourably with a similar study (Petrovic *et al*. 2006). All data presented below represent values corrected for SPE recovery in matrix samples.

Table 4-5: Performance of the HPLC-MS/MS method and SPE recovery for receiving water and STP effluent samples

Compound	Mean t_R (mins)	Instrumental detection limit (ng L^{-1})	Linear dynamic range (ng L^{-1})	Mean R^2	Mean precursor mass accuracy (ppm)	Mean (product(s)) mass accuracy (ppm)	SPE recovery DI H_2O (%)	SPE recovery matrix H_2O (%)
Diclofenac	19.5	5	0 - 1000	0.991	-16.6	6.1	65.8 (±12.9)	105.2 (±33.2)
Erythromycin	19.4	5	0 - 1000	0.983	6.5	-39.1 -33.6	93.8 (±11.0)	71.8 (±26.3)
Ibuprofen	20.1	25	0 - 1000	0.983	3.5	365.1	84.9 (±18.3)	61.2 (±17.6)
Mefenamic acid	20.7	5	0 - 1000	0.992	-8.1	18.7	87.0 (±14.5)	70.7 (±25.8)
Propranolol	16.2	5	0 - 1000	0.985	0.2	-20.2 -130.5	92.9 (±21.1)	81.2 (±24.2)

4.3.3 Monthly sampling

A total of 121 effluent and 125 receiving water samples were collected during the monthly sampling campaign (Tables 4-6 and 4-8 for summaries; Appendix B for raw data). The physicochemical data suggest that the rivers Aire and Calder were at moderate to good status based on Water Framework Directive criteria for pH and dissolved oxygen (DO) during the sampling campaign (Table 4-6). Notably, DO was elevated and electrical conductivity reduced at the two upstream semi-rural sites (Oxenhope and Ripponden) when compared to the more urbanised downstream locations. Overall, test substances demonstrated a trend of maximum pharmaceutical concentrations during the winter period, particularly in the winter 2010/11 (e.g. Figure 4-5). This trend was evident at all sites but was less pronounced at Oxenhope and Ripponden. Furthermore, propranolol differed from the other study compounds by demonstrating peak concentrations during winter 2010/11.

Table 4-6: Mean (±1 SD) physicochemical data during the monthly sampling campaign

Site	Channel	pH	Dissolved oxygen (mg L^{-1})	Electrical conductivity (µs cm^{-1})
Garforth Owlwood	Channel	7.7 ± 0.3	9.3 ± 0.5	1234.0 ± 465.3
	Effluent	7.4 ± 0.5	8.7 ± 0.6	1300.6 ± 318.3
Heaton Lodge	Channel	7.5 ± 0.1	9.9 ± 0.8	839.9 ± 183.6
	Effluent	7.0 ± 0.1	9.5 ± 0.5	2779.6 ± 556.1
Horbury Junction	Channel	7.5 ± 0.1	9.2 ± 1.0	959.6 ± 158.8
	Effluent	7.3 ± 0.1	7.4 ± 2.1	1068.0 ± 82.8
Knostrop	Channel	7.4 ± 0.2	9.8 ± 0.7	1520.1 ± 224.2
	Effluent	7.3 ± 0.2	9.5 ± 0.5	1534.7 ± 229.7
Oulton	Channel	7.8 ± 0.2	9.3 ± 1.0	1274.4 ± 229.7
	Effluent	7.6 ± 0.5	9.4 ± 0.5	1231.7 ± 180.4
Oxenhope	Channel	7.6 ± 0.1	10.4 ± 0.8	236.4 ± 22.2
	Effluent	6.8 ± 0.2	8.2 ± 0.6	718.9 ± 71.0
Ripponden	Channel	7.4 ± 0.2	10.3 ± 0.7	288.1 ± 19.5
	Effluent	6.5 ± 0.1	8.1 ± 1.5	622.4 ± 42.1

Table 4-7: Mean (±1 SD) receiving water pharmaceutical concentrations during the monthly sampling campaign

Site	Diclofenac[1]	Erythromycin	Ibuprofen	Mefenamic acid	Propranolol
Garforth Owlwood	589.6 ± 887.4 (100.0)	393.5 ± 475.7 (100.0)	779.6 ± 828.5 (93.8)	11.3 ± 7.7 (87.5)	37.1 ± 38.8 (68.8)
Heaton Lodge	156.9 ± 318.3 (100.0)	249.0 ± 278.9 (88.2)	320.2 ± 284.9 (82.4)	31.5 ± 32.6 (58.8)	10.5 ± 5.6 (70.6)
Horbury Junction	173.4±196.6 (94.1)	199.7 ± 251.2 (100.0)	1146.5 ± 863.8 (94.1)	4.5 ± 4.9 (35.3)	12.5 ± 10.3 (88.2)
Knostrop	272.8 ± 265.8 (100.0)	184.6 ± 302.2 (100.0)	302.2 ± 223.2 (100.0)	7.9 ± 8.6 (82.4)	15.9 ± 13.0 (94.1)
Oulton	231.3 ± 237.5 (100.0)	249.1 ± 287.9 (100.0)	793.7 ± 1035.4 (100.0)	6.9 ± 8.2 (77.8)	14.3 ± 11.5 (88.9)
Oxenhope	60.8 ± 93.5 (88.9)	398.8 ± 480.3 (88.9)	363.0 ± 318.7 (88.9)	2.5 (16.7)	14.5 ± 10.7 (77.8)
Ripponden	110.5 ± 148.1 (100.0)	240.4 ± 257.6 (78.6)	524.4 ± 585.6 (100.0)	107.8 (7.1)	9.7 ± 5.1 (71.4)
Detection frequency (%)	90.9	77.7	51.2	90.9	94.2

Table 4-8: Mean (±1 SD) effluent pharmaceutical concentrations during the monthly sampling campaign

Site	Diclofenac[1]	Erythromycin	Ibuprofen	Mefenamic acid	Propranolol
Garforth Owlwood	649.3 ± 957.5 (100.0)	402.8 ± 307.4 (94.1)	812.2 ± 848.9 (100.0)	10.6 ± 11.1 (94.1)	19.1 ± 11.6 (88.2)
Heaton Lodge	88.1 ± 86.6 (94.4)	143.4 ± 126.7 (94.4)	6165.1 ± 23388.8 (88.9)	21.8 ± 19.8 (61.1)	5.3 ± 6.1 (55.6)
Horbury Junction	103.4 ± 121.5 (100.0)	121.1 ± 108.4 (100.0)	3741.1 ± 13316.1 (88.9)	23.8 ± 28.5 (72.2)	4.1 ± 2.0 (77.8)
Knostrop	323.1 ± 307.3 (100.0)	229.5 ± 254.2 (100.0)	719.9 ± 1090.8 (100.0)	11.6 ± 11.1 (88.9)	30.0 ± 38.0 (94.4)
Oulton	187.1 ± 169.6 (100.0)	138.2 ± 132.1 (100.0)	456.5 ± 639.3 (94.4)	10.7 ± 8.0 (94.4)	8.4 ± 17.0 (83.3)
Oxenhope	39.0 ± 37.8 (66.7)	49.2 ± 40.1 (77.8)	99.7 ± 56.8 (83.3)	3.8 ± 2.8 (27.8)	2.9 ± 1.2 (44.4)
Ripponden	25.1 ± 18.2 (94.1)	33.7 ± 43.0 (94.1)	123.6 ± 96.9 (100.0)	6.3 ± 5.5 (29.4)	2.9 ± 1.2 (47.1)
Detection frequency (%)	92.8	93.6	92.8	66.4	69.6

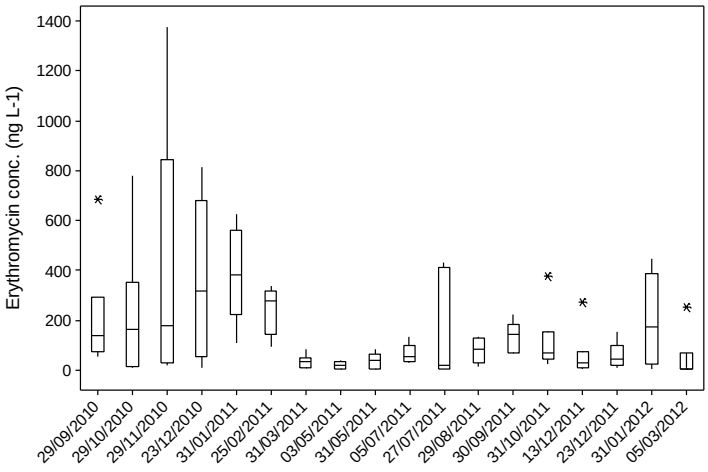


Figure 4-5: Example of seasonal trend in pharmaceutical concentrations [Values are erythromycin concentrations in receiving waters at 7 sites from the Aire and Calder catchments]

4.3.3.1 Relationship between flow and receiving water concentrations

Flow at the time of sampling was classified into low, normal or high flow based on flow duration curves which were available at all but one site (Garforth Owlwood). Significant differences between receiving water concentrations at different flows were present for all compounds except mefenamic acid (Table 4-9; Figure 4-6). Receiving water concentrations during moderate and low flow were consistently higher than those during high flow. However, frequencies of detection were consistently greatest during high flow for all compounds except mefenamic acid (Figure 4-7). During moderate and low flow compounds were detected less frequently but when present were at higher concentrations.

Table 4-9: Summary of statistical analysis comparing pharmaceutical concentrations from monthly sampling in the Aire and Calder catchments during differing flow conditions

Compound	Kruskal-Wallis H results	Median concentrations (ng L^{-1})[1]		
		Low flow (n=39)	Moderate flow (n=54)	High flow (n=13)
Erythromycin	H=10.01, DF=2 p<0.01	45.5^{B}	92.9^{A}	29.8^{B}
Propranolol*	H=7.26, DF=2 p=0.03	6.1^{A}	5.5^{A}	2.5^{A}
Mefenamic acid	H=1.79, DF=2 p=0.41	7.9^{A}	8.2^{A}	4.6^{A}
Ibuprofen*	H=10.79, DF=2 p<0.01	132.9^{A}	257.6^{A}	96.6^{A}
Diclofenac	H=7.75, DF=2 p=0.02	$43.4^{A\,B}$	96.5^{A}	33.7^{A}

Notes:
1. Results of pairwise comparisons between flow categories (Tukey's family error rate: 0.05). Categories that do not share a letter are significantly different.
* Note that Kruskal-Wallis H test for ibuprofen and propranolol indicated significant differences but pairwise comparisons did not.

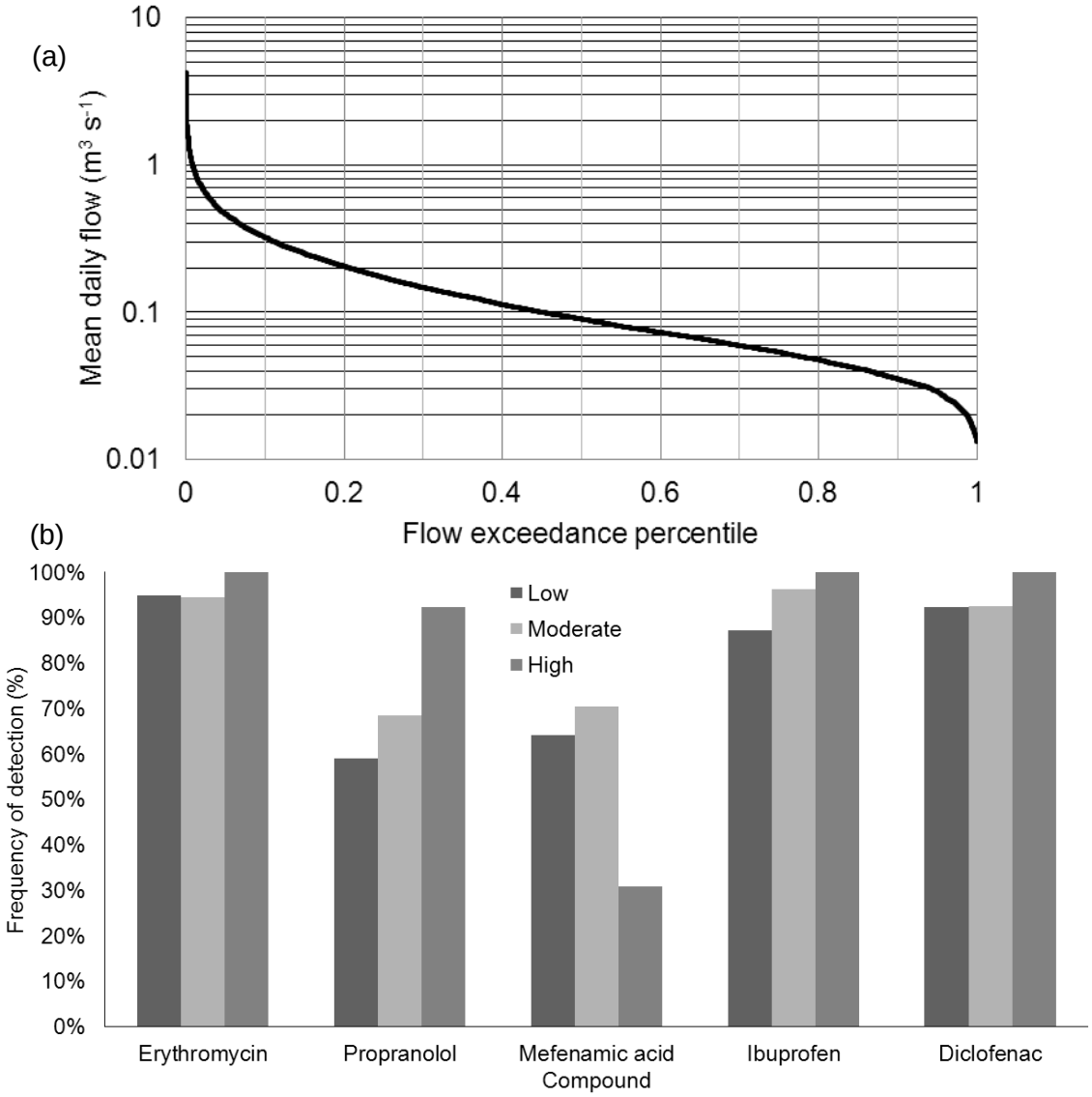


Figure 4-6: (a) example flow duration curve for Oulton Beck and (b) frequencies of detection for pharmaceutical compounds in the Aire and Calder catchments during low, moderate and high flow conditions

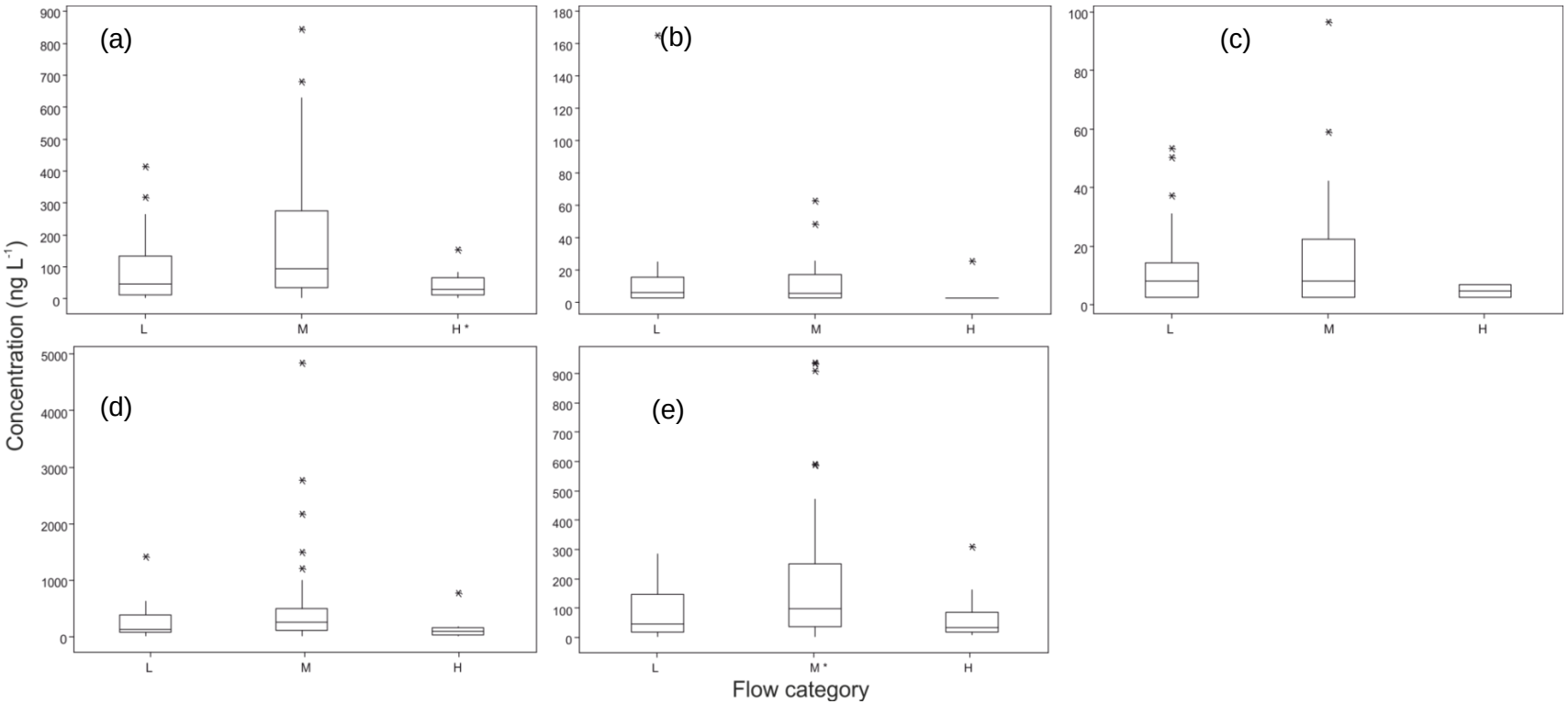


Figure 4-7: Boxplots of receiving water concentrations from the monthly sampling campaign classified by low, moderate or high flow for (a) erythromycin, (b) propranolol, (c) mefenamic acid, (d) ibuprofen and (e) diclofenac
(Note: two extreme outliers of ibuprofen (93 863 and 53 633 ng L^{-1}) were excluded from this image for clarity but were included in statistical analyses. Asterisk markers in the x-axis represent significant differences between flow categories; see Table 4-13 for statistical analyses)

4.3.3.2 Comparison of treatment technologies

Significant differences in concentrations between different STP treatment technologies were present for propranolol, ibuprofen and diclofenac (Table 4-10). Secondary BF and Tertiary A1 samples demonstrated median concentrations that were generally 2 to 3xs greater than respective concentrations from the Secondary AS STP in both receiving waters and effluent (Figure 4-8; 4-9).

Table 4-10: Summary of statistical comparisons of pharmaceutical concentrations between different STP treatment technologies

Compound	Channel	Effluent
Erythromycin	H=4.51 DF=2 P=0.11	H=3.08 DF=2 P=0.21
Propranolol	H=7.26 DF=2 P=0.03	H=0.04 DF=2 P=0.98
Mefenamic acid	H=3.75 DF=2 P=0.154	H=5.18 DF=2 P=0.08
Ibuprofen	H=11.86 DF=2 P<0.01	H=2.91 DF=2 P=0.23
Diclofenac	H=9.93 DF=2 P<0.01	H=11.36 DF=2 P<0.01

Note: pairwise comparisons with Tukey's family error rate of 0.05 did not reveal any significant differences

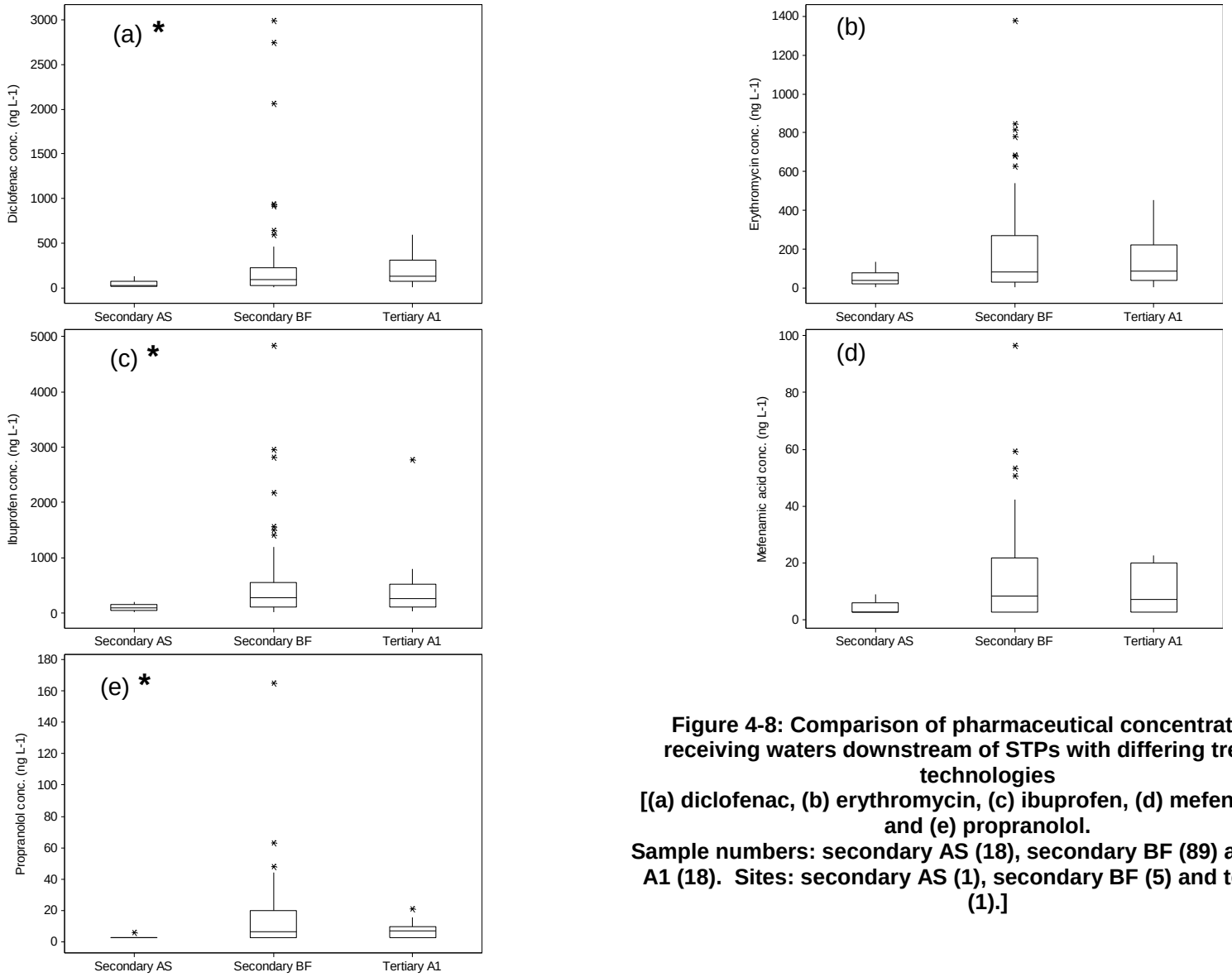


Figure 4-8: Comparison of pharmaceutical concentrations in receiving waters downstream of STPs with differing treatment technologies
[(a) diclofenac, (b) erythromycin, (c) ibuprofen, (d) mefenamic acid and (e) propranolol.
Sample numbers: secondary AS (18), secondary BF (89) and tertiary A1 (18). Sites: secondary AS (1), secondary BF (5) and tertiary A1 (1).]

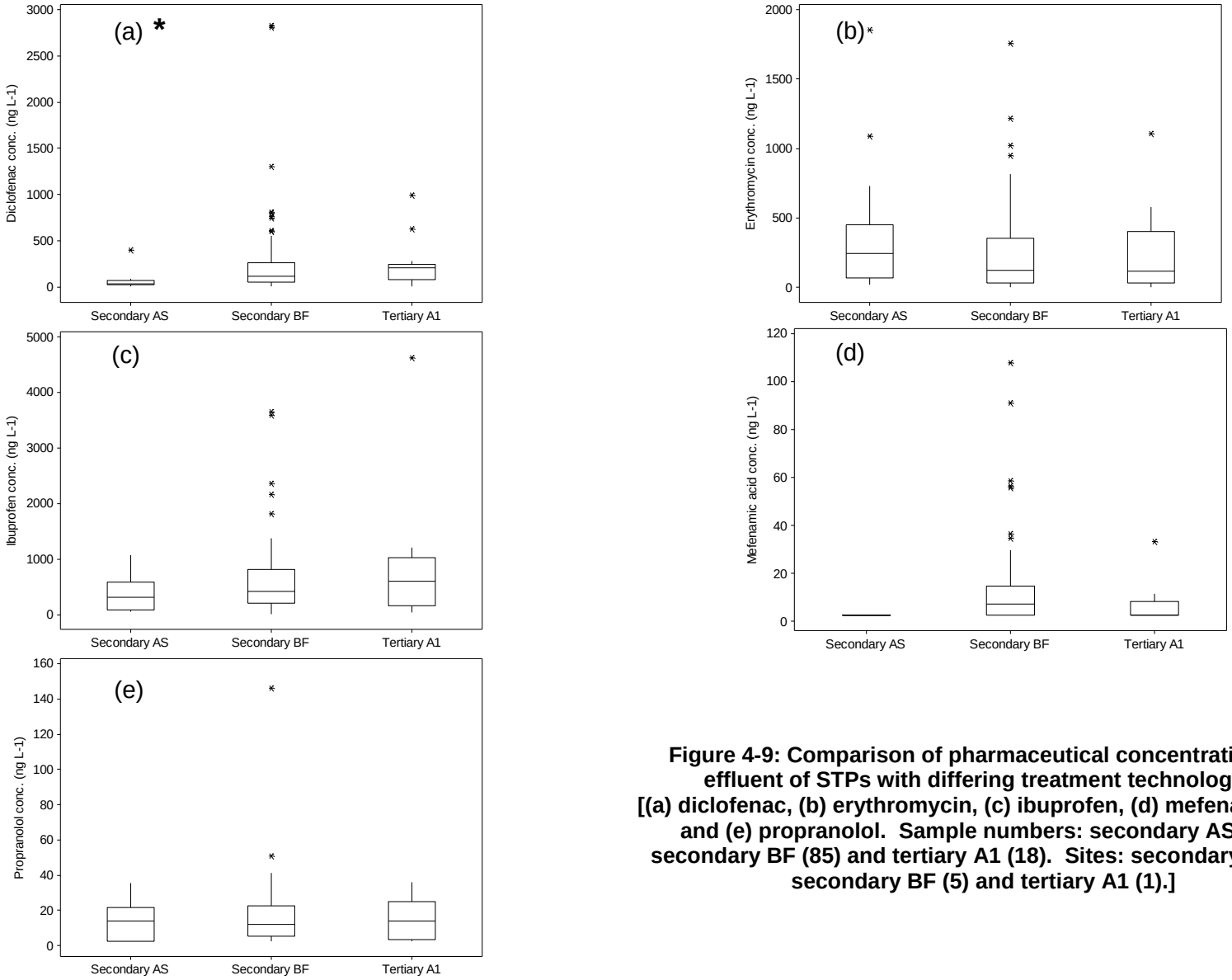


Figure 4-9: Comparison of pharmaceutical concentrations in effluent of STPs with differing treatment technologies [(a) diclofenac, (b) erythromycin, (c) ibuprofen, (d) mefenamic acid and (e) propranolol. Sample numbers: secondary AS (18), secondary BF (85) and tertiary A1 (18). Sites: secondary AS (1), secondary BF (5) and tertiary A1 (1).]

4.3.3.3 Comparison of sample matrix

Effluent samples demonstrated greater concentrations than receiving waters for all compounds except mefenamic acid. This relationship was highly significant for erythromycin (H=6.53, DF=1, p=0.01), propranolol (H=9.91, DF=1, p<0.01) and ibuprofen (H=10.81, DF=1, p<0.01) with effluent concentrations approximately 2 x greater than channel concentrations. This confirms STP effluent as a major source of pharmaceuticals to rivers.

4.3.4 CSO sampling

All five pharmaceutical compounds were present in CSO effluent albeit at generally lower detection frequencies compared to STP effluent and associated receiving waters (Table 4-11). Erythromycin concentrations in CSO effluent were significantly less than those in STP effluent (17.3 ng L^{-1} vs. 139.7 ng L^{-1}) but this was not the case for the other four compounds where concentrations were broadly similar between the two sample types.

Table 4-11: Results of pharmaceutical monitoring in combined sewer overflow effluent

Site	No. samples	Diclofenac	Erythromycin	Ibuprofen	Mefenamic acid	Propranolol
Newlay Lane	1	160.7	1602.7	75.5	-	-
Canal Road	2	186.8 ± 123.2	-	143.4 ± 190.3	18.8 ± 23.0	-
Commercial Road	4	74.0 ± 68.4	256.0 ± 407.5	2734.1 ± 4887.8	19.2	10.2 ± 10.9
Oulton Beck	7	388.3 ± 791.9	98.0 ± 128.6	2207.0 ± 5303.2	-	11.3 ± 15.2
CSO vs. STP effluent	-	H=1.88 p=0.17	H=5.55 p=0.02	H=1.37 p=0.24	H=0.92 p=0.34	H=1.86 p=0.17
Detection frequency (%)	-	85.7	64.3	100.0	21.4	35.7

4.3.5 Intensive diurnal sampling

Concentrations of all five pharmaceutical compounds sampled from Sheffield Beck (downstream of Garforth STP) and the River Aire (downstream of Oulton/Lemonroyd STP) varied by up to a factor of four within the same day (Table 4-12). Detection frequencies amongst these samples were 100 % for all compounds except for propranolol on the first sampling day (86 %). Mean channel concentrations were significantly greater at Garforth Owlwood for all compounds except propranolol. Although concentrations varied throughout the day there were no statistically significant temporal trends. Note that there was no rainfall for several days prior to or during the sampling campaign at either site. A check of the flow gauge record before and during the sampling campaign revealed flow conditions were stable.

4.3.6 River Aire reach monitoring

The data indicate no appreciable reduction in pharmaceutical concentrations along the 5 km study reach and in some cases mean concentrations fluctuate and increase slightly

along the reach (Figure 4-10). However, none of these concentration changes were statistically significant. The general trend amongst the sampling campaign was for reduced River Aire concentrations later in the year (October to November) when compared to spring and summer samples.

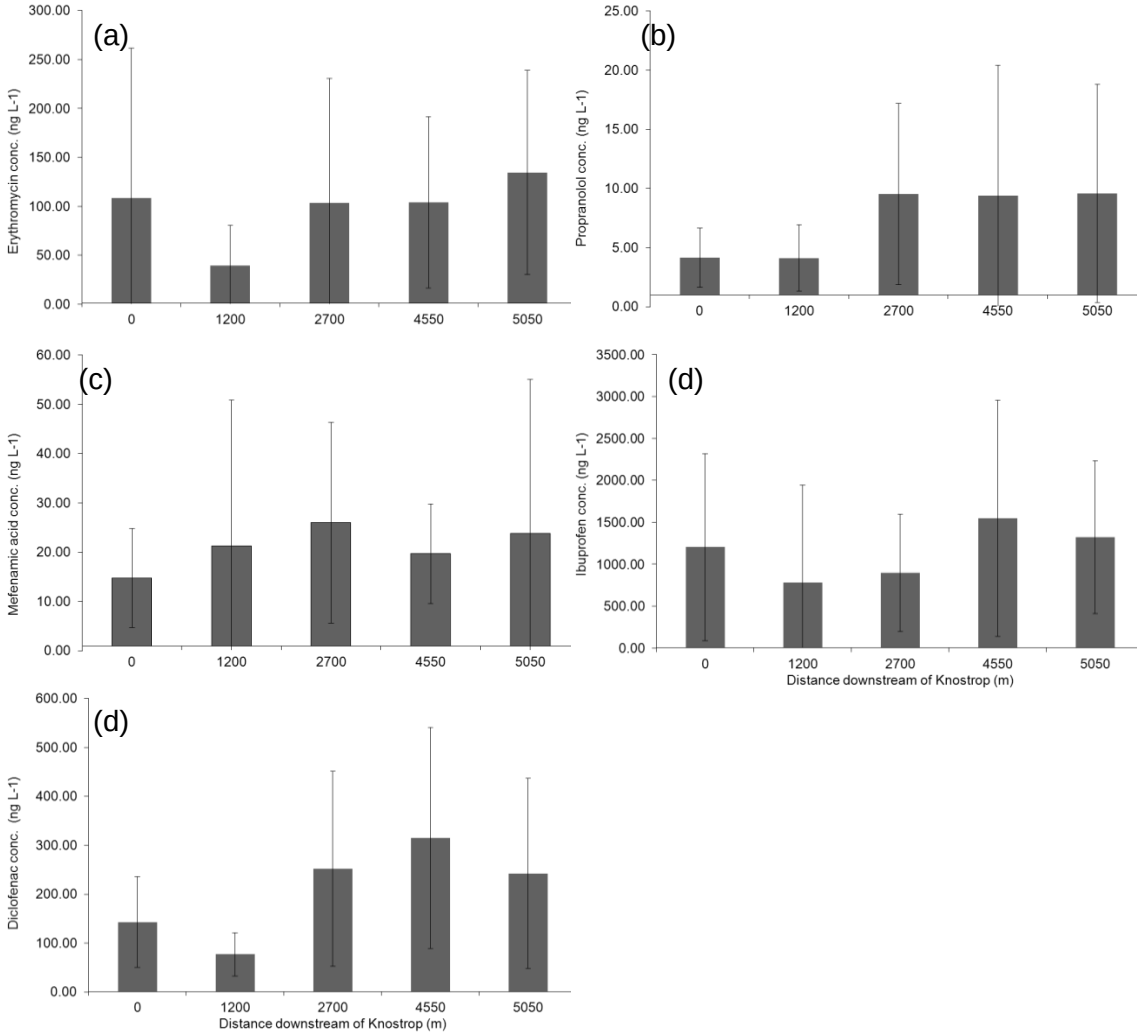


Figure 4-10: Downstream concentration profiles of (a) erythromycin, (b) propranolol, (c) mefenamic acid, (d) ibuprofen and (e) diclofenac along a 5 km stretch of the River Aire between two major STPs (Knostrop and Oulton)
[values are means of seven replicates and bars represent ± 1 SD; samples collected Spring-Autumn 2011]

Table 4-12: Mean (±1 SD) concentrations of pharmaceuticals (ng L^{-1}) in receiving water of two STPs during intensive diurnal sampling

Time	Garforth Owlwood					Oulton/Lemonroyd				
	Erythromycin	Propranolol	Mefenamic acid	Ibuprofen	Diclofenac	Erythromycin	Propranolol	Mefenamic acid	Ibuprofen	Diclofenac
0500	133.7 ± 1.6	2.5	30 ± 16.1	743.1 ± 68.02	1747.5 ± 407.4	66.1 ± 16.5	4.5 ± 2.8	8.2 ± 8.0	261.7 ± 29.3	479.6 ± 186.9
0800	146.3 ± 25.5	5.9 ± 4.8	58.2 ± 46.1	829.8 ± 31.7	2228.7 ± 196.6	51.2 ± 4.7	11.9	10.5 ± 1.6	464.7 ± 280.5	316.4 ± 34.5
1100	210.6 ± 38.04	13.4 ± 6.2	53.1 ± 6.4	1424.4 ± 734.8	3583.3 ± 1139.4	61.2 ± 1.7	4.5 ± 2.8	9.4 ± 0.3	362.5 ± 145.0	947.7 ± 240.1
1400	155.3 ± 8.4	7.35 ± 6.9	29.5 ± 4.7	1312.9 ± 903.7	2697.6 ± 947.5	68.8 ± 5.4	2.5	10.6 ± 2.4	449.0 ± 76.2	359.6 ± 243.7
1700	128.9 ± 20.6	7.2 ± 6.6	60.4 ± 39.1	1263.6 ± 14.1	2732.5 ± 737.0	67.3 ± 22.5	6.8 ± 1.9	18.5 ± 8.7	476.5 ± 314.5	438.4 ± 2.4
2000	202.1 ± 63.9	11.2 ± 0.8	72.4 ± 64.1	1300.7 ± 494.4	2912.1 ± 869.5	61.2 ± 9.6	2.5	16.8 ± 0.4	746.9 ± 431.1	557.7 ± 110.0
2300	162.7 ± 12.9	6.8 ± 6.1	42.5 ± 27.4	1796.4 ± 754.9	2848.0 ± 746.6	62.5 ± 9.1	2.5	13.1 ± 2.3	776.9 ± 491.9	206.9

Statistical comparison between sampling site and time[1]				
Erythromycin	Propranolol	Mefenamic acid	Ibuprofen	Diclofenac
Time: F=1.75, p=0.18 Site: F=125.33, p<0.001 Interaction: F=2.04, p=0.128	Time: F=1.03, p=0.45 Site: F=3.07, p=0.10 Interaction: F=1.62, p=0.22	Time: F=0.56, p=0.75 Site: F=14.94, p<0.01 Interaction: F=0.30, p=0.93	Time: F=1.27, p=0.33 Site: F=18.75, p<0.01 Interaction: F=0.37, p=0.89	Time: F=1.69, p=0.20 Site: F=105.32, p<0.001 Interaction: F=0.68, p=0.67

Notes:
1. General linear model: Concentration vs. Time Site (Time*Site)

4.3.6.1 Comparison of reach samples during differing flow conditions

Low and moderate flow samples demonstrated the highest median concentrations for all compounds except mefenamic acid and ibuprofen (Table 4-13). The difference between concentrations at the upstream and downstream sampling points were compared against daily mean flow ($m^3 s^{-1}$) on the day of sampling to analyse whether stream flow conditions had any effect on in-channel fate of each pharmaceutical compound. Low flow conditions demonstrated the greatest downstream reductions in concentrations for all compounds except ibuprofen (which demonstrated an increase) although these differences were not significant (Table 4-13).

Table 4-13: Summary of statistical analysis comparing pharmaceutical concentrations sampled along a 5km stretch of the River Aire during different flow conditions

Compound	Kruskal-Wallis H results	Median concentrations (ng L^{-1})[1]		
		Low flow	Moderate flow	High flow
Erythromycin	H=7.07, DF=2 p=0.03	30.4^{A}	86.4^{A}	17.5^{A}
Propranolol	H=2.12, DF=2 p=0.35	17.0^{A}	5.1^{A}	2.5^{A}
Mefenamic acid	H=7.38, DF=2 p=0.03	36.5$^{A\ B}$	12.0^{B}	22.8^{A}
Ibuprofen	H=1.48, DF=2 p=0.48	1506.7^{A}	553.7^{A}	582.7^{A}
Diclofenac	H=6.23, DF=2 p=0.04	454.3^{A}	143.3$^{A\ B}$	83.2^{B}
Compound	**Kruskal-Wallis H results**	**Downstream concentration change (ng L^{-1})[2]**		
		Low flow	**Moderate flow**	**High flow**
Erythromycin	H=2.89, DF=2, p=0.24	-288.9	-5.9	-112.7
Propranolol	H=2.25, DF=2 p=0.33	-27.4	-1.4	-1.5
Mefenamic acid	H=2.14, DF=2 p=0.34	-19.8	+3.4	-11.5
Ibuprofen	H=2.41, DF=2, p=0.30	+938.2	-134.5	+133.5
Diclofenac	H=2.25, DF=2 p=0.33	-409.2	-32.2	-49.7

Notes:
Results of pairwise comparisons between flow categories (Tukey's family error rate: 0.05). Categories that do not share a letter are significantly different.
Difference between farthest upstream and farthest downstream concentration (positive value indicates an increase in concentrations along the study reach, 5 km)

4.4 Discussion

4.4.1 HPLC-MS/MS method performance

Overall, the HPLC-MS/MS method performed well and compared favourably with a similar method also developed for UK STP effluent and receiving water samples (Petrovic *et al*., 2006). The precursor mass accuracies reported here compare well with those of Petrovic *et al*. (2006) which ranged from 1.4 ppm for erythromycin to 3.1 ppm for mefenamic acid. Product ion mass accuracies were generally poorer although still within acceptable ranges (≤20 ppm; EC (2002)). The slightly reduced product ion mass accuracy compared to Petrovic *et al*. (2006) may be explained by their use of ultra-performance liquid chromatography compared to HPLC in this study (Swartz, 2005).

SPE recoveries from de-ionised water and environmental samples compare favourably and fall within the range (≥60 % effluent; ≥80 % river) reported by Petrovic *et al*. (2006) for similar samples. These results further confirm that tandem MS detection coupled with effective HPLC separation is suitable for detecting trace levels of pharmaceuticals in complex aqueous environmental samples and is less susceptible to matrix effects.

4.4.2 Frequencies of detection

The dataset presented here represents a substantial contribution to the knowledge base of pharmaceutical occurrence in rivers at the UK and global scales. Pharmaceuticals were detected at very high frequencies in both receiving waters and STP effluent and to a lesser extent in CSO effluent across the Aire and Calder. The reduced detection frequencies in CSO effluent agree with the original hypothesis (**H_5**). The high detection frequencies confirm that STP and CSO effluent are the major source of pharmaceutical pollution in the Aire and Calder and that such pollution is ubiquitous in semi-rural and urban areas. Similarly high detection frequencies have been detected in the USA by Kolpin *et al*. (2002) at up to over 80 % for non-prescription drugs. They hypothesised that the high detection frequencies were due to high annual consumption values. This may also be the dominant cause in this study as diclofenac and ibuprofen are available over the counter in the UK. Furthermore, erythromycin, mefenamic acid and propranolol are widely prescribed in the UK with annual consumption volumes up to several tonnes (Ayscough et al., 2000; Jones et al., 2001; Webb, 2001; Jones et al., 2002; Sebastine & Wakeman, 2003).

Detection frequencies for the compounds monitored here were highly variable across other published studies. They ranged from 5 to 100 % for diclofenac (Comeau *et al*., 2008; Zhou *et al*., 2009), 3 to 100 % for erythromycin (Kolpin *et al*., 2004; Kasprzyk-Hordern *et al*., 2009), 1 to 100 % for ibuprofen (Bound & Voulvoulis, 2006; Focazio *et al*., 2008), 14 to 88 % for mefenamic acid (Kasprzyk-Hordern *et al*., 2009; López-Roldán *et al*., 2010) and 37 to 100 % for propranolol (Ashton *et al*. 2004).

Detection frequencies in the present study were broadly similar except for ibuprofen and erythromycin which were detected in the Aire and Calder at higher frequencies than previously reported. One explanation for this was the unfiltered samples used in this study which have previously been shown to yield greater detection frequencies (Kolpin *et al*., 2004). However, data collected by Hughes *et al*. (2013; Appendix A) showed that 25 % of studies used unfiltered samples but no obvious relationship between filtration and detection frequency or measured concentrations was present. Furthermore, direct comparisons between this study and others are not straightforward given differences in sampling sites, sample preparation and analytical techniques. Kolpin *et al*., 2002). In this study ibuprofen demonstrated similar detection frequencies to the other compounds

despite having a detection limit five times greater at 25 ng L^{-1}. Overall, pharmaceuticals were detected in the vast majority of samples for four compounds at frequencies similar or greater than previous UK and global studies highlighting the consistent presence of pharmaceuticals in effluent impacted rivers. Therefore, pharmaceuticals should be considered a ubiquitous pollutant in freshwater ecosystems receiving waste material from domestic settings.

4.4.3 Pharmaceutical concentrations in effluent and receiving waters

This study successfully identified all five study compounds in the effluent and receiving waters of seven typical UK STPs in the Aire and Calder catchments. Effluent concentrations were greater than receiving water concentrations for all compounds except mefenamic acid (6.4 ng L^{-1} effluent vs. 7.4 ng L^{-1} river) in agreement with the original hypothesis (**H_1**). Notably, the results identified new UK maximum concentrations for diclofenac, erythromycin and ibuprofen in receiving waters and diclofenac in STP effluent. Furthermore, it is believed this study was the first to quantify the presence of pharmaceuticals in UK CSO effluent. Median concentrations were lower than those reported by other UK studies for all compounds (Table 2-6; 2-7) apart from diclofenac. Contrastingly, the median receiving water concentrations reported here were greater than global medians for diclofenac, erythromycin and ibuprofen (Hughes *et al*., 2013; Appendix A) suggesting these are pollutants of particular concern in the UK. One possible reason for this is that these three compounds are very frequently prescribed in the UK up to 163 tonnes per year (Table 2-3).

One other possible reason for the identification of new maxima and elevated median concentrations is simply the number and type of samples collected as part of this study. From the 11 previous UK studies identified by Hughes *et al*. (2013), total sample numbers ranged from 142 to 348 for compared to 317 collected during this single study. Furthermore, the majority of UK studies conducted sampling over relatively short timescales (Ashton *et al*., 2004; Roberts & Thomas, 2006) whilst only two studies monitored for longer than six months (Kasprzyk-Hordern *et al*., 2008; 2009). As such, they are unlikely to capture the inherent temporal variability at short-term and seasonal time scales that has been captured as part of this study. Were the bulk of sample collection to be conducted during summer months then it is possible that winter maximum concentrations as identified in this study (Section 4.4.4) would be missed. The data presented here suggest that much further monitoring is required in similar heavily urbanised catchments in the UK such as those around Greater Manchester and the Midlands which have until now received little or no pharmaceutical monitoring effort. This monitoring may highlight certain catchments or locations that have particularly high levels of localised pollution that until now have not been accounted for.

Of further concern is the contamination of STP effluent and receiving waters with the metabolites and transformation products of pharmaceutical compounds. All five of the study compounds are excreted largely in the form of metabolites or conjugates (Table 4-3). Consequently, it is likely that substantial volumes of metabolites will also be present in the Aire and Calder at concentrations likely similar or in excess of the parent compounds measured here. This is a major concern given that certain metabolites have demonstrated increased toxicity compared to their parent compounds (Bedner & MacCrehan, 2005; La Farré *et al*., 2008; Lienert *et al*., 2007). Furthermore, some conjugated metabolites have the potential to be re-transformed into parent compounds under environmental conditions (Warman, 1981) representing a further potential sink for pharmaceutical pollution.

4.4.4 Seasonal trends in pharmaceutical concentrations

The data presented here show seasonal trends in concentrations with higher concentrations of all compounds during the winter months. This is in partial agreement with the original hypothesis (**H_2**). Similar winter peaks have been detected in Spanish rivers (Valcárcel *et al.*, 2013) attributed to reduced solar radiation and degradation at lower temperatures. However, Loraine & Pettigrove (2006) identified summer maximum concentrations in wastewater and raw drinking water for a range of effluent associated contaminants in Southern California. This was explained by the complex nature of drinking water supply relying on the import from an adjacent effluent dominated catchment during the summer months (Loraine & Pettigrove, 2006). No such imports are present in the Aire and Calder and so any seasonal trends must be caused by processes occurring within the catchments.

Winter peaks have also been observed for ibuprofen in the effluent of a Californian STP (Yu *et al.*, 2013). Sui *et al*. (2011) demonstrated winter peak concentrations for three different STP technologies in China. These authors attributed the reduced summer concentrations to a more efficient treatment process under warmer temperatures. MacLeod & Wong (2010) used passive samplers to monitor temporal trends in concentrations from a small STP. As in this study, winter concentrations were peaks were present. These were attributed to reduced attenuation at lower temperatures and changes in use patterns. Similar processes such as these may be operating in the Aire and Calder which would explain the higher winter concentrations.

MacLeod & Wong (2010) also highlight the susceptibility of smaller STPs (2000 p.e) to small changes in use patterns amongst the served population. They estimated that a single additional 200 mg dose of a compound would result in an effluent increase of 100 ng L^{-1} if the compound was un-metabolised. This is clearly an oversimplification but emphasises the importance of considering local factors when designing pharmaceutical

monitoring campaigns. The two smaller STPs monitored in this study should be considered a monitoring priority given their relatively small population equivalents (Oxenhope: 2295 p.e. and Ripponden 4495 p.e.).

A key finding of this study was winter peak concentrations in pharmaceuticals, particularly erythromycin. The macrolide group of antibiotics (including erythromycin) are amongst the most widely used and are consumed in greater volumes during the winter across Europe (Alexy *et al.*, 2006). Similar winter peaks in consumption have also been demonstrated for antibiotic consumption across the UK (Davey *et al.*, 2008). The consumption of newer broad-spectrum antibiotics (including macrolides) is increasing across Europe (Goossens *et al.*, 2005) which may be an additional driving factor of pharmaceutical pollution in rivers. One theory for the strong seasonal trends in antibiotic consumption is the winter prevalence of upper respiratory tract infections (Deschepper *et al.*, 2002). Metoprolol (a beta-blocker similar to propranolol) has demonstrated winter peak concentrations in STP effluent (Sui *et al*., 2011) which could be explained by the prevalence of higher blood pressure during the winter months (Brennan *et al.*, 1982). These increased rates of consumption during winter will lead to greater excretion to the sewer network and subsequent release to rivers which may partly explain the winter peaks found in this study. The above studies highlight the importance of considering prescription or consumption datasets when designing monitoring strategies for pharmaceuticals.

4.4.5 The relationship between pharmaceutical concentrations and flow

Kolpin *et al*. (2004) examined the relationship between flow and pharmaceutical concentrations around ten cities in Iowa, USA with similar population ranges to the Aire and Calder catchments (populations 2000 to 200 000). The frequency of detection was greatest in low flow samples for prescription drugs and antibiotics. Additionally, the urban contribution to pharmaceutical concentrations in rivers was dependent on flow with upstream-downstream concentrations only demonstrating significant differences during low and normal flow conditions (Kolpin *et al*., 2004). The data presented in this study show an opposite trend for greater detection frequencies during high flow conditions. One possible explanation is the additional input of unknown point sources (such as CSOs or sewer misconnections) which may be more active during such events (Ellis, 2006). Another possible explanation is reduced hydraulic retention time (HRT) within STPs during high flow/rainfall events or the activation of overflow/bypass mechanisms within the STPs themselves (Phillips *et al.*, 2012). Numerous studies have confirmed reduced removal efficiencies of hormones and other emerging contaminants during high flow (Fono & Sedlak, 2005; Johnson *et al.*, 2005) which may explain the greater detection frequencies found during high flows in this study.

Although Kolpin *et al*. (2004) found the greatest detection frequencies at high flow, the greatest measured concentrations were found during low flow conditions. The same was true in this study with concentrations up to 3 x greater during moderate or low flows. Higher concentrations in rivers during low flow events are likely to be a function of reduced dilution of STP effluent (Daughton, 2001) although this is further complicated by the dependence of in-stream degradation on flow conditions (Kunkel & Radke, 2011). Clearly, any future monitoring should seek to include periods of both low and high rainfall/flow.

4.4.6 Comparisons of STP treatment technologies

Effluent samples demonstrated greater concentrations than their respective channel samples further confirming that STP effluent is a major source of pharmaceutical pollution to rivers. The secondary AS STP displayed the lowest concentrations compared to secondary BF and Tertiary A1 for all compounds in both receiving waters and effluent. This trend was particularly prevalent in receiving waters where secondary AS concentrations were lower by up to 2 to 3xs. These results were in partial agreement with the original hypothesis (**H_3**) and with Kasprzyk-Hordern *et al*. (2009) who found concentrations in effluent and receiving waters from a Welsh secondary AS STP displayed consistently lower concentrations for all compounds except diclofenac compared to a secondary BF works. The results presented here agree with the wider research which shows variable but often poor removal of the study compounds during secondary BF treatment; the effects of additional tertiary treatment steps are variable often resulting in slight reductions in removal efficiency (Ternes, 1998; Kanda *et al.*, 2003; Carballa *et al.*, 2004; Jones *et al.*, 2005; Castiglioni *et al.*, 2006; Jones *et al.*, 2006; Spongberg & Witter, 2008; Terzic *et al.*, 2008; Lundström *et al.*, 2010; Tambosi *et al.*, 2010).

Given the absence of influent samples collected during this study it was not possible to make comparisons in removal efficiencies between STPs and as such any observed differences may be the result of site-specific factors. Notably, the comparatively low concentrations observed at the single secondary AS STP sampled may be explained by this works having the lowest population equivalent (2205 p.e.) of all the sampling sites. Influent concentrations have been shown to be strongly dependent on the contributing sewer catchment including the age demographics of the population served and the relative contribution of domestic vs. commercial and industrial wastewater (Yu *et al.*, 2013). The presence of hospitals within the catchment of a STP can have a significant influence on the characteristics of the influent, effluent and receiving waters (Escher *et al.*, 2011). Indeed, Yu *et al*. (2013) found significantly different influent concentrations of a range of pharmaceuticals (including diclofenac and

ibuprofen) in two apparently similar STPs, one serving three hospitals and the other just one. No data are available on hospitals or long-term care facilities within the STPs studied here although it is likely that the large STP downstream of Leeds (Knostrop) receives effluent from the major hospitals (Leeds General Infirmary and St. James' Hospital) which serve the large populations within Leeds and surrounding areas. Future detailed monitoring of influent, effluent and receiving waters will allow researchers to fully quantify the effects of STP technologies and operating parameters on pharmaceutical pollution loads to rivers.

4.4.7 Combined sewer overflows

Pharmaceuticals were detected in the majority of CSO samples except for mefenamic acid and propranolol. These results further confirm that CSOs are a source of pharmaceutical pollution to rivers and are the first to detect pharmaceuticals in the effluent of UK CSOs. Published studies examining the occurrence of pharmaceutical compounds in CSO effluent are rare despite the widespread acknowledgement of the detrimental impact they can have on water quality (Balmforth, 1990; Mulliss *et al.*, 1997; Lee & Bang, 2000; Lau *et al.*, 2002; Old *et al.*, 2003; Weyrauch *et al.*, 2010). Boyd *et al*. (2004) monitored canals receiving stormwater effluent in New Orleans where the majority of pharmaceuticals showed a positive correlation with rainfall. This which was contrary to the data presented by Kolpin *et al*. (2004). The likely reason for the increase during high rainfall was raw sewage contamination and the 'first-flush' phenomenon re-suspending raw sewage, which subsequently leaked into the storm water drains (Boyd *et al*. 2004). It is possible that similar flush processes operate in the Aire and Calder CSOs although further detailed monitoring and continuous sampling techniques would be required to confirm this.

The results presented here and elsewhere demonstrate the capacity for CSOs to contribute significant loads to receiving waters. Phillips *et al*. (2012) found that although CSOs account for a small proportion of discharge volume (10 %) in a USA sewer system they contribute 40-90 % of annual pollution loads for wastewater-associated micropollutants. They are a particularly dominant source for compounds that would be well removed during the sewage treatment process with CSO concentrations up to ten times greater than associated STP effluent values. This difference in concentration between STP and CSO effluent was present in this study for erythromycin (~10 x greater in STP effluent) agreeing with the original hypothesis (**H_5**) and that of Phillips *et al*. (2012) as erythromycin is generally well removed by secondary and tertiary STPs (Table 2-5). However, the other study compounds also demonstrate high removal rates but did not show significant differences in STP and CSO effluent concentrations. One possible explanation is that the lack of treatment removal in STP effluent is negated by additional

dilution by surface water runoff (Mulliss *et al*., 1997). A further factor in support of this hypothesis was the fact that the order of study compounds in terms of average concentrations was consistent across CSO and STP effluent and receiving waters with ibuprofen consistently detected at the highest concentrations and propranolol at the lowest. This same pattern was also evident across the various CSO monitoring sites.

Given their proven contribution to pharmaceutical pollution of rivers, CSOs should be seen as a major concern for water quality in the UK and across the world. It is estimated that there may be over 25 000 CSOs in the UK with up to a third of these classed as unsatisfactory under the terms of the Urban Waste Water Treatment Directive (Thompson RPM, 2006). There are at least 100 such CSOs within the Aire and Calder catchments alone (Yorkshire Water, pers. comm.). Furthermore, recent campaigns in the popular media have shown that a number of CSOs can discharge at very high frequencies, sometimes hundreds of times per year (BBC, 2009). There are suggestions that CSO effluent and other cross-connections or poorly operating sewer systems are the major contributor of pharmaceutical pollution in areas far from the STP effluent discharges (Ellis, 2006). Indeed, one of the CSOs (Oulton Beck) sampled as part of this study was observed to be active even during periods of dry weather suggesting that cross-connections in the sewer system may be contributing pharmaceutical pollution to rivers even when CSOs should be inactive. Furthermore, such non-STP point sources are not represented in the widely used risk assessment models such as GREAT-ER which may in turn underestimate receiving water pharmaceutical concentrations (Schowanek *et al.*, 2001; Schowanek & Webb, 2002). The poor performance of CSOs in the UK is a major concern to the UK water regulator Ofwat who in 2009 required the water industry to invest £ 985 million in reducing unsatisfactory discharges during overflow events (Ofwat, 2009). Despite such investment and attention, research into the contribution of CSOs to pharmaceutical pollution is still in its infancy and much more monitoring is required if the effects are to be fully understood.

4.4.8 Short-term temporal variations in pharmaceutical pollution

Data from this campaign demonstrated high degrees of diurnal variation with consistent peaks at late morning (8-11 am) and late evening (8-11 pm) for all five compounds at both STPS in agreement with the original hypothesis (**H_2**). The peak towards midday is in agreement with sampling conducted by Kanda *et al. (2003)* which found ibuprofen peaked in the middle of the day. This peak could be explained by the combination of individual wastewater pulses from households (i.e. flushing of toilets, showers, emptying of sinks etc.) in the morning coupled with a 3-4 hour delay due to passage through the sewer system and STP. Such a delay would yield maximum receiving water concentrations at approximately 11am (Ort *et al.*, 2010b). The same processes and

delays could also explain the peaks in late evening as people arrive home from work although to fully test this hypothesis then detailed sampling of influent, effluent and receiving waters would be required.

STP effluent concentrations are known to be highly variable over time due to fluctuating influent concentrations, release rates, retention times and operating conditions (Kanda *et al.*, 2003). Raw influent entering STPs is known to be even more variable due to potentially thousands of wastewater pulses per day, connectivity, storage and dispersion within the sewer system (Ort, 2006) which can result in large fluctuations (Ort, 2005). Typical peak sewer flow rates in a similar gravity based system as the ones studied here were at mid-morning (10 am to 12 pm) and late evening (8 to 10 pm) corresponding with other studies (Ort *et al.*, 2010b). Hydraulic pulses in the sewer network can propagate quickly through STPs and as such the effluent pattern of a STP can mirror that of its influent (Ort & Siegrist, 2009) but due to retention and mixing during the treatment process the variability of effluent concentrations is usually reduced (Ort *et al.*, 2010a). This was also mirrored in the variation of receiving water concentrations in this study which varied by up to a factor of three within 24 hours. Furthermore, dilution in receiving waters, fluctuating flow conditions and in-stream degradation processes may also serve to smooth some of the variability once pharmaceuticals enter rivers (Kolpin *et al*., 2003, Kunkel & Radke, 2003).

The timing of peak concentrations in late morning may have implications for freshwater ecosystems given that they coincide with peak photosynthetically active radiation and photosynthetic activity in rivers (Simonsen & Harremoës, 1978; Giller & Malmqvist, 1998). Propranolol demonstrates very high specific toxicity towards the photosynthetic inhibition of green algae over short exposures with an EC_{50} of 648 000 ng L^{-1} (Escher *et al.*, 2005). Antibiotics caused significant alterations to the photosynthetic pathways of plants which in turn caused reduced root growth and deformities at environmentally realistic concentrations (165 000 ng L^{-1} (Aristilde *et al.*, 2010)). Such disruptions to photosynthetic activity could have important implications for whole ecosystem processes. Fluctuations in photosynthetic activity influence pH and dissolved oxygen levels which in turn can impact on the diurnal cycling in the sorption characteristics of heavy metals (Bourg & Bertin, 1996; Nimick *et al.*, 2003). Therefore, much further monitoring is required to capture the short-term temporal variation in effluent and receiving waters which can in turn be used to inform more relevant ecotoxicological studies.

The short-term temporal variation demonstrated here and elsewhere (Kanda *et al.*, 2003; Sui *et al.*, 2011) has implications for longer-term monitoring campaigns which utilise repeated grab sampling techniques. If sites were sampled in the same order (and same approximate time) at each visit they may routinely capture or miss the daily peak

concentrations (Kanda et al. 2003). Future monitoring should seek to reduce this possible bias through simultaneous, continuous sampling or varying the site sampling order randomly at each visit.

4.4.9 Downstream profiles of pharmaceutical pollution

The results show a consistent presence of the study compounds along a 5 km reach despite there being no known effluent sources between the two STPs along this stretch of the River Aire. These results suggest that in-stream degradation processes along reaches of several kilometres are negligible and that exposure of freshwater ecosystems to pharmaceutical pollution will occur well beyond the locality of STP discharges. Therefore, the original hypothesis of significant attenuation along the study reach was rejected (**H_4**). However, the in-stream fate of pharmaceuticals is relatively poorly understood with few laboratory studies and even fewer conducted in the field (Radke *et al.*, 2010). Sabaliunas *et al*. (2003) found that the antimicrobial triclosan underwent approximately 54 % removal along a 1500 m stretch of the River Aire. However, direct comparisons between triclosan and the compounds studied here may be misleading as triclosan is generally more sorptive.

Radke *et al.* (2010) examined the in-stream degradation of four pharmaceuticals along a 14 km stretch of a German river. Diclofenac was most susceptible to direct photolysis, despite this, attenuation across the reach was just 5 to 15 % and of little quantitative relevance. Contrary to our results, downstream concentrations were consistently different from upstream concentrations (339 – 381 ng L^{-1} upstream c.f. 279 – 283 ng L^{-1} downstream) with greatest differences during dry weather. This was due to a longer travel time and greater solar radiation allowing for higher in-stream degradation. Overall, attenuation of diclofenac in German rivers during both low and peak flow conditions was not significant (approximately 5 %) suggesting that photolysis, sorption and abiotic/biotic degradation are not relevant removal mechanisms at these temporal and spatial scales. This is in agreement with the data presented here where overall attenuation is negligible or absent.

Direct photolysis is the dominant removal mechanism for diclofenac but not for ibuprofen for which sedimentation and sorption were thought to be the most important (Tixier *et al.*, 2003). Gross *et al*. (2004) demonstrated significant removal (up to 97 %) of ibuprofen along a 11 km stretch of a shallow Californian river due to reduced flow, biodegradation and microbial transformation (Lin *et al.*, 2006) and greater light penetration in the shallow river (Moss, 1988). Fono *et al*. (2006) measured high in-stream removal of ibuprofen along a >100 km reach of a Texan river. However, the large number of STP and CSO effluent discharges to the Aire and Calder (occurring every few

kilometres along the channel; Figure 4-2) mean that any such removal along a 100 km stretch is likely to be replaced by additional pollution at other point sources downstream.

Kunkel & Radke (2011) examined the in-stream removal of six pharmaceuticals (including diclofenac and ibuprofen) along a 16 km stretch of a small Swedish river. Ibuprofen was fully attenuated but diclofenac was not attenuated and in fact mass recoveries of this compound increased downstream. The lack of attenuation of diclofenac, which is susceptible to photolysis (Packer *et al.*, 2003), was attributed to high turbidity, riparian vegetation and cloudy weather. These factors may also explain why diclofenac was poorly attenuated along the Aire study reach although no such data are available to confirm this. Further comparisons between the small Swedish river and the River Aire are problematic given vast differences in catchment size, land use (the Swedish brook received no STP effluent) and channel dimensions. A similar dye tracer study along the River Aire would provide the best estimate of in-stream attenuation and would better inform environmental risk assessment for similar catchments.

Reductions in concentration along the downstream profile were most pronounced when low flow conditions were compared with moderate or high flow conditions. This is in agreement with Kolpin *et al*. (2004) who showed downstream change was most pronounced during low flow. However, such changes are likely to be highly site specific and one possible explanation for the apparent increased in-stream removal during high flow may be the number of overflows from the adjacent Aire and Calder Navigation canal. It is likely that these overflows will discharge greater volumes of relatively uncontaminated canal water to the Aire during high flow conditions diluting the pharmaceutical pollution in the Aire.

The results presented here and elsewhere highlight the need for caution when making assumptions about in-stream pharmaceutical removal with rates dependent very strongly on site specific factors, individual compound properties, latitude, season, weather, flow, turbidity, suspended sediment, hyporheic exchange, microbial communities and dissolved organic carbon concentrations (Kunkel & Radke, 2011). However, given the range of properties for the compounds studied here (Table 4-3) there was no appreciable difference in downstream fate between the compounds. This suggests that local factors play a dominant role in ultimate compound fate and transport. It is clear that over relatively short travel times and distances up to tens of kilometres the assumption of significant in-stream removal is unsafe (Kunkel & Radke, 2011, Radke *et* al., 2010). Furthermore, the data do not agree with the assumption in many risk assessments models (such as GREAT-ER) of a 1st order in-stream decay *i.e.* that the rate of in-stream decay is dependent on the concentration of the pharmaceutical (Schowanek *et al.*, 2001). Therefore, it is suggested that this assumption is revised and informed by laboratory or field fate data where available.

4.4.9.1 Physicochemical properties

Pharmaceuticals are comprised of myriad compounds with a wide range of physicochemical properties which play a significant role in determining their environmental fate and transport (Kunkel & Radke, 2011). All of the study compounds have positive pK_a values (Table 4-3) indicating they are unlikely to dissociate into ionic components at environmental temperature and pH ranges. This means the majority of the compounds will remain in their 'parent' form once they have entered rivers which may explain the high concentrations seen several kilometres downstream of the point source. Propranolol demonstrated the highest pK_a of the study compounds (9.4; Table 4-3) which is contrary to this compound displaying the lowest mean concentrations in both STP effluent and receiving waters. However, this may be explained by biodegradation within rivers as propranolol has demonstrated relatively short half-lives (0.4-1.8 days) in water/sediment systems (Ramil *et al.*, 2009).

Log K_{OW} is variable across compounds and a log K_{OW} > 4.5 is the threshold at which compounds are considered to be persistent and bioaccumulative (EMEA, 2006). This threshold is exceeded by mefenamic acid indicating a propensity to sorb to biological material. Sorption of mefenamic acid to solid phases during STP treatment may explain why this compound was detected at the lowest frequency and the second lowest mean concentration. Existing laboratory data appear to agree with this assumption showing mefenamic acid to be relatively well removed by secondary and tertiary STPS (87-100 %; Table 2-5). The other study compounds demonstrate moderately high log K_{OW} suggesting that accumulation within biological matter and other solid phases must be considered when evaluating fate and transport which can in turn influence their bioavailability and ultimately their toxicity to aquatic organisms (Mace, 2002). Conversely, ibuprofen demonstrates relatively low sorption coefficients (k_d and k_{OC}; Table 2-9) which may partly explain its variable but often low removal during sewage treatment (ranging from 0 to 100 %; Table 2-5). Clearly, simple correlations between physicochemical properties and ultimate occurrence, fate and transport of pharmaceuticals in freshwater systems are not appropriate. Of equal if not greater importance is the variable pharmaceutical consumption within the contributing sewer catchments and dynamic environmental conditions both during and after sewage treatment. Biological uptake is also highly variable but can be significant, particularly for NSAIDs given their sufficient solubility to avoid sorption to sludge in STPs but high enough lipophilicity to bioconcentrate (Brown *et al.*, 2007). Therefore, biological loads of pharmaceuticals should also been seen as monitoring priority.

The excretion rate of parent compounds after human consumption is generally very low for all compounds except mefenamic acid. Excreted metabolites are therefore of concern as they may potentially be present at concentrations several fold higher than

parent compounds (Ayscough *et al*., 2000) and certain metabolites can demonstrate increased toxicity e.g. paracetamol (Bedner & McCrehan, 2005). Phase II metabolism involves conjugation prior to excretion although some of these 'inactive' conjugates have the potential to be re-transformed into active or parent compounds after excretion (Warman, 1981). Occurrence and toxicity of pharmaceutical metabolites remains poorly studied despite a recommendation they be considered as part of the environmental risk assessment process (EMEA, 2006). Given, the variable physico-chemical properties of the compounds studied here it is likely that significant loads of parent and metabolised compounds are present within solid and biological phases of the Rivers Aire and Calder that were unaccounted for in this study. Consideration of these additional pollution burdens should be seen a future research priority.

4.5 Conclusions

The results presented here highlight the consistent presence of pharmaceutical compounds at high concentrations in the STP/CSO effluent and receiving waters of a typical UK catchment encompassing both semi-rural and urban environments. The results confirm STP effluent as the major source of pharmaceutical pollution to rivers. Concentrations were high relative to other global studies with new UK maximum concentrations detected for diclofenac, erythromycin and ibuprofen in receiving waters and diclofenac in STP effluent. Winter concentrations were generally greater than those from the spring/summer period demonstrating seasonal trends that other shorter monitoring campaigns may have missed. Compounds were detected more frequently during high flow conditions suggesting that reduced removal efficiency and overflow mechanisms in STPs may be contributing additional pollution loads at high flow. During low and moderate flow conditions, compounds were detected less frequently but at significantly greater concentrations perhaps due to reduced dilution. The high frequency of detection across all samples is of particular ecological concern given the potential for life-long, multigenerational exposure of aquatic organisms given the 'pseudo-persistence' arising from continually discharging STP effluent (Daughton, 2001).

This study also represents the first work examining the contribution of CSO effluent to UK rivers with concentrations up to a maximum of 10 060 ng L^{-1} for ibuprofen with variable but generally high detection frequencies. Concentrations measured in CSO effluent were of the same order as those in STP effluent except for erythromycin which was higher in CSO effluent. This suggests that CSOs may be a particularly important source for those compounds that are otherwise well removed by STP treatment (Phillips *et al*., 2012). Therefore, CSOs have the potential to be a major contributor of pharmaceutical pollution in rivers and much more work is needed to understand the implications for urban water quality. Furthermore, the poor degradation of the study

compounds along a 5 km stretch of the River Aire means that the assumption of rapid in-stream degradation is not widely applicable and is subject to local factors such as weather, turbidity and flow. Therefore, the general bias of current research to focus on areas immediately downstream of STP effluent discharges should be challenged. Concentrations have been shown to vary markedly across diurnal and seasonal timescales highlighting the importance of more intelligent, representative monitoring strategies in order to capture this variability (Ort *et al.*, 2010a; Ort *et al.*, 2010b; Hughes *et al.*, 2013). Continuous monitoring, composite samplers and passive integrative sampling techniques represent the best options to fully quantify the variable pharmaceutical levels in complex STP and receiving water systems (Zhang *et al.*, 2008; MacLeod & Wong, 2010; Jacquet *et al.*, 2012).

The concentrations detected here are relatively high for a developed western nation and demonstrate a low factor of safety for ecological risk (Crane *et al.*, 2006; Fent *et al.*, 2006; Enick & Moore, 2007; Santos *et al.*, 2010; Hughes *et al.*, 2013). Furthermore, pharmaceutical pollution of rivers is now of major policy concern at the European level with the inclusion of diclofenac (and the synthetic oestrogen, EE2) on the 'watch list' of Hazardous Substances under Annex X of the Water Framework Directive (EC, 2000; EC, 2012). 93 % of the receiving water samples analysed here would exceed the initial proposed EQS limit of 10 ng L^{-1} for diclofenac representing a potentially significant future cost and regulatory burden for the UK water industry (UK Parliament, 2012). These data suggest that pharmaceuticals represent a more pressing freshwater pollution problem than pesticides where only 6 % of samples exceeded the 100 ng L^{-1} threshold (EA, 2009c) stipulated by the WFD and EU Pesticide Directive (2009/128/EC).

Clearly, pharmaceuticals are consistent pollutants in both semi-rural and urban rivers and are likely to be present wherever waste from domestic consumption enters freshwater systems. Despite this, there remain many significant knowledge gaps in the spatiotemporal aspects of their occurrence in rivers, particularly the contribution of non-STP effluent sources such as CSOs. The inclusion of diclofenac on the ‘watch list’ of Priority Substances of the Water Framework Directive should be seen as an opportunity to expand research across the estimated 3000 pharmaceutical compounds currently approved for use in the UK alone (Ayscough *et al.*, 2000) as well as the myriad other chemicals consumed in domestic, commercial, industrial and hospital settings every day.

5 LETHAL AND SUBLETHAL EFFECTS OF PHARMACEUTICALS ON FRESHWATER SHRIMP (*GAMMARUS PULEX*)

5.1 Introduction

Pharmaceuticals are a major group of environmental contaminants found in soils, ground and surface waters and biota (Jones *et al.*, 2001; Kolpin *et al.*, 2002; Boxall, 2004; Ternes *et al.*, 2007; Daughton & Ruhoy, 2009b). There has been a recent focus on the effects of pharmaceuticals in aquatic ecosystems (Daughton, 2007) but these compounds are generally considered to pose minimal risk of acute toxicity to freshwater organisms given their low concentrations (generally ng to µg L^{-1}; (Hughes *et al.*, 2013). However, most of the ecotoxicological studies that underpin this position have been conducted over very short exposure periods (≤96 hours) and at relatively high concentrations. Although these tests are a good starting point and have value in producing relatively high volumes of reproducible and comparable data, they rarely represent exposure conditions in the freshwater environment. Extended exposures to more realistic concentrations are less common, perhaps given the associated costs and logistics. Several review articles indicate a reduced margin of safety for long-term toxicity manifesting in the environment with effects present at the µg L^{-1} level (Crane *et al.*, 2006; Fent *et al.*, 2006; Enick & Moore, 2007; Santos *et al.*, 2010; Hughes *et al.*, 2013). In fact, even many extended exposure studies do not meet the criteria to be properly classified as chronic tests (Suter, 1993; Newman & Unger, 2003).

Aquatic macroinvertebrates have been used widely to study toxicological effects of antibiotics, antidepressants, antiepileptics, cardiovascular drugs and painkillers (Crane *et al.*, 2006; Fent *et al.*, 2006; Enick & Moore, 2007; Santos *et al.*, 2010). However, tests have been conducted overwhelmingly using water fleas (Cladocera: *Daphnia magna*), a commonly used standard organism in ecotoxicology (OECD, 2004; OECD, 2008). *D. magna* has even been proposed as the sentinel aquatic invertebrate for future research in environmental metabolomics (Snape *et al.*, 2004). Despite this, the generic relevance of *D. magna* has been questioned, given its absence in most running waters where pharmaceutical pollution is most likely to be an issue (Koivisto, 1995; Stark *et al.*, 2004). Other widespread, abundant and functionally important taxa such as freshwater amphipods (e.g. *Gammarus pulex*) are less well studied (Macneil *et al.*, 1997) despite their long standing recognition as a suitably sensitive species for ecotoxicological research (McCahon & Pascoe, 1988).

The vast majority of ecotoxicological tests have been conducted using single compounds in isolation; a situation that is clearly not representative given the myriad other pollutants and stressors present in the aquatic environment (Mihaich *et al.*, 2005; Fent *et al.*, 2006). There have been a small number of studies examining the effects of

pharmaceutical mixtures (Cleuvers, 2003; Cleuvers, 2004; Dietrich *et al.*, 2010) and these have indicated that pharmaceuticals generally follow the concept of concentration addition, leading to effects well below their individual thresholds (Escher & Hermens, 2002; Escher *et al.*, 2005). More emphasis needs to be placed on mixture toxicity if we are to further our understanding of the complex effects in the aquatic environment (Backhaus, 2008). There is clearly a need for a more flexible, knowledge-based approach towards toxicity assessment (Länge & Dietrich, 2002) and to develop tests that include ecologically relevant species and endpoints (Koivisto, 1995; Kümmerer *et al.*, 2004b).

Given the low risk of acute or short-term toxicity, measuring endpoints such as growth, survival, feeding and reproduction may miss more subtle, sub-organismal effects (Daughton & Ternes, 1999). Despite such long standing concerns, these endpoints are still the most widely assessed in both acute and chronic ecotoxicology (Santos *et al.*, 2010). Daughton & Ternes (1999) and Brooks *et al.* (2003) recommended ecotoxicological testing incorporate compound or species specific biomarkers and bioassays to detect subtle effects. The rapidly expanding area of environmental omics techniques, which allow the objective and simultaneous measurement of hundreds or even thousands of responses, has considerable potential to identify sub-lethal effects and enable biomarker development (Viant, 2007).

Metabolomics may be able to detect minute but important disturbances that other techniques may miss (Daviss, 2005). This technique can now reliably quantify a large number of metabolites, small products of metabolism < 2.5 kDa in size (Fernie *et al.*, 2004; Preti, 2005). These can be used these to quantify an organisms baseline metabolic state (Viant, 2007) and in turn to contextualise physiological and metabolic changes caused by multiple stressors in the natural environment (Robertson, 2005).

The first published application of metabolomics to aquatic organisms revealed an ability to distinguish between healthy and diseased red abalone (*Haliotis rufescens)* (Viant *et al.*, 2003a). The method has since been applied successfully to detect the effects of pesticides, solvents, polycyclic aromatic hydrocarbons and synthetic oestrogens in several fish species (Viant, 2005; Samuelsson *et al.*, 2006; Viant *et al.*, 2006; Ekman *et al.*, 2008; Williams *et al.*, 2009). Environmental metabolomics demonstrates significant potential for addressing current issues in aquatic ecotoxicology (Viant, 2008) but to date only two studies have applied metabolomics to pharmaceuticals and aquatic organisms, namely antibiotic effects on *H. rufescens* (Viant *et al.*, 2003a) and propranolol on *D. magna* (Taylor, 2010). Neither of these studies included exposure concentrations that are representative of environmental conditions nor did they consider the effects of pharmaceutical mixtures.

5.1.1 Aims and hypotheses

The overall aim of the study was to assess the effect of extended (28 day) exposure to environmentally realistic concentrations of erythromycin (antibiotic), propranolol (beta-blocker) and their mixtures on the functionally important freshwater amphipod *Gammarus pulex (L.)*. Response measurements included growth, feeding, mortality and the whole organism metabolic profile. This included a first application of environmental NMR metabolomics to *G. pulex* to assess whether such techniques were applicable and able to differentiate between control organisms and those exposed to pharmaceuticals. The investigation was undertaken as a comparative study of pharmaceutical effects, and was not an attempt to gain a detailed biochemical understanding of the *G. pulex* metabolome. Specific objectives were:

- Measure the effect of extended exposures to erythromycin and propranolol on the growth and feeding of *G. pulex*;
- Measure the effects of these pharmaceuticals on the mortality of *G. pulex*;
- Apply NMR environmental metabolomics to quantify the sublethal, metabolic effects of extended pharmaceutical exposure;
- Combine the above pharmaceuticals and examine the lethal and sublethal effects of a pharmaceutical mixture.

It was hypothesised that (**H_1**) low-level exposure would not have a direct lethal effect on *G. pulex* but (**H_2**) prolonged exposure would cause significant reductions in sub-lethal endpoints (e.g. growth and feeding). It was also hypothesised that (**H_3**) these sub-lethal effects would be detectable via alterations to the whole organism metabolome. Finally, it was hypothesised (**H_4**) that lethal and sublethal effects of mixtures would be more pronounced than compounds acting in isolation.

5.2 Methodology

5.2.1 Experimental design

Erythromycin and propranolol were chosen as study compounds given their high environmental risk quotients (Jones *et al.*, 2002; Thompson, 2006; Yamamoto *et al.*, 2009) and their widespread occurrence in rivers worldwide (Hughes *et al*., 2013). Treatment concentrations were chosen as an indicator of likely exposures based on published maxima for UK rivers (erythromycin: 1000 ng L^{-1} (Hilton & Thomas, 2003); propranolol 215 ng L-1 (Ashton *et al.*, 2004)) and an indicator of worst-case exposure based on 100x this concentration. Low treatment concentrations were 200 and 1000 ng L^{-1} of propranolol and erythromycin respectively. High treatment concentrations were 20

000 and 100 000 ng L^{-1} respectively. High and low mixture treatments were binary combinations of the respective low and high treatments for the individual compounds.

Six pharmaceutical treatments and a control (CON), each replicated six times, were established at random across 42 0.8 L flasks; treatments included low and high treatments of erythromycin (LT ERY and HT ERY), propranolol (LT PRO and HT PRO) and the equivalent binary mixtures (LT MIX and HT MIX), with 0.2 g of Beech (*Fagus sylvatica*) leaf litter provided in each flask for feeding. *F. sylvatica* feed material was used as this was the predominant leaf species at Silsden Beck from where test organisms were sourced.

The exposure matrix water was sourced from Silsden Beck, West Yorkshire (UK; OSNGR SE 04461 47504). The site was chosen due to it being situated upstream of any STP effluent inputs, thereby reducing the potential for contamination by the study compounds. A 1 L grab sample of water from Silsden Beck was analysed *a priori* by HPLC-MS/MS indicated that all five of the preliminary study compounds were below detection limits (5ng L^{-1} for diclofenac, erythromycin, mefenamic acid and propranolol and 25 ng L^{-1} for ibuprofen).

The test was a modified static renewal exposure conducted with *Gammarus pulex* over 28 days; further details on the test organisms and laboratory acclimatisation are available in Appendix C. After 28 days, each surviving individual *G. pulex* was flash frozen in liquid nitrogen, weighed and stored at -20°C prior to extraction. Flash freezing to kill the individuals instantly was crucial as stress associated with a more prolonged death (e.g. preserving in 70% ethanol) would impact on the metabolome. Remaining beech leaf feed material was immediately dried, weighed and combusted (to determine Ash Free Dry Mass) for analysis of feeding rates.

5.2.1.1 Sample extraction

The sample extraction procedure was based on that used for earthworm (*Lumbricus rubellus*) tissue by Bundy *et al*. (2007). Whole organism extracts have been proven to be more discriminatory than specific fluid or tissue extracts such as hemolymph (Taylor, 2010). Each individual was homogenised using an Omni TH homogeniser (Omni International, GA, USA) in 1.8 mL of ice-cold 60 % acetonitrile solution (v/v, D_2O), vortexed and centrifuged (16000 g, 10 min); the supernatant was pipetted and dried in a vacuum concentrator. Each sample was rehydrated in 0.6 mL of an NMR buffer; this was 100 % D_2O, 100 mM phosphate buffer (30.5 mL Na_2HPO_4 and 19.5 mL NaH_2PO_4 in 50 mL D_2O), pH 7.0, and 0.25 mM sodium (trimethylsilyl)[2,2,3,3-2H4]-proprionate (TSP). The rehydrated samples were centrifuged (3 min, 16000 g), and 0.60 mL was transferred to a 5 mm NMR tube for analysis.

5.2.2 Data analysis

Percentage change in *G. pulex* and feed material mass from day 0 to day 28 were tested using the non-parametric Kruskal-Wallis H test after arcsine transformation of proportional data. Differences in mortality were tested using life tables and calculating the non-parametric Wilcoxon (Gehan) test using PASW Statistics 17.0 (SPSS Inc., IBM).

5.2.3 NMR spectroscopy

NMR spectroscopy was chosen for its high reproducibility and the ready availability of an appropriate instrument with qualified and trained operators. Samples were analysed at 293 K on a Varian Unity Inova 500 spectrometer (Varian Inc., Palo Alto, California, USA) with a field strength of 11.7 T and a proton resonance frequency of 499.97 MHz, equipped with a 5 mm ^{1}H [^{15}N - ^{31}P] inverse detection probe. The data were acquired in 8192 pairs data points across a spectral width of 6 kHz, with an acquisition time of 1.36 s. Spectra were acquired with water presaturation, with an initial 1.0 s longitudinal relaxation delay and a 1.5 s presaturation time. A 45 ^{0} RF pulse was used and 400 transients were acquired for each sample. All these delays together resulted in a total relaxation time of more than 3 T_1 (T_1 = the longitudinal spin-lattice relaxation time). The spectra were processed using ACD Labs software (v.12.01, Advanced Chemistry Development, Inc., (ACD/Labs), Toronto, Canada). An exponential apodization function of 1.0 Hz was applied to the free induction decay, and the data were zero-filled to 32 768 real points prior to Fourier transformation. The spectra were manually phase-corrected, baseline corrected (first-order polynomial) and referenced to TSP (0 ppm). The data were reduced (0.005 ppm bins) within the ACD package having first defined dark regions (0.000-0.180 TSP, 4.700-5.160 residual water, 7.760-7.960 histidine and 8.400-8.500 formate), normalising to total integral sum of the remaining regions. Spectra were overlaid and inspected individually; three samples were identified as erroneous and were removed from subsequent analysis (one each of control, high erythromycin and high mixture).

5.2.4 Multivariate data analysis

Spectral data were analysed using multivariate statistical methods in SIMCA 13 (Umetrics Inc., Umea, Sweden). All data were pareto scaled and partial least-squares discriminant analysis (PLS-DA) models were created for pairwise comparisons between the control and each pharmaceutical treatment (Nicholson *et al.*, 2002). The number of PLS-DA axes fitted was determined automatically in SIMCA 13. These models were validated using the permutation testing routine in SIMCA (i.e. 20 randomly permuted models were compared to the 'correct' *i.e.* non-random model) and those which had suitable permutation plots were kept for detailed scrutiny. 20 permutations were deemed sufficient given the relatively low number of samples in the study. Q^2 ('goodness of

predictability', i.e. the ability of the model to predict the class of new samples) and Y^2 ('goodness of fit', i.e. the degree of variability between classes explained by the model) were also used to evaluate the models suitability.

Accepted models were subjected to further internal validation by manually excluding each sample, rebuilding the PLS-DA model and using this to predict the class of the excluded sample. The percentage of correct and incorrect classifications using these validation models was recorded (see (Westerhuis *et al.*, 2008)). 1-D loadings plots for each PLS-DA were examined visually to identify chemical shifts of metabolites driving differences between the treatment groups (e.g. Figure 5-1). Where possible these peaks were assigned by comparison to published chemical shifts (Viant *et al.*, 2003a; Rosenblum *et al.*, 2006) and the Human Metabolome Database (Wishart *et al.*, 2009). A number of chemical shifts found to be significant could not be assigned to specific metabolites using these databases. However using a 2D TOCSY NMR spectrum it was possible to establish whether these shifts were for hydrogens in the same or different molecules (Table 5-2).

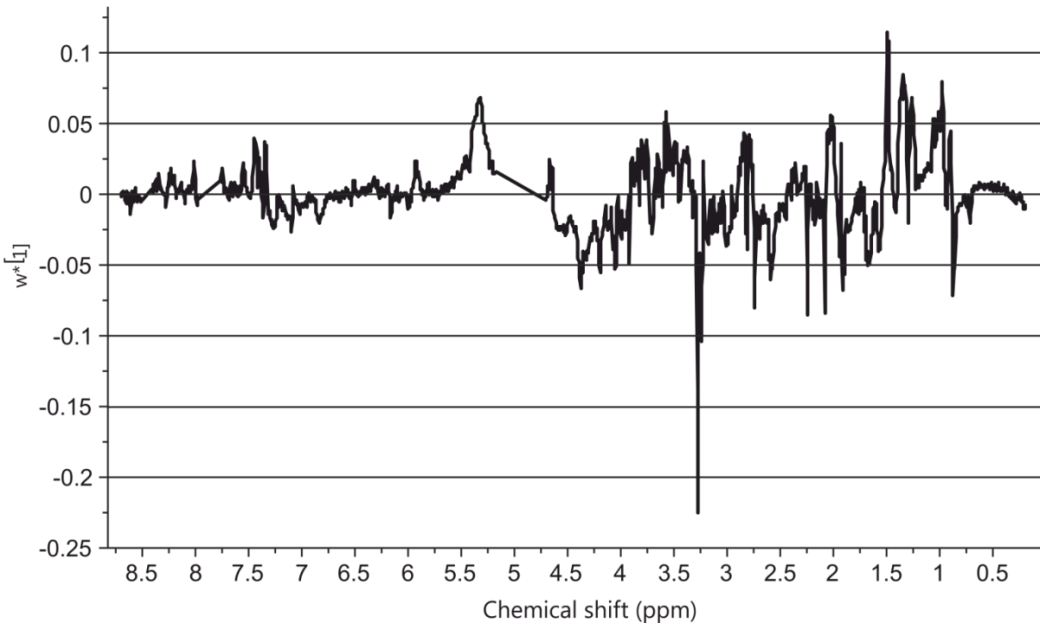


Figure 5-1: Sample 1-D loadings plot for PLS-DA model of control vs. high treatment erythromycin

Univariate analyses of the key chemical shift bins responding to treatment differences were conducted in Minitab 16 (Minitab Ltd.) and PASW Statistics 17.0. Specifically, bins were tested for normality and subjected to either t-test, Mann-Whitney U or one-way ANOVA and considered significant where $p<0.05$. Changes in metabolite levels relative to control samples were calculated by dividing the average bin intensity for the treatment group against the average for control samples (>1 indicated a specific treatment caused enrichment; <1 caused depletion) (Ekman *et al.*, 2008).

5.3 Results

5.3.1 Initial test conditions

There were no significant differences in *G. pulex* mass at day 0 across treatments (F=0.19, DF=6, 35, p=0.98; Appendix C1). Dissolved oxygen was maintained consistently at or close to saturation (≥9.8 mg L^{-1}) throughout the exposure period and although there were some slight fluctuations between treatments these were not significant (F=2.16, DF=6, p=0.06). pH remained within the circum-neutral range of 7.4 – 7.9 and conductivity within 426 – 472 µs cm^{-1}. Statistically significant differences were observed for pH (F=6.48, DF=6, p<0.001) although the magnitude of these (range 7.33 to 7.95) changes was small and unlikely to affect the fitness of *G. pulex*. Mean temperature during exposure was 7.7 ± 0.3 °C.

5.3.2 G. pulex growth and feeding

Based upon the average initial masses of *G. pulex* across all treatments (26.4 mg ±11.4 mg), their estimated age was 150 days prior to exposure, although this is strongly dependent on gender (Sutcliffe *et al.*, 1981). However, growth differences between control and all treatments were not significant (Figure 5-2, H=3.18, DF=6, 162, p=0.79). Furthermore, there were no statistically significant differences in feeding between treatments (Figure 5-3, H=7.48, DF=6, 35, p=0.28).

5.3.3 G. pulex mortality

Mortality increased consistently over time in all six treatments compared to the control (Figure 5-4). Treatments started to demonstrate significantly increased mortality from the control at days 15-17 (HT MIX: W=4.06, p=0.04; LT ERY: W=4.33, p=0.04 and HT ERY: W=5.74, p=0.02) except for LT MIX which diverged at day 27 (W=3.98, p<0.05). Neither of the two propranolol treatments demonstrated significant changes although they did follow a similar trend of increased mortality. Mean mortality was 3-4x higher than control in all treatments (except LT PRO) from day 16 onwards and this was maintained until the end of the experiment.

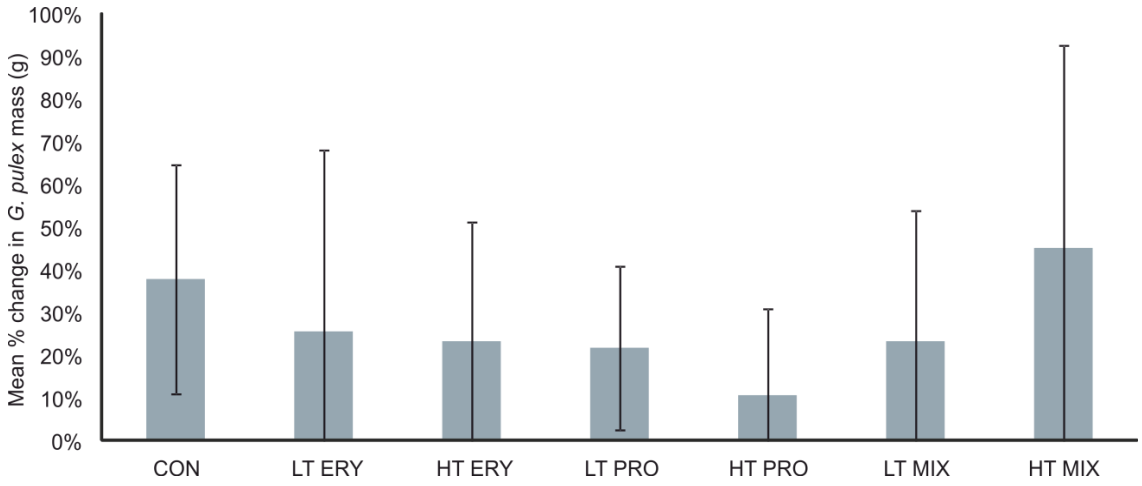


Figure 5-2: Mean % increase in mass of *G. pulex* during a 28 day exposure to erythromycin, propranolol and their binary mixture
[values are means (n=6; error bars are ± 1 SD)]

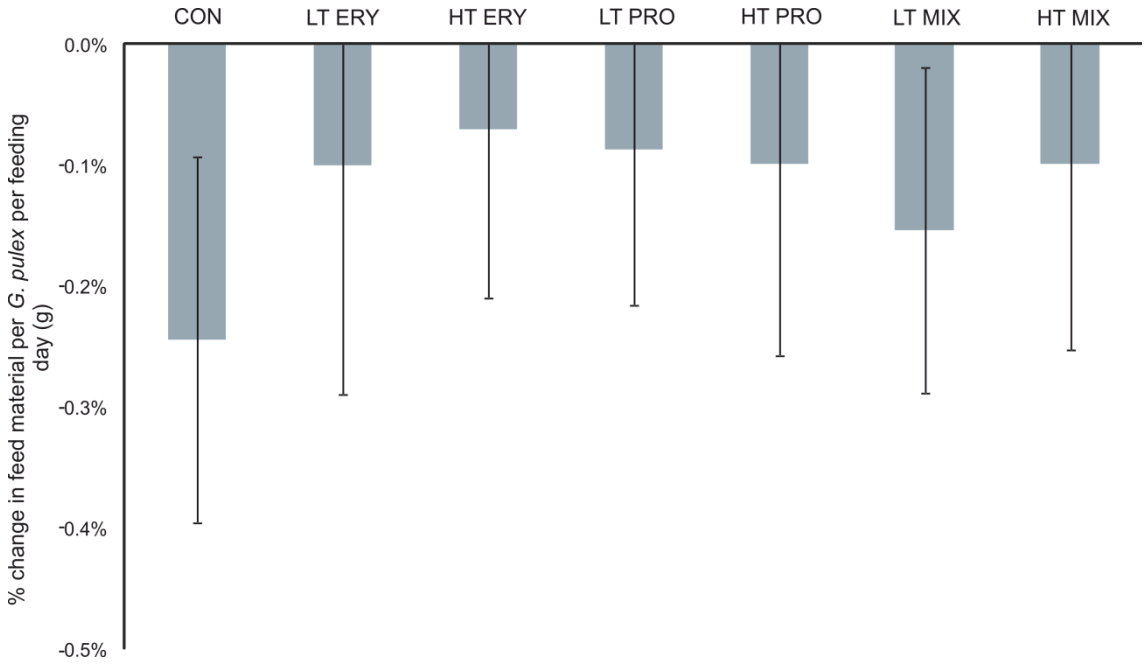


Figure 5-3: Mean (±1 SD) % mass loss of *F. sylvatica* litter normalised by the number of 'feeding days'
[i.e. the total of the number of days each individual survived]

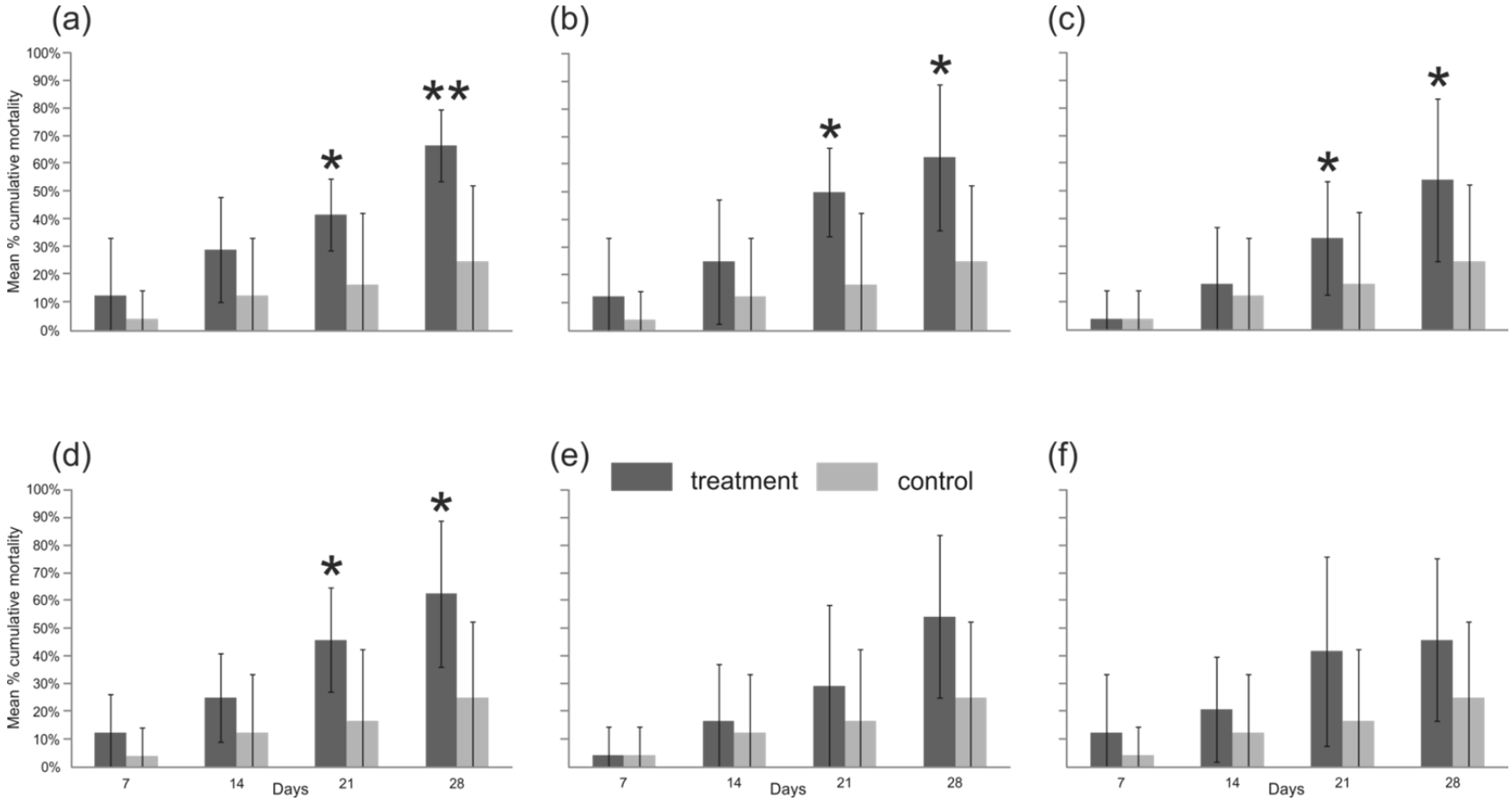


Figure 5-4: Cumulative % mortality of *G. pulex* during a 28 day static renewal exposure to (a) LT ERY, (b) HT ERY, (c) LT MIX, (d) HT MIX, (e) LT PRO and (f) HT PRO
[values are means (n=6); error bars are ± 1 SD; asterisks represent significant difference from CON (* < 0.05, ** < 0.01)]

5.3.4 Environmental metabolomics

It was possible to obtain good quality NMR spectra from the whole organism aqueous extract of *G. pulex* and several metabolites were identified (Figure 5-5) enabling comparison between the various pharmaceutical treatments. The whole organism *G. pulex* metabolomes were able to differentiate between controls and all treatments with changes in several of the assigned metabolites. When compared pairwise with the controls, the PLS-DA scores plots show separation between controls and all high treatments and for low treatment propranolol (Figure 5-6 a-e). Separation was also evident between the high and low treatments of each compound when considered apart from the controls (Figure 5-6 f-g). Characteristic of all the scores plots was the greater variability amongst controls when compared to individual treatments.

The model parameters showed the ability of PLS-DA to account for a significant proportion of the variability between treatments (R^2Y generally >0.50) (Table 5-1). R^2Y were particularly strong for pairwise comparisons between controls and high treatment erythromycin/propranolol (> 0.37) and between low and high treatments of the same compound (> 0.50). The goodness of predictability (Q^2) indicated usable, although somewhat noisy, models which were particularly strong for comparing between controls and high treatment levels of erythromycin and propranolol (> 0.30). Internal validation showed strong predictability for controls and high treatments across all compounds (> 69 %) although this was much reduced when classifying low treatment samples (55 % and 0 % for low treatment propranolol and erythromycin respectively). However, predictability was improved when comparing between low and high treatments without controls (≥ 0.77).

Table 5-1: PLS-DA model parameters indicating the degree of variability explained (R^2Y) and their predictive power for 'new' samples (Q^2)

Model description	No. model vectors	R^2Y	Q^2	Prediction accuracy (%) (no. samples)
CON vs. HT ERY	3	0.887	0.558	CON 100%, HTERY 100% Total 100% (25)
CON vs. LT ERY vs. HT ERY	1	0.282	0.198	CON 88%, LTERY 0%, HTERY 88% Total 67% (33)
CON vs. HT MIX	1	0.375	0.172	CON 94%, HTMIX 69%, Total 85% (26)
CON vs. HT PRO	1	0.569	0.300	CON 94%, HTPRO 69%, Total 85% (30)
CON vs. LT PRO vs. HT PRO	2	0.404	0.142	CON 88%, LTPRO 55%, HTPRO 69%, Total 73% (41)
LT ERY vs. HT ERY	2	0.842	0.552	LTERY 100%, HTERY 100%, Total 100% (16)
LT MIX vs. HT MIX	1	0.537	0.259	LTMIX 82%, HTMIX 89%, Total 85% (20)
LT PRO vs. HT PRO	1	0.506	0.235	LTPRO 82%, HTPRO 77%, Total 79% (24)

Prediction accuracy was estimated using SIMCAs internal misclassification tables

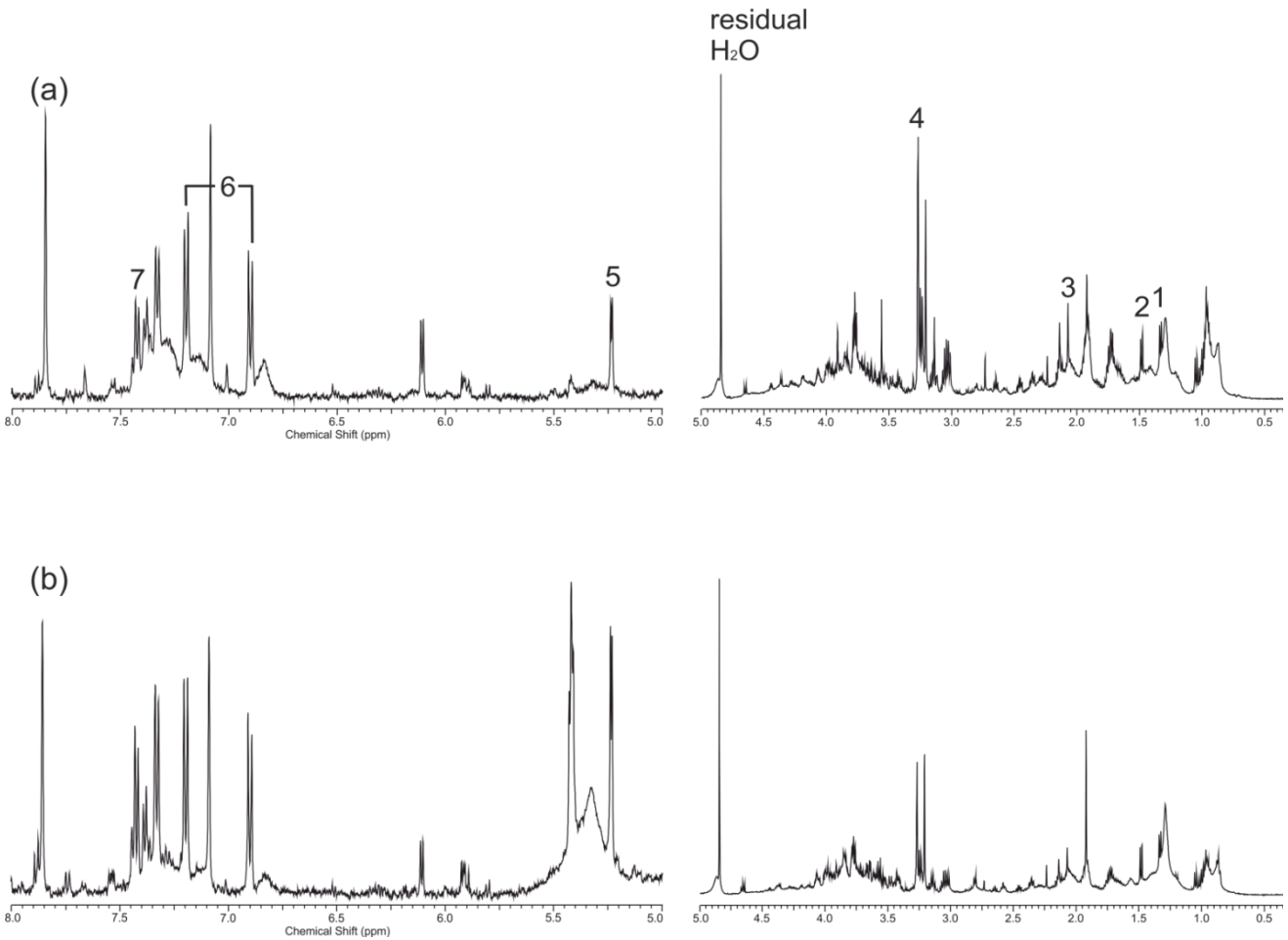


Figure 5-5: ^{1}H NMR spectrum from (a) control and (b) high treatment erythromycin samples *G. pulex* with some key metabolite peaks identified
(1 lactate, 2 alanine, 3 acetone, 4 trimethyl amine N-oxide (TMAO), 5 glycogen, 6 tyrosine , 7 phenylalanine)

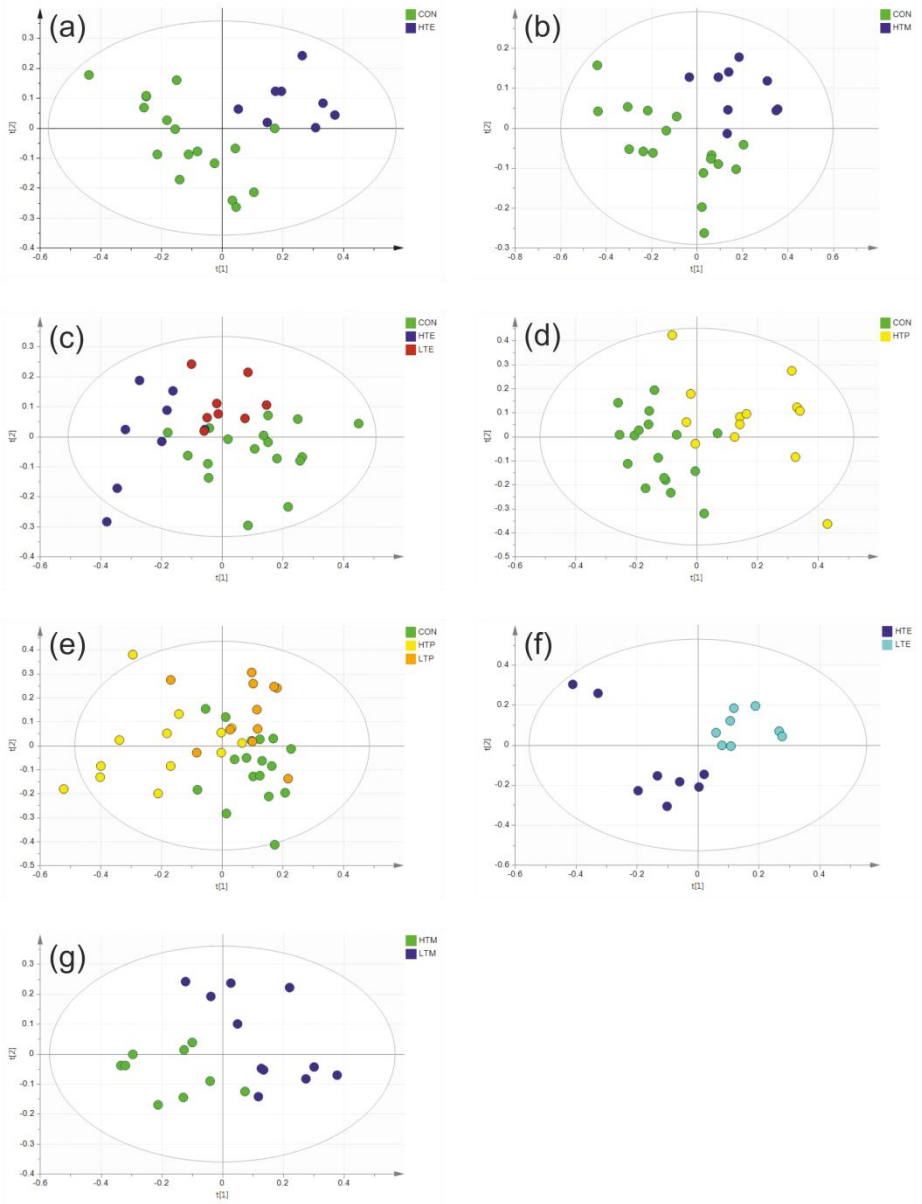


Figure 5-6: PLS-DA scores plot comparing controls to (a) high erythromycin, (b) high mixture, (c) low and high erythromycin, (d) high propranolol, (e) low and high propranolol and pairwise comparisons between high and low treatments of (f) erythromycin and (g) mixture
[Models show first two model vectors; 2nd vectors were fitted manually to aid visual comparisons for (b), (c), (d) and (g). Only separation along the first vector t[1] should be considered in these figures]

Examination of the scores and loadings plots (Figure 5-1; 5-6) highlighted several metabolites that were driving the differences between the controls and treatment groups (Table 5-2). High treatment levels of erythromycin and propranolol (200 000 and 1 000 000 ng L^{-1} respectively) generally caused greater relative changes in metabolite signals when compared to their low treatment equivalents.

Table 5-2: Relative metabolite changes[1] against controls from whole organism extracts of pharmaceutical exposed G. pulex

Metabolite (chemical shifts, ppm)	LT ERY	HT ERY	LT PRO	HT PRO	LT MIX	HT MIX
Leucine (0.875)	1.02	0.90	1.12*	1.00	1.01	1.06
Unknown 1 (0.965)	0.96	1.23	1.01	1.27	1.05	**1.27***
Unknown 2 [2] (1.275)	1.07	1.05	1.19	1.00	1.03	0.96
(1.585)	1.00	0.94	1.12	1.02	1.01	0.95
(2.595)	0.97	**0.69*****	1.15	0.85	0.99	1.04
Lactate (1.345)	1.06	**1.27****	1.05	**1.20****	1.01	0.93
Alanine (1.485)	1.07	**1.37***	0.97	**1.34***	1.04	1.20
Acetone (2.235)	0.62	0.54	0.66	0.60	0.57	**0.51***
Unknown 3 [2] (2.735)	0.99	**0.75****	1.08	**0.67*****	0.99	1.03
(4.105)	1.12	**0.89***	1.06	1.05	0.99	1.06
(4.375)	0.98	**0.70*****	1.08	**0.83***	0.98	1.05
Unknown 4 [2] (2.795)	1.09	0.93	**1.22****	0.93	1.06	0.99
(4.015)	1.02	0.96	1.11	1.05	1.02	1.05
TMAO (3.275)	1.05	**0.71****	0.83	1.27	0.99	1.12
Unknown 5 (4.645)	1.02	**0.88***	1.12	1.04	1.08	**1.15***
Glycogen (5.325)	**1.67***	**2.39***	**1.49***	**2.00***	1.46	0.85
Phenylalanine (7.325)	1.00	1.23	1.06	1.21	1.13	**1.25***
(7.395)	1.05	1.26	1.10	1.33	**1.18***	**1.35***
(7.425)	0.99	1.43	1.07	1.27	**1.17***	**1.31***

[1.] Relative changes were calculated by dividing the treatment group mean metabolite intensity by the mean intensity for control samples. Changes in **bold** indicate a significant difference between treatment and control, number of asterisks denotes the degree of significance (* < 0.05, ** < 0.01, *** > 0.0001);

[2.] 2D TOCSY NMR experiment confirmed these shifts belonged to the same unknown molecules.

5.4 Discussion

The aim of this experiment was to quantify the lethal, sublethal and metabolic effects of extended exposure to environmentally realistic concentrations of erythromycin and propranolol. Overall, substantial increases in mortality of *G. pulex* and perturbations to the metabolome were found as a result of pharmaceutical exposure. No concurrent effects on growth or feeding of *G. pulex* were evident. Although the results indicate some slight differences between treatments in flask DO, pH and EC, the magnitude of these differences was relatively small and unlikely to affect the fitness of the *G. pulex* as they are well within the ranges of tolerance demonstrated by G*. pulex* in the environment (Meijering, 1991). As such, this study confirms the suitability of *G. pulex* as an ecotoxicological test species that is both amenable to laboratory culture and sufficiently sensitive to provide reliable quantification of environmental risk. This work represents the first application of an extended exposure to a simple pharmaceutical mixture using *G. pulex* and the first application of NMR metabolomics to this species allowing

characterisation of the lethal and sublethal effects of pharmaceutical exposure on this functionally important freshwater macroinvertebrate.

5.4.1 Effects of erythromycin and propranolol on growth and feeding

It has long been established that growth and feeding of freshwater amphipods are sensitive indicators of a variety of stressors including metals (Naylor, 1989; Maltby & Naylor, 1990), sewage effluent (Maltby *et al.*, 1990), mine drainage (Macedo-Sousa *et al.*, 2007) and nutrients (Alonso & Camargo, 2004; Alonso & Camargo, 2008). The anti-histamine cimetidine has been shown to reduce the growth and biomass of other gammarids (*Gammarus fasciatus*) at environmentally realistic concentrations (Hoppe *et al.*, 2012). Here the exposure to low levels of erythromycin, propranolol and their binary mixture for a period of 28 days indicated a similar albeit not statistically significant trend of reduced growth in *G. pulex*. There was also some indication that exposed *G. pulex* may have been feeding at a reduced rate but this was not significant and therefore hypothesis $\mathbf{H_2}$ was rejected. Feeding and growth of *G. pulex* may be strongly dependent on size and gender so future studies should seek to standardise by these factors (Willoughby & Sutcliffe, 1976; Sutcliffe *et al.*, 1981). However, this was not possible during this experiment due to the risk of excess stress and injury. One way to remedy this in future studies would be to obtain pre-copulatory pairs of *G. pulex* or to set up large numbers of excess replicates and retain the genital region for post-mortem identification of gender.

Sublethal effects of pharmaceuticals on the behaviour and activity of other amphipod species have been established at concentrations similar to those reported here (De Lange *et al.*, 2006; De Lange *et al.*, 2009), albeit with different compound classes (antidepressants and anti-inflammatories). Brooks *et al*. (2003) showed that the beta-blockers reduced the heart rate and respiration of cladocerans below the concentrations required to affect survival or growth. This suggests that further work is necessary which combines evaluations of growth and feeding with other more sensitive sublethal behavioural and physiological assays.

5.4.2 Effects of erythromycin and propranolol on mortality

Clear and consistent increases in mortality were observed for erythromycin and mixture treatments after 7-14 days contrary to hypothesis $\mathbf{H_1}$. This compound caused a doubling in mortality after relatively long exposures (when compared to existing research) at environmentally relevant concentrations. Lethal concentrations for erythromycin (≥1000 ng L^{-1}) demonstrated in this study were up to four orders of magnitude below those demonstrated in short-term exposure tests of other aquatic invertebrates (Table 5-3). Other freshwater taxa such as green algae appear to be more sensitive than *D. magna* but even these effects manifest at levels 60x the nominal concentration of the lowest

exposure reported here. This demonstrates that longer experimental exposures are likely to change the conclusions drawn from short-term experiments substantially. As such future ecotoxicological testing should be conducted over longer time periods to better represent actual environmental exposures. However, short-term experiments remain useful for producing high volumes of reproducible data and may be appropriate in simulating a high concentration 'pulse' of pharmaceuticals in STP effluent. Furthermore, the results highlight the importance of using appropriate sensitive species when conducting ecotoxicological tests (Posthuma, 2002).

Although propranolol exposed *G. pulex* did not demonstrate significantly increased mortality, the data appeared to follow the same general trend exhibited by erythromycin and the mixture. This suggests that propranolol warrants further examination for its effects resulting from extended exposures to environmentally realistic concentrations.

Table 5-3: Summary of published ecotoxicological data for erythromycin and propranolol to aquatic taxa

Compound	Effective concentration in other studies (ng L^{-1})	Exposure duration (days)	Species tested	Reference
Erythromycin	211 000 000	2	*D. magna*	(Webb, 2001; Kim *et al.*, 2009)
	60 000	4	Green algae	
	>100 000 000	1	*Thamnocephalus platyurus*	
Propranolol	800 000	2	*Ceriodaphnia dubia*	(Huggett, 2002; Ferrari *et al.*, 2004; Kim *et al.*, 2009)
	1 600 000	2	*D. magna*	
	50 000	21	*D. magna*	
	10 310 000	1	*T. platyurus*	

The low treatment mixture exhibited a mortality increase double that of controls (and similar to erythromycin treatments). The results suggest that erythromycin and propranolol did not interact to produce enhanced effects during this study and therefore hypothesis **H_4** was rejected. This is contrary to previous work which has shown that the effects of some pharmaceuticals, when combined, can elicit even greater effects than when present alone (Cleuvers, 2003; Cleuvers, 2004). However the lack of interaction demonstrated here is probably due to the fundamentally different target receptors between antibiotics and beta-blockers with erythromycin targeting prokaryotic cells and propranolol targeting eukaryotes. Nevertheless, some studies have shown that pharmaceuticals with different target receptors can interact and that these interactions vary with exposure (Pomati *et al.*, 2006). Furthermore, some pharmaceuticals demonstrate non-polar narcosis which is not dependent upon the presence of specific receptors and as such remains a concern when considering the exposure to complex mixtures in freshwater environments (Cleuvers, 2003). Studies examining the toxicity of

simple or complex pharmaceutical mixtures are relatively sparse although there has been much increased attention over recent years (Brain *et al.*, 2004a; Brain *et al.*, 2004b; Brain *et al.*, 2005). Clearly, the issue of pharmaceutical mixtures is highly complex and there are many unanswered questions. Mixture studies like the one presented here and elsewhere are the first step in improving understanding.

Studies examining the effects of pharmaceuticals on *G. pulex* are relatively sparse and no studies are available reporting increased mortality although non-lethal effects have received increased attention over recent years. De Lange *et al*. (2006) exposed *G. pulex* to realistic concentrations of fluoxetine (antidepressant) and ibuprofen and found significant reductions in movement. However, these experiments had very short exposure durations of just two hours. A further study (De Lange *et al.*, 2009) demonstrated increased ventilation at low levels of the same compounds but increased activity at high levels (1000 – 1 000 000 ng L^{-1}). Some studies have demonstrated the effects of metals (including copper, lead and thallium) on *G. pulex* mortality with acutely lethal concentrations in the sub mg L^{-1} range (Gerhardt, 1995; Ozalp *et al.*, 2011). Sublethal responses in ventilation and locomotion were also present for these metals. Pesticides have been shown to exhibit acute lethal effects at low µg L^{-1} concentrations although the exposure periods for such studies were less than 96 hours (Taylor *et al.*, 1994; Crane *et al.*, 1995). Behavioural (e.g. invertebrate drift) and lethal effects have also been demonstrated over a range of time periods (<1 h to 10 days) for *G. pulex* exposed to complex industrial effluent and acidification (Taylor *et al.*, 1994; Gerhardt, 1996). Generally, the concentrations required for more 'traditional' pollutants to cause lethal and sublethal effects are greater than those required for pharmaceuticals. The body of research on *G. pulex* response to stressors suggest it is an ecologically sensitive indicator species for a wide range of aquatic pollutants, including pharmaceuticals. The results presented here lend support to its use for detecting effects of pharmaceuticals with effective mortality evident at concentrations well below those reported for the much more widely used test species, *D. magna*.

The lack of samples testing nominal concentrations in the exposure matrix means it was not possible to fully establish a dose-response relationship for mortality at specific concentrations of pharmaceuticals. However, the experimental design allowed comparisons between exposures to high and low levels of each pharmaceutical and their comparisons against control conditions. Further work evaluating additional exposure levels with measured exposure concentrations should be the next research step. The three-fold increase in mortality demonstrated here may be sufficient to manifest effects at the population level (Brain *et al.*, 2004a; Brain *et al.*, 2004b). Given the important role that *G. pulex* plays in the processing of organic matter, increased mortality may have serious secondary implications for leaf litter decomposition and nutrient cycling in

freshwater ecosystems (Macneil *et al.*, 1997), in addition to implications for its predators and the wider food web. These potential widespread negative effects on populations and ecosystem functioning due to pharmaceutical pollution should be seen as a future research priority.

5.4.3 Effects of erythromycin and propranolol on the metabolome

Environmental NMR metabolomics has been applied to a range of organisms and has shown considerable potential in the field of biomarker development (Viant *et al.*, 2003; Viant, 2007). This study represents the first application of environmental metabolomics in studying the effects of pharmaceuticals on *G. pulex*. This study has established that ^{1}H NMR metabolomics can successfully distinguish the whole organism metabolic profile of *G. pulex* and identify differences in individuals exposed to erythromycin and propranolol when compared to controls. Therefore, hypothesis **H_3** was accepted. Furthermore, this technique has identified several metabolites which can together serve as potential biomarkers of pharmaceutical exposure.

Models showed a good ability to distinguish and predict between controls and high treatments across all three exposures. However, this ability was weakened when low treatments were included with such samples. This suggests that any effects on the metabolome caused by low treatments are not distinguishable from normal metabolic 'noise' in the controls. This trend is consistent for all compounds and suggests a metabolic response that increases with exposure concentration *i.e.* dose response. However, further work is needed to establish a true dose-response relationship and nominal exposure concentrations.

Discriminating stress effects from metabolic 'noise' (due to diet, age, gender etc.) is a major challenge in environmental metabolomics although phenotypic anchoring can help reduce this (Viant, 2007). One possible explanation for this 'noise' is the lack of standardisation by gender, (Viant, 2007); however this was not possible during this study due to the excessive stress to which *G. pulex* would be subjected. High levels of noise have been demonstrated in other laboratory studies even in cultured populations (Hines *et al.*, 2007). Phenotypic anchoring of *G. pulex* is particularly important for future studies because sexual dimorphism in this species can influence growth, feeding and metabolism (Sutcliffe *et al.*, 1981; Greenwood & Adams, 1984). Furthermore, changes in the molt and sexual stages of *G. pulex* may have occurred during the exposure period which are likely to cause changes in the metabolome. These differing stages are known to have varying susceptibility to toxicants such as heavy metals (Geffard *et al.*, 2010). Another possible explanation for noise is parasitism; microsporidian parasites have been shown to alter the activity, aggression and micro-scale distribution of Gammarids (MacNeil *et al.*, 2003; Fielding *et al.*, 2005). However, all individuals were collected from

the same river to reduce the likelihood of infection/non-infection being a confounding factor.

Several metabolites were found to be significantly altered by pharmaceutical exposure and all (except TMAO) appear to be related to fluctuations in exertion (anaerobic metabolism), stress response and the resultant demand on energy stores (glycogen). These are perhaps indicators of a general sub-lethal stress response and increased energy requirements in order for *G. pulex* to combat the sublethal toxic action of pharmaceuticals.

Exposure to erythromycin caused significant changes in the chemical shift region identified as TMAO; this compound has been widely identified as an osmolyte in aquatic crustaceans playing a crucial role in regulating fluid balance and maintaining cell volume (Yancey, 2005). In addition to their roles as osmolytes, methylamines such as TMAO have demonstrated the ability to counteract the negative effects, such as protein destabilisation, of urea and salt accumulation within cells (Yancey, 2001). Intracellular levels of TMAO appear to increase in the presence of a stressor, such as urea, pressure, temperature and possibly pharmaceuticals and this can itself be detrimental by over stabilising proteins and inhibiting their function (Yancey *et al.*, 1982).

Erythromycin displays its therapeutic bacteriostatic activity by disrupting protein synthesis and related processes that are critical for survival and reproduction (Tenson *et al.*, 2003) but this mechanism of action is only likely to be present in prokaryotic cells. If similar receptors were present in *Gammarus pulex* the ultimate effects may be vastly different making comparisons difficult. However, freshwater amphipods such as *G. pulex* and *Asellus aquaticus* have been shown to have endosymbiotic relationships with gut bacteria which contribute to the digestive process (Harris, 1993; Zimmer & Bartholme, 2003; Wang *et al.*, 2007). Were such a symbiotic relationship to be disrupted by antibiotics then the feeding, fitness and growth of *Gammarus pulex* could be negatively affected. Furthermore, *G. pulex* selectively choose food sources based on the level of pre-conditioning by fungal and bacterial communities which can significantly alter feeding rates (Gulis & Suberkropp, 2003a; 2003b; Woodward *et al*., 2012). Antibiotic compounds have been shown to disrupt this pre-conditioning and food selection at environmentally relevant concentrations (Bundschuh *et al*., 2009; Hahn & Schulz, 2007). Therefore, it is possible that antibiotics exert an indirect effect on *G. pulex* metabolism by altering food selection, feeding rates and digestion processes within the gut. This represents an interesting avenue for future research.

One other possible explanation for the observed effect is the accumulation of the pharmaceutical compounds (or their breakdown products) in cells which disrupts the osmotic balance causing alterations in TMAO. TMAO did not appear to be involved in general stress response mechanisms (associated with the other metabolites) and may

be responding more specifically to the pharmaceuticals. This could indicate that TMAO is a useful metabolite in the study of sub-lethal impacts of pharmaceuticals in freshwater invertebrates but further evidence from other studies would be needed to support this hypothesis.

Exposure to erythromycin caused significant increases in lactate, alanine and glycogen. Lactate is known to accumulate in the muscles and liver after significant exertion (Phillips *et al.*, 1977). Exposure of fish to environmentally realistic concentrations of copper and synthetic oestrogens caused increased hepatic concentrations of lactate indicative of anaerobic metabolism (Ekman *et al.*, 2008; Santos *et al.*, 2009). Alanine is produced by the muscles and transported to the liver during exertion (Salway, 2004). Exposure to very high levels (1.4 mg L^{-1}) of propranolol have been shown to illicit small but significant elevations (+16 %) in alanine levels from whole organism extracts of *D. magna* (Taylor, 2010); however this concentration is extremely high and not representative of those likely to be encountered in the environment.

Exposure to the high and low mixtures caused an increase in phenylalanine. Phenylalanine is an indispensable amino acid which in humans may be involved in the 'fight or flight' response and is linked with growth and metabolic processes and stress control via tyrosine (Lehnert & Wurtman, 1993). This amino acid has been identified in the whole organism metabolome of *D. magna* and was shown to increase slightly (2.1 %) upon exposure to high levels of propranolol (Taylor, 2010). The greater enhancement of phenylalanine present may perhaps be explained by the longer exposure period in this study (28 days) compared to the above study (one day).

Glycogen (elevated in erythromycin and propranolol exposed individuals) is a polysaccharide energy store and is the principle storage form of glucose in most animals (Sedlmeier, 1982). Existing research presents contrasting results when examining glycogen storage in response to external stressors. Exposure of dog whelks (*Nucella lapillusi)* to cadmium caused increased storage in male individuals (Leung & Furness, 2001) but tributyltin caused a depletion in the freshwater shrimp (*Caridina rajadhari*) (Nagabhushanam *et al.*, 1991). It is hypothesised that depletion of energy reserves is caused by increased demand in response to toxic stress. However, continuous availability of food (as in this study) may offset this demand on the glycogen store and explain why the exposed *G. pulex* displayed only a slight increase in glycogen levels. One study has shown the potential for low levels of insecticides to initially elevate glycogen levels in field crabs (*Barytelphusa guerini*) followed by significant decreases after 72 hours (Reddy *et al.*, 1991). This indicates these crabs have mechanisms to initially increase glycogen storage and then draw upon these excess energy stores in response to the stressor; a process that may be mirrored by *G. pulex* in this study.

Despite the inherent noise and lack of phenotypic anchoring, ^{1}H NMR metabolomics has proved a powerful technique capable of differentiating between pharmaceutical treatments and identifying subtle, sublethal effects in *G. pulex*. Toxicities of these compounds can be reliably distinguished between some treatments and controls with minimal classification error rates. The next step in environmental metabolomics should be the continued testing of *G. pulex* alongside other important freshwater species – both individually and as part of interacting communities (e.g. within realistic food webs (Brown *et al.*, 2011)). Testing should include other pharmaceuticals and wider chemical pollutants in order to create extensive databases of metabolic responses (Taylor, 2010). However, there remain considerable obstacles in understanding the underlying biochemical significance of changes in the metabolome given the complex interactions between metabolites and the lack of knowledge of how these function in lower organisms (Bundy *et al.*, 2009).

5.5 Conclusions

The results presented here demonstrate the potential for extended exposure to low levels of pharmaceuticals to cause significant sublethal effects in freshwater macroinvertebrates that would likely go undetected by standard short-term toxicological experiments. Furthermore, they highlight the vital importance of using appropriate experimental design, sensitive species and sufficiently long exposures if a full understanding of the risk posed by pharmaceutical pollution is to be gained. The novel application of ^{1}H NMR metabolomics has demonstrated its strong potential in understanding the sublethal effects of emerging contaminants in aquatic ecosystems and in the field of biomarker development. The demonstrated effects of pharmaceuticals will have implications for the water and pharmaceutical industries, managers, regulators and policy makers. In particular, the low-level effects of emerging contaminants may be a major barrier to meeting legislative requirements such as the ‘good status’ required by the EU Water Framework Directive (EC, 2000). Future policy and management strategies should consider pharmaceuticals as widespread and potentially damaging pollutants in freshwater ecosystems.

6 THE IMPACT OF PHARMACEUTICALS ON LEAF LITTER DECOMPOSITION IN RIVERS

6.1 Introduction

Pharmaceuticals are widely present as pollutants in the environment and have been detected in soils, rivers, groundwater, biota and drinking water across most of the globe (Daughton & Ruhoy, 2009b; Hughes *et al.*, 2013). Knowledge of pharmaceutical occurrence is growing rapidly but studies of their effects on freshwater organisms and ecosystems are relatively sparse (Fent *et al.*, 2006; Kümmerer, 2009a). In particular, much existing ecotoxicological research has focused on short-term exposure to high, environmentally unrealistic concentrations of pharmaceuticals using a small number of standard test species and endpoints mostly focused around organismal level metrics (e.g. feeding, growth, reproduction, mobility, mortality). Such procedures are useful in generating high volumes of reliable and reproducible effects data but their ecological relevance is limited and the risk of such acute toxicity is generally low. Even more recently, research effort has shifted towards understanding the effects of long-term, low-level exposure to pharmaceuticals in freshwater ecosystems where the risk of negative implications is much higher (Fent *et al.*, 2006; Kümmerer, 2008a; Santos *et al.*, 2010; Hughes *et al.*, 2013). Despite this, most work still examines the effects at the individual organism level with very few studies attempting to address the complex issue of pharmaceutical mixtures and their implications for structural and functional aspects of freshwater ecosystems (Hughes *et al.*, 2013).

Scientists and policy makers need reliable indicators of integrity to assess the state of ecosystems and to alleviate negative impacts resulting from anthropogenic stressors (Gessner & Chauvet, 2002). Ecosystem integrity is composed of structural and functional components and the two are intimately linked; pattern (structure) determines process (function) and *vice versa* and so both must be considered (Matthews *et al.*, 1982; Bunn *et al.*, 1999). Functional measures are integrative over time, space and organisational levels and are much less dependent on the presence of specific species sets and are therefore not restricted to particular geographical settings. Furthermore, it is possible for changes in structure to occur with no corresponding change in processes and *vice versa*. Despite this bioassessment is overwhelmingly based on structural measures alone (Matthews *et al.*, 1982; Gessner & Chauvet, 2002).

An often cited concern in the use of functional indicators for monitoring stressor impacts is that inherent environmental variability will mask stressor response (Hill *et al.*, 2000). However, Niemi *et al. (1993)* reviewed a variety of chemical and biological (structural and functional) measures of ecosystem recovery to a range of stressors (temperature, ammonia, heavy metals and pesticides) and found that functional

measures were generally more sensitive (requiring lower samples sizes and reduced costs) in detecting impact and recovery in stream ecosystems. Despite this, the application of functional endpoints in studying the effects of pharmaceuticals and other emerging contaminants in freshwater ecosystems is sparse (Santos *et al.*, 2010; Hughes *et al.*, 2013). These studies fail to draw upon the well-developed 'tool kit' of methods available in aquatic ecology (Rosi-Marshall & Royer, 2012).

The input of allochthonous carbon in the form of coarse particulate organic matter (CPOM; i.e. particles >1mm in size) is a major and often dominant energy source in freshwater ecosystems (Conners & Naiman, 1984; Richardson, 1991; Webster *et al.*, 1995; Giller & Malmqvist, 1998). The decomposition of CPOM (including leaf litter) involves three vital processes: direct leaching of dissolved organic carbon (DOC), microbial and fungal decomposition, and biological and mechanical fragmentation ('shredding') by macroinvertebrates (Webster & Benfield, 1986). These processes are interrelated and mechanical shredding increases surface area of leaf litter available for microbial colonisation (Giller & Malmqvist, 1998). Colonisation by microbial and fungal communities can in turn affect mechanical breakdown by influencing food selection by shredder macroinvertebrates perhaps by rendering leaf litter more palatable and by increasing the energy content of the food as shredders consume both leaf material and the microbial colonisers (Merritt *et al.*, 1984; Graça, 1993; Giller & Malmqvist, 1998; Graça *et al.*, 2001). Studies have shown that fungi dominate over bacteria in these microbial communities and contribute most towards microbial decomposition rates (Gulis & Suberkropp, 2003a; Gulis & Suberkropp, 2003b). Without these processes combining to decompose leaf litter, the associated carbon would be unavailable at higher trophic levels and downstream transport would be reduced (Giller & Malmqvist, 1998). For example, disruptions to the input and processing of leaf litter during large experimental manipulations has been shown to decrease secondary production by up to 80 % (Wallace *et al.*, 1997). The decomposition of leaf litter is a key ecosystem function with important implications for energy flow and nutrient cycling with the potential for this process to act as a bottom-up control cascading to higher trophic levels (Wallace *et al.*, 1997; Giller & Malmqvist, 1998). Despite this, almost nothing is known about the impacts of emerging contaminants, such as pharmaceuticals on this key ecosystem process.

There are numerous studies documenting the impact of anthropogenic change on the decomposition rates of leaf litter in rivers (Table 6-1). These studies demonstrate that leaf litter decomposition (including the colonisation by bacteria, fungi and macroinvertebrates) is sensitive to a wide range of environmental stressors. In particular, moderate nutrient enrichment appears to stimulate decomposition whereas high levels of nutrients, heavy metals or specific chemical compounds (e.g. pesticides) can be inhibitory. Studies have shown that leaf litter colonised under different pollutant

loads, but then incubated under the same reference conditions, can decompose at different rates (Medeiros *et al.*, 2010). This approach is potentially useful for examining the effects of stressors on microbial colonisation in controlled laboratory conditions, prior to transplanting leaf packs to 'natural' rivers to evaluate subsequent macroinvertebrate colonisation and breakdown.

Laboratory studies examining the effects of pharmaceuticals on leaf litter breakdown are rare and have focused on food selection by shredders following conditioning by antibiotics (Table 6-1; (Hahn & Schulz, 2007; Bundschuh *et al.*, 2009)). These studies show contradictory effects of certain antibiotic mixtures on food selection and decomposition but comparisons between experiments may be misleading given the different compounds and test species used. Currently, there are no studies available examining the effects of non-antibiotic pharmaceutical compounds or mixtures of different compound types on leaf litter decomposition. In addition, no studies have combined structural and functional aspects of this process (i.e. quantifying the impact of pharmaceuticals on decomposition rates and the associated colonisation of leaf litter by macroinvertebrates) using both laboratory and *in situ* techniques. A further limitation of existing studies is that they have used relatively high exposure concentrations over short exposure periods; concentrations ≥2000 ng L^{-1} up to 20 days. Therefore, further studies are necessary which examine frequently observed environmental concentrations over longer exposure periods. This study aimed to address these knowledge gaps and further our understanding of pharmaceutical effects on leaf litter breakdown in rivers.

Table 6-1: Examples of anthropogenic stressor effects on aspects of leaf litter decomposition in freshwaters

Stressor	Effects	Reference
Land use		
Intensive agricultural land use (crops and industrial animal rearing)	Invertebrate mediated decomposition reduced in most intensively farmed catchments, microbial decomposition unaffected	(Piscart *et al.*, 2009)
Nutrient enrichment		
Agricultural runoff	High phosphate reduced fungal diversity but increased decomposition	(Sridhar *et al.*, 2009)
	Ammonium, phosphate and nitrite reduced invertebrate mediated decomposition but nitrate increased it	(Feio *et al.*, 2010)
N and P	Fungal and bacterial colonisation, biomass and decomposition increased at elevated nutrient levels,	(Gulis & Suberkropp, 2003a; 2003b)
	No effect on bacterial colonisation when fungi excluded but decomposition was reduced	(Woodward *et al.*, 2012)
Low level eutrophication (N and P)	Decomposition reduced at extreme low (nutrient limitation) and high ends of nutrient gradient. Strong stimulation at moderate nutrient levels	(Gulis *et al.*, 2006)
	Decomposition, fungal biomass, sporulation and invertebrate abundance and richness elevated in eutrophic streams	
Heavy metals		
Mine drainage: As, Fe, Mn and Zn	Fungal mediated breakdown unaffected but sporulation increased at polluted sites. Macroinvertebrate decomposition reduced in polluted sites.	(Medeiros *et al.*, 2008)
		(Medeiros *et al.*, 2010)
Cu	Fungal biomass increased in polluted streams but sporulation decreased	(Roussel *et al.*, 2008)
	Reduced decomposition rates and macroinvertebrate (incl. *Gammarus pulex*) abundance in polluted streams. Fungal communities unaffected	
Fungicides		
Tebuconazole	Reduced decomposition and microbial biomass	(Artigas *et al.*, 2012)
Propiconazole	Reduced microbial decomposition and microbial biomass. Increased macroinvertebrate decomposition	(Rasmussen *et al.*, 2012)
Insecticides		
Alphacypermethrin	Reduced macroinvertebrate decomposition	(Rasmussen *et al.*, 2012)
Pharmaceuticals		
Antibiotic mixture (erythromycin, clarithromycin, roxithromycin, sulfamethoxazole and trimethoprim)	Increased food selection and macroinvertebrate (*Gammarus fossarum*) decomposition of antibiotic exposed leaves. Increased fungal colonisation but microbial decomposition unaffected. ≥2000 ng L^{1} exposure (20 days).	(Bundschuh *et al.*, 2009)
Antibiotic mixture (oxytetracycline and sulfadiazine)	*Gammarus pulex* selected non-exposed leaves. No effect of microbial colonisation or decomposition. ≥2000 ng L^{-1} (10 days)	(Hahn & Schulz, 2007)

6.2 Aims, objectives and hypotheses

The aim of this study was to quantify the effects of low-level extended exposure to five pharmaceutical compounds both in isolation and as part of a mixture on leaf litter decomposition during the microbial pre-conditioning phase and during subsequent in-stream breakdown. Specific objectives were:

- Understand the effects of environmentally relevant concentrations of pharmaceutical compounds on the microbially and macroinvertebrate mediated decomposition of leaf litter in freshwater ecosystems;

- Understand whether pharmaceutical exposed pre-conditioned leaf litter is colonised and decomposed at different rates compared to non-exposed leaf litter when returned to an unpolluted stream;

- Understand the effects of different compounds acting as a combined mixture on the above processes;

- Target erythromycin for detailed study using the above techniques to improve on previous studies showing the propensity for antibiotics to affect decomposition (Hahn & Schulz, 2007; Bundschuh *et al.*, 2009).

This study aimed specifically to test the following hypotheses: (**H_1**) pharmaceuticals disrupt the colonisation and pre-conditioning of leaf material by fungal and microbial communities resulting in measurable changes in decomposition rates (Bundschuh *et al.*, 2009). Changes to the pre-conditioning of leaf litter will alter the colonisation of leaf litter in streams and subsequent decomposition rates (**H_2**). Important macroinvertebrate shredder species such as *Gammarus pulex* and *Asellus aquaticus* will actively select leaf material colonised in the absence of pharmaceuticals (**H_3**) (Hahn & Schulz, 2007).

6.3 Methodology

6.3.1 Site selection

The site chosen for the *in situ* phases was Silsden Beck immediately downstream of Silsden Reservoir, Silsden, West Yorkshire (OSNGR SE 04461 47504; Figure 6-1). The site was chosen due to it being situated upstream of any STP effluent inputs, thereby reducing the potential for contamination by the study compounds. A 1 L grab sample of water from Silsden Beck was analysed *a priori* by HPLC-MS/MS indicated that all five of the preliminary study compounds were below detection limits (5ng L^{-1} for diclofenac, erythromycin, mefenamic acid and propranolol and 25 ng L^{-1} for ibuprofen). Furthermore, the regulated nature of the stream made this an ideal choice for the *in situ* studies due to the stable channel platform, hydraulics and flow regime. Wooded banks

(predominantly beech, *Fagus sylvatica*) supported a community of shredder macroinvertebrates (particularly *Gammarus pulex* and *Asellus aquaticus*); an initial kick net survey found >50 individuals per kick sample).

6.3.2 Chemicals and materials

Glassware and vessels were disinfected then pre-rinsed with 100 % methanol and DI H_2O prior to the experiments. HPLC-grade methanol was supplied by Fisher Scientific UK Limited (Loughborough, UK). Deionised water was generated with a Purite Select HP160/BP/IT deioniser. All pharmaceuticals were of the highest purity available (>95%) and supplied by Sigma-Aldrich Company Ltd (Dorset, UK). Individual stock standard solutions were prepared on a weight basis in 100 mL of 100 % methanol and stored in the dark at -20 °C until used. A fresh working mixture solution of all pharmaceuticals was prepared by appropriate dilution of the individual stocks in 100 % methanol immediately before each experimental run and used as working standard solutions. Working standards were also stored in the dark at -20 °C between uses.

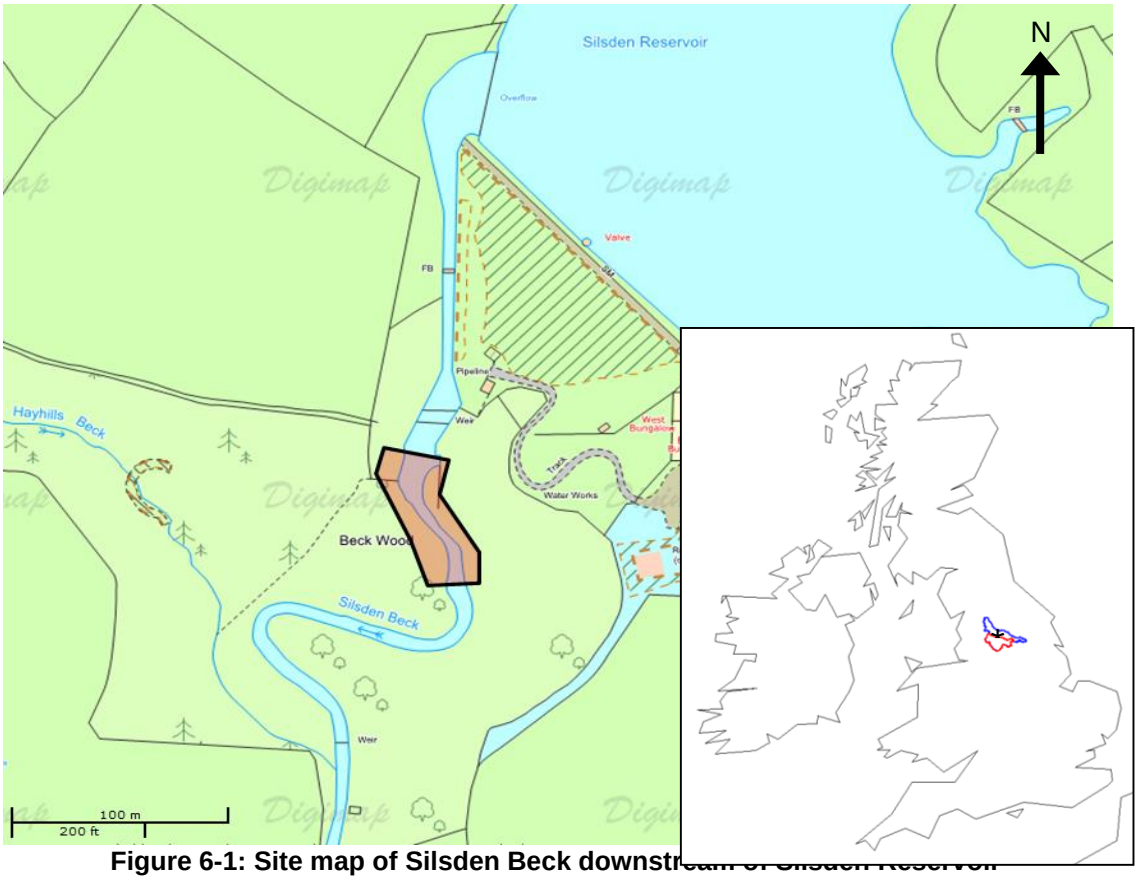


Figure 6-1: Site map of Silsden Beck downstream of Silsden Reservoir [highlighted area indicates location of leaf pack placement; OSNGR SE 04461 47504) © Crown copyright/database right 2010. An Ordnance Survey/EDINA supplied service]

6.3.3 Collection and preparation of leaf material

Abscised beech (*F. sylvatica*) leaves were collected adjacent to Silsden Beck in September 2010. After collection, these leaves were soaked in de-ionised water for 2 days to wash off any extraneous soil or mineral matter on the surface of the leaves (which may affect mass loss calculations), prior to drying at 55 ºC in an oven for 48 hours (Benfield, 2006). The leaves were then stored in sealed polythene bags in dark, dry conditions at room temperature until use. River water used in the laboratory experiments was collected from Silsden Beck into pre-cleaned 25 L plastic containers. Water was filtered through a 0.064 mm sieve to remove any coarse sediment or invertebrates and stored at 7.7 °C in a controlled temperature room prior to use. Invertebrates were removed to ensure any breakdown in the laboratory was mediated by microbial communities only.

6.3.4 Laboratory pre-conditioning phase

6.3.4.1 Individual compound study

The experimental setup for this study included 14 treatments (Table 6-2). Nominal pharmaceutical concentrations were based on the maximum published concentrations in UK rivers (for LT) (Ashton *et al*., 2004) which were multiplied by a factor of 100 to yield

the HT concentrations. The rationale for these values was to ascertain whether environmentally realistic concentrations could affect litter breakdown and whether a more conservative estimate of higher concentrations (i.e. several orders of magnitude greater than measured maxima) would yield a more pronounced effect to gain some knowledge of potential dose-response relationships. Furthermore, the evaluation of specific mixtures will serve to highlight any potential additive, synergistic or antagonistic interactions between the five study compounds.

Table 6-2: Summary of treatment schedule for the five compound leaf litter degradation experiment

Compound	Re-treatment frequency (days) [1]	Concentration (ng/L)	
		LT[2]	HT[3]
Diclofenac (DIC)	8	600	600 000
Erythromycin (ERY)	11	1000	1 000 000
Ibuprofen (IBU)	6	5000	5 000 000
Mefenamic acid (MEF)	3	400	400 000
Propranolol (PRO)	5	200	200 000
Low treatment mixture (LT MIX)	3 – 11	Sum of all LT	n/a
High treatment mixture (HT MIX)	3 – 11	n/a	Sum of all HT
Methanol control (MeOH)	Solvent control treated with equivalent volume of 100% methanol only		
Control (CON)	Untreated Silsden Beck water		

Notes:
1. Based on conservative estimates of environmental freshwater half-lives
2. LT: low treatment based on UK maxima
3. HT: high treatment = 100 * LT

Control and HT MIX tanks also contained artificial leaf packs

2 g samples (±0.04 g) of dried beech leaves were weighed using a 4 d.p. analytical balance and placed within 1000 µm stiff mesh leaf packs (EFE UK Ltd., Cornwall, UK). 2 g of black plastic (pre-rinsed and leached in de-ionised water for 7 days) replica leaves were also assembled into 12 'artificial' leaf packs. Twelve beech leaf packs were then randomly placed in each of 14 separate glass aquaria (total n = 168); the artificial leaf packs were placed in the control and HT MIX aquaria (n=6 in each). Each aquaria contained 20 L of water from Silsden Beck. This water was equilibrated for temperature and dissolved oxygen over 7 days prior to the addition of leaf packs. Throughout equilibration and exposure the aquaria received constant aeration (Hailea ACO-9602 air pumps, Guangdong Hailea Group Co. Ltd., Guangdong, China) at 7.7 ±0.3 °C and a light: dark photoperiod of 12:12 hours. The experiments were conducted in all-glass 45 x 38 x 30 cm Clear-seal aquaria (Clear-seal Ltd., Birmingham, UK).

After equilibration, treatment of 12 tanks with pharmaceutical compounds was achieved by pipetting the appropriate volume of pharmaceutical working solution directly into the tank and then stirring thoroughly with a glass rod. No further mixing was conducted as the aeration was observed daily and found to mix the entire tank adequately. The treatment approach was a modified static-renewal design whereby each tank was re-treated with working pharmaceutical solutions during the 8 week laboratory exposure period. This modified static-renewal approach was adopted in order

to simulate the ‘pseudo-persistence’ of pharmaceuticals in the environment and allowing for losses due to hydrolysis, photolysis, adsorption and biological uptake (Daughton, 2001). The re-treatment frequency was determined by examining the literature for relevant environmental half-lives and renewing the treatment solution at the number of days coinciding with each compounds half-life (Tixier *et al.*, 2003; Castiglioni *et al.*, 2004; Löffler *et al.*, 2005; Alonso & Camargo, 2008; Yamamoto *et al.*, 2009). For example, erythromycin was found to have a half-life of approximately 11 days; on day 0 the erythromycin tanks were treated with the appropriate working solution. After 11 days the tanks were renewed to ‘top up’ the concentration back to nominal level. This re-treatment schedule is summarised in Table 6-2.

Each tank received the same volume of working solution but with varying concentrations depending on treatment compound and level. A solvent-only control (methanol) was also established to evaluate any potential confounding effects. The total treatment period in the controlled temperature room was eight weeks during the period August to October 2010. The *in situ* treatment phase totalled an additional eight weeks from the period October to December 2010. An initial pilot study under identical conditions showed eight weeks was sufficient to obtain small but quantifiable changes in leaf pack mass.

6.3.4.2 Detailed erythromycin study

This study focused on a single compound - the macrolide antibiotic erythromycin - as previous studies of other antibiotics to have significant impacts on food selection and decomposition (Hahn & Schulz, 2007; Bundschuh *et al.*, 2009). Four treatment levels were adopted (Table 6-3) to cover a much wider range of exposure concentrations based on published UK river concentrations (for VLT and LT: (Ashton *et al.*, 2004)) and higher concentrations (HT and VHT) to represent worst-case exposures. Treatment conditions, exposure period and leaf material preparation were identical to the individual compound study. Treatment in the laboratory was carried out over eight weeks during the period April to May 2011 with an additional eight weeks *in situ* during May to July 2011.

Table 6-3: Summary of treatment schedule for the detailed erythromycin leaf litter degradation experiment

Treatment level	Nominal concentration (ng/L)
Very low treatment (VLT)	100
Low treatment (LT)	1,000
High treatment (HT)	100,000
Very high treatment (VHT)	1,000,000
Solvent control (MeOH)	16.4 mL methanol
Control (CON)	n/a

6.3.5 *In situ* phase

After the eight week pre-conditioning phase the leaf packs in each tank (n=12) were removed. Six (hereafter termed pre-conditioning packs) were selected randomly for immediate analysis in the laboratory and the remaining packs (hereafter termed *in situ* packs) were transported to the Silsden Beck site. A total of 84 leaf packs (6 replicates across 14 treatments) were placed in the stream. The packs were anchored to the stream bed using house bricks held in place with steel bar. Suitable locations in the stream were identified (i.e. stretches that were deep enough to cover the whole pack and with slower flows favoured by *Gammarus* and other shredders) and the packs were randomly assigned to these locations. The leaf packs were left *in situ* for a total period of eight weeks and a number of checks were made throughout this time to monitor for any loss of packs due to vandalism or high flow events. Temperature in the stream during this eight week period was recorded at 15-minute intervals using a Tinytag Plus 2 data logger (Gemini Data Loggers UK Ltd., Chichester, UK) as temperature is known to be a major controlling factor on leaf litter decomposition (Petersen & Cummins, 1974). Mean stream temperature for the individual compound study was 7.2 (±0.6) and 17.0 (±0.5) °C for the detailed erythromycin study.

6.3.6 Analysis of leaf packs

This section describes the process of measuring change in leaf litter mass and organic matter due to decomposition processes in the laboratory and *in situ*. After retrieval from Silsden Beck, the leaf packs were opened and rinsed with deionised water onto a 0.064 mm sieve to remove any macroinvertebrates which were then preserved with 70 % methylated spirit for subsequent identification of taxa to family level under stereo microscope. More specifically, *Gammarus pulex* and *Asellus aquaticus* were identified and counted as these were found to be the most dominant shredder macroinvertebrate species at Silsden Beck. The leaf packs were oven dried at 55 ºC for 48 hours. A 0.5 g (± 0.1 g) subsample of the leaf pack and the 1 cm discs were combusted in the furnace at 550 ºC for 1 hour in order to calculate ash free dry mass (AFDM) and % organic matter (%OM). This procedure was also followed for the leaf packs treated in the laboratory immediately after their removal from the treatment tanks. Handling loss control packs

(n=6) were also made and subjected to the same transport and handling procedures to account for potential losses during handling of the fragile dry leaf tissue (Benfield, 2006) and the final mass changes for treatment packs were corrected as necessary.

6.3.7 Statistical analysis

All univariate and multivariate analyses were conducted in Minitab 16 (Minitab Inc., PA., USA) and PASW Statistics 17.0 (SPSS Inc., IBM). All data were tested for normality using the Anderson-Darling test and then the most powerful appropriate parametric (t-test, ANOVA or General Linear Model) or non-parametric (Mann-Whitney or Kruskal-Wallis) statistical tests were used. All proportional data were arcsine transformed prior to comparisons. Macroinvertebrate diversity indices (species richness, Bray-Curtis D, Fisher's Alpha, Shannon H Index) were calculated using Species Diversity and Richness v2.65 (Pisces Conservation, Lymington) and analysis of similarity (ANOSIM using Bray-Curtis distance over 9999 runs) was calculated using PAST (Hammer *et al.*, 2001). Two-dimensional non-metric multidimensional scaling (NMDS) plots were constructed in PAST using the Bray-Curtis distance measure (Rabinowitz, 1975).

6.4 Results

6.4.1 Individual compound study

6.4.1.1 Mass and organic matter change

Mass loss was greater in the *in situ* decomposition packs when compared to the laboratory pre-conditioning phase but pharmaceutical treatment caused no significant differences from controls in either phase. Significant differences in AFDM change and %OM between the pre-conditioning and *in situ* phases were present (Table 6-4; Figure 6-2). Controls packs lost less AFDM and organic matter than all but three pre-conditioning treatments (HT-DIC, HT-MIX and HT-PRO) and all of the *in situ* treatments but these differences were not significant.

Table 6-4: Summary of statistical analysis of mass and organic matter change in *F. sylvatica* leaf packs exposed to diclofenac, erythromycin, ibuprofen, mefenamic acid, propranolol and their mixture during a 16 week laboratory and *in situ* experiment

Comparison	AFDM change		%OM	
Pre-conditioning vs. in situ	-14% vs. -19% (H=61.75, DF=1, p>0.0001)		93% vs. 87% (H=97.93, DF=1, p>0.0001)	
Treatment comparisons	Pre-conditioning	In situ	Pre-conditioning	In situ
	H=21.12, DF=12, p=0.05*	H=11.09, DF=12, p=0.52	H=18.28, DF=12, p=0.11	H=15.34, DF=12, p=0.22

* Statistically significant difference between treatments, pairwise comparisons against control (family error rate: 0.05) showed no differences.

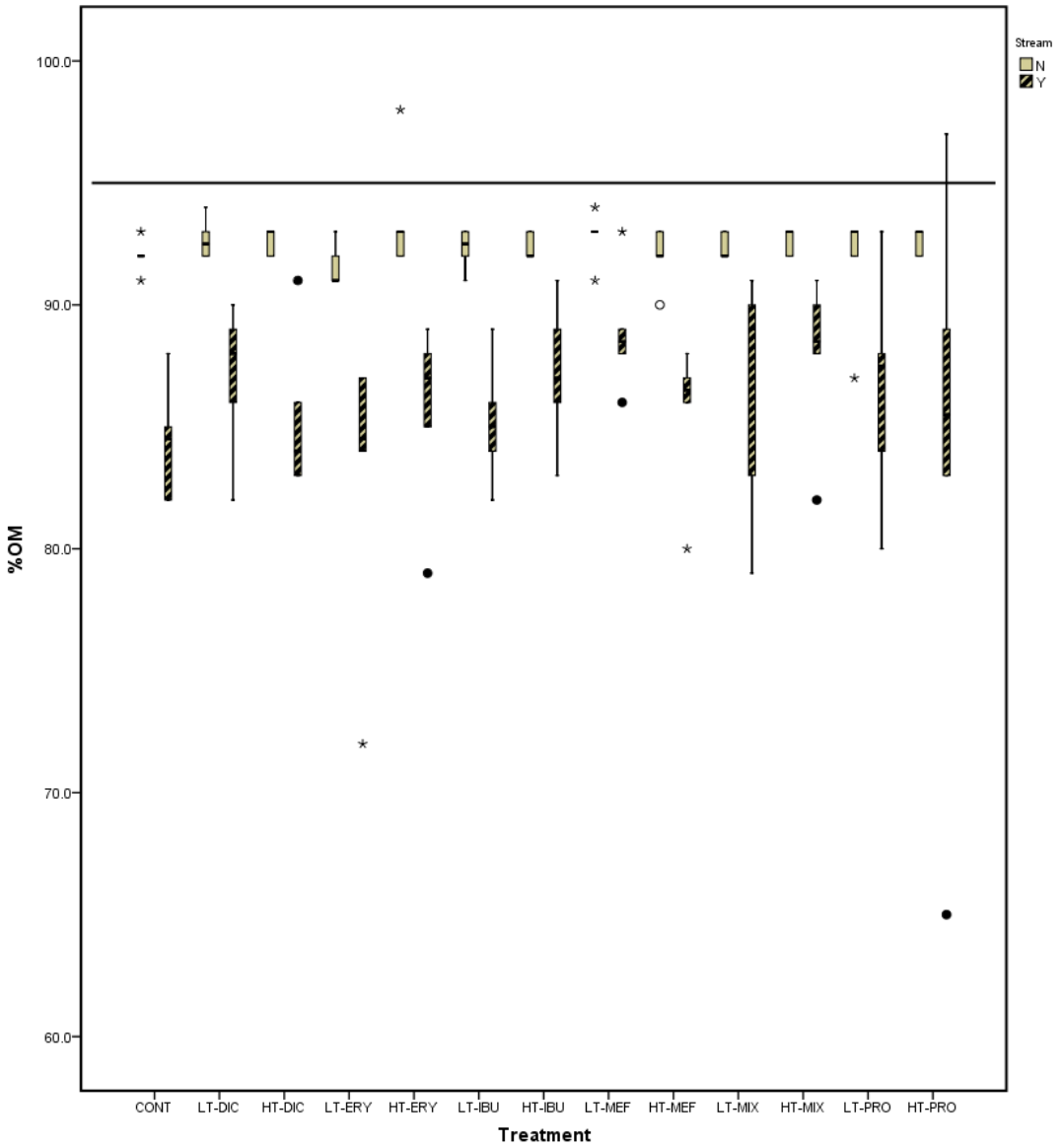


Figure 6-2: Percent organic matter in *F. sylvatica* leaf pack mass after 8 week laboratory exposure to five pharmaceuticals and their additive mixtures followed by eight weeks in situ in a stream
[black line represents initial %OM of untreated leaf packs at 94.1%. Bars represent mean from n=6 replicates; error bars are ±1 SD; CONT: control, DIC: diclofenac, ERY: erythromycin, MIX: mixture, MEF: mefenamic acid, PRO: propranolol, LT: low treatment, HT: high treatment. N indicates laboratory-only exposure, Y indicates additional *in situ* exposure]

6.4.1.2 Macroinvertebrate colonisation

In situ leaf packs were colonised predominantly by Chironomidae with smaller numbers of *A. aquaticus,* Oligochaeta, Plecoptera and Simuliidae (Table 6-5). Total abundance, species richness, dominant taxa and numbers of individual taxa varied but were not significantly different between treatments. Mean total abundance in artificial leaf packs (6.2) was lower than the *F. sylvatica* leaf packs (13.5); although this difference was not significant (H=18.50, DF=13, p=0.14). The dominant taxon in all treatments was Chironomidae.

Table 6-5: Mean (±1 SD) macroinvertebrate community metrics for leaf litter packs from the individual compound study

Treatment	Total abundance	Taxonomic richness	Berger-Parker D (%)	Abundance *A. aquaticus*	Abundance *G. pulex*
ART	6.2 ±4.6	2.8 ± 1.6	68.5 ± 19.8	0.5 ± 0.5	0.1 ± 0.3
CON	12.2 ± 4.1	4.2 ± 0.8	63.7 ± 10.0	0.3 ± 0.5	0.2 ± 0.4
LT DIC	14.2 ± 7.6	3.2 ± 1.2	68.3 ± 16.9	0.5 ± 0.5	0.0 ± 0.0
HT DIC	11.5 ± 5.1	3.7 ± 1.4	57.2 ± 11.4	1.0 ± 1.7	0.0 ± 0.0
LT ERY	14.3 ± 9.3	3.2 ± 1.2	72.7 ± 16.4	0.7 ± 0.8	0.0 ± 0.0
HT ERY	14.0 ± 3.7	3.8 ± 0.4	64.6 ± 17.4	1.2 ± 1.3	0.4 ± 0.5
LT IBU	13.8 ± 5.3	4.0 ± 0.6	56.7 ± 11.1	1.0 ± 2.4	0.2 ± 0.4
HT IBU	17.5 ± 4.1	4.0 ± 1.8	58.1 ± 17.4	2.8 ± 3.1	0.3 ± 0.8
LT MEF	12.3 ± 9.0	4.2 ± 1.6	49.9 ± 13.5	1.5 ± 1.6	0.0 ± 0.0
HT MEF	13.3 ± 2.3	3.8 ± 1.3	70.7 ± 13.8	0.8 ± 0.8	0.0 ± 0.0
LT PRO	13.0 ± 6.3	3.7 ± 1.5	67.7 ± 19.6	0.3 ± 0.5	0.0 ± 0.0
HT PRO	13.7 ± 5.9	3.5 ± 0.5	64.2 ± 13.7	1.2 ± 1.2	0.2 ± 0.4
LT MIX	13.3 ± 6.3	4.8 ± 1.8	57.6 ± 9.8	1.5 ± 1.0	0.2 ± 0.4
HT MIX	12.5 ± 7.8	3.0 ± 1.7	79.6 ± 17.2	0.3 ± 0.5	0.3 ± 0.8

One-way ANOSIM showed no significant dissimilarity in macroinvertebrate colonisation across all treatments (R=-0.003, p=0.50) but pairwise comparisons between treatments did highlight some significant dissimilarities. In particular, HT-IBU showed significant dissimilarity when compared to CON (p=0.02), HT-MIX (p=0.01) and LT-IBU (p=0.04). A two-dimensional non-metric multidimensional scaling (NMDS) plot showed no obvious clustering of treatments**.** The calculated Kruskal's stress value was 0.156 which is indicative of a good, interpretable ordination (Rabinowitz, 1975).

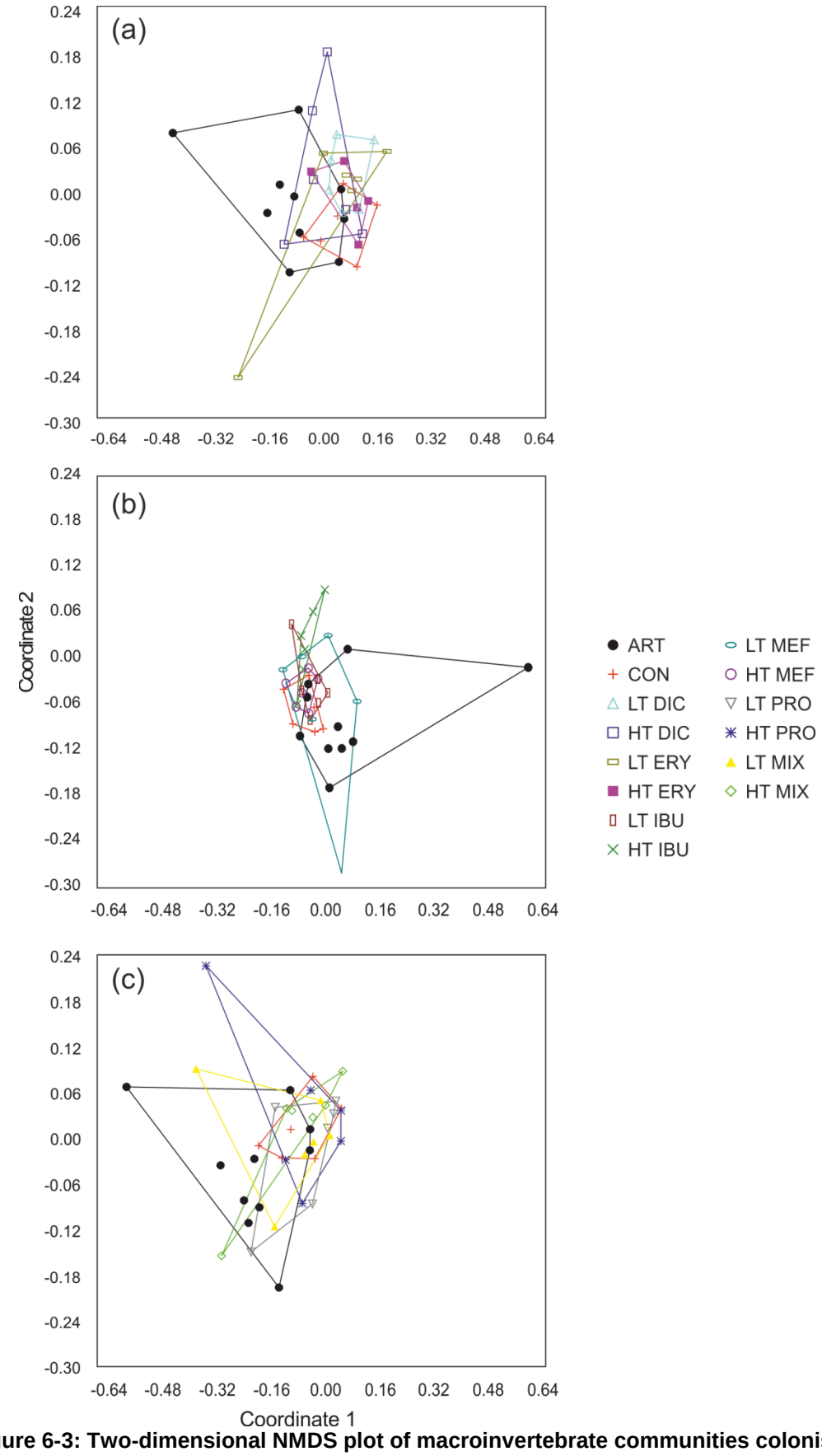


Figure 6-3: Two-dimensional NMDS plot of macroinvertebrate communities colonising real and artificial leaf packs exposed to (a) diclofenac and erythromycin, (b) ibuprofen and mefenamic acid and (c) propranolol and mixtures (treatment abbreviations in table 6-2; similarity measure = Bray-Curtis. Kruskal's stress for whole dataset = 0.156)

6.4.2 Erythromycin study

6.4.2.1 Organic matter change

There were significant differences in the AFDM change at the end of the pre-conditioning and *in situ* phases (H=53.22, DF=1, p<0.001) with an average reduction of 45 % after *in situ* compared to 14 % for the pre-conditioning phase. However, there were no significant differences between treatments in the pre-conditioning (H=9.80, DF=5, p=0.08) or *in situ* phases (H1.08, DF=5, p=0.96).

Differences in %OM between the pre-conditioning and *in situ* phases and between treatment levels showed clearly reduced organic matter content within *in situ* packs (88%) when compared to the pre-conditioning phase (90%) and this difference was significant (H=28.22, DF=1, p<0.001; Figure 6-4). There were no significant differences in %OM between treatments during pre-conditioning (H=4.89, DF=5, p=0.43) but there were significant differences after the *in situ* phase (H=12.24, DF=5, p=0.03). High treatment displayed significantly lower %OM than both the very low (W=34.0, p=0.04) and very high treatments (W=33.0, p=0.03). Low treatment exposed leaves also showed significantly lower %OM than both the very low (W=24.0, p=0.02) and very high treatments (W=21.0, p<0.01). There were no differences between control leaf packs and any of the treatments and pairwise comparison between control and the methanol solvent control indicated no difference (W=43.0, DF=1, p=0.58).

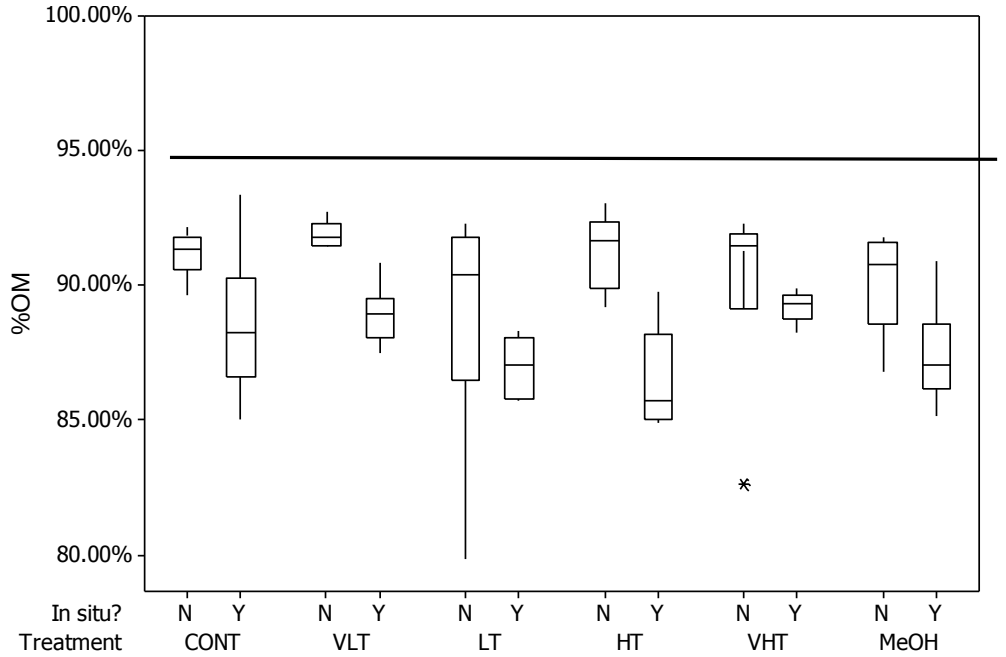


Figure 6-4: % organic matter in *F. sylvatica* leaf packs after 8 week laboratory tank and 8 week in situ stream exposure to four concentrations of erythromycin [line represents initial %OM of untreated leaf packs at 94.1%. Bars represent mean values from n=6 replicates, error bars are ±1 SD; CON: control, MeOH: methanol solvent control, VLT: very low treatment, LT: low treatment, HT: high treatment, VHT: very high treatment]

6.4.2.2 Macroinvertebrate colonisation

Colonised leaf packs were dominated mostly by *A. aquaticus* followed by *G. pulex* and Chironomidae. *A. aquaticus* was the dominant taxon in all but three of the leaf packs (Table 6-6). HT showed significantly elevated species richness when compared to CON (H=19.14, DF=5, p<0.01). Comparisons of individual taxa, Berger-Parker D and % EPT showed no significant differences between the treatments. Across all treatments there was a marginal significant difference in the abundance of *A. aquaticus* (H=10.99, DF=5, p=0.05) but not *G. pulex* (H=5.89, DF=5, p=0.32).

Table 6-6: Mean (±1 SD) macroinvertebrate community metrics for litter packs from the detailed erythromycin study

Treatment	Total abundance	Species richness	Berger-Parker D (%)	Abundance *A. aquaticus*	Abundance *G. pulex*
CON	82.5 ± 22.9	4.3 ± 0.5	69.9 ± 10.1	58.3 ± 20.9	4.2 ± 1.3
MeOH	64.7 ± 15.6	6.0 ± 0.9	48.4 ± 16.6	29.0 ± 22.8	11.7 ± 8.9
VLT	93.8 ± 34.4	5.0 ± 0.0	64.6 ± 20.8	64.7 ± 38.4	7.8 ± 6.1
LT	67.2 ± 18.1	6.0 ± 0.9	53.3 ± 6.5	31.8 ± 10.9	7.3 ± 5.9
HT	79.2 ± 56.3	6.8 ± 0.4 **	51.4 ± 15.5	47.7 ± 53.9	10.7 ± 4.8
VHT	88.7 ± 27.3	5.8 ± 1.7	67.4 ± 13.4	62.2 ± 29.9	6.5 ± 2.2

** indicates significant difference from control (p<0.01)

One-way ANOSIM (using Bray-Curtis distance) of the whole macroinvertebrate colonisation dataset confirmed statistically significant dissimilarity between treatments (R=0.10, p=0.04). Pairwise comparisons highlighted significant dissimilarity between controls and HT (R=0.49, p<0.01), LT (R= 0.19, p=0.02) and the methanol solvent control (R= 0.21, p=0.05). The difference between the control and solvent control packs was driven by *G. pulex* (control mean abundance: 4.2. vs. methanol abundance 11.2) and *A. aquaticus* (58.3 vs. 29.0). The solvent control was not significantly different to any other treatment. HT was also significantly different to VHT (R= 0.29, p=0.04). Two-dimensional NMDS showed some clustering of control packs around the origin with the majority of low and high treatment packs different to these (Figure 6-5) confirming the dissimilarities found by ANOSIM. The calculated Kruskal's stress value was 0.11 indicating a useful ordination.

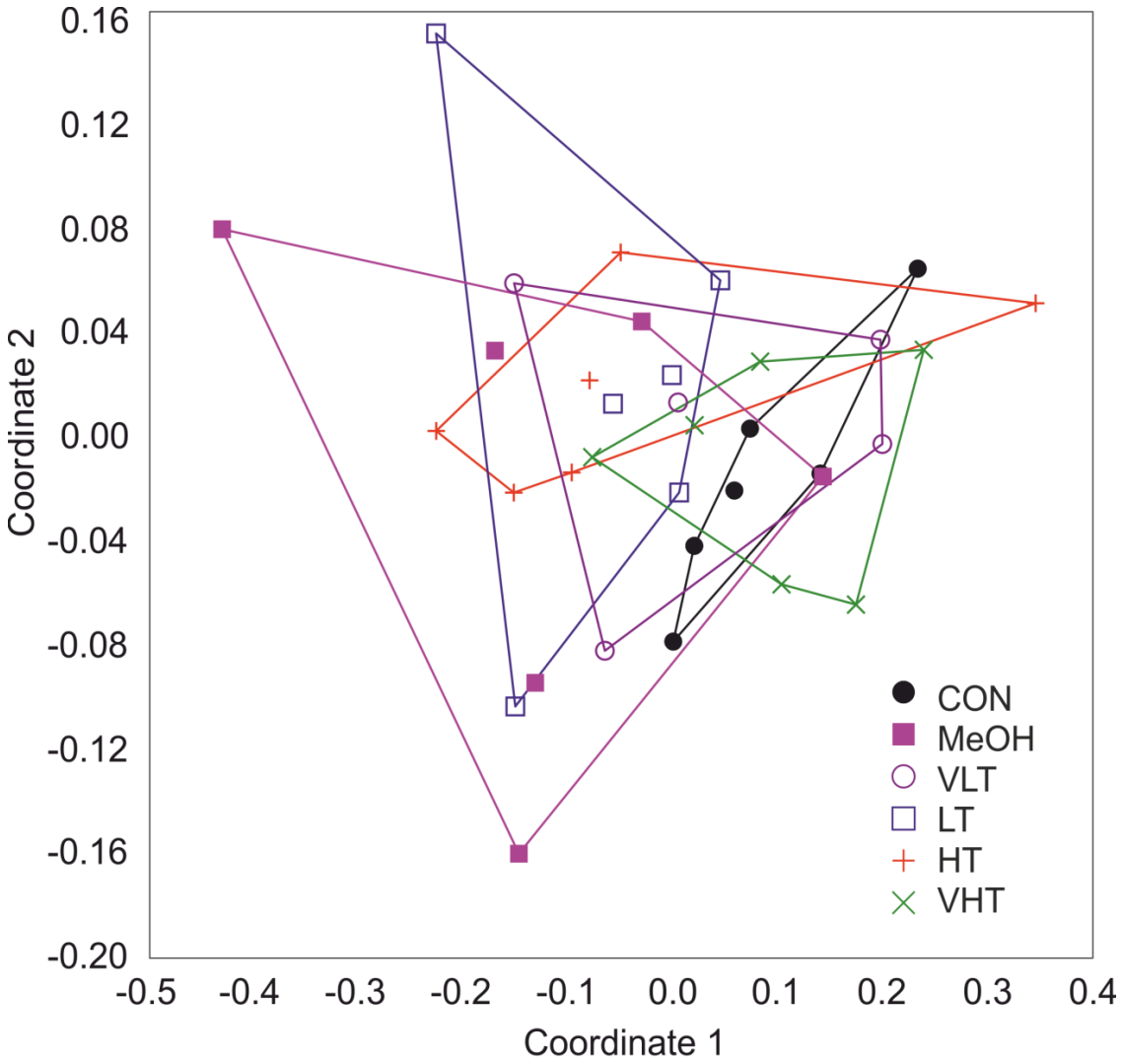


Figure 6-5: Two-dimensional NMDS plot of macroinvertebrate communities colonising leaf packs exposed to four treatment levels of erythromycin (for treatment abbreviations see Table 6-3; similarity measure = Bray-Curtis; Kruskal's stress for whole dataset = 0.1142)

6.5 Discussion

Pharmaceuticals are a widespread pollutant found in soils, groundwater and surface waters across the globe (Daughton & Ruhoy, 2009b; Hughes *et al.*, 2013). Existing research on their effects in freshwater ecosystems indicates a low risk of toxicity but the full implications of long-term, low-level exposure are poorly understood (Fent *et al.*, 2006; Kümmerer, 2008a; Kümmerer, 2009a; Santos *et al.*, 2010). The margin of safety for long-term or chronic effects is much smaller than that for short-term effects and in some cases environmental concentrations are known to exceed those necessary to cause negative effects (Hughes *et al.*, 2013). However, the use of structural and functional indicators to quantify the effects of pharmaceutical exposure is extremely limited although the small amount of research already conducted has highlighted their usefulness and sensitivity in detecting such effects.

6.5.1 Leaf litter decomposition

The loss of organic matter was generally low in both the laboratory phase (< 5 % decrease) and the *in situ* phase (< 10 % decrease). The similarity in mass loss between the two phases confirms the large contribution that microbial communities make to total decomposition rates (Irons *et al.*, 1994). Decomposer microbial communities have been shown to grow actively on leaf litter even at temperatures close to freezing and so they are likely to have played a major role during *in situ* decomposition in both the individual compound and detailed erythromycin studies (Barlocher & Kendrick, 1974). Therefore, the differences in decomposition rates between the two experiments in this study is probably due to the much lower abundance of colonising macroinvertebrates (particularly *A. aquaticus* and *G. pulex*) during the autumn-winter exposure of individual compound study packs. The results also indicate consistently higher abundances in leaf packs when compared to artificial packs; this suggests that macroinvertebrates (particularly shredders) are preferentially colonising the *F. sylvatica* packs for their food value above and beyond any value as merely a microhabitat (Richardson, 1992).

6.5.2 Impact of individual compounds on leaf litter decomposition

When the five compounds were tested in isolation at environmentally relevant concentrations no significant differences in leaf litter decomposition (whether mediated by microbes or macroinvertebrates) or macroinvertebrate colonisation were found. Therefore hypotheses $\mathbf{H_1}$ and $\mathbf{H_2}$ were rejected. This was the first study to examine such effects of pharmaceuticals other than antibiotics on this important ecosystem process and these results suggest that low levels of the study compounds do not pose a risk to leaf litter decomposition in freshwater ecosystems. However, decomposition rates in this study were generally low both in the pre-conditioning and *in situ* phases. The results further confirm the contribution that microbial communities make to total decomposition (14 % mass change in 4 weeks microbial cf. 20 % changes in 8 weeks microbial and macroinvertebrate; (Irons *et al.*, 1994)). The low breakdown rates may be partly explained by the use of *F. sylvatica* leaves which are known to be one of the slowest degrading leaf species in temperate streams (Cummins *et al.*, 1973; Petersen & Cummins, 1974). Furthermore, the timing of *in situ* exposure (October to December) will contribute to slower decomposition rates as water temperature is known to be a major controlling factor on this process (Petersen & Cummins, 1974). Seasonal cycles in macroinvertebrate density are well established in temperate streams not prone to disturbance, with peaks often present in summer and autumn due to the domination of particular taxa (Hynes, 1970). Associated cycles in ecosystem functioning have also been observed (Stoneburner & Smock, 1979; Mulholland & Lenat, 1992) which may mean that leaf litter decomposition (alongside other ecosystem functions) may be more

or less sensitive to pharmaceutical exposure at different times of the year. Repeated studies using a variety of different leaf species and at varying times throughout the year are needed to determine whether there are impacts in the sensitivity of macroinvertebrate and microbial communities across the seasons.

Colonisation of leaf packs in this study highlighted some differences between pharmaceutical exposed packs and controls, particularly those exposed to higher treatments of erythromycin and ibuprofen. Therefore hypothesis **H_3** was partially accepted. The differences appear to have been driven by elevated levels of the shredder *A. aquaticus* as well as Oligochaeta sp. in ibuprofen treated leaf packs. This broadly agrees with the preferential selection by shredders of antibiotic exposed leaf discs demonstrated previously in laboratory studies (Bundschuh *et al.*, 2009). *G. pulex* in this study showed a tendency to select leaf packs exposed to higher levels of erythromycin and ibuprofen but the tendency was not as strong as for *A. aquaticus*. *Gammarus* species are selective consumers capable of differentiating between conditioned and unconditioned leaves of various sources (Barlocher & Kendrick, 1975; Arsuffi & Suberkropp, 1989; Graça *et al.*, 2001). This muted response by *G. pulex* could perhaps be explained by their demonstrated preference of other leaf species over beech (*F. sylvatica*); Friberg & Jacobsen (1994) showed that *Fagus* leaves were the least preferred by *Gammarus* in a laboratory study. However, in this study the choice of *F. sylvatica* leaf material was justified given this species dominance in the riparian vegetation community at Silsden Beck. Furthermore, there were some differences in macroinvertebrate communities between the controls and solvent controls which may have masked any response in the pharmaceutical treatments. Further experiments may be necessary utilising exposure techniques which do not require carrier solvents such as methanol (see Dietrich *et al*., 2010 and Chapter 7).

Studies examining the effects of the non-antibiotic compounds (diclofenac, ibuprofen, mefenamic acid and propranolol) on food selection by macroinvertebrates and leaf litter decomposition are not available but some work has been carried out on other aspects of ecosystem structure and functioning. Brain *et al.* (2004a) demonstrated small impacts on the growth and pigmentation of two aquatic plant species caused by a mixture of eight pharmaceuticals over 7 and 35 days exposures. However, lowest observable effects concentrations were relatively high (>30 µg L^{-1}) compared with the concentrations tested during this study and the changes in growth were unlikely to be ecologically significant. An examination of 25 different pharmaceuticals (including ibuprofen) was performed using still water mesocosms (Brain *et al.*, 2004b) which found that a concentration greater than 1 mg L^{-1} of ibuprofen was necessary to affect the growth and pigmentation of *Lemna gibba*. This agrees with the finding here which indicated a concentration greater than 5 mg L^{-1} was necessary to affect leaf litter decomposition.

However, the margin of safety is low when compared to the maximum concentration of 0.31 mg L^{-1} detected in a Turkish river (Loos *et al.*, 2009; Hughes *et al.*, 2013). The results presented here and elsewhere indicate that environmentally realistic concentrations of diclofenac, ibuprofen, mefenamic acid and propranolol are unlikely to pose a risk to leaf litter decomposition in freshwater ecosystems. However, much more research is necessary which examines pharmaceutical effects on ecosystem functioning using relevant exposure conditions. Furthermore, margins of safety may be significantly reduced for the more widely used painkillers (namely diclofenac and ibuprofen) which have been identified in freshwaters at very high concentrations (up to 18 740 and 31 323 ng L^{-1} respectively; (Hughes *et al.*, 2013)).

6.5.3 Impact of erythromycin on leaf litter decomposition

The results presented here indicate that long-term exposure to environmentally realistic concentrations of erythromycin acting in isolation is unlikely to affect the decomposition of beech leaf litter in freshwater ecosystems. However, erythromycin is most likely to be present in the environment as part of a complex, dynamic mixture of pharmaceuticals and other contaminants (Fent, 2008) and as such may still pose a risk to leaf litter decomposition.

This was the first study to examine the effects of erythromycin on leaf decomposition in isolated detail using *in situ* techniques. Two other studies are available which examine the effects of antibiotic mixtures on food selection and decomposition rates of macroinvertebrates and microbial communities although both were conducted solely in the laboratory (Hahn & Schulz, 2007; Bundschuh *et al.*, 2009). A mixture of five antibiotics (erythromycin, clarithromycin, roxithromycin, sulfamethoxazole and trimethoprim; ≥2000 ng L^{-1}) promoted colonisation by macroinvertebrates and microbial communities and increased decomposition rates (Bundschuh *et al.*, 2009). This agrees with the results presented here which show that the shredder *A. aquaticus* actively preferred leaves pre-conditioned in the presence of erythromycin (100 000 ng L^{-1}). However, the magnitude of these differences was low and did not manifest in changes at the functional level. Direct comparisons between experiments must be performed with caution as differences in exposure conditions and leaf or macroinvertebrate species used may have a significant impact on end results. For example, exposure of a different gammarid species (*Gammarus fossarum*) to an alternative antibiotic mixture (oxytetracycline and sulfadiazine) showed opposite effects on food selection and no overall effect on decomposition rates (Hahn & Schulz, 2007). More work is necessary which combines parallel exposures of different leaf and macroinvertebrate species to a range of antibiotic compounds and mixtures if reliable comparisons of specific compound toxicity are to be made.

Brain *et al*. (2005) examined the effects of a mixture of antibiotics at similar concentrations to those tested here (oxytetracycline, chlortetracycline, tetracycline and doxycycline; ≥9000 ng L^{-1} for 35 days). Significant reductions in somatic endpoints were found at concentrations less than 9000 ng L^{-1} although one species (*Myriophyllum sibiricum*) was up to 57 times more sensitive than *L. gibba*. Long-term exposure (30 days) to erythromycin in mixture with other antibiotics caused some alterations to the sex ratio of *D. magna* at concentrations ≥10 000 ng L^{-1}. Notably, these previous studies did not conduct parallel exposures to individual compounds so it is not yet impossible to say what contribution erythromycin had on the overall effects seen. Furthermore the differences in sensitivity highlight the importance of using appropriately sensitive species and indices during ecotoxicological testing.

6.5.4 Impact of mixtures on leaf litter decomposition

Pharmaceuticals are not present in rivers in isolation but rather alongside myriad other chemicals and pollutants that form complex and dynamic mixtures (Fent, 2008). Most studies have examined the effects of mixtures on individual organisms (macroinvertebrates and aquatic plants) with compounds generally demonstrating concentration addition (CA). This is crucial as it allows a mixture to exert toxic effects below the threshold required from individual compounds (Cleuvers, 2003; Cleuvers, 2004; Escher *et al.*, 2005). Despite this, only a limited number of studies have addressed the issue of pharmaceutical mixtures and to the best of the author's knowledge none have examined the effects of non-antibiotic pharmaceutical mixtures on leaf litter decomposition.

Antibiotic mixtures have been shown to decrease leaf litter decomposition and alter food selection by invertebrates (Hahn & Schulz, 2007; Bundschuh *et al.*, 2009) at concentrations similar to those tested here. Cleuvers (2003) showed that diclofenac and ibuprofen acting in combination elicited much stronger effects on the immobilisation of *D. magna* and growth of the chlorophyte *Desmodesmus subspicatus* than when these compounds were tested in isolation. A further analysis of a mixture of four non-steroidal anti-inflammatory drugs (including diclofenac and ibuprofen) confirmed concentration addition (Cleuvers, 2004). However, these studies were conducted at much higher concentrations (≥10 mg L^{-1}) than those tested here and so their environmental relevance is limited. Dietrich *et al.* (2010) found that a mixture of four pharmaceuticals (carbamazepine, diclofenac, metoprolol and 17α-ethinylestradiol (EE2)) at environmentally relevant concentrations (up to 1200 ng L^{-1} for pharmaceuticals and 0.1 ng L^{-1} for EE2) disrupted the moulting behaviour of *G. fossarum*. A similar study exposed freshwater snails (*Lymnaea stagnalis*) to a total mixture of 16 compounds of varying classes (antibiotics, cardiovascular, antihypertensive and psychiatric) for three days at

relevant concentrations (10 to 500 ng L^{-1}) showed the potential for complex mixtures to disrupt their immunocompetence to a similar extent to that caused by exposure to treated sewage effluent (Gust *et al.*, 2013). Other studies have demonstrated lethal and sublethal effects of simple and complex pharmaceutical mixtures on aquatic mesocosms although generally at very high concentrations (Brain *et al.*, 2004a; Brain *et al.*, 2004b; Brain *et al.*, 2005; Flaherty & Dodson, 2005; Laird *et al.*, 2007).

This and other studies represent the first vital steps in improving our understanding of the complex effects of pharmaceutical mixtures on aquatic organisms. However, further work needs to be carried out which combines the use of functionally important organisms (e.g. *Gammarus* spp.) with environmentally relevant concentrations and sufficiently long exposure periods to maximise the environmental realism of such studies. Furthermore, many of the above studies did not conduct parallel exposures of mixtures and individual compounds which limited their ability to quantify the contribution each pharmaceutical to overall mixture toxicity. The simple mixture tested in this study represents an important early step in such research although actual environmental mixtures are much more complex perhaps containing thousands of contaminants from a range of sources (Fent, 2008).

Although the data presented here did not indicate significant effects of the tested mixture on leaf litter decomposition or microbial and macroinvertebrate colonisation other studies have demonstrated lethal and sublethal effects of pharmaceutical mixtures at concentrations below those caused by individual compounds. The probable reason for other researchers identifying significant effects was their use of relatively simple organismal and sub-organismal endpoints (e.g. growth, mortality, motility etc.) compared to the inherently complex structural and functional endpoints employed in this study which can display higher degrees of variability (Hill *et al.*, 2000). However, structural and functional endpoints have distinct advantages both in terms of their ecological relevance and their integration an ecosystems response over time and space (Gessner & Chauvet, 2002). Despite the lack of effects demonstrated in this study, research elsewhere indicates that further studies into the effects of pharmaceuticals on leaf litter decomposition are necessary.

6.5.5 Future research

Due to analytical and cost constraints this study was unable to quantify the composition or change in the microbial and fungal communities colonising the leaf packs which is of particular importance in the degradation of leaf material in streams (Webster and Benfield 1986). As such, any further studies should attempt to estimate bacterial abundance using epifluorescence microscopy (Buesing, 2005) and fungal biomass using the indicator ergosterol (Mille-Lindblom *et al.*, 2004). This is particularly important as

bacterial communities have been shown to develop tolerance against certain antibiotics over relatively short exposure periods (Schmitt *et al.*, 2005). Furthermore, competitive release of fungal communities due to inhibition of bacteria by antibiotic compounds can have implications for food selection and decomposition by macroinvertebrates (Gulis & Suberkropp, 2003b). Quantification of fungal and microbial communities will help in understanding the importance of these interactions on overall decomposition rates. One promising area of research is metagenomics which allows the rapid characterisation of microbial diversity in environmental samples. This work has already been applied to characterise the diversity and functional roles of microbial communities in large rivers (Ghai *et al.*, 2011) and in understanding the effects of pollutants of microbial communities (George *et al.*, 2010).

6.6 Conclusions

The results presented here indicate the potential for environmentally realistic concentrations of pharmaceuticals to alter the colonisation of leaf litter by macroinvertebrates in temperate, low-order streams. However, no effects were observed due to exposure of the other study compounds nor were any significant or ecologically relevant effects seen on leaf litter decomposition rates. This suggests that low levels of these five pharmaceuticals do not pose a risk to leaf litter decomposition. However, there remain significant knowledge gaps in understanding the effects of pharmaceuticals at low-levels over extended periods and as part of complex mixtures on this and other important aspects of freshwater ecosystem functioning. Future assessments of ecosystem health and their response to stressors such as pharmaceuticals should utilise both established structural indices as well as functional measures if a full understanding of the risk pharmaceuticals pose in freshwaters is to be obtained. This is of particular importance as any disruptions to leaf litter degradation, sediment metabolism and nutrient cycling have the potential to cascade throughout the ecosystem causing additional indirect effects at higher trophic levels and downstream (Wallace *et al.*, 1997).

The slowing in improvement of biological water quality indices in UK rivers (EA, 2012) may in part be due to the structural and functional disruption caused by pharmaceuticals (alongside the myriad other emerging contaminants and aquatic pollutants). This is of concern to policy makers, catchment managers and the water industry across the UK and Europe if rivers are to meet the requirements of 'good ecological status' mandated by the European Water Framework Directive (EC, 2000). The focus of water quality interventions may be changing in the future with the proposed inclusion of diclofenac (alongside 47 other chemicals) on the list of Hazardous Substances (EC, 2012). This could mean that increased attention is paid to these

compounds by regulators and water managers in the coming years but should be seen as an opportunity to broaden the research and policy focus to the thousands of other emerging contaminants that are present within rivers.

7 THE IMPACT OF PHARMACEUTICALS ON RESPIRATION AND NUTRIENT DYNAMICS IN RIVER SEDIMENTS

7.1 Introduction

The integrity of any ecosystem is measured by assessing both structural and functional components which are intimately linked. Any assessment of ecosystem integrity or attempt to understand the impact of anthropogenic stressors must consider both of these aspects (Matthews *et al.*, 1982; Bunn *et al.*, 1999; Gessner & Chauvet, 2002). Functional indices have numerous advantages over their structural counterparts such as being integrative over space and time and not being reliant on the presence of specific species sets yet despite these advantages, bioassessment is overwhelmingly based on structural measures alone (Matthews *et al.*, 1982; Gessner & Chauvet, 2002). In particular, the application of functional endpoints in studying the effects of emerging contaminants (including pharmaceuticals) is not widespread and most studies focus on single-species examinations of toxicity which fail to draw upon the well-developed tool kit of methods available in aquatic ecology (Rosi-Marshall & Royer, 2012). Community respiration (CR) and sediment respiration (SR) in particular have been shown to be highly sensitive to a range of environmental stressors (Hill *et al.*, 1998; Hill *et al.*, 2000). These functional endpoints, along with nutrient cycling in freshwater sediment systems show promise as indicators of ecosystem stress caused by emerging contaminants. Despite this, the application of these indicators to pharmaceutical pollution in rivers is sparse and the potential effects for freshwater ecosystem functioning are poorly understood.

Stream sediments are biologically active zones which modify the chemistry of water passing through them, acting as both sinks and sources of nutrients from both upwelling groundwater and infiltrating stream water (Rutherford & Hynes, 1987). Processes within stream sediments can increase overall ecosystem efficiency by allowing more rapid recycling of nutrients that would otherwise be lost to downstream transport (Battin, 1999; Battin *et al.*, 2003). The majority of metabolic activity in small streams occurs within bed sediments, either at the sediment-water interface or in the deeper pore waters where there is a hyporheic zone (Sobczak & Findlay, 2002). Therefore, the microbial processes within sediments play a major role in the cycling of both carbon and other nutrients in rivers with potential wider catchment impacts manifested through changes in the downstream fluxes of dissolved organic carbon (DOC) (Rutherford & Hynes, 1987). Carbon supplied to stream sediments may come in the form of coarse or fine particulate organic carbon (POC) and the direct transport of DOC from stream or groundwater (Findlay, 1995). POC can be directly utilised by invertebrates as a food source but this is not often the case for DOC where some form of microbial transformation into a particulate form is necessary. This DOC represents

a major energy source for bacterial communities in freshwater sediments (Miller, 1987). These communities are responsible for significant energy fluxes and nutrient cycling both within the hyporheic zone and at the sediment/water interface (Giller & Malmqvist, 1998) and any disruptions to these communities by emerging contaminants may have significant impacts on ecosystem-level processes.

The relatively slow velocity of pore waters can allow ample time for the microbial metabolism of bioavailable solutes. Indeed, Sobczak & Findlay (2002) have shown that DOC losses along hyporheic flow paths can be as high as 50 %. This depends predominantly on microbial metabolism and less so on other parameters such as dilution, residence time and initial DOC concentrations. Biotic uptake, predominantly at the sediment-water interface, is considered to be the dominant removal mechanism for DOC in streams (Dahm, 1981; Findlay & Sobczak, 1996; Tank *et al.*, 2010). The fundamental importance of sediment microbial communities in retaining DOC and rendering otherwise refractory carbon available to higher trophic levels (via its incorporation in bacterial biomass) is known as the 'microbial loop' and is well recognised both in marine (Fenchel, 2008) and freshwater systems (Meyer, 1994). Rosenfeld & Roff (1991) have shown that, alongside their role in the microbial loop, respiration by benthic microbial communities (particularly bacteria) can exceed whole stream primary production in forested streams. This is a relatively common phenomenon, where ER exceeds gross primary production (GPP) resulting in a net heterotrophic stream which consumes more organic carbon than it produces (Mulholland *et al.*, 2001). Smith (1973) estimated that bacteria were responsible for the majority of sediment respiration (accounting for 30 to 60 %) whereas macrofaunal respiration accounted for just 5 to 26 %. Therefore any change in benthic respiration caused by disruption or inhibition of sediment microbial communities has the potential to significantly alter the stream production: respiration (P/R) ratio (Wilson *et al.*, 2004).

The disruption of microbial cycling in sediments can cause the sediment to become a carbon sink rendering nutrients unavailable for secondary productivity which will have knock-on effects via food web interactions (Burton, 1991). The communities present within sediment pore waters (particularly microorganisms) therefore play a major role in both the respiration of organic matter in sediments and nutrient cycling. Therefore, pharmaceuticals (particularly those designed to target microorganisms such as antibiotics and antifungals) have the potential to alter or disrupt these sediment dwelling organisms which in turn may impact on the important ecosystem functions they perform (Likens, 2004). However, studies examining the effects of pharmaceuticals on the respiration and nutrient cycling of freshwater sediments are sparse and there is a pressing need for further research in this area if potential disruption to the functioning of freshwater ecosystems is to be understood.

Studies comparing CR to other functional attributes in stream ecosystems have shown it is the most sensitive to a range of environmental stressors such as pH, heavy metals and organic chemicals (Matthews *et al.*, 1982). For example, Crossey *et al. (*1988*)* showed that CR was significantly increased (by a factor of 2-3) in a Montana stream experiencing chronic heavy metal pollution but structural indices did not show any difference between control and impact sites. Bunn *et al*. (1999) found that both GPP and CR were sensitive to changes in catchment land use. As the above studies indicate, the use of CR or similar endpoints as indicators of stress caused by 'traditional' stressors is relatively widespread but their use in examining the effects of pharmaceutical pollution is much more limited.

Bunch & Burnot (2011) examined the effects of ibuprofen and paracetamol at environmentally realistic concentrations (100 to 10 000 ng L^{-1}) on sediment respiration and nitrate uptake using both *in vitro* and *in situ* techniques. The *in vitro* exposures demonstrated increased respiration rates at low levels of both pharmaceuticals and a slight inhibition at the highest exposure to paracetamol. Furthermore, lower levels of paracetamol appeared to enhance nitrate uptake. None of the above differences were evident in the more variable *in situ* experiments although this may be due to the long exposure history to pharmaceuticals at the site yielding greater adaptation and tolerance in the microbial community (Bunch & Burnot, 2011). The picture is further complicated as different pharmaceutical compounds may be stimulatory (due to the uptake of pharmaceuticals as a nutritive source) or inhibitory (due to direct toxic effects), although this is dependent on compound-specific properties and can change depending on exposure concentrations (Lawrence *et al.*, 2005). These few studies indicate the potential for low levels of pharmaceutical compounds to have a significant impact on stream microbial activity and the cycling of nutrients which may have further implications for autotrophic production and algal blooms (Dodds & Welch, 2000). Therefore, further research is required to fully understand the effects of environmentally relevant concentrations of a broader range of pharmaceutical compounds on processes within freshwater sediments. Significant knowledge gaps remain, particularly in testing the effects of pharmaceutical classes other than antibiotics (e.g. painkillers, antidepressants, cardiovascular drugs and cancer treatments). Furthermore, the majority of existing studies have examined pharmaceutical compounds in isolation and have not addressed the issue of complex, dynamic mixtures of such substances (Backhaus, 2008). Mixture toxicity is a key concern in pharmaceutical pollution research as many compounds act additively in combination allowing them to demonstrate toxic effects at levels well below those shown for individual compounds (e.g. Cleuvers, 2003).

7.2 Aims and hypotheses

The aim of this study was to quantify the effects of low-level extended exposure to two pharmaceutical compounds (erythromycin and propranolol; both in isolation and as part of a mixture) on respiration and nutrient cycling in freshwater sediments. Specific objectives were:

- Measure the effects of erythromycin and propranolol on the degradation of organic matter in artificial freshwater sediments over a 7 day period;
- Evaluate the effects of the same pharmaceuticals on the respiration rates of freshwater sediments;
- Quantify the effects of pharmaceuticals on the exchange of dissolved carbon, nitrogen and phosphorous between surface sediments and overlying waters;
- Quantify the effects of the binary mixture of erythromycin and propranolol on the above processes.

The following hypotheses were tested: (**H_1**) pharmaceuticals (particularly antibiotics) disrupt the functioning of bacterial communities living in sediments, resulting in a reduction of the processing of organic matter (**H_2**). This disruption will also induce a reduction of microbial community respiration rates (**H_3**). Changes to microbial respiration in sediments will in turn reduce the microbial cycling of dissolved carbon, nitrogen and phosphorous and subsequently their exchange with the overlying water column (**H_4**). The effects will be more pronounced when sediments are exposed to a mixture as opposed to pharmaceutical compounds in isolation due to additive effects.

7.3 Methodology

7.3.1 Chemicals and materials

All glassware and vessels were thoroughly disinfected and pre-rinsed with 100 % methanol and deionised water prior to each use. HPLC-grade methanol was supplied by Fisher Scientific UK Limited (Loughborough, UK). Deionised water was supplied by a Purite Select HP160/BP/IT deioniser. All pharmaceuticals were of the highest purity available (>99%) and supplied by Sigma-Aldrich Company Ltd (Dorset, UK). Individual stock standard solutions were prepared on a weight basis in 100 mL of 100 % methanol and stored in the dark at -20 °C until used. A fresh working mixture solution of all pharmaceuticals was prepared by appropriate dilution of the individual stocks in 100 % methanol immediately before each experimental run and used as working standard solutions. Working standards were also stored in the dark at -20 °C between uses.

7.3.2 Preparation of sediment

The site chosen for the collection of natural sediment and pre-conditioning of artificial sediment was Silsden Beck, immediately downstream of Silsden Reservoir, Silsden, West Yorkshire (OSNGR SE 0446147504). An initial survey of Silsden Beck showed that the depth and quantity of sediment in this stretch was insufficient for experimental use. However, this site was retained due to its situation upstream of any STP effluent inputs, thereby reducing the potential for contamination by the human pharmaceutical study compounds. Furthermore, the regulated nature of the stream (i.e. directly below Silsden Reservoir) made it ideal for *in situ* studies. A preliminary analysis of a 1 L water grab sample from Silsden Beck by HPLC-MS/MS indicated that all five of the preliminary study compounds were below detection limits (<5 ng L^{-1}).

25 kg sacks of gravel (<10 mm grain size) and sand (average grain size 0.2 mm) were purchased and leached thoroughly in deionised water for seven days (water was replaced daily), to minimise any potential contaminants. After leaching, the sand and gravel were placed in hessian bags and anchored on the Silsden Beck stream bed for a period of 21 days to allow microbial communities to develop similar to previous studies (Hill & Perrotte, 1995). Slower flowing areas were chosen for placement of the sediments (for example in deeper pools, inside edges of meanders and downstream of large boulders) where most deposition was likely to occur and hydraulic forces were less likely to dislodge the sacks. A Tinytag temperature logger was secured alongside the hessian sacks which recorded temperature at 15-minute intervals. After retrieval, the sediment was transported immediately to the laboratory in stream water to be used in the experiments.

Six samples of streambed sediment were collected from random locations within the stream for quantification of organic matter content. The top 2-3 cm of natural sediment were sampled as this represents the biologically and hydrodynamically active portion (Burton, 1991). These samples were then composited to homogenise the spatial variability of sediment composition across the site and obtain a more representative sample of mean conditions. The composite sample was sieved (Shelton & Capel, 1994) and washed into clean plastic bottles using stream water. Any coarse particulate organic matter (CPOM) or macroinvertebrates were removed. Natural and artificial sediment samples were transported back to the laboratory and stored in the dark at 4°C. Each time artificial and natural sediment were collected, subsamples (n=3) of each size fraction were taken, dried and ashed to calculate % organic matter (%OM) and ash free dry mass (AFDM) following the method outlined in Hill *et al.* (1998). This was done in order to compare %OM between the natural and artificial sediments.

Artificial sediments were homogenised to reduce any variations in colonisation by microbial communities between the outer and inner areas of the sacks. 350 g (± 1.24

g) each of sand and gravel was then added to a 0.97 L clear glass flask and filled with stream water. Flasks were aerated using air pumps (Hailea ACO-9602 air pumps, Guangdong Hailea Group Co. Ltd., Guangdong, China) and kept at 7.0 °C for seven days with a light: dark photoperiod of 12:12hrs to equilibrate the sediment samples and maintain dissolved oxygen concentrations at saturation.

7.3.3 Experimental design

Each experiment used pre-conditioned sand and gravel to simulate natural river sediment. Artificial sediment was used in order to minimise inter-experimental differences in sediment composition and organic matter content; the preparation of this material is detailed in Section 7.3.2. Four concentrations each of erythromycin, propranolol and their equivalent binary mixture were employed during a seven day static non-renewal test in order to measure changes in respiration rate, sediment organic matter content and dissolved nutrient concentrations (Table 7-1). The exposure concentrations ranged from environmentally realistic concentrations (based on published UK maximum river concentrations) to much higher concentrations (2 orders of magnitude greater than the UK maxima) intended to simulate a worst-case exposure. The experimental apparatus consisted of 0.97 L clear glass flasks containing 700 g (±5.9 g) of artificial sediment overlain by river water obtained from Silsden Beck (see Chapter 6 for site details). Each compound was tested in turn in a self-contained experimental run which consisted of four pharmaceutical treatments plus one sediment control and one water-only control, each with six replicates (n=36). The experiment was conducted three times in succession, once each for erythromycin, propranolol and the mixture because space and equipment restraints meant it was not possible to conduct experimental runs simultaneously. Due to the sequential experimental runs it was necessary to standardise against control values in order to make comparisons between runs. Total exposure period for all runs was seven days and was conducted in the dark at a mean temperature of 7.0 °C (±0.6 °C).

These study compounds and exposure levels were chosen based on previously published environmental monitoring and risk assessment studies which highlighted their potential to cause negative effects in freshwater systems (Jacobsen & Berglind, 1988; Jones *et al.*, 2002; Ashton *et al.*, 2004; Thompson RPM, 2006; Yamamoto *et al.*, 2009). Furthermore, experiments have indicated their potential to readily sorb and persist in sediment systems and alter the structure of associated bacterial communities (Kim & Cerniglia, 2005; Jones *et al.*, 2006; Ramil *et al.*, 2009).

Table 7-1: Exposure concentrations for sediment metabolism experiment

Compound	Concentration (ng L^{-1})			
	VLT	LT1	HT	VHT
Control (CON)	n/a	n/a	n/a	n/a
Native-water only control (H2O)	n/a	n/a	n/a	n/a

Erythromycin (ERY)	100	1000	100000	1000000
Propranolol (PRO)	20	200	20000	200000
Mixture (MIX)	100 + 20	1000 + 200	100000 + 20000	1000000 + 200000

Notes:
VLT = very low treatment (LT / 10)
LT = low treatment (UK river max)
HT = high treatment (LT * 100)
VHT = very high treatment (LT * 1000)

7.3.4 Treatment with pharmaceuticals

After the period of equilibration a static non-renewal exposure of the overlying water to the target pharmaceutical compounds was conducted over a period of seven days across a range of four environmentally realistic and higher concentrations (Table 7-1). Exposure was achieved by pipetting the appropriate amount of the relevant pharmaceutical working standard solution into 5 mL glass beaker and allowing the methanol to evaporate. Given the very low vapour pressures of the crystalline form of both pharmaceutical compounds, the loss of the compounds to evaporation was deemed to be negligible. This glass beaker was placed into the sediment flask and the overlying water thoroughly mixed. The advantage of this exposure technique is it removes the need for a carrier solvent such as methanol which could be a confounding effect and would otherwise require a solvent control (Dietrich *et al.*, 2010). Empty glass beakers were placed into control flasks.

After treatment with pharmaceuticals, the flasks were filled completely and PTFE lids fitted leaving no air-filled headspace in order to minimise the effect of oxygen diffusion through the free water surface. Flasks were placed in the dark and incubated at a temperature of 7.1 °C (±0.3). Measurements of dissolved oxygen (DO), water temperature, pH and electrical conductivity (EC) were taken twice daily for seven days using an Intellical LDO probe and HACH HQ30d multi-meter (HACH Company, CO, USA). The LDO probe calibration was checked with a 0 % DO solution (1 % sodium sulphite in de-ionised water, m/v) and any minor corrections required (max. ±0.03 mg L^{-1}) were performed manually. 50 mL water column samples were taken immediately following treatment and at exposure termination for analysis of DOC and nutrients. Subsamples of the surface layer (top 2-3 cm) of artificial sediment were taken immediately prior to and after 7 days for calculation of %OM and AFDM.

7.3.5 Analysis of dissolved organic carbon, nitrogen and phosphorous

All water samples were filtered through a 0.45 µm membrane and analysed within 12 hours; filtration was performed to ensure that results represent the dissolved phase only. Subsamples for carbon analysis were placed into 1.5 mL capped glass vials and analysed on an Analytik Jena Multi N/C 2100 measuring carbon by the differential method (Fukushima *et al.*, 1996) with a detection limit of 5.95 mg L^{-1}. 12 mL subsamples

for ammonium (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N) and phosphate (PO_4-P) were placed in test tubes for colorimetric analysis with a four-channel Skalar SAN++ autoanalyser. Detection limits for ammonium, nitrate, nitrite and phosphate were 0.015, 0.270, 0.017 and 0.015 mg L^{-1} respectively. Dissolved nitrate was calculated as the difference between total N and nitrite concentrations.

7.3.6 Statistical analysis

All univariate and multivariate analyses were conducted in Minitab 16 (Minitab Inc., PA., USA). All data were tested for normality using the Anderson-Darling test and then selecting the most powerful appropriate parametric (t-test or ANOVA) or non-parametric (Mann-Whitney, Kruskal-Wallis or Generalised Linear Model) statistical tests. All proportional data were arcsine transformed prior to comparisons in order to satisfy the assumption of normal distribution. Pairwise tests were corrected for multiple comparisons using a Tukey's family error rate of 5 % or using Dunnett's test to compare against controls where appropriate.

Mean OM retention from each treatment was tested with linear regression vs. $\log_{10}$ exposure concentration. Comparisons of OM retention between experimental runs was complicated by the differing levels of retention in each experimental control. Therefore, it was necessary to standardise for this variation. This was achieved by expressing OM retention as a proportion of each individual experimental control. This standardisation was also necessary for experimental comparisons of nutrient exchange as initial nutrient conditions appeared to vary between experimental runs.

Generalised Linear Models were used to compare differences in respiration rates against treatment and timestep in the form: respiration rate vs. treatment, time (treatment*time). The final term in this model evaluates the degree of interaction between the treatment and time within the statistical mode. Following this, non-parametric Kruskal-Wallis H tests were used to compare differences between treatments at individual timesteps.

7.4 Results

7.4.1 Characteristics of sediment

The organic content of the artificially colonised sediment was substantially lower than natural in-stream sediments of all size fractions (Table 7-2). The artificial 10mm gravel contained 6.4 % of the organic matter content of the nearest size fraction (natural >2mm) and artificial sand contained 12.1 % of the organic content of the equivalent 0.15 mm natural sediment. Although organic content was significantly lower in the artificial sediment, it was deemed sufficient for experimental purposes and was greater than uncolonised artificial sediment.

Table 7-2: Organic matter content of artificial and natural sediment collected from Silsden Beck in summer 2011

Size fraction	Mean % OM (± sd)
Natural >2 mm	5.81 ± 0.71
Natural >1 mm	4.55 ± 0.74
Natural >0.5 mm	4.53 ± 1.27
Natural >0.15 mm	4.86 ± 1.50
Artificial 10 mm gravel	0.37 ± 0.17
Artificial 0.2 mm sand	0.59 ± 0.18
Uncolonised artificial 10 mm	0.04 ± 0.01
Uncolonised artificial 0.2 mm	0.03 ± 0.02

Notes:
n=3
natural sediment collected January 2011 from Silsden Beck

7.4.2 Sediment organic matter content

Significant differences from control organic matter retention were observed for artificial sediments exposed to erythromycin (H=13.03, DF=4, p=0.01) and the mixture (H=19.41, DF=4, p<0.01) but not for propranolol (H=2.86, DF=4, p=0.58; Figure 7-1). Specifically, slight increases in OM retention were present at the highest exposure for erythromycin and at all but the lowest exposure for the mixture (Table 7-3). Furthermore, OM retention appeared to increase with exposure dose for the mixture treatment. The linear regression of OM retention vs. $\log_{10}$ exposure concentration was shown to be statistically significant (R^2=94.8, F=54.43, DF=4, p<0.01; Figure 7-2) but this same relationship was not present for erythromycin (p=0.29) or propranolol (p=0.71).

Table 7-3: Summary of statistical analysis organic matter retention of artificial sediments exposed to erythromycin, propranolol and mixture

Treatment concentration	Erythromycin	Propranolol	Mixture
	Comparison of OM retention against control value[1]		
VLT	↑8% (W=29.0, p=0.13)	↑25% (W=36.0, p=0.69)	↑11% (W=27.0, p=0.07)
LT	↑11% (W=25.0, p=0.03)	↓6% (W=44.0, p=0.47)	↑26% (W=23.0, p=0.01)
HT	↑3% (W=34.0, p=0.47)	↑28% (W=38.0, p=0.94)	↑49% (W=21.0, p<0.01)
VHT	↑14% (W=23.0, p=0.01)	↑4% (W=36.0, DF=0.69)	↑46% (W=21.0, p<0.01)
Treatment concentration	**Comparison between compounds[2]**		
VLT	ERY 145%, PRO 118%, MIX 108% (H=1.31, DF=2, p=0.52)		
LT	ERY 145%, **PRO 92%,** MIX 144% (H=10.98, DF=2, p<0.01)		
HT	ERY 111%, PRO 132%, MIX 184% (H=6.88, DF=2, p=0.03)		
VHT	ERY 160%, **PRO 105%,** MIX 180% (H=11.59, DF=2, p<0.01)		

Notes:
1. Values represent mean differences in OM retention relative to control mean
2. Values represent mean OM retention standardised by the mean control OM retention for each compound experiment. **Bold** text indicates significant difference between compound exposures.

There was a large degree of variation in control OM retention between the three experiments (Figure 7-1; H=13.05, DF=2, p<0.01) and so any comparisons between the experiments had to be standardised for this variation (Figure 7-3). Differences in OM retention between compound exposures were statistically significant at all treatment levels except VLT (Generalised Linear Model: F=8.46, DF=2, p<0.01; Table 7-3). The mixture sediments were characterised by the highest levels of OM retention across all treatments except the very low exposure and propranolol generally displaying the lowest levels of OM retention.

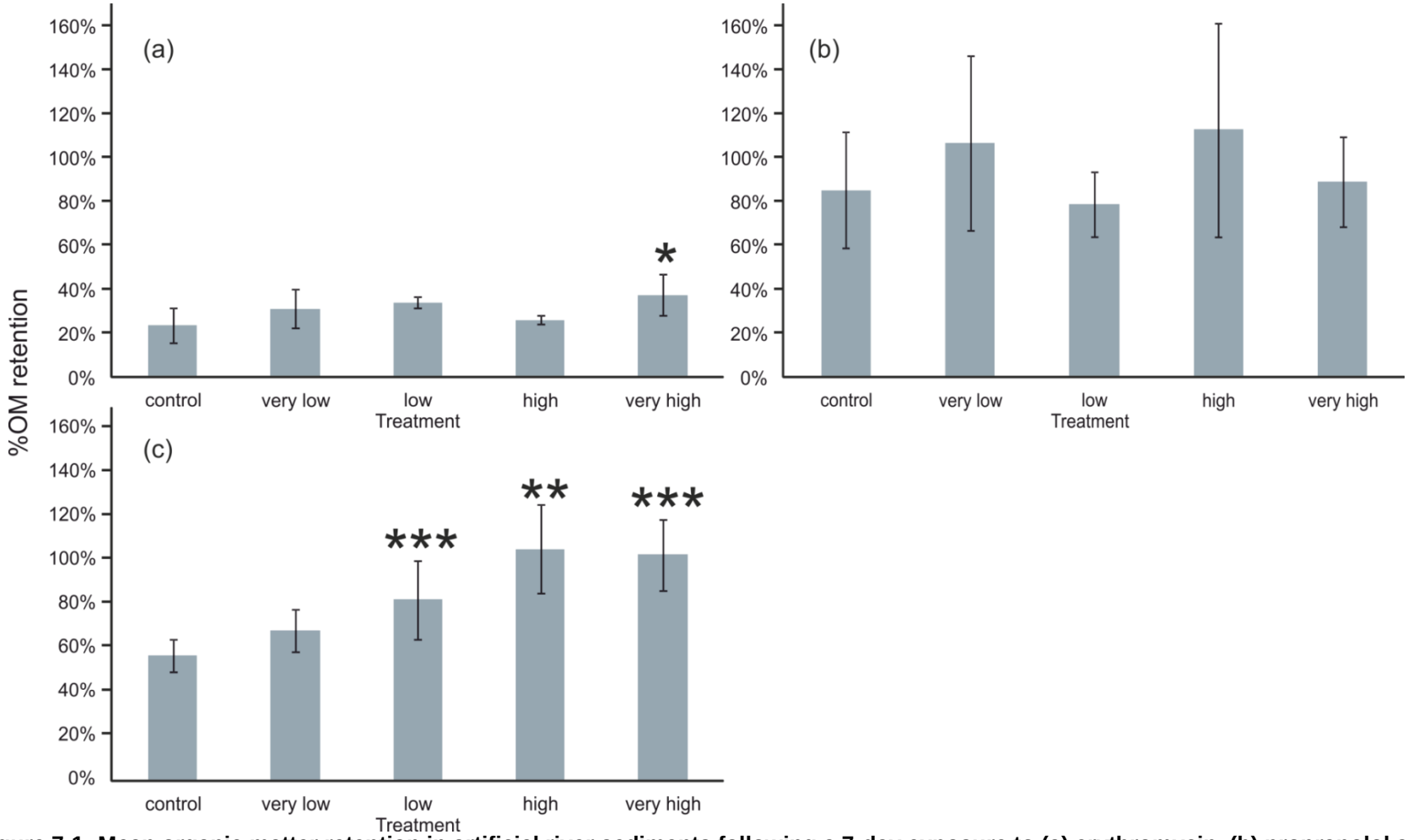


Figure 7-1: Mean organic matter retention in artificial river sediments following a 7-day exposure to (a) erythromycin, (b) propranolol and (c) a binary mixture of erythromycin and propranolol
[%OM retention = %OM at day 7 / initial %OM at day 0]
[Bars represent mean from n=6 replicates, error bars represent ±1 SD; asterisks indicate values significantly different from control: * < 0.05, ** < 0.01, * < 0.001]**

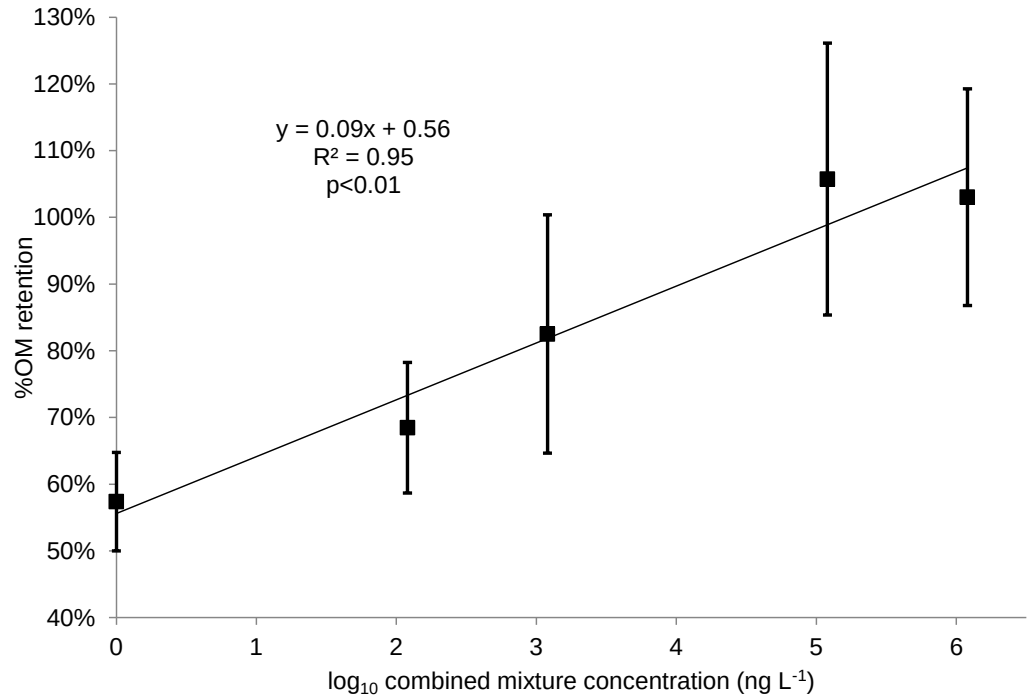


Figure 7-2: Linear regression of OM retention vs. log_{10} concentration of artificial sediment following a 7 day exposure to a mixture of erythromycin and propranolol [points represent mean values from 6 replicates], error bars represent ±1 SD]

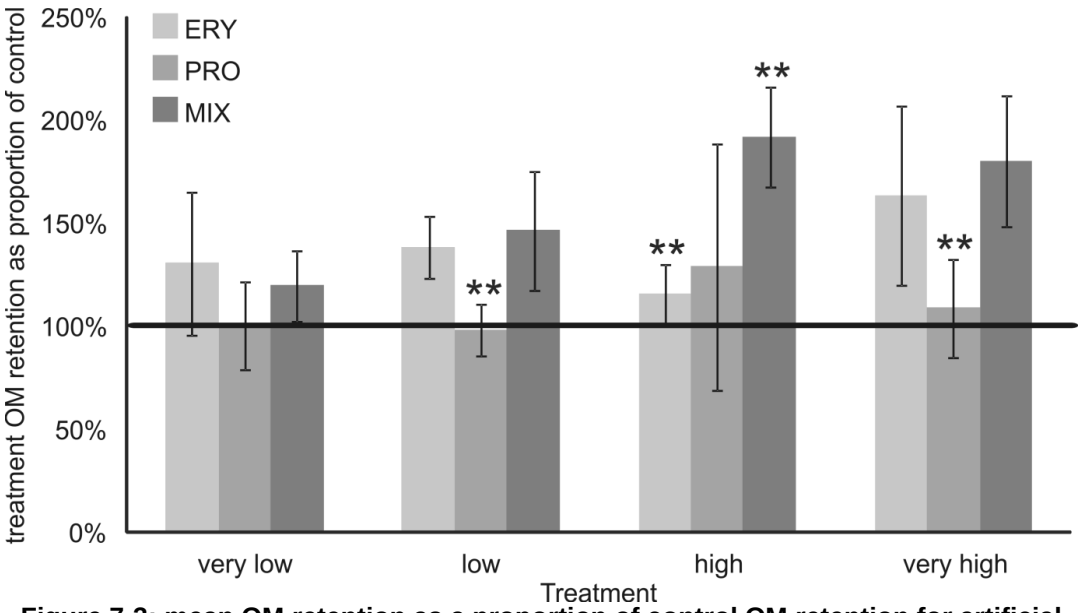


Figure 7-3: mean OM retention as a proportion of control OM retention for artificial sediments exposed to four treatment levels of erythromycin, propranolol and a mixture [Bars represent mean from n=6 replicates, error bars represent ±1 SD; asterisks indicate values significantly OM retention between experiments: * < 0.05, ** < 0.01, * < 0.001; the solid line represents 100% *i.e.* no change in OM during the experiment, values >100% indicate a gain in OM and values <100% a reduction]**

7.4.3 Sediment respiration rates

Significant differences in respiration rates were present between erythromycin treatments. Treatments were compared pairwise against the control across the whole exposure period which showed the very high concentration erythromycin exposed sediments respired at a significantly reduced rate (Table 7-4). When respiration rates

were examined daily, no significant differences between treatments were present. Overall, erythromycin exposed sediments respired at a greater rate compared to the propranolol and mixture sediments (Figure 7-4).

Comparison of respiration rates for propranolol was significant between treatments (F=21.09, DF=4, p<0.001) with significant pairwise differences between the control and both low and very high treatments. When daily rates were examined low treatment exposed sediments respired at a significantly reduced rate compared to the control at 72 hours and 96 hours. Respiration rates between mixture treatments showed no significant difference from the controls either across the whole seven days or at individual time steps.

Table 7-4: Analysis of mean (±1 SD) respiration rates of artificial sediments exposed to erythromycin, propranolol and mixture

Timestep (hours)	Erythromycin	Propranolol	Mixture
Whole dataset[1]	(F=4.63, DF=4, p<0.01) VHT reduced from control	(F=22.07, DF=4, p<0.001) VHT increased and LT reduced from control	F=1.94, DF=4, p=0.11)
24[2]	H=10.15, DF=4, p=0.04 None different from control	H=13.20, DF=4, p=0.01 None different from control	H=2.05, DF=4, p=0.73
32	H=7.18, DF=4, p=0.13	H=13.49, DF=4, p<0.01 VHT increased from control	
48	H=2.01, DF=4, p=0.73	H=11.81, DF=4, p=0.02 None different from control	H=4.04, DF=4, p=0.40
55	H=1.93, DF=4, p=0.74	H=10.70, DF=4, p=0.03 None different from control	
72	H=4.00, DF=4, p=0.41	H=15.88, DF=4, p<0.01 LT reduced from control	H=4.58, DF=4, p=0.33
79	H=3.21, DF=4, p=0.52	H=9.99, DF=4, p=0.04 None different from control	
96	H=1.62, DF=4, p=0.81	H=11.55, DF=4, p-0.02 LT reduced from control	H=3.34, DF=4, p=0.50
102	H=4.33, DF=4, p=0.36	H=11.75, DF=4, p=0.02 LT reduced from control	
168	H=10.90, DF=4, p = 0.03 None different from control	H=5.84, DF=4, p=0.21	H=4.60, DF=4, p=0.33

Notes:
Generalised Linear Model of all treatments and timesteps: respiration rate vs. treatment, time (treatment * time). There was no significant interaction between treatment and time in the GLM for any of the three experiments.
Kruskal-Walls H tests comparing treatments at each individual timestep (24 to 168 hours)

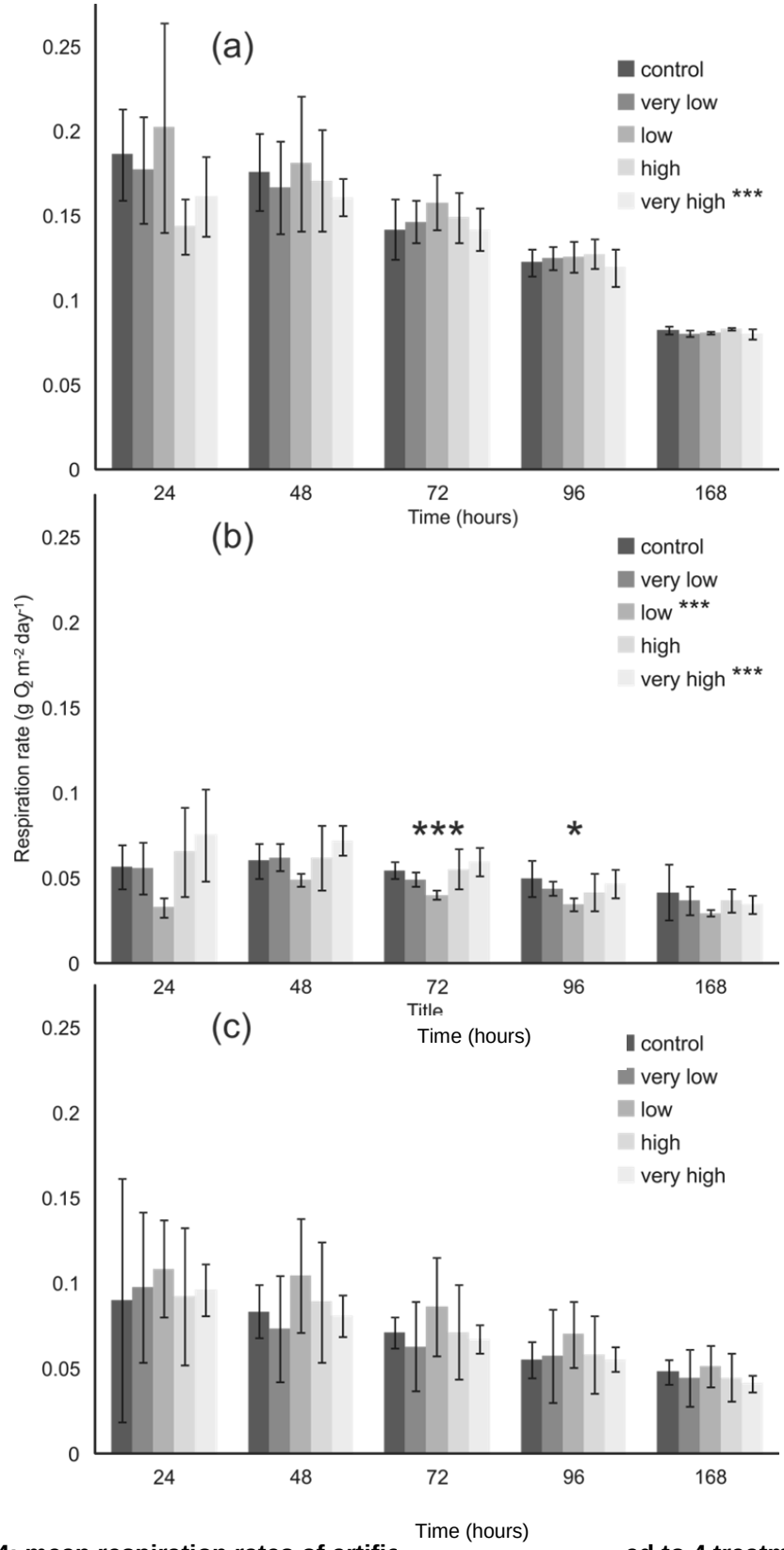


Figure 7-4: mean respiration rates of artific . ed to 4 treatment levels of (a) erythromycin, (b) propranolol and (c) their binary mixture over a 7 day period [Bars represent mean from n=6 replicates, error bars represent ±1 SD; asterisks indicate values significantly different from control: * < 0.05, ** < 0.01, * < 0.001. Asterisks next to legend indicate differences across the whole exposure whereas asterisks above bars indicate differences at that specific timestep. For further details see Table 7-4]**

7.4.4 Dissolved organic carbon, nitrate and phosphate

Exposure of sediments to erythromycin resulted in significant differences in nitrate, ammonium and phosphate exchange with overlying water. In particular, high and very high treatments showed increased ammonium and decreased phosphate exchange over control levels (Table 7-5; Figure 7-5). Propranolol exposed sediments showed significant differences in phosphate exchange with the high treatment (200 000 ng L^{-1}) demonstrating increased levels compared to control. Mixture exposed sediments at low treatment levels demonstrated reduced DOC exchange when compared to controls.

Differences between experimental control values for nutrient and DOC exchange were present for NO_2-N (H=8.02, DF=2, p=0.02), NO_3-N (H=7.03, DF=2, p=0.03) and DOC (H=12.23, DF=2, p<0.01). After standardisation, erythromycin exposed sediments demonstrated significant differences in NO_2-N, NH_4-N and DOC when compared to propranolol and mixture exposed sediments. Propranolol exposed sediments demonstrated reduced NO_3-N exchange when compared to erythromycin and mixture.

Table 7-5: Statistical analysis of nutrient and carbon change of artificial sediments exposed to erythromycin, propranolol and their mixture for seven days

Analyte	Difference from control		
	Erythromycin	Propranolol	Mixture
NO_2-N	H=6.89, DF=4, p=0.14	H=2.90, DF=4, p=0.58	H=3.82, DF=4, p=0.43
NO_3-N	H=10.55, DF=4, p=0.03 No differences from control	H=1.80, DF=4, p=0.77	H=6.89, DF=4, p=0.14
NH_4-N	H=14.44, DF=4, p=0.01 VHT increased from control	H=1.00, DF=4, p=0.91	H=7.50, DF=4, p=0.11
PO_4-P	H=9.56, DF=4, p<0.05 HT reduced from control	H=9.56, DF=4, p<0.05 HT elevated from control	H=3.40, DF=4, p=0.49
DOC	H=7.66, DF=4, p=0.105	H=2.88, DF=4, p=0.58	H=10.31, DF=4, p=0.04 LT reduced from control
Analyte	**Difference between experiments**		
NO_2-N	F=44.57, DF=2, p<0.001 ERY increased c.f. PRO and MIX		
NO_3-N	F=30.96, DF=2, p<0.001 PRO reduced c.f. ERY and MIX		
NH_4-N	F=3.80, DF=2, p=0.03 ERY reduced c.f. MIX		
PO_4-P	F=3.19, DF=2, p<0.05 No differences with Tukey's comparisons		
DOC	F=131.65, DF=2, p<0.001 ERY reduced c.f. PRO and MIX		

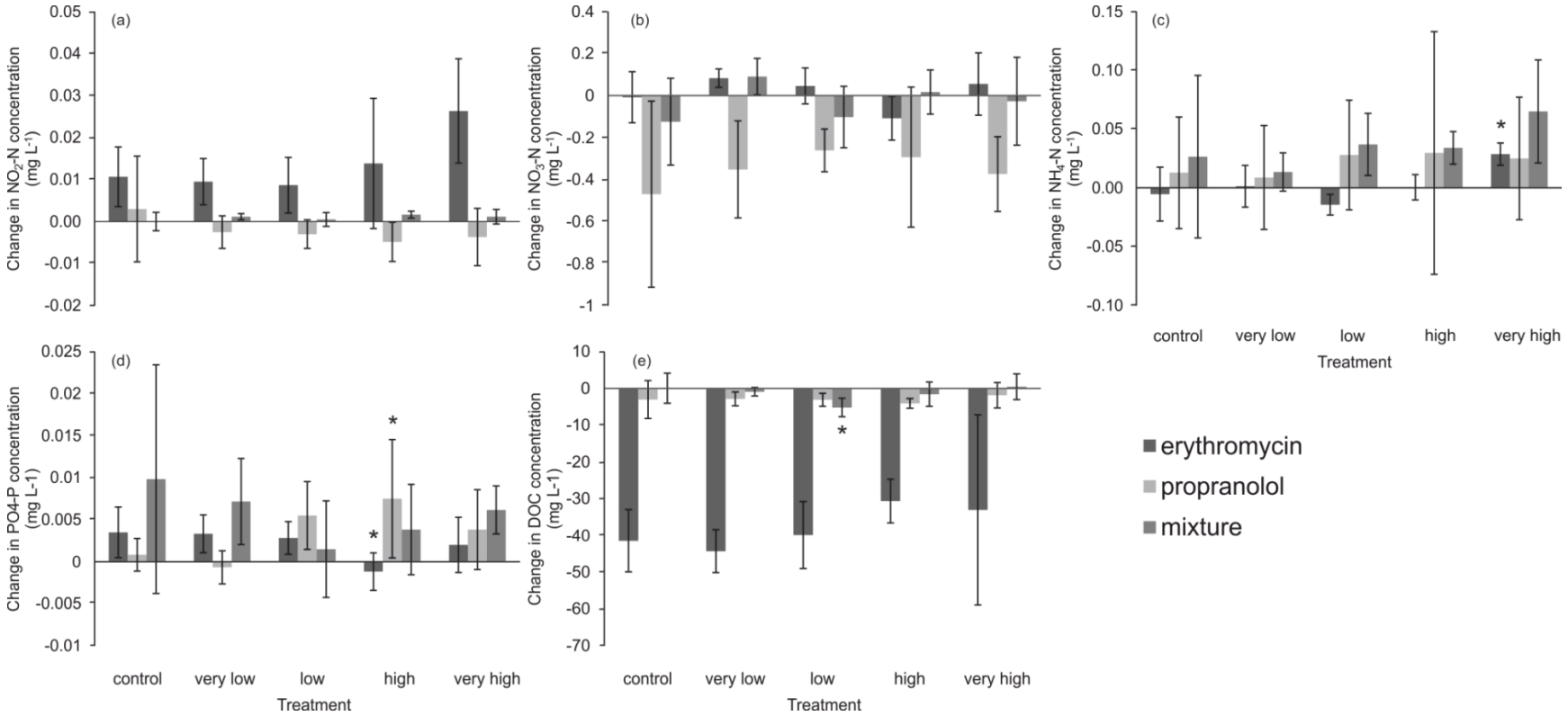


Figure 7-5: Mean changes in (a) NO_2^--N, (b) NO_3^--N, (c) NH_4+-N, (d) PO_4^--P and (e) DOC concentrations in the overlying water of artificial sediment exposed to 4 treatments of erythromycin, propranolol and their binary mixture over a 7 day period
[Bars represent mean from n=6 replicates, error bars represent ±1 SD; asterisks indicate values significantly different from control: * < 0.05]

7.5 Discussion

7.5.1 The effects of pharmaceuticals on respiration and organic matter degradation in sediments

The results presented here demonstrate the capacity for pharmaceuticals to significantly disrupt the processing of organic matter and nutrients in freshwater sediments which may represent a wider threat to the functioning of freshwater ecosystems with implications for catchment-wide water quality. The contamination of river sediments by a variety of pollutants (e.g. metals, organic chemicals, mine drainage and nutrients) is widespread (Ryther & Dunstan, 1971; Rutherford & Hynes, 1987; Atlas & Bartha, 1997; Palmer, 1997; Neal *et al.*, 2005; Byrne *et al.*, 2010; Colas *et al.*, 2013). These contaminated sediments act as both a diffuse store for further contamination downstream and also represent a serious threat to the integrity of freshwater ecosystem biodiversity and ecosystem functioning (Colas *et al*., 2013). This is of concern because sediments (and their associated microbial and invertebrate communities) play a vital role in organic matter decomposition, nutrient cycling, and pollutant degradation (Groffman & Bohlen, 1999; Austen *et al.*, 2002; Colas *et al.*, 2013).

Up to 80 % of the organic material fixed by primary producers flows to the detrital food chain (i.e. sediment biota (Odum, 1983)) which in turn recycles nutrients and carbon making these available at higher trophic levels (Groffman & Bohlen, 1999). The importance of microbial communities in processing carbon is well documented and is known as 'the microbial loop' (Meyer, 1994; Fenchel, 2008) and the potential alterations to key microbial processes demonstrated in this study may have serious implications for the wider freshwater ecosystem. Covich *et al*. (1999) describe these communities as 'invisible' because they live below the surface whilst performing vital functions for the integrity of freshwater ecosystems and the supply of freshwater resources. Clearly, disruption to the structure and function of sediment dwelling microbial communities as demonstrated in this study can have significant implications for the whole freshwater ecosystem.

Sediments exposed to high but environmentally relevant levels of erythromycin (≥100 µg L^{-1}) and the mixture in this study demonstrated up to 50 % increases in the retention of organic matter (i.e. a reduction in organic matter degradation) which supports the original hypothesis (**H_1**). Other studies have shown similar functional inhibition, for example Näslund *et al*. (2008) showed that the presence of the antibiotic ciprofloxacin (>200 µg L^{-1}) significantly reduced the mineralisation of the polycyclic aromatic hydrocarbon pyrene over a 77 day period; the degree of inhibition was shown to be significantly correlated with ciprofloxacin concentration. This finding is in agreement with

the results presented here which showed a significant linear relationship between mixture exposure concentration and OM retention. The linear relationship suggested a dose-dependent response for the effects of an erythromycin and propranolol mixture on organic matter degradation. Disruptions to organic matter degradation can also have knock-on effects for the fate and release of contaminants bound to the organic phase (Holden & Firestone, 1997). For example, studies have shown that the degree of OM degradation can influence the loads of dissolved, bioavailable heavy metals in overlying waters (Rae & Allen, 1993; Masson *et al.*, 2011). The reductions in OM degradation demonstrated in this study may disrupt the degradation, exchange and bioavailability of other sediment-associated contaminants by allowing them to remain bound to the solid phase.

The research examining the effect of pharmaceuticals on microbial respiration is sparse and no published studies have evaluated the effects of erythromycin and propranolol on this important ecosystem function. In this study, sediments exposed to very high levels of erythromycin (1 000 000 ng L^{-1}) and low levels of propranolol (≥200 ng L^{-1}) demonstrated reduced respiration rates when compared to controls in agreement with hypothesis **H_2**. This finding is supported by Girardi *et al*. (2011) who demonstrated that high levels of the antibiotic ciprofloxacin (20 mg L^{-1} over 25 days) inhibited microbial respiration in both soil and water microcosms. Disruptions to sediment respiration by other pollutants are well documented, for example, increased nutrient levels and STP effluent have been shown to stimulate respiration (Ryther & Dunstan, 1971; Ingendahl *et al.*, 2002). Furthermore, ibuprofen and paracetamol demonstrated a similar stimulation at low concentrations. Higher concentrations of paracetamol (up to 10 000 ng L^{-1}) proved inhibitory whereas the stimulatory effect of ibuprofen was present even at the highest concentrations (Bunch & Bernot, 2011). These results agree with those demonstrated in this study which highlighted a brief stimulation of respiration caused by very high levels of propranolol (200 000 ng L^{-1}) during the first 32 hours of exposure. This stimulation may be explained by microbial communities possessing the ability to chemically alter some pharmaceutical compounds and use them as a nutritive source thus increasing respiration rates (Bradley *et al.*, 2007). At higher concentrations direct toxicity may override this stimulatory effect. Clearly, measurement of sediment respiration is an appropriate method for examining the effects of emerging contaminants on ecosystem functioning.

The net impact (stimulatory or inhibitory) of pharmaceuticals is highly dependent upon specific compound properties and exposure concentrations (Lawrence *et al.*, 2005). *In situ* tests in pharmaceutical or wastewater impacted streams have so far not demonstrated such effects on microbial respiration. This may be explained by the capacity of such microbial communities to adapt and develop tolerance as a result of a

long exposure history to specific stressors (Spain, 1983; Liebert *et al.*, 1991). The stimulation in community respiration is one of the expected trends in stressed ecosystems as energy is diverted from growth and production to maintenance and stressor response (Odum, 1985). However, the opposite effect was observed in this study with a general decrease in respiration caused by erythromycin and propranolol which may be due to direct toxicity of these compounds on microorganisms (Lawrence *et al*., 2005). The general decrease in respiration (R) observed in this study may lead to a disruption in the production: respiration (P/R) ratio which tends towards balance in non-stressed ecosystems. Odum (1985) suggests P/R as a more sensitive and appropriate tool in measuring ecosystem stress and so any future studies examining the effects of pharmaceuticals on freshwater sediment functioning should examine both production and community respiration rates.

Environmentally relevant concentrations of sulphonamide and tetracycline antibiotics have also been shown to significantly inhibit bacterial respiration and $Fe_{(III)}$ reduction in soils (Thiele-Bruhn & Beck, 2005). These disruptions to important ecosystem functions in both matrices were accompanied by reductions in bacterial abundance or shifts in the bacterial community composition. In some cases these changes are accompanied by increased dominance of fungal biomass (Thiele-Bruhn & Beck, 2005). Lawrence *et al*. (2005) demonstrated that 10 000 ng L^{-1} of carbamazepine, furosemide and ibuprofen caused shifts in the structure of riverine biofilm communities. All four compounds suppressed cyanobacteria and bacterial biomass. These results demonstrate the ability for pharmaceuticals to exhibit both nutrient-like and toxic effects in freshwater microbial communities (Lawrence *et al*., 2005).

These linkages between ecosystem functioning and biodiversity (e.g. bacterial/fungal diversity or macroinvertebrate species richness) are poorly understood but it is likely that diverse communities are important for functions such as community respiration (Bell *et al.*, 2005). Freshwater sediments are of particular concern as the microbial communities inhabiting them have been shown to be susceptible to disruption by antibiotic compounds (e.g. Girardi *et al*., 2011). No data on the structure of microbial communities were available for this study but it is likely that the changes in respiration rates were caused by a shift in the makeup of respiring bacteria and other microorganisms. Clearly, any further research examining the effects of pharmaceuticals on sediment microbial communities should consider both structural and functional aspects if a full understanding is to be gained.

7.5.2 The effect of pharmaceutical mixtures on sediment functioning

Pharmaceuticals are not present in isolation in the aquatic environment but rather form part of dynamic, multi-component mixtures containing a vast array of other

pharmaceuticals and their metabolites and myriad other organic and inorganic pollutants (Fent, 2008). Despite this, the research into the environmental effects of pharmaceutical mixtures is relatively sparse. This study represents the first investigation of the effects of a simple pharmaceutical mixture on important aspects of ecosystem functioning in rivers.

Sediments exposed to the binary mixture of erythromycin and propranolol displayed significant reductions in DOC exchange and organic matter degradation at concentrations up to three orders of magnitude below those displayed when these compounds were tested in isolation (200 and 1000 ng L^{-1} in mixture c.f. 20 000 and 1 000 000 ng L^{-1} in isolation). This suggests some form of interaction between these two compounds which allows them to elicit effects below their individual threshold levels for effects and partially confirms the original hypothesis (**H_4**). These compounds may follow the concentration addition model between erythromycin and propranolol despite their differing mechanisms of action (MOA) (Cedergreen *et al.*, 2008); most existing work on pharmaceutical mixtures suggests they follow this model. For example, Cleuvers (2003; 2004) showed that anti-inflammatory and other drugs acted additively on *Daphnia magna* and *Lemna minor*, with mixtures demonstrating up to five fold increases in negative response at the same concentration of individual compounds.

Other studies suggest that for some compounds, particularly those with very different MOAs (Backhaus, 2008), the Independent Action model may be more appropriate (Escher *et al.*, 2005). Dyer *et al*. (2000) suggest that the cumulative baseline toxicity (narcosis) of very complex mixtures may dominate over the independent effects of specific MOAs, particularly at low concentrations and as such the concentration addition model has been recommended unless specific evidence indicates otherwise (Cleuvers, 2003). The results presented here appear to agree with this assumption although they and others have key limitations

- Different degradation kinetics between compounds means the relative concentrations in the mixture will vary over time;

- Inability to infer the effects of individual compounds from the mixture when no parallel studies of individual compounds are conducted. This was addressed as part of this study by conducting identical exposures of mixtures and individual compounds;

- The mixture is static with no compounds being added or lost whereas environmental mixtures are dynamic in time;

- Toxicity can only be inferred for the specific mixture(s) tested for and the results are not applicable for different mixtures;

- The mixtures are relatively simple (generally containing <10 compounds) and do not reflect the highly complex nature of environmental mixtures containing potentially thousands of compounds.

Clearly, the situation of contaminant mixtures is highly complex and much more research is necessary if we are to fully understand potential negative effects on the structure and functioning of freshwater ecosystems. One promising area of research is metagenomics which allows the rapid characterisation of the structure and functional capacity of microbial communities in environmental samples. Such techniques have already been successfully applied to characterise microbial communities in large rivers (Ghai *et al.*, 2011) and in understanding the effects of pollutants on such communities (George *et al.*, 2010). These emerging techniques should be combined with more traditional endpoints of effects at the individual, structural and functional level to fully understand the risks of pharmaceutical pollution. Furthermore, the contamination of sediments has been shown to disrupt macroinvertebrate communities which can in turn have implications for other important ecosystem functions such as leaf litter breakdown (Colas *et al.*, 2013), which in itself may be directly affected by pharmaceutical pollution (Bundschuh *et al*., 2009; Hahn & Schulz, 2007). Therefore, future research on pharmaceuticals in riverine sediments should also consider macroinvertebrate assemblages as these too play a key role. The results presented here and elsewhere highlight the need to consider both structural and functional aspects of ecosystem integrity when evaluating the effects of pharmaceutical mixtures in freshwaters.

7.5.3 Dissolved carbon and nutrient cycling

Carbon, nitrogen and phosphorous are vital elements in aquatic ecosystems and play an important role in determining their level of productivity (Giller & Malmqvist, 1998). Freshwater sediments, particularly associated microbial communities, have a major role in the cycling of nutrients and carbon and rendering these available to higher trophic levels (Groffman & Bohlen, 1999). In the current study, small but significant disruptions to the cycling of key nutrients between sediment and overlying water appear to have been caused by low level pharmaceutical exposure, supporting hypothesis **H_3**. Erythromycin exposed sediments demonstrated increased ammonium exchange and decreased phosphate exchange when compared to controls albeit at relatively high levels (≥ 100 µg L^{-1}). Changes in NH_4-N exchange with overlying waters are indicative of disruption of sediment microbial communities as the majority of N released from sediments via microbial action is in the form of NH_4-N (Kemp *et al.*, 1990). This suggests that erythromycin may stimulate the bacteria responsible for converting mineralised nitrogen to NH_4-N whilst inhibiting those responsible for the interconversion of organic

phosphorous to the mobile phase (PO_4-P; Boström *et al*. (1988)). Disruptions to NH_4-N exchange have important implications for freshwater ecosystems as ammonium is an important source of nitrogen for numerous plant species and also supplies energy for nitrifying bacteria. The resulting mineralised nitrate is a very important source of nitrogen for phytoplankton (Rheinheimer, 1985).

The opposite trend was true for propranolol exposed sediments where the production of PO_4-P by sediment microbial communities appeared to be enhanced. A possible explanation for this may be the direct release of phosphorous from bacteria upon cell death (lysis; see Montigny & Prairie (1993)). The disruption of cell membranes via baseline toxicity (narcosis) has been suggested as an important toxic mechanism for pharmaceuticals in aquatic systems (Dyer *et al*., 2000) and such disruption may lead to the release of phosphorous held within bacterial cells.

Enhancements in nutrient uptake have been demonstrated by Bunch & Bernot (2011) which showed increased NO_3-N uptake by sediments exposed to low concentrations of paracetamol (55 and 110 ng L-1) although much higher concentrations inhibited uptake (5055 and 10 000 ng L-1). No differences were present in NO_3-N uptake were present in this study. The lack of any effect of erythromycin on the exchange of nitrite (NO_2-N) and nitrate (NO_3-N) may be explained by the fact that the majority of nitrifying bacteria (e.g. *Nitrobacter* and *Nitrosomonas*) are gram-negative (Skerman *et al.*, 1980; Chain *et al.*, 2003) whereas erythromycin targets gram-positive bacteria. Disruptions to nutrient cycling caused by a variety of stressors are well documented (Blann *et al.*, 2009; Cataldo *et al.*, 2012; Trimmer *et al.*, 2012) and Odum (1985) suggested that disruptions to nutrient cycling are one of the key features of a stressed ecosystem. In particular, vertical cycling (between water and sediment decreases) causing increased downstream transport and nutrient loss from the ecosystem. A variety of factors can cause increased nutrient loss (Margalef, 1975) and the changes caused by pharmaceuticals in this study may play a role in altering downstream transport of nutrients.

Costanzo *et al.* (2005) demonstrated significantly reduced denitrification rates of laboratory exposed marine sediments to high concentrations (1000 µg L^{-1}) of the antibiotics erythromycin, clarithromycin and amoxicillin. Denitrifying bacteria may be particularly susceptible to the above compounds given they specifically target such bacteria (Costanzo *et al.*, 2005). Evidence (albeit anecdotal) of the effect of antibiotics on nitrogen cycling bacteria is widespread in the aquarium industry which relies on such bacteria in biological filters. Negative effects have also been demonstrated on nitrogen cycling processes in STPs although at very high concentrations (Luis Campos *et al*., 2001). The results presented here and elsewhere demonstrate the potential for

environmentally realistic concentrations of antibiotics to illicit small but varying and significant effects on nitrogen and phosphorous cycling in aquatic sediments.

Microbial activity represents an interesting avenue for the effects testing of trace organic chemicals such as pharmaceuticals because they can be both toxic but also act as a nutritive source which act to simultaneously stimulate and inhibit the overall community (Bunch & Bernot, 2011). Whether or not pharmaceuticals are net stimulatory or inhibitory is likely to depend strongly on individual compound properties and identifying these mechanisms of microbial response should be seen as a research priority (Lawrence *et al.*, 2005). Changes to nutrient cycling by pharmaceutical exposed microbial communities, such as that demonstrated in this study, may have cascading effects on whole freshwater ecosystems by altering the amount and availability of NO_3-N and NH_4-N for other organisms and perhaps decreasing the potential for the mitigation of nutrient pollution by microbes (Bunch & Bernot, 2011). This is of particular concern in rivers affected by STP effluent where high concentrations of both nitrogen and pharmaceutical compounds are likely to occur alongside each other (Paul & Meyer, 2001; Glassmeyer *et al.*, 2005).

Sediments exposed to the pharmaceutical mixture at low levels (200 and 1000 ng L^{-1} of erythromycin and propranolol respectively) demonstrated a net reduction in overlying water DOC concentrations when compared to the controls. This suggests that mixture exposed sediments assimilated a greater amount of carbon from the water column when compared to controls. However, this is not in agreement with the respiration data which showed mixture exposed sediments respired at a reduced rate which would suggest a reduction in the amount of carbon assimilated from the overlying water. Clearly, these processes are complex with the availability and fate of carbon and nutrients (particularly N) and their effects on respiration strongly linked (Trimmer *et al*., 2012). However, any disruptions to the cycling and availability of carbon can have significant impacts on contaminant transport, energy supply, nutrient cycling and also drinking water treatment (Stewart & Wetzel, 1981; Wetzel, 1992; Alarcon-Herrera *et al.*, 1994; Lawlor & Tipping, 2003). Furthermore, alterations to the export of terrestrially-derived carbon to estuaries and oceans may significantly alter their energy and nutrient regimes (Raymond & Bauer, 2001). Emerging technologies such as metagenomics may play a role in understanding how contaminants affect the structure and functional capacity of sediment microbial communities (e.g. Ghai *et al*., 2011).

The results presented here demonstrate the capacity of low levels of pharmaceuticals to have significant impact on the exchange of nutrients and carbon between freshwater sediments and the overlying water. These changes (although significant) represent only small perturbations when compared to the rates of exchange demonstrated by control sediments. However, much more research is required on the

effects of emerging contaminants on such processes given the important role freshwaters play in global carbon and nutrient cycling where even small disruptions can lead to significant effects (Trimmer *et al.*, 2012).

7.6 Conclusions

The results presented here indicate the potential for pharmaceuticals, in low but environmentally realistic concentrations over extended exposure periods, to alter important aspects of ecosystem functioning in freshwater sediment systems and their exchange of key nutrients with the water column. This has potential implications for secondary production and the downstream transport of carbon, nitrogen and phosphorous. Future assessments of ecosystem health and their response to stressors such as pharmaceuticals should utilise both established structural indices as well as functional measures tested here if a full understanding of the risk pharmaceuticals pose in freshwaters is to be obtained. This is of particular importance in the context of the results presented here given the distinct possibility of disruptions to sediment metabolism and nutrient cycling to cascade causing additional indirect effects at higher trophic levels and downstream (Wallace *et al.*, 1997). Of particular concern is the identification of the additive effects of low level pharmaceutical exposure when acting as part of a mixture. This demonstrates the potential for pharmaceuticals to elicit negative effects on freshwater ecosystem function below their individual effects thresholds and may mean that existing ecotoxicological research of individual compounds has underestimated the risk that pharmaceuticals pose.

8 RESEARCH SYNTHESIS, WIDER IMPLICATIONS AND FUTURE OPPORTUNITIES

8.1 Research synthesis

This thesis has examined the occurrence and effects of widely used human-use pharmaceuticals in freshwater ecosystems. The primary goals of the research were to: 1) review and critique the state of the art in the subject, 2) quantify the presence and spatiotemporal variation of pharmaceutical compounds in STP effluent, CSO effluent and receiving waters, 3) examine the lethal, sublethal and metabolic effects of extended exposure of freshwater macroinvertebrates to pharmaceuticals, and 4) understand the effects of these compounds on some important aspects of ecosystem structure and functioning.

Pharmaceuticals enter freshwater ecosystems via a variety of complex sources, pathways and receptors with consumption, disposal, sewage treatment, climate, risk assessment, policy and in-stream physicochemical, biotic and ecological processes all playing a key role in the ultimate occurrence, fate and effects of pharmaceuticals in rivers (Figure 8-1). An increasing global population with improving quality of life will be accompanied by increased consumption of pharmaceuticals worldwide which is likely to be the key driver of pharmaceutical pollution in coming decades (Daughton, 2003). The environmental impact of increased consumption is unlikely to be offset in improvements to sewage treatment and may worsen key public health issues particularly that of antibiotic resistance (APUA, 2010; Figure 8-1). Despite the requirement for environmental risk assessment of new pharmaceuticals, there remain large knowledge gaps in terms of the occurrence and effects of hundreds, if not thousands, of pharmaceuticals in rivers (Pascoe *et al*. 2003). These have the potential to disrupt biodiversity and the vital ecosystem services provided by river ecosystems upon which human society relies (Costanza *et al*., 1997, Vorosmarty *et al*., 2010).

Notably, this body of work has addressed several key components of this complex system and has shown that pharmaceuticals are consistently present throughout both the semi-rural and urban reaches of the Aire and Calder catchments and demonstrate high degrees of variation across diurnal and seasonal as well as reach and catchment scales. Pharmaceuticals also demonstrated significant lethal and sub-lethal metabolic effects on the important freshwater amphipod *Gammarus pulex* when exposed for extended periods at environmentally relevant concentrations. This highlights the pressing need for similar investigations using other functionally important freshwater taxa including macroinvertebrates and fish. Similar exposures to pharmaceuticals appeared to cause no significant effects on the microbially or macroinvertebrate mediated decomposition of leaf litter in freshwaters. Both stimulatory

and inhibitory effects on the degradation of organic matter, nutrient exchange and respiration in freshwater sediments were also observed. More specific key findings in relation to the individual research questions can be summarised as follows:

Aim 1: What is the current spatial and compound coverage of existing pharmaceutical pollution research and how reliable/representative are data obtained from these studies?

Research into pharmaceutical pollution of rivers has expanded rapidly from the late 1990s onwards aided by advancements in analytical techniques. However, the existing research is heavily biased towards Western Europe, North America and parts of China and furthermore spatially clustered within these countries around a relatively small number of urban areas. Very little or no research has been conducted in Africa, central Asia and South America. Research effort has focused on a small majority of commonly studied compounds leaving large numbers of potentially high risk compounds poorly investigated. The sampling techniques frequently employed during such research are poor for capturing the high degree of temporal and spatial variability associated with pharmaceutical pollution. Measured concentrations in rivers often approached or exceeded levels known to cause acute or chronic toxic effects in freshwater organisms with fish and macroinvertebrates at particular risk from antibiotics, antidepressants, cardiovascular drugs and painkillers. This overlap between measured occurrence and effects concentrations should inform future risk assessment and regulatory systems to ensure that any conclusions are drawn from environmentally relevant data and will provide an adequate degree of protection to freshwater ecosystems. The database of pharmaceutical occurrence assembled as part of this chapter should serve as a useful tool for industry professionals, academics and regulators in prioritising future research into both the occurrence and effects of pharmaceutical pollution in rivers.

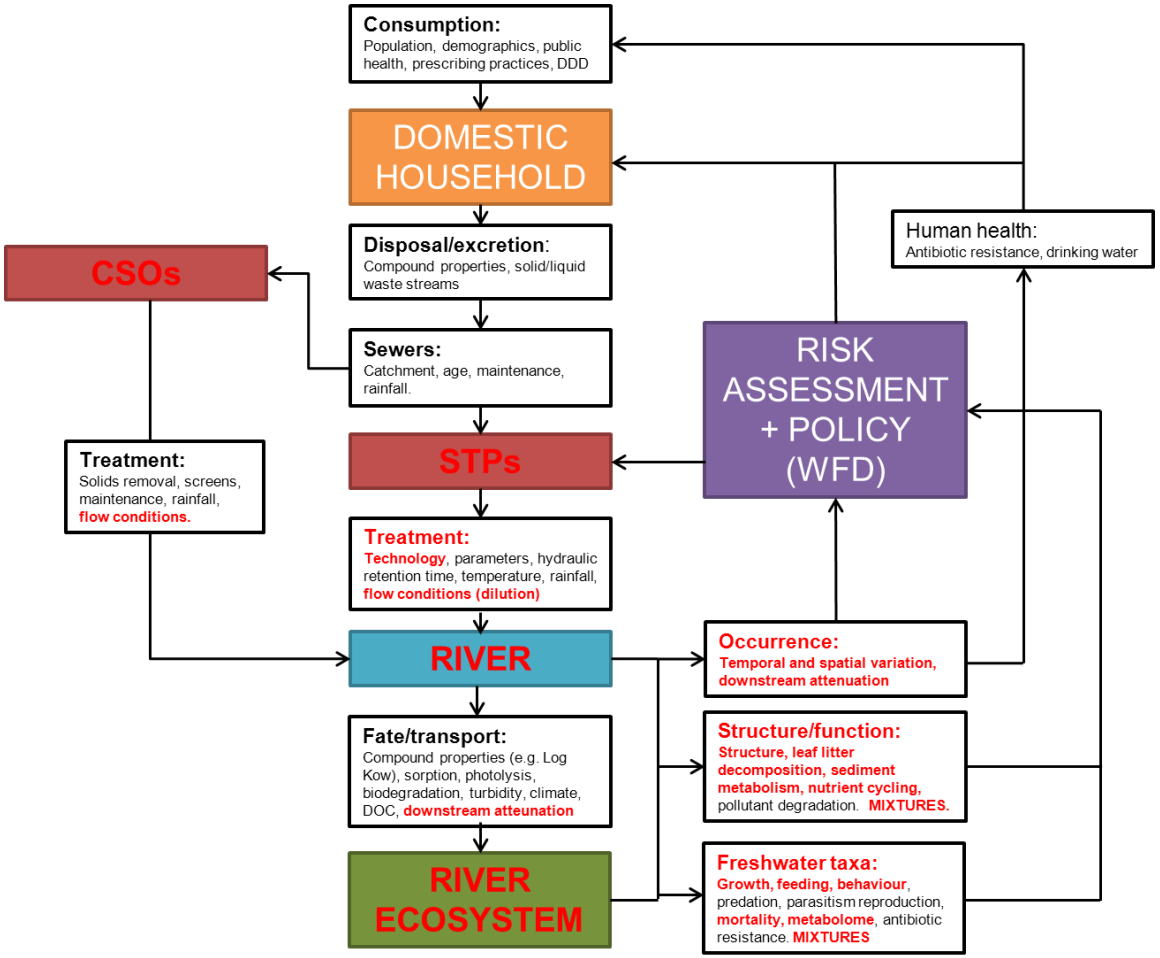


Figure 8-1: Conceptual summary of the pharmaceutical pollution of rivers via STP and CSO effluent [coloured boxes represent key reservoirs of pharmaceutical pollution, white boxes represent the key pathways and processes operating between and within these reservoirs. Text in bold red indicates research questions that have been specifically addressed as part of this thesis]

Aim 2: At what concentrations do pharmaceuticals occur in STP effluent, CSO effluent and receiving waters and how do these levels vary over temporal (diurnal and seasonal) and spatial (reach and catchment) scales?

This work has confirmed STP effluent as a major source of pharmaceutical pollution to receiving waters and has further identified CSO effluent as a significant secondary source (Figure 8-1). Significant spatial and temporal variation was evident across both STP effluent and receiving water concentrations (see Figure 8-2 for a conceptual summary of temporal changes). Conceptually, changes over hourly and diurnal scales are driven by peaks in domestic activity and individual wastewater pulses in the morning and mid to late evening. Variability may also be expressed at weekly scales in relation to higher levels of daily domestic consumption during the weekend when people are not at work. Finally, seasonal variability is driven by altering patterns of pharmaceutical consumption in relation to seasonal illnesses and also by changes in the removal efficiencies and receiving water dilution dependent on temperature, weather and flow.

Periods of high flow demonstrated greater frequencies of detection for pharmaceuticals but at generally lower concentrations than moderate or low flow conditions, suggesting that dilution may be an important mechanism for reducing ultimate receiving water concentrations at high flows. The greater detection frequencies suggest that STP overflow/bypasses or reduced STP removal efficiencies may also be acting as additional pharmaceutical sources to rivers at high flows. The consistent presence of pharmaceuticals in rivers with little or no attenuation along a 5 km study reach challenges some previous assumptions about in-stream degradation of such compounds and highlight the importance of locally relevant studies of pharmaceutical transport and fate in river ecosystems. In particular, local weather and flow conditions, turbidity, vegetation and suspended sediment may play a key role in transport and fate processes (Kunkel & Radke, 2011; Figure 8-3).

Seasonal trends were present in the receiving water and STP effluent concentrations of all compounds with winter months generally exhibiting elevated concentrations, which would be missed by shorter sampling campaigns (Figure 8-2). For example, Chapter 3 of this thesis (Hughes *et al*., 2013) showed that just 13 % of studies collected samples for 12 months or more suggesting that the majority of existing occurrence data is not accounting for such seasonal trends. Pharmaceuticals were confirmed as ubiquitous in both STP effluent, receiving waters and to a lesser extent in CSO effluent. New UK maximum concentrations were identified for diclofenac, erythromycin and ibuprofen (4388, 1378 and 93 863 ng L^{-1} respectively) in receiving waters and diclofenac in STP effluent (2830 ng L^{-1}). These concentrations represent a reduced margin of environmental safety for erythromycin and propranolol particularly

with some evidence of negative consequences for algae, invertebrates and fish at the low or sub µg L^{-1} range (Crane *et al*., 2006; Enick & Moore, 2007; Hughes *et al*., 2013; Santos *et al*., 2010). Furthermore, the consistent identification of multiple compounds within samples confirms the presence of pharmaceuticals as part of complex mixtures, which represents an additional risk for likely ecotoxicological effects. Finally, diclofenac was identified with a median receiving water concentration of 416 ng L^{-1} well in excess of the proposed Environmental Quality Standard limit of 10 ng L^{-1} for European Union member states (EC, 2012). This limit has now been postponed but if this were adopted after the current five year review period then it is considered that pharmaceutical pollution prevention will represent a significant financial and regulatory cost for the Environment Agency and UK water industry (UK Parliament, 2012).

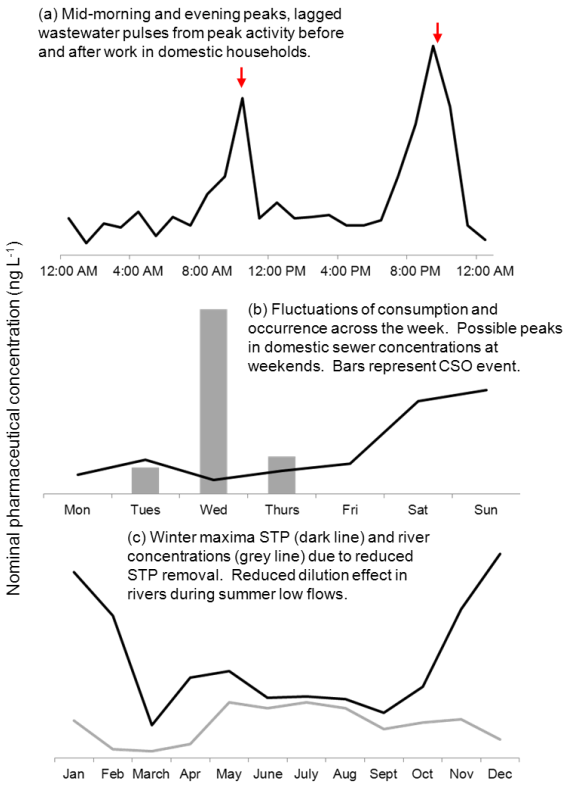


Figure 8-2: Conceptual summary of temporal changes in pharmaceutical concentrations in STP and CSO effluent and receiving waters across (a) diurnal, (b) weekly and (c) seasonal timescales

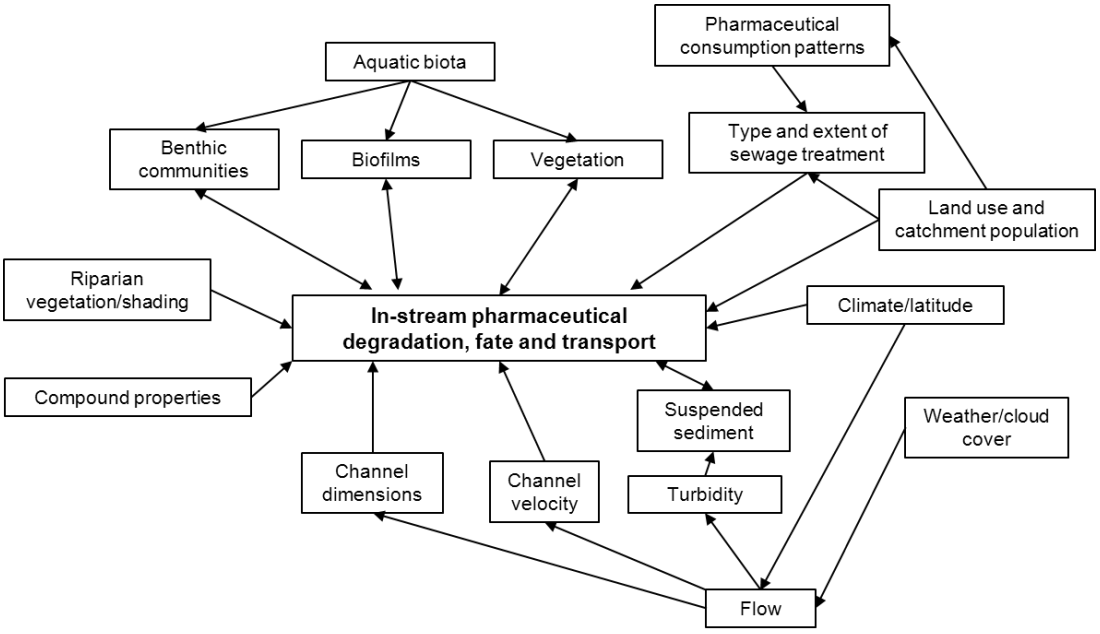


Figure 8-3: Summary of key processes and parameters affecting the fate and transport of pharmaceuticals in rivers
[note double headed arrows indicate feedbacks]

Aim 3: What lethal and sublethal impact does extended exposure to environmentally relevant concentrations of pharmaceuticals have on important freshwater taxa?

A 28 day exposure to environmentally realistic concentrations of erythromycin and a mixture with propranolol caused more than a doubling in mortality of the functionally important freshwater amphipod *Gammarus pulex* compared to controls but did not disrupt growth or feeding to a significant extent (Figure 8-1). Propranolol acting in isolation showed no significant effects on mortality, feeding or growth although similar patterns of effects to erythromycin and the mixture were observed. NMR spectroscopy environmental metabolomics was identified as a suitably sensitive technique for detecting sublethal effects of pharmaceuticals on the metabolome of *G. pulex* with potential future applications in biomarker development centred on the osmolyte TMAO. Surviving individuals demonstrated sublethal alterations to the metabolome associated with energy storage and metabolism. Extended exposure tests coupled with relatively straightforward NMR metabolomics analysis represents a promising area for future research into the effects of long-term low level exposure to pharmaceuticals on important freshwater taxa. Crucially, concentrations that have been readily detected in UK and global rivers were shown to elicit significant lethal and sublethal effects on *G. pulex*. Disruptions to the fitness, feeding rates, populations and communities of important shredders such as *G. pulex* are likely to have knock-on implications for leaf litter decomposition in rivers. Such impacts and potential linkages between individual lethal

and sublethal effects and wider ecosystem functioning are considered in the next section (Figure 8-4).

Aim 4: What impact does exposure to pharmaceuticals have on important aspects of ecosystem functioning?

The results highlight the importance of both microbial and macroinvertebrate communities in the decomposition of *Fagus sylvatica* leaves in freshwater ecosystems. However, environmentally relevant concentrations of five pharmaceuticals were found to have no significant effect on the microbially or macroinvertebrate mediated decomposition of leaf litter during both the autumn/winter and summer periods. However, the shredder *Asellus aquaticus* and Oligochaeta preferentially colonised leaf litter exposed to higher concentrations of erythromycin and ibuprofen which shows that shredders are selective feeders capable of differentiating between available foods based on their exposure history to pharmaceuticals. An examination of five pharmaceuticals in a mixture also showed no effects on leaf litter decomposition and colonisation by macroinvertebrates (Figure 8-1).

However, Chapter 5 (Aim 3) did indicate some significant sublethal and lethal effects on the important freshwater shredder *G. pulex* which may have the potential to propagate effects on wider ecosystem functioning (Figure 8-4). For example, direct mortality of *G. pulex* will reduce the overall decomposition rate attributable to shredders and sublethal changes to metabolism, energy storage and feeding may also alter shredder feeding rates, food selection and ultimately decomposition rates. In turn, shredder food selection and decomposition is altered by pharmaceuticals via the indirect effects of fungal and bacterial colonisation of leaf litter. Clearly, these effects are complex and mediated across food webs via bacterial, fungal and invertebrate predators which must be examined using a combination of single species laboratory tests and more complex experiments examining ecosystem structure and function. The experiments presented in Chapters 5 and 6 of this thesis represent important early steps in examining the integrated effects of pharmaceuticals in this way.

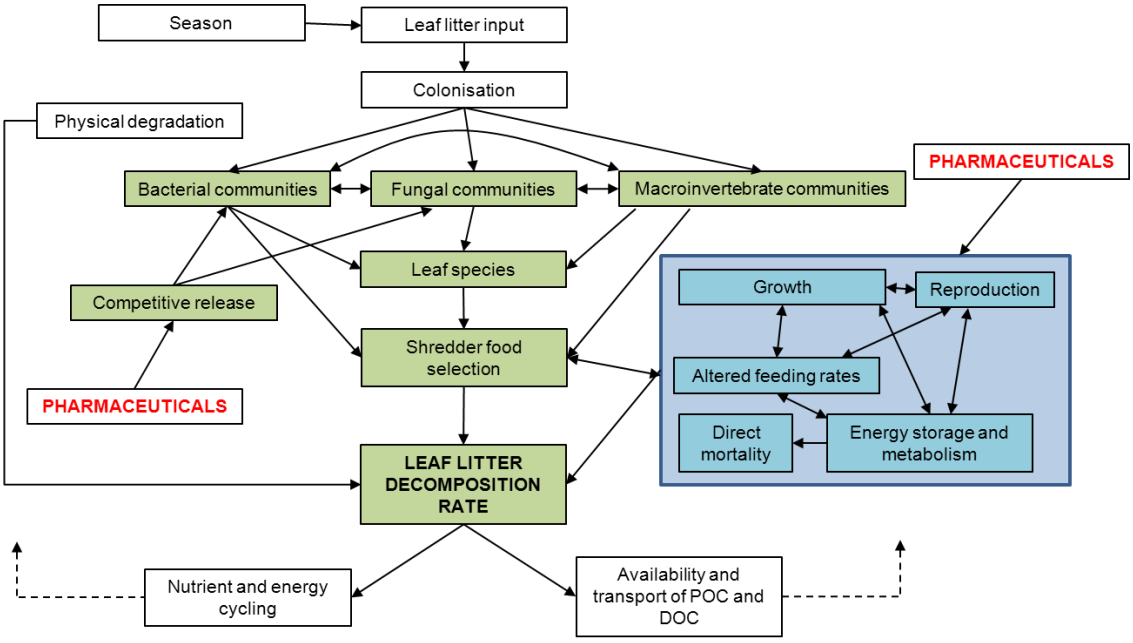


Figure 8-4: Conceptual summary outlining the linkage between the effects of pharmaceuticals on single shredder species and leaf litter decomposition in rivers [green boxes highlight aspects considered in Chapters 6 and 7 of this thesis and similar studies, blue boxes highlight aspects considered by Chapter 5 and others for effects on individual organisms]

Freshwater sediments exposed to high but environmentally relevant concentrations of erythromycin demonstrated reduced organic matter processing compared to controls with some suggestions of a dose-response relationship. Very high levels of erythromycin and low levels of propranolol caused a reduction in the rate of respiration of freshwater sediments. Some short term stimulation of respiration was also observed at high levels of propranolol. This may have been caused by microbial communities utilising pharmaceutical compounds as a nutritive source overriding any direct toxic effects. Erythromycin exposed sediments demonstrated increased ammonium and reduced phosphate exchange with overlying waters whereas phosphate exchange was enhanced in propranolol exposed sediments. No effects on dissolved organic carbon (DOC) exchange were observed in the individual compound exposures. Disruptions to ammonium exchange is important as this is the dominant source of nitrogen for many aquatic plant species and supplies energy for important nitrifying bacteria (Rheinheimer, 1985).

Sediments exposed to binary mixtures of erythromycin and propranolol demonstrated reductions in OM processing and DOC exchange up to three orders of magnitude below those displayed by pharmaceuticals acting in isolation (Figure 8-1). This suggests some form of interaction or additivity between the toxic effects of these compounds despite their differing mechanisms of action. Disruptions to DOC cycling have important consequences for contaminant transport, energy supply, nutrient cycling and drinking water treatment (Stewart & Wetzel, 1981; Wetzel, 1992; Alarcon-Herrera et

al., 1994; Lawlor & Tipping, 2003) and perhaps even global carbon cycles (Raymond & Bauer, 2001). The identification of significant effects on the functioning of microbial communities in sediments was contrary to the lack of effects identified on leaf litter decomposition in Chapter 6. This was likely due to the relative importance of fungal and bacterial communities within each of the functional processes. The preconditioning and decomposition of leaf litter is dominated by fungi (Gulis & Suberkropp, 2003a; 2003b) and competitive release of fungal communities may override any negative effects caused to bacterial communities by the presence of pharmaceuticals (Bundschuh *et al*., 2009). Contrary to this, respiration in sediments is dominated by bacteria (Smith, 1973) and as such, the capacity for fungi to maintain overall sediment respiration is reduced when pharmaceuticals exert a direct toxic effect on bacterial communities.

Laboratory examination of sediment respiration rates was identified as an appropriately sensitive tool for examining the effects of pharmaceuticals on freshwater ecosystem functioning. However, these interactions are complex and the magnitudes of change relatively small suggesting further work is required to fully understand the implications for wider freshwater ecosystems.

8.2 Implications and future research

This thesis has increased knowledge on the occurrence and effects of pharmaceuticals in freshwater ecosystems highlighting their consistent presence across the semi-rural to urban continuum and their potential to cause subtle but potentially significant effects on the structure and functioning of freshwater ecosystems. The research raises concerns surrounding the release of pharmaceutical compounds alongside the myriad other chemicals consumed and disposed of in domestic and commercial settings that routinely enter rivers via the sewage system. The work provides new information that can inform future management and policy decisions within the arenas of water quality and emerging contaminants. For example, the data presented on spatial and temporal variation in pharmaceutical concentrations will aid in the design of future monitoring campaigns capable of capturing such variability. The novel effects experiments presented here highlight pharmaceutical levels at significant lethal, sublethal and ecosystem-level effects manifest themselves and as such may prove useful in future risk assessment studies for new or existing pharmaceutical compounds. This work also identifies many important areas to prioritise for further research including:

8.2.1 Pharmaceutical pollution of rivers via STP and CSO effluent

Pharmaceutical pollution of rivers is now of major policy concern at the European level with the proposed inclusion of diclofenac (and the synthetic oestrogen, EE2) on the list of Hazardous Substances under Annex X of the Water Framework Directive (EC, 2000;

EC, 2012). An initial proposal suggested a mandatory environmental quality standard (EQS) for diclofenac of 10 ng L^{-1} which would have been exceeded by over 93 % (180 out of 194) of samples in this study representing a significant potential future cost for the UK water industry and Environment Agency. The proposed EQS was later dropped and diclofenac is now merely listed as a Priority Substance without an EQS. This decision was based on knowledge gaps and ambiguities in both the environmental occurrence and effects of diclofenac in European rivers (UK Parliament, 2012). Such a high exceedance of the proposed EQS compares poorly with pesticide pollution where only 6 % of samples exceeded the 100 ng L^{-1} EQS (EA, 2009c).

Clearly, if an EQS of 100 ng L^{-1} were mandated in the future, significant investment would be required by water companies to meet this target. The costs of additional advanced waste water treatment required were estimated at £27 billion over 20 years (UK Parliament, 2012). These costs would predominantly arise from the need to upgrade a substantial number of STPs in the UK with advanced treatment technologies capable of removing diclofenac (UK Parliament, 2012). This thesis has highlighted (Section 2.4.3.5) that technologies that are efficient at removing particular compounds often result in the same or reduced removal efficiencies for other pharmaceutical compounds. Ozonation of STP effluent perhaps represents the most promising treatment option (Zhang *et al*., 2010) although this would have significant associated capital and energy costs (Heberer, 2008). However, the estimated £27 billion investment required would not just reduce the pollution burden of diclofenac but would also drive remediation of many other compounds and help to reduce their combined effects in freshwater ecosystems. Therefore, any proposed or future EQS for diclofenac or similar pharmaceuticals should be seen as an opportunity to drastically improve the quality of STP effluent discharged to rivers considering all domestically consumed substances and not just individual pharmaceutical compounds. Furthermore, a cost of £27 billion over 20 years is equal to just over £1.35 billion per year which is low compared to the total of £10.4 billion spent annually on prescription and OTC drugs in England alone (see Section 2.3). Finally, such an investment compares favourably with current UK water industry investments. In the period 2010-2015 £22 billion was invested, a large proportion of which was into sewage treatment plants or associated environmental improvements (Ofwat, 2009).

The data presented in Chapters 3 and 4 of this thesis highlight the high degree of spatiotemporal variability in pharmaceutical pollution across the globe. This confirms the need for more intelligent, representative monitoring strategies that are capable of capturing spatiotemporal variation in order to fully understand the true extent of pharmaceutical pollution in rivers (Hughes *et al*., 2013; Ort *et al*., 2010a; 2010b). Continuous monitoring, composite samplers and passive integrative sampling

techniques represent the best options to fully quantify the variable pharmaceutical levels in complex STP and receiving water systems (Zhang *et al.*, 2008; MacLeod & Wong, 2010; Jacquet *et al.*, 2012). Future monitoring studies should provide adequate details on their sampling techniques and strategies and monitoring sites in order to facilitate meaningful comparisons between studies. Such monitoring campaigns should address both long-term seasonal trends and the short term diurnal trends identified here and elsewhere (Kanda *et al*., 2003; Ort *et al*., 2010a; 2010b).

An ideal monitoring campaign would consider temporal variation at hourly, diurnal and weekly scales using continuous autosampling techniques and at seasonal scales using repeated grab sampling and passive integrative samplers (Figure 8-5). Spatial variability should be addressed at a range of scales including at key locations within sewer catchments, perhaps by direct sampling of sewers for untreated wastewater. Catchment scale monitoring should consider isolated rural areas where agricultural runoff (Kay *et al*., 2004), sewer misconnections (Ellis, 2006) or septic tanks may contribute low levels (USEPA, 2010) and smaller semi-rural STPs which may pose a particular risk (MacLead & Wong, 2010). Reach sampling examining the downstream fate and transport of pharmaceuticals should be conducted across both rural and urban settings. Comparisons should be made between STPs encompassing a range of populations and technologies with consideration of both influent and effluent to allow quantification of STP removal efficiencies. STPs serving populations with differing demographics, and those containing large hospitals or care facilities should also be included (Daughton, 2003; Kümmerer, 2001a). Finally, future monitoring studies should seek to expand the coverage of compound classes to those that until now have been poorly studied or demonstrate significant environmental risk (Hughes *et al*., 2013).

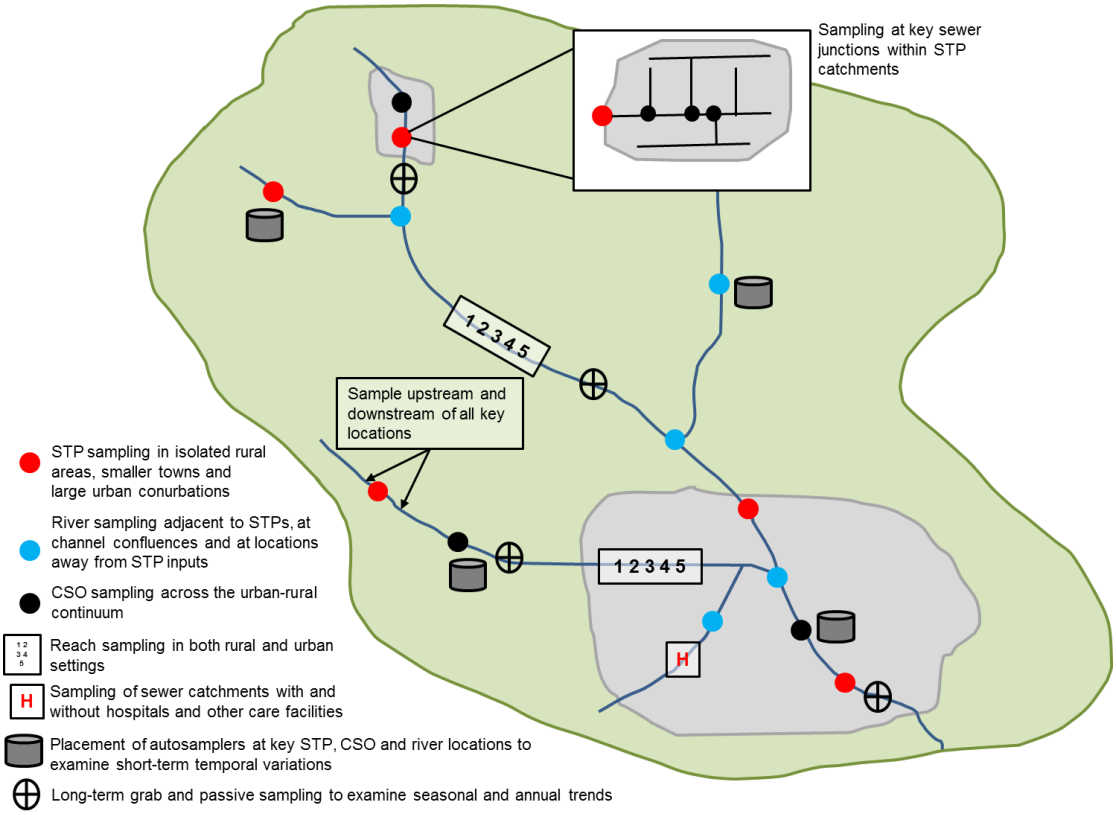


Figure 8-5: Schematic of idealised pharmaceutical monitoring project

More novel monitoring techniques (biosensors) are now becoming available which use enzymes or other biomolecules that can detect analytes such as pesticides in extremely low concentrations (e.g. 10^{-10} M) in environmental samples (Vakurov *et al.*, 2004; Palchetti *et al.*, 2009). Molecularly imprinted polymers (MIPs) mimic the natural action of enzymes and are designed to recognise and retain specific template molecules and have shown great promise in improving the extraction of analytes (i.e. SPE) in complex samples (Tse-Sum-Bui & Haupt, 2010). MIPs have already been successfully applied to the extraction and analysis of pharmaceuticals (including diclofenac and ibuprofen) in surface water, waste water and sediment and show great promise in dramatically improving sample extraction and limits of detection (Qiao *et al.*, 2006; Sun *et al.*, 2008; Demeestere *et al.*, 2010; Duan *et al.*, 2013). However, SPE still represents a useful and straightforward method of sample cleanup and preconcentration which is confirmed by the low limits of detection (5 ng L^{-1}) achieved in this thesis and elsewhere (Petrovic *et al*., 2006).

The lack of downstream attenuation over a reach of approximately 5 km suggests that sampling effort should also address stretches of river not within the immediate vicinity of known waste water or STP effluent discharges. A recent prioritisation exercise for antibiotics and anti-cancer drugs suggested that monitoring effort be focused around the major European urban areas (Oldenkamp *et al.*, 2013). However, other studies have suggested that smaller STPs in semi-rural areas or smaller towns should be monitored

as these may be more susceptible to pharmaceutical pollution (Macleod & Wong, 2010). Clearly, much further monitoring is required but risk assessment models (e.g. GREAT-ER; Schowanek *et al*., 2001) should fully account for smaller STPs and in-stream fate/transport processes ideally through the collection of locally relevant laboratory and field data. Metabolites and transformation products should also be seen as a research priority for both their environmental occurrence and ecotoxicology given their potential to demonstrate similar and even enhanced toxicity compared to their parent compounds (La Farré *et al*., 2008). Furthermore, they are likely to be present in freshwater systems at concentrations comparable or greater than parent compounds given that the compounds studied here are excreted predominantly in the form or metabolised or conjugated transformation products (Chapter 4).

8.2.2 The effects of pharmaceuticals on important freshwater taxa and the structure and function of freshwater ecosystems

The sublethal and lethal effects demonstrated here on *G. pulex*, sediment metabolism, nutrient exchange and colonisation of leaf litter were all manifested at concentrations previously measured in the surface waters of the UK and similar western developed nations (Chapter 4; Calisto & Esteves, 2009; Hughes *et al*., 2013; Santos *et al*., 2010). Furthermore, these effects concentrations have been exceeded by several orders of magnitude in studies examining polluted rivers in China, India and the United States (Larsson *et al*., 2007, Li *et al*., 2008; Phillips *et al*., 2010) demonstrating the distinct likelihood that such complex and negative effects are occurring in freshwater ecosystems across the globe.

8.2.2.1 Leaf litter decomposition

Although the results presented here did not demonstrate effects of pharmaceuticals on leaf litter decomposition, both inhibitory and stimulatory responses have been demonstrated elsewhere (Bundschuh *et al*., 2009; Hahn & Schulz, 2007) which suggests that much further research is required to reconcile reasons for these differences. Research effort should be expanded to focus on previously unstudied antibiotics and other high risk pharmaceutical groups (e.g. antidepressants), different leaf species, longer exposure periods and more complex mixtures of pharmaceuticals and other emerging contaminants. Further examination of the fungal and microbial contribution to leaf litter colonisation and decomposition should also be a priority as it is likely that these elements are more susceptible to the presence of pharmaceuticals, particularly antibiotics (Schmitt *et al*., 2005). Complex competitive mechanisms between fungi and bacteria have been demonstrated which can have knock-on effects for decomposition rates and food selection by macroinvertebrates (Gulis & Suberkropp, 2003b). Techniques for estimating bacterial abundance (e.g. epifluorescence

microscopy; Buesing, 2005) and fungal biomass using the indicator ergosterol (Mille-Lindblom *et al*., 2004) are available and should be applied to unravel these intricate but important microbial systems. Environmental metagenomics is a further promising research area that has already proven capable in characterising diverse microbial communities in large rivers and the effects of pollutants on them (George *et al*., 2010; Ghai *et al*., 2011).

8.2.2.2 Sediment respiration and nutrient cycling

Disruptions to the turnover of particulate and dissolved carbon and nutrients in freshwater sediments and the water column has implications for whole ecosystem efficiency by altering the degree and duration of storage within sediments (Battin, 1999; Battin *et al*., 2003) and also wider catchment scale impacts manifested through changes in downstream nutrient and carbon fluxes (Rutherford & Hynes, 1987). Disruptions to the cycling of DOC will also have implications for the supply of carbon and energy to higher trophic levels through the 'microbial loop' (Meyer, 1994) and the transport of organic pollutants such as pesticides (Carter & Suffet, 1982) and pharmaceuticals (Figure 8-6). Changes to carbon cycling and transport within rivers has important implications as storage within river systems and the transport of carbon to shallow ocean sediments are a major component of the global carbon cycle (Hope *et al.*, 1994). Future research in this area should also expand to include other high risk pharmaceutical groups, particularly antibiotics, given the important role that microbial communities play in these processes (Rutherford & Hynes, 1987). Quantification of changes to microbial communities using the same techniques for leaf litter mentioned earlier should also be considered.

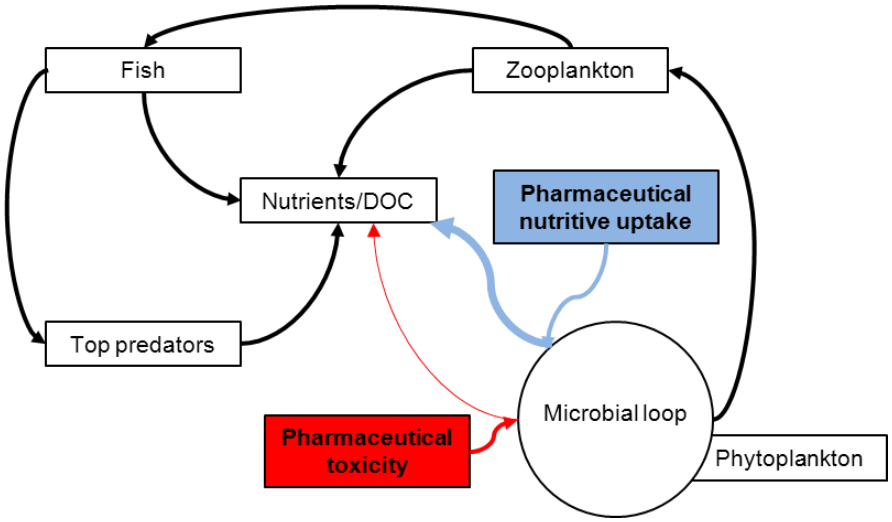


Figure 8-6: The effect of pharmaceuticals on the microbial loop [modified from Moloney *et al*., 2011, line thickness indicates process turnover affected by pharmaceutical pollution]

A combination of laboratory and *in situ* techniques will allow researchers to understand how effects are mediated under variable environmental conditions (Boynton *et al.*, 1981; Hedin, 1990; Wagner-Dobler *et al.*, 1992; Mermillod-Blondin *et al.*, 2005). Finally, the use of microcosms and mesocosms should be considered which will allow the evaluation of effects from the individual scale, through to populations, communities and ecosystem functioning mediated via complex food webs (Brown *et al*., 2011). Microcosms containing simple freshwater ecosystems have already been successfully applied in identifying the effects of pharmaceuticals on algae, macrophytes, invertebrates and fish with some effects at the population and community level (e.g. Brain *et al*., 2004; Brodin *et al*., 2013; Richards *et al*., 2004). However, their use should be expanded to bridge the gap between more simplified laboratory experiments and the complex, dynamic exposure conditions exhibited in the freshwater environment allowing researchers to examine effects across multiple trophic levels.

8.2.2.3 Environmental metabolomics

NMR spectroscopic environmental metabolomics was identified in this thesis as a suitably sensitive technique to identify sub-lethal effects of pharmaceuticals on freshwater taxa. As such, this technique should be expanded to examine the effects of other pharmaceutical groups that demonstrate a high risk of toxicity to freshwater macroinvertebrates and fish such as painkillers, antidepressants and cardiovascular drugs (Hughes *et* al., 2013; Jones *et al*., 2002). However, future studies should ensure to employ phenotypic anchoring by size, age and gender wherever possible as noise associated with these factors is one of the key limitations of metabolomics techniques (Hines *et al*., 2007; Viant, 2007). Furthermore, sexual dimorphism in Gammarids is known to influence growth and feeding (Sutcliffe *et al.*, 1981; Greenwood & Adams, 1984) which in turn will have implications for their metabolic profile. Expansion of these techniques to other sensitive freshwater taxa such as mayfly (Ephemeroptera) and stonefly larvae (Plecoptera) should also be considered (Peterson *et al.*, 2001; Arciszewski *et al.*, 2011). The significantly increased mortality of *G. pulex* should be studied further to examine whether such changes would be significant at the population level. A variety of population models have been developed which may prove useful in answering this question and may also help in identifying sensitive life stages (Sauer & Pendleton, 2002).

8.3 Conclusions

Human-use pharmaceuticals have been found to be ubiquitous and important pollutants in both semi-rural and urban river systems capable of eliciting significant lethal, sublethal and metabolic effects on important freshwater taxa and aspects of ecosystem

structure and functioning. Continued research into the occurrence of pharmaceuticals in STP effluent and freshwaters should use the widely developing toolkit of sampling techniques and strategies in order to fully quantify spatiotemporal variation of pharmaceuticals and their associated metabolites and transformation products. Ecotoxicological research should aim to maximise environmental relevance and to combine standard laboratory procedures with more novel investigations of metabolomics and ecosystem structure and function both in the laboratory and *in situ*.

Until the last ten years, the main thrust of water quality improvement was the reduction of organic pollution and nutrients in the effluent of STPs. With the introduction of the Water Framework Directive (EC, 2000), regulatory effort has switched to a focus on more integrated, biological indicators of water quality. If the targets of this Directive are ever to be met, then the myriad stressors affecting water quality beyond nutrients, organic pollution and hydromorphological alteration must be addressed. This includes pharmaceuticals and the thousands of emerging contaminants that routinely enter rivers due to consumption in normal domestic settings. This work provides valuable new information on their occurrence and effects that can be used towards this goal.

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10 APPENDICES

10.1.1 Appendix A1: Global pharmaceutical occurrence database

This appendix presents the full database of pharmaceutical occurrence in global rivers assembled as part of the below paper:

Hughes, S. R., P. Kay and L. E. Brown (2013). "Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems." *Environmental Science & Technology* **47**(2): 661-677.

The full database is available on the attached CD-ROM. Alternatively, an excel spreadsheet version of the database and associated reference list is available online by following the below instructions:

To access the database, please go to http://www.wateratleeds.org/ and log into the Associate Members Area (on right hand side of page):
Username: pharmadata
Password: openaccess
Once you are logged in, cut and paste this link into the address bar: http://www.wateratleeds.org/members-area/paper-reviews-and-materials/quality/

10.2 Appendix B: Pharmaceutical monitoring data

10.2.1 Appendix B1: Aire and Calder gauge information

Table B-1: Summary of Environment Agency flow gauges used in this study

Station name (ID)	Watercourse	Record start date	OS NGR	QMED[1] (m^3/s)
Oulton Fleet Weir (8079)	River Aire	September 1985	SE 38100 28100	11.24
Farrer Lane (8075)	Oulton Beck	November 1986	SE 36500 28100	0.09
Armley (8009)	River Aire	January 1972	SE 28057 34020	8.83
Keighley (8109)	River Worth	December 1980	SE 63690 40866	0.77
Ripponden (8172)	River Ryburn	March 1981	SE 35470 18938	0.32
Heaton Lodge (8096)	River Calder	March 1996	SE 17915 20591	Median stage 0.30m
Horbury (8100)	River Calder	June 2000	SE 29012 17748	Median stage 0.34m

10.2.2 Appendix B2: Sample physicochemical data

Table B-2: Physicochemical data from routine monitoring samples

Site	Matrix	Date	pH	DO (mg/L)	Conductivity (µs/cm)
Garforth Owlwood	Channel	31/03/2011	7.32	9.12	1536
	Effluent channel	31/03/2011	7.08	8.61	1578
Heaton Lodge	Channel	31/03/2011	7.70	10.74	872
	Effluent	31/03/2011	7.10	9.61	2540
Horbury Junction	Channel	31/03/2011	7.50	9.49	977
	Effluent channel	31/03/2011	7.20	8.00	1099
Knostrop	Channel	31/03/2011	7.10	9.60	1869
	Effluent channel	31/03/2011	7.00	9.69	1898
Oulton	Channel	31/03/2011	7.50	9.75	1466
	Effluent	31/03/2011	7.10	9.88	1414
Oxenhope No. 2	Channel	31/03/2011	7.70	11.21	259
	Effluent	31/03/2011	7.07	8.12	796
Ripponden Wood	Channel	31/03/2011	7.30	10.95	297
	Effluent	31/03/2011	6.40	8.25	584
Garforth Owlwood	Channel	03/05/2011	7.50	9.74	1579
	Effluent channel	03/05/2011	7.30	9.10	1612
Heaton Lodge	Channel	03/05/2011	7.50	10.85	1121
	Effluent	03/05/2011	7.00	9.90	3560
Horbury Junction	Channel	03/05/2011	7.50	10.10	1195

	Effluent channel	03/05/2011	7.10	8.51	1148
Knostrop	Channel	03/05/2011	7.49	11.13	1667
	Effluent channel	03/05/2011	7.20	10.06	1682
Oulton	Channel	03/05/2011	7.66	9.90	1569
	Effluent	03/05/2011	7.25	9.85	1476
Oxenhope No. 2	Channel	03/05/2011	7.70	11.21	259
	Effluent	03/05/2011	7.07	8.12	796
Ripponden Wood	Channel	03/05/2011	7.30	10.95	297
	Effluent	03/05/2011	6.40	8.25	584

Garforth Owlwood	Channel	31/05/2011		9.07	1261
	Effluent channel	31/05/2011		8.47	1263
Heaton Lodge	Channel	31/05/2011		10.04	682
	Effluent	31/05/2011		9.93	2280
Horbury Junction	Channel	31/05/2011		9.97	901
	Effluent channel	31/05/2011		9.16	918
Knostrop	Channel	31/05/2011		10.16	1270
	Effluent channel	31/05/2011		10.01	1287
Oulton	Channel	31/05/2011		9.71	1224
	Effluent	31/05/2011		9.55	1318
Oxenhope No. 2	Channel	31/05/2011		10.67	254
	Effluent	31/05/2011		9.22	763
Ripponden Wood	Channel	31/05/2011		10.92	325
	Effluent	31/05/2011		10.85	663

Garforth Owlwood	Channel	05/07/2011	7.90	9.12	1375
	Effluent channel	05/07/2011	7.90	8.98	1381
Heaton Lodge	Channel	05/07/2011	7.30		990
	Effluent	05/07/2011	7.20		2017
Horbury Junction	Channel	05/07/2011	7.52		1073
	Effluent channel	05/07/2011	7.40		1105
Knostrop	Channel	05/07/2011	7.50		1508
	Effluent channel	05/07/2011	7.40		1504
Oulton	Channel	05/07/2011	8.00		1069

	Effluent	05/07/2011	8.30		907
Oxenhope No. 2	Channel	05/07/2011	7.60		208.1
	Effluent	05/07/2011	6.70		725
Ripponden Wood	Channel	05/07/2011	7.60		275
	Effluent	05/07/2011	6.60		646

Garforth Owlwood	Channel	27/07/2011	8.13	9.67	1342
	Effluent channel	27/07/2011	8.03	9.57	1285
Heaton Lodge	Channel	27/07/2011	7.51	9.08	867
	Effluent	27/07/2011	6.88	8.73	3180
Horbury Junction	Channel	27/07/2011	7.52	8.68	1004
	Effluent channel	27/07/2011	7.39	7.87	1067
Knostrop	Channel	27/07/2011	7.54	9.33	1555
	Effluent channel	27/07/2011	7.44	9.09	1564
Oulton	Channel	27/07/2011	7.76	8.54	1184
	Effluent	27/07/2011	8.03	9.52	799
Oxenhope No. 2	Channel	27/07/2011	7.61	9.63	224
	Effluent	27/07/2011	6.59	7.92	686
Ripponden Wood	Channel	27/07/2011	7.76	9.60	271
	Effluent	27/07/2011	6.55	7.66	680

Garforth Owlwood	Channel	30/09/2011	7.53	9.77	210.9
	Effluent channel	30/09/2011	6.73	7.52	649
Heaton Lodge	Channel	30/09/2011	7.51	9.16	770
	Effluent	30/09/2011	7.19	9.1	3210
Horbury Junction	Channel	30/09/2011	7.42	7.53	871
	Effluent channel	30/09/2011	7.45	3.31	1140
Knostrop	Channel	30/09/2011	7.57	9.21	1557
	Effluent channel	30/09/2011	7.39	8.98	1585
Oulton	Channel	30/09/2011	7.82	8.86	1272
	Effluent	30/09/2011	7.35	8.52	1409
Oxenhope No. 2	Channel	30/09/2011	7.53	9.77	210.9
	Effluent	30/09/2011	6.73	7.52	649
Ripponden Wood	Channel	30/09/2011	7.28	9.85	278

	Effluent	30/09/2011	6.47	6.21	624

Garforth Owlwood	Channel	31/10/2011	7.71	8.33	1334
	Effluent channel	31/10/2011	7.47	8.84	1336
Heaton Lodge	Channel	31/10/2011	7.36	9.74	577
	Effluent	31/10/2011	6.90	9.62	2670
Horbury Junction	Channel	31/10/2011	7.37	9.19	696
	Effluent channel	31/10/2011	7.25	7.72	999
Knostrop	Channel	31/10/2011	7.41	9.48	1215
	Effluent channel	31/10/2011	7.17	9.09	1223
Oulton	Channel	31/10/2011	7.80	9.16	1137
	Effluent	31/10/2011	7.27	9.04	1299
Oxenhope No. 2	Channel	31/10/2011	7.38	9.66	240
	Effluent	31/10/2011	6.80	8.54	617
Ripponden Wood	Channel	31/10/2011	7.33	9.64	274
	Effluent	31/10/2011	6.37	7.55	576

Garforth Owlwood	Channel	01/11/2011	8.00	9.50	293.00
	Effluent channel	01/11/2011	8.80	9.40	841.00
Heaton Lodge	Channel	01/11/2011	8.50	10.90	1015.00
	Effluent	01/11/2011	7.00	9.90	3410.00
Horbury Junction	Channel	01/11/2011	8.70	9.80	1102.00
	Effluent channel	01/11/2011	8.00	4.00	1005.00
Knostrop	Channel	01/11/2011	9.00	10.20	1332.00
	Effluent channel	01/11/2011	7.40	11.00	1865.00
Oulton	Channel	01/11/2011	8.90	9.70	1197.00
	Effluent	01/11/2011	8.00	9.70	1422.00
Oxenhope No. 2	Channel	01/11/2011	8.70	10.30	252.00
	Effluent	01/11/2011	7.10	9.50	728.00
Ripponden Wood	Channel	01/11/2011	8.10	10.60	294.00
	Effluent	01/11/2011	7.30	8.10	668.00

Garforth Owlwood	Channel	01/12/2011	8.20	9.20	804
	Effluent channel	01/12/2011	7.60	9.30	1304

Heaton Lodge	Channel	01/12/2011	8.00	10.10	1021
	Effluent	01/12/2011	7.10	9.90	3041
Horbury Junction	Channel	01/12/2011	9.00	10.00	855
	Effluent channel	01/12/2011	8.00	8.00	1018
Knostrop	Channel	01/12/2011	8.60	10.10	1522
	Effluent channel	01/12/2011	7.40	11.00	1441
Oulton	Channel	01/12/2011	8.30	9.40	1274
	Effluent	01/12/2011	8.10	9.20	951
Oxenhope No. 2	Channel	01/12/2011	8.10	11.00	209
	Effluent	01/12/2011	7.40	8.10	663
Ripponden Wood	Channel	01/12/2011	8.30	10.00	322
	Effluent	01/12/2011	7.00	9.00	667

Garforth Owlwood	Channel	01/01/2012	9	9.6	615
	Effluent channel	01/01/2012	7.8	8.6	867
Heaton Lodge	Channel	01/01/2012	8.00	10.8	781
	Effluent	01/01/2012	7.30	9	3501
Horbury Junction	Channel	01/01/2012	8.80	10.60	864
	Effluent channel	01/01/2012	8.20	7.50	923
Knostrop	Channel	01/01/2012	8.90	10.60	1463
	Effluent channel	01/01/2012	7.10	10.40	1305
Oulton	Channel	01/01/2012	8.10	9.50	1360
	Effluent	01/01/2012	8.00	9.40	978
Oxenhope No. 2	Channel	01/01/2012	8.50	11.40	221
	Effluent	01/01/2012	7.20	9.00	633
Ripponden Wood	Channel	01/01/2012	8.40	10.30	311
	Effluent	01/01/2012	7.20	8.40	679

Garforth Owlwood	Channel	01/02/2012	8.6	9.2	999
	Effluent channel	01/02/2012	8.1	8.2	1249
Heaton Lodge	Channel	01/02/2012	8.50	11	1037
	Effluent	01/02/2012	7.70	9.2	2680
Horbury Junction	Channel	01/02/2012	8.20	9.10	725
	Effluent channel	01/02/2012	8.30	5.20	1126

Knostrop	Channel	01/02/2012	8.40	11.00	1779
	Effluent channel	01/02/2012	7.10	9.00	1291
Oulton	Channel	01/02/2012	8.70	9.80	1385
	Effluent	01/02/2012	8.40	9.40	800
Oxenhope No. 2	Channel	01/02/2012	8.70	11.00	244
	Effluent	01/02/2012	7.30	9.40	757
Ripponden Wood	Channel	01/02/2012	8.70	10.20	284
	Effluent	01/02/2012	7.70	9.30	638

Table B-3: Physicochemical data from River Aire reach samples

Sample	Distance D/S (m)	Date	pH	DO (mg/L)	Conductivity (µs/cm)
AIRE001	0	13/06/2011	7.8	9.66	1375
AIRE002	100	13/06/2011	7.6		
AIRE003	200	13/06/2011	7.8	9.42	1273
AIRE004	300	13/06/2011	8.1	8.53	1217
AIRE005	400	13/06/2011	8.3	8.87	1193
AIRE001	0	04/07/2011	8	9.07	1377
AIRE002	100	04/07/2011	7.9		
AIRE003	200	04/07/2011	7.9	8.98	1395
AIRE004	300	04/07/2011	7.7	8.92	1410
AIRE005	400	04/07/2011	7.7	9.03	1344
AIRE001	0	11/07/2011	8.1	10.02	794
AIRE002	100	11/07/2011	8.1		
AIRE003	200	11/07/2011	8	9.92	907
AIRE004	300	11/07/2011	7.8	9.23	921
AIRE005	400	11/07/2011	7.9	9.22	991
AIRE001	0	13/10/2011	8.08	10.48	428
AIRE002	100	13/10/2011	8.13	10.22	448
AIRE003	200	13/10/2011	7.97	9.99	473
AIRE004	300	13/10/2011	7.99	9.95	472
AIRE005	400	13/10/2011	7.97	10.09	568
AIRE001	0	20/10/2011	8.09	10.47	702
AIRE002	100	20/10/2011	8.1	10.58	779
AIRE003	200	20/10/2011	7.9	10.61	831
AIRE004	300	20/10/2011	8.05	10.32	806
AIRE005	400	20/10/2011	7.97	10.64	793
AIRE001	0	17/11/2011	7.87	10.27	1022

AIRE002	100	17/11/2011	7.92	10.21	1075
AIRE003	200	17/11/2011	7.77	10.09	801
AIRE004	300	17/11/2011	7.96	9.65	1099
AIRE005	400	17/11/2011	7.88	9.67	1090

Table B-4: Physicochemical data from intensive diurnal river samples

Garforth 19/7/11				Oulton 19/07/2011			
Time	pH	DO	Cond	Time	pH	DO	Cond
05:05:00	8.05	9.45	1277	05:45:00	7.77	9.04	1122
07:50:00	8.01	9.13	1242	08:25:00	7.8	9.4	1043
11:05:00	7.9	9.44	1225	11:35:00	7.9	8.89	986
13:50:00	8.1	9.28	1219	14:20:00	8	9.35	956
16:50:00	8.3	9.05	1233	17:20:00	8	9.36	970
19:55:00	8.1	9.6	1265	20:25:00	7.96	9.88	986
22:35:00	8.08	9.43	1279	23:10:00	7.92	9.64	974
Garforth 25/7/11				Oulton 25/7/11			
Time	pH	DO	Cond	Time	pH	DO	Cond
05:05:00	7.4	9.42	1352	05:40:00	7.1	8.21	1175
07:55:00	7.3	9.83	1360	08:30:00	7.3	8.2	1152
11:15:00	7.3	10.02	1352	11:50:00	7.1	8.39	1164
13:50:00	7.9	9.31	1325	14:25:00	7.4	8.89	1141
17:15:00	7.1	9.43	1331	17:45:00	7	9.55	1116
19:55:00	7.7	9.65	1327	20:25:00	7.7	9.87	1144
22:50:00	8	9.98	1343	23:20:00	7.7	10.41	1173

10.2.3 Appendix B3: Pharmaceutical concentration data

Table B-5: Erythromycin concentrations (ng L^{-1}) in STP effluent and receiving waters of the Rivers Aire and Calder (2010 to 2012)

	Garforth Owlwood		Heaton Lodge		Horbury Junction		Knostrop Lower		Oulton/Lemonroyd		Oxenhope No. 2		Ripponden	
Month	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel
September 2010	812.7	684.7	396.3	138.0	777.5	293.7	266.2	213.7	27.5	76.2	67.9	100.3	687.5	55.1
October 2010	1757.2	778.5	407.7	64.0	793.1	165.7	372.1	350.2	1105.6	183.1	1089.2	13.6	280.8	8.7
November 2010	329.7	1377.8	10.7	179.5	19.0	161.3	30.5	844.5	40.5	278.2	64.3	19.6	96.5	29.8
December 2010	947.9	814.5	585.6	317.0	38.0	242.9	465.6	678.0	300.0	356.6	nd	56.3	nd	7.9
January 2011	44.0	539.3	12.0	262.2	27.3	317.8	15.6	627.2	36.1	450.5	256.5	110.1		nd
February 2011		190.6		337.8	88.4	275.9		292.3	487.9	92.9	416.4		8.2	
March 2011	8.4	39.0	27.7	9.3	62.2	86.0	31.1	34.9	52.9	28.9	76.1	51.4	89.4	7.1
April 2011	8.9	38.3	nd	11.2	2.5	27.8	33.3	31.2	45.0	2.5	21.0	nd	nd	2.5
May 2011	24.6	2.5	nd	nd		40.4	5.0	81.5	2.5	45.5	nd	nd	nd	2.5
June 2011	701.1	nd	1020.1	42.5	25.0	53.1	6.7	30.8	390.2	254.9	1856.8	131.7	44.8	61.9
July 2011	69.3	430.9	2.5	412.7	214.4	8.4	1215.9	2.5	51.4	2.5	731.7	21.3	782.0	30.2
August 2011	161.5	60.7	102.0	112.3	286.5	131.0	54.2	132.2	409.1	85.5	232.5	16.9	113.3	29.0
September 2011	252.8	142.4	398.9	225.5	105.5	182.9	80.8	126.2	184.5	164.8	161.2	70.1	170.3	69.5
October 2011	360.3	375.5	157.7	68.2	184.9	154.7	105.2	69.7	401.9	92.8	461.8	43.2	130.0	23.4
November 2011	19.2	272.2	135.5	30.9	2.5	20.7	2.5	74.1	10.9	50.8	62.5	2.5		10.9
December 2011			133.0	81.6	291.6	11.7	6.6	152.4	23.8	58.4	409.4	29.8		20.1
January 2012	597.9	447.6	274.9	139.8	422.4	2.5	302.5	386.6	581.0	260.6	190.3	22.4	241.7	173.8
February 2012	200.0	250.5	70.9	5.3	55.1	2.5	143.9	2.5	333.2	2.5	282.8	nd		7.1

Notes: limit of detection (LOD) was 5 ng L^{-1}. Values of 2.5 above indicate samples where a signal was present at a signal: noise ratio > 3 but below the LOD, indicating the sample was detected but not quantified. nd indicates no signal detected.

Table B-6: Propranolol concentrations (ng L^{-1}) in STP effluent and receiving waters of the Rivers Aire and Calder (2010 to 2012)

	Garforth Owlwood		Heaton Lodge		Horbury Junction		Knostrop Lower		Oulton/Lemonroyd		Oxenhope No. 2		Ripponden	
Month	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel
September 2010	17.7	14.2	9.7	2.5	9.1	7.2	22.3	24.0	27.5	2.5	nd	2.5	12.1	nd
October 2010	37.5	16.4	19.4	nd	7.9	2.5	26.7	24.7	32.7	10.1	25.0	5.8	5.4	nd
November 2010	2.5	15.9	nd	6.2	nd	2.5	nd	19.8	nd	9.2	nd	nd	16.7	nd
December 2010	24.1	12.9	12.9	nd	31.3	nd	41.2	16.7	28.3	6.8	nd	nd	nd	nd
January 2011	nd	34.2	5.4	7.6	2.5	7.4	2.5	63.0	6.1	20.8	20.0	2.5		nd
February 2011		19.3		2.5	19.6	5.5		17.4	16.2	6.7	20.8		2.5	
March 2011	nd	2.5	2.5	2.5	6.4	nd	2.5	6.2	5.7	2.5	2.5	nd	5.7	nd
April 2011	nd	2.5	nd	nd	nd	2.5	2.5	6.1	5.8	nd	2.5	nd	nd	nd
May 2011	nd	nd	nd	nd		nd	2.5	25.3	2.5	7.7	nd	nd	nd	nd
June 2011	39.2	nd	19.9	nd	2.5	2.5	8.6	24.3	2.5	25.1	35.4	2.5	nd	2.5
July 2011	12.9	33.2		21.8	34.5	6.1	34.8	nd	nd	nd	8.7	nd	12.0	2.5
August 2011	29.2	18.1	13.2	2.5	6.9	nd	17.4	18.3	36.2	6.9	16.9	nd	18.4	2.5
September 2011	146.4	28.3	12.0	2.5	22.9	5.4	22.5	165.0	15.1	15.6	27.1	2.5	7.8	6.0
October 2011	32.6	12.9		2.5	11.3	5.3	26.0	21.0	16.8	2.5	11.2	nd	10.1	nd
November 2011	nd	22.0	11.0	nd	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5		2.5
December 2011			8.9	2.5	9.6	2.5	2.5	25.2	2.5	2.5	20.0	2.5		2.5
January 2012	51.0	44.0	8.1	nd	15.4	2.5	26.6	48.0	14.8	5.2	7.5	2.5	6.3	2.5
February 2012	15.0	9.8	2.5	nd	5.0	2.5	13.9	2.5	13.6	nd	2.5	nd		2.5

Notes: limit of detection (LOD) was 5 ng L^{-1}. Values of 2.5 above indicate samples where a signal was present at a signal: noise ratio > 3 but below the LOD, indicating the sample was detected but not quantified. nd indicates no signal detected.

Table B-7: Mefenamic acid concentrations (ng L^{-1}) in STP effluent and receiving waters of the Rivers Aire and Calder (2010 to 2012)

	Garforth Owlwood		Heaton Lodge		Horbury Junction		Knostrop Lower		Oulton/Lemonroyd		Oxenhope No. 2		Ripponden	
Month	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel
September 2010	29.6	29.9	56.3	nd	nd	96.6	10.5	8.0	2.5	14.6	2.5	nd	107.8	nd
October 2010	23.8	40.5		nd	nd	nd	11.9	21.9	33.3	22.4	nd	nd	nd	2.5
November 2010	6.9	18.4	nd	6.3	nd	59.1	2.5	21.2	2.5	22.6	nd	2.5	nd	nd
December 2010	14.6	16.4	55.6	53.4	14.5	nd	34.7	38.9	11.7	22.2	nd	2.5	nd	2.5
January 2011	nd	2.5	36.6	11.2	2.5	14.8	2.5	10.6	2.5	17.8	2.5	2.5		2.5
February 2011		2.5		37.9	nd	37.6		2.5	2.5	22.3	nd		nd	
March 2011	nd	2.5	58.7	25.7	nd	42.3	2.5	2.5	nd	6.5	nd	nd	nd	nd
April 2011	15.5	nd	2.5	37.2	nd	2.5	2.5	12.3	10.0	12.2	nd	nd	nd	nd
May 2011	2.5	2.5	nd	2.5		2.5	2.5	2.5	2.5	2.5	nd	nd	nd	nd
June 2011	13.2	12.4	nd	nd	nd	22.2	10.1	31.2	7.3	7.9	nd	nd	nd	nd
July 2011	11.3	5.3	6.1	10.5	2.5	7.4	nd	2.5	nd	2.5	nd	8.9	nd	9.4
August 2011	8.6	2.5	2.5	2.5	nd	2.5	nd	7.2	6.7	nd	nd	nd	nd	nd
September 2011	6.1	11.0	91.2	50.5	2.5	16.5	8.8	nd	7.7	7.0	nd	nd	nd	nd
October 2011	2.5	2.5	2.5	2.5		2.5	7.1	nd	2.5	2.5	nd	nd	nd	nd
November 2011	8.1	9.5	nd	nd	nd	nd	nd	2.5	nd	6.9	nd	nd		nd
December 2011			nd	nd	2.5	nd	2.5	6.7	nd	2.5	nd	nd		nd
January 2012	6.7	9.0	nd	nd	2.5	nd	10.5	8.4	2.5	2.5	nd	nd	nd	nd
February 2012	8.3	2.5	2.5	nd	nd	2.5	2.5	6.4	2.5	6.6	2.5	2.5		14.6

Notes: limit of detection (LOD) was 5 ng L^{-1}. Values of 2.5 above indicate samples where a signal was present at a signal: noise ratio > 3 but below the LOD, indicating the sample was detected but not quantified. nd indicates no signal detected.

Table B-8: Ibuprofen concentrations (ng L^{-1}) in STP effluent and receiving waters of the Rivers Aire and Calder (2010 to 2012)

	Garforth Owlwood		Heaton Lodge		Horbury Junction		Knostrop Lower		Oulton/Lemonroyd		Oxenhope No. 2		Ripponden	
Month	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel
September 2010	729.3	597.1	482.1	93863.1	1817.7	1195.1	236.6	280.2	126.9	196.4	126.9	205.2	2363.8	92.0
October 2010	1368.1	824.7	nd	nd	1356.3	53633.9	277.2	248.5	876.5	255.7	583.0	81.5	350.2	12.5
November 2010	639.1	2960.3	nd	nd	1359.2	2171.7	387.5	1495.5	1017.3	486.8	111.4	127.2	135.6	186.7
December 2010	3592.9	2821.0	nd	1409.2	3644.7	nd	536.1	4838.4	4617.1	2770.0	nd	151.2	912.3	115.4
January 2011	631.4	1565.9	949.4	627.5	1055.2	454.7	544.8	995.7	1183.9	801.4	1068.2	147.8		180.4
February 2011		554.7		259.5	706.0	225.5		808.1	891.0	495.2	951.4		432.1	
March 2011	468.0	417.7	360.6	207.7	1042.2	397.9	345.3	557.2	322.0	488.4	403.8	nd	549.3	144.3
April 2011	645.2	375.7	877.1	619.8	281.2	282.5	433.2	518.4	1026.3	435.0	583.4	133.9	386.2	52.5
May 2011	565.8	830.6	280.3	327.7		344.1	298.8	448.4	474.3	625.6	nd	73.5	191.6	115.8
June 2011	603.6	606.0	381.9	585.6	847.1	511.7	198.8	597.1	47.5	137.5	91.0	125.0	251.8	131.8
July 2011	45.8	452.4	191.8	86.7	129.0	141.1	12.5	234.3	164.1	27.9	75.7	62.4	828.5	114.5
August 2011	298.3	213.2	192.3	168.0	316.1	nd	92.9	93.2	153.7	nd	319.3	nd	53.8	358.4
September 2011	394.6	311.5	197.3	108.7	2164.2	215.8	33.9	52.3	192.5	75.2	65.9	26.2	459.0	126.6
October 2011	743.6	416.0	43.9	109.7	938.9	54.6	178.1	115.7	518.3	117.1	54.9	38.3	310.9	12.5
November 2011	nd	52.3	34.3	35.8	451.8	62.1	232.7	140.8	692.3	168.6	96.4	12.5		12.5
December 2011			319.4	105.3	928.1	31.7	863.2	762.5	740.5	51.3	394.6	167.2		96.6
January 2012	578.8	449.1	93.9	64.1	1305.4	122.1	453.5	454.7	1209.8	97.5	305.3	46.9	117.1	36.9
February 2012	389.7	359.4	77.9	62.7	nd	12.5	12.5	317.4	33.3	530.3	576.2	97.2		312.7

Notes: limit of detection (LOD) was 25 ng L^{-1}. Values of 12.5 above indicate samples where a signal was present at a signal: noise ratio > 3 but below the LOD, indicating the sample was detected but not quantified. nd indicates no signal detected.

Table B-9: Diclofenac concentrations (ng L^{-1}) in STP effluent and receiving waters of the Rivers Aire and Calder (2010 to 2012)

	Garforth Owlwood		Heaton Lodge		Horbury Junction		Knostrop		Oulton/Lemonroyd		Oxenhope		Ripponden	
Month	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel	Effluent	Channel
September 2010	2811.2	2990.7	139.9	198.7	526.6	116.0	768.9	936.7	230.2	591.5	85.4	81.5	411.4	76.4
October 2010	2830.4	2746.6	521.1	39.4	556.6	339.2	797.2	933.4	994.4	347.4	400.6	87.5	332.3	31.3
November 2010	612.5	2057.5	120.9	284.3	138.7	455.8	151.8	909.6	162.2	471.1	79.5	10.1	62.8	29.2
December 2010	744.7	635.1	1301.7	163.6	600.5	88.1	806.4	588.5	631.2	428.3	nd	123.5	380.8	nd
January 2011	136.4	451.7	78.1	97.5	54.6	74.2	154.1	393.5	238.8	293.2	45.9	35.8		47.5
February 2011		161.3		35.6	71.1	41.9		174.6	217.0	177.9	54.8		77.0	
March 2011	168.3	86.5	45.7	44.3	56.0	121.7	87.9	125.0	19.2	162.2	nd	nd	113.0	19.6
April 2011	187.0	9.6	98.4	246.8	20.7	33.6	155.6	202.2	207.2	145.6	26.7	17.3	12.2	2.5
May 2011	101.3	67.7	19.5	34.9		31.9	60.6	103.2	99.6	67.9	9.2	10.0	2.5	16.1
June 2011	172.3	168.4	50.9	47.2	86.5	104.4	194.0	245.7	22.4	61.4	35.0	28.2	9.3	20.8
July 2011	187.5	157.2	51.0	143.8	182.2	231.6	13.3	11.7	9.6	7.1	22.8	23.0	87.6	42.6
August 2011	228.2	165.9	29.8	56.4	92.5	7.6	178.0	209.2	256.0	14.8	34.0	nd	13.9	24.9
September 2011	224.2	322.0	29.4	20.5	43.0	66.5	373.6	47.7	281.4	186.0	19.2	nd	14.3	7.4
October 2011	265.6	189.6	25.2	18.6	95.6	25.8	244.7	108.4	208.9	73.0	23.5	nd	16.5	5.8
November 2011	305.1	389.1	11.5	25.8	54.7	53.2	71.1	160.0	220.1	101.2	10.7	9.1		20.6
December 2011			115.0	26.8	105.1	16.3	288.2	308.4	187.2	69.4	27.8	33.6		16.0
January 2012	260.3	221.9	18.8	nd	90.1	51.4	282.3	234.7	160.1	79.3	27.9	nd	14.0	19.5
February 2012	198.5	217.5	10.2	13.0	nd	2.5	10.6	123.4	17.9	89.9	70.0	8.4		21.3

Notes: limit of detection (LOD) was 5 ng L^{-1}. Values of 2.5 above indicate samples where a signal was present at a signal: noise ratio > 3 but below the LOD, indicating the sample was detected but not quantified. nd indicates no signal detected.

10.3 Appendix C: Environmental metabolomics

10.3.1 Appendix C1: *Gammarus pulex* collection and acclimatisation

Gammarus pulex is a common species of freshwater amphipod widespread across Europe and has been shown to be suitably sensitive and ecologically relevant for use in ecotoxicological testing (Alonso et al., 2010). Live G. pulex were collected in November 2011 from Silsden Beck, West Yorkshire (OSNGR SE 0446147504). This site was chosen because of the known abundance of G. pulex and its location above any known point sources likely to contain pharmaceutical contaminated effluent. A preliminary analysis of a 1L water grab sample from Silsden Beck by HPLC-MS/MS indicated that both of the preliminary study compounds were below detection limits.

After collection, G. pulex were transported to a constant temperature laboratory immediately and acclimatised in 10L glass aquaria with Silsden Beck water for a period of 7 days; the water was oxygenated throughout using air pumps (Hailea Group Company Ltd., China). On day 7, individual G. pulex were weighed wet then placed at random in groups of four into 42x 1L clear glass treatment flasks containing 0.5L of Silsden Beck water, thus giving a total of 168 individuals. Wet weighing was achieved by carefully placing individuals into a pre-weighed flask of stream water on a 4d.p. balance.

After being placed into flasks, they were then allowed to acclimatise for a further 3 days; during this total 10 day period the G. pulex were fed to excess with beech (Fagus sylvatica) leaves which had been pre-conditioned in Silsden Beck stream water for a period of four weeks. F. sylvatica was chosen as the feed material as it was predominant along the banks of Silsden Beck and as such would form the majority of the G. pulex diet at this site. The 10 day acclimatisation was intended to allow recovery from the effects of stress caused during the capture or weighing process. Immediately prior to treatment, excess feed material was removed and each flask was provided with 0.2 g (±0.06) of pre-conditioned beech leaves to provide both food and shelter during the exposure period. Pilot studies demonstrated that 0.2g of feed material would be sufficient for the 28d exposure period. During acclimatisation and treatment mean water temperature was 7.7 (±0.3) °C and a 12:12 light: dark cycle was maintained throughout.

10.3.2 Appendix C2: *Gammarus pulex* masses and physicochemical data

Table S1: Treatment schedule and physicochemical data for 28 day exposure of *G. pulex* to erythromycin and propranolol

Treatment	Nominal conc. (ng L^{-1})	Mean day 0 *G. pulex* mass (mg)	Mean dissolved oxygen (mg L^{-1})	Mean pH	Mean EC (µs cm^{-1}±SD)
Control (Silsden Beck water)	n/a	26.8 (±13.0)	10.1 (±0.8)	7.7 (±0.0)	433.5 (±12.8)
Low treatment erythromycin	1000	25.9 (±9.6)	10.4 (±0.3)	7.8 (±0.1)	446.1 (±27.5)
High treatment erythromycin	100 000	26.0 (±10.0)	9.8 (±0.8)	7.8 (±0.1)	435.3 (±24.1)
Low treatment propranolol	200	24.6 (±9.9)	10.6 (±0.1)	7.7 (±0.1)	439.1 (±23.5)
High treatment propranolol	20 000	27.2 (±11.8)	10.5 (±0.1)	7.8 (±0.1)	464.7 (±27.0)
Low treatment mixture	1000 + 200	26.9 (±11.9)	10.7 (±0.1)	7.9 (±0.0)	472.6 (±28.1)
High treatment mixture	100 000 + 20 000	27.8 (±13.3)	10.2 (±1.0)	7.4 (±0.3)	426 (±7.9)
Notes: All values are means ± 1 standard deviation					

10.3.3 Appendix C3: NMR spectra

The raw NMR spectra in ACD Labs format are included on the attached CD-ROM for reference.