Environmental risk assessment and human pharmaceuticals: limitations and future improvements

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Abstract

A cocktail of human pharmaceuticals contaminate surface waters worldwide in the ng-µgl^{-1} range. Adverse effects on non target organisms including endocrine disruption and alterations in behaviour and growth have been reported. All new pharmaceuticals require an environmental risk assessment (ERA) prior to market authorisation. The aims for this research were to (1) assess the limitations of the current ERA by comparing crude and refined predicted environmental concentrations (PECs) used in ERAs with measured environmental concentrations (MECs) from the literature; (2) interview key people working in the field of risk assessment and ecotoxicology of pharmaceuticals in order to establish expert opinion in the area; (3) to establish whether bioinformatics databases can be used as a potential tool to aid ecotoxicological tests for use in ERAs.

The scientific literature was data mined for environmental concentration data and compared with calculated PECs for ten pharmaceuticals carbamazepine, diclofenac, 17α ethinyl estradiol, fluoxetine, gemfibrozil, ibuprofen, paracetamol, propranolol, tamoxifen and Trimethoprim. An engagement exercise through questionnaire based interviews with representatives of regulatory bodies, water companies and pharmaceutical companies as well as academics involved in ecotoxicology was undertaken to establish experts' views on pharmaceutical risk assessment and management. A genomic search for human drug target homologues in aquatic species for the ten selected pharmaceuticals was undertaken. Molecular docking experiments on two pharmaceuticals, diclofenac and ibuprofen were carried out for human drug target homologues in *Daphnia pulex*, (water flea) *Oncorhynchus mykiss* (rainbow trout), *Salmo salar* (Atlantic salmon) and *Danio rerio* (zebra fish).

The current environmental risk assessment may be insufficient to protect the aquatic environment. PECs can underestimate MECs due to the simplicity of the calculations and the assumptions underpinning them. The interviewees regarded the exposure assessment of the ERA including the PEC calculation as inaccurate and recommended using exposure modelling computer software as a potential solution. The bulk of the scientific literature had substantial deficiencies in the reporting of environmental data; setting reporting standards for peer reviewed journals may make such data more useful for regulators and policy makers. Interviewees felt that the current ecotoxicity tests would benefit from a more intelligent approach incorporating

the mode of action of the drug. The bioinformatics results show that protein drug targets are highly conserved in some aquatic organisms but not others. The molecular docking results indicate that the cyclooxygenase (COX 2) primary drug target homologues are probably functional in *O.mykiss*, *S.salar* and *D.rerio* but not *D.pulex*. It appears from this data that bioinformatics and molecular docking indeed may be a useful tool to aid ecotoxicology tests by informing choice on relevant chronic test endpoints and directing sensitive species selection. Such techniques might contribute to more appropriately targeted ecotoxicity testing. Interviewees felt that the 10 ngl^{-1} action limit was an inappropriate mechanism to trigger ecotoxicological tests. The pharmaceuticals data analysis shows that many existing pharmaceuticals regularly exceed the 10 ngl^{-1} action limit for ecotoxicological assessment. A system of prioritisation is required to assess the need for retrospective risk assessment of these medicines.

This thesis provides an original analysis of the current environmental risk assessment of human pharmaceuticals and makes recommendations for improvements. A novel application of molecular docking utilizing the mode of action of the pharmaceutical has the potential to aid and direct ecotoxicological tests.

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Authors Declaration

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

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List of EU Directives and Guidelines

- **Directive 2004/27/EC** amending **Directive 2001/83/EC** on the Community code relating to medicinal products for human use

- **EMEA//CHMP/SWP/4447/00** Guideline on the environmental risk assessment of medicinal products for human use.

- **Directive 2009/128/EC** Establishing a framework for Community action to achieve the sustainable use of pesticides.

- **Directive 2000/60/EC** of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy or, in short, the EU Water Framework Directive (WFD).

Abbreviations

DEFRA	Department for Environment, Food and Rural Affairs
DDD	defined daily dose
DOSEai:	maximum daily dose consumed per inhabitant ($mg.hab^{-1}.day^{-1}$)
EA	Environment Agency
EE2	17α ethinyl estradiol
EMEA	European Medicines agency
ERA	environmental risk assessment
Fpen	market penetration factor
Log K_{ow}	octanol-water partition coefficient
LOD	limit of detection
LOEC	lowest effect concentration
LOQ	limit of quantification
MEC	measured environmental concentration
MoA	mode of action
NA	not available
ND	not detected
NOEC	no effect concentration
NSAID	non steroidal anti inflammatory drug
OECD	Organisation for Economic Co-operation and Development
PBT	persistence, bioaccumulation, toxicity
PEC	predicted environmental concentration
PNEC	predicted no effect concentration
REACH	Registration, Evaluation Authorisation and Restriction of Chemicals
RQ	risk quotient
SD	standard deviation
SSRI	elective serotonin reuptake inhibitor
STP	sewage treatment plant
QSAR	quantitative structure activity relationship
WASTEWinhab	Amount of wastewater per inhabitant per day

Section 1

Introduction

1.1 Background

Human pharmaceuticals have been suspected to be pollutants of the aquatic environment since the 1970's (Hignite & Azarnoff, 1977; Kummerer, 2010). It is only over the last 20 years that advances in analytical chemistry techniques have confirmed this and led to a wealth of information and data on pharmaceuticals in water courses. A substantial number of publications have reported concentrations of some 180 different human drugs in surface waters worldwide in the ng-μgl^{-1} range (Sadezky *et al*., 2010). These pharmaceuticals, however, still only represent a small proportion of medicines currently licensed in the UK, which is thought to be over 3000 (Redshaw *et al*., 2008). Pharmaceuticals have also been reported in sewage influent and effluent, sewage sludge, agricultural land, groundwater, estuarine and marine waters, reservoirs, drinking water and landfill leachate (Roberts & Thomas, 2006; Kasprzyk-Hordern *et al*, 2009; Mompelat *et al*., 2009; Lapworth *et al*., 2012; Eggen *et al*., 2010).

1.2 Sources of exposure

Routes of entry of drugs into water courses include disposal of unused medicines down the sink or toilet, pharmaceutical manufacture, hospital effluent, landfill leachate, land run off from agricultural sewage sludge application and veterinary use (Kummerer, 2009). There is no accurate quantitative data on the contribution from each of these sources to contaminant levels in aquatic environments (Roig & Touraud, 2010). However, the main source is thought to be human usage (Cunningham *et al*., 2006; Sanderson *et al*., 2003). Pharmaceuticals are designed to avoid degradation by the body in order to have the desired therapeutic effect (Fent *et al*., 2006). They are excreted in urine and faeces as parent compound, conjugates and metabolites (Carlsson *et al*., 2006, Herberer, 2002). These are not fully removed by sewage treatment facilities and are discharged into water courses, where they persist (Calisto & Esteves, 2009) and may accumulate. In fact as much as 80% of the total load of pharmaceuticals entering sewage treatment plants (STPs) may be discharged into surface waters (Zabczynski *et al*., 2010).

1.3 Ecotoxicological effects

Aquatic organisms are exposed to a continuous cocktail of human pharmaceuticals. At least a dozen different pharmaceuticals have been measured in a single surface water sample (Daughton & Brooks, 2011). This is highly likely to be a substantial underestimate because of

limitations in analysis. Human pharmaceuticals are designed to have a specific biological effect (Schmitt *et al*., 2010). This effect can often disrupt key biological functions in aquatic organisms such as reproduction and growth (Fent *et al*., 2006). Despite the high toxicity and high potency of some pharmaceuticals, only one major effect on aquatic organisms has come to light. The presence of the synthetic hormone contraceptive 17α ethinylestradiol (EE2) in sewage effluent and surface waters has been clearly linked with the endocrine disruption of fish and frogs (Gyllenhammar, 2009; Caldwell *et al*., 2008). The presence of intersex fish was discovered as far back as 1976 within STP settlement lagoons in the UK (Sumpter & Johnson, 2008). It is still unknown exactly to what extent synthetic hormones such as EE2 effect feminisation of male fish compared with naturally occurring oestrogens such as oestrone; however, it is thought to play a major role (Sumpter, 2010). Fish are particularly sensitive to EE2, the predicted no effect concentration (PNEC) for EE2 is <1 ngl^{-1} (Lange *et al*., 2001; Caldwell *et al*., 2008). This detrimental effect on aquatic organisms was not foreseen. However, it is important to highlight that the mode of action (MoA) for EE2 is via the oestrogen receptor which is highly conserved in other vertebrates i.e. other than human, such as fish (Christen *et al*., 2010).

Veterinary medicines have also been the cause of a dramatic detrimental effect on non target organisms. The use of diclofenac in cattle has caused a major decline in vultures in India and Pakistan. The *Gyps* genus of vulture were surprisingly sensitive to residues of diclofenac in deceased carrion on which they fed, leading to acute renal failure and visceral gout (Oaks *et al*., 2004). Diclofenac has since been withdrawn as a veterinary medicine (Kumar, 2006). However it is still used widely as an analgesic in human medicine; it is persistent through sewage treatment and is regularly detected in effluent and surface waters around the world (Hoeger *et al*., 2005).

Despite the longevity of exposure of aquatic organisms to a wide variety of human drugs, notable adverse effects are surprisingly rare. The reason for this may be that the concentrations in aquatic ecosystems are far too low to show acute toxic effects. Acute effects data show that generally, an effect concentration of over 1 mgl^{-1} is required to induce mortality in aquatic organisms (Crane *et al*., 2006; Fent *et al*., 2006). It is now widely accepted that the route of exposure is of a continuous chronic nature and this is beginning to be reflected in the ecotoxicological publications in the literature.

A number of reviews have been published which summarise ecotoxicological effects of human pharmaceuticals, for example, Santos *et al*., (2009) and Fent *et al*., (2006). There are

several examples of chronic effects on aquatic organisms at environmentally relevant concentrations. The antidepressant fluoxetine (Prozac) has been shown to effect innate behavioural responses of fish at environmentally relevant concentrations (Painter *et al*., 2009; Schultz *et al*., 2011). Alterations in reproduction patterns have also been observed (Brooks *et al*., 2003). The beta blocker propranolol has been shown to inhibit egg laying in fish at environmentally relevant concentrations (Huggett *et al*., 2002). The anti-convulsive carbamazepine has been shown to effect antioxidant defence systems in fish brains (Li *et al*., 2010). However Fent *et al*., (2006) concluded that for most pharmaceuticals, chronic lowest effect concentrations (LOECs) were two orders of magnitude higher than maximal reported STP effluent concentrations.

These examples of potential adverse effects on non target organisms highlight the uncertainties regarding the effects of pharmaceuticals in the aquatic environment. Environmental policy is based on the precautionary principle and prevention principles (Kampa *et al*., 2010). Prevention principles are more complicated for pharmaceuticals than for other chemical pollutants, such as pesticides, because they are required for human health benefits. This means that unlike veterinary use of diclofenac, sales and usage of human medicines cannot be restricted on environmental grounds. This means that end of pipe solutions, i.e. wastewater treatment, must be considered as a control measure. The precautionary principle emphasises that, where evidence of a threat to the health of the environment exists, scientific uncertainty must not be allowed to delay reasonable forms of management action (Kampa *et al*., 2010). The detrimental effects of human pharmaceuticals such as EE2 on aquatic organisms and mounting evidence of other subtle chronic effects on behaviour, health and reproduction may soon be sufficient to require precautionary action to manage the effects in the environment, despite scientific uncertainty over impacts. This potential for effects has led to the development of an environmental risk assessment (ERA) of human pharmaceuticals as part of the licensing procedure.

1.4 Environmental risk assessment

Environmental risk assessment (ERA) of human medicines appeared as part of the marketing authorisation process in the 1990s, however, detailed risk assessment was only carried out in exceptional cases prior to 2004 (Holzmann, 2005). The current environmental regulation of human pharmaceutical products in Europe is laid out by Directive 2004/27/EC. This states

that an ERA should accompany any application for marketing authorisation of a medicinal product for human use. The guidelines for the ERA procedure in the European Union are set by the European Agency for the Evaluation of Medicinal Products (EMEA) and contained in EMEA//CHMP/SWP/4447/00, which came into force December 2006. It is a tiered process beginning with an initial prediction of environmental concentration (PEC) with an action limit for further ecotoxicological risk assessment of 10 ngl^{-1}. If the PEC exceeds this action limit, phase II of the ERA is invoked. This involves the calculation of a risk quotient (RQ), the ratio between the PEC and a predicted no effect concentration (PNEC). The PNEC is derived from ecotoxicological tests which should include acute and chronic data from organisms of at least three trophic levels; usually algae, *Daphnia* and fish.

Since the introduction of the ERA in Europe several papers have been published assessing its usefulness/ fitness for purpose and level of protection for aquatic organisms (Ferrari *et al*., 2004; Bound & Voulvoulis, 2004; Kuster *et al*., 2009). This has led to certain amount of identification of its limitations and recommendations for its improvement. These include aspects related to the overall procedure, effects assessments and exposure assessments.

1.4.1 General limitations of the ERA

One of the limitations with the ERA is that authorisation of a new medicine cannot be turned down on an environmental basis. Even if a substantial risk to the environment is perceived, it is assumed that the benefit to human health far outweighs any environmental damage. There are no guidelines for mitigation of a perceived environmental problem in the current authorisation process aside from a statement on the package leaflet which should read:

> *"Medicines should not be disposed of via wastewater or household waste. Ask your pharmacist how to dispose of medicines no longer required. These measures will help to protect the environment."*

Since the main source of pharmaceuticals is thought to be from human usage and not disposal, this control measure does not adequately address the problem.

Another key shortfall of the current ERA requirements is that it only applies to new medicines requiring authorisation. A large proportion of medicines were authorised prior to 2006 and have therefore not undergone an ERA. It has been recommended that in accordance with

other environmental legislation for industrial chemicals, (Registration, Evaluation Authorisation and Restriction of Chemicals, REACH) and pesticides (The Pesticides Framework Directive 2009/128/EC) that retrospective ERA for human medicines should be performed using a system of prioritisation (SRU, 2007). Sweden has embarked on a fairly comprehensive prioritisation strategy for pharmaceuticals (Wennmalm & Gunnarsson, 2009). However, no such system of ranking and prioritisation for retrospective risk analysis of pharmaceuticals has been applied in the UK. The Environment Agency attempted to prioritise pharmaceuticals of environmental concern and undertook a monitoring study in 2003 (EA, 2003). Unfortunately a lack of effects data, especially chronic effects data, and a lack of analytical chemistry techniques for measuring the compounds of highest concern restricted its effectiveness.

It is known that pharmaceuticals occur as mixtures in the environment. In human medicine the potential for synergistic, additive and antagonistic effects of combinations of drugs is considered of high importance, however this is not the case with the ERA. Several compounds in the aquatic environment may affect the same metabolic pathway or process in non target organisms. This could lead to effects in aquatic organisms that would not occur if exposed to a compound in isolation. The current ERA does not take into account mixture effects of different pharmaceuticals. For many therapeutic classes of pharmaceuticals more than one product available with the same mode of action (MoA). Examples include non steroidal anti inflammatory drugs (NSAIDs) and antidepressants such as selective serotonin reuptake inhibitors (SSRIs). These drugs have been shown to have combined effects that are much higher than the individual drug in isolation would show. These classes of drugs have been shown to follow a concentration addition model (Christensen *et al*., 2007; Cleuvers, 2004).

The action limit of 10 ngl^{-1} has also come under some scrutiny on its level and as a mechanism for risk assessment. The level was set using mainly acute effects data with an assessment factor applied (Schmitt *et al*., 2009). The application of assessment factors to account for acute to chronic effects has been shown to be flawed (Roig, 2010). The use of an action limit that terminates risk assessment for compounds which have a PEC of less than 10 ngl^{-1} may lead to potentially toxic substances being overlooked. This is not wholly unlikely considering the case of EE2 and the lack of scientific knowledge about the effects of and exposure to, many human pharmaceuticals. Although 10 ngl^{-1} is considered to be at least two orders of magnitude below the therapeutic dose for most medicines, the effects of active compounds cannot be excluded.

Impacts with pesticides have been shown in the low ngl^{-1} range (Steur-Laurisden *et al*., 2000; Kampa *et al*., 2010).

1.4.2 Limitations of the ERA for exposure assessment

On the exposure assessments side of the ERA several problems have been identified. These include some of the assumptions that are made in calculation of the PEC. These include the dilution factor default value of 10 and the wastewater production per person per day default of 200L (Tarazona *et al*., 2009). PECs are based on an assumption that 1% of the population will consume the drug and is termed the market penetration factor (Fpen). This does not reflect the actual level of consumption after market authorisation. The PEC calculation also assumes the same market penetration across all the countries in Europe and that consumption is equal across a country and over the course of the whole year. The PEC calculation also neglects to account for degradation processes in the environment such as photolysis and microbial degradation (Mompelat *et al*., 2010). The potential inaccuracies of the PEC calculation has led to some comparison of PECs with measured environmental concentrations (MECs) with mixed results (Coetsier *et al*, 2009; Liebig *et al*., 2006; Bound & Voulvoulis, 2006; Carballa *et al*., 2008). Refinements can be made to the initial crude PEC if it is over the 10 ngl^{-1} action limit. These include removal by sewage treatment and metabolism by the body. The reliability of estimating these is problematic (Tauxe-Wuersch *et al*., 2005; Santos *et al*., 2009; Carballa *et al*., 2008). The EMEA guidelines recommend using the SimpleTreat computer package to estimate removal by STPs however this package is mainly based on the octanol-water partition coefficient (log K_{ow}) which has been shown to be a poor indicator of actual adsorption of pharmaceuticals to sewage sludge (Besse & Garric, 2010; Fent *et al*., 2006).

1.4.3 Limitations of the ERA for ecotoxicological effects assessment

The ecotoxicological effects calculation, i.e. the PNEC has also come under criticism for its lack of incorporation of the MoA of the drug (Boxall & Greenwood, 2010; Poynton *et al*., 2008; Sanderson & Thomsen, 2009) and the limited number of species used for its derived level (Besse & Garric, 2010). It has been recommended that some pharmaceuticals should undergo effects tests even if the PEC was below the action limit of 10 ngl^{-1}. Reasons for this include: potential for persistence, bioaccumulation, carcinogenic, mutagenic or reproductive effects, high potency or low therapeutic margin, known toxic effects of structurally similar compounds and

new classes of therapeutics which may have unknown ecotoxicological effects (Schmitt *et al*., 2009).

1.5 Integration of expert knowledge into ERA

In the EU precautionary management for the release of pharmaceuticals into the aquatic environment is governed by an ERA. Since the new guidelines for this ERA were introduced in 2006, six years ago, it is considered prudent to assess its efficiency and effectiveness for the protection of the aquatic environment. In order to gain useful insights into issues related to human pharmaceuticals in the aquatic environment and the associated ERA, expert knowledge (i.e. scientific knowledge) holder engagement is a useful tool. It provides a technique to gather valuable information and opinion from different standpoints in an area of high scientific uncertainty such as pharmaceutical impact on aquatic systems.

Stakeholder consultations, including communication with experts, are an essential component of risk management and are important for developing policies (Daughton, 2003a & b). Expert knowledge can often provide valuable information for assessing environmental problems beyond that contained in the peer reviewed literature (Reed, 2008). In a stakeholder engagement exercise in 2006, a key finding was that engagement by multiple levels of government and multiple stakeholders, including experts, holds much promise as a tool to improve management of pharmaceuticals in the environment (Doerr-MacEwen & Haight, 2006).

Over the last 10 years the UK Government has strongly promoted the more effective use of science to inform policy-making and regulation (Holmes & Clark, 2008). The Cabinet Office (1999) consider that a core competency of good policy-making is using the "best available evidence from a wide range of sources" including evidence from "expert knowledge and the critical evidence held in the minds of front line staff in departments, agencies and local authorities and those to whom the policy is directed''. Effective access to information and expertise is a necessary precursor to the use of science to inform policy-making and regulation (Holmes & Clark, 2008). External experts (including researchers, consultants and experts in other Government departments and agencies) are an important source of scientific advice (Holmes & Clark, 2008). These experts synthesise and interpret information for policymakers and their involvement may lend credibility to ensuing policy decisions. The involvement of diverse experts can also lead to a more comprehensive understanding of ecological hazards and

can improve problem formulation by generating an ecologically robust set of information on which to base the subsequent, more technical ERA (Dana *et al*., 2012). The participatory ERA process can also increase the transparency of the ERA by exposing the logic and rationale for decisions made at each step (Dana *et al*., 2012).

One of the aims of this research was to interview relevant individuals who can provide expert knowledge and valuable information on the successes and failings of the current ERA. The individuals that were targeted were involved directly with the ERA procedure including academics, water company managers, government agency staff and pharmaceutical company employees. Engagement with these experts may provide novel insights into the performance of the ERA and provide new knowledge on how it could be improved in a practical and applicable way. It is considered that it would be largely unhelpful to engage with a range of other stakeholders such as shareholders, pharmacists, doctors and the general public as they would have little knowledge or understanding of the complex guidelines which comprise the ERA.

1.6 Bioinformatics

Pharmaceuticals are different to some chemical pollutants in that they are designed to have a specific biological effect (Christen *et al*., 2010; Dorne *et al*., 2007; Kar & Roy, 2010). This means that traditional ecotoxicity tests using mortality as an end point might underestimate potential chronic effects in the environment and therefore give an underestimate of effects concentrations (Crane *et al*., 2006). Standard tests even when using chronic end points such as reproduction still do not incorporate, in most cases, the mechanism or mode of action (MoA) of a pharmaceutical compound. Many chronic ecotoxicological studies using MoA related end points have revealed NOEC concentrations that are substantially lower than traditional studies (Crane *et al*., 2006; Boxall & Greenwood, 2010). Another potential shortfall is that a relatively narrow variety of species are used in the ecotoxicological tests recommended by OECD guidelines and the EMEA guidelines. These may not incorporate the most sensitive species that could be exposed in water courses. This fact is supported by chronic effects studies on species that are not currently included in the guidelines. For example, a study by Meredith-Williams *et al*., (2012) showed a substantial difference in the uptake and bioconcentration of pharmaceuticals across three species of aquatic invertebrates. Mussels, not currently considered in ERAs have also been

shown to be surprisingly sensitive to some drugs such as fluoxetine (Bringolf *et al*., 2010). Serotoninergic antidepressants have been shown to have effect concentrations that ranged over several orders of magnitude in crustaceans and algae (Henry *et al*., 2004; Johnson *et al*., 2007). This has also been highlighted by the unexpected and surprisingly high sensitivity of vultures in Asia to diclofenac (Oaks *et al*., 2004).

These problems have led many authors to propose an intelligent testing strategy for pharmaceuticals. This would incorporate MoA based chronic tests and endpoints. The use of 'omics' based approaches using extrapolation of human and mammalian data has been suggested as a method for predicting environmental effects for risk assessment (Gunnarsson *et al*., 2008; Christen *et al*., 2010; Berninger & Brooks, 2010; Boxall & Greenwood, 2010). Schmitt *et al*., (2009) recommended that chronic test end points should reflect the MoA of the drug or known side effects and also that effects testing should be carried out regardless of the PEC when drug target structures are conserved across species.

Human pharmaceuticals target specific proteins and metabolic pathways that might be highly conserved in other species. Evolutionary conservation of drug targets could prove a useful method for guiding the ERA by identifying sensitive species and interpreting the relevance of existing toxicological data (Besse & Garric, 2010). If a drug has a specific MoA in a human then this same MoA may also be occurring in other organisms. For example the beta blocker propranolol may cause cardiovascular effects in fish (Owen *et al*., 2007, 2009) and drugs such as statins may also break down cholesterol in fish as well as humans (Ellesat *et al*., 2010). Sequence conservation in drug targets has been proposed as a potential guide for selecting end points for toxicity studies and also to select a range of species that may be sensitive (Christen *et al*., 2010; Gunnarsson *et al*., 2008; Kostich & Lazorchak, 2008).

It is important to note that the existence of a similar protein sequence in an organism does not automatically mean that the human MoA of the drug will occur. In a study by Gunnarsson *et al*., (2008), a high number of conserved drug targets were identified in other species. In order to make this information relevant to ecotoxicological tests and ERAs further work on the 3D structure of the proteins was needed to predict drug protein interactions. Besse & Garric, (2010) identified four ways that bioinformatics information could aid and direct ecotoxicological tests for ERA:

1. Identification of drugs with the most potential to elicit adverse effects on non-target organisms.
2. Interpretation and assessment of ecotoxicological and pharmacological data.
3. Improvement of the possibilities to identify which pharmaceuticals may pose a risk to a certain type of species (or identification of specific sensitive species to certain compounds).
4. Selection of relevant species and/or end points for ecotoxicological studies.

1.7 Hypothesis

1. The environmental risk assessment (ERA) for human pharmaceuticals and use of predicted environmental concentrations may be inadequate to protect the aquatic environment.
2. Bioinformatics and molecular docking may be a potential tool to aid and direct the ERA of human pharmaceuticals through a focus on mode of action.

1.8 Aims

1.8.1 Overarching aim

Assess the current effectiveness of the environmental risk assessment of pharmaceuticals (ERA) and make recommendations for improvements.

1.8.2 Specific objectives

1. Assess the reliability of the predicted environmental concentration (PEC) used for environmental risk assessments (ERA) in relation to reported pharmaceutical environmental concentration data.
2. Investigate reporting standards in peer reviewed literature for data on pharmaceuticals in aquatic systems and their potential use in monitoring and ERA.
3. Establish whether currently available bioinformatics databases are a potential tool to aid ecotoxicological testing as part of risk assessment.
4. Examine expert opinion, obtained through interviews, on risk assessment and risk management of pharmaceuticals.

1.9 Thesis structure

Section 1: Introduction

Introduction, Aims, Hypothesis and Thesis Outline

Section 2: Pharmaceutical Data Analysis

Addresses **Specific objectives 1 & 2** of this thesis by providing an analysis of:

1. The environmental concentration data for pharmaceuticals in aquatic environments and STPs including the efficiency of sewage treatment on removal of pharmaceuticals and the effect population size on sewage effluent concentrations.
2. The reliability and robustness of predicted environmental concentrations (PECs) in the context of reported measured environmental concentrations (MECs) and the limitations of current methods for calculating crude and refined PECs for human pharmaceuticals in water bodies.
3. The scientific literature for its utility in making environmental concentration data reported in scientific journals useful to environmental risk assessors and regulators.

The rationale for this section was to investigate the effectiveness of the first stage of the ERA relating to the initial calculation for exposure of pharmaceuticals in the aquatic environment. This initial stage currently dictates whether further environmental risk assessment including ecotoxicological tests should be performed. This is the pivotal point at which it is decided if there is the possibility of an environmental risk and therefore it is essential to ascertain if this is a reliable and robust mechanism to protect the environment.

Environmental risk assessment should be seen as an ongoing process and therefore it is necessary to periodically review its effectiveness. Therefore it is important that the reporting of environmental concentration data in the scientific peer reviewed literature is made useable to environmental risk assessors and policy makers.

Section 3: Interviews

This section addresses **Specific objective 3** by presenting the results and analysis of eleven in depth interviews with representatives of regulatory bodies, academics, the pharmaceutical industry and water industry. The results provide views, opinions and recommendations on the successes, failings, limitations of and future improvements to the current environmental risk assessment of pharmaceuticals in the environment. This section links both the work performed in Section 2 on exposure concentrations, predicted and measured (i.e. the initial stage of the ERA) and Section 4, the potential for bioinformatics to aid and direct ecotoxicological risk assessment (the second stage of the ERA) thereby addressing on the overarching aim of the thesis.

Section 4: Bioinformatics

Section 4 addresses **Specific objectives 4** by assessing the potential application of current bioinformatics databases and molecular docking to direct ecotoxicity tests. A BLAST search for drug target homologues in aquatic organisms is included. Two drugs diclofenac and ibuprofen were used to investigate the ability of the molecular docking package AutoDock to predict interactions with drug target homologues in aquatic species. The rationale for this section and aim 4 was to address the lack of incorporation of the mode of action (MoA) of pharmaceuticals when ecotoxicological effects are assessed as part of the ERA. Bioinformatics may provide a useful improvement that could be made to the ERA to protect the aquatic environment.

Section 5: Conclusion

Tests the central hypotheses and overarching aim of the thesis by assessing the effectiveness of the ERA and concludes by making recommendations for improvements to the ERA for human pharmaceuticals in freshwater systems.

Section 2

Pharmaceutical Data Analysis

2.1 Aim of Section 2

This chapter will collate and analyse data on concentrations of ten selected pharmaceuticals in the aquatic environment including surface waters, sewage effluent, groundwater, marine and estuarine water and drinking water. The efficiency of sewage treatment for removal of pharmaceuticals and the effect population size has on sewage effluent concentrations is investigated. The reliability and robustness of predicted environmental concentrations (PECs) in the context of reported measured environmental concentrations (MECs) will be examined and the limitations of current methods for calculating crude and refined PECs for human pharmaceuticals in water bodies will be assessed.

2.2 Novelty of work performed in Section 2

There is disagreement in the scientific peer reviewed literature on the reliability of PECs for human pharmaceuticals. In this section the direct PEC and MEC comparisons for human pharmaceuticals in surface waters from the literature in Europe were evaluated. Prescription data was also used to calculate a PEC for England which was compared to all the MEC data published to date. Rather than comparing a crude or refined PEC to measurements taken in a single water body, this work aims to compare crude PECs to more than a decade of published environmental data not previously considered as a single body of work. This section also provides a novel analysis of the relationship between population size and sewage effluent concentrations of human pharmaceuticals. This work concludes that the current PEC calculation in the EMEA ERA guidelines is not always precautionary and conservative.

Unfortunately a novel meta analysis of MECs was not possible due to a lack of reporting standards in the peer reviewed scientific literature. The environmental concentration data gathered during this work leads to a novel set of recommendations for reporting environmental concentration data in peer reviewed scientific journals.

2.3 Introduction

Despite a considerable amount of published data on environmental concentrations of human pharmaceuticals the fate and effects of these micro pollutants is still largely unknown.

Routes of entry of human drugs into water courses include disposal of unused medicines via the sink or toilet, pharmaceutical manufacture, hospital effluent, landfill leachate and land run off from agricultural sewage sludge application. However, the main route of entry of pharmaceuticals into water bodies is thought to be through incomplete metabolism by the body (Ellis, 2006; Cunningham *et al*., 2006; Sanderson *et al*., 2003) and subsequent discharge in sewage effluent.

Factors that have a major effect on drug concentrations in surface waters, therefore, relate predominantly to this source. Thus the amount of pharmaceutical consumed by local populations is significant, as is the percentage excreted as parent compound or conjugates in urine and faeces and the pharmaceutical removal efficiency of the STP (Jones *et al*., 2005). Other influences on final concentration include the volume of the receiving water body and degradation processes in the environment (Fatta-Kassinos *et al*., 2011; Loffler *et al*., 2005).

In the EU any application to licence a new medicine must be accompanied by an ERA. In 2006 the European Agency for the Evaluation of Medicinal Products (EMEA) published a revised guideline for ERA of human pharmaceuticals (EMEA/CHMP/SWP/4447/00). It is general practice for an ERA of any substance to begin with a conservative predicted environmental concentration (PEC). The calculation for the initial PEC for human pharmaceuticals often termed the crude PEC is specified in EMEA guidelines. If the PEC exceeds the action limit of 10 ngl^{-1} then a second phase of the ERA is triggered and the PEC is refined with consideration of relevant data on metabolism, excretion, degradability and persistence. This refined PEC value is compared to the predicted no effect concentration (PNEC), which is derived from existing ecotoxicological data. If the ratio termed the risk quotient, is greater than 1 the PEC and PNEC are further refined using substance and compartment specific tests.

The crude PEC calculation assumes that: 1% of the population consume the maximum daily dose of a drug; 100% of pharmaceuticals prescribed are consumed evenly across the population; and that over the year, 100% of the parent compound is excreted and no removal occurs during sewage treatment. Despite the apparent simplistic and uncertain nature of this method there are two reasons why it is necessary. The first is that the actual consumption data is not available until a drug has been licensed so an estimate of usage must be made. The second is that it is impractical to measure the environmental concentrations of all the pharmaceuticals that

are marketed annually in all the water bodies that may be affected. Given these limitations it is necessary to estimate the exposure concentrations (Besse & Garric, 2008; Kostich *et al*., 2010).

Several studies on the accuracy of the PEC calculation have been performed and the evidence is somewhat contradictory, some have found PEC and measured environmental concentrations (MEC) values to be in good agreement (Besse & Garric, 2008) while others have shown this not to be the case (Coetsier *et al*., 2009). Liebig *et al*., 2006 demonstrated that PECs calculated on the basis of human metabolic removal and removal by sewage treatment were very close to measured environmental values, but noted that exposure assessments should always result in PECs that are higher than environmental concentrations. Refined PECs have, in fact, also been found to underestimate MECs for several pharmaceuticals (Bound & Voulvoulis, 2006). Indeed, Morasch *et al*., (2010) found some MEC/PEC ratios to be greater than 10. The uncertainty over PEC leaves the EMEA ERA open to criticism and some workers have suggested that the ERAs are insufficiently robust to protect aquatic environments (Ferrari *et al*., 2004). Undoubtedly, the PEC calculation must be conservative and precautionary to protect the environment. If a pharmaceuticals environmental concentration is underestimated then unforeseen adverse effects on non target organisms may occur and remain un-investigated.

2.4 Methods

2.4.1 Data collection

A comprehensive and systematic search of peer reviewed literature; books, UK government (e.g. Department for the Environment, Food and Rural Affairs, DEFRA), EU Commission reports and Environment Agency (EA) reports was undertaken. Online databases 'National Centre for Biotechnology Information' (www.ncbi.nlm.nih.gov) and 'Science Direct' (www.sciencedirect.com) were used initially with search terms such as 'pharmaceutical AND environment', 'pharmaceutical AND pollution' followed by specific searches relating to each of the selected pharmaceuticals. The matrixes included in the initial search were: Surface waters including rivers, streams, lakes, reservoirs, coastal waters, sea water, ground water, soil, landfill leachate, soil, drinking water, sewage effluent, influent and sludge. The initial search included all pharmaceuticals from all therapeutic classes. This resulted in approximately 500 relevant articles. The references provided in each publication were also examined.

The information and data extracted from relevant publications were: pharmaceutical, therapeutic class, matrix (surface water, effluent etc), mean (and standard deviation), median, minimum, maximum, single reported pharmaceutical concentrations, sample size, country and location of the detection, analytical method used and the detection limit of that method, sewage treatment employed, date of sampling, flow of the receiving waters, population size served by STP or population base of surrounding area, flow/ quantity of effluent discharge and any reported weather conditions.

Data on pharmaceuticals chemical structure, physical and chemical data and consumption data were found using: Drug Bank (www.drugbank.ca); Centre for Coastal Environmental Health and Bimolecular Research, pharmaceuticals in the environment database (PEIAR) (www.chbr.noaa.gov/peiar); RxList (www.rxlist.com); and UK Department of Health Prescriptions cost analysis 2008 (www.ic.nhs.uk/webfiles/publications/PCA%202008/PCA%202008v2.pdf).

2.4.2 Data analysis

2.4.2.1 Concentrations in surface waters and sewage effluent

Ten pharmaceuticals were selected for potential meta analysis on the basis of being representative of each major therapeutic class, population usage (highly prescribed) (Department of Health prescription cost analysis, 2008), wealth of environmental concentration data from the literature search (see appendix 1), high concentrations detected in the environment and known or potential (suspected) ecotoxicological impacts. The ten pharmaceuticals were: carbamazepine (anticonvulsive), diclofenac (non steroidal anti-inflammatory), 17α-ethinylestradiol (EE2) (contraceptive), fluoxetine (Prozac) (antidepressant), gemfibrozil (lipid regulator), ibuprofen (non steroidal anti-inflammatory), paracetamol (analgesic), propranolol (ß-blocker), tamoxifen (cancer drug) and trimethoprim (antibiotic). Measurements were included regardless of their collection method (grab sample or 24 hour composite) and analytical method used, in order to increase the amount of data available for analysis.

Originally, it was hoped that a meta analysis of the ten selected pharmaceuticals could be performed using the guidelines in the Cochrane Systematic Reviews of Interventions (2008). This is an accepted protocol for meta analysis data on medical health practices. The Cochrane Review can be used to analyse results from different studies. The method involves collection of mean values, standard deviation (SD) and sample size of data collected in each study. The intention was to compare the data collected on environmental concentrations of the ten selected pharmaceuticals to assess the effect of different parameters such as weather (season), river flow, sewage treatment type and population size.

The mean concentration values from the literature were extracted from the complete MEC data set (see appendix 1). Where the mean value was not supplied, some authors, on request, also supplied raw data for mean calculations (see acknowledgements). If enough raw data was available in the original publication (or supplied by email), the mean and SD were calculated. Where positive detections were found below the limit of quantification for that method, a figure of half the detection limit was used as in methods used in Ashton *et al*., (2004).

Unfortunately the data available in the literature was not generally appropriate for use with the Cochrane Review computer package (see Section 2.4.5 on reporting standards). Sufficient data was available to compare the mean (reported or calculated from the data) reported concentrations in surface water and sewage effluent for four of the selected pharmaceuticals:

carbamazepine, diclofenac, gemfibrozil and ibuprofen. These were selected for further study. Mean of means calculations on these pharmaceuticals included all the reported concentrations. For non detections (ND) a value of zero was included in the calculation. For the other six pharmaceuticals; paracetamol, trimethoprim, tamoxifen, EE2, fluoxetine and propranolol all MEC measurements were used for analysis including; single reported measurements, mean, median, max and minimum concentrations.

2.4.2.2 Sewage treatment efficiency

The efficiencies of different sewage treatment technologies including: primary sedimentation, secondary biological, activated sludge, biological trickling filter, tertiary treatments, settlement lagoons, chlorination and UV for removal of pharmaceuticals was assessed. Four pharmaceuticals were selected for this study because there was the greatest amount of data available on their removal by sewage treatment. The efficiency of sewage treatment for carbamazepine, diclofenac, ibuprofen and gemfibrozil was determined by calculating the change in concentration between sewage influent and effluent at individual sewage treatment plants (STP) or pilot plants, using the equation:

$[C_{influent} - C_{effluent}]/C_{influent} \times 100$.

2.4.2.3 Pharmaceutical dilution in receiving waters

The dilution effect of pharmaceutical concentration from sewage effluent outfall to receiving waters was investigated. Measurements from final effluent and downstream/upstream receiving water concentrations for carbamazepine, diclofenac, gemfibrozil and ibuprofen were analysed. The dilution in pharmaceutical concentration from sewage effluent outfall to the receiving waters was calculated using the equation:

$[C_{effluent} - C_{surface\ water}]/C_{effluent} \times 100$.

2.4.2.4 Population size and effluent concentration correlations

Microsoft Office Excel was used to calculate statistical correlations between population size and concentration of pharmaceutical in sewage effluent for four pharmaceuticals: carbamazepine, diclofenac, ibuprofen and gemfibrozil using a 95% confidence limit of the mean (n varies with dataset). These were performed first using all data without separation of sewage treatments and then between broad categories of sewage treatment (see above).

2.4.3 Action limit for environmental risk assessment

The frequency that nine of the selected pharmaceuticals were reported as present in surface waters above the 10 ngl^{-1} action limit for risk assessment set by the EMEA guidelines was assessed using mean MECs (see above). EE2 was not included in this assessment as concentrations are rarely detected at this level.

2.4.4 Calculation of PECs for comparison with MECs

Two surface water PECs were calculated using the Committee on Medicinal Products for Human Use (CHMP), 2006 guidelines for each of the selected pharmaceuticals. The first PEC calculations were performed using all the default values including the default market penetration factor (Fpen) set out in the guidelines (Eq 1). The second set of PEC calculations involved the substitution of the Fpen of 1% for actual prescription data for England in 2008.

2.4.4.1 Crude PEC using default market penetration factor (Fpen)

The first equation (Eq 1) was used to calculate a crude PEC (PEC 1). The only variable between different pharmaceuticals when using Eq1 is the maximum daily dose. This data was obtained from RxList. The default Fpen of 1% is based on a wide range of individual market penetration factors from German consumption data in 2001 (EMEA CHMP, 2006).

[Eq 1] Crude PEC (EMEA guidelines, 2006)

$$PEC_{SURFACEWATER} = \frac{DOSEai * Fpen}{WASTEWinhab * Dilution}$$

DOSEai: Maximum daily dose consumed per inhabitant ($mg.hab^{-1}.day^{-1}$)
Fpen: Percentage of market penetration (default value = 1%)
WASTEWinhab: Amount of wastewater per inhabitant per day ($l.hab^{-1}.day^{-1}$; default value = 200L)
Dilution: dilution factor from STP to surface water (default value = 10)

2.4.4.2 Crude PEC calculations using prescription data

The EMEA guidelines advise that if marketing or consumption data is available then PEC calculations can be performed using this data. Consumption data for England were collected for the majority of pharmaceuticals in this study from the Department of Health cost analysis 2008 (NHS, 2008). The IMS™ sales data from 2004, obtained from DEFRA, (2007), were used for paracetamol and ibuprofen. The latter provides the total amounts of active ingredient sold, which are generally more realistic than prescription data for these two pharmaceuticals because of their high over the counter sales (DEFRA, 2007). The population of England in 2008 was 51.5 million (UK National Statistics, 2008).

[Eq 2] Crude PEC using consumption data

$$\text{PEC surface water} = \frac{\text{Consumption [mg*year}^{-1}\text{]}}{\text{WASTEWinhab [l/inh*d]} * \text{Dilution} * 365\ \text{d*year}^{-1} * \text{inhabitants [inhab]}}$$

(See equation above for definition of terms)

2.4.5 Critical analysis of reporting methods and standards in the literature

The quality of reporting environmental concentration data in peer reviewed scientific journals was too poor to perform a meta analysis on amalgamated data from articles. This led to a novel analysis of reporting methods and standards of human pharmaceuticals. Proposed reporting criteria (see below) were selected on the basis of information that was regularly missing from publications which was critical for performing a meta analysis of environmental concentration data. Missing information related to population size, sewage treatment, flow rates, season, and pharmaceutical persistence in water bodies. Meta analysis is a recognised and valuable tool in medical interventions and could be an equally useful tool in environmental protection.

Reporting standards for peer reviewed publications on the selected ten pharmaceuticals were assessed. In total 128 articles were analysed for the presence or absence of the following information as an indication of reporting standard:

1. Date, month or season for sampling.

In order to assess how seasonal changes may affect environmental concentrations of pharmaceuticals it is necessary to know the date that samples were collected. Date of sampling is especially important for assessing the reliability of predicted concentrations (PECs) as sales/prescription data may vary season to season or year to year.

2. Sample size or number of samples. (Frequency of detection was not counted if the overall sample size was not provided.)

It was intended that the "The Cochrane Review" would be used as the method for performing a meta analysis of environmental concentration data. This method uses the statistical mean in order to compare data sets from different studies. The sample size for each mean is required in order to apply weighting according to the size of the study and the contribution each study makes to the overall finding. For example a mean of 3 samples is not as statistically significant as a mean of 40 samples. The sample size is therefore important for assessing the weight of evidence that a study provides.

3. Statistics including median, mean, standard deviation, 90th percentile, minimum, maximum or a range of concentrations for the selected pharmaceuticals.

In order to perform a meta analysis of data from different studies, statistical uniformity is needed. The Cochrane Review uses the mean and standard deviation for this purpose. The statistical method used in each publication was recorded during data collection.

4. Replicate samples either taken on a different date or time at the same location, at a different sewage treatment plant (STP), different location on the river or more than one river in the same location. Composite samples were also included as a repeat.

A single measurement does not provide robust information with which to draw conclusions about the concentrations of pharmaceuticals in a water body.

5. Location of sampling site for surface water e.g. upstream or downstream from sewage outfall.

When examining the effects that sources of pharmaceuticals have on concentrations in water bodies it is necessary to know the location of the sampling site. In theory concentrations of

pharmaceuticals should be lower upstream of the sewage effluent outfall, highest at the sewage discharge point and then decrease downstream of the discharge point. In order to ascertain if this is the case it is necessary to know the location of sewage effluent discharge points and hence the location of the sampling site.

6. Distance from a sewage outfall and position of the sampling location.

Changes in pharmaceutical with distance from the sewage outfall location might provide information on persistence of pharmaceuticals in water bodies. It is desirable to know what downstream dilution effects are taking place on sewage effluent discharge points downstream. This is important for examination of the dilution default of 10 used in PEC calculations.

7. Population served or capacity of the STP relevant to the sample location.

In order to calculate a predicted concentration for a pharmaceutical in the environment the total amount of the pharmaceutical consumed over the year is divided by the size of population. It is therefore important to determine if the size of population served by an STP affected the concentration of the pharmaceutical in the final sewage effluent.

8. Specification of sewage treatment process.

In order to assess the efficiency of different sewage treatments for the removal of pharmaceuticals and hence the affect on concentrations in receiving water bodies it is necessary to know the type of sewage treatment employed.

9. Average discharge of effluent or the quantity of sewage treated.

If the average discharge of effluent is provided it is possible to calculate the environmental load using the concentration of pharmaceutical in sewage effluent.

10. For surface waters the average river flow rate or flow rate at the time of sampling.

Changes in pharmaceutical concentration may be directly related to the flow rate of the river. This data is useful for calculation of average and maximal loads based on high and low flow data.

11. Limit of detection (LOD) for the analytical method. (A limit of quantification (LOQ) was considered valid.)

This information is required to assess the limitations of the analytical chemistry method used and ascertain the reliability and robustness of the results.

The articles were scored against the criteria and the percentage of articles providing each category of information calculated.

2.5 Results

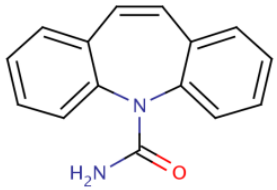
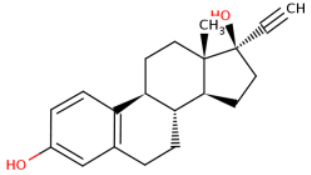
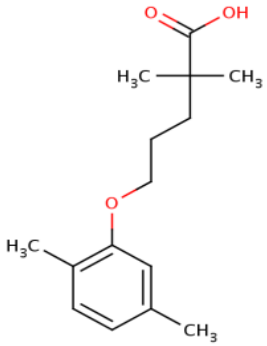
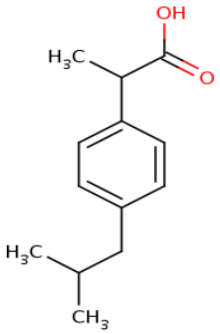
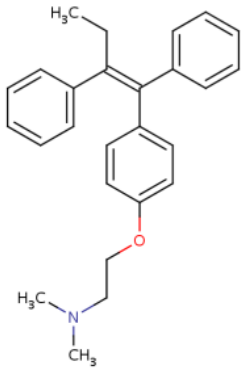
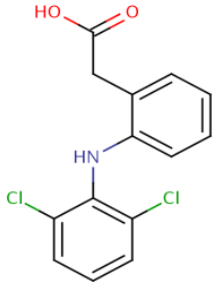
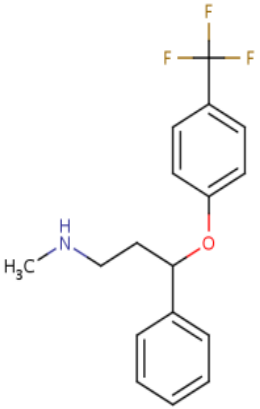
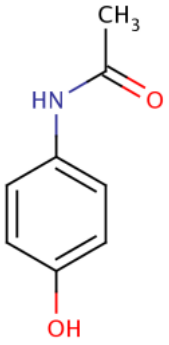
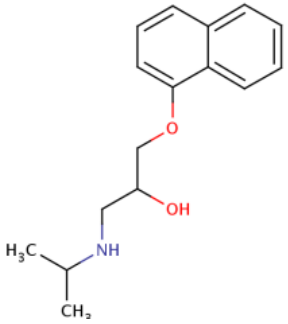
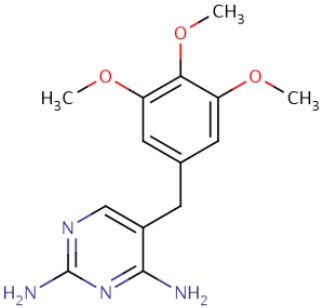
2.5.1 Physical, chemical and sales data for selected pharmaceuticals

Structure, physical and chemical information, octanol-water partition coefficient (log K_{ow}), water solubility and prescription amounts in England, (2008) in numbers and weight (kg) were collected for each of the ten selected pharmaceuticals; carbamazepine, diclofenac, EE2, fluoxetine, gemfibrozil, ibuprofen, paracetamol, propranolol, tamoxifen and trimethoprim (Table 2.1 & Table 2.2).

Table 2.1 Physical, chemical and prescription data
([a]: www.chbr.noaa.gov/peiar/; [b]: www.drugbank.com; [c]: IMS data DEFRA, 2004; [d]: Oakes *et al*., 2010; [e]: Mompleat *et al*., 2010; OTC: over the counter sales)

Pharmaceutical	Therapeutic use	Log k_{ow}	Water solubility (mg ml^{-1})	Consumption data, England, 2008	
				(prescriptions in 1000's)	(kg)
Carbamazepine CAS -298-46-4	anti-convulsive anti-neuralgic anti-manic anti-diuretic anti-psychotic	2.25[e]	0.0177[a]	24,025	45,705kg
Diclofenac CAS -15307-86-5	analgesic	4.51[e]	2.43[a]	71,935	26,442.7
Ethinylestradiol CAS -57-63-6	contraceptive	3.67	11.3[ab]	-	0.08
Fluoxetine CAS-54910-89-3	anti-depressant anti-obsessional	3.82 - 4.67 [d]	14[a]	5,034.5	4435.4
Gemfibrozil CAS-2581-30-0	anti-hyperlipidemic	3.9[e]	10[a]	20.6	755.3
Ibuprofen CAS-15687-27-1	analgesic	3.97[e]	0.021[a]	OTC	330,292[c]
Paracetamol CAS-103-90-2	analgesic	0.46[e]	14[a]	OTC	3,534,737[c]
Propranolol CAS-318-98-9	anti-hypertensive/diuretic beta-adrenergic blocking agent	- 0.45[e]	0.07[a]	2,732.1	7,784.5
Tamoxifen CAS-10540-29-1	anti-estrogen cancer drug	6.3[e]	0.0002[b]	641.1	521.4
Trimethoprim CAS – 738-70-5	antibiotic	0.91[e]	0.3-0.4[a] 12.1[b]	3,203.4	9736.6

Table 2.2 Chemical structure of selected pharmaceuticals

Carbamazepine	**Ethinylestradiol**	**Gemfibrozil**	**Ibuprofen**	**Tamoxifen**
				
Diclofenac	**Fluoxetine**	**Paracetamol**	**Propranolol**	**Trimethoprim**
				

2.5.2 Surface water concentrations in freshwater systems

Mean surface water concentrations were collected or calculated for four pharmaceuticals, diclofenac, carbamazepine, ibuprofen and gemfibrozil. The concentrations varied with pharmaceutical but all were in the ng – μgl^{-1} range (Fig 2.1-2.5). A summary of the data for all four of these pharmaceuticals separated between Europe and outside of Europe is also provided (Fig 2.1). Mean measurements of these four pharmaceuticals regularly differed more than an order of magnitude (Fig 2.5). The variation between measured concentrations was to a degree that meant the results could only be shown on a logarithmic scale. The standard deviations of total means were too large to be shown graphically when calculating the total mean (Fig 2.1). Mean reported concentrations for these pharmaceuticals ranged from 0.1 ngl^{-1} to 2.1 μgl^{-1}. All four pharmaceuticals had total mean of means concentrations between 10 and 100 ngl^{-1} and all had some mean detections over 100 ngl^{-1}. Mean ibuprofen concentrations exceeded 1 μgl^{-1} on more than one occasion (Fig 2.4). Measured surface water concentrations for all four pharmaceuticals in Europe (Austria, Finland, France, Germany, Romania, Spain, Switzerland, UK) appeared representative of the situation outside of Europe (data from USA, Canada, China and Brazil) (Fig 2.1).

In studies of surface water, where a mean concentration was obtained carbamazepine was almost always present. The highest mean concentration reported for this pharmaceutical was 675 ngl^{-1} in France (Coetsier *et al*., 2009) and the overall mean of all mean reported concentrations worldwide using all the mean data was 54 ngl^{-1} with a standard deviation of 106.3 ngl^{-1} (Fig 2.2). The mean outside of Europe was 31 ngl^{-1} and inside Europe was 68 $ngl^{-}1$ (Fig 2.1). Only twice, in Romania, was the mean calculated as less than the limit of detection for that method (see appendix 2 for raw data). A mean concentration of carbamazepine in surface waters in England was not found in the literature although maximum concentrations have been reported between 7 ngl^{-1} (Kasprzyk-Hordern *et al*., 2008) and 647 ngl^{-1} (Kaspryk-Horndern *et al*., 2009) in Wales. The mean concentration value for carbamazepine appears to exceed 10 ngl^{-1} (ERA action limit) in 69.5% of sampling campaigns for surface waters worldwide and 73% within Europe (see appendix 2 for raw data).

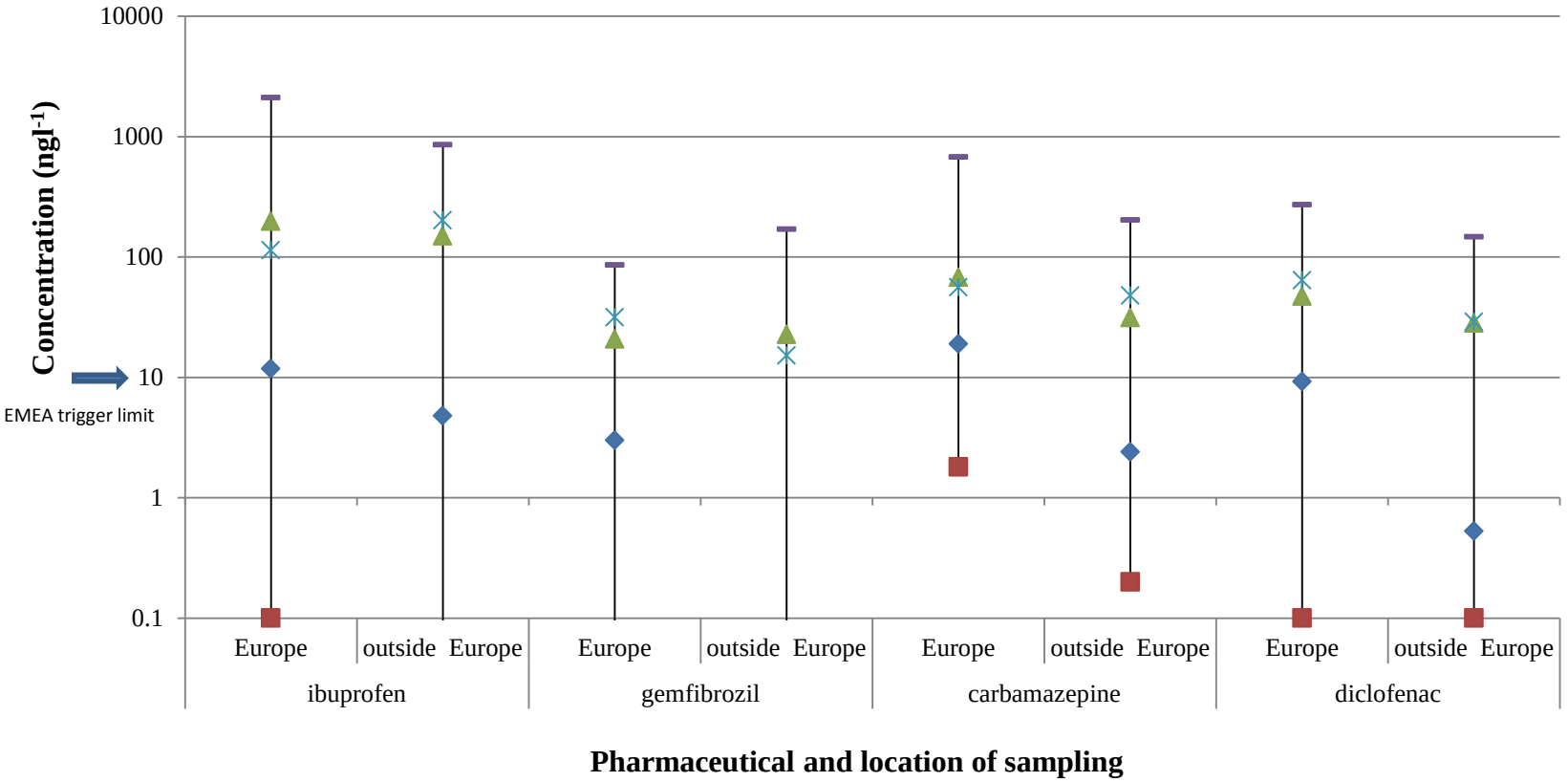


Fig 2.1 Summary of reported and calculated mean measured environmental concencentrations (MECs) in surface waters from the literature. For a breakdown of this data see Figs 2.2-2.5. (European data includes measurements taken in: Austria, Finland, France, Germany, Romania, Spain, Switzerland, UK. Outside Europe data includes measurements taken in Canada, China, Brazil, USA). For raw data see appendix 1. ◆25th percentile ■min ▲total mean —max ✳75th percentile

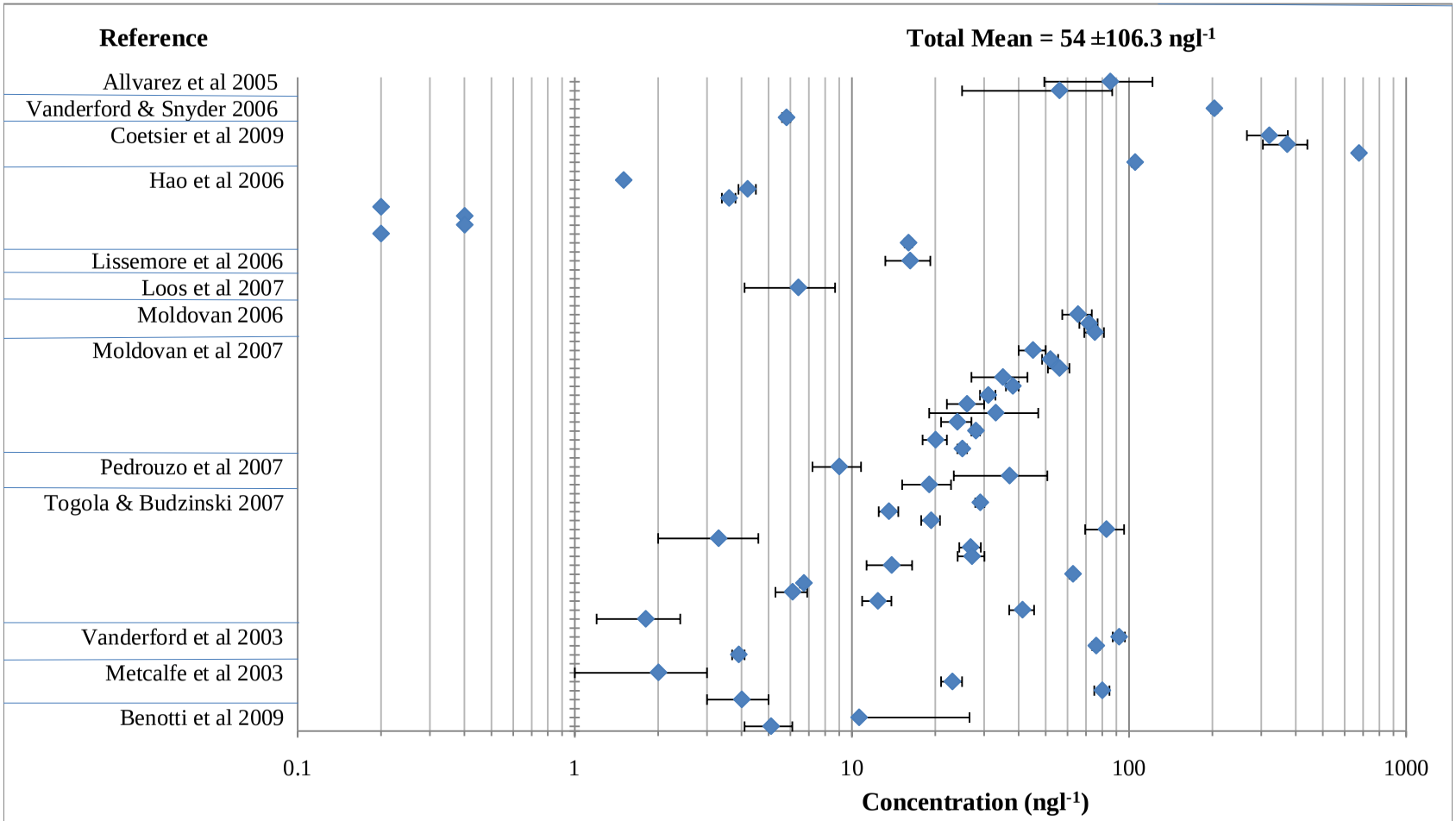


Fig 2.2 Mean surface water concentrations of carbamazepine reported in the literature. Dataset does not contain any mean concentrations below the limit of detection or quantification (LOD/LOQ). For raw data and means calculations see appendix 2. Multiple mean measurements for the same reference indicate samples taken at different locations or times.

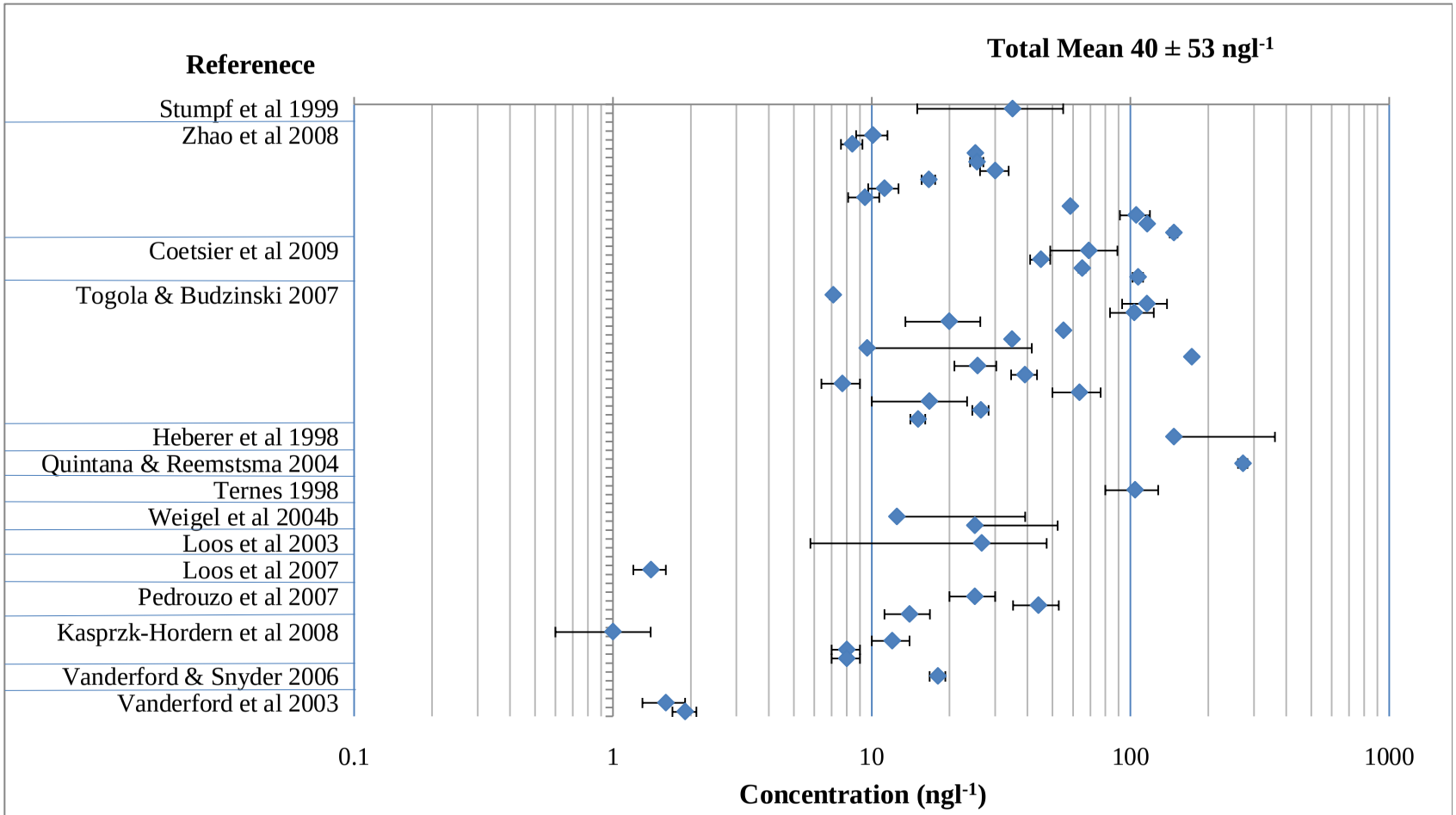


Fig 2.3 Mean surface water concentrations of diclofenac reported in the literature. Dataset does not contain any mean concentrations below the limit of detection or quantification (LOD/LOQ). For raw data and means calculations see appendix 3. Multiple mean measurements for the same reference taken in different locations or times.

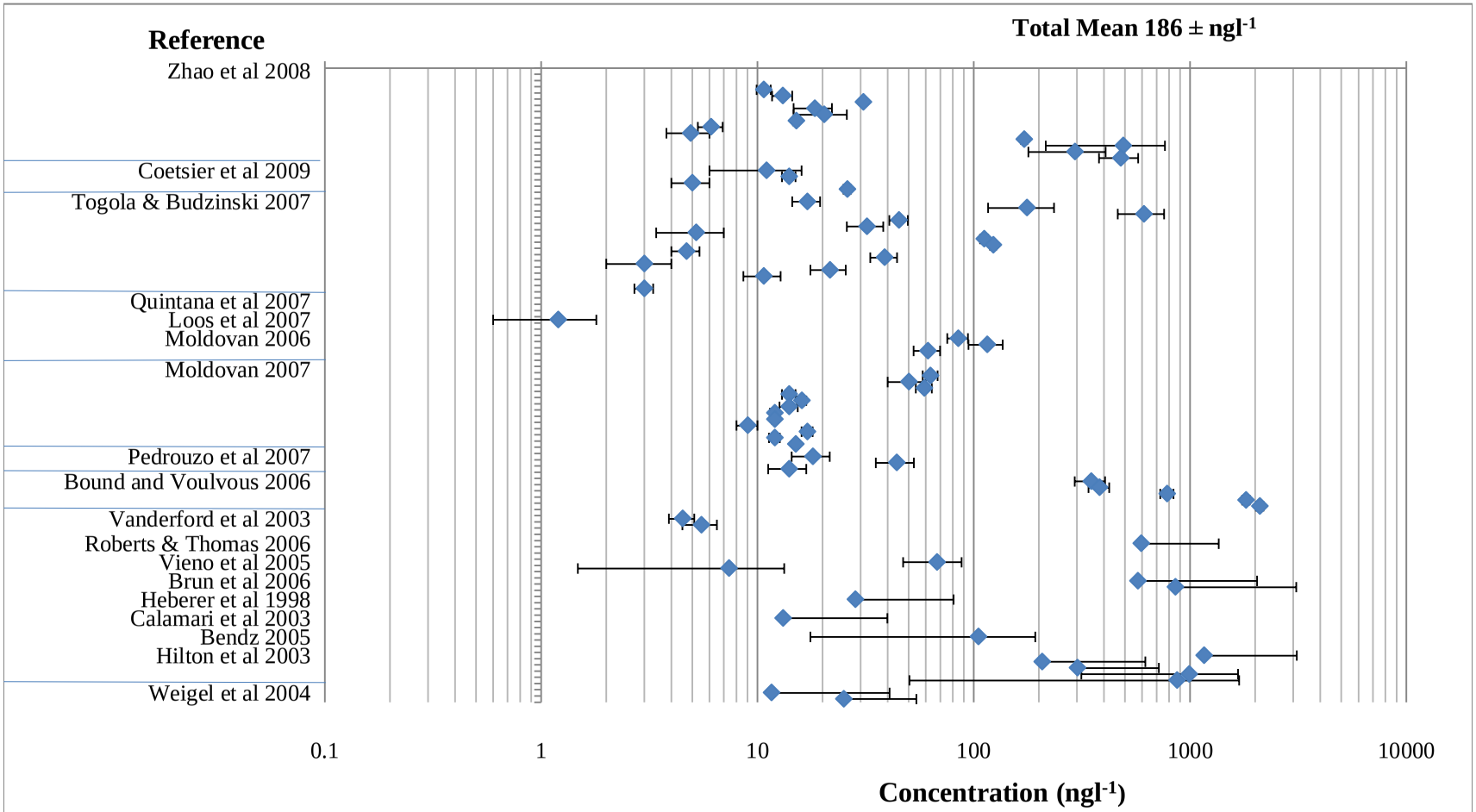


Fig 2.4 Mean surface water concentrations of ibuprofen reported in the literature. Multiple points for the same reference indicate smples taken at different locations or times (see appendix 4 for raw data). Data set includes 8 mean concentrations below the limit of detection or quantification (LOD/LOQ) not shown on the graph due to logarithmic scale but included in the total mean concentration calculation.

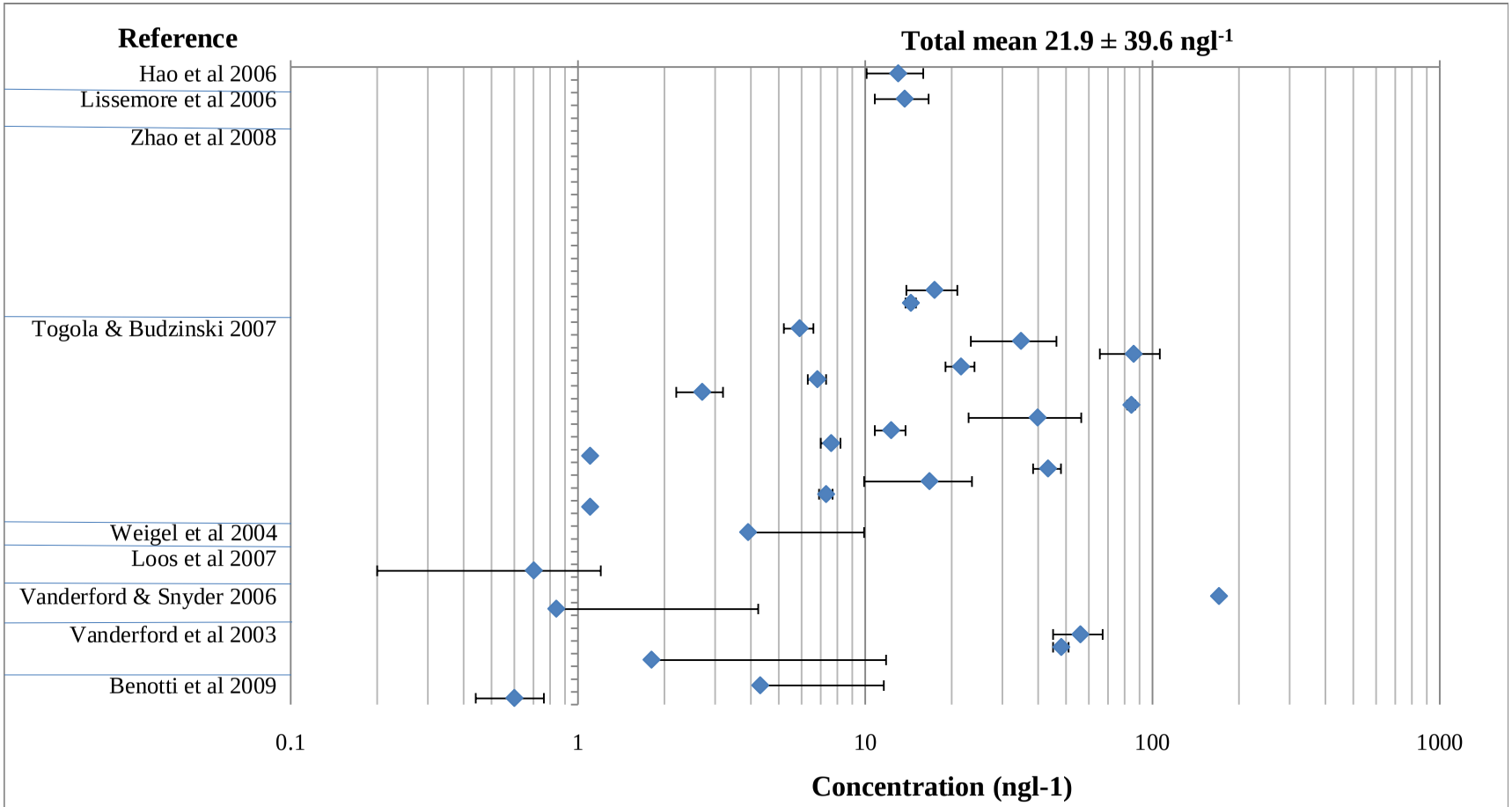


Fig 2.5 Mean surface water concentrations of gemfibrozil reported in the literature. Multiple points for the same reference indicate samples taken at different locations or times. Dataset includes 16 mean concentrations below the limit of detection or quantification (LOD/LOQ) which are not shown the the graph due to the logarithmic scale but included in the total mean calculation. For raw data see appendix 5.

Diclofenac had a total mean surface water concentration in Europe of 47.2 ngl^{-1} and 28.2 ngl^{-1} for the rest of the world (Fig 2.1). The highest mean concentration of diclofenac was 272 ngl^{-1} measured in Berlin in 2003 (Quintana & Reemtsma, 2004) (Fig 2.3). The mean concentrations of diclofenac fell below the detection limit in 14.3% of surface water sampling events (see appendix 3 for raw data). The average of all mean concentrations calculated was 40 ngl^{-1} four times the ERA action limit. The standard deviation about the total mean was 53 ngl^{-1} (Fig 2.3).

Ibuprofen showed the largest variation in concentration and the highest mean concentration in surface waters of the four drugs (Fig 2.4). Its highest mean concentration was 2.1μgl^{-1} (Fig 2.4) was measured in the UK in 2006 (Bound & Voulvoulis, 2006). Non detections (or detections below the method's detection limit) (see appendix 4 for raw data) occurred in 10% of published mean results and was incorporated into the mean of mean surface water concentration, which was 186 ngl^{-1} (SD 385 ngl^{-1}) (Fig 2.4), nearly 19 times the ERA action limit.

The total mean surface water concentration in Europe for gemfibrozil was 24.9 ngl^{-1} and 49.5 ngl^{-1} for outside of Europe (Fig 2.1). The overall total mean was 35.3 ngl^{-1} with a standard deviation of 45.6 ngl^{-1} (Fig 2.5). A highest mean concentration of gemfibrozil was measured in an urban waterway in the USA of 170 ngl^{-1} (Vanderford & Snyder, 2006) (see appendix 5 for raw data). The mean concentration of gemfibrozil exceeded 10 ngl^{-1} in over 50% of the published literature. Overall there was much less data for gemfibrozil. A published mean concentration was not available and insufficient raw data has been measured in order to calculate one for the UK.

The ranges of concentrations of trimethoprim (Fig 2.6), paracetamol (Fig 2.7), propranolol (Fig 2.8) and tamoxifen (Fig 2.9) were all quite variable. There was much less data for tamoxifen with only two countries, France and the UK investigating the environmental concentration of this pharmaceutical. Trimethoprim, paracetamol and propranolol were regularly detected in water courses worldwide often exceeding 10 ngl^{-1}, the trigger limit for risk assessment. Paracetamol concentrations exceeded 1μgl^{-1}, 100 times the trigger limit in several countries (UK, Canada, USA) (Fig 2.7). Maximum concentrations of 2.4μgl^{-1} in the River Taff, UK, 1km upstream from the STP (Kasprzyk-

Hordern *et al*., 2008) and 10µgl^{-1} in the USA (Kolpin *et al*., 2002) have been reported for this analgesic.

There was limited data for concentrations of fluoxetine (Table 2.3) and EE2 (Table 2.4) in surface waters but reported concentrations were always in the ngl^{-1} range. The highest reported concentration of fluoxetine was a mean of 20 ngl^{-1} in the USA (Shultz & Furlong, 2008) however a measurement 3 ngl^{-1} also in the U.S.A (Benotti *et al*., 2009) is more representative of general findings (Table 2.3). EE2 surface water concentrations have been measured, in a reconnaissance exercise studying over 90 rivers and streams in the USA, at a maximum of 831 ngl^{-1} and a median of 73 ngl^{-1} (Kolpin *et al*., 2002) (Table 2.4). However reported concentrations in other published literature were usually below the detection limit of the method or around 0.5- 4 ngl^{-1}.

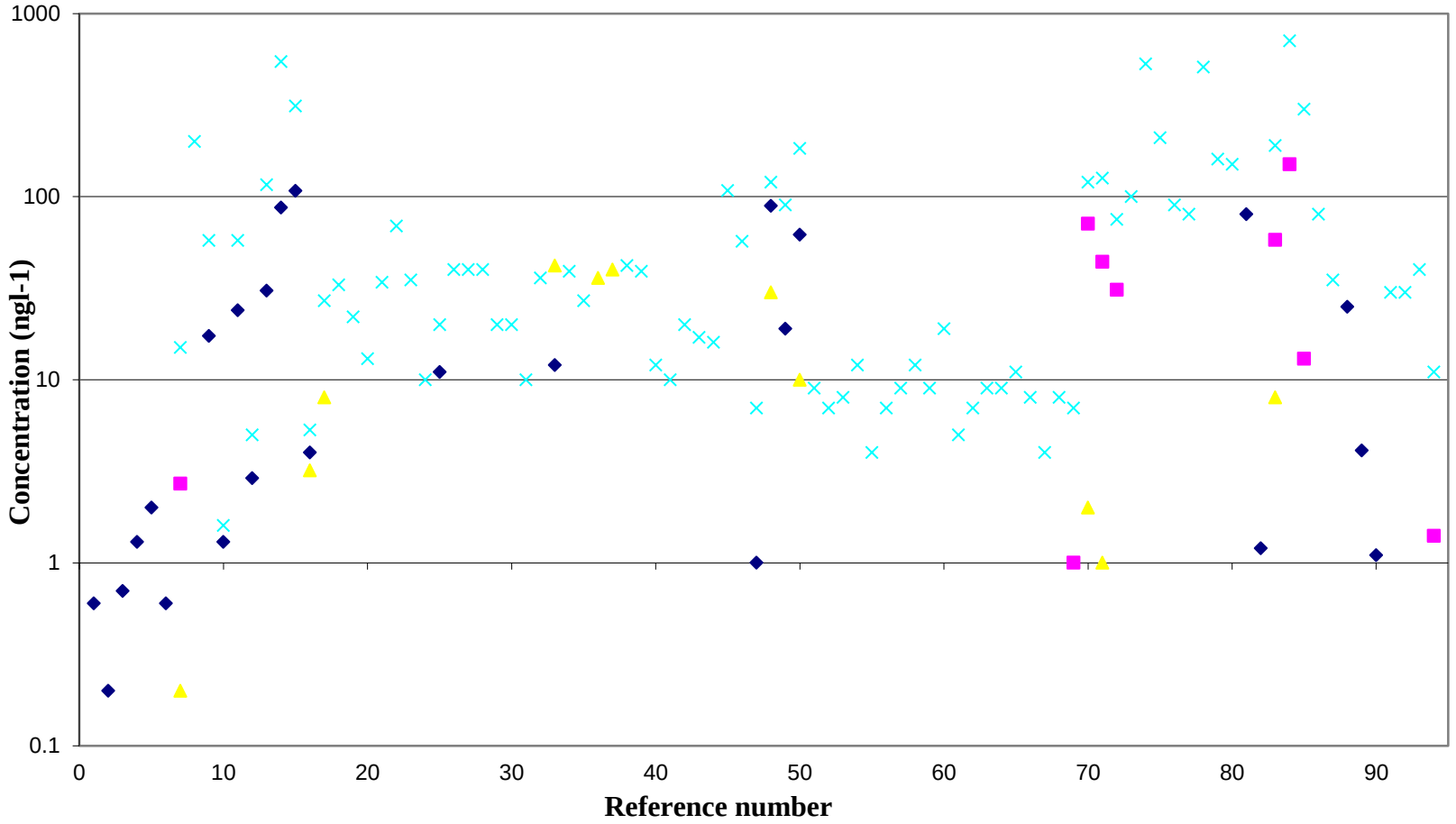


Fig 2.6 Measured environmental concentrations (MECs) of trimethoprim in surface water. Individual points represent MECs reported in the literature. Only positive detections are shown due to logarithmic scale. Data set includes 74 measurements below the limit of detection or quantification (LOD/LOQ). For raw data and references see appendix 1. ◆ Mean ■ Median ▲ Minimum × Maximum

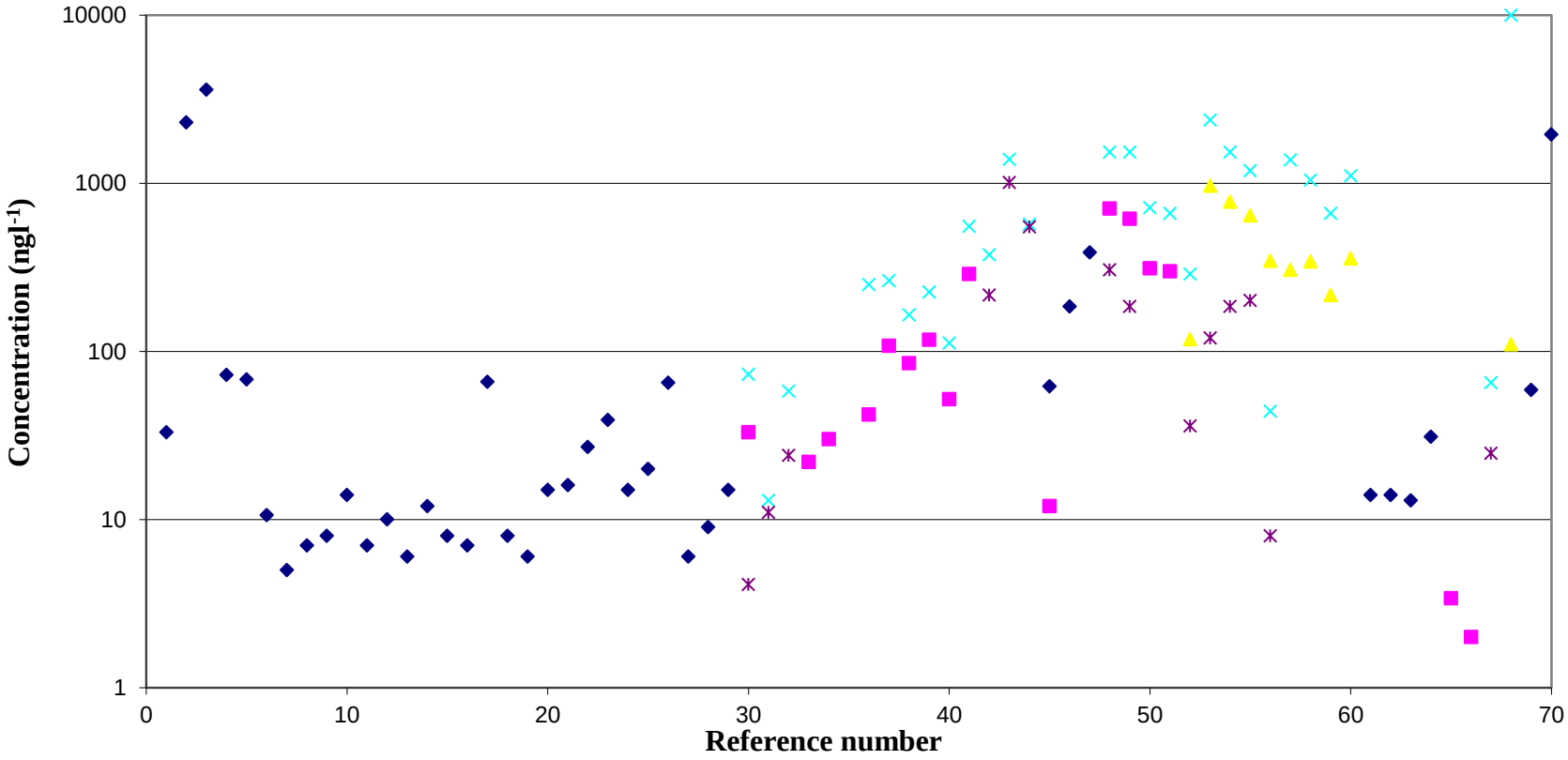


Fig 2.7 Measured environmental concentrations (MECs) of paracetamol in surface water. Individual points represent MECs reported in the literature. Only positive detections are shown due to logarithmic scale. Data set includes 18 non detections (ND) and 54 detections below the limit of quantification (LOQ). For raw data and references see appendix 1.

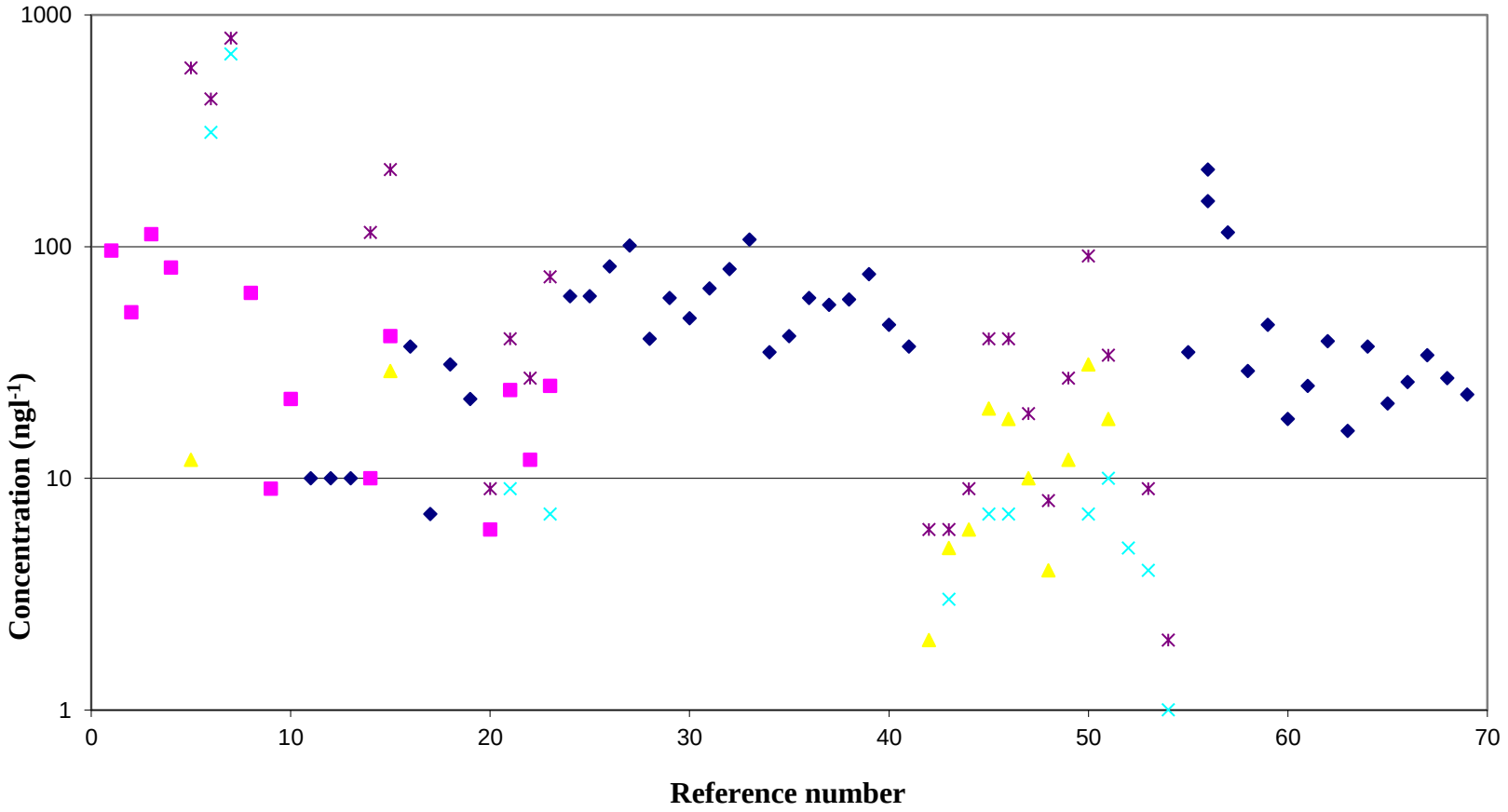


Fig 2.8 Measured environmental concentrations (MECs) of propranolol in surface water. Individual points represent MECs reported in the literature. Only positive detections are shown due to logarithmic scale. Data set includes 4 non detections (ND) and 31 measurements below the limit of quantification (LOQ). For raw data and references see appendix 1.

◆ Invidual MEC ■ Mean ▲ Median × Minimum ✳ Maximum

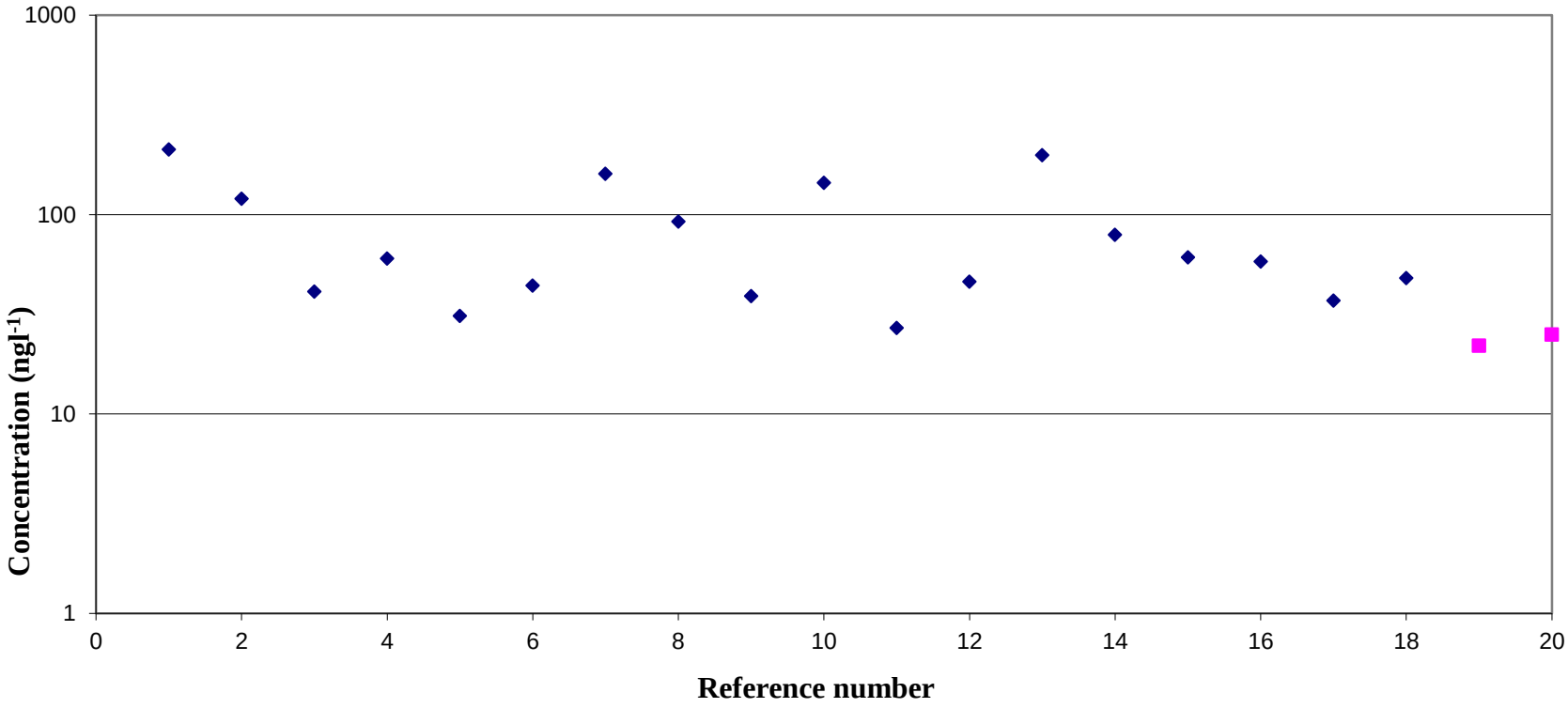


Fig 2.9 Measured environmental concentrations (MECs) of tamoxifen in surface water. Individual points represent MECs reported in the literature. Only positive detections are shown due to logarithmic scale. Data set includes 31 measurements below the limit of detection or quantification (LOD/LOQ). For raw data and references see appendix 1. ◆ Individual MEC ■ Mean

Table 2.3 Measured environmental concentrations of fluoxetine in surface water (ngl^{-1}) (ND: non detection; bld: below detection limit)

Mean	Median	Minimum	Maximum	Reference
ND				Kim *et al*., 2007
bld	bld	bld	bld	Gros *et al*., 2006
	0.8		3	Benotti *et al*., 2009
<18	<18	<18	<18	Alvarez *et al*., 2005
ND	ND	ND	ND	Batt *et al*., 2008
2.6				Vanderford & Snyder, 2006
<0.50				Vanderford & Snyder, 2006
12				Schultz & Furlong, 2008
20				Schultz & Furlong, 2008
12				Schultz & Furlong, 2008
5.5				Vanderford *et al*., 2003
ND	ND	ND		Vanderford *et al*., 2003
	14	bld	44	Gonzalez-Alonso *et al*., 2010

Table 2.4 Measured environmental concentrations of EE2 in surface water (ngl^{-1}) (ND: non detection)

Mean	Median	Minimum	Maximum	Reference
ND				Kim *et al*., 2007
	73		831	Kolpin *et al*., 2002
ND	ND	ND	ND	Zhang *et al*., 2007
	1.4		1.4	Benotti *et al*., 2008
ND			1	Peng *et al*., 2008
<1.0				Vanderford *et al*., 2003
			ND	Zuccato *et al*., 2005
ND				Zuehlke *et al*., 2004

Despite the wide variation in surface water concentration data for all 9 of the pharmaceuticals investigated from the published literature frequently had a mean measured concentration above the 10 ngl^{-1} action limit set by the EMEA for further environmental risk assessment (Table 2.5).

Table 2.5 Percentage of mean surface water concentrations greater than the 10 ngl^{-1} environmental risk assessment action limit. Only mean measurements used for comparison. ([a]: Brazil, Canada, China, Korea, Taiwan and the USA. [b]: Austria, France, Finland, Germany, Greece, Hungary, Italy, Netherlands, Norway, Poland, Romania, Slovenia, Spain, Sweden, Switzerland, and UK.)

Pharmaceutical	Worldwide[a+ b]		Europe[b]	
	Total mean reported or calculated concentrations	Percentage >10 ng/L	Total mean reported or calculated concentrations	Percentage >10 ng/L
carbamazepine	59	**69.5**	37	**73**
diclofenac	49	**77.6**	33	**78.8**
fluoxetine	14	**21.4**	1	**0**
gemfibrozil	44	**38.6**	18	**44.4**
ibuprofen	79	**74.7**	47	**79.7**
paracetamol	16	**87.5**	13	**100**
propranolol	18	**61.1**	24	**54.2**
trimethoprim	26	**46.2**	12	**75**
tamoxifen	6	**33.3**	6	**33.3**

Mean concentrations of carbamazepine, diclofenac, gemfibrozil, ibuprofen, paracetamol, propranolol and trimethoprim were all above the trigger limit in more than 50% of samplings in Europe. Mean concentrations of tamoxifen were over 10 ngl^{-1} in Europe in 33% of the studies. Fluoxetine was not measured above 10 ngl^{-1} in Europe but has exceeded this limit elsewhere in the world. Insufficient data was available for EE2, but findings show that generally concentrations of this drug were small.

2.5.3 Sewage effluent concentrations

Mean concentrations of carbamazepine, diclofenac, ibuprofen and gemfibrozil in sewage effluent were very varied (Fig 2.10-2.15). A summary graph of mean MEC data for these four pharmaceuticals is also provided with European data separated from data outside of Europe (Fig 2.10). Again a logarithmic scale had to be used to display the data graphically. The total means for these pharmaceuticals ranged from 34 ngl^{-1} (gemfibrozil) and 3.5 µgl^{-1} (ibuprofen) (Fig 2.10). The maximum mean across the four pharmaceuticals

was for ibuprofen at 16.5µgl^{-1} (Fig 2.10) and the lowest was below the detection limits or not detected (see appendix 6-9). Apart from concentrations of gemfibrozil outside of Europe, the 75th percentile of the means for all four drugs was above 100 ngl^{-1} and concentrations regularly exceed 1 µgl^{-1}(Fig 2.10).

Carbamazepine is ubiquitously detected in sewage effluent worldwide. Mean reported concentrations ranged from 7 ngl^{-1} (Canada) (Metcalfe *et al*., 2003) to 2.5 µgl^{-1} (France) (Togola & Budzinski, 2007) with a total mean of all mean concentrations measured of 544.6 ngl^{-1} (standard deviation, 524.2 ngl^{-1}) (Fig 2.11). A mean effluent concentration of carbamazepine has not been measured in sewage effluent in the UK although a maximum of 4.6µgl^{-1} has been reported in Wales (Kasprzyk-Hordern *et al*., 2009) (Fig 2.11) (see appendix 6 for raw data). Total mean concentrations inside and outside Europe were over 100 ngl^{-1} (Fig 2.11) exceeding the ERA trigger limit when the dilution factor of 10 (see PEC Eq 1) from sewage effluent to sewage outfall was applied.

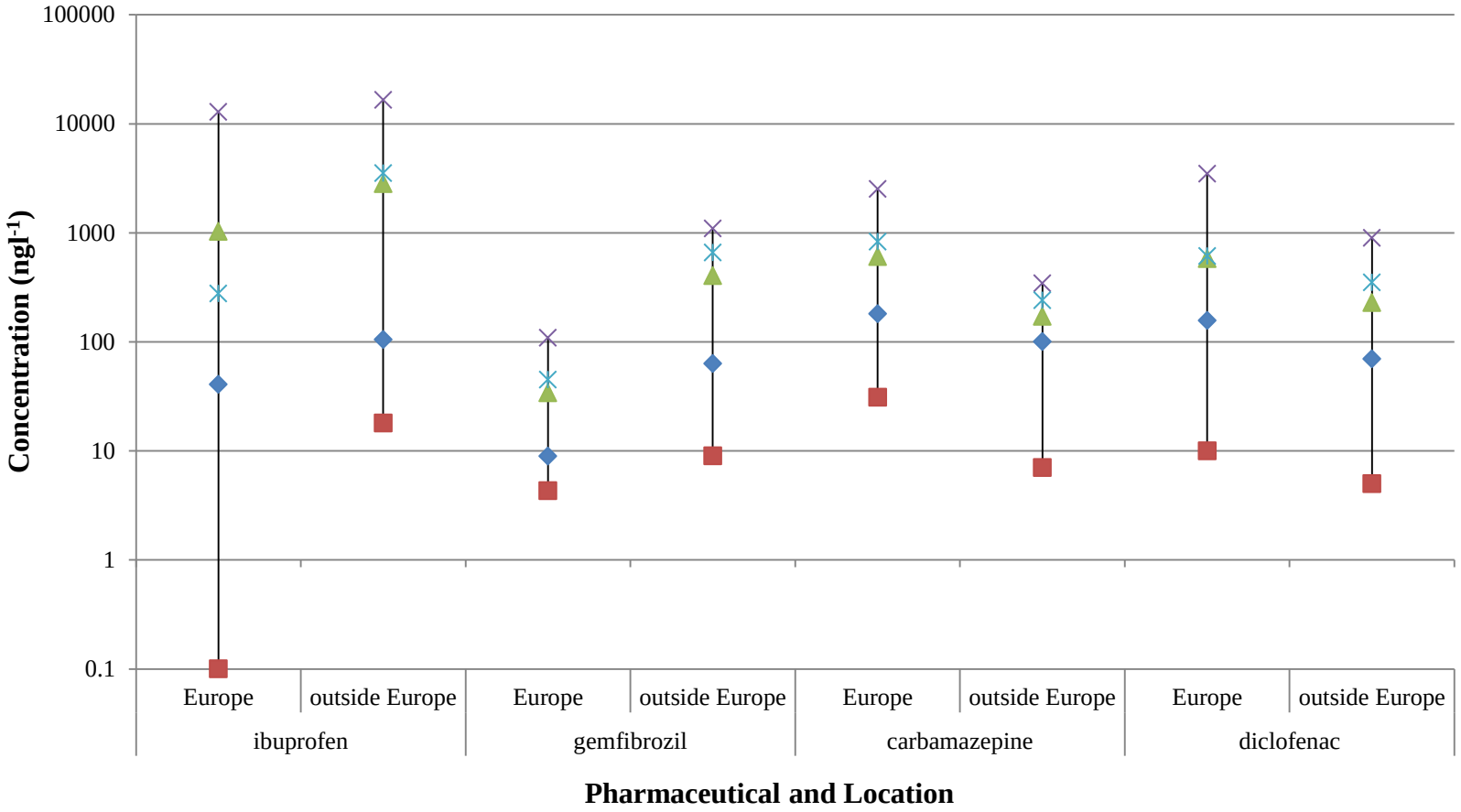


Fig 2.10 Summary of reported and calculated mean sewage effluent concentrations from the literature. For a breakdown of this data see Figs 2.11-2.14. (European data includes measurements taken in: Austria, Finland, France, Germany, Romania, Spain, Switzerland, UK. Outside Europe data includes measurements taken in Canada, China, Brazil, USA). For raw data see appendix 1. ◆25th percentile ■min ▲total mean ×max ✳75th percentile

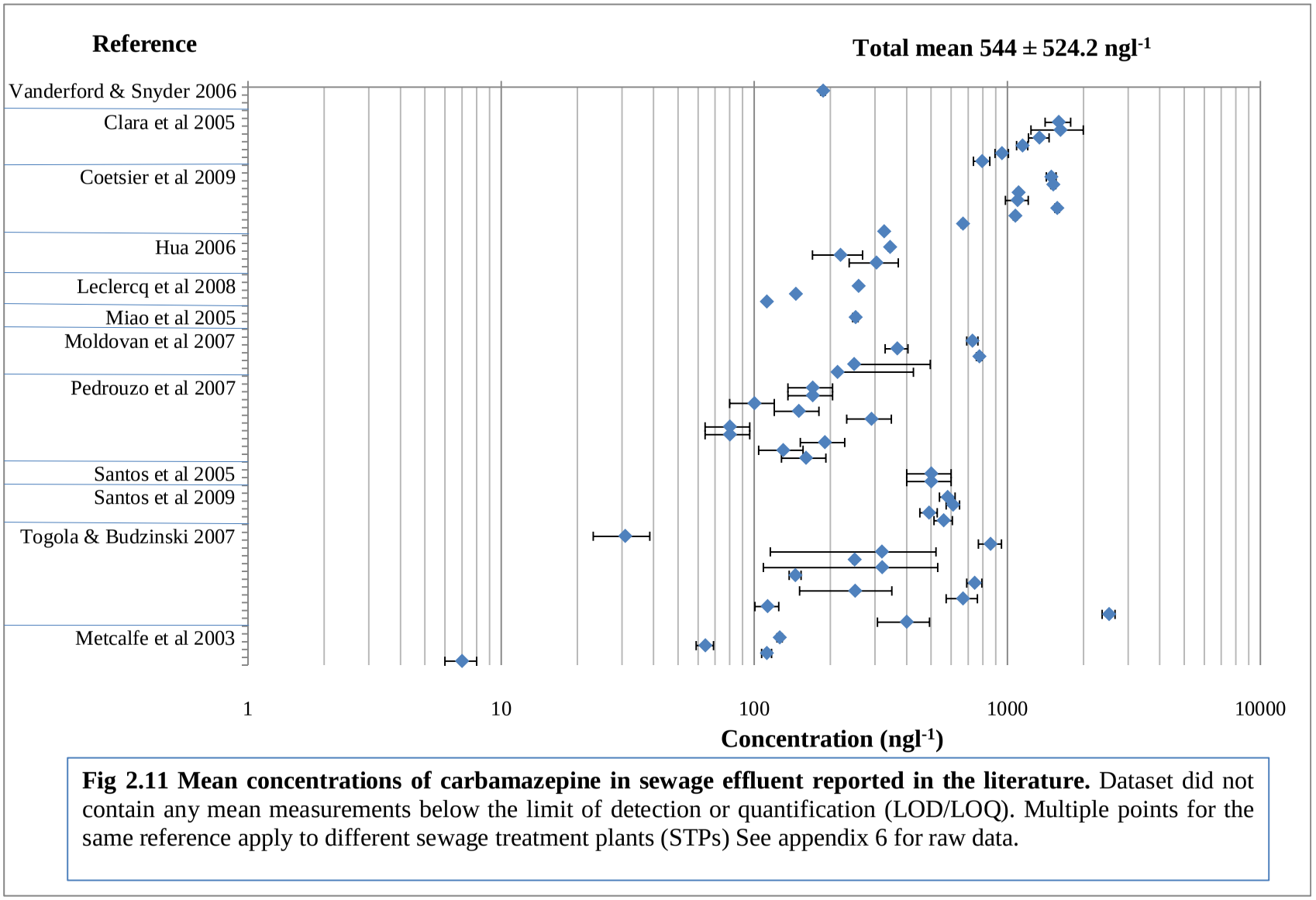


Fig 2.11 Mean concentrations of carbamazepine in sewage effluent reported in the literature. Dataset did not contain any mean measurements below the limit of detection or quantification (LOD/LOQ). Multiple points for the same reference apply to different sewage treatment plants (STPs) See appendix 6 for raw data.

Diclofenac had a maximum mean concentration of 3.5µgl^{-1} (Austria) (Clara *et al*., 2005), a minimum mean concentration of 5 ngl^{-1} (Canada) (Metcalfe *et al*., 2003), and an overall mean of mean concentrations reported or calculated of 505 ngl^{-1} (standard deviation, 640.8 ngl^{-1} in sewage effluent (Fig 2.12.) The European total mean concentration was 577 ngl^{-1} in Europe and 230 ngl^{-1} outside of Europe (Fig 2.10). In the UK the mean concentration of diclofenac in sewage effluent ranged between 382.7 ngl^{-1} (Roberts & Thomas, 2006) and 714 ngl^{-1} (Hilton & Thomas, 2003) (Fig 2.12) (see appendix 7 for raw data).

Ibuprofen had a detection frequency of 92% in sewage effluent (see appendix 8 for raw data). The mean concentration ranges from ND (Togola & Budzinski, 2007) (not shown on graph due to logarithmic scale) to 16.5 µgl^{-1} (Canada) (Metcalfe *et al*., 2003) with a total average mean of 1.47 µgl^{-1} (SD 3.2µgl^{-1}) (Fig 2.13). In the UK reported mean concentrations range from 213 ngl^{-1} (Bound & Voulvoulis, 2006) to 12.8 µgl^{-1} (Roberts & Thomas, 2006) (Fig 2.13 & appendix 8). In Europe the total mean was 1 µgl^{-1} but was higher outside of Europe at 2.8 µgl^{-1} (Fig 2.10).

Much less data exists in the literature for gemfibrozil than carbamazepine, ibuprofen and diclofenac. Only four studies were found where a mean concentration was presented or could be calculated from raw data and none of these were in the UK (see appendix 9 for raw data). The range of means was 4.3 ngl^{-1} to 1.1 µgl^{-1} in sewage effluent with an overall average of 233.4 ngl^{-1} (SD 329.3 ngl^{-1}) (Fig 2.14). The total mean in Europe was 34 ngl^{-1} (Fig 2.10) and is based on only one study in France (Togola & Budzinski, 2007). The total mean for MECs outside of Europe of was 402.4 ngl^{-1} based on 3 studies, 2 from Canada (Brun *et al*., 2006; Hua, 2006) and 1 from the USA (Vanderford & Snyder, 2006) (Fig 2.10).

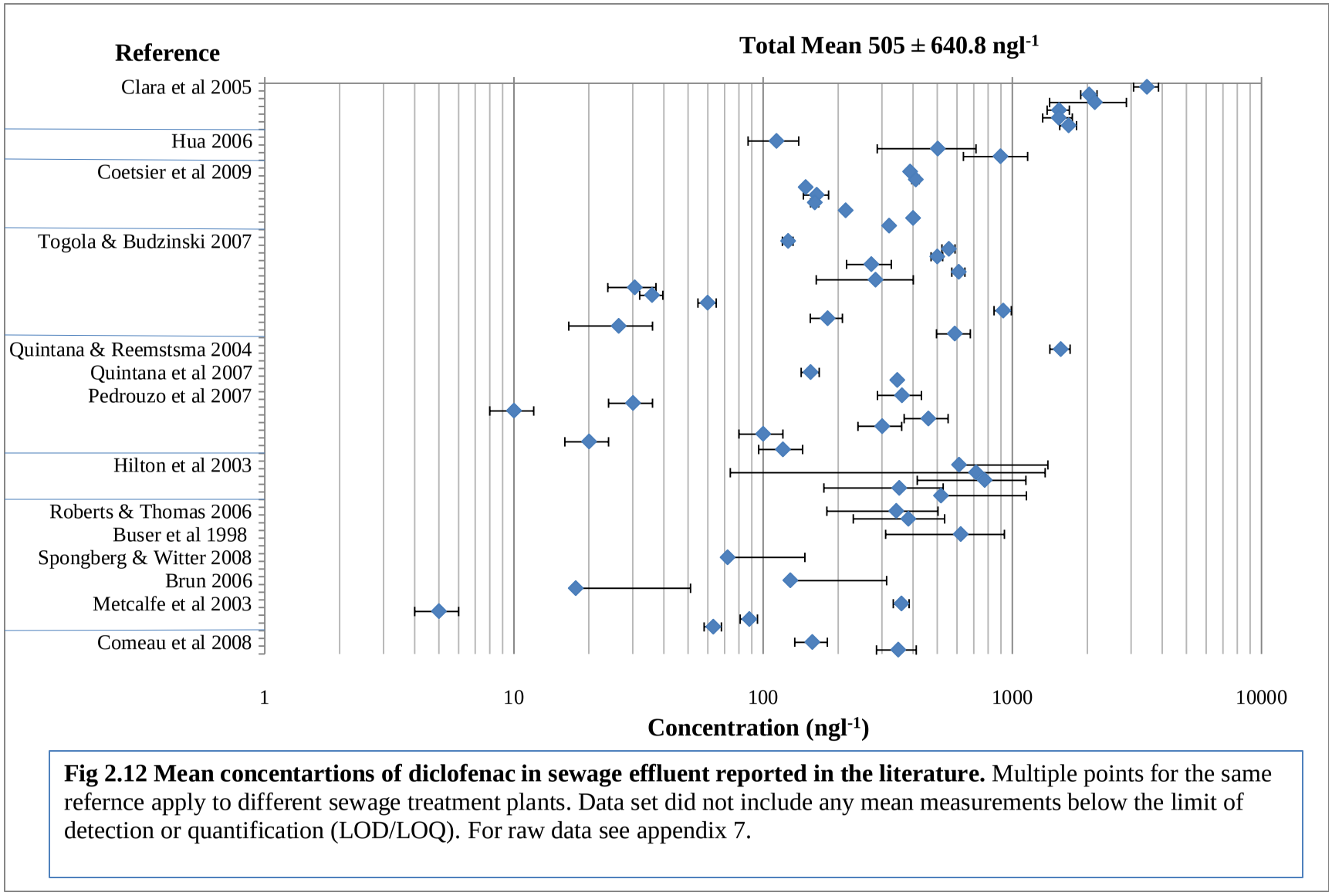


Fig 2.12 Mean concentartions of diclofenac in sewage effluent reported in the literature. Multiple points for the same refernce apply to different sewage treatment plants. Data set did not include any mean measurements below the limit of detection or quantification (LOD/LOQ). For raw data see appendix 7.

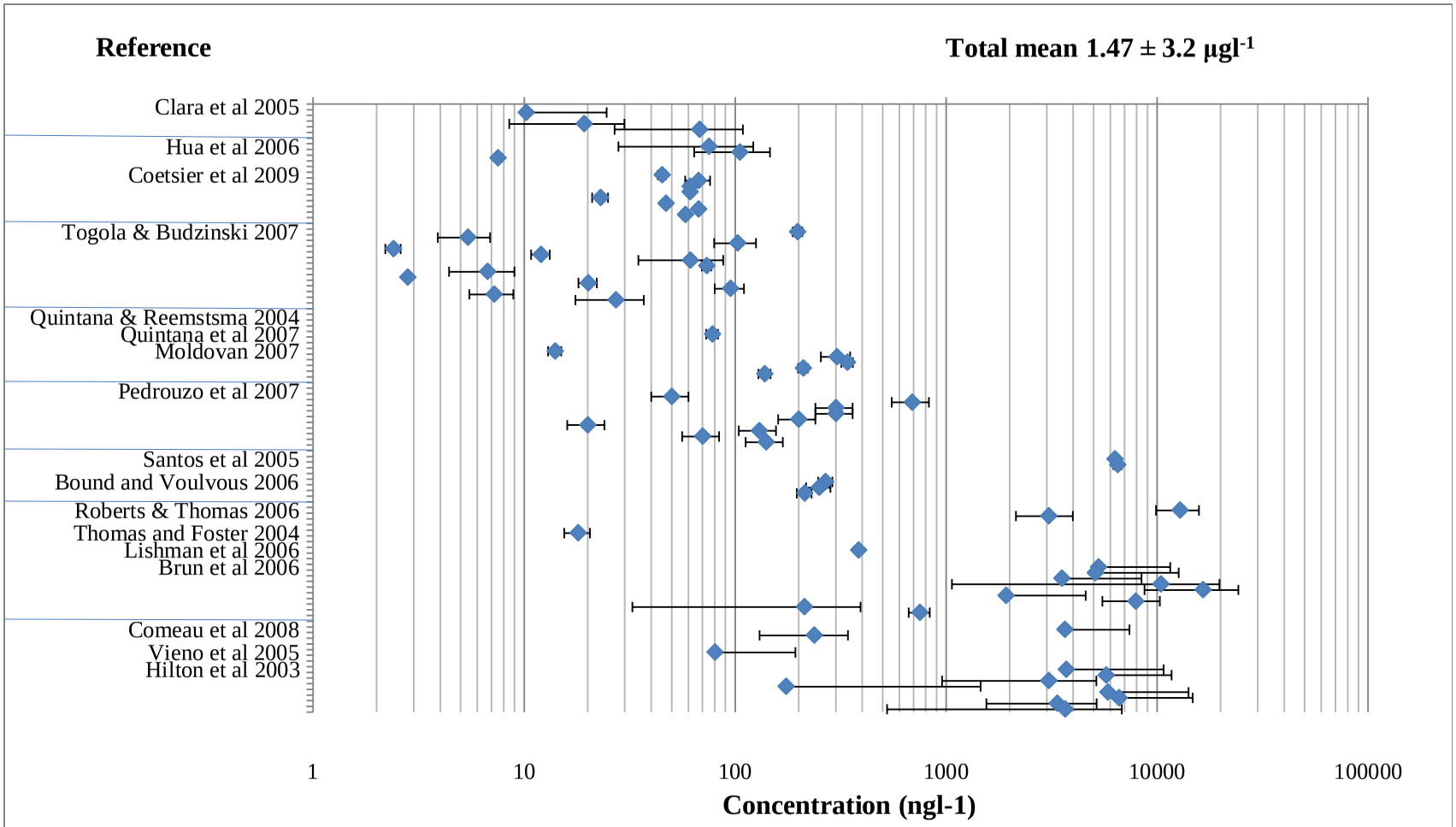


Fig 2.13 Mean concentrations of ibuprofen in sewage effluent. Positive detections only shown on graph due to logarithmic scale. Data set contains 4 non detections and 2 measurements below the limit of quantification. Multiple points for the same reference apply to different sewage treatment plants (STPs). Fow raw data and calculations see appendix 8.

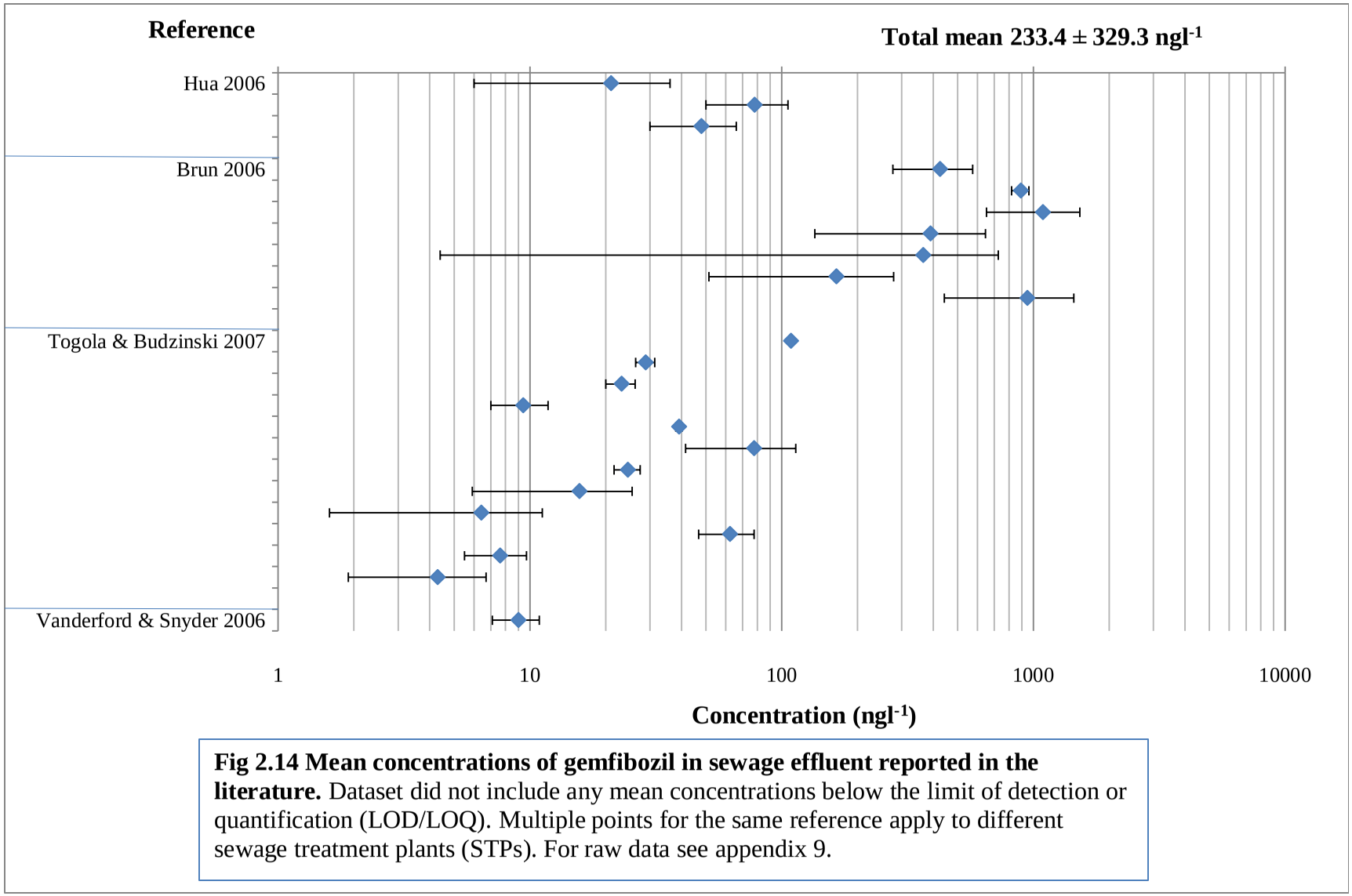


Fig 2.14 Mean concentrations of gemfibozil in sewage effluent reported in the literature. Dataset did not include any mean concentrations below the limit of detection or quantification (LOD/LOQ). Multiple points for the same reference apply to different sewage treatment plants (STPs). For raw data see appendix 9.

Paracetamol ranged in concentration from ND (not detected) to 24.5µgl^{-1} within sewage effluent (Fig 2.15). Non detections cannot be seen on the graph due to the logarithmic scale (see appendix 1 for raw data). The highest concentration was reported in the UK (Kasprzyk-Hordern *et al*., 2009) (Fig 2.15). The mean reported concentrations ranged from 9.5 ngl^{-1} to 11.7 µgl^{-1} and approximately half of all measurements reported for paracetamol in final sewage effluent worldwide were zero or below the detection limit (see appendix 1 for raw data).

The range of reported mean concentrations in sewage effluent for propranolol was between zero (Pedrouzo *et al*., 2007) (Spain) and 560 ngl^{-1} (France) (Coetsier *et al*., 2009) (Fig 2.16). The latter was also the highest mean concentration of propranolol in sewage effluent found in the literature. The majority of detections fell between 10 and 1000 ngl^{-1}. Non detections of propranolol were reported four times in 90 sampling events worldwide.

Trimethoprim concentrations in sewage effluent ranged from zero or below detection limit in 16% of sampling events (see appendix 1 for raw data). The highest reported concentration was 7.9 µgl^{-1} (Fig 2.17) which was a mean measurement from the USA in 2007 (Batt *et al*., 2007). The maximum recorded concentration was not published in this article. Concentrations of trimethoprim exceeded 1µgl^{-1} regularly across of variety of countries worldwide (Fig 2.17) (see appendix 1 for raw data).

There is a lack of data for measurements of the anti-cancer drug tamoxifen in sewage effluents. Tamoxifen has been measured in sewage effluent in just 3 studies, one in France (Coetsier *et al*., 2009) and two in the UK (Hilton *et al*., 2003; Roberts & Thomas, 2006). The maximum reported concentration was 740 ngl^{-1} (Roberts & Thomas, 2006) (Fig 2.18) and non detections accounted for 78% of sampling events (see appendix 1 for raw data).

There is also lack of data for measured concentrations of EE2 and fluoxetine in sewage effluent. A maximum concentration of 43 ngl^{-1} of EE2 (Soliman *et al*., 2004) and a mean of 7 ngl^{-1} (Desbrow *et al*., 1998) has been reported (Table 2.6). For fluoxetine, a mean concentration of 560 ngl^{-1} was reported in sewage effluent in the USA (Benotti *et al*., 2007) and a maximum of 73 ngl^{-1} in the U.S.A (Batt *et al*., 2006) (Table 2.7).

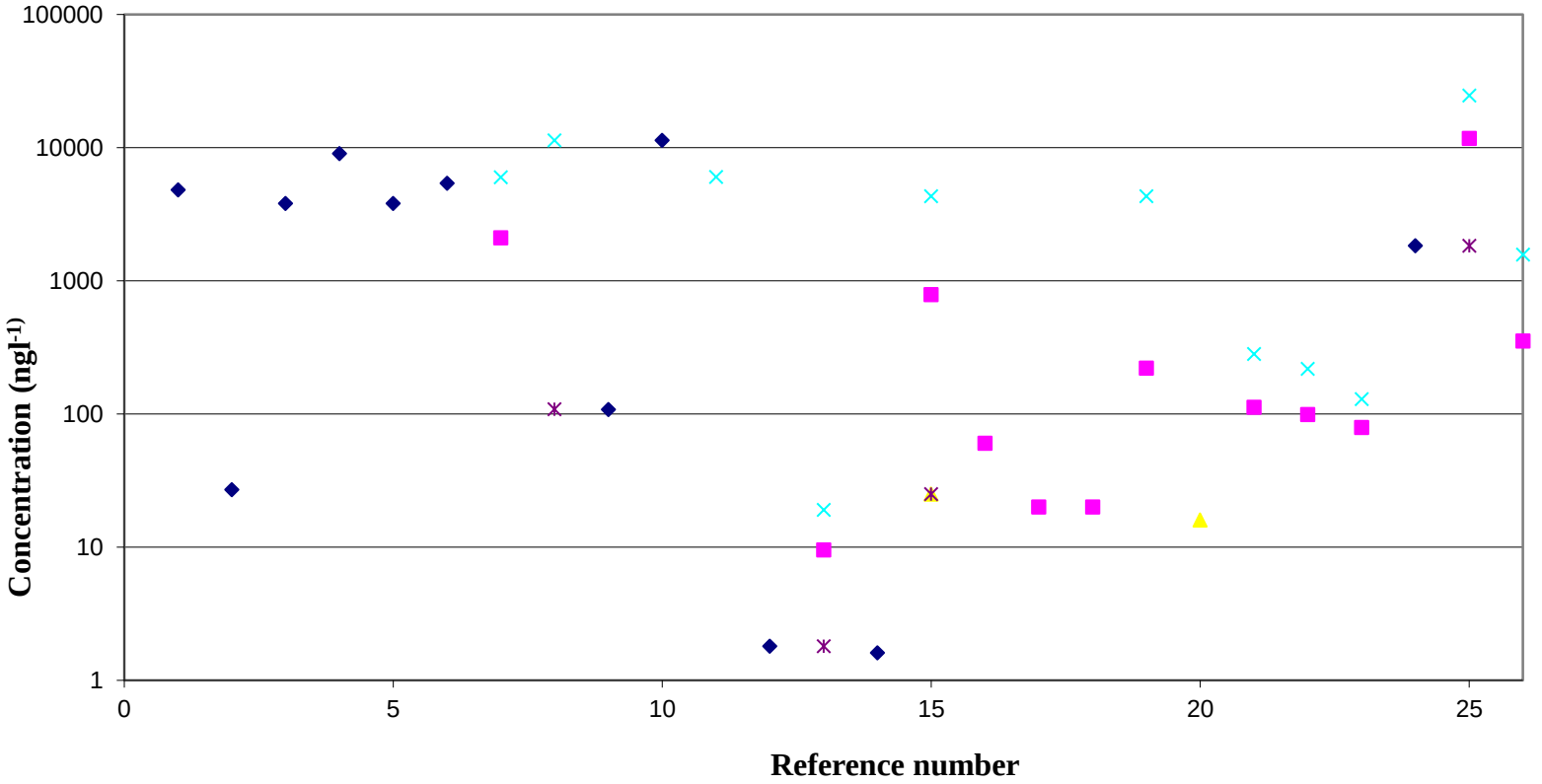


Fig 2.15 Measured environmental concentrations of paracetamol in sewage effluent. Individual points represent reported concentrations in sewage effluent from the literature. Only positive detections are shown on the graph due to the logarithmic scale. Dataset includes 33 non detections (ND) or detections below the limit of quantification (LOQ). For raw data and references see appendix 1. ◆ Individual MEC ■ Mean ▲ Median × Maximum ✳ Minimum

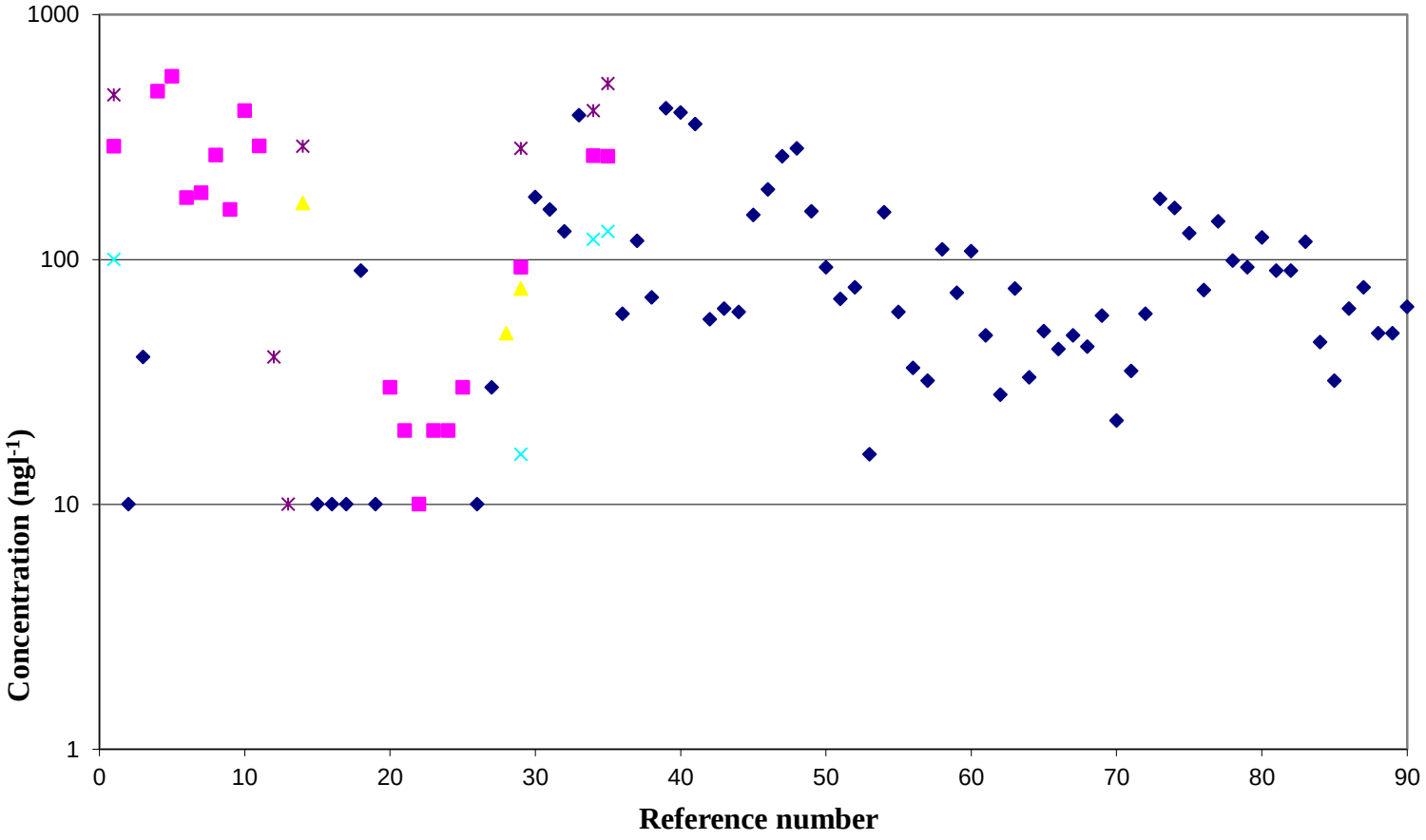


Fig 2.16 Measured environmental concentrations of propranolol in sewage effluent. Individual points represent reported concentrations in sewage effluent from the literature. Only positive detections are shown on the graph due to the logarithmic scale. Dataset includes 4 non detections (ND) or detections below the limit of quantification (LOQ). For raw data and references see appendix 1. ◆ Individual MEC ■ Mean ▲ Median × Minimum ✳ Maximum

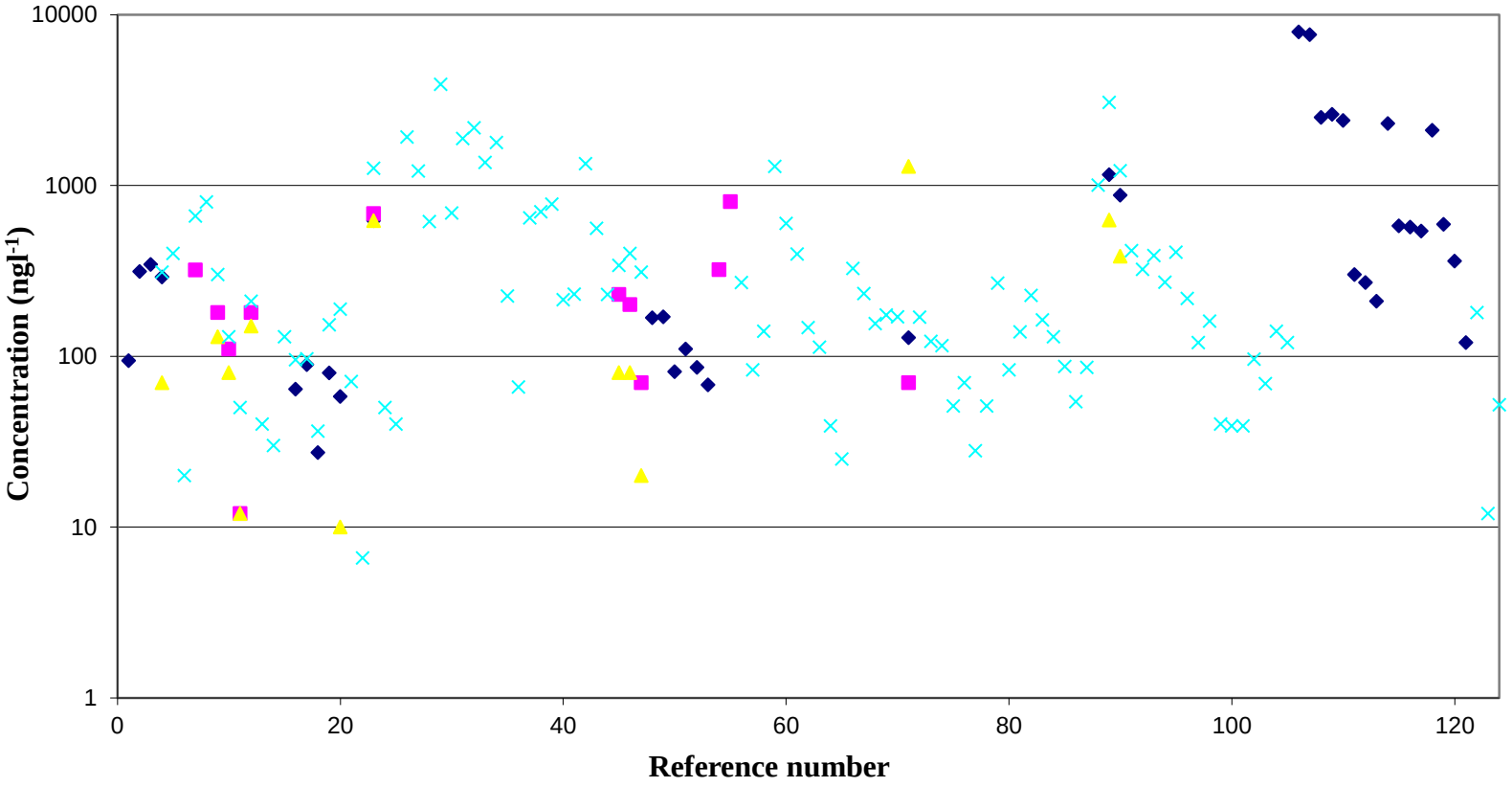


Fig 2.17 Measured environmental concentrations of trimethoprim in sewage effluent. Individual points represent reported concentrations in sewage effluent from the literature. Only positive detections are shown on the graph due to the logarithmic scale. Dataset includes 20 non detections (ND) or detections below the limit of quantification (LOQ). For raw data and references see appendix 1. ◆ Mean ■ Median ▲ Minimum × Maximum

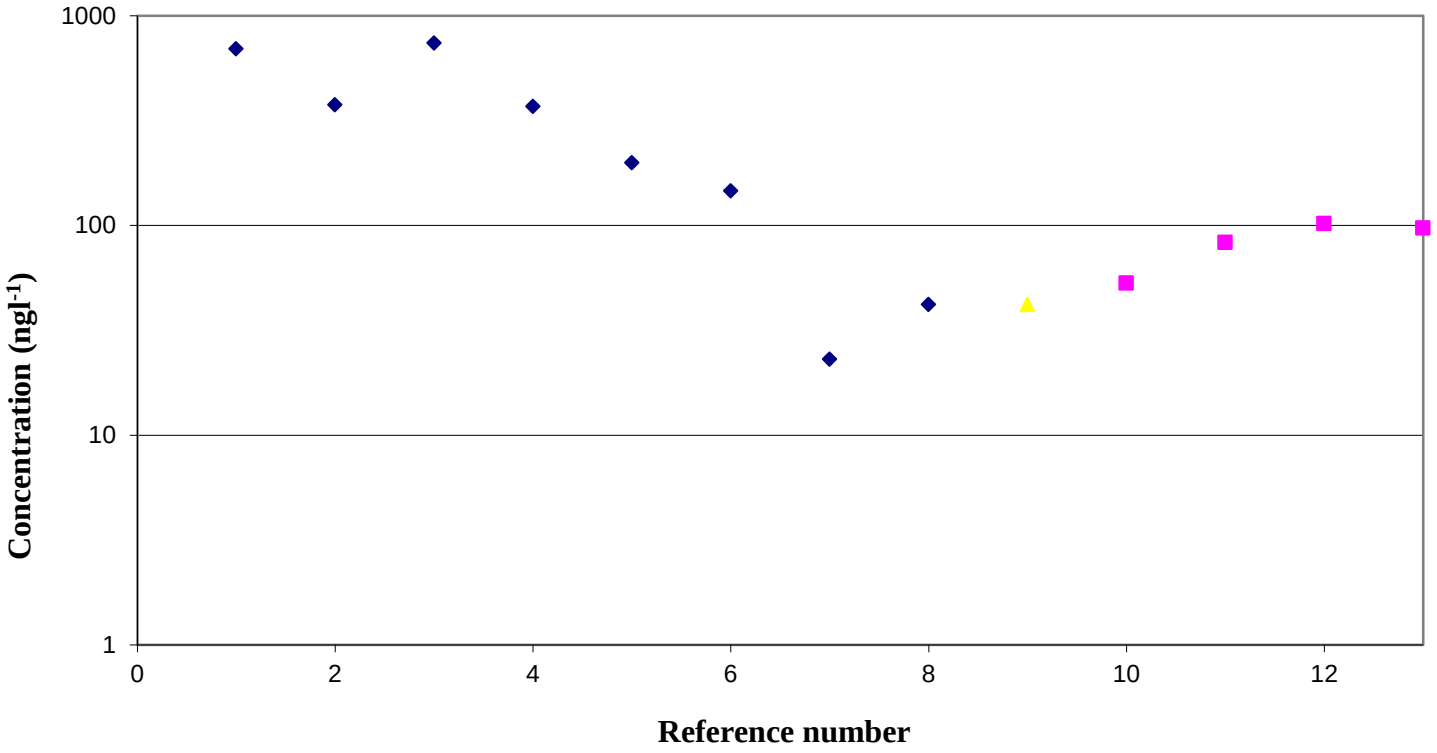


Fig 2.18 Measured environmental concentrations of tamoxifen in sewage effluent. Individual points represent reported concentrations in sewage effluent from the literature. Only positive detections are shown on the graph due to the logarithmic scale. Dataset includes 33 non detections (ND) or detections below the limit of quantification (LOQ). For raw data and references see appendix 1.

◆ Individual MEC ■ Mean ▲ Maximum

Table 2.6 Reported concentrations of 17 α ethinylestradiol (EE2) in sewage effluent (ngl^{-1}) (ND: non detections)

Mean	Median	Minimum	Maximum	Reference
7				Desbrow *et al*., 1998
ND				Desbrow *et al*., 1998
4.3				Desbrow *et al*., 1998
0.6				Desbrow *et al*., 1998
1.9				Desbrow *et al*., 1998
0.2				Desbrow *et al*., 1998
0.6				Desbrow *et al*., 1998
0.8				Desbrow *et al*., 1998
1.3				Kim *et al*., 2007
			43	Soliman *et al*., 2004
			31	Soliman *et al*., 2004
			40	Soliman *et al*., 2004
<0.3	<0.3	<0.3	<0.3	Thomas, 2007
ND				Trenholm *et al*., 2006
ND			1	Peng *et al*., 2008
				Zuccato *et al*., 2005
2				Zuehlke *et al*., 2004
0.4				Zuehlke *et al*., 2004

Table 2.7 Reported concentrations of fluoxetine in sewage effluent (ngl^{-1}) (ND: non detection; bld: below detection limit)

Mean	Median	Minimum	Maximum	Reference
		0	64	Hua *et al*., 2006
<60				Hua *et al*., 2006
bld	bld	bld	bld	Gros *et a*l., 2006
			1.7	Kim *et al*., 2007
	ND	ND	ND	Trenholm *et al*., 2006
			1.7	Trenholm *et al*., 2006
	ND	ND	ND	Trenholm *et al*., 2006
			bld	Vasskog *et al*., 2006
			1.2	Vasskog *et al*., 2006
			1.3	Vasskog *et al*., 2006
		40	73	Batt *et al*., 2008
560				Benotti & Brownawell, 2007
25				Vanderford & Snyder 2006
ND				Jones-Lepp *et al*., 2004
58				Schultz & Furlong, 2008

2.5.4 Removal of pharmaceuticals by sewage treatment

The efficiency of sewage treatment plants in the removal of four pharmaceuticals carbamazepine, diclofenac, ibuprofen and gemfibrozil was assessed. Differences in removal efficiency were striking (Table 2.8). The percent removal of these four drugs varied not only between different sewage treatments, but also between different STPs employing similar treatments (see appendix 10 for raw data). In fact removal efficiency varied day to day at the same STP. The results show that pharmaceuticals both increased as well as decreased in concentration after sewage treatment (Table 2.8).

The removal efficiency for diclofenac was quite varied between sewage treatment plants and their practices. Removal efficiencies varied from 4.7% (secondary treatment) to 99.8% (tertiary treatment with chlorination). Increases in concentration have also been reported. Even when treatments were similar at different STPs the removal efficiency varied, a range of 7.1 - 77% removal to a 143% increase was found for diclofenac concentration in final effluent after conventional activated sludge treatment (Table 2.8).

Carbamazepine was very persistent in all sewage treatments and regularly increased in concentration. Carbamazepine seemed highly resistant to removal with STPs achieving removal efficiency of between 0 to 30% and a maximal reported increase in concentration of 43.1%.

Ibuprofen removal ranged from 0 to 100%. Several increases in concentration of ibuprofen have been reported (Table 2.8). There was a lack of data for gemfibrozil but available figures suggest its removal can vary from 0 to 100% An increase in concentration for gemfibrozil after sewage treatment in a lagoon has also been reported (Lishman *et al*., 2006) (See appendix 10 for raw data).

Table 2.8 Sewage treatment efficiencies

(Percent removal or increase calculated by $[C_{influent} - C_{effluent}]/C_{influent} \times 100$).
References: Lishman *et al*., 2006; Clara *et al*., 2005; Gomez *et al*., 2007; Kasprzyk-Hordern *et al*., 2009; Radjenovic *et al*., 2009; Bendz *et al*., 2005; Stumpf *et al*., 1999; Ternes *et al*., 1998; Buser *et al*., 1998; Sebok *et al*., 2008; Thomas *et al*., 2007; Spongberg & Witter 2008; Kasprzyk-Hordern *et al*.,2008; Quintana *et al*., 2007; Vanderford & Snyder 2006; Kimura *et al*., 2007; Santos *et al*., 2005; Santos *et al*., 2007; Santos *et al*., 2009; Carballa *et al*., 2004; Buser 1999; Roberts & Thomas 2006; Sebok *et al*., 2008; Metcalfe *et al*., 2003; Weigel *et al*., 2004; Benotti & Brownawell 2007; Leclerq *et al*., 2008; Miao *et al*., 2005; Vieno *et* al., 2006; Zhou *et al*., 2009)

Pharmaceutical	Sewage Treatment	Removal (%)	Increase (%)
diclofenac	activated sludge	7.1-77	143
	activated sludge & trickling filter	9	
	trickling filter	9	75.7
	secondary (not specified)	4.7-51.6	128.1
	Tertiary	55.1	
	tertiary and chlorination	99.8	
	membrane bioreactor	32.9-50.6	6.6
ibuprofen	Primary	0	13.3
	activated sludge	75-100	4.4
	biological trickling filter	22-93.9	
	secondary (not specified)	64.6-80.7	52.8
	secondary & disinfection	51.5-100	100
	activated sludge & biological trickling filter	44.6	
	activated sludge & biological trickling filter & UV	86.8	
	membrane bioreactor	90-99.2	
	Tertiary	96.5-100	104.1
	Lagoon	98.7-100	
carbamazepine	activated sludge	0-30	43.1
	activated sludge + UV	29.5	
gemfibrozil	activated sludge	0-74.6	
	Lagoon		127
	tertiary and chlorination	99.8	
	biological trickling filter	16	
	secondary (not specified)	81-96	

2.5.5 Population size and pharmaceutical concentration

Sewage effluent is thought to be the main source of pharmaceuticals in the aquatic environment. The concentrations of four pharmaceuticals in sewage effluent were examined in regard to the number of people served by an STP. There was no significant correlation (95% confidence limits) between concentrations of ibuprofen (n=56), gemfibrozil (n=25), diclofenac (n=53) and carbamazepine (n=65) in final sewage effluent and the size of population served by the STP when all sewage treatment technologies were pooled (Fig 2.19 – 2.21). When the data was broken down between four broad categories of sewage treatment; secondary biological, tertiary, ultra violet disinfection and final chlorination, there was still no positive correlation at 90% confidence limits between population size and levels of carbamazepine, diclofenac and ibuprofen in final effluent. Gemfibrozil was the only pharmaceutical to show a positive correlation, (95% confidence limit), between increasing population size and increasing pharmaceutical concentration in secondary biological (25 degrees of freedom) and activated sludge sewage effluent (Fig 2.2) (11 degrees of freedom) (See appendix 11 for raw data).

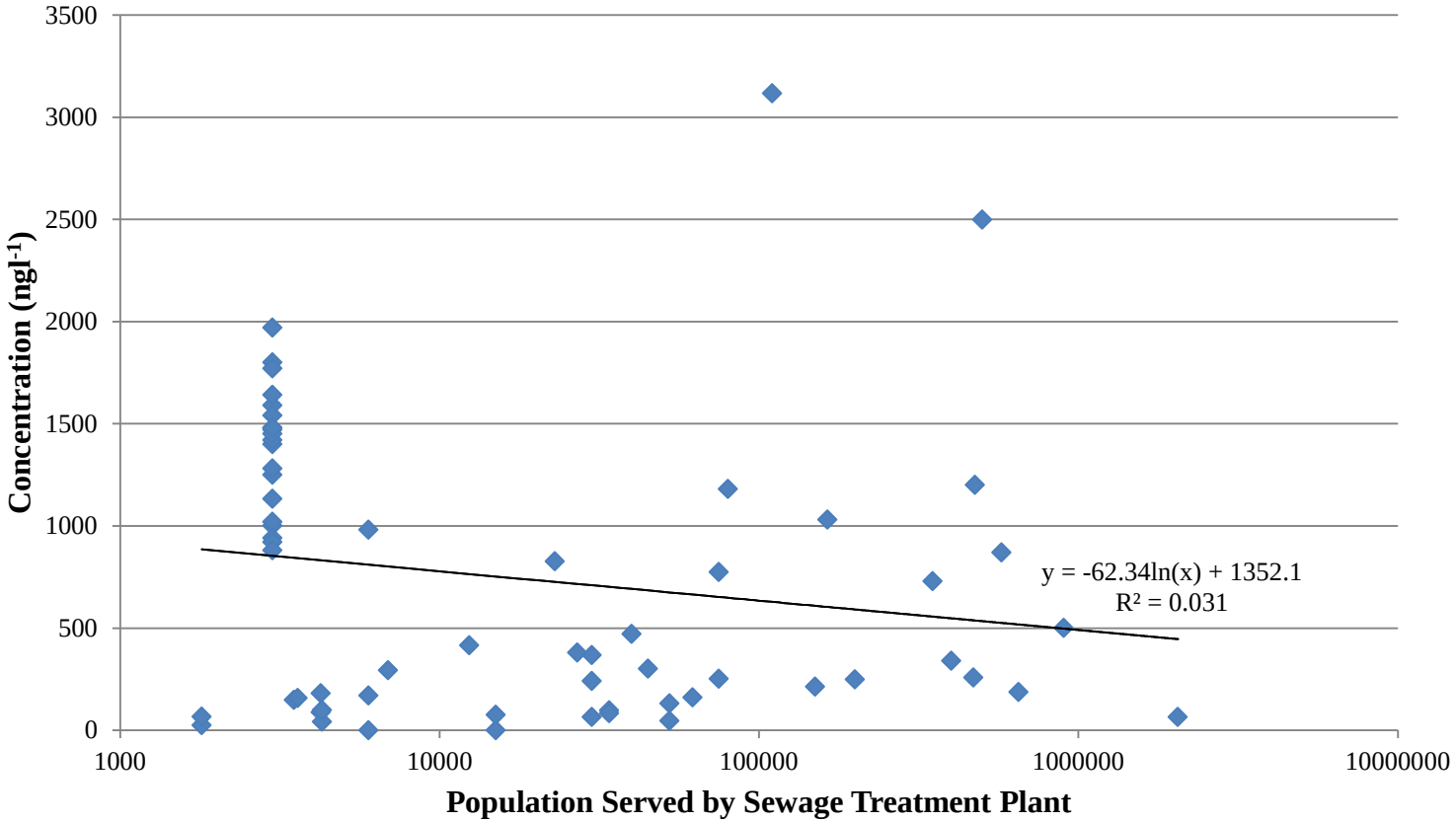


Fig 2.19 Reported concentrations of carbamazepine in sewage effluent and capacity of sewage treatment plant. Correlation between the size of population serviced by the sewage treatment plant and carbamazepine concentration was not significant at 95% (n=65). (for raw data see appendix 11). A logarithmic scale used to incorporate wide ranging data.

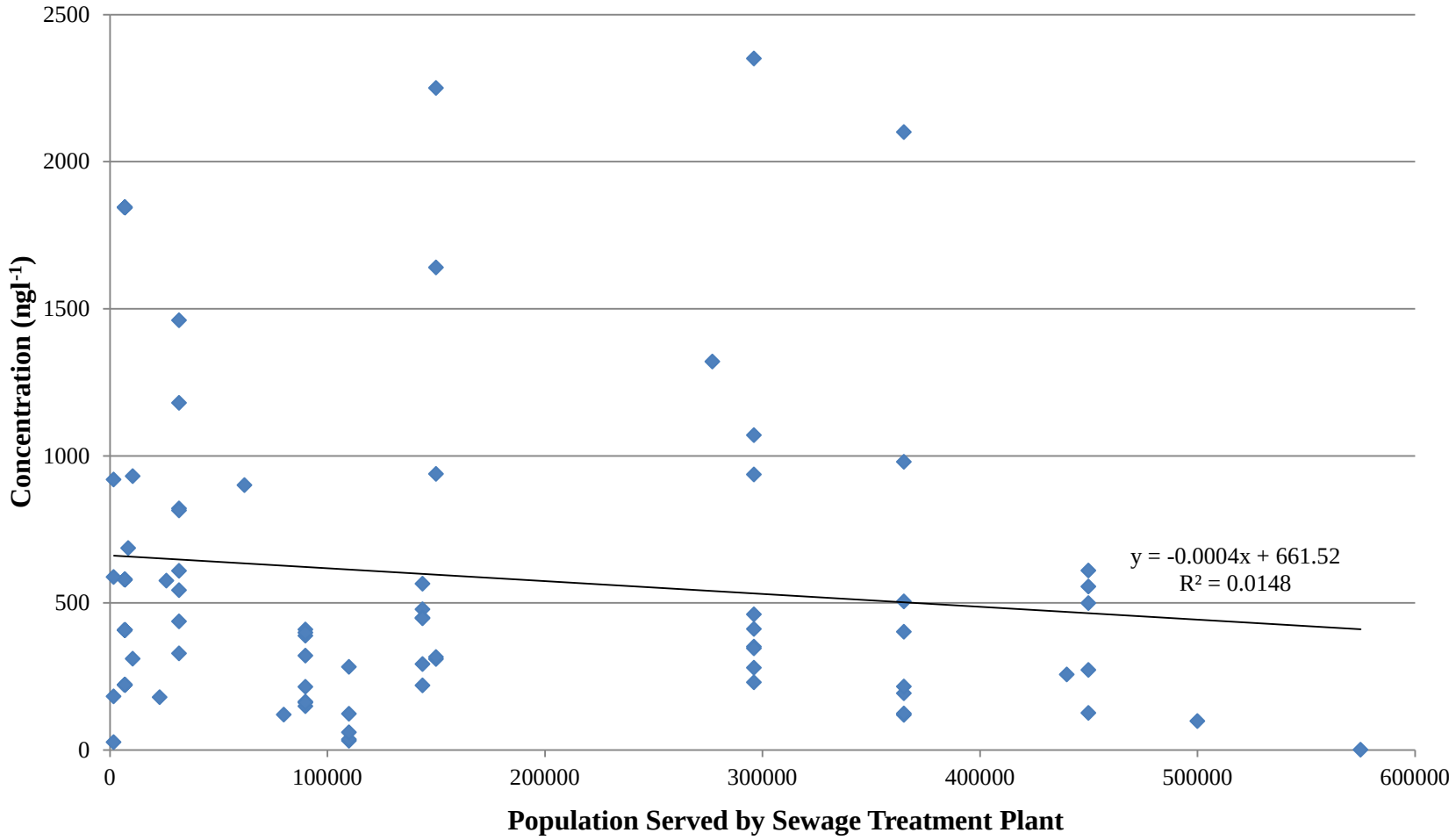


Fig 2.20. **Reported concentrations of diclofenac in sewage effluent and capacity of sewage treatment plant**. Correlation between the size of population serviced by the sewage treatment plant and diclofenac concentration was not significant at 95% (n=53). (for raw data see appendix 11).

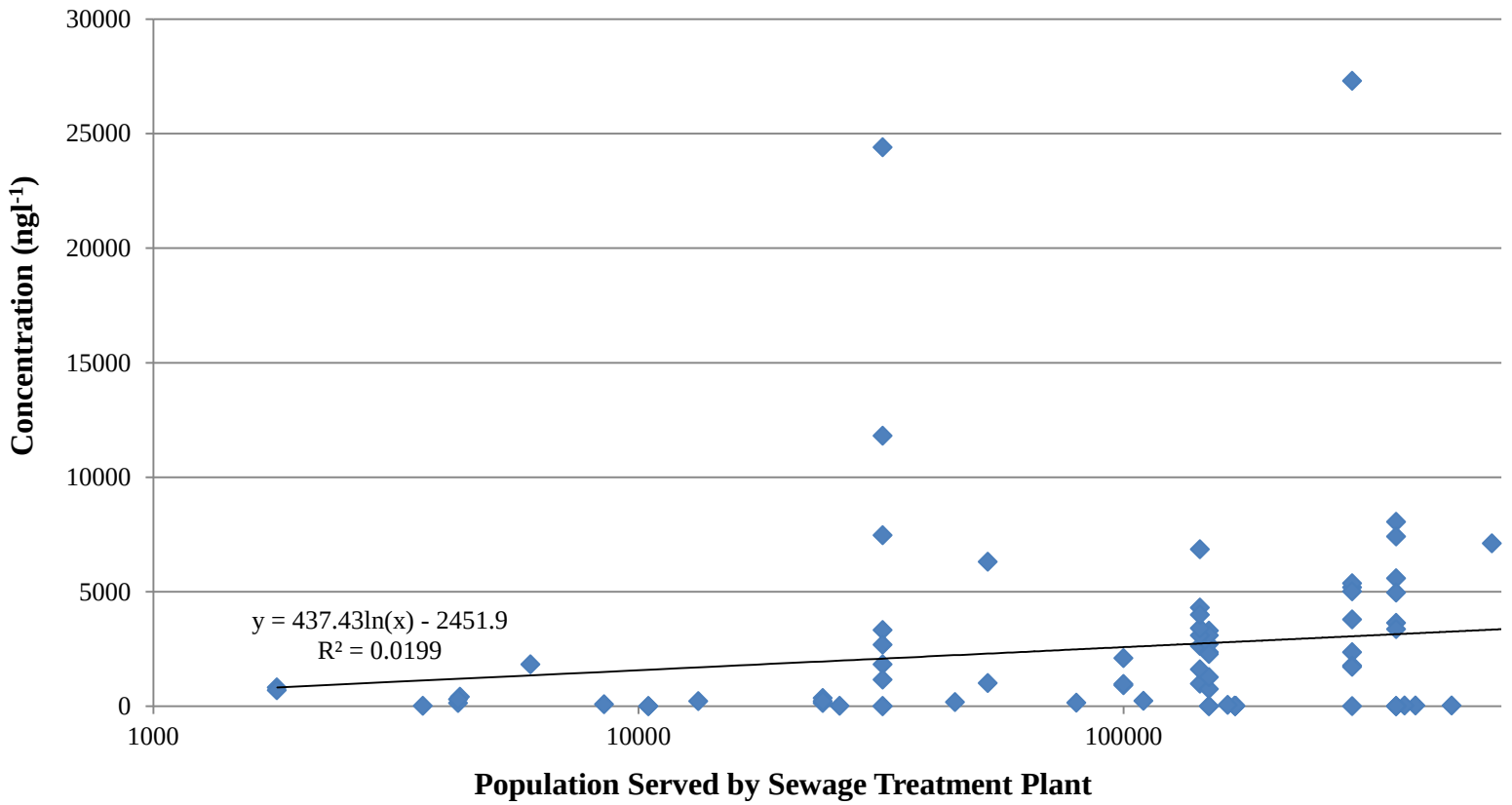


Fig 2.21 Reported concentrations of ibuprofen in sewage effluent and capacity of sewage treatment plant. Correlation between the size of population serviced by the sewage treatment plant and ibuprofen concentration was not significant at 95% (n=56). (for raw data see appendix 11). A logarithmic scale used to incorporate wide ranging data.

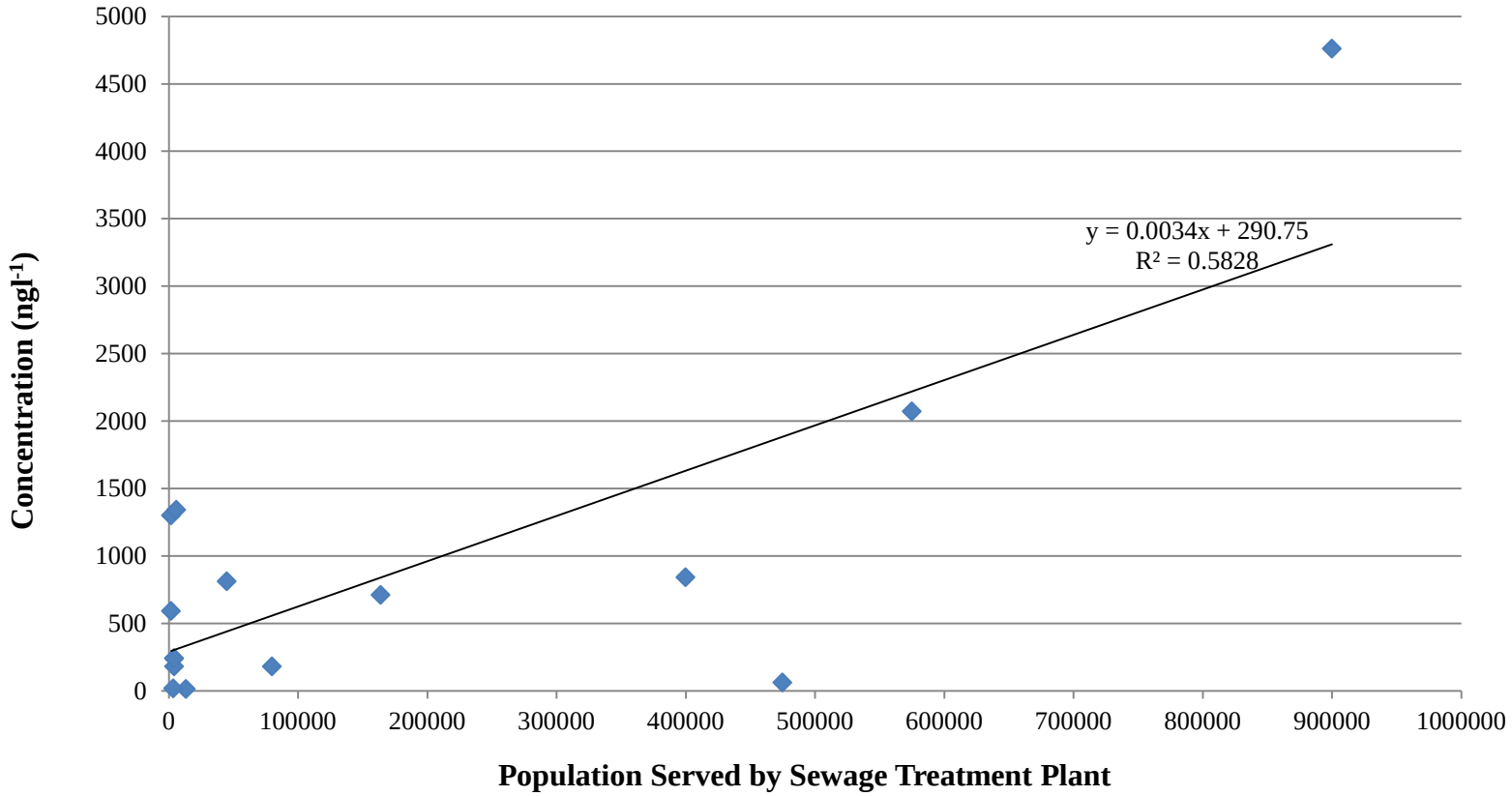


Fig 2.22 Reported concentrations of gemfibrozil in sewage effluent and capacity of sewage treatment plant. Correlation between concentrations of gemfibrozil and the size of the poulation served by the Sewage treatment plant was significant at 95% (n= 25) (for raw data see appendix 11).

2.5.6 Comparison of sewage effluent and receiving waters pharmaceutical concentrations

The results of this study show that the concentration of the four investigated pharmaceuticals, carbamazepine (Fig 2.23a), diclofenac (Fig 2.23b), ibuprofen (Fig 2.23c) and gemfibrozil (Fig 2.23d) increased as well as decreased in receiving waters downstream from the sewage outfall in comparison to final sewage effluent concentration. Despite this finding, on the whole results indicated that the majority of surface water concentrations were less than the sewage outfall. However the results also show that the concentration was not reduced by 90% of the original concentration in all cases and therefore surface water dilution was less than the default factor of ten specified in the EMEA guidelines for calculation of predicted surface water concentrations.

Interestingly, when comparing the total mean concentrations calculated for surface water (Fig 2.2-2.5) with the total mean concentrations calculated for sewage effluent (Fig 2.11-2.14), the difference was approximately an order of magnitude. This indicates that overall that sewage outfall dilution was probably around a factor 10. However, this does not account for variations in dilution in different water bodies.

It was not possible to assess the effect of distance downstream from sewage outfall due to the lack of data in the published literature. The distance from sewage outfall downstream was not always specified and analysis of a range of distances downstream and upstream was rarely performed.

The data available in the published literature was too varied and inconsistent to compare the effect of season on surface water concentration. Data was often provided over a long monitoring period for example a mean of 12 samplings over a 6 month period. Data about other sewage discharges, river flow, rainfall, temperature and pH was not usually stated.

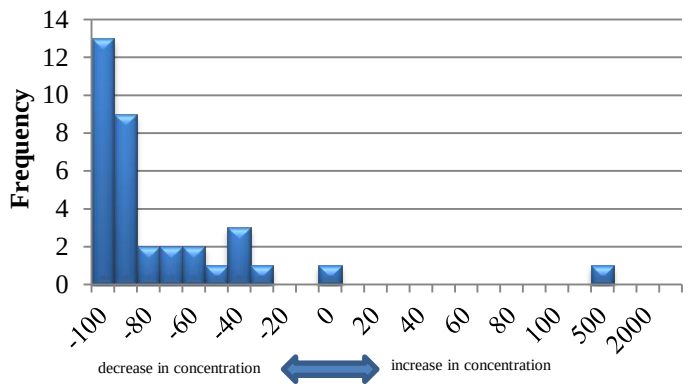


Fig 2.23a Carbamazepine

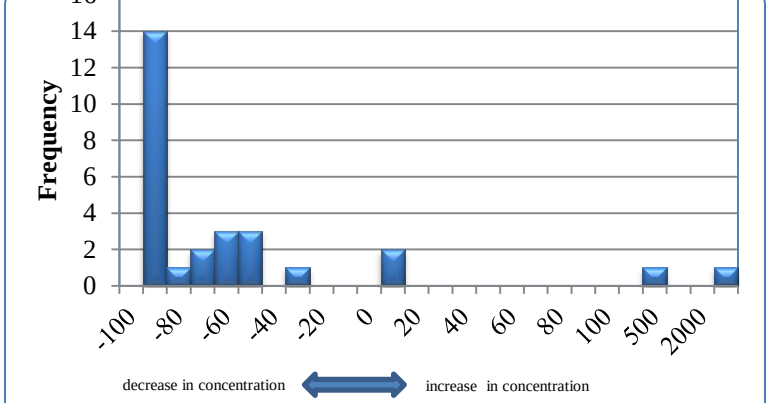


Fig 2.23b Diclofenac

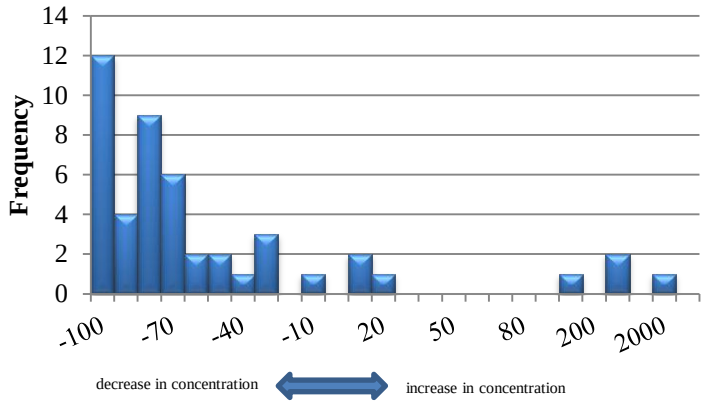


Fig 2.23c Ibuprofen

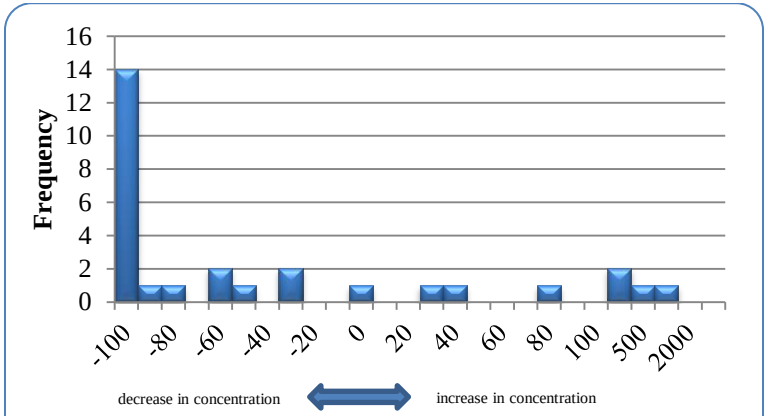


Fig 2.23d Gemfibrozil

Fig 2.23 a-2.23d Surface water dilution, shown as percent change in concentration from sewage outfall to receiving surface water

2.5.7 Comparison of predicted and measured pharmaceutical concentrations

Accurately predicting concentrations of pharmaceuticals in the environment is an integral part of any environmental risk assessment. To assess the validity of the current calculation used to predict surface water concentrations several investigations were performed.

Firstly the published literature was searched for studies where the predicted environmental concentration (PEC) for surface water of one of the ten selected pharmaceuticals selected for investigation was compared to a measured environmental concentration (MEC). Only 6, out of over 500 articles reporting concentrations of human pharmaceuticals in surface waters, directly compared surface water PECs and MECs in Europe for carbamazepine, diclofenac, ibuprofen, paracetamol, propranolol, tamoxifen and trimethoprim (Table 2.9). No data comparisons were found for gemfibrozil or fluoxetine. Two of these six studies found the MEC never exceeded the PEC (Ashton *et al*., 2004; Letzel *et al*., 2009). However Letzel *et al*., (2009) reported a PEC that had not been refined for metabolism, removal during sewage treatment or environmental degradation, equal to the MEC.

Overall, nearly 40% of MECs (9 out of 24) exceeded the related PECs. Paracetamol (Bound & Voulvoulis, 2006), ibuprofen (Bound & Vouvoulis, 2006; Castiglioni *et al*., 2004), diclofenac (Coetsier *et al*., 2009), propranolol (Ferrari *et al*., 2004), tamoxifen (Coetsier *et al*., 2009) and carbamazepine (Coetsier *et al*., 2009 and Ferrari *et al*., 2004) were all detected above the PEC at least once (Table 2.9).

The crude calculation of the PEC, however, in general was predominantly above or aligned to the MEC calculation. When the MEC exceeded the PEC it was regularly only by the maximum recorded concentration. In the case of ibuprofen, paracetamol and tamoxifen PECs refined for loss due to metabolism in the body, sewage treatment or environmental degradation tended to be underestimates of MECs (Table 2.9).

Table 2.9 Predicted (PECs) and Measured (MECs) concentrations from the literature (ngl^{-1})
(N = no; Y = yes; ND = not detected; STP: sewage treatment plant)

Pharmaceutical	Country	Reference	PEC	MEC (mean)	MEC (Max)	MEC (median)	MEC>PEC	Refinements
paracetamol	UK	Ashton *et al*., 2004	76400	nd	nd		N	
	UK	Bound & Voulvoulis, 2006	210	100	550		Y	Phase 11B EMEA
	UK	Bound & Voulvoulis, 2006	20,000	100	550		N	Phase 1 EMEA
	UK	Bound & Voulvoulis,2006	64,690	100	550		N	Phase 11A EMEA
ibuprofen	UK	Ashton *et al*., 2004	10800	1105	5044		N	
	UK	Bound & Voulvoulis, 2006	65	370	3080		Y	Phase 11B EMEA
	UK	Bound & Voulvoulis, 2006	6000	370	3080		N	Phase 1 EMEA
	UK	Bound & Voulvoulis, 2006	4310	370	3080		N	Phase 11A EMEA
	France	Coetsier *et al*., 2009	101		26	13	N	excretion & removal in STP
	Italy	Castigliglioni, 2004	45	7	9.8		N	
	Italy	Castigliglioni, 2004	3	7	9.8		Y	excretion and half life
diclofenac	UK	Ashton *et al*., 2004	1090	154	568		N	
	France	Coetsier *et al*., 2009	72		107	67	Y	excretion & removal in STP
	Germany	Ferrari *et a*l., 2004	1810		1200		N	
	Germany	Letzel *et al*., 2008	140	10.4	140		N	
propranolol	UK	Ashton *et al*., 2004	365	41	215		N	
	UK	Bound & Voulvoulis 2006	<2	<4	<4		Y	Phase 11B EMEA
	France	Coetsier *et al*., 2009	121		113	89	N	excretion & removal in STP
	Germany	Ferrari *et al*., 2004	120		590		Y	
tamoxifen	UK	Ashton *et al*., 2004	63	ND	ND		N	
	France	Coetsier *et al*., 2009	7		25	11	Y	excretion & removal in STP
trimethoprim	UK	Ashton *et al*., 2004	289	12	42		N	
carbamazepine	France	Coetsier *et al*., 2009	156		675	346	Y	excretion & removal in STP
	Germany	Ferrari *et al*., 2004	1930		2100		Y	

The relationship between PECs and MECs was investigated further by applying the equations in the EMEAs guidelines to the ten investigated pharmaceuticals using first Eq 1 and then Eq 2 (see methods Section 2.4.4.1). These equations gave substantially different PEC values (Table 2.10). Equation 1 was set out in the EMEA CHMP guidelines (2006) and despite the assumptions and crude nature of the calculation, the resultant PECs were predominantly above or in line with MECs found in the literature (Table 2.11). There were some exceptions, however. Several MECs for diclofenac exceeded the calculated 1 μgl^{-1} PEC, including MECs of 15 μgl^{-1} (Jux *et al.*, 2002) and 1.2 μgl^{-1} (Ternes *et al*., 1998). Carbamazepine exceeded the PEC of 4 μgl^{-1} once, with a maximum concentration of 7.1 μgl^{-1} reported in Germany (Weigel *et al*., 2004).

There were no reports of trimethoprim exceeding the PEC of 1 μgl^{-1} but MECs fell in the same range e.g. 0.7 μgl^{-1} (Kolpin *et al*., 2002) and 0.5 µg/L (Batt *et al*., 2008). These two measurements were made in the USA but are still relevant to consider in respect of Eq 1 since the only variable is maximum daily dose with no location specific data incorporated. Default data for the other parameters is specified in the guidelines. The calculated PEC 1 of 0.2 μgl^{-1} for tamoxifen matched two measurements in a UK study by Roberts and Thomas, (2006). No MECs were found in the literature above the calculated PEC 1 for gemfibrozil, fluoxetine, propranolol, ibuprofen or paracetamol.

Table 2.10 Predicted environmental concentrations (PECs) for England

PEC 1 calculated using the default market penetration factor of 1%, PEC 2 calculated using prescription data in England (2008) ([a]Dose ai = maximum daily dose).

Pharmaceutical	Dose ai[a] (mg)	PEC 1 (μgl^{-1})	Consumption (Kg)	PEC 2 (μgl^{-1})
carbamazepine	800	4	45705	1.22
diclofenac	200	1	26442.7	0.70
gemfibrozil	1200	6	755.3	0.02
fluoxetine	80	0.4	4435.4	0.12
trimethoprim	200	1	9736.6	0.26
tamoxifen	40	0.2	521.4	0.01
propranolol	640	3.2	7784.5	0.21
ibuprofen	3200	16	330292	8.79
paracetamol	3900	19.5	3534737	94.02

Table 2.11 Measured environmental concentrations (MECs) for England (ngl^{-1})

(E = effluent; SW = surface water; - = not analysed; MECs exceeding PEC 2 are highlighted in bold).

Reference	Matrix	carbamazepine	diclofenac	trimethoprim	tamoxifen	propranolol	ibuprofen	paracetamol
Hilton *et al*., 2003	E	–	**2350**	**1290**	**42**	**284**	**27,300**	–
Roberts & Thomas, 2006	E	–	598	**414**	**740**	**414**	**15,778**	<20
Hilton & Thomas 2003	E	–	460	**270**	<10	180	3,800	<50
Bound & Voulvoulis, 2006	E	–	–	–	–	–	391	281
Zhou *et al*., 2009	E	1061	176	–	–	135	–	–
Zhang *et a*l., 2008	E	652	85			72		
Hilton *et al*., 2003	SW	–	568	42	<10	**215**	5,040	–
Roberts & Thomas, 2006	SW	–		19	**198**	107	2370	<20
Bound & Voulvoulis, 2006	SW	–	–	–	–	<4	3080	555
Hilton & Thomas, 2003	SW	–	91	39	<10	37	<20	<50
Zhou *et a*l., 2009	SW	>350	<50	–	–	>50	–	–
Zhang *et al*., 2008	SW	>200	25			<25		
Thomas & Hilton, 2004	SW	–	195	**569**	**71**	56	928	–

When Eq 2 (consumption/prescription data) was used to calculate the PEC, a much smaller value was obtained than for Eq 1 PEC 1, except in the case of paracetamol (Table 2.10). This is a reflection of Paracetamol having a higher market penetration factor than the default 1%. The PECs derived from Eq 2 were calculated using actual consumption data for 2008 in England and then compared with the maximum concentrations reported in the literature between 2000 and 2011. A total of 8 studies from 2003-2011 were found (Table 2.11). It should be noted that sales of drugs differ year to year and the prescription data collected for 2008 may differ from precise prescription amounts in each of the years in which data was reported. However this was the most recent prescription data available.

Maximum surface water concentrations have been reported above the PECs generated in Eq 2 (PEC 2) for tamoxifen, trimethoprim and propranolol (Table 2.11). No MEC data was available for either gemfibrozil or fluoxetine representing a substantial knowledge gap. Both had PECs greater than 10 ngl^{-1} the action limit for further risk assessment.

2.5.7.1 Site specific MEC and PEC comparison

In order to provide a more robust comparison of the PECs and MECs, it was desirable to assess information on the specific locations used to sample for surface water pharmaceutical concentrations. However, in general the articles containing concentration data lacked the information necessary (see Table 2.12). The dilution of effluent to receiving water was not specified in any of the publications despite common analysis of effluent and receiving water pharmaceutical concentrations. The capacity of the STP was however, often supplied. According to the EMEA guidelines PECs for local surface water concentration can be refined by the following equation: as:

[*Eq 3*]

$$PEC_{SURFACEWATER} = \frac{Elocal_{water} * F_{stp\ water}}{WASTEWinhab * CAPACITYstp * Factor * Dilution}$$

Where:
$Elocal_{water} = DOSEai * F_{excreta} * F_{pen} * CAPACITYstp$
Fstp water = Fraction of emission directed to surface water (calculated by simple Treat)
WASTEWinhab = wastewater production per person per day (default 200L)
CAPACITYstp = capacity of local sewage treatment plant
Dilution = dilution factor (default 10)
Factor = Factor taking the adsorption to suspended matter into account

The information required to carry out further refinements of the PECs for local water using the equation was not available in the majority of publications (Table 2.12). In particular the **Fstp water** (Fraction of emission directed to surface water) and **Factor** (Factor taking the adsorption to suspended matter into account) data was not included in any of the articles examined. It appears imperative that the standards of reporting for environmental concentration data in peer reviewed journals is improved in order to make data usable for environmental risk assessments and policy development (see Section 2.5.9).

Reference	Pharmaceutical	LOD	Location	Dilution	CAPACITYstp	Date	Sample Size	Sampling Method
Roberts & Thomas, 2006	propranolol tamoxifen ibuprofen trimethoprim	10 10 10 10	River Tyne (6 sites)	NP	NP	14th June	Triplicate (2.7L)	12hr composite
Thomas & Hilton, 2004	Diclofenac Ibuprofen Paracetamol propranolol Tamoxifen trimethoprim	8 8 20 4 4 4	River Tyne River Tees River Mersey River Thames	NP	NP	October & November 2002	2.7L	grab
Bound & Voulvoulis, 2006	Ibuprofen Paracetamol propranolol	2-4	River Thames & a small unnamed river in the South East	NP	1.8million & 150,000	2003-2004 (12 week period)	47 samples & 13 samples (1L)	Composite and grab
Zhang *et al*., 2008	Propranolol Carbamazepine Diclofenac	6-487 pg/L (not specified for each pharmaceutical or method)	River Ouse, West Sussex	NP	NP	23-27 October	NP	POCIS & spot samples
Hilton *et al.*, 2003	Trimethoprim Paracetamol Ibuprofen Diclofenac Propranolol Tamoxifen	10 50 20 20 10 10	Corby Great Billing East Hyde Harpenden Ryemeads	NP	150,000 296,100 143,801 31,905 365,071	May-July 2002	Sampled once each month (2.7L)	Grab
Zhou *et al*., 2009	Carbamazepine Diclofenac propranolol	1-288 pg/L (not specified for individual pharmaceuticals	River Ouse, West Sussex	NP	162,619	November 2006	NP	NP

Table 2.12 Information availability in published literature for measured concentrations of pharmaceuticals in surface waters (MECS). Fstp water = Fraction of emission directed to surface water and **Factor** (Factor taking the adsorption to suspended matter into account) was not included in any of the articles examined. (LOD; limit of detection; NP: not provided, POCIS: polar organic integrative passive sampler, PE: population equivalents).

2.5.8 Reported concentrations of pharmaceuticals in other matrixes

Carbamazepine, diclofenac, ibuprofen and gemfibrozil have been detected in coastal and marine waters and drinking water (Table 2.13).

Table 2.13 Range of concentrations of pharmaceuticals reported in drinking water, groundwater and marine water (ngl^{-1})

	Carbamazepine	Diclofenac	Gemfibrozil	Ibuprofen
Coastal & marine water	0 - 56.3	0 - 195	0 - 53	0 - 928
Ground water	<5 – 1260	0 - 900	0 - <20	0 - 200
Drinking water	43.2	2.5	0 - 70	0 - 1350

Generally there was a lack of data for measured concentrations of pharmaceuticals in drinking water, groundwater and marine waters. The maximum concentration for carbamazepine detected in drinking water was 43.2 ngl^{-1} in the Mediterranean, France (Togola & Budzinski, 2008). In the same study a range of concentrations for the coastal waters in the Mediterranean was ND-56.3 ngl^{-1} (Table 2.13). This is the only report of carbamazepine in marine or coastal waters. However, in a more comprehensive study of drinking water in the USA, including 18 different locations, carbamazepine was detected at a median level of 6 ngl^{-1} and a maximum of 18 ngl^{-1} (Benotti *et al*., 2009). The maximum concentration of carbamazepine measured in groundwater after infiltration with treated sewage effluent was 1260 ngl^{-1} (Kreuzinger *et al*., 2004). Although this was the highest reported figure it was representative of general findings (Drewes *et al*., 2003, Sacher *et al*., 2001).

Diclofenac has been detected at a maximal concentration of 900 ngl^{-1} in groundwater (Kreuzinger *et al*., 2004). However, it was not detected in this matrix in over half ofsamples analysed. Diclofenac has been detected in drinking water at maximal concentrations of 2.5 ngl^{-1} in the USA and 6 ngl^{-1} in Germany (Jones *et al*., 2005). The highest marine and coastal measurement of diclofenac was 195 ngl^{-1} in the Mersey estuary in the UK (Thomas & Hilton, 2004). This study sampled 22 UK estuaries, 15 of which were below the detection limit of 8 ng l^{-1} for diclofenac. A similarly high

concentration of 191 ngl^{-1} was also found in the Tees. Another study of coastal waters in Norway did not find concentrations of diclofenac above 0.7 ngl^{-1} (Weigel *et al*., 2004). In Canada the maximum concentration was 6 ngl^{-1} (Comeau *et al*., 2008).

Maximum concentrations of ibuprofen have been detected in estuarine water of the Thames, UK at 928 ngl^{-1} (Thomas & Hilton, 2004), in Canada's coastal waters at 230 ngl^{-1} (Comeau *et al*., 2008) and in Norway at 0.7 ngl^{-1} (Weigel *et al*., 2004). Ibuprofen has been rarely detected in groundwater although a maximum detection of 200 ngl^{-1} was recorded in Berlin (Herberer *et al*., 1998). Ibuprofen has been detected in drinking water at a concentration of 1.35 $\mu g\ l^{-1}$ (Loraine & Pettigrove, 2006).

Gemfibrozil was detected in drinking water in 7 of 18 samples at a median concentration of 0.48 ngl^{-1} and a maximum of 2.1 ngl^{-1} in the USA (Benotti *et al*., 2009). There were four other studies of gemfibrozil in drinking water, two non detections, a maximum detection of 0.8 ngl^{-1} (Loo's *et al*., 2007) and a highest reported concentration of 70 ngl^{-1} (Tauber, 2003). There has been no analysis of gemfibrozil in the UK. Only one sea water analysis of gemfibrozil has been undertaken, in the coastal waters of Canada. Here gemfibrozil was detected 3 times out of 31 samples at a maximum concentration of 53 ngl^{-1} (Comeau *et al*., 2008). Gemfibrozil has not been detected above the detection limit in groundwater.

2.5.9 Critical analysis of reporting methods and standards for pharmaceuticals in surface waters and sewage.

Meaningful interpretation of data from peer reviewed literature for pharmaceuticals in the aquatic environment was severely hampered by poor reporting standards. Generally all reports had important information missing. Only 4 out of 128 articles (3%) published in peer reviewed literature met the criteria to allow useful analysis of environmental fate, mobility and longevity of pharmaceuticals (Table 2.13). Even then 3 of these articles only examined sewage effluent and not the receiving waters. Distance from sewage outfall and direction of water flow criteria therefore were not applicable in these cases.

The articles always adequately described the detection methods and generally a detection limit or quantification limit for that method was provided. The main difference between sample collections was grab or composite samples. Some publications used both, some did not state which approach was used. Statistical analysis of results varied with publication. The mean was most commonly used statistical analysis accounting for 38% of publications, 63% of these calculated a standard deviation about the mean. Many other publications reported the maximum, minimum, range or median concentration found. There were, however, a large number of publications which reported a single grab sample measurement. The sample size was specified in less than half of articles examined. The method of sewage treatment was stated in 66% of cases however the detail in which it was presented varied considerably. Some articles examined concentrations of pharmaceuticals in sewage effluent without ever mentioning the treatment type. One fifth of publications did not include a date or season for their sampling. Other criteria and parameters which were rarely included in articles were biological oxygen demand, sewage retention times, hydraulic retention times, age of sewage, temperature, pH and time of day samples were taken.

Table 2.14 Publications meeting the sound science criteria

Criteria	Publications (%)
Date	80
Mean	38
Standard deviation	24
Sample size	46
Replicates performed	18
Direction of water flow	50
Distance from sewage outfall	57
Population size	74
Sewage treatment	66
Flow rate of effluent or surface water	47
All criteria met	3

2.6 Discussion

The data mining exercise performed for environmental occurrence and fate data for the ten pharmaceuticals investigated revealed that they are frequent pollutants of the aquatic environment. They have all been detected in the ng - μgl^{-1} range in surface waters and sewage effluents worldwide.

2.6.1 Surface water concentrations in freshwater systems

Concentrations of pharmaceuticals in surface waters vary considerably from non detections to several micrograms per litre (Fig 2.1-2.5). Although data is not available for every one of the 3000 or so licensed pharmaceuticals, the results indicate that they will be present in parts per trillion to parts per billion range in surface waters wherever they are being consumed. The findings show that data can vary by an order of magnitude from one sample to another (Fig 2.1-2.5). This was not only the case between MECs from different water bodies but also when the same water body was sampled at different times of day, e.g. the measurements taken in a study by Zhou *et al*., (2009). MECs were compiled from over 15 countries in 3 continents and therefore it is fair to conclude that this situation is congruous worldwide. The variation in concentrations of pharmaceuticals in surface waters is best highlighted by the fact that, when mean MECs for carbamazepine, diclofenac, ibuprofen and gemfibrozil were analysed, the standard deviation for these results was so large that it could not be shown on the graph (Fig 2.1). This finding brings into question the applicability of using a default PEC to represent the concentration of a pharmaceutical in any water body in Europe (see Section 2.6.4).

Carbamazepine (Fig 2.2), diclofenac (Fig 2.3), ibuprofen (Fig 2.4), gemfibrozil (Fig 2.5), trimethoprim (Fig 2.6), paracetamol (Fig 2.7) and propranolol (Fig 2.8.) are regularly detected in surface waters worldwide. Considerably less data is available for tamoxifen but it was detected in studies where it has been measured (Fig 2.9). This highlights a data need for this anti-cancer drug especially in light of the fact that this compound targets the same receptor as EE2, the oestrogen receptor (see Section 4) and has log k_{ow} of 6.3 (Table 2.1). With reference to the OSPAR Convention, any pharmaceutical with a log K_{ow} of > 4.5 should be screened for persistence, bioconcentration and toxicity (PBT; European Chemical Bureau, 2003). Despite the wide

variation in surface water concentration, eight out of the ten pharmaceuticals investigated from the published literature frequently had a mean measured concentration above the 10 ngl^{-1} action limit set by the EMEA for further environmental risk assessment (Table 2.5). This finding is important when considering the effects these compounds may be having on non target organisms. If mean concentrations are regularly above 10 ngl^{-1}, then maximal concentrations will be far greater. This finding indicates a need for retrospective risk assessment of these pharmaceuticals. Chronic ecotoxicity studies need to be performed for these compounds in order to determine the PNEC and the RQ so that the environmental concentrations can be put into context. Retrospective risk assessment for these compounds would help reduce the scientific uncertainty about what effects they may be having on aquatic ecosystems and determine whether action should be taken to mitigate these risks.

There was a lack of surface water concentration data for ethinylestradiol (EE2) and fluoxetine (Table 2.3 & 2.4). Available data reveals that these two pharmaceuticals did not generally exceed the 10 ngl^{-1} action limit. This is not surprising for EE2 since the therapeutic dose is relatively small in comparison to other drugs. The recommended daily dose for EE2 is 0.03 mgs (RX list), and is only prescribed to women of reproductive age. The average excretion of EE2 per person per day is 0.89µg and sewage treatment removes 65-85% of this (Jobling *et al*., 2006). The recommended initial adult daily dose for adults for fluoxetine is much higher than EE2 at 20 mgs (RX list) and the excretion rate of fluoxetine is reportedly between 17 and 25% (Carballa *et al*., 2008). Removal in tertiary sewage treatment plants has been shown to be greater than 90% (Zorita *et al*., 2008). Environmental concentrations of both pharmaceuticals are likely to be small as a consequence. This was supported by the MECs reported in the literature (Table 2.3 & 2.4). The method detection limits reported for these compounds were generally quite low e.g. 0.5 ngl^{-1} for both compounds (Peng *et al*., 2008; Vanderford & Snyder, 2006). This suggests that these compounds are often not present in surface waters.

Both these compounds are endocrine disrupters which have an effect on hormone regulation. EE2 has attracted a lot of attention over the past decade and has been studied extensively. It is a highly potent endocrine disrupter shown to cause intersex characteristics in fish downstream from sewage treatment plants (Jobling *et al*., 2002) and

has been shown to cause adverse affects on non target organisms at concentrations as at less than 1 ngl^{-1} (Caldwell *et al*., 2000). This indicates a disparity between the MEC data and the effects observed in the environment. One potential reason for this may be that naturally occurring oestrogens such as oestriol and oestrone are producing combined endocrine disrupting effects in wildlife (Liu *et al*., 2010).

Fluoxetine is in the top 100 prescribed drugs in the UK and USA and is also attracting interest due to environmental concerns. Several chronic ecotoxicity studies have been published recently indicating that fluoxetine may cause effects on behaviour and reproduction of aquatic organisms (Painter *et al*., 2009; Foster *et al*., 2010; De-Lange *et al*., 2006; Mennigen *et al*., 2010). In an ERA performed by Oakes *et al*., (2010) an RQ of greater than 1 was obtained, indicating a potential risk to the environment for fluoxetine. However the data collected in this study indicate that it is rarely detected in surface water (Table 2.3).

Fluoxetine's principal metabolite is norfluoxetine, which is also pharmaceutically active and considered to be more potent than the parent compound (Fong & Molnar, 2008). The findings of this study show that this is one of the only metabolites of pharmaceuticals that have been measured in the environment (see appendix 1). The Environment Agency produced a briefing note in April 2005 for fluoxetine, which concluded that if monitoring revealed fluoxetine or its metabolite, norfluoxetine are present in rivers in England and Wales they will be at levels too low to cause acute impacts (EA, 2005). However, it was considered that monitoring data for rivers and sewage works in England and Wales was needed. At present this has not been carried out.

The ERA of human pharmaceuticals currently only applies to applications for marketing authorisation of new medicines. Licensing applications for carbamazepine, diclofenac, gemfibrozil, ibuprofen, paracetamol, propranolol, trimethoprim and tamoxifen as new medicines would require phase 11 of the ERA including ecotoxicological examination as the findings of this study show that they are all present in the environment at concentrations above the 10 ngl^{-1} action limit. The EMEA ERA processes any pharmaceuticals which are known to affect reproduction of vertebrates and lower animals are exempt from the action limit of 10 ngl^{-1}. Consequently fluoxetine and EE2 would require the second phase of ERA if being licensed today. Presently no official

retrospective risk assessments have been performed for any existing pharmaceuticals in the UK (Private communication with the EA). However the EA (England and Wales) has concluded that the weight of evidence for endocrine disruption in fish is sufficient to develop a risk management strategy for estrogenically active effluents that discharge to the aquatic environment (Gross-Sorokin *et al*., 2006).

The Environment Agency conducted an extensive study in 2003 on pharmaceuticals in surface waters in the south of England. The conclusion of this was that concentrations of pharmaceuticals were too low to be of environmental concern. The study was hampered by two issues, a lack of analytical methods for measuring many pharmaceuticals that had been prioritised as potentially problematic and a lack of chronic ecotoxicity data for the majority of the pharmaceuticals investigated. The study only related impacts to acute toxicity data, which does not reflect the actual exposure situation. The situation for aquatic organisms to human pharmaceuticals is a chronic low level continual exposure to a considerable mixture of drugs.

2.6.1.1 Degradation

The rate of degradation of pharmaceuticals in the environment is a contributing factor in their surface water concentration. Breakdown of some pharmaceuticals e.g. diclofenac and propranolol are dependant on sunlight, (Buser *et al*., 1998; Yamamoto *et al.*, 2009) while this is not the case for others e.g. fluoxetine (Kwon & Armbrust., 2006). The findings of this research show that diclofenac is detected regularly in surface waters at an overall total mean concentration of 40.7 ngl^{-1} and a maximum mean of 272 ngl^{-1} (Fig 2.3) despite a rapid half life of less than one day (Zuccato *et al*., 2000). This suggests that although photolysis is an important degradation mechanism, continual discharge, environmental distribution affecting exposure to sunlight and the possibility of accumulation in some environmental compartments are contributing factors to the overall surface water concentration. This finding supports the theory that the continuous input of pharmaceuticals causes a pseudo persistence effect even when they are readily degraded (Daughton & Ternes, 1999).

Ibuprofen was also frequently detected at high concentrations in the aquatic environment (Fig 2.4) even though it also has a reported environmental half life of <1day

(Castiglioni *et al*., 2004). High consumption of ibuprofen is probably the main reason for its high detection rate (Table 2.1). A half life of 660-9900 hours has been reported in photolysis experiments, considerably more than diclofenac. In fact biodegradation by microorganisms is thought to be a more important removal mechanism for this pharmaceutical (Yamamoto *et al*., 2009).

Adsorption to sediments may occur for some pharmaceuticals effectively removing them from the water body. Octanol-water partition coefficients (log K_{ow}) have been shown to be poor indicators of actual adsorption of pharmaceuticals because of their ionic nature (Williams *et al*., 2006; Oppel *et al*., 2004). Carbamazepine has a low sorption coefficient (Scheytt *et al*., 2005). However, nearly seventy times the concentration of carbamazepine was measured in river sediment compared to the water column in the USA (Thacker, 2005). Diclofenac, ibuprofen and trimethoprim have also been shown to have some sorption to particles (Khunjar & Love, 2011). Fluoxetine also has a low sorption coefficient (Monterio, 2008), which is below the action limit for terrestrial risk assessment. However, it has been found in high concentrations in sludge and sediments (Kwon & Armbrust, 2006). This may account for the lack of detections and low concentrations measured in surface waters found in this study. Fluoxetine is one of the most persistent pharmaceuticals in the environment raising concerns about accumulation (Redshaw *et al*., 2008). It is resistant to bacterial biodegradation, photolysis and hydrolysis in the environment and has a half-life greater than 100 days (Kwon & Armbrust, 2006).

The degradability and persistence of pharmaceuticals in water and sediment is of great importance to chronic exposure of aquatic organisms however there is little data available on degradation (Calisto & Esteves, 2009). It is crucial that pharmaceutical sediment concentrations and their bioavailability to aquatic organisms are assessed.

2.6.2 Sewage effluent

The results of this comprehensive study found that all ten of the pharmaceuticals investigated were regularly detected in sewage effluents worldwide. The concentrations reported are quite varied although always within the ng –$\mu g l^{-1}$ range. The most likely explanation for such variability in effluent concentration data is sewage treatment

efficiency and is probably one reason for the difficultly in accurately predicting surface water concentrations (see below).

Sewage treatments appear not to remove pharmaceuticals effectively. Differences in removal efficiency were striking (Table 2.8). The removal of carbamazepine, diclofenac, ibuprofen and gemfibrozil by different STPs was calculated wherever possible within individual studies reported in the literature. The results show that removal efficiency varied between STP and treatment type, (biological, physico-chemical). The season and weather conditions within the same plant also affected efficiency (Santos *et al*., 2009; Gomez *et al*., 2007). The results show that removal efficiency varied substantially even for the same pharmaceutical, at the same plant under the same conditions (Tauxe-Wuersch *et al*., 2005).

One finding of this study was that STP technologies and methods vary considerably and that these variations can affect the removal efficiencies of pharmaceuticals (Table 2.8). This means it is difficult to make accurate predictions about removal rates of pharmaceuticals after sewage treatment. Not only do removal efficiencies vary between different drugs, removal rates for the same pharmaceutical differ due to a variety of factors. These include the treatment level employed, (primary, secondary, tertiary), the method (activated sludge, trickling filter, lagoon etc) hydraulic retention times (HRT), sludge retention times (SRT), biological oxygen demand (BOD), pH, and temperature (Zabczynski *et al*., 2010; Zorita *et al*., 2009). These continually altering variables at the same STP and between STPs make pharmaceutical removal predictions very difficult and complex. These variables are not taken into account sufficiently well when applying STP removal to PECs. For example the results from 19 separate studies where sewage treatment removal could be calculated for diclofenac, percent removal ranged from as little as 4.7 % in a modern 3 stage biological plant to 99.8% after tertiary treatment and chlorination (Table 2.8 & appendix 10). One of the reasons for this variation could be that diclofenac removal is dependant on sludge retention time and is only significantly degraded when SRT was at least 8 days (Kreuzinger *et al*., 2004). Six studies revealed an increase in concentration of diclofenac after sewage treatment. The highest calculated was an increase of 143% in a study by Lishman *et al*., (2006) (appendix 10). Increases in concentration of the other three

pharmaceuticals for which sewage treatment efficiency was assessed were also found (Table 2.8). Carbamazepine increases have been regularly reported (Santos *et al*., 2009; Clara *et al*., 2003) (appendix 10). There were fewer increases in concentration found for gemfibrozil and ibuprofen; the possible reasons for these increases are discussed below.

In general ibuprofen removal in secondary treatment STPs was quite high, usually around 80-90% and 100% in more than one study (Table 2.8). This finding indicates that high MECs in surface waters are probably due to the high consumption of this pharmaceutical (see Section 2.6.1) rather than recalcitrance to STP removal. However, the fact that some low removal efficiencies and some increases in concentration have been reported (Table 2.8) means that caution needs to be taken when incorporating STP removal into PEC calculations. Ibuprofen has shown low adsorption to sewage sludge (Horsing *et al*., 2011), meaning that high removal efficiencies are likely to be due to microbial degradation for this pharmaceutical. Tertiary settlement lagoons appear to be a poor removal system for some pharmaceuticals like ibuprofen in comparison to microbiologically active systems like activated sludge. In a recent study by Horsing *et al*., (2011), to determine sewage sludge sorption, tamoxifen was so readily adsorbed to the glass bottles that it was not possible to calculate sewage sludge adsorption. These two extremes in adsorption potential of pharmaceuticals highlight some of the difficulties when estimating removal in STPs.

Carbamazepine seems highly resistant to removal with STPs achieving removal efficiencies of between 0 and 30% (Table 2.8). In fact, none of the sewage treatment technologies employed removed carbamazepine effectively. It is therefore considered that STP upgrades would probably not decrease the environmental concentrations of this drug.

There was a lack of data for STP removal of gemfibrozil but available figures suggest its removal can vary from 0 to 100% (appendix 10). This highlights a potential research need. However, if a removal efficiency of zero occurs at any STP, then a removal rate of zero must be assumed when calculating a PEC for ERA in order to provide a precautionary and worst case scenario.

In the publications reviewed the authors generally analysed the liquid phase of the influent or effluent, and rarely measured the concentration in solids or sludge. This may be of greater relevance for some pharmaceuticals than others due to differences in

adsorption to solid particles. Fluoxetine is known to have a high adsorption to sludge but paracetamol, ibuprofen and diclofenac and gemfibrozil are thought to have little tendency to bind and will occur mainly in the aqueous phase (Zabcynski *et al*., 2010). One of the difficulties with predicting sorption of pharmaceuticals is that they tend to be ionic, therefore, sorption may increase at lower pH (Ternes *et al*., 2004). High quantities of fluoxetine were found in bio solids produced at an STP (4.7 $mgkg^{-1}$) (Kinney *et a*l., 2006). Hydrophobic EE2 adsorbs readily to digested sludge with no significant degradation. Temes *et al*., (2002) reported a concentration of 17 ngg^{-1} of EE2 in sewage sludge, which is quite high considering the small quantities of EE2 entering sewage treatment plants (see above). A high adsorption to sludge could pose risks from run off in ground and surface water when sludge is applied to agricultural fields.

Removal of pharmaceuticals during sewage treatment is assumed to be zero in the initial crude PEC calculation for an ERA but can be included as a refinement. In this study, increases in pharmaceutical concentration after sewage treatment were often found. This has been reported previously for diclofenac (Zorita *et al*., 2009; Heberer & Feldman, 2005) and for carbamazepine (Vieno *et al*., 2006). This finding means that any refinements made to the PEC for STP removal may over estimate actual removal. In order to be precautionary it may not be appropriate to refine PECs for STP removal for some pharmaceuticals.

It is thought increases in concentration may be the result of microbial activity during secondary sewage treatment (Panter *et al*., 1999; Gros *et al*., 2010). During metabolism some pharmaceuticals such as EE2 and carbamazepine become conjugated with glucuronide groups. The faecal bacterium *Escherichia coli* produces very large amounts of the enzyme ß-glucuridase (Ternes, 1998). It is likely, therefore, that these glucuronides are readily cleaved reproducing the parent compound and hence increasing the concentration after sewage treatment (Calisto & Esteves 2009; Bound & Voulvoulis 2006; D'Ascenzo *et al*., 2003). Apparent increases in concentrations, though, may be due to other reasons. The detection method may produce errors e.g. suppression of the MS/MS detector signal due to the high concentrations of pharmaceutical in the raw waste water effluent, can result in apparent increases in concentration after STP treatment

(Roberts & Thomas, 2006). So some care may be needed to interpret these data and reproducing the results is important.

Wide-ranging concentrations of pharmaceuticals measured in sewage may be caused in part by the time of day the sample was taken. Tracking of influent sample to final effluent can be problematic when quantifying removal efficiencies. The method used to collect the sample may also affect concentration measurements, for example, a 24 hour composite sample may provide different data than a grab sample.

All this makes including STP degradation in refined PEC calculations very difficult. The EMEA state that STP modeling using the Simple Treat model described in the European Union System for the Evaluation of Substances (EUSES) can be used, incorporating adsorption of substances to sewage sludge in STPs, using the data from the estimation of the adsorption coefficient (OECD 106), and the test for ready biodegradability in the STP (OECD 301). It can be seen from the calculations in this study illustrating the differences with the variety of sewage processes and the variation in concentrations from the same STP (Table 2.8 & appendix 10), that the impact of the sewage treatment process is complex and dependant on a number of constantly changing variables. It could therefore, be difficult to get robust and accurate data to use in a modeling software package.

The EMEA recommend using the SimpleTreat package to estimate removal of pharmaceuticals in STPs as part of the PEC refinements for an ERA. However this package may not be particularly accurate. A large variability in removal prediction has been found when incorporating parameters such as: sewage flow, degradation rates, pH and dissociation constants when using SimpleTreat. The problem may be because it is a steady state model describing a highly non-steady system (Kah & Brown, 2011). Another criticism of Simple Treat is that it uses log k_{ow} to predict partitioning which has been shown to be a poor indicator of sewage sludge adsorption because pharmaceuticals are generally polar ionic compounds (Williams *et al*., 2006). Further investigation into the reliability of the SimpleTreat model in light of the sewage treatment removal analysis done here, is an important research need.

The sheer variety of STP practices and combinations of treatment types may also complicate matters in calculating refined PECs. Many STPs can run two different

treatment streams simultaneously e.g. activated sludge and trickling filter beds. Individual STP practices vary throughout the year with sludge and hydraulic retention times varying from week to week within a plant. Such variations have significant impacts on removal efficiency. For example, increased residence times have been shown to increase degradation of ibuprofen significantly (Tauxe-Wuersch *et al.,* 2005). Individual treatment processes vary substantially in efficiency as well. Santos *et al.,* (2009) found high variability in removal of the same pharmaceutical at different STPs despite them all using activated sludge.

Clearly, accurately predicting pharmaceutical removal by STPs is difficult. An underestimation of the environmental concentration in a study by Morasch *et al*., (2010) was caused by over estimation of the removal of pharmaceuticals during sewage treatment gathered from the literature. Organisms present in the receiving waters, of course, are exposed to these fluxes in concentration suggesting any assumed average concentration may not represent the real exposure situation. PEC calculations involving sewage treatment removal are problematic and clearly should be used with extreme caution. The variation in removal and the measured increases in concentration of the selected pharmaceuticals during sewage treatment (Table 2.8) meant that no refinement to PECs 1 & 2 could be made.

2.6.3 Population size and pharmaceutical concentrations

The greatest contribution of pharmaceuticals in the environment is thought to be through human use and subsequent excretion. Theoretically, the number of people served by an STP should affect the amount of pharmaceutical discharged. Population is not used in the EMEA guidelines (Eq 1) instead a default Fpen (market penetration factor) of 0.01 is used. It is, however, incorporated when consumption data is used (Eq 2) and when local surface water PECs are calculated (Eq 3).

There was no correlation between size of population served by an STP (capacity) and effluent concentration of selected pharmaceuticals, except in the case of gemfibrozil (see Section 2.5.5 & Fig 2.19-2.22). The reasons for no clear relationship between size of population served and outfall drug concentrations are unclear. Differences in sampling

procedures (see above) used by the researchers compared in the calculation of correlation may have had an effect, as might differences in biological oxygen demand (BOD) and pH of the effluent (Horsing *et al*., 2010). The hydraulic retention times (HRT) and sewage retention times (SRT) (Zabcynski *et al*., 2010) may all have an impact through altering STP efficiency. In light of the fact that concentrations in effluent are highly variable with time, season, weather etc (see above) it is unlikely that a correlation would be found. Gros *et al*., (2010) also found no obvious correlation between the size of population served and the total load of pharmaceuticals at seven activated sludge STPs. While a higher ibuprofen MEC was found in a river surrounded by a small population base compared to a highly populated area in West London (Bound & Vouvoulais, 2006). In contrast, others have found that STPs serving larger populations result in the highest environmental loads (Ashton *et al.*, 2004).

Lack of a clear relationship may be influenced by factors not related to STPs. Some regions may have a higher usage of certain drugs depending on the average age of the community (Kostich *et al*., 2010). Consumption of certain drugs is also dependant on the time of year. For example, the use of paracetamol and decongestants increases in the winter months, and an increased consumption of antihistamines (for hay fever) in the summer. There are also large discrepancies in excretion rates of pharmaceuticals. For example variation in diclofenac excretion has been reported from 2-75% and 1-61% for carbamazepine (Carballa *et al*., 2008) between individuals. The initial crude PEC accounts for this by usually applying a conservative assumption of 100% excretion. However, if the risk quotient (RQ) exceeds 1, then refinements for human metabolism may be made. Any refinements of this type may lead to an underestimate of the actual environmental concentration.

The general lack of correlation between population served by an STP and effluent pharmaceutical concentrations poses a difficult problem when deriving an accurate PEC for any ERA. Usually the calculation for predicted surface water concentration involves an estimate of usage and population size. This figure is divided equally across the country and per person. If there is no correlation between the population size and the quantity of pharmaceutical discharged into the environment then this method breaks down. Letzel *et al*., (2009), however, used load per capita data from nine different STPs in Germany to

calculate a surface water PEC for diclofenac. These exactly matched the highest MEC in receiving waters.

ERAs are performed prior to the medicinal product being marketed, which means that an estimate of sales volume is used for PEC calculations. Trends in prescriptions and usage change year on year as new pharmaceuticals become available, their popularity changes or side effects become apparent. Pharmaceuticals are also consumed in different amounts country to country for example sales of fluoxetine in the UK are three times that of Germany (Oakes *et al*., 2010) therefore a PEC in one country may not be valid for another. Over time an active ingredient may be included in several brands, which could change any PEC. This can occur when a pharmaceutical comes off patent or new uses for a new drug are discovered (Sannella *et al*., 2008). In reality the crude PEC is only applicable prior to marketing of a drug. This study indicates that after market authorisation a new PEC should be calculated based on consumption data annually and that each PEC should be calculated on a regional basis. Changes in PEC are important as they will alter the associated risk quotient (RQ) which is calculated by dividing the PEC by the predicted no effect concentration (PNEC) from ecotoxicity tests. If the RQ is over 1 then an ecotoxicological risk is perceived in the ERA.

2.6.4 Predicted environmental concentrations (PECs)

The crude calculation of the PEC was in general above or aligned to MECs (Table 2.9). When the MEC exceeded the PEC it was commonly only by the maximum recorded concentration. This suggests that the crude PEC calculation does produce a realistic or precautionary estimate of environmental concentration perhaps surprising given the assumptions it makes. The concern however, is that the PEC may be an underestimate of concentrations when refinements are made for loss due to metabolism in the body, sewage treatment or environmental degradation. Letzel *et al*., (2008) reported a PEC, which had not been refined for metabolism, removal during sewage treatment or environmental degradation which was equal to the MEC. This is an important finding because according to the guidelines refinements to the PEC could justifiably be made thereby reducing the prediction to a value below that which has been detected in the surface water. Ibuprofen, paracetamol and tamoxifen were all measured above the PEC

when these refinements were made (Table 2.9). The reason for this may be errors in the calculation of refined PECs arising from inaccurate or unreliable data being used in the refinements (Castiglioni *et al*., 2004; Bound & Voulvoulis, 2006).

Other authors have made similar findings from data in the literature. Ferrari *et al*., (2004) found that unrefined PEC and MEC for carbamazepine were generally similar, while Letzel *et al*., (2008) found the maximum MEC for diclofenac equivalent to an unrefined PEC. In a comprehensive study, Zhang & Geissen., (2010) calculated refined sewage effluent PECs for 68 countries using sales volumes, metabolism, disposal, municipal water withdrawal (in place of wastewater production) and removal in STPs for carbamazepine and compared these to effluent concentrations recorded for the same countries in the literature. They found that although effluent MECs were in a comparable range they often exceeded PECs. Predicted concentrations were generally not below MECs in this analysis of literature (Table 2.11), but it is important nevertheless that the measured concentrations always fall below the prediction in order to ensure that non-target organisms and ecosystem system and function as a whole are protected. This was certainly not always the case. The variation in MECs highlights the importance of calculating a precautionary and conservative PEC and the potential need for monitoring of some pharmaceuticals.

Despite the assumptions and crude nature of Eq 1 which includes no actual sales or consumption data, the resultant PECs were predominantly above or in line with MECs found in the literature. However there were some exceptions, several MECs did exceed the PECs. This suggests the PEC calculation may not be sufficiently conservative and that some of the default parameters need reconsideration.

PECs calculated using prescription and sales data were lower than the crude PECs using the default market penetration factor (Fpen) of 1% except in the case of paracetamol (Table 2.10). This is a reflection of paracetamol having a higher market penetration factor than the default 1%. It appears, therefore, that the default Fpen was a precautionary figure for the majority of pharmaceuticals analysed. In the absence of marketing data, however, it remains important that a realistic market penetration factor be obtained using similar licensed drug sales data or drug manufacture market penetration predictions to obtain a consumption figure in place of a default Fpen. This will strengthen

the resilience of PEC calculations prior to marketing. The fact that some licensed pharmaceuticals have been detected in surface waters above the 10 ngl^{-1} action limit for further risk assessment leads to the conclusion that accurate prediction is an important requirement for the protection of the environment and that PEC calculations should be revisited after marketing.

The most recent prescription data available for England was from 2008. Unfortunately surface water MECs for England were not available in 2008 for direct comparison with PECs calculated using this data (Table 2.11). The lowest PECs generated in all the equations, therefore, were compared to maximum concentrations reported in the literature in England from a total of 8 studies between 2000 and 2011. Maximum surface water concentrations have been reported above these PECs for only three of the pharmaceuticals investigated; tamoxifen, trimethoprim and propranolol (Table 2.11). It should be noted that trimethoprim is also sold in combination with another antibiotic sulfamethoxazole as co-trimoxazole. The calculation for trimethoprim proportion of the active ingredients for this product was not specified in the prescription analysis and therefore not incorporated in the PEC thereby underestimating actual consumption. However co-trimoxazole accounted for approximately 2% of total prescriptions containing trimethoprim. No MEC data was available for either gemfibrozil or fluoxetine representing a substantial knowledge gap. Both had PECs greater than 10 ngl^{-1} (using all four calculations) the action limit for further risk assessment and both have chronic ecotoxicity effects (Nentwig, 2007; Painter *et al*., 2009; 2008, Mimeault *et al*., 2005).

In order to gain a more accurate picture of the reliability of PECs a much more tailored study should be performed. Sales of drugs differ year to year and the prescription data collected for 2008 may differ from precise prescription amounts in each of the years in which data was reported. This lack of data and a lack of any investigation for some pharmaceuticals mean that the analysis is inconclusive. It would appear that although maximal concentrations occasionally exceed PECs, overall PECs are higher than measured environmental concentrations. However, MECs do occasionally exceed PECs and therefore they are not the worst case scenario they purport to be.

2.6.5 Pharmaceutical PECs, effluent concentrations and surface water dilution effects

The derived surface water PECs were compared to reported effluent concentrations. Concentrations of diclofenac, trimethoprim, ibuprofen (Hilton *et al*., 2003) and tamoxifen (Roberts & Thomas 2006) in effluent have all been reported at levels exceeding PECs 1 & 2 (Table 2.11). Propranolol has been reported above PEC 2 where consumption data was used (Roberts & Thomas 2006) but has not exceeded the PEC generated in Eq 1.

The EMEA guideline for a PEC calculation includes a default value of 10 for the dilution from sewage effluent to surface water. When this was applied to the trimethoprim and tamoxifen effluent concentrations they still exceeded the surface water PEC 2 (consumption data). This was not the case for the Eq 1 PECs using the default Fpen. Trimethoprim has been measured in sewage effluent in Wales at a maximum concentration of 3.05 $\mu g l^{-1}$ (Kasprzyk-Hordern *et al*., 2009), as the consumption of trimethoprim is similar in Wales and England (Welsh Government, 2011) the PEC (Eq 2) is exceeded by over 10 times with the dilution factor applied. The sales of co-trimoxazole alone cannot account for this. It should be noted that the PECs 1 & 2, have not been refined either for excretion or removal in STP. Moreover, PECs of course should be greater than MECs in order to be conservative, precautionary and offer adequate protection for water bodies.

The validity of a dilution factor of 10 was investigated for carbamazepine, diclofenac, ibuprofen and gemfibrozil. It was found that concentrations of these pharmaceuticals could increase as well as decrease downstream from a sewage outfall. This may be due to other sources of pharmaceuticals such as release from other sewage treatment plants, untreated sewage, storm overflow events, misconnections, pharmaceutical manufacture and even desorption from sediment. The underlying concentration of a drug in a water body through previous contamination, lack of natural degradation and potential accumulation in different environmental compartments are important factors to be considered when estimating surface water concentrations. The PEC calculation is based on new inputs of pharmaceuticals and is not inclusive of existing concentrations. On one occasion gemfibrozil was not detected in the sewage

effluent but was found at concentrations of 1.3µgl^{-1} in the receiving water (Metcalfe *et al*., 2003). Despite this finding, on the whole results indicated that the majority of surface water concentrations were less than the sewage outfall. However, the results also show that the concentration was not reduced by 90% of the original concentration and therefore surface water dilution was less than the default factor of ten.

Clearly a default dilution does not reflect reality well. There was also a notable lack of studies that investigated MECs at more than one point downstream from a sewage outfall. There are a number of factors that might make the use of a default dilution factor problematic. Firstly, because the dilution factor for each river is different. For example, the dilution factor in the Ebro river was found to be 30-40 times but the river Agra in Pamplona was closer to 5 (Gros *et al*., 2010) and the river Gardon in the south of France was found to be nearer 3(Coetsier *et al*., 2009). The dilution factor can change daily because of the volume of water in the river fluctuates with flow and weather conditions. Another factor is the effect of existing pharmaceutical surface water concentrations. Even when daily fluctuations in receiving water body volume were applied to calculate an accurate dilution factor, large variations in day to day MECs were still recorded (Ter Laak *et al*., 2010). The variation in reliability of the dilution factor seems to be large. Although the findings in this study suggest the dilution factor is an underestimate in some cases as well as often overestimating concentrations.

Clearly the use of a default dilution factor of 10 is appropriate for some water bodies e.g. those with dilution factors >10 and becomes a problem only when the water bodies dilution effect drops below this. Inevitably there will be variability in dilution both seasonal e.g. dry periods, and potentially daily with peak flow times at the STP e.g. early mornings. Low flow occurrences maybe become an increasing problem with climate change. It may be appropriate to identify worst-case scenarios for the dilution effect of a water body when calculating a refined PEC given this variability and the need for a precautionary stance. Alternatively a lower default dilution factor might be set, but this will not be appropriate where water bodies have large dilution capacities.

Drug consumption and sales figures are important parameters for the calculation of refined PECs. This investigation highlights two issues here. First, the default market penetration factor, which may be greater than 1%, is problematic. For over the counter

medicines actual sales volumes are unknown in the UK. The market penetration factor for paracetamol must be higher than 1% because the PEC generated in Eq 2 using consumption data is much higher than Eq 1 using the default 1%. Grung *et al*., (2008) also found the default Fpen of 1% to be an underestimate for paracetamol and ibuprofen in Norway where excellent sales records are maintained. The actual sales volume for an unlicensed medicine is unknown, of course.

Second, the official prescription or sales data may not actually reflect the amount consumed (Halling-Sorensen, 2000). The assumption made in PEC calculations is 100% consumption. This may overestimate the PEC but is precautionary. Propranolol for instance has a 30% non compliance rate (Mulleners *et al*., 1998) and in the UK approximately half of prescriptions may be unfinished (Bound and Voulvoulis, 2005). There is some variation with country, however, the Swedish Medicinal Products Agency (2004) claim that 90% of pharmaceuticals sold in Sweden are consumed. Unused drugs kept in the home or disposed in the bin will, of course, reduce the concentration going to water bodies, but disposal via the sink or toilet with no degradation in the body will tend to increase environmental loads. Accounting for societal effects in PEC calculations is clearly problematic.

There are no precise sales statistics in most countries for pharmaceuticals used in hospitals or for over the counter drugs (Grung *et al*., 2008; Castiglioni *et al*., 2004; Calamari *et al*., 2003). PECs calculated with prescription data for paracetamol and ibuprofen were found to be significantly lower than river MECs although the use of production figures for paracetamol significantly increased the PECs making them more representative (Bound & Voulvoulis, 2006). It appears that production figures may be a more reliable data set for over the counter drugs. In the same study, however, the maximum MEC for salbutamol was 1200 times greater than the PEC even though this drug is not available without prescription. Clearly other factors are at play.

The consumption and disposal of pharmaceuticals, of course, is directly related to the concentration in sewage effluent and surface waters (Ashton *et al.,* 2004; Ter Laak *et al.,* 2010). It is, therefore, critical to find accurate production, sales and consumption data to generate a reliable PECs. The EMEA PEC calculation provides a 200L per day per person default value for wastewater production. This may not be a valid assumption for

some countries and can vary with location. For example, 150L for France and 140L for Germany are probably more realistic figures (Ferrari *et al*., 2004), whereas in Norway 216L of wastewater is produced per person per day (Grung *et. al.*, 2008). Such values would increase the PEC by up to 30%. It appears that wastewater production should be determined on a location by location basis if realistic PEC values are to be calculated.

Finally it cannot be discounted that one of the problems with comparing PEC and MECs is the quality of the analytical data. Despite advances in analytical chemistry over the last 20 years there still remain difficulties with collecting accurate data on pharmaceuticals in the environment at these miniscule concentrations. On occasion concentrations can be reported which could not realistically be present in the environment because the quantity required is simply not manufactured. Metcalfe *et al*., (2010) calculated a raw sewage PEC for fluoxetine which is not available without prescription based on sales data of 32 ngl^{-1} which was exceeded by a measured concentration nearly 3 times (91 ngl^{-1}). There is evidence of studies which have compared different laboratories abilities to analyse water samples with worrying results (Van Leeuwen *et al*., 2006). Bound & Voulvoulis, 2006 report an average error of 13% for measured concentrations. As mentioned above it was thought that increases in sewage concentration may be due to suppression of the MS/MS signal.

One of the problems is the complexity of the variables required to accurately predict environmental concentrations. GREAT-ER and *P*hate are computer packages which use geo-referenced river models to predict environmental concentrations of pollutants. They are easy to use, cost effective and can incorporate processes such as STP removal and dilution in rivers. Diclofenac and several beta blockers have had successful exposure rate predictions using these models (Johnson *et al*., 2007; Alder *et al*., 2010). Although the lack of accurate STP removal efficiency and consumption data still remains a limitation.

The fact remains that when considering the expense of determining environmental concentrations, inaccuracies with measurement and the sheer number of compounds and water bodies, PECs are necessary and crucial for environmental risk assessment. They are a valuable first indicator of environmental risk for a pharmaceutical or chemical. They can help prioritise pollutants to avoid unnecessary toxicology experiments. It is

paramount therefore that the data which is entered into these calculations is accurate and reliable. When the data used in calculations is reliable and accurate, and assumptions are minimised and precautionary, the resultant estimate could be more accurate than available analytical chemical techniques.

2.6.6 Pharmaceuticals in other matrices

The most important findings in regard to other matrixes were that there is still a lack of research into pharmaceuticals in drinking water, ground water and marine waters. However, there are reports of several pharmaceuticals being detected in all three of these matrixes (Table 2.13).

Currently under ERA procedures for pharmaceuticals, even if an environmental risk is identified, a pharmaceutical cannot be restricted for human use. It is human health that is paramount above any environmental concern. The presence of pharmaceuticals in groundwater and drinking water also has potential health implications as well as environmental ones. Pharmaceuticals can enter drinking water through abstraction from surface water as well as ground water. The few measurements of drugs available in the literature, however, have been far below the therapeutic dose in finished drinking water (Table 2.13). The concern here is that certain drugs such as anti cancer medicines may be damaging to vulnerable groups such as pregnant women and children (Rowney *et al*., 2009). Despite the long established existence of pharmaceuticals in river water used for abstraction, there is insufficient data on concentrations and almost no data available on occurrence in drinking water. There are no reports of tamoxifen, fluoxetine, trimethoprim and EE2 being detected in drinking water so far. However, this may be due to the lack of testing rather than their non existence. The incidence of pharmaceuticals in surface water suggests their presence in abstracted water. Although no detections of propranolol have been reported in drinking water, a concentration of 50 ngl^{-1} was recorded in a reservoir in Sweden (Ferrari *et al*., 2003). Also 18 ng/l of atenolol another beta blocker has been detected in drinking water (Thomas *et al*., 2007). Reported concentrations of pharmaceuticals in drinking water suggest that concentrations above 10 ngl^{-1} can occur. However, there is no quality standard or limit set for pharmaceutical concentration in drinking water. The Drinking Water Inspectorate, (2007) used probability modelling to

estimate concentrations in drinking water and concluded they were generally equal to 100 ngl^{-1}. In fact data suggests that concentrations can exceed 100 ngl^{-1}. Although 100 ngl^{-1} is reported to be below the therapeutic dose for most pharmaceuticals (Choi *et al*., 2008), chronic exposure data is rarely available. Some pharmaceuticals may work in conjunction causing additive or combined effects that may lower the active concentration of individual pharmaceuticals. Combinations may be complicated and impacts unforeseen. There are no analytical methods for measurement for all 3000 pharmaceuticals on sale and their associated metabolites. It is important that these methods are developed in order to monitor levels in river and drinking water. It may be appropriate for pharmaceutical companies to be required to develop analytical detection methods for their products and establish their metabolites as part of the ERA required for licensing.

2.6.7 Critical analysis of reporting methods and standards for pharmaceuticals in the environment.

A considerable number of papers have been published on environmental concentrations of pharmaceuticals. This number must now be in excess of 1000, not including ecotoxicological assessment studies of pharmaceuticals. This is a valuable and substantial contribution to knowledge on the fate and effects of drugs in the environment. Monitoring studies are time consuming and costly exercises, making it paramount that the data generated in these studies should be accurate, reliable and complete.

Measurement of pharmaceuticals in water provides a useful case study to determine the reporting standards on pollutants in peer reviewed articles. It is clear from the analysis here of 128 articles reporting pharmaceutical concentrations in freshwater and effluents that in the vast majority of cases significant environmental data and statistical analysis was missing from the papers (Table 2.14). The lack of specific sampling, statistical and environmental data renders concentration data almost meaningless. The ranges of concentrations of pharmaceuticals reported worldwide are quite wide and the environmental and sampling variables need to be specified in order to allow useful analysis. Published data provided must be of a scientifically acceptable standard enabling an assessment of the environmental risks (Kuster *et al*., 2009). Method validation was a common reason for measuring pharmaceutical concentrations in water in

many of the surveyed articles, however, even in this context the lack of repeat measurements and statistical analysis means that the error associated with the use of the technique on field samples cannot be established. Overall a clear opportunity to add invaluable information to build a comprehensive picture of the fate of pharmaceuticals in the environment is being missed. It is important that literature data provides valuable supplementary information for risk assessment processes (Kuster *et al*., 2009). It is essential that policy makers have access to a high quality and wide ranging evidence base. Decision makers must be confident that evidence is robust and stands up to challenges of credibility, reliability and objectivity (H M Government, Guidelines on Scientific Analysis in Policy Making, 2005). Over the last ten years the UK government has strongly promoted the more effective use of science to inform policy-making and regulation (Holmes & Clark, 2008). Yet from this analysis much of the information available in the academic literature is not of an appropriate standard to provide 'sound' scientific evidence. Setting standards for academic reporting of environmental and health information that go beyond peer review to provide policy makers a sound evidence base for decisions seems essential. A set of standards for reporting environmental pollutant data in peer reviewed literature may help provide a basis for informed policy and regulatory decision making. This would also ensure that the body of knowledge could expand. It is a shame when work becomes meaningless because simple information is missing. A set of uniform standards for reporting of concentrations of all pollutants not just pharmaceuticals would enable valuable science to inform policy and regulation decision making.

'WikiPharma' is a new database set up to provide free publically available ecotoxicity data for pharmaceuticals (Molander *et al*., 2009). The database uses information from peer reviewed journals as a quality control. It is an excellent example of how peer reviewed journal research articles can be used to provide ecotoxicological information or other scientific knowledge on existing and novel pollutants. It is essential that this type of database provides reliable and robust data. Only 'sound science' can support policy and regulatory controls.

In the current climate of fiscal constraint continued pressure on research funding and on the work of regulators is inevitable (McEldowney *et al*., 2010). Setting reporting

standards for academic research would not only provide a sound evidence base for policy decisions but would help provide value for money in research outcomes for funders.

The results of the critical reporting standards analysis has led to the development of a list of criteria for data that should be available in publications of environmental concentration data in aquatic environments:

1. **Uniform statistical analysis including mean, standard deviation and sample size.**

This would enable comparisons to be made between studies and meta analysis of concentration data possible.

2. **Access to raw data in a supplementary section. This should be available on line permanently.**

Access to raw data would make expensive monitoring data generally available and further statistical analysis possible. Meta analysis could be performed between studies.

3. **Description of sewage treatment processes discharging effluent into water bodies.**

The effectiveness of different sewage treatment processes for pharmaceutical removal could be assessed and more rigorous sewage removal refinement applied in PEC calculations.

4. **Sewage effluent to surface water dilution factor at the time of sampling. It would be helpful to supply a low flow and an average dilution factor for the water body analysed.**

This would enable risk assessment of the average dilution for a water body and also worst case scenarios. The time of sampling may not reflect the range of dilutions in that water body.

5. **Population size serviced by STP, specifically population equivalent to take into account industrial contribution on population.**

The local water PEC calculation in the EMEA documents requires a figure for the capacity of the STP. Population size is an important parameter when assessing predicted concentrations.

6. **Specific data on location. Including distance from sewage outfall, direction of water flow and other discharges into the water body and their location.**

This type of information would enable assessments to be made on the persistence of pharmaceuticals in water bodies.

7. **Specific time and date of sampling.**

This information would enable daily and seasonal changes in pharmaceuticals effluents and surface waters to be assessed. This information could help explain reasons for fluctuations in pharmaceutical concentrations.

8. **LOD or LOQ for the method used.**
9. **Season and weather conditions at time of sampling.**

Changes in pharmaceutical concentration in water bodies and removal by STPs may be highly dependent on weather conditions. In order to determine the effects that decreased temperature or increased rainfall may have it is necessary to know the weather conditions at the time of sampling. This information may allow comparisons across different studies.

Some of this data could be included in the publication itself, other material in an on-line resource as appropriate. Other reporting standards should be developed by knowledge exchange between, scientific journals, academics and environmental regulators to establish any other information that would make environmental data produced fit for use by regulators and policy makers.

2.7 Conclusion

A great deal of data is available on pharmaceutical concentrations in surface water and sewage effluent. Analysis of this data shows that concentrations between different pharmaceuticals, water bodies and sewage treatment plants are wide ranging. The most surprising and interesting feature being that concentrations often vary for the same pharmaceutical in the same water body or STP by an order of magnitude. Sewage treatment does not remove pharmaceuticals efficiently and concentrations may increase after treatment. The situation for non target organisms in water courses appears to be a continuous exposure to a large number of human pharmaceuticals at fluctuating concentrations.

PEC calculations are a vital stage of ERA of pharmaceuticals (Coetsier *et al*., 2009; Ginebreda *et al*., 2010). There are questions, however, over current guidelines for PEC calculations. Although MECs are commonly in range of PECs, they are not precautionary enough as they are sometimes exceeded. This disconnect between PEC and MEC may find its basis in some of the assumptions made in calculating PECs. Some of these may need revising, including the surface water dilution factor of 10 and the 200 L per day wastewater production volume. Standardizing drug consumption over the population over the year is questionable and there are clear inaccuracies in consumption, sales and excretion data. There appear also to be overestimates of sewage treatment removal.

There are analytical problems associated with measuring small pharmaceutical concentrations in water bodies, however, which may raise questions over the accuracy of some reported MECs. An indicator that analytical problems may be occurring is that environmental concentrations can be measured far above that which could have entered the environment. Comparison of different laboratories also suggests differences in the accuracy of measuring pharmaceuticals in water samples (Farre *et al*., 2008). Such difficulties in measuring MECs, however, do not undermine the argument that the assumptions made for PEC calculation should be valid and representative of the factors effecting pharmaceutical concentrations in individual countries, localities and water bodies. PECs are a valuable first indicator of environmental risk for a pharmaceutical, can help prioritise pollutants to avoid unnecessary toxicology experiments and provide a

margin of safety for aquatic ecosystems. They should provide a realistic estimate of the maximum possible exposure of a water body to a pharmaceutical. At present there can be no great confidence that this is so.

Peer reviewed literature provides a valuable wealth of information on environmental and ecotoxicological issues. In the current financial climate this source of knowledge should be used to its full potential. With a robust, reliable set of reporting standards in academic publications for data on environmental pollutants, the opportunity is presented to provide sound science to underpin and inform regulation and policy.

Section 3
Interview Analysis

3.1 Aim for Section 3

The aims of the work performed in this section were to engage key individuals involved in environmental protection related to human pharmaceuticals, including representatives of water companies, pharmaceutical companies, regulatory organisations and academics. Different perspectives were sought on, the potential inadequacies and successes of the ERA and the proposed solutions and mitigation mechanisms that could minimise the risks to the aquatic environment. These were then examined to determine where there was consensus and differences about the best route for environmental protection from pharmaceuticals.

3.2 Introduction

Pharmaceuticals are frequent pollutants of the aquatic environment (Section 2) with a continual input of low concentrations of a large (3000+) mixture of compounds that are designed to cause a specific biological effect (Section 4). Two pharmaceuticals, one for human use and one used for both human and veterinary medicine ethinylestradiol (Jobling *et al*., 2002) and diclofenac (Oakes *et al*., 2004) respectively have been shown to cause serious adverse effects on wildlife. New research on the chronic effects of human pharmaceuticals on aquatic wildlife is being published all the time. Recent publications include: Alterations of gene regulation in fish brain after propranolol exposure (Lorenzi *et al*., 2010) and enzymatic stress in gills, liver and muscles of fish after carbamazepine exposure (Malarvizhi *et al*., 2012). This situation has led to the development of an ERA for human pharmaceuticals (EMEA, 2006).

In accordance with Article 8(3) of Directive 2001/83/EC, as amended, an ERA should include the evaluation of the potential environmental risks posed by a medicinal product and an environmental impact assessment. This should also include an evaluation of the positive therapeutic effects of the medicinal product in relation to the risks associated with undesirable effects on the environment. Precautionary management action should be considered to reduce the release of pharmaceuticals into the environment and should include shareholders, stakeholders, consumers, pharmacists and medical practitioners in order to find solutions (Kummerer, 2009). Expert knowledge can often provide valuable information for the assessment of environmental problems and help fill in knowledge gaps that may be present in the peer reviewed literature (Doerr-MacEwen & Haight, 2006; Human & Davies, 2010). Recently, there have been calls among decision makers, interest groups, citizens, and scientists for more science-based

environmental policy (Johnson *et al*., 1999; Sarewitz *et al*., 2000). Including scientists in early stages of policy formation will improve the quality of complex policy decisions and facilitate the resolution of environmental decisions by providing objective scientific information to policymakers (Mazur, 1981; Steel *et al*., 2004). Over the last 10 years the UK Government has strongly promoted the more effective use of science to inform policy-making and regulation (Holmes & Clark, 2008). The Cabinet Office (1999) summarises the core competencies of good policy-making including using evidence which is described as the "best available evidence from a wide range of sources and involves key stakeholders at an early stage''. This should include evidence from stakeholder consultation, expert knowledge and the critical evidence held in the minds of front line staff in departments, agencies and local authorities and those to whom the policy is directed. Effective access to information and expertise is a necessary precursor to the use of science to inform policy-making and regulation (Holmes & Clark, 2008). External experts (including researchers, consultants and experts in other Government departments and agencies) are an important source of scientific advice (Holmes & Clark, 2008). These experts synthesise and interpret information for policymakers and their involvement may lend credibility to the ensuing policy decision. The addition of diverse experts can also lead to a more comprehensive understanding of ecological hazards and can improve problem formulation by generating an ecologically robust set of information on which to base the subsequent, more technical environmental risk assessment (Dana *et al*., 2012). The participatory ERA process can also increase the transparency of the ERA by exposing the logic and rationale for decisions made at each step (Dana *et al*., 2012).

Hajer (2003) identifies an 'institutional void' between policy and practice in the field. In recent decades, there has been increased interest on participation in environmental decision making (Reed, 2008; Hansen & Mäenpää, 2007; Abelson *et al*., 2007). Techniques such as interviews, analyses of reports and minutes from meetings can increase the probability of an in-depth understanding of the science process and is important for decision making (Blackstock *et al*., 2007). By focusing upon different expert knowledge, deeper insights can be gained into the day to day management and governance of environmental problems (Bracken & Oughton, 2012). Policy makers should use the best available evidence from research and legitimate sources of knowledge, such as expert knowledge, when making decisions on environmental policy and management (Bracken & Oughton, 2012).

This leads to the conclusion that engagement with expert knowledge holders including pharmaceutical company employees, water company managers, government bodies and academics that are involved in and are knowledgeable about the current ERA for human pharmaceuticals may be beneficial for gaining insight into its effectiveness and for its development into the future. In depth knowledge of the ERA protocol and guidelines was required in order to answer the in depth interview questions, so other stakeholders, e.g. pharmacists, doctors and shareholders were not sort for interview.

There are a number of potential inadequacies with the current ERA of pharmaceuticals. These include, issues around the 10 ngl^{-1} action limit for ecotoxicological assessments, the accuracy of PEC calculations (Section 2), the spectrum of ecotoxicology tests applied (Section 4), a lack of monitoring, a lack of retrospective ERAs for existing drugs, a lack of mitigation measures and ultimately the fact that at present a pharmaceutical cannot be refused for human use on environmental grounds. Therefore, engagement with environmental regulators from water companies, pharmaceutical companies, government and academia could provide valuable insight and potential remedies for the shortfalls in the current ERA. This section assesses opinions provided by experts working in the field of pharmaceuticals on a number of issues outlined below.

3.2.1 The 10 ngl^{-1} action limit

The 10 ngl^{-1} action limit for ecotoxicological assessment may be set at an inappropriate level. Having the same trigger limit for all pharmaceuticals may not be appropriate. A concentration of 10 ngl^{-1} may be insufficiently precautionary for some pharmaceuticals and set at an unnecessarily high level for others. For example pharmaceuticals such as EE2 are highly potent and are known to adversely affect fish at concentrations <1 ngl^{-1} (Lange *et al*., 2001; Caldwell *et al*., 2008). However, many toxicity studies report effect concentrations for other pharmaceuticals at levels of an order of magnitude or more above 10 ngl^{-1} (Santos *et al*., 2009).

3.2.2 Retrospective ERAs

Most of the pharmaceuticals in this study have been reported in surface waters at concentrations far higher than 10 ngl^{-1} (Section 2). If these were new medicines they would require an ERA prior to market authorisation, however, current legislation does not require retrospective risk assessment of pharmaceuticals. Many authors recommend that ERAs should be

performed for some existing medicines (Agerstrand *et al*., 2009). As there are a considerable number of existing pharmaceuticals which were authorised prior to the requirement for an ERA, it has been suggested that a prioritisation or ranking strategy is the most pragmatic way to decide which medicines may pose a risk to the environment (Roos *et al*., 2012).

3.2.3 PECs

The generation of PECs is an essential stage of the ERA however their calculation involves many assumptions and uncertainties (Section 1). The current PEC calculation lacks the real world complexities of the parameters involved in the exposure of pharmaceuticals. Without sufficient data for removal by sewage treatment, dilution factors of specific water bodies, environmental degradation and projected consumption data it will always be an inaccurate estimate of what exposure concentrations might be (Section 2) (Coetsier *et al*., 2009; Bound & Voulvoulis, 2006). This leads to a degree of uncertainty when using PECs to assess the risks to the environment including species populations and communities they support. The development of computer packages for estimating environmental exposure is one potential way of reducing scientific uncertainty as they provide a range of probable concentrations and incorporate much more information on site specific factors such as hydrological information, season and pH (Johnson *et al*., 2007).

3.2.4 Mitigation

The precautionary principle emphasizes that where evidence of a threat to the health of the environment exists, scientific uncertainty must not be allowed to delay reasonable forms of management action (CEPA 1999, Quijano 2003, United Nations General Assembly 1992). However, management action and mitigation strategies tend to be one of the key areas lacking in the environmental management of pharmaceuticals that have been found to present a potential risk in an ERA. Pharmaceuticals cannot be restricted for human medical use on environmental grounds. Currently the only suggestion in the EMEA, (2006) guidelines in light of an environmental concern is to include a statement on the package leaflet which should read:

> *"Medicines should not be disposed of via wastewater or household waste. Ask your pharmacist how to dispose of medicines no longer required. These measures will help to protect the environment."*

As human consumption and subsequent excretion is thought to be the main source of pharmaceuticals in the aquatic environment (Cunningham *et al*., 2006; Sanderson *et al*., 2003). This measure will not reduce the environmental input of pharmaceuticals from human consumption; therefore, other control measures need to be considered. Upgrading of sewage treatment facilities with tertiary treatments such as activated charcoal and ozonation could reduce exposure (Wang *et al*., 2009; Schaar *et al*., 2010; Schroder *et al*., 2012). However, the wide ranging capabilities of different sewage treatments for removing pharmaceuticals means this may not be the most suitable option (Zabcynski *et al*., 2010). The question then arises as to who should pay for any improvements if needed. The polluter pays principle also known as extended producer responsibility (EPR) is defined by OECD Environment Directorate, Paris, France (2006) as:

> *a concept where manufacturers and importers of products should bear a significant degree of responsibility for the environmental impacts of their products throughout the product life-cycle, including upstream impacts inherent in the selection of materials for the products, impacts from manufacturers' production process itself, and downstream impacts from the use and disposal of the products. Producers accept their responsibility when designing their products to minimise life-cycle environmental impacts, and when accepting legal, physical or socio-economic responsibility for environmental impacts that cannot be eliminated by design.*

It is necessary therefore, to establish who is the polluter, the pharmaceuticals industry, the water industry or the consumer?

3.2.5 Green Pharmacy

A further proposal for the reduction of pharmaceuticals in the environment is green pharmacy. The term green pharmacy stems from the concept of green chemistry. Green chemistry, describes the development of more environmentally acceptable and sustainable chemical processes and products (Greenwood *et al*., 2010). Green pharmacy is the design of pharmaceutical products and processes that eliminate or significantly reduce the use and generation of hazardous substances and the prevention or reduction of environmental safety and heath impacts at the source (Clark, 2009). It has been recommended by several authors that green pharmacy should incorporate the whole life cycle of the drug including, design, manufacture,

transport, prescription, sales, storage, usage, disposal, sewage treatment and environmental degradation or persistence. The European Parliament and the European Commission agreed in 2001 that within a generation chemicals should be produced and applied that do not have any impact on the environment (EU Parliament and EU-Commission, 2002). This should also hold true for pharmaceuticals (Kummerer, 2010). The concept of green pharmacy falls into three broad categories or stages where the environmental protection should be considered. These are green manufacture, environmental stewardship and environmentally friendly design of pharmaceuticals (Daughton & Ruhoy, 2008; Kummerer, 2007; Clark *et al*., 2010; Greenwood *et al*., 2010).

3.2.5.1 Green Manufacture

Firstly the green manufacture and synthesis of pharmaceutical compounds, including the reduction of hazardous waste products and energy costs is an important element of green pharmacy. This can include novel green catalytic methods, reduced solvent use, waste minimisation and elimination of hazardous reagents, production of enantimerically pure compounds and reduction of production steps. This is one of the aspects of green pharmacy that is being incorporated into pharmaceutical production. Cleaner synthesis methods and green production methods are being adopted by the pharmaceutical industry. Merck and GlaxoSmithKline have established best practice awards to reward their staff for embracing these initiatives (Greenwood *et al*., 2010). Green improvements in the manufacture and production of pharmaceuticals have been applied to new products and to existing drugs. The new green synthesis of ibuprofen in 1992 reduced the process steps from six to three and virtually eliminated all waste products by recovery and recycling (www.greenchemex.org). The synthesis of sildenafil citrate (Viagra) has had new green chemistry techniques applied to reduce the amount of waste created during its production from over 1000kg to 6kg per 1kg of product (Kuzemko *et al*., 2007).

3.2.5.2 Take back schemes

The second aspect of green pharmacy is to consider environmental stewardship of pharmaceuticals, ‘cradle to grave’ or including sustainability as well ‘cradle to cradle’. Two authors Christian Daughton and Ilene Ruhoy have written extensively on this topic. Post marketing surveillance of pharmaceuticals by medical, pharmaceutical, pharmacy and regulatory

industries to track adverse effects is termed pharmacovigilance (Blake *et al*., 2012). They believe that pharmacovigilance should be extended to include environmental issues such as adverse effects on wildlife (Daughton & Ruhoy, 2008; 2011). This would involve consumers, pharmacists, manufacturers, prescribing physicians and veterinarians taking action to reduce the introduction and release of pharmaceuticals into the environment, its pioneers believe they do not need to invoke the precautionary principle to justify such a programme. Take back schemes involving classification and labelling are heavily reliant on communication and education of those involved including doctors, pharmacists and the general public (Daughton & Ruhoy, 2008). Directive 2004/27/EC requires that take back schemes are available for unused and expired medicine. Reference to these collection schemes are to be acknowledged on the package label or leaflet. This is to target not only environmentally friendly disposal of medications but also prevents build up of products in the home which can lead to accidental poisonings. The effectiveness of these schemes is unknown; however, surveys have indicated that potentially fifty percent of prescribed medication is not taken and that many consumers do not return unused medication to the pharmacy (Grass & Lalande, 2005). Take back schemes are one of the more deliverable solutions when considering prevention of pharmaceutical exposure. Although it is unknown what contribution they make to the overall problem, source control is widely recognised as having a large potential for reduction (Vidaurre *et al*., 2010). The key problem with the success of these schemes seems to stem from a lack of public awareness and clarity (Kampa *et al*., 2010). It has been identified that a fundamental part of this aspect of green pharmacy is a need to engage with doctors, manufacturers, water companies and environmental regulators to address the different issues at each stage of drugs life cycle (Greenwood *et al*., 2010).

3.2.5.3 'Benign by Design'

One aspect of green pharmacy is based on the concept that drugs should be sustainable from the very beginning 'benign by design' (Kummerer, 2007). It may be possible to 'design' pharmaceuticals to limit their adverse environmental effects while retaining their therapeutic benefits. The idea is that a drug should be stable enough to survive degradation by the body in order to have the desired therapeutic effect but should also biodegrade to harmless elements once in the environment (Kummerer, 2007). Khetan & Collins, (2007) have suggested methods for

green chemistry such as including a 'chemical switch' to be built into the drug that would lead to rapid decomposition outside the body or attachment of affinity groups that could lead to quantitative sorption onto a particular support to be used in STPs. This is the aspect of green pharmacy that has seen the least research and progress. There are few examples of benign drug design. One is the anti-cancer drug ifosamide, which is highly recalcitrant, however by attaching sugar molecules, another compound glufosfamide is obtained which retains the therapeutic activity of ifosamide but is much more biodegradable (Kummerer *et al*., 2000; Keil, 2008).

3.2.6 Ecotoxicology tests

It was found (see Sections 2 & 4) that there was a distinct lack of data for some aspects of the ecotoxicological assessment part of the ERA. This included gaps for chronic effects data using MoA related end points and mixture effects for pharmaceuticals (Agerstrand *et al*., 2009). Ecotoxicology also uses a narrow spectrum of species that may not reflect the most sensitive organisms in exposed habitats. There is also lack of data on routes of exposure including bioconcentration and transport through food webs (Carbonell *et al*., 2000; Boxall & Greenwood, 2010; Nfon *et al*., 2011).

Standardized ecotoxicology tests may underestimate the effects of pharmaceuticals. This is because traditional tests do not incorporate the MoA of the drug or a mixture of compounds which may produce antagonistic, synergistic or additive effects. For example Cleuvers, (2008) found toxicity of a mixture of analgesics decreased reproduction by 100% but had no effect on survival. Tests, however, could be based usefully on MoA, for example, the induction of vitellogenin in fish by oestrogens (Sumpter *et al*., 2006).

Metabolites of pharmaceuticals that can be produced by breakdown products through the human body, sewage treatment, microorganisms in the environment or by sunlight (Liu *et al*., 2009; Wei *et al*., 2011) may also be toxic to aquatic organisms. There is virtually no data in the literature on the toxicity of pharmaceutical metabolites. Biotransformation products of pharmaceuticals in the environment, i.e. intermediate products that resist total mineralisation, can be more stable than the parent compound thus increasing their potential for accumulation (Kummerer, 2009).

3.3 Method

Structured in depth interviews were conducted with eleven people who either had expert knowledge of the environmental issues related to pollution of the aquatic environment by human pharmaceuticals or worked in relevant industries and governmental bodies. These included, a pharmaceutical company, a water company, The Department for the Environment, Farming and Rural Affairs (DEFRA), the Environment Agency (EA), an environmental consulting company, and 5 academics from a NERC funded research council and 2 universities.

The interviewees were recruited by formal letter. Ten of the interviews were conducted in person and recorded. One interviewee responded by email. The interviewees gave written permission for anonymous responses to be recorded and used in this thesis.

Participants were selected due to their contribution to the peer reviewed literature on the subject of pharmaceuticals in the environment, recommendations by colleagues, or by their organisations. Interviewees were chosen in order to explore the length and depth of views of a variety of expert knowledge holders rather than randomly selected to generate statistical information.

All the interviewees were asked ten main open ended questions and then if time allowed six further short supplementary questions. Most interviews lasted between thirty minutes and one hour. Interviewees were offered a transcription of the interview and a copy of the results analysis of all the interviews when completed as an incentive for taking part.

The interviews were fully transcribed (Appendix 13) and comments were collated into broad topic areas including: PECs, the 10 ngl^{-1} action limit, retrospective risk assessment, the precautionary principle, ecotoxicity tests, the polluter pays principle and mitigation strategies.

3.3.1 Interview questions

Current EU Environmental risk assessment

1. Do you think that the current EU guidelines for environmental risk assessment of human pharmaceuticals (laid out by Article 8(3) of directive 2001/83/EC as amended including guidelines EMEA//CHMP/SWP/4447/00) sufficient to protect aquatic organisms and ecosystems? Can you explain why?

2. Is the 10 ngl^{-1} trigger limit in Europe an appropriate mechanism and set at an adequate level to evoke the second phase of environmental risk assessment and ecotoxicity testing?

3. What are your views on the accuracy of predicted environmental concentrations? (3a. what are your thoughts about computer packages using GIS for example for estimating environmental concentrations?)

Remedies/mitigation

4. What are your opinions on monitoring of environmental concentrations of human pharmaceuticals?

5. If a substantial ecotoxicological risk for a human pharmaceutical is identified what strategies can be employed to mitigate the risks to the environment?

6. Sewage treatment methods have different capacities for removing different pharmaceuticals. Do you think that upgrading sewage treatment plants is the most effective way to reduce pollution by pharmaceuticals? Is it necessary? Who should pay for this?

7. One of the routes of entry for human pharmaceuticals is disposal down sinks and WC's. Although not the most important route it is one of the more easily remedied. For example 30% of Propranolol (beta blocker) is never taken, potentially due to the side effects. In a survey by Bound & Voulvoulis, (2005) it was found half of respondents (400) did not finish prescriptions.
    a. Should healthcare professionals take more responsibility over non compliance of medicines and take steps to monitor this?
    b. Should health care professionals make people aware of correct disposal of unused medicines when prescribing?
    c. Should dispensing pharmacies make people aware of correct disposal of unused medicines?

Ecotoxicity testing

8. Do you think the current guidelines for the ecotoxicity testing of human pharmaceuticals are sufficient to protect ecosystems?

9. What would be the obstacles for pharmaceutical companies in sharing information about human and animal toxicity results obtained during drug discovery and development to aid and direct ecotoxicity tests for environmental risk assessment? For example read across data?

10. What do you think about the potential of computer generated packages to predict ecotoxicity? For example QSAR packages.

Supplementary questions

11. Does the trigger limit need more scientific evidence including chronic ecotoxicity data to support its level?

12. Do you think PECS are an appropriate tool for environmental risk assessment

13. ERA is only required for new medicines. Do you think we need to conduct retrospective environmental risk assessments? If so who should be responsible for this? Which pharmaceuticals and why?

14. Do you believe there sufficient scientific uncertainty of environmental risk of human pharmaceuticals to invoke the precautionary principle?

15. Pharmaceutical metabolites can also be toxic. Should pharmaceutical companies be required to identify the metabolites of new pharmaceuticals?

16. Should there be incentives for pharmaceutical companies to design green pharmaceuticals?

3.4 Results and Discussion

The results of the interview analysis provided an interesting insight into the different views of people involved in the regulation and control of the release of pharmaceuticals into the environment. The areas covered are the current ERA, its successes, limitations and ideas for improvement, the 10 ngl^{-1} action limit, mitigation of risks including STP upgrades, take back schemes and green pharmacy.

3.4.1 Current EU environmental risk assessment

The interviewees identified several shortfalls with the current environmental risk assessment of human pharmaceuticals and made suggestions for improvements that could be made to better protect ecosystems. Criticisms of the current ERA and suggestions for improvements that could be made fell into two main categories, effects and exposure.

3.4.1.1 Ecotoxicological effects

Positive aspects of the effects assessment were related to the development of a risk assessment for pharmaceuticals and the fact that in 2006 the guidelines shifted from a focus on acute effects to chronic effects of pharmaceuticals including a full lifecycle assessment on *Daphnia* and algae and a sub chronic, partial lifecycle effects assessment on one fish (EMEA, 2006). This change better reflects the exposure route of pharmaceuticals (Section 2). One academic interviewee said:

> *"The effects data side is good; we have over a decade of effects data and so can do fairly good species distribution effects."*

One EA interviewee commented:

> *"The progression of the risk assessment has been quite good especially since historically environmental data on pharmaceutical compounds was limited."*

In regard to the current set of ecotoxicological tests required for an ERA, opinions were very mixed. The water company interviewee thought that the current ecotoxicity tests were sufficient and stated:

"That's a reasonable spread of tests, how many companies do you want to put out of business by doing long term chronic mutigenerational tests at varying concentrations."

Financial burden is of course a very important consideration when performing ecotoxicity tests. It is obviously not possible to test all pharmaceuticals on all species including all possible end points because chronic tests are time consuming and too expensive to conduct on a compound by compound basis. Another academic interviewee concurred by saying:

"To be honest it's quite nice to have new drugs developed for our health."

Although a different academic interviewee thought that:

"The cost is a tad small compared to the overall cost of developing a new pharmaceutical."

The respondent from the environmental consultancy agency stated:

"Yes – as long as assessors can request further studies beyond fish, algae and daphnids if there is evidence for a mode of action that might not be covered by these taxonomic groups. This should not be seen as carte blanche for the more bonkers regulators from certain Member States to feel that they can ask for lots of unnecessary testing on weird critters!"

3.4.1.1.1 Intelligent ecotoxicity testing

Regulators have to take a pragmatic approach; a standard suite of tests that are relatively cheap and can be done by standard ecotoxicology companies is required for an ERA. However, seven interviewees voiced negative opinions about with the current effects testing regime. Six of these interviewees raised one of the key criticisms with the current ecotoxicology tests. Despite the move toward chronic ecotoxicology tests, the tests were still very traditional and standardised for all chemicals. Pharmaceuticals are designed to have a specific MoA and that this needed to be reflected in the ecotoxicology tests. It was thought by three academics, the pharmaceutical company interviewee, and the EA interviewees that the idea of intelligent testing or targeted ecotoxicology testing should be developed. This should incorporate the use of chronic test end

points that reflect the MoA of the drug and any known side effects. Four respondents thought there was an opportunity to make better use of mammalian toxicology data. One academic interviewee said:

> *"The specific mode of action might mean they pose a greater risk than standard tests reveal. There is a risk of underestimates of hazard."*

Another academic interviewee commented:

> *"Intelligent ecotoxicity incorporating mode of action in the human might be relevant to the wider environment and then testing more appropriately with that information. Rather than testing a drug that targets depression on algae."*

A third academic interviewee thought that:

> *"Chronic and sub lethal effects assays should be focussed on the known side effects of those drugs, and known mode of action…for example the chronic growth test isn't particularly helpful because growth isn't a particular end point for pharmaceuticals."*

One EA interviewee said:

> *"The concern is the unwitting mode of action effects that might arise, that you don't pick up from the rather narrow array of ecotox tests. It's a blunt instrument."*

The choice of ecotoxicological test end points can change the no effect concentration (NOEC) substantially. It is likely that short term growth and reproduction responses are not related to target-mediated responses, which may be observed at concentrations that are orders of magnitude lower than the standardized chronic toxicological benchmark concentrations (Boxall & Greenwood 2010). Stanley *et al*., (2007) and Valenti *et al*., (2009) reported lower effect concentrations for feeding behaviour of fish, a response related to the mammalian therapeutic MoA of the antidepressants fluoxetine and sertraline respectively, than the effect concentration for growth (Berniger & Brooks, 2010).

The responses of the interviewees in respect to a more intelligent chronic test strategy related to the MoA of the drug supports the validity of the work carried out in Section 4. The MoA of pharmaceuticals as a potential guide for choice of chronic test end points is clearly desirable and would better reflect the effects that may be occurring on non target organisms after pharmaceutical exposure. Bioinformatics and molecular docking could provide a useful tool to aid the choice of chronic test end points in ecotoxicity tests (Section 4). A recent study on the effects of the selective serotonin reuptake inhibitor (SSRI) anti-depressant sertraline on fish found a combination of a serotonin receptor binding assay and observations of behaviour to be a useful ecotoxicity strategy. It was found that a decrease in the binding of serotonin to the serotonin receptor was linked with shelter seeking behaviour of fish after sertraline exposure (Valenti *et al*., 2012). This study highlights the validity of using bioinformatics information such as that carried out in Section 4 to make predictions about pharmaceutical effects based on conservation of drug target sequence homology.

3.4.1.1.2 Laboratory versus field

Another point that was made about the limitations of current ecotoxicological tests was that tests are carried out in the laboratory and may not reflect the real life situation. One academic interviewee thought:

> *"A general criticism of ecotox is that it's carried out in laboratories and they don't look at what the real effect might be in the field. Might be lesser or greater effect depending on how the other environmental variables effect how the animal is affected. Combinations of stressors, mixtures and physical stressors"*

Many environmental stressors occur in the real environment which are not replicated in the laboratory such as: combinations of mixtures of pharmaceuticals and/or other compounds, fluctuating concentrations (Section 2), changes in bioavailability, environmental degradation or sorption to sediments, natural changes in environmental conditions, effects on other organisms which can alter ecosystem dynamics (e.g. predators, competitors and organisms that are food sources) and anthropogenic stressors such as the presence of other pollutants. Current ecotoxicology tests do not reflect all the routes of exposure and in reality there is a general lack of data on routes of exposure including trophic magnification and bioconcentration. The near

extinction of vultures caused by the veterinary drug diclofenac highlighted evidence of this concern. The guidelines for ERA (EMEA/CHMP/SWP/4447/00) state that an assessment factor (AF) of 10 should be applied to the PNEC to account for laboratory to field impact extrapolation. Currently there is a lack of evidence in the literature that this AF is set at an appropriate level to account for differences in laboratory to field effect concentrations for pharmaceuticals and highlights a potential research need.

3.4.1.1.3 Species selection

Three respondents mentioned a need for an increase in the numbers of species that are used in ecotoxicity tests. Molluscs were highlighted as a species that should be included on more than one occasion. The DEFRA interviewee said:

> *"We do need to increase the numbers of species across the board. We haven't got any tests for molluscs and we know that they are susceptible to compounds. We are trying to develop them but it takes time. There's huge groups of which we have no idea."*

The pharmaceutical company interviewee mentioned that:

> *"Some mollusc species seem to be more sensitive than some of the other species. I know that's true for propranolol and fluoxetine."*

Molluscs and mussels have been highlighted as a species that should be covered by ecotoxicity testing of chemicals and pharmaceuticals but which are not currently (Rittschof & McClellan-Green, 2005). Several studies have found that molluscs may be sensitive to propranolol and fluoxetine (Lazzara *et al*., 2012; Bringolf *et al*., 2010; Ericson *et al*., 2010). Conservation of drug targets in molluscs may also lead to similar a MoA occurring in these organisms (Section 4). Clearly molluscs should be considered as an important addition to ecotoxicity tests.

Selection of sensitive test species is important for ecotoxicity studies. At present the test species selection is quite narrow and may not reflect the species that are likely to be exposed. Bioinformatics and molecular docking techniques may be a useful tool to identify species that have conserved drug targets that could be used in MoA directed chronic tests (Section 4).

3.4.1.1.4 QSARs

A number of new approaches are being developed using quality structure activity relationships (QSARs) for chemicals. These bioinformatics computer packages can help make predictions about toxicity, persistence, biodegradation and lipophilicity of organic compounds (Jager & Kooijman, 2009; Mekenyen *et al*., 2005). They provide structural alerts using non test data, existing databases and assignments of modes of actions for compounds (Jager & Kooijman, 2009; Clark *et al*., 2010). The general consensus from all interviewees was that present computer generated packages for predictions of toxicity were poor. Three academic interviewees commented that:

> *"To date they have failed abysmally, impossible to think of an example where a QSAR has predicted something we didn't know previously."*

> *"It's an obvious one to look at but the messages I've had about their effectiveness have been very mixed."*

> *"I think they are getting better. QSARs are predicting chemical properties base on structures and whether something would degrade quickly or whether it would adsorb, sediment phase. Toxicity is trickier, I wouldn't trust it."*

One of the main drawbacks for using current QSARs to predict toxicity is that they predict acute toxic effects and this is not appropriate for human pharmaceuticals (Carlsson *et al*, 2006; Fent *et al.*, 2006). One EA interviewee said:

> *"We tried that and it was probably a bit dodgy. We did that in the prioritisation work five or six years ago. They tend to focus on acute effects and that's not the issue here. Also they only really tell you with confidence something about baseline toxicity and we are dealing with specific effects."*

Although they have been shown as satisfactory to predict acute toxicity (Torapova *et al*., 2012), QSARs have been shown to be inaccurate tools for predicting chronic toxicity or acute chronic toxicity ratios (Dom *et al*., 2012). Seven of the interviewee's were sceptical about QSARs at present but were optimistic about their potential in the future. Six interviewees hoped that they

could become a useful tool for predicting persistence, bioaccumulation and toxicity (PBT) or providing guidance for ecotoxicity testing. The government interviewee thought that:

> *"A lot of potential but they need to be used carefully though. They can give you an indication of likely toxicological impacts. Still have to go out and do the science after. A good basis for starting. I see them as plugged into an intelligent ecotox strategy."*

The water company interviewee asked:

> *"Have we got enough experience to have faith in what they are telling us? Another screen….another tool in the box."*

One academic interviewee expressed the view that:

> *"I think that all of the computer generated packages have something to contribute. No single test or computer model will give all the information. Part to play especially in the context of new substances, can't test all of them, on all organisms. Computer modelling has a role to play in establishing a prioritisation, sometimes you miss things."*

The pharmaceutical company interviewee believed that:

> *"In the short to medium term they've got a long way to go. In the longer term I think they've got a lot to offer. For ecotox tools like genomic tools, looking at pathways for gene expression, protein expression…they might point you to the type of definitive studies that you need to do and the types of endpoints…As you are sequencing more data from a larger range of species, the likelihood that if you know the mode of action in man, what the end point, what the gene target is, you can see whether that target exists in the wider wildlife and the most appropriate species. Although I don't see us changing a lot of what we do, we may supplement it with more intelligent end points to direct the types of studies we are doing and maybe encompass a range of different species."*

Another academic interviewee thought that:

"They are potentially very good as we got them from the medical community. If there was a group of compounds you could use QSARS its pharmaceuticals."

The overall impression was that although the environmental community like the idea of QSARs, the reality is that at present QSARs are not capable of predicting PBT. Biodegradation systems using QSAR systems may be flawed because they do not mimic real conditions (Greenwood *et al*., 2010). A recent study on using QSARs for predicting PBT within REACH (Registration, Evaluation and Authorisation of Chemical Substances) legislation found that widely used QSAR databases might have some errors (Zachary & Greenway, 2009). The KNAPPE Workshop, (2008) also concluded that current QSAR approaches are probably inappropriate for use on pharmaceuticals (Boxhall & Greenwood, 2010). Lienert *et al*., (2007) found there were limitations with QSAR modelling for hydrophilic and ionisable drugs.

It can be seen from the views expressed by the interviewees that they hoped QSARs could become a useful tool for risk assessment but could not replace traditional ecotoxicity tests. The comments from the pharmaceutical interviewee were supportive of the bioinformatics work carried out in Section 4. Bioinformatics and molecular docking packages such as AutoDock could provide a tool to guide the selection of sensitive organisms and MoA related end points for toxicity tests. Although QSARs still need substantial development and validation to be used as an accurate predictor for toxicity, it is clear from the comments from interviewees that these tools are desirable. The potential exists to use bioinformatics to supplement ecotoxicity tests.

3.4.1.2 Exposure

Five interviewees thought the exposure data side of the ERA needed to be improved. One reason for this was the lack of data for degradation in the environment, degradation during sewage treatment and human metabolism. The pharmaceutical company interviewee commented:

"The current exposure model does not account for ionisable compounds or compounds that have a charge that may shift depending on pH or ionic strength. Chemicals with a polar charge or low log K_{ow} (octanol water coefficient) may not fit the exposure model."

The same interviewee also believed that:

"Bio concentration estimates based on log K_{ow} may be inappropriate for some drugs, so we could be overestimating or underestimating the bio concentration potential. Some of the pharmaceutical industry is starting to look at this."

The octanol water coefficient log K_{ow} is used as an indicator for adsorption and bioconcentration. In fact it appears that log K_{ow} is a poor descriptor of environmental behaviour for either sorption to sediments, sewage sludge or bioaccumulation. This is because the majority of pharmaceuticals are polar ionisable compounds (Besse & Garric, 2010). For example ciprofloxacin has a reported log Kow of -1.74 (Brooks *et al*., 2003) and hence would be assumed to adsorb to sludge very poorly and be disregarded for PBT assessment in the ERA guidelines as it is well below the 4.5 threshold limit. However, ciprofloxacin adsorbs strongly to sewage sludge (Golet *et al*., 2002). A further issue with the use of log K_{ow} for these assessments is the high variation in reported figures in the literature. For example a log K_{ow} as low as 1.57 (Brooks *et al*., 2003) and as high as 4.6 (Oaks *et al*., 2010) has been reported for fluoxetine. It is thought that pH corrected log D_{ow} may be a better indicator for sewage sludge adsorption (Kah & Brown, 2008). It has also been found that the liposome/water distribution ratio ($D_{lip\text{-}water}$) may be a more useful descriptor for predicting bioaccumulation and toxicity as there is very little ionic strength dependence for the partitioning of compounds across liposome membranes (Nakamura *et al*., 2008). The problem with using K_{ow} for estimating removal of pharmaceuticals during sewage treatment are apparent in models such as SimpleTreat, which is the model recommended in the EMEA guidance for the ERA of pharmaceuticals to estimate removal during sewage treatment. These models are not able to handle ionised compounds or characterize biodegradation rates accurately (Seth *et al*., 2008).

Another problem with predicting exposure that was highlighted by interviewees was that the current ERA assumes there is no metabolism in the patient in the initial PEC calculation. However, a number of medicines are highly metabolised. The pharmaceutical company interviewee said:

"One or two drugs we know will degrade to something else within the STP, and some drugs are hydrolytically unstable and fall apart quite quickly in STPs, therefore the active ingredient is not always the compound we should be testing."

This comment covers two important points, one is that the lack of inclusion of metabolism data means that the exposure models will overestimate concentrations of pharmaceuticals in the environment and therefore produce a worst case scenario which is perhaps highly precautionary. However, the other point is more of a worry in regard to protecting the environment in that the ERA does not adequately consider situations where not only the parent compound will enter rivers and streams but their breakdown products will also enter water bodies. The impact and fate of these metabolites is not known and should be examined.

3.4.1.3 Retrospective risk assessment

Currently the EMEA guidelines for environmental risk assessment of pharmaceuticals only apply to the registration of new medicines. Retrospective risk assessment for pharmaceuticals is not currently required. However, the scientific literature contains several ERAs for pharmaceuticals that were licensed prior to 2006 which may pose a potential risk to the environment. Some examples are: mefenamic acid (anti inflammatory) (Tauxe-Wuersch *et al*., 2005), ibuprofen, paracetamol and acetylsalicyclic acid (Stuer-Lauridsen *et al*., 2000), propranolol (Miege *et al*., 2006) ciprofloxacin, diclofenac (Grung *et al*., 2008) and fluoxetine (Oaks *et al*., 2010). The findings of these studies are not, however, fed into any regulatory procedure. This issue was touched upon during the interviews. One academic commented:

> *"We are not good in the UK with dealing with the findings. We need to ensure follow through to action."*

In 2003 the EA produced a report on pharmaceuticals in the environment (EA, 2003). This report was essentially a retrospective ERA for pharmaceuticals starting with a priority system, however, a lack of effects data and scarcity of methods for measuring the chemicals identified as priority led to a considerable amount of uncertainty about the risks in the report. One EA interviewee said:

> *"We had our fingers burnt here, we are happy to do it but the problem came when we got into discussions with the pharmaceuticals industry, they weren't keen on picking up the challenge. It wasn't helped by the fact that we had to make so many assumptions about the risks."*

Eight interviewees were in favour of retrospective ERA of pharmaceuticals. It was thought that a system for prioritisation was the key to this issue because of the high numbers of pharmaceuticals involved. Besse & Garric, (2010) stated that prioritization of pharmaceuticals is necessary due to the high numbers of pharmaceuticals hindering the possibility of assessing the ecotoxicity of every compound. There is a need to accurately assess exposure and the environmental effects. The consultant interviewee commented:

> *"Yes, there should be an ERA during reauthorisation of older medicines, paid for by the authorisation holder. There could be an appropriate sales volume cut off for this so that minor but useful medicines are not removed from the market."*

One academic interviewee expressed the view that:

> *"It's sensible to know what the environmental risk is with all the drugs we use today…I would like to see that"*

Another academic interviewee highlighted the fact that:

> *"The difficulty is how to rank the pharmaceuticals"*

The pharmaceutical company interviewee believed that:

> *"For existing products I think some prioritisation is needed, there are some data gaps that need to be filled."*

It is apparent from these findings that retrospective ERA for pharmaceuticals is a failing of the current regulations. The introduction of a prioritization scheme is an important starting point for implementation of any retrospective analysis. One example put forward for prioritisation is that of Sweden which is based on biodegradability, potential for bioaccumulation and toxicity. It is based on both hazard and associated risk (Agerstrand & Ruden, 2009). Many other suggestions by other authors are being proposed for prioritisation systems (Besse & Garric, 2008; Kumar & Xagoraraki, 2010; Roos *et al*., 2012; Cooper *et al*., 2008).

The Water Framework Directive 2000/60/EC (WFD) may be a mechanism by which retrospective risk assessment and environmental monitoring of priority pharmaceuticals may be achieved (von der Ohe *et al*., 2011). WFD requires "good chemical status" of water bodies in the EU by 2015. WFD contains a defined list of priority substances that can adversely affect the ecosystem. This list does not contain any human pharmaceuticals at present, however, the list is to be reviewed every four years. WFD put emphasis on the precautionary principle stating that especially in identifying priority hazardous substances, any potential adverse effects of the product should be taken into account and should lead to scientific assessment of the risk (recital 11 & 44, WFD). Inclusion on the priority list is governed by toxic, persistent and bio accumulative characteristics. On this basis three pharmaceuticals have been suggested for inclusion by the German Environment Agency; carbamazepine, diclofenac and ibuprofen (Kampa *et al*., 2010). However, in a recent press release it was stated that EE2 and diclofenac are to be added to the next updated list (European Commission, 2012).

3.4.1.4 Trigger limits

Six interviewees did not agree with the 10 ngl^{-1} trigger limit. The pharmaceutical interviewee commented:

> *"For the most part I can live with it. I have some issues around the market penetration factor…for most drugs; one percent is extremely over protective for other compounds it's extremely under protective."*

There were several reasons given for disagreeing with either the trigger limits level or having a set limit at all. The main reason given was that EE2 has a detrimental effect on aquatic organisms at concentrations below 10 ngl^{-1}. In fact five interviewees, (3 academics, the government and the water company interviewees) mentioned this point. Although the ERA has a caveat for endocrine disrupting substances that mean they must conduct the second phase of the ecotoxicological tests, it was thought that the limit was insufficiently precautionary because EE2 has an effect at 0.3 ngl^{-1} and could kill a fish at 10 ngl^{-1} (Caldwell *et al*., 2008). If the requirement for an ERA had been in existence when EE2 was first marketed it would not have been picked up as a potential problem because concentrations of EE2 would never reach 10 ngl^{-1} in surface waters simply because of its high potency and the low tonnage marketed. The fact that

ERA does exist now presents an opportunity to learn important lessons from past mistakes by not discounting compounds which may act at low concentrations but are not endocrine disrupting substances.

Analytical chemistry techniques may not be reliable or sensitive enough to measure compounds as low as either 10 ngl^{-1}or more importantly at concentrations that can cause ecotoxicological effects i.e. analytical chemistry detection limits are too high for some compounds (Johnson *et al*., 2008). One academic interviewee believed that:

> *"It should be a trigger limit that's based on the ability to detect the material."*

Another academic interviewee agreed:

> *"There are some pharmaceuticals that are actually quite difficult to measure at ten nanograms per litre."*

The water company interviewee concurred:

> *"I suspect that for a lot of these compounds for analytical chemistry to get down to ten nanograms is rather optimistic"*

In fact there are several problems with analytical chemistry techniques that have been highlighted in the literature. The fact that techniques cannot measure as low as 10 ngl^{-1} is one. Analytical chemistry measurements for pharmaceutical compounds can be unreliable and may be inaccurate at very low levels or in difficult matrixes such as sewage. For example, results of pharmaceutical measurements in the literature have been discredited in the case of EE2 (Johnson *et al*., 2008). An inter-laboratory exercise showed that measurements of organic contaminants can differ by two orders of magnitude despite state of the art equipment (Van Leeuwen *et al*., 2006). In regard to these issues with the capabilities of analytical chemistry, one academic interviewee thought:

"One big caveat to threshold values if we are using them if we are using them for enforcement or some way as part of the regulatory process it has to be something we can measure."

Two academics disagreed with having a trigger value at all. One believed that:

"No regulation based on an arbitrary number for any chemical whatsoever is scientifically sustainable."

The other academic agreed:

"I don't think you should have a single number for any trigger because…ethinylestradiol affects fish at 1 ngl^{-1} or even less. It's always dangerous to have a single trigger value."

Incorporating the MoA of each pharmaceutical in the ERA was a common thread among many of the interviewees (see above). Another recommendation by an academic interviewee was:

"The trigger limit should bet set at a level that incorporates data on the bioavailability and blood or plasma circulating concentrations of the pharmaceuticals in organisms."

This could well be considered if pharmaceuticals were examined on a case by case basis and should incorporate data from non mammalian trials for blood circulation levels. This type of approach could help prevent unnecessary animal tests for ecotoxicological purposes. Despite these criticisms the water company interviewee commented:

"You can always criticise but you have to start somewhere"

The government interviewee expressed the view that:

"Trigger limits are useful"

and the pharmaceutical company interviewee thought that:

"It's fair to have a trigger limit but people need to be aware of the short comings."

It appears that trigger limits are intrinsically flawed. It would seem a more intelligent approach is to establish what the effect concentration is first by conducting some ecotoxicological tests. These tests should be conducted on a compound by compound basis, incorporate the MoA of the drug and potentially mammalian toxicology data. The other crucial aspect of setting exposure limits for pharmaceutical relates to analytical chemistry techniques. If methods are not sensitive enough to measure concentrations below the effect concentration then potentially other techniques for estimating environmental concentrations need to be employed as well.

3.4.1.5 Predicted environmental concentrations (PECs), measuring, modelling and monitoring

There are several issues with the current PEC calculation used in ERAs (Section 2). The problems are the default values it contains i.e. the default market penetration factor (Fpen) of 1%, the default dilution factor of 10, the default figure for waste water produced per person per day (200L), the assumptions that it makes i.e. an even distribution across the geographical area, even consumption over a whole year, the maximum daily dose is taken by every user and the refinements that can be made for metabolism or sewage treatment removal. Many of these problems were mentioned by the interviewee's. The water company interviewee believed that PECs:

"Require some pretty heroic assumptions."

The government interviewee concurred:

"They have to be taken with a good dose of scientific salt"

The pharmaceutical company interviewee expressed a similar view:

"It's a bit of an art as much as a science"

The pharmaceutical interviewee also highlighted that the 1% default market penetration factor was often either an underestimate or overestimate of what actual sales would be (for quote see section 3.4.2). The pharmaceutical company interviewee then said:

> *"My problem comes in a little bit more when a drug will go off patent, at that point there is an assumption made by the regulators that those generics will move in to that sector and that they will just take up that market share that the branded company had and that's not always the case because the price comes down and the market can grow"*

This means that a PEC or MEC could increase some years after the ERA was conducted and a new drug was licensed. Therefore, it would seem reasonable to require companies which market generics to also conduct an ERA once a patent has expired. The pharmaceutical company interviewee also had an issue with the maximum daily dose used in the PEC calculation.

> *"The maximum daily dose is an assumed daily dose whereas some therapies you might take intermittently or for a short period until you are better."*

This may seem somewhat over protective for some pharmaceuticals, however, the ERA has to reflect a worst case scenario and once you incorporate factors relating to differences in regimes of therapy you could incorporate more uncertainty into the PEC.

Other criticisms of the PEC calculation that echo the findings from Section 2, were that the dilution factor of ten is often not precautionary enough for some water bodies especially during dry seasons. Some drugs are likely to have an uneven distribution across the UK landscape due to factors such as increased use of some drugs in more elderly populated areas and also near hospitals or nursing homes. Towns with a high population elderly people can discharge considerably more compounds such as anti-inflammatory drugs (Ahonen *et al*., 1992). These factors could lead to regional hot spots. The pharmaceutical interviewee commented:

> *"In the UK we can get a grip on what the total tonnages or total kg per medicine that are prescribed but we don't know the geographical distribution between different healthcare trusts and different areas. This can result in uneven distribution across the UK landscape. So you can get regional hot spots."*

An EA interviewee thought:

> *"It involves a lot of assumptions in terms of how much is used. How much of the market things are going to take up……things that have been picked up previously are in terms of hot spots."*

The water company interviewee said:

> *"If you are working from usage, you are making a lot of assumptions about how much is broken down even before it gets to sewage treatment works, what the breakdown is through the works, what different breakdown you get through different treatment processes, and then what dilution you've got."*

An academic expressed the view that:

> *"Many areas or seasons may have a dilution factor a lot less than 10"*

With regards to initial PEC refinements, the interviewee from the pharmaceutical company commented that:

> *"We rarely refine our PECs and tend to leave them as the crude initial worst case figure for water." This was partly because drugs rarely pass the ready biodegradation study and therefore a default rate constant for sewage treatment removal cannot be calculated to refine a PEC and the water sediment study fails to predict fate in a river."*

It was interesting to note that this interviewee also mentioned that:

> *"For all our products at the moment we don't have a risk quotient greater than 1 and we only have one that is greater than 0.1."*

This means that no refinements are required as all the compounds pass the ERA. Despite the criticisms on PECs there were several positive comments. Four interviewees felt that PECs were

a necessary part of risk analysis that allows us to make a decision. Academic interviewees believed:

> *"They are just an estimate; they give you a 'ball park figure'."*

> *"Risk assessment requires an estimate of exposure and that at present there is a lack of an alternative."*

> *"PEC estimates are usually rather poor, but that's fine so long as they err in a conservative direction during early tiers of a risk assessment."*

The consultancy interviewee said:

> *"Yes – I don't know what else you can use. Surely the question should be about the way in which the PECs are derived. They need to be quick, cheap and highly conservative at lower tiers and more realistic (therefore much more expensive) at higher tiers in a risk assessment framework."*

3.4.1.5.1 Exposure modelling

Exposure modelling packages have the potential to reduce some of the uncertainties that the assumptions in the PEC calculations (dilution factors and even geographical usage) and deficiencies with analytical chemistry techniques create. Several exposure modelling packages have been developed to predict environmental concentrations of organic compounds. These include European Union for Evaluation of Substances (EUSES), Geography-referenced Regional Exposure Assessment Tool for European Rivers (GREAT-ER) and Pharmaceutical Assessment and Transport Evaluation (*Ph*ATE). Overall interviewee's views on exposure modelling packages were quite positive. Four academic interviewees commented:

> *"Well, I think if it's done properly, it's excellent and possibly almost better than measurements."*

> *"PECs are more reliable than measured concentrations."*

"I am confident that concentrations they predict are a reasonable starting point."

"If the models are used appropriately they provide very useful information."

One of the advantages with exposure modelling packages was that you can change the parameters of the model. For example you can change the flow of the water body, temperature and pH. Another advantage is that the models provide a range of concentrations, so that you can find out the difference in low or high flow situations. A single measurement at one time, on one particular day, at one location doesn't provide as much information. It is important to be able to put measurements into context. It was also thought that exposure models could overcome some of the difficulties with the reliability and accuracy of analytical chemistry especially when measuring at such low concentrations and in difficult matrices such as sewage. Finally they are quick to run and cheap to do. Problems with the use of these packages included: possibility of overestimates, some uncertainties with knowledge about fate and behaviour, choosing the right model and an understanding of the models limitations. The consultancy interviewee commented:

"Packages such as GREAT-ER are fine, as long as they are not used as "black boxes" with little chance of understanding how input data are converted into pretty-looking outputs."

Geo-referenced point source water quality models such as *Ph*ATE and GREAT-ER incorporate factors such as geography, hydrology and dilution and complex information such as sewage discharge and abstraction points and annual low and high river flow information (Cunningham *et al*., 2011; Price *et al*., 2009). The European Union System for the Evaluation of Substances (EUSES) is a multimedia model that can predict the fate of organic chemicals in water, air, soil and sediment and is used in chemical risk assessment in the EU (Schwartz *et al*., 1998). The greatest value of such models is the ability to compare the fate and partitioning of chemicals to one another, but they are much less suitable for predicting real world surface water concentrations (Johnson *et al*., 2008). A small scale targeted monitoring study should always be used to corroborate a models prediction (Johnson *et al*., 2008).

Without detailed knowledge of fate and behaviour, PECs will always be an estimate. Models such as GREAT-ER and *Ph*ATE hold much promise for offering a more detailed and informative predicted range of environmental concentrations of pharmaceuticals and could assist with some of the limitations associated with analytical chemistry techniques. However, they need to be used with some caution. The answers that they provide are only as reliable as the data that is fed into the model. Therefore some environmental measurements will still need to be taken and some monitoring may need to be performed. The most sensible solution appears to be a combined approach incorporating some modelling and some measurements. Although it seems that a definitive answer on exposure cannot always be reached, the most important feature of PECs must be that they are precautionary and represent a worst case scenario. The findings from Section 2 indicate that this is not currently always the case which is unsurprising considering the assumptions that the PEC calculation makes.

3.4.1.5.2 Monitoring

There were several valid points made that highlighted the problems with monitoring of pharmaceuticals. There was a general feeling that monitoring for its own sake was a waste of time and that some indication of an adverse impact needed to be evident in order to warrant a monitoring campaign. The consultancy interviewee stated:

> *"I see little point in monitoring for its own sake. There would need to be a good reason to require monitoring of effluents or receiving waters, such as unexplained adverse effects below a discharge."*

The water company interviewee asked:

> *"How much effort are you going to throw into monitoring before you think you have an effect? If there no effect then what's the problem?"*

There are a lot of pharmaceuticals on the market and it would be clearly unfeasible and probably unnecessary to monitor all of them. Monitoring any pollutant is very expensive so it is important to establish that a compound does in fact pose a risk before starting a monitoring campaign. The most sensible way is probably to prioritise or rank pharmaceuticals to establish

which may warrant monitoring. It is essential to identify at as an early stage as possible those compounds that are of the highest priority so that monitoring can be focused and not wasted on products that do not pose a risk (Greenwood *et al*., 2010).One academic expressed the view that:

> *"You really have to target which compounds you are most concerned with. The compounds I would have the greatest curiosity knowing what's out there and in what concentrations is the groups of cancer chemotherapy, cytotoxic drugs and anything to do with hormonally acting drugs."*

Once it is established that monitoring of a certain compound is required, the second problem is deciding where the measurements should be taken. A concentration of a pharmaceutical in a water body in one location doesn't tell you what concentrations will be in other water bodies or for that matter what concentrations are likely to be further up or downstream. Differences in hydrology, degradation, and particulate binding also need to be considered. The findings in the data analysis performed in this work (see Section 2) highlighted these problems. Many researchers do not examine different locations when testing for pharmaceuticals and may only take a single grab sample at sewage outfall. This gives you limited information about the actual exposure situation in the bulk of the water body. Potentially the use of modelling software packages could be used in conjunction with a monitoring campaign. Although modelling needs validating with some actual measurements, modelling can be a useful tool to tell you where to measure, and help develop a monitoring strategy. A combination of measuring and modelling would provide the most robust approach to the risk assessment of micro contaminants in the freshwater environment and scientists in both disciplines should cooperate more closely to achieve this (Johnson *et al*., 2008). If diclofenac and EE2 are added to the list of priority pollutants as part of WFD, monitoring of these compounds will become a reality. One academic said:

> *"There needs to be a balance between monitoring strategy and some link with a modelling prediction of where to look."*

The next problem is choosing an analytical chemistry technique that is reliable and robust enough to measure concentrations especially when they are likely to be very low, as in the case of EE2. One academic interviewee thought that:

"Analytical chemistry has some limitations that have to be recognised."

Important factors mentioned by interviewees when considering monitoring of pharmaceuticals were that it is difficult, very expensive and time consuming. The methods can be quite difficult to develop and perform and the subsequent data analysis requires some expertise. Another academic commented:

"Its expensive, analysis is difficult and the results are probably unreliable."

A lot of measurements are required for robust results that could be used in an ERA. A third academic interviewee said:

"The techniques don't lend themselves to cheap rapid robust methods that you can use in a regulatory environment with high throughput."

And then suggested:

"What we can do is a total measurement of a particular type of compound, for example we had a total pesticide concentration in the water."

A total concentration measurement of similarly acting pharmaceuticals may be useful. This could also be applied when thinking about ranking pharmaceuticals for monitoring by environmental concern i.e. by treating similar compounds as a group (Greenwood *et al*., 2010). However, Kummerer, (2009) advises against this strategy because even small changes in chemical structure may have significant impacts on solubility, polarity, toxicity and MoA. Bioinformatics and molecular docking packages could be extremely valuable for this type of approach. The work carried out in Section 4 highlights the potential of molecular docking to establish whether different pharmaceuticals bind to the same target protein and thus act in a

similar way. If they do as in the case of ibuprofen and diclofenac then potentially a total concentration of these drugs could be more useful than individual amounts. It is important to also consider whether these compounds act in an additive or synergistic manner or in an antagonist manner. If drugs can cancel out or lower the effects of other drugs then perhaps a total concentration is not particularly helpful when considering what effects may be occurring on non target organisms.

Bioassays were mentioned as an alternative to doing chemical monitoring. One academic said:

> *"If we were to use a type of bioassay for example a vitellogenin response that would integrate some of the signals from multiple substances, both estrogenic substances, SSRIs and other substances to be more additive."*

Many substances have estrogenic properties like EE2. The effect concentrations and environmental concentrations are very low making it difficult for analytical chemistry measurements to be accurate (see above). The use of biomarkers may hold some promise for offering a more informative assessment of what effects a considerable mixture of pharmaceuticals may be having on aquatic ecosystems. One example of a potential biomarker is the vitellogenin response. Vitellogenin 1 and 3 are particularly expressed when male zebra fish are exposed to a concentrations of EE2 greater than 10 ngl^{-1} (Martyniuk *et al*., 2007). Induction of vitellogenin has also been demonstrated as an indicator of oestrogenic activity of mianserin (serotonergic antidepressant) in the aquatic environment (Van de Ven *et al*., 2006). Another potential biomarker is the cytochrome P450 which has an essential function in the metabolism of many pharmaceuticals. Expression levels of this can be used to indicate exposure to pharmaceuticals (Hong *et al*., 2007). The bioinformatics results in Section 4 indicate that cyclooxygenase enzymes would be inhibited in three species of fish (and potentially more) leading to reduced prostaglandin production. Experimental work has supported this finding (Mehinto *et al*., 2010). Bioinformatics may be valuable for the indication of suitable biomarkers. Bio assays such as ELISA (enzyme linked immunosorbent assays) offer specificity and accuracy at low concentrations and could be used to measure selected biomarkers. One proposal to help with development of techniques for bio monitoring was improving communication between pharmaceutical companies and ecotoxicologists and regulators. An academic interviewee said:

"The science community need to talk to the drug companies with regards to techniques that have been used in the drug development process that we could use for bio monitoring for example bioassays, antibody assays, ELISA and cell based assays. There's no need to reinvent the wheel. There needs to be a mechanism to release the protocol without releasing any confidential, commercial information that's in the dossier for that drug. These problems have been met before with workshops."

The pharmaceutical company interviewee said that they did not actively do any monitoring of pharmaceutical concentrations apart from at manufacturing facilities. However, they did compare their PECs to measured concentration data from the literature and would revise a PEC in the light of a higher MEC in order to be more precautionary. As an example of this the interviewee mentioned that recently they set a PEC for a generic drug using published MEC data because a substantial quantity of monitoring data was available for that product. This was interesting because although the other interviewees felt that monitoring was not warranted and potentially a waste of time and money, the pharmaceutical company were making use of data that was produced from monitoring campaigns.

One of the problems that the pharmaceutical company regularly encountered when using the peer reviewed literature to set PECs and PNECs was a lack of reliable data in many publications. This was problematic on the exposure and the effects side. The pharmaceutical company interviewee said:

"80-90% of data that is published we can't use for regulatory purposes."

This echoes the findings during this study in which the majority of publications in the peer reviewed literature lacked important information that would make exposure concentration data fit for analysis (see Section 2). The non governmental organisation, ECETOC (The European Centre for Ecotoxicology and Toxicology of Chemicals) is addressing these issues by providing a mechanism for scientists to come together and publish things around what a good, robust monitoring program might look like and encourages people to follow these guidelines (Kuster *et al*., 2009). The development of standards and guidelines for publication of environmental data is

of paramount importance if work carried out by the academic community is to be used for regulatory purposes.

The EA interviewees and the government interviewee thought that some monitoring was warranted and thought that a 2 year post market monitoring campaign was a good idea. The government respondent said:

> *"For new pharmaceuticals that are coming on stream and for those we know little about, I think there is a good case for monitoring those."*

One suggestion from the EA was that a condition to licence for post market monitoring could be imposed on pharmaceutical companies especially if the PEC and PNEC were quite close. This is often applied to other chemicals such as pesticides. This would seem like a sensible idea as currently once a pharmaceutical is licensed and released into the environment, risk assessment ends. In fact ERA needs to be a more ongoing process that incorporates the entire life cycle of the drug.

It appears that the most practical approach for monitoring of pharmaceuticals is to begin with a system of prioritisation to establish compounds of concern. This should include existing pharmaceuticals and new pharmaceuticals. When compounds that pose a potential risk are identified and it is established that they pose a risk to the environment, a monitoring campaign should be devised. It is at this point before any measurements are taken that a set of standards which make the monitoring campaign meaningful and sound needs to be developed. Modelling software could then be used in order to establish where to take measurements.

3.4.2 Mitigation

Although the EU and the US have devised ERAs for pharmaceuticals over the past decade, risk mitigation measures are still somewhat lacking. An academic expressed the view that:

> *"The requirement for environmental data or risk doesn't preclude their registration. New drugs even if they have an environmental issue wouldn't be turned down on that basis."ERA does not work because the requirement for environmental data does not*

preclude their registration meaning that new drugs wouldn't be turned down on an environmental basis."

This is one of the crucial deficiencies of the current ERA. Even if an environmental problem is identified this would not prevent the drug being licensed because it is for human medical use. Human health is of course the priority. The choice was often seen as a cost to the environment versus benefit to human health analysis. Another academic interviewee said:

"The main thing is, understanding what the concentrations will be, where, and making a societal judgement on it……benefit versus harm."

The consultancy respondent said:

"I would first exhaust all possible tiers in an ecological risk assessment to ensure that simpler assessments are not over-precautionary. If risks still remain, then a socioeconomic assessment should show what the costs to the environment might be versus benefits to human health. Depending on the balance between these one might wish to put restrictions on use."

The issue with any environmental harm versus human health benefit is what level of environmental damage should actually outweigh the benefit of human health? The government interviewee said:

"In the case of ethinylestradiol the societal benefits are very huge in terms of population control."

The pharmaceutical company interviewee commented that:

"The cost benefit and environmental benefit analysis is starting to come into the medicines application from either later this year or next year. The environmental consideration is coming in, it still won't block it, but it just means it's moving higher up."

This issue led to some discussions about what strategies could be put in place to reduce an ecotoxicological risk if identified without preventing human use of a drug. Mitigation suggestions included use of the drug in a targeted way, for example restricting use to hospitals, and then incorporating extra treatment stages for hospital sewage. This was mentioned by several interviewees. An academic asked:

> *"Can we use the drug in a targeted way, only in hospitals and we are going to put hospital waste through extra layers of treatment?"*

A suggestion by another academic was the potential of substitution of a pharmaceutical compound with a more environmentally friendly one:

> *"If say a particular beta blocker caused rotifers to swim upside down or commit suicide, could say well, there are ten other beta blockers on the market and there's no evidence to say that this one is better, so substitute."*

If there are several drugs that treat the same medical problem with similar results, the less environmentally problematic one could be chosen. This is the method that has been adopted in Sweden. The Swedish Association of Pharmacy Industries (LIF), Apoteket, the Swedish Association of Local Authorities and Regions and Stockholm County Council have developed a voluntary scheme to promote consideration of persistence, bioaccumulation, toxicity, environmental hazard and associated risks of pharmaceuticals. Committees establish recommendations for choice of drug for each clinical condition based on firstly medical aspects and secondly environmental classification. Although the system is voluntary it has been having a positive effect on both the procurement of bulk drugs in hospitals and on individual prescribers (Wennmalm & Gunnarsson, 2010; Clark *et al*., 2010). The Swedish system has gained considerable attention from other countries and there is a likelihood that the scheme will be rolled out across Europe in future (Clark *et al*., 2010).

One academic interviewee mentioned trying to reduce exposure at the drug delivery stage. This could be done by reducing the prescribed dose by increasing the half life of the drug in the body. If a serious environmental risk profile emerged the dose could be reduced to once a day. The pharmaceutical company interviewee expressed the view that:

"Intravenous treatment with drugs is much more effective and could reduce the amount required but is very unpopular with the public."

The pharmaceutical company interviewee also said:

"Labelling is the other alternative you've got…But we've always got that concern that if you were to put on the message that this drug could seriously affect the health of fishes you've got the risk that the patient wont take the medicine so that could impact on that person."

It appears that this may not however be the case. In Sweden pharmaceutical producers were initially concerned that some patients would not take medicines which might cause negative environmental impacts; however, no such effects have been observed (Wennmalm & Gunnarsson, 2010).

Generally ideas for mitigation are somewhat limited with adoption of the Swedish system for classification and substitution being the most practical approach. This is also a system that has been actually implemented and seems to be having some success. The questions then focussed on other mitigation strategies including upgrading of STPs and pharmaceutical take back schemes.

3.4.2.1 Improving sewage treatment

Upgrading STPs is likely to be one of the most straightforward and workable ways to reduce the input of medicines to receiving waters because sewage effluent is considered to be the main source. However, all respondents thought that it was not a good option because of the associated costs. It was highlighted that at present the evidence was not strong enough that pharmaceuticals in British rivers are adversely affecting wildlife. It was considered that the risk had to be tangible and not theoretical in order to take this action. It was thought that it was important to exhaust alternatives first, such as detailed ecological risk assessment. The reasons for STP upgrades being unpopular were the cost and environmental burden of increased carbon emissions. The water company interviewee believed that:

"The environmental costs of something like activated carbon are huge…..energy costs and carbon emissions are real issues for us."

At present sewage treatment is relatively cheap and quite efficient. More environmental impact may be produced than removed by advanced sewage treatment technologies (Wenzel *et al*., 2008). Respondents often highlighted the fact that removing the last trace amount of a compound is the most expensive option. The water company interviewee asked:

"For every order of magnitude you drop in detection limit the cost escalates 100 fold. On a good day with bolt on's you can get below 1 ngl^{-1} for ethinyl estradiol, but is it worth it?"

This is a valid point. The cost of extra treatment steps could be expensive financially and environmentally in terms of carbon emissions. If EE2 can adversely effects fish at concentrations as low as 0.3 ngl^{-1}(Caldwell *et al*., 2008), it may not be possible to reduce emissions to a level below this even with a substantial amount of extra sewage treatment. Another point that was raised by the pharmaceutical company interviewee was:

"I think sewage treatment; obviously connectivity has gone up over the years. In terms of standards discharges have always improved with time."

An academic interviewee considered:

"The advantage is that, (and loads of money is being spent already) is that it should deal with a wide range of organic chemicals and inorganic ones, phosphorous and nitrogen levels and anything you can imagine basically."

These are also valid points. There is already a substantial amount of money being spent on sewage treatment improvements in the UK. Thames Water embarked on a £675m project to improve its 5 STPs (www.thameswater.co.uk/cps/rde/xchg/corp/hs.xsl/10094.htm). Removal of organic compounds and inorganic substances such as nutrients, nitrogen and phosphorous is required to meet good ecological status in water bodies for WFD.

Suggestions for reducing the cost of STP upgrades if found to be necessary included: only applying further processes if the population age in the area was quite high and therefore had an increased usage of pharmaceuticals or if the population size was very large; rather than adding expensive tertiary treatments such as UV or charcoal you could extend the biological process itself, increasing residence times for instance and additional treatments could be switched off at certain times of year, i.e. when there was a greater dilution in the receiving water.

It was also mentioned that STP treatments can be substance specific, some compounds might pass through and not be suitable for treatment. Upgrading STPs, then, might not tackle substances of concern especially if that concern is related to their lack of degradability and persistence.

3.4.2.2 Take back schemes and pharmaecovigilance

One of the routes of entry of human pharmaceuticals into the aquatic environment is disposal down sinks and WC's. Although it is thought not to be the most important route it is one of the more easily remedied. In a survey by Bound & Voulvoulis, (2005) it was found half of respondents (400) did not finish prescriptions. The first point that was made in respect of take back schemes was that it would be useful to know what contribution disposal of unused medicines down the drain actually makes to the overall exposure situation. Two academics said:

> *"I don't think that we do definitively know that it's not the major route of exposure. There's no data in the literature. People assume inappropriate exposure is not a major contributor, and it may not be."*

> *"I would like to know more about what happens to unused pharmaceuticals, what is the size of the problem, I'd like to have data on that."*

When taking into consideration the fact that medicines disposed of in this way will not be degraded by metabolism, they could alter a PEC refined for excretion rates substantially. All interviewees felt that more should be done to address the issue because source control was a better and more easily remedied solution than end of pipe control. Respondents thought that both physicians and pharmacies should take more of an active role in communicating the importance

of correct disposal of unused medicines and remind people that they must be returned to the pharmacy. One academic interviewee said:

> *"Doctors and pharmacists should take considerably more responsibility, it's managed effectively in other countries and I don't know why we can't manage it in this country."*

The pharmaceutical company interviewee mentioned the importance of the media in this issue by saying that a highly popular TV soap opera had recently portrayed a character flushing their anti-depressants down the toilet. This sends the wrong message to the general public. The water company interviewee commented:

> *"We are generally running campaigns, bin it don't flush it."*

Take back schemes are an important way to decrease pollution of the aquatic environment of pharmaceuticals. The views of the interviewees indicate that this is not being currently achieved adequately. The media clearly has a part to play in reminding the public about returning medications to the pharmacy. Some countries run occasional newspaper adverts to remind people as well as ensuring that doctors and pharmacies return medications. Sporadic campaigns on top of permanent take back schemes, calling for consumers to return medicines can help raise awareness (Vidaurre *et al*., 2010). There is a clear research need here for more accurate data on the contribution that used medicines make to overall exposure.

Environmental stewardship is an important aspect of green pharmacy. A potential way to achieve this is through 'pharmaecovigilance' (Daughton & Ruhoy, 2011). This would see the current system of pharmacovigilance of drugs which monitors adverse medical effects after market authorisation being extended to include environmental issues. This includes factors such as prescription, disposal and take back schemes. Some of the factors that should be considered for pharmecovigillance were mentioned by interviewees. This included factors such as smaller initial prescriptions. One academic interviewee said:

> *"Packaging should be smaller. If you know twenty percent is not generally taken, reduce the size of the prescription, less wastage. It's in everyone's interests to save money."*

Three interviewees thought that doctors should take more responsibility with reminding the patients about returning unused medication but also to think carefully about what and how much of a drug they are prescribing. The pharmaceutical company interviewee gave an example:

> *"The first line of defence for high cholesterol within the UK is simvastatin, but it is known that a great number of people do not respond to this drug so are then prescribed atorvastatin (Lipitor). When this happens the remaining simvastatin is not requested back by the GP, too much is often prescribed originally as most things are prescribed on a 28 day cycle. A smaller initial prescription of simvastatin should be given initially to see if the patient responds."*

In a stakeholder engagement exercise in 2006, interviewees were uncertain about the feasibility of convincing doctors to reduce prescription rates when patients expect to be given a prescription when they visit the doctor and that the doctors are subject to considerable advertising pressure from the pharmaceutical industry to do so (Doerr-MacEwen & Haight, 2006). Correct disposal, i.e. incineration is an important issue with regards to unused medications. However, it was thought by some interviewees that pharmacies on the whole did not appreciate people returning unused medication because they had to pay for disposal. An EA interviewee believed that:

> *"The pharmacy will simply flush it down the drain anyway."*

One of the key points made was that by nature people are pretty lazy, forget and cannot be bothered to return unused drugs. One academic interviewee suggested:

> *"There are some very practical things we could do but don't. GP surgeries should have a return bin, every surgery."*

This would have two advantages in that it could be anonymous, therefore people could drop the unused medicines into the bin without having to admit to the GP that they hadn't taken or finished the prescription. The other advantage would be that people could do this whenever they

went to the doctors. When asking the water company interviewee what they thought of this idea they asked:

"What about supermarkets?"

This obviously has drawbacks in that someone would have to take responsibility for safe collection and disposal. However, most large supermarkets do have pharmacies, so if the return bin was at the pharmacy they could take responsibility. The real advantage to supermarkets is that people visit them regularly.

Take back schemes for unused or expired medicines are required by EU legislation, Directive 2004/27/EC. Reference to collection schemes should be made on the label or package leaflet. However the direction may simply be to ask the pharmacist about disposal and not give actual direction about how to dispose of the medication. In a survey of 28 European countries, only 30% could provide data on the performance of their take back schemes (Taylor & Poulmair, 2007). France and Sweden are considered to have the most comprehensive take back schemes. In France a take back scheme CYCLAMED has been in force since 1993, which collected, and redistributed unused medications to non government organisations (NGOs) for third world countries (Vidaurre *et al*., 2010). However, France encountered problems with fraudulent resale of medications. Since 2008 pharmacists must collect unused medicines, free of charge for incineration. Sweden also incinerates all the returned pharmaceuticals (Vidaurre *et al*., 2010).

The "Swedish model" 2005 is a collaboration of the Swedish Association of Pharmacy Industries (LIF), Swedish Medicinal Products Agency, Apoteket, the Swedish Association of Local Authorities and Regions and Stockholm County Council. It is the most comprehensive classification and labelling scheme within the EU, information on environmental impacts is made public on websites and in booklets (Vidaurre *et al*., 2010). The system is voluntary but has a high compliance with the expert group's recommendations informing doctors and patients. Sweden is unique from other countries in Europe in that until January 2010 all pharmacies belonged to one company, Apoteket. This meant that they could exert a big influence over informing doctors and the public about environmental hazards and associated risks of pharmaceuticals, and promote return schemes in their pharmacies. The system is based on ranking of pharmaceuticals for environmental harm including PBT assessment, (persistence

bioaccumulation and toxicity) to aquatic organisms. Factors such as therapeutic efficiency, side effects and price are also used to select drugs that are recommended for use by the healthcare system.

There seems to be some discrepancies in the data for the success of take back schemes. CYCLAMED reports collection of 5.7% of medicines sold annually. However, the European Federation of Pharmaceutical Industries and Associations estimate a recovery rate of 80% for the take back schemes in France (Taylor & Poulmair, 2007). Grass & Lalande, (2005) have estimated that 50% of medicines sold are not consumed in France. There is an obvious discrepancy in estimates of effectiveness of the scheme. Sweden report that 73% of the population return unused medicines to the pharmacy (Apoteket, 2006). All this leads to the conclusion that more information is required on the success of these return schemes. This should be combined with data on the contribution to pharmaceutical concentrations made by unused medicines.

3.4.3 Pharmaceutical companies and sharing information

A higher degree of communication between and transparency of pharmaceutical companies and environmental regulators, risk assessors and ecotoxicologists could improve the risk assessment of pharmaceuticals. Nine interviewees agreed that a sharing of information especially in respect to ecotoxicological techniques would be beneficial. An academic interviewee agreed:

"In principle it's a good idea, why do the tests more than once?"

Interviewees were asked what the limitations would be with sharing information obtained during drug development and mammalian toxicology tests. The main problems highlighted were linked intellectual property rights, confidentiality and patent infringements. It was thought, however, that there should be no problem with sharing this information, it was covered by patent and should be in the public domain, the more openness and access the better. The consultancy interviewee expressed the view:

"In most cases there shouldn't be any real obstacles, however, regulators need to find a way to encourage this information release."

An academic interviewee believed that:

> *"There should be no stumbling blocks at all; it's just the neuroses of companies that feel the information should be confidential. Covered by patent so should be in the public domain. Many companies are moving toward this. It's a man power, cost of doing issue."*

Three respondents said that it was entirely up to the pharmaceutical company whether they wanted to release this information because it was theirs, they had paid for it and patent time was small already. There was also a concern that if some of the mammalian data were published it could worry end users unduly. An academic interviewee believed that:

> *"It shouldn't be in the public domain, an LD50 for lab rats would worry patients."*

Three interviewees felt that it was important to encourage pharmaceutical companies to share this information with regulators and the ecotoxicology community in a way that protected their need for confidentiality. It was mentioned that making this data available can take time, money and man power. The pharmaceutical industry interviewee said:

> *"Things are changing from regulatory perspective. I've been led to believe that from next year the ERAs are going public through the Commission and the same may be happening with the tox data. I think the regulatory pressures mean that transparency is going to be lot greater. I believe our risk assessments and our data will start appearing on our web pages. PECs will be published based on a worst case for Europe, I can't tell you the country. We will include the data that contributed to the PEC and PNEC, test species and the guideline. A lot of that data sits on a Swedish site at the moment. More scientific scrutiny of the studies is the only thing."*

An increase in communication between pharmaceutical companies, regulators and ecotoxicologists would be a positive step in terms of increasing the efficiency of ERAs. This could be especially valuable in the development of ecotoxicological techniques and bioassays for monitoring purposes. The release of mammalian toxicology data could provide further scope for assessing the potential use of this data for 'read across' to ecotoxicological assessment. The

interviewee responses indicate that pharmaceutical companies are beginning to do this. In order for an increase in communication and data flow to be successful regulators need to explore mechanisms to release this data to ecotoxicological and regulatory communities without worrying the general public.

3.4.4 Metabolites

Seven interviewees were in favour of pharmaceutical companies identifying the metabolites of new pharmaceuticals including the consultancy interviewee, the government interviewee , the water company interviewee all agreed and 4 academics. An EA interviewee said:

> *"This is something that is picked up quite late in the risk assessment; there is a mention right at the end. I think metabolites should be looked at a little earlier in the process, the industry should indicate key metabolites and these should go through the process."*

The other EA interviewee asked:

> *"Are we overstating it? It's unlikely that a drug would be more potent after it's been metabolised."*

One academic interviewee considered:

> *"Huge task…interesting scientific issue but not the most important thing to be doing."*

Another academic thought that:

> *"Desirable but often questionable how much benefit we'd get from this. I can't think of a pharmaceutical breakdown product that we are concerned about."*

The pharmaceutical company interviewee commented:

> *"We have to identify them in terms of the human breakdown products. For environmental ones we do something called the water sediment study CD308. Anything that's formed at*

greater than 10% in that study we have to make an attempt to identify it. Sometimes we can't. We might test the metabolite, we fill in data gaps sometimes"

The current ERA states that pharmaceutical metabolites should be identified if they are produced at levels greater than 10% of the parent compound; however, this was a final stage of the process. It would seem logical that identification of any major metabolites should be performed much earlier on in the risk assessment process and that these should undergo a risk analysis.

One relevant point that was made several times was that, there were no metabolites at present that have been highlighted as ecotoxic. This could be through a lack of data or research into pharmaceutical metabolites and breakdown products. The KNAPPE workshop also found that limited data is available on the ecotoxicity of pharmaceutical metabolites (KNAPPE, 2010). However, some pharmaceutical metabolites could be toxic. The key metabolite of fluoxetine, nor fluoxetine has been shown to be more toxic than fluoxetine in bioassays with the protozoan *Spirostomum ambiguum* and the crustacean *Thamnocephalus platyurus* (Nalecz-Jawecki, 2007). Another rare example is a key metabolite of the Tamiflu vaccine, which has been shown to increase the overall toxicity when in a mixture with the parent compound (Escher *et al*., 2010).

One of the problems with incorporating the metabolites into the ERA is the sheer number of new compounds that may be formed through metabolism and transformation during sewage treatment or in the environment by biotic or abiotic processes. There is potential to use QSARs to make predictions about the relative toxicity of a metabolite in comparison to the parent compound. Information on factors that make pesticide transformation products more ecotoxic than the parent compound have been identified (Boxall & Greenwood, 2010; Belfroid *et al*., 1998; Neuwoehner *et al*., 2010). These factors include alterations in a compounds properties which might increase lipophilicity or dissociation behaviour. This could be incorporated into QSARs. This type of tool could then be applied to pharmaceutical metabolites as a first screen to predict an increase in toxicity (Boxall & Greenwood, 2010).

3.4.5 Precautionary Principle

The precautionary principle emphasizes that where evidence of a threat to the health of the environment exists, scientific uncertainty must not be allowed to delay reasonable forms of management action (CEPA 1999, Quijano 2003, United Nations General Assembly 1992). All

respondents thought that it was unnecessary to invoke the precautionary principle and that in general we were already being precautionary in having a risk assessment. The water company interviewee thought:

> *"I get nervous about being over precautionary, safety factors etc."*

The consultancy interviewee believed that:

> *"No – I don't think that there is any more uncertainty about human pharmaceuticals than for other substances – indeed there is probably a lot less. The precautionary principle (and I mean the one adopted by the EC, not some half-arsed version espoused by certain NGOs) should only be invoked if there are likely to be widespread, serious and irreversible effects on the environment. I can't honestly see this being the case for any human medicine."*

In some respects the precautionary principle has been already been applied to pharmaceuticals in the aquatic environment in the fact that an ERA must be performed prior to licensing. However, the findings of this research indicate that there are several limitations with the current ERA guidelines. One of the key problems is that even in the event of an environmental risk being identified; market authorisation cannot be refused because the drug is for human use. The only option is to attempt to reduce the exposure, however, mitigation measures for reducing pharmaceutical exposure are still quite limited (see above). There is also lack of any retrospective risk assessment for old medicines. In light of the problems encountered from environmental exposure of diclofenac and EE2 this is something that needs to be addressed. The results of these expert knowledge holder interviews support this finding. Although respondents did not feel that scientific uncertainty was sufficient to invoke the precautionary principle, responses to earlier questions clearly indicate that there are gaps to be filled in order to increase the protection of the environment from human pharmaceuticals.

3.4.6 Polluter pays principle

The critical problem with applying the polluter pays principle to pharmaceuticals as pollutants is identifying who is the polluter. This was reflected in the responses of the

interviewees. In respect to who should pay for monitoring of pharmaceuticals one academic interviewee said:

> *"At the moment when the drug company are preparing their dossier on their new drug they will pay for all the toxicology tests and clinical trials and it would be a relatively small cost in the overall cost for them to also build in an environmental package, they will pay for the ERA but not necessarily the monitoring that follows on from that. I think that the principal that the polluter pays should still apply to pharmaceuticals as it does to other companies….. It makes sense that they should make a contribution to monitoring ….we shouldn't be reliant on the Environment Agency to take a sample. The downside is that the regulator doesn't have any control over when the samples are taken or quality of the work.*"

In response to possible retrospective ERAs, the pharmaceutical company interviewee expressed the view that:

> *"Who should pay? It is the inventor and those with a financial issue. Lily is picking up ninety five percent of the burden for the pharmacovigilance of things like Prozac and they don't actually make that much money from it anymore. The generic industry needs to raise their game as part of the pie issue."*

However, in regards to who should pay for improvements in sewage treatment it was widely accepted that water companies should pay and pass the costs on to the consumer. The water company interviewee stated that:

> *"If we have to put in a new stage of treatment, it is termed a new obligation; this goes into our five year programming and is reflected in our pricing that is charged to customers."*

The pharmaceutical company interviewee responded:

> *"For 1 or 2 drugs that may be a problem the water utilities companies may have to start investing in tertiary treatments. The tax payer will have to pay for that and at that point I think*

society and politicians need to start thinking is the investment going to be addressing a real and significant risk or is it a theoretical risk? That's something that the pharmaceutical company can't do alone we are just one of many stakeholders in that debate."

An academic interviewee said:

"The principal is already accepted that it's the water user that should pay as opposed to the pharmaceutical company"

Does this therefore mean that the drug consumer is the polluter? Of course it is the consumer that will pay regardless of whether the cost comes from an increase in the price of medicine or an increase in the cost of clean water. Most interviewees felt that society did not want increased water bills. Another academic interviewee said:

"Would I like higher quality effluents in rivers? Absolutely yes. Would I be willing to pay for it? Absolutely yes, but society probably wouldn't."

The question of who is polluter would seem to be unresolved with expert opinion divided. However, it would seem that different aspects of the problem could be broken down and assigned to different companies. Monitoring and retrospective ERAs could be potentially be the pharmaceutical companies, including producers of generics, responsibility. Whereas, improvements in water quality arising from upgrades in sewage and water treatment, should be paid for by the water companies. DEFRA and the EA could be responsible for any monitoring that is required under WFD. This approach will require cooperation and collaboration from all of these organisations if pollution by pharmaceuticals is to be reduced. The final bill will of course sit with the consumer and the tax payer.

3.4.7 Green pharmacy

The final question was about the potential of green pharmacy to reduce the risks of human pharmaceuticals to the environment. It received quite a mixed response. Two interviewees (the water company and an academic) thought that the idea of green pharmacy was ridiculous.

However, two interviewees thought that pharmaceutical companies already considered the environmental impacts of their products especially in regards to public perception and affect on share prices. The development of green pharmaceuticals is already being pursued by the industry (Doerr-MacEwen & Haight, 2006). One of the key problems with developing environmentally friendly drugs is that they need to be stable enough to persist in the body long enough to take effect. Easy degradation should be taken into account even before a pharmaceuticals synthesis (benign by design) (Kummerer, 2009). Although there is little progress so far in the benign by design approach there is an increasing consensus that the earlier the environmental impact of a pharmaceutical product is considered in the development process the greater the chance of adopting prevention and minimisation techniques (Greenwood *et al*., 2010). Several drugs have had their manufacturing processes improved to be more environmentally friendly. For example ibuprofen, had its manufacturing process redeveloped which reduced the catalytic steps from 6 to 3 (EPA, 2012) to reduce impacts. Atorvastatin has had greener reaction conditions developed which increased yield and reduced waste and sertraline had its manufacturing process streamlined to reduce consumption of energy and raw materials while doubling yield (Greenchemex, 20120. The pharmaceutical industry is spending much time and money trying to reduce exposure at source. For example production of more enantiomerically pure drugs (Daughton *et al*., 2003). An example of a more benign by design drug, is the anti tumour drug ifosamide, which is highly recalcitrant, however by attaching sugar molecules, another compound glufosfamide is obtained which retains the therapeutic activity of ifosamide but is much more biodegradable (Kummerer *et al*., 2000; Keil, 2008).

Six interviewees thought that no financial incentives should be given to pharmaceutical companies for greener drug design. An increase in patent length has been suggested as an incentive that pharmaceutical companies would like, however, the pharmaceutical company interviewee thought that most of the incentives put forward for greener drugs were unlikely to come to fruition. One academic interviewee summed up green pharmacy in a positive way by saying:

> *"Yes we should work together we need pharmaceuticals and we need environmental protection. No financial incentives."*

There has been a distinct change in the way new pharmaceuticals are 'discovered'. Instead of observations of biological activity of natural products, there has been a shift towards the use of genomics involving drug target definition from gene interrogation methods. The understanding of the molecular basis of disease and the introduction of high throughput technologies for chemical and biological screening presents an opportunity to consider environmental aspects of a compound at the lead candidate stage. It is widely held that the environmental stability of a compound should be considered early on in the development stage, i.e. ten years before market authorisation (Sumpter, 2010). The sooner environmental impact is considered during the development of pharmaceuticals the greater the chance of adopting prevention and minimisation techniques (Butters *et al*., 2006). Bioinformatics databases and QSARs could be utilised at this stage to make predictions about lipophilicity and PBT. Once lead compounds have been identified structural alerts based on human toxicity could be used to disregard compounds which have PBT potential (Clark *et al*., 2010). For example fluorine is incorporated into many pharmaceuticals such as fluoxetine. Some drugs such as lipitor and fluoxetine contain very strong fluorine-carbon bonds which make them much more resistant to degradation in the body in order for them to reach their drug targets (Muller *et al*., 2007). The carbon-fluorine bond also decreases the biodegradability of compounds in the environment (Muller *et al*., 2007). Greener pharmaceuticals should not contain fluorine or other halogens (Sumpter, 2010). Although environmental aspects are not the priority for pharmaceutical companies, green pharmacy is becoming an increasingly important consideration (Lubick, 2008).

'Benign by design' can be tackled in two ways. Can you design persistence out or build degradation in to a compound? The pharmacokinetic and pharmacodynamic profile or a drug can be optimized, so why cant the environmental degradation? In contrast to one of the more recalcitrant pharmaceuticals carbamazepine, another anti epileptic drug valporic acid has been used worldwide for over 40 years and is fully mineralized by environmental bacteria (Yu *et al*., 2006; Kummerer, 2010; Kummerer, 2007).

Expert computer systems have been developed that screen molecules for medical properties and environmental properties such as biodegradation (Kummerer, 2010), e.g. QSPRs (quantitative structure-property relationship) for the prediction of persistence of small organic molecules (Papa & Gramatica, 2008).

The advent of genomics in the drug development process with specific target definition means that bioinformatics and molecular docking packages are an important tool in the drug development process (Schoichet *et al*., 2002). For example the recent development of the anti viral drug for bird flu used this technology (Nguyen *et al*., 2009). The development of anticancer drugs is also an example of where molecular docking packages are being utilized (Mukherjee & Majumder, 2009). The bioinformatics work (Section 4) shows that these packages could also be used to predict toxicity of pharmaceuticals on aquatic organisms and aid and direct laboratory ecotoxicity tests.

3.5 Conclusion

The results of the expert knowledge holder interviews provide a novel insight into the views of regulatory bodies, the pharmaceutical industry, water companies and academics that have substantial knowledge on the limitations of the ERA of pharmaceuticals. The main recommendations for improvements to risk assessment included a need for some retrospective ERAs for existing pharmaceuticals based on some prioritisation. Interviewees identified a need to reduce the scientific uncertainties created by the current PEC calculation for exposure potentially by modelling of environmental concentrations with the use of computer software. This was thought especially important in the context of monitoring. The 10 ngl^{-1} action limit was seen as an inappropriate mechanism for risk assessment of pharmaceuticals by many interviewees. It would seem that a more effective method would be to consider each pharmaceutical on a case by case basis considering aspects such as mode of action and PBT. It would be desirable to increase the effectiveness of QSARs for this task. Computer packages may also offer a valuable tool to aid and direct ecotoxicity tests by providing a more intelligent approach for selecting mode of action related test end points and choice of sensitive test species.

Green pharmacy still has some way to go with regards to 'benign by design' solutions; however, environmental stewardship was seen by many as a practical and achievable measure to reduce exposure. Research into the contribution that unused medicines make in the environment and the effectiveness of take back schemes is needed.

In conclusion the key messages that can be drawn from the interview analysis are:

- Pharmaceuticals are designed to have a specific biological effect and this should be reflected by the ecotoxicology tests incorporating the mode of action.
- An increase in the spread of species included in ecotoxicity tests would be beneficial especially in the case of molluscs.
- Development of QSARs and computer generated packages for prediction of toxic effects is highly desirable but at present provides poor indication of chronic ecotoxicological effects.
- Retrospective environmental risk assessment of pharmaceuticals licensed before 2006 needs to be performed using a system of prioritisation.

- Having a trigger limit for ecotoxicological risk assessment is flawed because some drugs such as ethinylestradiol have effects at concentrations lower than 10 ngl^{-1} .
- PEC calculations need to be revised to take into account 'hot spots' caused by high usage of drugs in some areas or at certain times of year and low effluent to surface water dilution for some rivers.
- Exposure modelling would aid environmental risk assessment.
- Monitoring of pharmaceutical concentrations is expensive and time consuming and considered mostly unnecessary unless an adverse ecological effect is identified or the PEC and PNECs were very close in value.
- STP upgrades are seen as an unfavourable option to decrease the input of pharmaceuticals into the environment because of associated financial and environmental (carbon emissions) costs.
- Increasing public awareness about disposal of unused medicines is needed.
- Increased communication between pharmaceutical companies, ecotoxicologists and regulators would help protect the environment.

The in depth interviews with expert knowledge holders provided valuable insights into the limitations of the current ERA. The results provide information on where research should be focussed and improvements that need to be made to better protect the environment. The outcomes from the interviews reflect the real value of expert knowledge for policy development in developing techniques to protect the environment and for improving the ERA of pharmaceuticals.

Section 4

Bioinformatics, Ecotoxicology and Environmental Risk Assessment of Pharmaceuticals

4.1 Aim of Section 4

The aims of this section of work are to: 1) investigate the usefulness of bioinformatics genome databases and analysis tools for identifying drug target homologues in aquatic species; 2) investigate the ability of molecular docking software to predict binding of drugs to identified target protein homologues in aquatic species; 3) evaluate the potential of current bioinformatics technology as a tool to aid and direct ERA of human pharmaceuticals by improving selection of sensitive species, informing choice on appropriate methodologies and appropriate end points for toxicology tests; 4) discuss the potential application of bioinformatics and molecular docking as a tool in green pharmaceutical design.

4.2 Introduction

Pharmaceuticals are designed to have a particular therapeutic effect. They target specific metabolic and molecular pathways and proteins as part of their MoA (Christen *et al*., 2010; Dorne *et al*., 2006; Kar & Roy, 2010). Due to their presence in the aquatic environment (Section 2) they may adversely affect non-target vertebrates and invertebrates. These organisms may have a number of identical or similar proteins to humans and these may act as unintended drug targets (Christen *et al*., 2010).

4.2.1 Ecotoxicology and environmental risk assessment

Concentrations of pharmaceuticals in sewage effluent and surface waters are generally low i.e. in the ngl^{-1} to μgl^{-1} range (Section 2) and are usually at least an order of magnitude below the amount required to produce an acute toxic effect (Kummerer, 2009). However the detrimental effects of the synthetic contraceptive 17α ethinylestradiol (EE2) have been well documented. EE2 causes intersex characteristics in fish downstream from sewage treatment outfalls (Jobling *et al*., 2006). This pharmaceutical is highly potent at small concentrations. In fact as little as 1 ngl^{-1} has been shown to cause vitellogenin production in male fish, a precursor to egg production (Länge *et al*., 2001; Lattier *et al*., 2002; Parrott & Blunt, 2005) and the zebra fish (*Danio rerio*) showed complete reproductive failure at 10 ngl^{-1} (Segner *et al*., 2003).

Despite the pseudo persistent nature of aquatic organism exposure to human pharmaceuticals, this has been the only major in-situ toxicological effect that has come to light so far. One of the concerns related to this environmental problem is that it was unanticipated.

Retrospective analysis of course, suggests this effect was likely to occur because hormone receptor targets are highly conserved in fish. EE2 binds to oestrogen receptor proteins with high affinity in fish as well as in humans (Campbell *et al*., 1994). Another important factor in this case is that the problem was not caused by a non specific toxic effect but rather an effect directly related to the action of the drug in humans. This ecotoxicological effect highlights the possibility that other well conserved drug targets in non target species may also be affected by human pharmaceuticals (Gunnarsson *et al*., 2008).

The constant input of low concentrations of pharmaceuticals into surface waters mean that the exposure of and toxicity to aquatic organisms is chronic rather than acute. To complicate matters further organisms are not exposed to a single drug in isolation but to a considerable mixture of chemicals. Thus may produce additive or synergistic or antagonistic effects, especially if compounds affect the same metabolic pathway or target protein (Schnell *et al*., 2009). There is a distinct lack of data on the long term, full lifecycle and multigenerational chronic ecotoxicity of pharmaceuticals and mixtures (Dietrich *et al*., 2010). This is due to a variety of factors. Chronic ecotoxicity tests are time consuming and expensive. There can be difficulties in selecting appropriate and informative end points based on known pharmacological properties of the pharmaceutical (Ankley *et al*., 2007). Choice of organisms is difficult because it is unknown which species would be most sensitive to a particular drug. It is unfeasible and unethical to extensively test great numbers of organisms. As a result protecting the aquatic environment requires knowledge about conserved drug targets in exposed organisms. This is critical for assessing possible ecotoxicological effects, selection of potentially sensitive species and development of more efficient test strategies (Kostich & Lazorchak, 2008; Seiler, 2002).

The current guidelines for risk assessment in the EU (EMEA, 2006) incorporate ecotoxicological data using the OECD guidelines. Since 2006 the EMEA has recommended using chronic effects data, this is rarely available prior to 2006 and as a consequence acute affects data is used. Standard long term toxicity tests are performed on three trophic levels. This should include full life cycle tests on daphnia and algae; and a semi chronic early life stage on one fish. The most sensitive of these organisms is used to predict the no-effect concentration (PNEC) in surface water. If the ratio between the PEC (see Section 2) and the PNEC is less than 1 there is no need for further ecotoxicological testing. If this ratio is above 1 then a tailored risk assessment taking into account the MoA is required. This is also true for pharmaceuticals that are

highly potent with a PNEC of 10 ngl^{-1} or below such as EE2 and levonorgesterel (synthetic progesterone). Despite EU guidelines requiring relevant toxicity data, there is still little focus on targeted test strategies (Gunnarsson *et al*., 2008). Prospective testing could be made more powerful by including targeted test strategies based on known pharmacological properties of the tested pharmaceutical (Ankly *et al*., 2007). There is a need for a long-term focus on specific modes of action for pharmaceuticals to decrease uncertainties (Fent *et al*., 2006).

4.2.2 Ecotoxicology and Green Pharmacy

Bioinformatics and molecular docking packages could be beneficial to achieving green pharmacy in several ways. Of course an ideal situation for the environment would be no adverse impacts on any of the life cycle stages of organisms by human pharmaceuticals, including all aspects of their manufacture, use and disposal. This would begin with a compound that was designed to be harmless environmentally i.e. the 'benign by design' approach (Kummerer, 2007). Bioinformatics computer packages such as quantity structure activity relationship's (QSARs) may be a practical way to help with approaches such as this. For example, to help inform the decision making process of drug development at the lead candidate stage by considering environmental aspects using predictions about environmental persistence, bioconcentration and toxicity of compounds (Jager & Kooijman, 2009; Mekenyen *et al*., 2005). However, this type of approach is in its infancy and as yet there are limited examples of 'benign by design' compounds (see conclusion for further discussion).

A more pragmatic approach may be to reduce environmental impact of pharmaceuticals at the risk assessment stage. Some authors have suggested that ecotoxicity testing of pharmaceuticals could be made more intelligent by incorporating the MoA (Boxall, 2004). This could provide more relevant information about effects that are actually likely to occur when non-target organisms are exposed to pharmaceuticals. The current standardised suite of ecotoxicological tests outlined above does not reflect the actual environmental situation in regard to pharmaceuticals well. Because aquatic organisms are exposed to continual low doses of a vast number of pharmaceuticals the general toxic effects investigated in the laboratory, especially acute effects, are unlikely to be observed in the real environment. In n order to move nearer to the goal of green pharmacy, i.e. a situation where no detrimental effects are caused to non target organisms, it is essential to know what the actual effects of low concentrations of mixtures of

pharmaceuticals may be. The choice of an appropriate toxicological end point is paramount. If a low concentration of a mixture of analgesics reduces prostaglandin production in humans, then they may cause a reduction in prostaglandin production in a fish.

4.2.3 Bioinformatics and molecular docking

Bioinformatics databases such as the NCBI database contain thousands of protein sequences from an increasing selection of organisms. It is possible to take the known drug target protein amino acid sequence and use BLAST to search for homologues i.e. similar protein sequences in other organisms. Nine pharmaceuticals were chosen for investigation: carbamazepine, diclofenac, ibuprofen, paracetamol, tamoxifen, propranolol, gemfibrozil, EE2 and fluoxetine. The rationale for this selection was a high detection rate in the aquatic environment (Section 2), coverage of the main therapeutic classes and high prescription rate (see Table 2.1). The MoA and drug target information for the analgesics (diclofenac, ibuprofen and paracetamol) and the other selected pharmaceuticals is displayed (Table 4.1 & Table 4.2 respectively).

Once protein homologues have been identified it is then possible to model the 3 dimensional structures of these proteins based on existing crystallised structures using modelling software such as Swiss Model. These free accessible databases and software make it possible to find aquatic organisms that may react to with drugs in a similar way to humans. This information could be highly relevant to environmental toxicologists.

Molecular docking computational software can be used to predict the orientation of one molecule to another when bound together to form a stable complex (Lengauer & Rarey, 1996). Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between the two molecules. The associations between biologically relevant molecules such as proteins, nucleic acids, carbohydrates, and lipids play a central role in signal transduction. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced (e.g., agonism vs antagonism). Docking is useful for predicting both the strength and type of signal produced. Molecular docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to predict the

affinity and activity of the small molecule. Hence docking plays an important role in the rational design of drugs (Kitchen *et al*., 2004).

Table 4.1 Drug targets and mode of action for analgesic pharmaceuticals investigated
(Information collected from DrugBank, 2012)

Pharmaceutical	Drug Targets	Mode of Action
diclofenac	[1] Prostaglandin G/H synthase 1 precursor (COX1) [2] Prostaglandin G/H synthase 2 precursor (COX2) [3] Transthyretin precursor	The antiinflammatory effects of diclofenac are believed to be due to inhibition of leukocyte migration and the enzyme cyclooxygenase (COX-1 and COX-2), leading to the peripheral inhibition of prostaglandin synthesis. As prostaglandins sensitize pain receptors, inhibition of their synthesis is responsible for the analgesic effects of diclofenac. Antipyretic effects may be due to action on the hypothalamus, resulting in peripheral dilation, increased cutaneous blood flow, and subsequent heat dissipation.
ibuprofen	[1] Prostaglandin G/H synthase 1 precursor (COX1) [2] Prostaglandin G/H synthase 2 precursor (COX2) [3] Serum albumin precursor	The exact mechanism of action of ibuprofen is unknown. Ibuprofen is a non-selective inhibitor of cyclooxygenase, an enzyme involved in prostaglandin synthesis via the arachidonic acid pathway. Its pharmacological effects are believed to be due to inhibition cylooxygenase-2 (COX-2) which decreases the synthesis of prostaglandins involved in mediating inflammation, pain, fever and swelling. Antipyretic effects may be due to action on the hypothalamus, resulting in an increased peripheral blood flow, vasodilation, and subsequent heat dissipation. Inhibition of COX-1 is thought to cause some of the side effects of ibuprofen including GI ulceration. Ibuprofen is administered as a racemic mixture. The R-enantiomer undergoes extensive interconversion to the S-enantiomer *in vivo*. The S-enantiomer is believed to be the more pharmacologically active enantiomer.
paracetamol	[1] Prostaglandin G/H synthase 1 precursor (COX1) [2] Prostaglandin G/H synthase 2 precursor (COX2)	Paracetamol is thought to act primarily in the CNS, increasing the pain threshold by inhibiting both isoforms of cyclooxygenase, COX-1, COX-2, and COX-3 enzymes involved in prostaglandin (PG) synthesis. Unlike NSAIDs, paracetamol does not inhibit cyclooxygenase in peripheral tissues and, thus, has no peripheral anti-inflammatory affects. While aspirin acts as an irreversible inhibitor of COX and directly blocks the enzyme's active site, studies have found that paracetamol indirectly blocks COX, and that this blockade is ineffective in the presence of peroxides. This might explain why paracetamol is effective in the central nervous system and in endothelial cells but not in platelets and immune cells which have high levels of peroxides. Studies also report data suggesting that paracetamol selectively blocks a variant of the COX enzyme that is different from the known variants COX-1 and COX-2. This enzyme is now referred to as COX-3. Its exact mechanism of action is still poorly understood, but future research may provide further insight into how it works. The antipyretic properties of paracetamol are likely due to direct effects on the heat-regulating centers of the hypothalamus resulting in peripheral vasodilation, sweating and hence heat dissipation.

Table 4.2 Drug targets and mode of action for selected pharmaceuticals
(Information collected from DrugBank, 2012)

Pharmaceutical	Drug targets	Mode of Action
carbamazepine	Sodium chanel protein type 5 subunit alpha	Carbamazepine inhibits sustained repetitive firing by blocking use-dependent sodium channels. Pain relief is believed to be associated with blockade of synaptic transmission in the trigeminal nucleus and seizure control with reduction of post-tetanic potentiation of synaptic transmission in the spinal cord. Carbamazepine also possesses anticholinergic, central antidiuretic, antiarrhythmic, muscle relaxant, antidepressant (possibly through blockade of norepinephrine release), sedative, and neuromuscular-blocking properties.
ethinyl estradiol	[1] Estrogen receptor [2] Orphan nuclear receptor	Estrogens diffuse into their target cells and interact with a protein receptor. Target cells include the female reproductive tract, the mammary gland, the hypothalamus, and the pituitary. Estrogens increase the hepatic synthesis of sex hormone binding globulin (SHBG), thyroid-binding globulin (TBG), and other serum proteins and suppress follicle-stimulating hormone (FSH) from the anterior pituitary. This cascade is initiated by initially binding to the estrogen receptors. The combination of an estrogen with a progestin suppresses the hypothalamic-pituitary system, decreasing the secretion of gonadotropin-releasing hormone (GnRH).
fluoxetine	Sodium dependant serotonin transporter	Metabolized to norfluoxetine, fluoxetine is a selective serotonin-reuptake inhibitor (SSRI), it blocks the reuptake of serotonin at the serotonin reuptake pump of the neuronal membrane, enhancing the actions of serotonin on $5HT_{1A}$ autoreceptors. SSRIs bind with significantly less affinity to histamine, acetylcholine, and norepinephrine receptors than tricyclic antidepressant drugs.
gemfibrozil	[1] Peroxisome proliferator-activated receptor alpha [2] Lipoprotein lipase precursor [3] Solute carrier organic anion transporter family member 1B1	Gemfibrozil increases the activity of extrahepatic lipoprotein lipase (LL), thereby increasing lipoprotein triglyceride lipolysis. It does so by activating Peroxisome proliferator-activated receptor-alpha (PPARα) 'transcription factor ligand', a receptor that is involved in metabolism of carbohydrates and fats, as well as adipose tissue differentiation. This increase in the synthesis of lipoprotein lipase thereby increases the clearance of triglycerides. Chylomicrons are degraded, VLDLs are converted to LDLs, and LDLs are converted to HDL. This is accompanied by a slight increase in secretion of lipids into the bile and ultimately the intestine. Gemfibrozil also inhibits the synthesis and increases the clearance of apolipoprotein B, a carrier molecule for VLDL
propranolol	[1]Beta- 1- adrenergenic receptor [2]5-hydroxytryptamine 1A receptor [3] Beta- 2- adrenergenic receptor [4] 5-hydroxytryptamine 1B receptor [5] beta- 3- adrenergenic receptor	Propranolol competes with sympathomimetic neurotransmitters such as catecholamines for binding at beta(1)-adrenergic receptors in the heart, inhibiting sympathetic stimulation. This results in a reduction in resting heart rate, cardiac output, systolic and diastolic blood pressure, and reflex orthostatic hypotension.
tamoxifen	[1] estrogen receptor [2] estrogen receptor beta [3] epoxide hydrolase [4] multidrug resistance protein 1 [5] thymidine phosphorylase	Tamoxifen binds to estrogen receptors (ER), inducing a conformational change in the receptor. This results in a blockage or change in the expression of estrogen dependent genes. The prolonged binding of tamoxifen to the nuclear chromatin of these results in reduced DNA polymerase activity, impaired thymidine utilization, blockade of estradiol uptake, and decreased estrogen response. It is likely that tamoxifen interacts with other coactivators or corepressors in the tissue and binds with different estrogen receptors, ER-alpha or ER-beta, producing both estrogenic and antiestrogenic effects

4.2.4 Choice of pharmaceuticals for molecular docking

Two pharmaceuticals were chosen for molecular docking, diclofenac and ibuprofen. These two compounds were selected for several reasons. Firstly, they were found to be ubiquitous pollutants of the aquatic environment (see Section 2). A second reason was that diclofenac has been shown to seriously adversely affect non target organisms i.e. the mass decline of the vulture population in Asia (Oaks *et al*., 2004) (see below). There is also increasing evidence in the literature that diclofenac may cause subtle chronic effects on aquatic organisms (Mehinto *et al*., 2010; Schwaiger *et al*., 2004; Hoeger *et al*., 2005). Chronic ecotoxicity studies have reported that exposure to environmentally relevant concentrations of diclofenac at 0.5 and 1 μgl^{-1}/L can result in adverse affects in various organs and possibly compromise the health of fish (Schwaiger *et al*., 2004; Trieskorn *et al*., 2004; Hoeger *et al*.,. 2005; Mehinto *et al*., 2010). The PEC results from Section 2 provide a crude PEC of 1 μgl^{-1} and a prescription data PEC as low as 0.7 μgl^{-1}. MECs of 2.35 μgl^{-1} in sewage effluent and 0.57 μgl^{-1} in surface waters have been detected in the UK (Hilton *et al*., 2003) and concentrations as high as 2.3 μgl^{-1} in surface waters in Germany (Jux *et al*., 2002). It seems that chronic effects may be occurring. According to the EMEA guidelines an assessment factor of 10 should be applied to the NOEC to account for inter/intra species variations and lab data to field impact extrapolation. If the NOEC for diclofenac is 0.05 μgl^{-1} (with assessment factor), and the PEC (from section 2) is 1 μgl^{-1} the risk quotient for PEC/PNEC ratio would be 20. This far exceeds the limit of 1 for further risk assessment and indicates a strong possibility of an environmental risk. This value is considerably higher than reported previously by Carlsson *et al*., (2006) who derived a PEC/PNEC quotient of 4.8 using the current ERA. It has been highlighted that recent release from prescription only will increase use and the environmental concentration of diclofenac (Mehinto *et al*., 2010).

It was also desirable to investigate two drugs that target the same drug receptor for potential synergistic or additive effects. Diclofenac and ibuprofen both target the same cyclooxygenase (COX) enzymes (Table 4.1). Finally, in order to test the feasibility of using molecular docking as part of an intelligent ecotoxicological approach for risk assessment, it was necessary to have the presence of similar drug target homologues in

aquatic organisms that have been well characterised in humans. Thus providing a suitable template on which to model aquatic species protein homologues.

Diclofenac is a non steroidal anti-inflammatory drug (NSAID) which is widely used in veterinary and human medicine. In humans it acts by inhibiting the cyclooxygenase (COX) enzymes (Vane & Botting, 1998) (Fig 4.1). These enzymes catalyse the synthesis of prostaglandins which are involved in inflammation, blood flow regulation, platelet aggregation and secretion of gastric mucus (Mutschler, 1996; Sali, 2005). Diclofenac was found to occur ubiquitously in sewage effluents and removal during sewage treatment was highly varied and not at a level sufficient to prevent its continued detection in surface waters worldwide (Section 2). Diclofenac has also been the cause of a major decline of vultures in India and Pakistan due to its use in veterinary medicine. Vultures feed on the dead carcasses of cattle treated with diclofenac causing renal failure and visceral gout (Oaks *et al*., 2004). The reasons for this are also related to its designed mode of action, and adverse renal side effects are common in human patients (Taggart *et al*., 2007; Banks *et al*., 1995).

Ibuprofen is another NSAID extensively used in human medicine and available cheaply without prescription. It was found to be ubiquitous in sewage effluents despite treatment removing over ninety percent in most activated sludge STPs (Section 2). This apparent persistence is probably due to its high usage across the world. Mean concentrations in surface waters can be as high as 2.1μgl^{-1}. Ibuprofen acts upon the same metabolic pathway as diclofenac (Fig 4.1) and also targets and inhibits COX enzymes (and therefore there is a strong possibility of additive, synergistic or antagonistic effects between ibuprofen and diclofenac).

The other pharmaceuticals investigated during this work were unfortunately unsuitable for molecular docking studies. A lack of ecotoxicity data in the scientific literature on the mode of action for tamoxifen, gemfibrozil, carbamazepine and propranolol prevented their use in molecular docking experiments. Fluoxetine as yet lacks a crystallized human serotonin receptor structure on which to model homologues and was therefore also unsuitable for molecular docking experiments at this time.

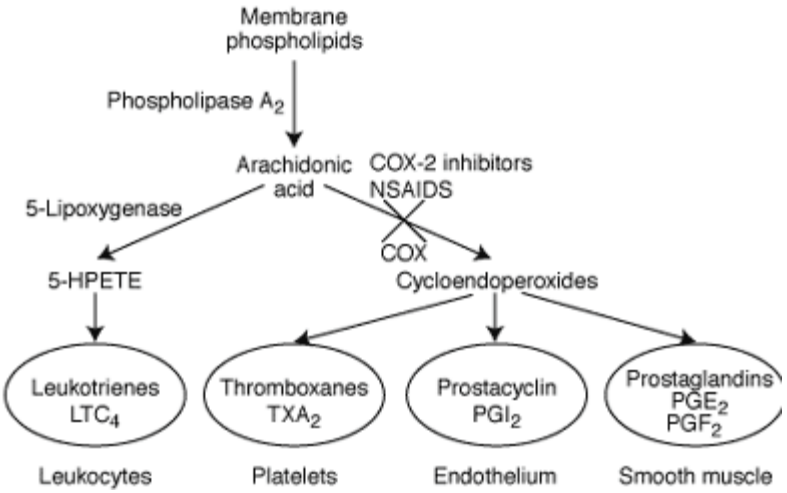


Fig 4.1 Metabolic pathway for the NSAIDs diclofenac and ibuprofen (adapted from Pharmacotherapy, 2003)

Investigation into the presence of drug target homologues in other species could provide evidence of a likelihood of ecotoxicological impacts; however, the presence of the gene target does not mean that the expressed protein will be functional. Small changes in the amino acid sequence may change the proteins 3D structure substantially. Molecular docking experiments *in-silico* may provide more scientific evidence that a protein homologue will bind to and respond in a similar way to the human type or conversely that a protein is sufficiently different that interaction between it and the drug is unlikely.

4.3 Method

4.3.1 Software and data sources

The bioinformatics software and databases in this study are all freely available for academic use and include:

- ***Drug Bank*** (http://drugbank.ca/) a unique bioinformatics and cheminformatics resource that combines detailed drug (i.e. chemical, pharmacological and pharmaceutical) data with comprehensive drug target (i.e. sequence, structure, and pathway) information. The database contains 6707 drug entries including 1436 FDA-approved small molecule drugs, 134 FDA-approved biotech (protein/peptide) drugs, 83 nutraceuticals and 5086 experimental drugs. Additionally, 4228 non-redundant protein (e.g. drug target/enzyme/transporter/carrier) sequences are linked to these drug entries. Each drug card entry contains more than 150 data fields with half of the information devoted to drug/chemical data and the other half to drug target or protein data.
- ***NCBI BLAST*** (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Basic Local Alignment Search Tool (BLAST) identifies regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.
- ***RCSB Protein Data Bank*** (http://www.pdb.org/pdb/home/home.do). An information portal to biological macromolecular structures. It holds over 77101 structures mainly determined by x-ray diffraction and solution NMR.
- ***Swiss Model*** (http://swissmodel.expasy.org/). A fully automated protein structure homology-modeling server, accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer). The purpose of this server is to make protein modeling accessible to all biochemists and molecular biologists across the world.
- ***CLUSTAL W Multiple sequence alignment*** (www.genome.jp/tools/clustalw/).

ClustalW2 is a general purpose multiple sequence alignment program for DNA or proteins. This tool can be used to align amino acid sequences in order to locate conserved binding domains and enzymatic active sites.

- ***AutoDock 4.0***. A molecular docking software package (The Scripps Research Institute, (www.scripps.edu). The package is used in combination with AutoDock Tools (ADT) - an accessory programme that allows the user to interact with AutoDock from a Graphic User Interface (GUI). AutoDock is a suite of automated docking tools designed to predict how small molecules/ligands such as substrates or drug candidates, bind to a receptor/protein of known 3D structure. AutoDock consists of three separate programmes: AutoDock which performs the docking of the ligand to a set of grids describing the target protein; AutoGrid pre-calculates these grids describing the target protein and AutoTors determines which bonds will be treated as rotatable in the ligand.

4.3.2 Drug target protein identification and gene sequence homology search

Drug target proteins for ten selected pharmaceuticals: carbamazepine, diclofenac, ethinyl estradiol, fluoxetine, gemfibrozil, ibuprofen, paracetamol, propranolol, tamoxifen and trimethoprim were identified using Drug Bank (for pharmaceutical selection rationale see Section 2.2 methods). The amino acid protein sequences for the drug targets were then collected in the FASTA format. This is a standard text-based format originating from the FASTA software package that represents nucleotides or amino acids as single-letter codes in either nucleotide or peptide sequences respectively. The format also allows for sequence names and comments to precede the sequences. Some drugs have more than one protein target, where this was the case all the protein targets were collected.

All available genomes were searched for similar, potentially conserved protein sequences to each of the drug target protein sequences using BLAST at the NCBI. Using a heuristic method, BLAST finds homologous sequences, not by comparing either sequence in its entirety, but rather by locating short matches between the two sequences. A standard BLAST search only returns the top 20 taxa that are matches. These taxa were

then excluded from subsequent searches. The results were then filtered for aquatic organisms including fish, frogs and invertebrates.

4.3.3 Creation of 3D protein models

The order of drug targets listed in DrugBank generally reflects their importance regarding therapeutic indication or physiological effect (Wishart *et al*., 2008). The primary drug target protein for the NSAID (non-steroidal anti-inflammatory drug) diclofenac was identified as prostaglandin endoperoxide H synthase 2 (PGHS2) (also called cyclooxygenase 2 (COX2). COX2 is also a drug target protein for another pharmaceutical investigated, ibuprofen (NSAID). The human COX2 (*h*COX2) sequence was used in a BLAST sequence homology search to find similar proteins in aquatic organisms. Atlantic salmon (*Salmo salar*), zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) and the water flea (*Daphnia pulex*) were selected due to their use in ecotoxicity testing and the presence of a high homology protein identified by BLAST search. The four FASTA sequences: *s*COX2 (*S.salar*), *z*COX2 (*D.rerio*), *t*COX2 (*O.mykiss*) and *d*COX2 (*D.pulex*) were submitted to Swiss model in the automated mode for the creation of 3D protein models. The models were based on a known crystallized molecular structure of sheep COX2 PDB 1PXX obtained from RCSB protein data bank. The resulting structures were saved in the PDB format needed for molecular docking.

4.3.4 Molecular Docking

The PDB files for diclofenac and ibuprofen were docked using AutoDock 4.0 with the five protein models *h*COX2, *z*COX2, *s*COX2, *d*COX2 and *t*COX2. The following procedure was used:

- *Preparing the ligand and macromolecule files for AutoDock*:

  The PDB files created in Swiss model were prepared using the GUI (graphic user interface) of ADT in order to limit imperfections in the PDB files e.g. missing hydrogen atoms, multiple molecules and added water. First all the hydrogen atoms were removed from the macromolecule files (*h*COX2, *z*COX2, *s*COX2, *d*COX2 and *t*COX2). Then polar hydrogen's were restored. ADT then checked whether the molecule had charges, if not ADT checked whether the molecule was a peptide. If the molecule was found to be a peptide, Kollman charges were

added, otherwise Gasteiger charges were added. Finally solvation parameters were added and the files saved with .pdbqs extension (where '*q*' and '*s*' represent charge and solvation, respectively).

The ligand files for diclofenac and ibuprofen were also read in ADT, all the hydrogens and charges were added and the non-polar hydrogens merged and saved with .pdbqs extension. ADT then automatically determined the best root, which is defined as the fixed portion of the ligand from which rotatable branches sprout. Next the rotatable bonds in the ligand were defined, making all amide bonds non-rotatable and the number of active torsions was set to fewest atoms. The ligand file was then saved with ligand out .pdbq extension (*q* representing charge).

- *Preparing the grid parameter file*:

  For the calculation of docking interaction energy, a three-dimensional box (grid) was created in which the target location (suspected enzyme active binding site) of the protein molecule was enclosed. The grid volume was large enough to allow the ligand to rotate freely, even with its most fully extended conformation. The grid box size was set to 64000 total grid points, with 40 points in each of the x, y, z directions. The spacing was set to 0.375 Angstrom.

  The location of the suspected enzyme binding site on the human COX enzyme model were obtained by opening the *h*cox pdb file in text pad and finding the co-ordinates for amino acid residues SER530 and TYR385, thought to be involved in binding diclofenac (Rowlinson *et al*., 2003). The multiple sequence alignment program Clustalw was used to locate these conserved residues on the homologues, *z*COX2, *s*COX2, *d*COX2 and *t*COX2. Once identified the co-ordinates of the residues involved were found by opening the PDB files in text pad. The parameters required to create the grid were stored in the grid parameter file with molecule .gpf extension.

- *Preparing the docking parameter file*:

  The docking parameter file, which instructs AutoDock about the ligand to move, the map files to use, and other properties defined for the ligand was created. AutoDock's Lamarckian genetic algorithm (LGA) was the algorithm used for the

docking file, which was stored with the .dpf extension. Finally, the AutoDock job was run from the GUI (graphical user interface) created at the University of Westminster. Each docking experiment was run 102 times and the results stored in docking log files with the .dlg extension. The best 10 dlg docking files, i.e. those with the lowest energy binding scores in docked ligand complexes were chosen. These were then read in the ADT viewer. A conformation instance was created for each docked result found in the docking log. A conformation represents a specific state of the ligand and has either a particular set of state variables from which all the ligand atoms' co-ordinates can be computed *or* the co-ordinates themselves. Conformations also have energies: docked energy, binding energy, and possibly per atom electrostatic and van der waals energies. AutoDock 4 computes the free energy of binding and reports a detailed energy breakdown.

4.4 Results

4.3.1 Drug targets and modes of action

A total of twenty FASTA protein sequences were collected to enter into separate BLAST sequence homology searches (Table 4.3-4.6). The results of the BLAST search are presented as percent sequence identity (I) and probability of this identity occurring by chance (E value). The lower the E value the less likely that the sequence similarity could occur by chance, zero being the best result.

Several of the drugs were found to share the same mode of action and drug target protein (Table 4.3 & 4.4). This is not surprising for pharmaceuticals in the same therapeutic class. Ibuprofen, diclofenac and paracetamol are all analgesics and all bind to the same target proteins, the COX 1 and COX 2 enzymes (Table 4.4). The oestrogen receptor was the primary drug target for the synthetic hormone contraceptive ethinyl estradiol (EE2) and also the anti-cancer drug tamoxifen (Table 4.3). The other pharmaceuticals investigated had different protein drug targets determined by their therapeutic class. Propranolol is a beta blocker, gemfibrozil is a lipid regulator and carbamazepine is an anti-convulsive. Fluoxetine has the same mode of action and drug target as other selective serotonin reuptake inhibitors (SSRIs) such as citalopram, escitalopram, zimelidine but these were outside the scope of this study (Table 4.6).

The BLAST homology data was also evaluated against relevant ecotoxicity data in the literature for drug receptor mediated MoA responses (Tables 4.3 – 4.6). Chronic MoA ecotoxicity data was found to be extremely limited for most pharmaceuticals with most organisms selected in the BLAST homology search. Due to the fact that only one aquatic organism (*D.rerio*) had a complete genome sequenced it was not possible to determine whether other aquatic organisms lack the primary drug receptor and hence may be less sensitive to exposure.

4.4.2 Drug target sequence homology

The results of the BLAST homology search show that lower vertebrates such as fish and frogs have significant protein sequence homology with human drug target proteins. There was much less sequence homology with invertebrates. Algae never appeared in the results of the blast sequence homology search for any of the twenty drug

target proteins of any the pharmaceuticals investigated. *Daphnia* (water flea), regularly used in ecotoxicological testing only rarely showed significant sequence homology with human drug target proteins. It is unknown at present whether this is due to a lack of sequence homology or that a limited number of genomes are fully sequenced. All of the human drug targets showed significant sequence homology with several fish and often with *Xenopus laevis* (African clawed frog) and *Xenopus tropicalis* (western clawed frog) (Table 4.3-4.6).

Table 4.3 17α ethinylestradiol and tamoxifen drug target sequence homology results

I: percent sequence identity; **E value**: the probability of this identity occurring by chance; **Ecotoxicity**, **YES** = relevant literature to support mode of action (MoA) effects of the particular pharmaceutical on the primary drug target receptor; NA: Ecotoxicity data not available. (Urbatzka *et al*., 2007[1]; Pettersson *et al*., 2006[2]; Velasco-Santamaría *et al*., 2009[3]; Salierno & Kane, 2009[4]; Schwaiger *et al*., 2000[5]; Solé *et al*., 2000[5]; Pérez *et al*., 2012[7]; Mortensen & Arukwe, 2007[8]; Notch & Mayer, 2011[9])

Pharmaceutical	Drug Target	Organism	I	E value	Ecotoxicology
ethinyl estradiol tamoxifen	estrogen receptor	*Pseudemys nelson* (Red Belly turtle)	79	0.0	NA
		Leidochelys olivacea (Olive Ridley turtle)	78	0.0	NA
		Crocodylus niloticus (Crocodile)	78	0.0	NA
		Alligator mississippiensis (Alligator)	77	0.0	NA
		Taeniopygia guttata (Zebra Finch)	78	0.0	NA
		Anas platyrhynchos (Mallard duck)	80	0.0	NA
		Xenopus laevis (African clawed frog)	69	0.0	YES[1]
		Xenopus tropicalis (Western clawed frog)	69	0.0	YES[2]
		Pleurodeles walt (Spanish ribbed newt)	68	0.0	NA
		*protopterus annectens (*West African lungfish)	59	0.0	NA
		Atractosteus tropicus (Tropical gar)	58	0.0	NA
		Acipenser schrenckii (Sturgeon)	59	7.0e-168	NA
		Zoarces viviparus (Eelpout)	54	3.0e-148	YES[3]
		Pimephales promelas (Fathead minnow)	57	6.0e-148	YES[4]
		*Tanichthys albonubes (*Mountain minnow)	58	2.0e-147	NA
		Cyprinus carpio (Common carp)	55	3.0e-146	YES[5,6]
		Odontesthes bonariensis (Peejerry fish)	51	4.0e-146	YES[7]
		Salmo salar (Atlantic salmon)	52	2.0e-145	YES[8]
		Danio rerio (Zebra fish)	58	2e-170	YES[9]
ethinyl estradiol	orphan nuclear receptor	*Danio rerio* (Zebra fish)	51	4.0e-105	
		Xenopus laevis (African clawed frog)	49	4.0e-104	
		Xenopus tropicalis (Western clawed frog)	51	3.0e-103	
		Fundulus heteroclitus (Mummichog)	44	8.0e-97	
		Oncorhynchus mykiss (Rainbow trout)	45	2.0e-95	
		Tetraodon nigroviridis (Pufferfish)	43	3.0e-86	
		Oryzias latipes (Medaka fish)	42	6.0e-86	
		Paralichtys olivaceus (Bastard halibut)	42	4.0e-85	
		Salmo salar (Atlantic salmon)	42	2.0e-83	
		Cyprinus carpio (Common carp)	42	6.0e-82	
		Petromyzon marinus (Sea Lamprey)	41	7.0e-81	
		Callorhinus ursinus (Seal)	40	8.0e-76	
		Pimephales promelas (Fathead minnow)	47	7.0e-73	
tamoxifen	estrogen receptor beta	*Taeniopygia guttata* (Zebra Finch)	74	0.0	
		Xenopus tropicalis (Western clawed frog)	69	0.0	
		Xenopus laevis (African clawed frog)	69	0.0	
		Ornithorhynchus anatinus (Platypus)	66	0.0	
		Protopterus annecten (African lugfish)	64	0.0	
		Protopterus dolloi (Spotted lugfish)	63	0.0	
		Squalus acanthias (Spiny dogfish)	63	0.0	
tamoxifen	multidrug resistance protein 1	*Xenopus laevis* (African clawed frog)	68	0.0	
		Ornithorhynchus anatinus (Platypus)	66	0.0	
		Platichthys flesus (Flounder)	63	0.0	
		Danio rerio (Zebra fish)	57	0.0	
		Xenopus tropicalis (Western clawed frog)	56	0.0	
		Branchiostoma floridae (Lancelet)	59	0.0	
		Trioplax adhaerens (Placozoa)	53	0.0	
		Taeniopygia guttata (Zebra Finch)	51	0.0	
		Raja erinacea (Little skate)	50	0.0	
tamoxifen	epoxide hydrolase	*Xenopus tropicalis* (Western clawed frog)	56	0.0	
		Xenopus laevis (African clawed frog)	56	0.0	
		Ornithorhynchus anatinus (Platypus)	60	1.0e-174	
		Danio rerio (Zebra fish)	50	3.0e-162	
		Salmo salar (Atlantic salmon)	47	9.0e-158	
		Trioplax adhaerens (Placozoa)	42	2.0e-138	
		Strongylocentrotus purpuratus) (Sea Urchin)	44	1.0e-135	
		Tetraodon nigroviridis) (Pufferfish)	46	3.0e-134	
		Ciona intestinalis) (Sea squirt)	45	2.0e-123	
		Nematostella vectensis) (Sea anaemonae)	39	5.0e-111	
		Branchiostoma floridae (Lancelet)	42	3.0e-61	

The oestrogen receptor primary protein drug target for the anti-cancer drug tamoxifen and the synthetic hormone EE2 showed very high sequence identity with a number of non target organisms (Table 4.3). Fish species included: Commercially relevant fish *Oncorhynchus mykiss* (rainbow trout) and *Salmo salar* (Atlantic salmon), British freshwater fish *Cyprinus carpio* (Common carp) and *Rultilus rutilus* (Roach) and ecotoxicological test fish *Danio rerio* (zebra fish) and *Pimephales promelas* (fathead minnow). Other organisms that have an oestrogen receptor with significant sequence identity to the human form included turtles, frogs, newt and crocodile.

The COX 1 and 2 target proteins for the analgesics diclofenac, ibuprofen and paracetamol were found to have significant similarity to the human forms including *Oncorhynchus mykiss* (rainbow trout), *Salmo salar* (Atlantic salmon), *Micropogonias undulates* (Atlantic croaker), *Danio rerio* (zebra fish), *Tetraodon nigroviridis* (pufferfish), *Ornithorhynchus anatinus* (platypus) and the frogs, *Xenopus laevis* (African clawed frog) and *Xenopus tropicalis* (Western clawed frog) (Table 4.4). A COX enzyme or protein with sequence homology was not found for the *Pimphales promelas* (fathead minnow), one of the species of fish regularly used in ecotoxicology. *Daphnia pulex* also produces a COX2 enzyme with 46% sequence homology with the COX2 human enzyme.

The primary drug target for gemfibrozil showed a high sequence homology with several fish including the British freshwater fish *Cyprinus carpio* (common Carp) and the ecotoxicological test relevant fish *D.rerio* (Table 4.5). Other organisms with high sequence homology included the frog *X. laevis* and *Anas platyrhynchos* (Mallard duck), *Ornithorhynchus anatinus* and *Crocodylus niloticus* (crocodile).

Propranolol had five identified drug target proteins. The primary target beta 1-adrenergenic receptor had significant sequence identity with several proteins in other species including the British freshwater fish, *C. carpio*, commercially relevant fish *S.salar* and *O.mykiss* and the ecotoxicological test fish *P.promelas* and *D.rerio* (Table 4.5). High sequence identity was also found with *O.anatinus* (Platypus) and *X.laevis* (African clawed frog).

The sodium channel protein drug target for carbamazepine showed a high sequence homology with *O.mykiss* (72%) and *D.rerio* (64%) but not with the *S.salar* or *P.promelas* (Table 4.6), indicating a strong possibility that a similar mode of action may

be occurring in some fish but not others (see discussion). The frog *X.laevis* also had a protein with 52% similarity (Table 4.6). It appears possible that frogs may be responding to this recalcitrant (section 2) anti- convulsive in the environment.

The anti-depressant fluoxetine drug target the sodium dependant serotonin transporter showed high sequence homology with several fish and both the frogs that have genome sequences available, *X.tropicalis* and *X.laevis* (Table 4.6). Therefore environmental exposure to fluoxetine will probably cause disruption in serotonin metabolism and function in these organisms.

Table 4.4 Analgesic drug target sequence homology results

(I: percent sequence identity; E value: the probability of this identity occurring by chance; Ecotoxicity, YES = relevant literature to support mode of action (MoA) effects of the particular pharmaceutical on the primary drug target receptor, references: David & Pancharatna, 2009[1]; Han *et al*., 2010[2]; Heckmann *et al*., 2007[3]; Mehinto *et al*., 2010[4]; NA: Ecotoxicity data not available).

Pharmaceutical	Drug Target	Organism	I	E value	Ecotoxicity
diclofenac ibuprofen paracetamol	Prostaglandin G/H synthase 1 precursor (COX1)	*Ornithorhynchus anatinus* (Platypus)	76	0.0	NA
		Taeniopygia guttata (Zebra Finch)	76	0.0	NA
		Xenopus laevis (African clawed frog)	74	0.0	NA
		Micropogonias undulates (Atlantic croaker)	69	0.0	NA
		Fundulus heteroclitus (Mummichog)	68	0.0	NA
		Myoxocephalus octodecemspinosus (Longhorn sculpin)	68	0.0	NA
		Salvelinus fontinalis (Brook trout)	72	0.0	NA
		Salmo salar (Atlantic salmon)	69	0.0	NA
		Danio rerio (Zebrafish)	68	0.0	YES[1]
		Squalus acanthias (Spiny dogfish)	67	0.0	NA
		Tetraodon nigroviridis (Pufferfish)	67	0.0	NA
		Xenopus tropicalis (Western clawed frog)	63	0.0	NA
		Oryzias latipes (Medaka fish)	64	0.0	YES[2]
		Dapnnia.magna	31	3.2	YES[3]
		Daphnia pulex	32	8.6	NA
diclofenac ibuprofen paracetamol	Prostaglandin G/H synthase 2 precursor (COX2)	*Ornithorhynchus anatinus* (Platypus)	86	0.0	NA
		Xenopus tropicalis (Western clawed frog)	74	0.0	NA
		Xenopus laevis (African clawed frog)	74	0.0	NA
		Taeniopygia guttata (Zebra Finch)	81	0.0	NA
		Danio rerio (Zebrafish)	75	0.0	YES[1]
		Fundulus heteroclitus (Mummichog)	72	0.0	NA
		Oncorhynchus mykiss (Rainbow trout)	72	0.0	YES[4]
		Myoxocephalus octodecemspinosus (Longhorn sculpin)	71	0.0	NA
		Micropogonias undulates (Atlantic croaker)	71	0.0	NA
		Salvelinus fontinalis (Brook trout)	71	0.0	NA
		Dicentrarchus labrax (European Seabass)	73	0.0	NA
		Myxine glutinosa (Hagfish)	63	0.0	NA
		Squalus acanthias (Spiny dogfish)	62	0.0	NA
		Salmo salar (Atlantic salmon)	67	0.0	NA
		Oryzias latipes (Medaka fish)	73	0.0	YES[2]
		Daphnia pulex	46	0.0	NA
diclofenac	Transthyretin precursor	*Ornithorhynchus anatinu)* (Platypus)	65	7.0e-53	
		Anas platyrhynchos (Mallard Duck)	70	4.0e-56	
		Xenopus laevis (African clawed frog)	58	2.0e44	
		Taeniopygia guttata (Zebra Finch)	68	4.0e-52	
		Rana catesbeiana (Bullfrog)	56	8.0e-41	
		Xenopus tropicalis (Western clawed frog)	59	7.0e-39	
		Perca flavescens (Yellow perch)	54	1.0e37	
		Cyprinus carpio (Common carp)	51	2.0e-35	
		Sparus aurata (Gilt-Head bream)	51	4.0e-35	
		Danio rerio (Zebrafish)	48	6.0e-34	
		Epinephelus coioides Orange spotted grouper)	50	1.0e-33	
ibuprofen	Serum albumin precursor	*Ornithorhynchus anatinus* (Platypus0	61	4.0e-150	
		Taeniopygia guttata (Zebra Finch)	44	1.0e-147	
		Bombina maxima (Giant fire bellied toad)	40	5.0e-138	
		Ambystoma maculatum (Salamander)	40	9.0e-136	
		Xenopus tropicalis (Western clawed frog)	39	2.0e-130	
		Xenopus laevis (African clawed frog)	39	2.0e-127	
		Oncorhynchus mykiss (Rainbow trout)	35	7.0e-24	

Table 4.5 Gemfibrozil and propranolol drug target sequence homology results

(I: percent sequence identity; E value: the probability of this identity occurring by chance; Ecotoxicity: YES = Ecotoxicity data available in the literature to support mode of action effects of the particular pharmaceutical on the primary drug target receptor: Finn *et al*., 2012[1]; Bartram *et al*., 2012[2]; Petersen *et al*., 2013[3]; NA: ecotoxicity data not available).

Pharmaceutical	Drug Target	Organism	I	E value	Ecotoxicity
Propranolol	Beta- 1-adrenergenic receptor	*Ornithorhynchus anatinus* (Platypus)	65	4.0e-157	NA
		Xenopus laevis (African clawed frog)	61	4.0e-138	NA
		Tetraodon nigroviridis (Pufferfish)	61	2.0e-127	NA
		Danio rerio (Zebra fish)	55	5.0e-127	YES[1]
		Oncorhynchus mykiss (Rainbow trout)	51	5.0e-109	YES[2,3]
		Salmo salar (Atlantic salmon)	51	1.0e-104	NA
		Pimphales promelas (Fathead minnow)	58	9e-156	NA
		Cyprinus carpio (Common carp)	50	5e-120	NA
Propranolol	5-hydroxytryptamine 1A receptor	*Taeniopygia guttata* (Zebra Finch)	79	0.0	
		Danio rerio (Zebra fish)	75	1.0e-173	
		Platichthys flesus (European flounder)	76	9.0e-169	
		Tetraodon nigroviridis (Pufferfish)	69	6.0e-164	
		Opsanus beta (Gulf Toad fish)	70	1.0e-163	
		Xenopus laevis (African clawed frog)	70	7.0e-155	
		*Lates calcarifer (*Barramundi)	67	4.0e-109	
		Branchiostoma floridae (Lancelet)	44	2.0e-88	
		Mizuhopecten yessoensis (Scallop)	39	7.0e-84	
		Aplysia californica (Sea slug)	41	5.0e-82	
		Helisoma trivolvis (Freshwater snail)	40	8.0e-79	
		Procambarus clarkii (Freshwater crayfish)	38	9.0e-76	
		Panulirus interruptus (California spiny lobster)	39	2.0e-71	
		Macrobrachium rosenbergii (Giant prawn)	39	4.0e-68	
propranolol	β-2 adrenergenic receptor	*Galemys pyrenaicus* (Pyrenean Desman)	94	2.0e-153	
		Sylvilagus floridanus (American Beaver)	93	8.0e-152	
Propranolol	5-hydroxytryptamine 1B receptor	*Taeniopygia guttata* (Zebra Finch)	87	0.0	
		Tetraodon nigroviridis (Pufferfish)	69	3.0e-146	
		Danio rerio (Zebra fish)	64	3.0e-138	
		Ornithorhynchus anatinus (Platypus)	58	6.0e-111	
		Branchiostoma floridae (Lancelet)	43		
propranolol	beta- 3-adrenergenic receptor	*Oncorhynchus mykiss* (Rainbow trout)	53	4.0e-95	
		Salmo salar (Atlantic salmon)	53	2.0e-91	
		Xenopus laevis (African clawed frog)	52	2.0e-90	
		Ornithorhynchus anatinus (Platypus)	53	3.0e-90	
Gemfibrozil	Peroxisome proliferator-activated receptor alpha	*Taeniopygia guttata* (Zebra Finch)	88	0.0	NA
		Anas platyrhynchos (Mallard duck)	88	0.0	NA
		Xenopus laevis (African clawed frog)	81	0.0	NA
		Ornithorhynchus anatinus (Platypus)	90	0.0	NA
		Pagrus major (Red seabream)	73	0.0	NA
		Lateolabrax japonicas (Sea perch)	73	0.0	NA
		Salmo salar (Atlantic salmon)	73	0.0	NA
		Ctenopharyngodon idella (Grass carp)	72	0.0	NA
		Danio rerio (Zebra fish)	71	0.0	NA
		Tetraodon nigroviridis (Pufferfish)	77	0.0	NA
		Crocodylus niloticus (Crocodile)	92	7.0e-180	NA
		Sparus aurata (Sea bream)	65	2.0e-177	NA
		Dentex dentex (Dentex fish)	64	1.0e-174	NA
		Rachycentron canadum (Cobia fish)	64	1.0e-174	NA
		Pleuronectes platessa (Plaice)	64	1.0e-168	NA
		Orizias latipes (Medaka fish)	59	6.0e-164	NA
		Sparus aurata (Gilthead bream)	65	1.0e-155	NA
		Cyprinus carpio (Common Carp)	67	6e-96	NA
gemfibrozil	Lipoprotein lipase precursor	*Ornithorhynchus anatinus* (Platypus)	85	0.0	
		Anas platyrhynchos (Mallard duck)	76	0.0	
		Danio rerio (Zebra fish)	61	1.0e-177	
		Ctenopharyngodon idella (Grass carp)	62	2.0e-175	
		Cyprinus carpio (Common carp)	63	1.0e-173	
		Oncorhynchus mykiss (Rainbow trout)	62	2.0e-172	
		Dicentrarchus labrax (Seabass)	63	3.0e-172	
		Sparus aurata (Gilthead bream)	62	2.0e-170	
		Thunnus niloticus (Bluefin Tuna)	56	2.0e-148	
		Xenopus laevis (African clawed frog)	50	6.0e-126	

Table 4.6 Fluoxetine and carbamazepine drug target sequence homology results
(**I**: percent sequence identity; **E value**: the probability of this identity occurring by chance; **Ecotoxicity**: **YES** = Ecotoxicity data available in the literature to support mode of action (MoA) effects of the particular pharmaceutical on the primary drug target receptor, references: Lister *et al*., 2011[1]; Painter *et al*., 2009[2]; Schultz *et al*., 20113; Conners *et al*., 2009[4]; Li *et al*., 2009[5]; **NA**: Ecotoxicity data not available)

Pharmaceutical	Drug Target	Organism	I	E value	Ecotoxicity
fluoxetine citalopram escitalopram zimelidine	Sodium dependant serotonin transporter	*Oncorhynchus mykiss* (Rainbow trout)	34	8.4	NA
		Salmo salar (Atlantic salmon)	44	2.0e-137	NA
		Danio rerio (Zebra fish)	69	0.0	YES[1]
		Pimphales promelas (Fathead minnow)	53	1.0e-40	YES[2,3]
		Xenopus laevis (African clawed frog)	44	4.0e-142	YES[4]
		Xenopus tropicalis (Western clawed frog)	42	1.0e-139	NA
carbamazepine	Sodium chanel protein type 5 subunit alpha	*Oncorhynchus mykiss* (Rainbow trout)	72	0.0	YES[5]
		Salmo salar (Atlantic salmon)	24	3.0e-07	NA
		Daphnia magna	35	6.8	NA
		Danio rerio (Zebra fish)	64	0.0	NA
		Pimphales promelas (Fathead minnow)	22	3.8	NA
		Takifugu rubripes (Japanese killifish)	19	4.0e-06	NA
		Xenopus laevis (African clawed frog)	52	2.0e-159	NA
		Hydra vulgaris (Freshwater hydroid)	33	2.0e-60	NA
		Lymnaea stagnalis (Pond snail)	32	1.0e-86	NA

4.4.3 Multiple sequence alignment (COX2)

Two drugs were chosen to test the potential for using molecular docking as an aid in ecotoxicology tests of pharmaceuticals. These were diclofenac and ibuprofen (for drug choice rationale see Section 4.1.4). Sequence alignment is useful for locating conserved regions in the amino acid sequence of proteins. Conserved regions often form the binding sites of enzymes. The homologues of five species were compared to the primary drug target for diclofenac and ibuprofen, the COX 2 enzyme. These species were: *Ovis aries* (sheep), because it was this species that was used to obtain the crystal structure of COX2 (Rowlinson *et al*., 2004), *O.mykiss* (used in several ecotoxicological studies (Hoeger *et al*., 2004; Schwaiger *et al*., 2004), *S.salar*, *D.rerio* and *D.pulex* (regularly used in ecotoxicity tests). As a COX2 homologue for *P.promelas* was not found in the BLAST search, this ecotoxicological test species was not included. The CLUSTAL W multiple sequence alignment for the *H.sapiens*, (human), *D.rerio*, *O.mykiss*, *S.salar* and *D.pulex* and *O.aries* (sheep) COX2 homologues are displayed (Fig 4.2). The multiple sequence alignment shows the differences in numbering of amino acid residues that can occur when aligning sequences. The amino acid residues found to be important for binding of diclofenac in the crystal structure (Rowlinson *et al*., 2004) and other NSAIDs (Pouplana *et al*., 2002) by hydrogen bonding are highlighted in red. The amino acid residues

arginine (R), tyrosine (Y), and serine (S) are contained in highly conserved regions of the protein sequence and form part of the binding pocket. The molecular docking experiments with ibuprofen and diclofenac with the *D.pulex* COX2 homologue were unsuccessful potentially due to a difference in amino acid at residue 525 from leucine to isoleucine (highlighted in blue) (Fig 4.2).

```
Ovis aries              SRSHLIESPPTYNVHYSYKSWEAFSNLSYYTRALPPVPDDCPTPMGVKGRKELPDSKEVV 163
Homo sapiens            SRSHLIDSPPTYNADYGYKSWEAFSNLSYYTRALPPVPDDCPTPLGVKGKKQLPDSNEIV 164
Danio rerio             SRAHLIDSPPTFNADYGYKSWEAYSNLSYYTRTLPPVPRDCPTPMGVAGKKELPDVKMLA 166
Oncorhynchus mykiss     PRSHLVDSPPTYNADYGYKSWEAYSNLFYYTRTLPPLPKDCPTPMGTAGRAVLPDVKLVV 167
Salmo salar             VRSNLIPSPPTFNSKYGYLSWESYSNVSYYTRILPPVPEDCPTPMGTKGKSVLPDPKLVV 178
Daphnia pulex           SRGAAIQSPPRFNSGHDYITTQSHFNTSYYARSLPPVPQHCPTPMGVAGHGELPDIDELA 171

Ovis  aries             FKLKFDPELLFN-QQFQYQNRIAAEFNTLYHWHPLLPDVFQIDGQEYNYQQFIYNNSVLL 400
Homo sapiens            FKLKFDPELLFN-KQFQYQNRIAAEFNTLYHWHPLLPDTFQIHDQKYNYQQFIYNNSILL 401
Danio rerio             FKLKFDPELLFN-ERFQYQNRISSEFNTLYHWHPLMPDDFHIQDEVYNYQQFLFNTSILT 403
Oncorhynchus mykiss     FQLKFDPELLFN-QRFQYQNRIAAEFNTLYHWHPLMPETFSIEDRAYTYPQFVFNNSLVT 404
Salmo salar             LDLKFDPVLLFK-STFQYRNRIAVEFKQLYHWHPLMPDSFHIDGDVVPYSQFMFNTSIVT 415
Daphnia pulex           VKLSYDPELLRDEPQFQFSNRIHVEFAHLYHWHPMAPEAITLGNNTYTLEQMSFSTKTVA 410

Ovis aries              ESFEELTG-EKEMAAELEALYGDIDAMELYPALLVEKPAPDAIFGETMVEAGAPFSLKGL 519
Homo sapiens            ESFEELTG-EKEMSAELEALYGDIDAVELYPALLVEKPRPDAIFGETMVEVGAPFSLKGL 520
Danio rerio             RSFEEMTG-EKEMAAELEEMYGDVDAVELYAGLLVEKPRSNAIFGETMVEMGAPYSLKGL 522
Oncorhynchus mykiss     TSFEDLTG-ETELAAELESLYGDVDAVELYPGLLVERPRPNAVFGETMVEMGAPYSLKGL 523
Salmo  salar            TSFSDFTG-EEEIARELEELYGDIDALEFYPAIMLEKTRPNAIFGESMVEMGAPFSLKGL 534
Daphnia pulex           TSFMELTGGDVDLSRQLDKLYGDIDALEFYPGMLLEKS-DSSVTPFTMVNIGGPYAIKGM 528

Ovis aries              MGNPICSPEYWKPSTFGGEVGFKIINTASIQSLICSNVKG--CPFTSFSVQDA----HLT 573
Homo sapiens            MGNVICSPAYWKPSTFGGEVGFQIINTASIQSLICNNVKG--CPFTSFSVPDP----ELI 574
Danio rerio             MGNPICSPEYWKPSTFGGKVGFEIVNSASLQNLVCNNVNGP-CPMASFYVPNV----KDS 577
Oncorhynchus mykiss     LGNPICSPEYWMPSTFGGSVGFDILNTASLERLVCNNVKGS-CPMVSFQVPDF----LRA 578
Salmo  salar            LGNPICSPEYWKPSTFGGQTGFDIVNSASLERLVCLNTNW--CPYVAFNVPPA----GQE 588
Daphnia pulex           MANPISSPHYWKPSTFGGPVGFDIVKSTTIKDLFCRNMKPGECGHIAFHLPTTEGQSQQQ 588
```

Fig 4.2 CLUSTAL W multiple sequence alignment of COX 2 enzymes
(Conserved residues involved in binding of diclofenac and ibuprofen arginine, tyrosine and serine highlighted red; mutation in leucine to isoleucine in *Daphnia pulex* highlighted blue)

4.4.4 Molecular docking

Molecular docking experiments were then performed for the human, *O.mykiss*, *S.salar*, *D.rerio* and *D.pulex* COX2 homologues with diclofenac and ibuprofen. All of the

dockings were successful except *D.pulex*. Diclofenac and ibuprofen failed to bind to the *D.pulex* COX2 homologues. This suggests that although the protein was of high similarity to the human form, some change in the residue sequence must prevent it from binding diclofenac or ibuprofen. This could be due to a change in the leucine residue at position 525 (Fig 4.2). This leucine residue can be seen in the binding pocket very close to the location of the docked diclofenac and ibuprofen molecules (Leu525 in Fig 4.13; Leu 531 in Fig 4.15; Leu 522 in Fig 4.17; Leu 517 in Fig 4.19; Leu521 in Fig 4.21).

For each of the eight separate successful docking experiments the ten best (i.e. the ten lowest binding energies) docked molecules (COX2) and ligands (diclofenac and ibuprofen) all docked in the same binding pocket (Fig 4.3-4.10). The results show that in each case the ten best docked drugs were positioned in the same orientation directly on top of one another. This indicates the reliability and the reproducibility of the results. The free energy of binding for each of the separate experiments ranged from -7.62 to -5.9 $kjmol^{-1}$ (Table 4.7). The free energy of binding for each of the drugs to each COX 2 homologue was very similar within each separate experiment. Ibuprofen had a lower free energy of binding than diclofenac for all the COX2 homologues apart from *D.rerio*. The reason for this is unknown.

Three amino acid residues were identified as important for binding of diclofenac and ibuprofen in the COX 2 binding pocket. Hydrogen bonding between occurred between either arginine, tryrosine or serine and the drug molecules diclofenac or ibuprofen. In all the dockings hydrogen bonding occurred between one or more of these residues and the drugs. An example of the hydrogen bonding between each of the docked drugs and the different COX homologues are displayed (Fig 4.11, 4.13, 4.15, 4.17, 4.19, 4.21, 4.23, and 4.25). The positioning of the drug binding site for each drug and COX 2 homologue is also shown (Fig 4.12, 4.14, 4.16, 4.18, 4.20, 4.22, and 4.24). The results of the docking experiments (Fig 4.11-4.24) show that binding between the human COX 2 enzyme and diclofenac and ibuprofen are in the same binding pocket. The hydrogen bonding that occurs is similar for all these experiments indicating a strong possibility that these three fish would all respond to diclofenac and ibuprofen in the same way as humans. These dockings also show that ibuprofen and diclofenac are bound to the same amino

acid residues in the same binding pocket of the COX 2 enzyme indicating a likelihood that these two drugs could have a concentration addition effect.

Table 4.7 The ten best free energy of binding (kjmol^{-1}) for diclofenac and ibuprofen to COX 2 enzymes

Organism	Diclofenac	Ibuprofen		Organism	Diclofenac	Ibuprofen
H.sapiens	-5.16	-7.58		***S.salar***	-6.26	-7.36
	-4.99	-7.59			-6.23	-7.33
	-4.79	-7.565			-6.19	-7.34
	-4.65	-7.57			-6.15	-7.37
	-4.62	-7.56			-6.15	-7.35
	-4.61	-7.56			-6.13	-7.38
	-4.6	-7.56			-6.12	-7.34
	-4.56	-7.59			-6.08	-7.34
	-4.47	-7.57			-6.07	-7.35
	-4.47	-7.56			-6.07	-7.45
O.mykiss	-6.62	-5.88		***D.rerio***	-5.13	-7.59
	-6.6	-5.88			-5.08	-7.59
	-6.57	-5.88			-4.91	-7.58
	-6.52	-5.89			-4.9	-7.58
	-6.51	-5.88			-4.86	-7.58
	-6.47	-5.88			-4.85	-7.58
	-6.47	-5.9			-4.84	-7.62
	-6.47	-5.9			-4.81	-7.59
	-6.45	-5.88			-4.79	-7.61
	-6.44	-5.9			-4.74	-7.58

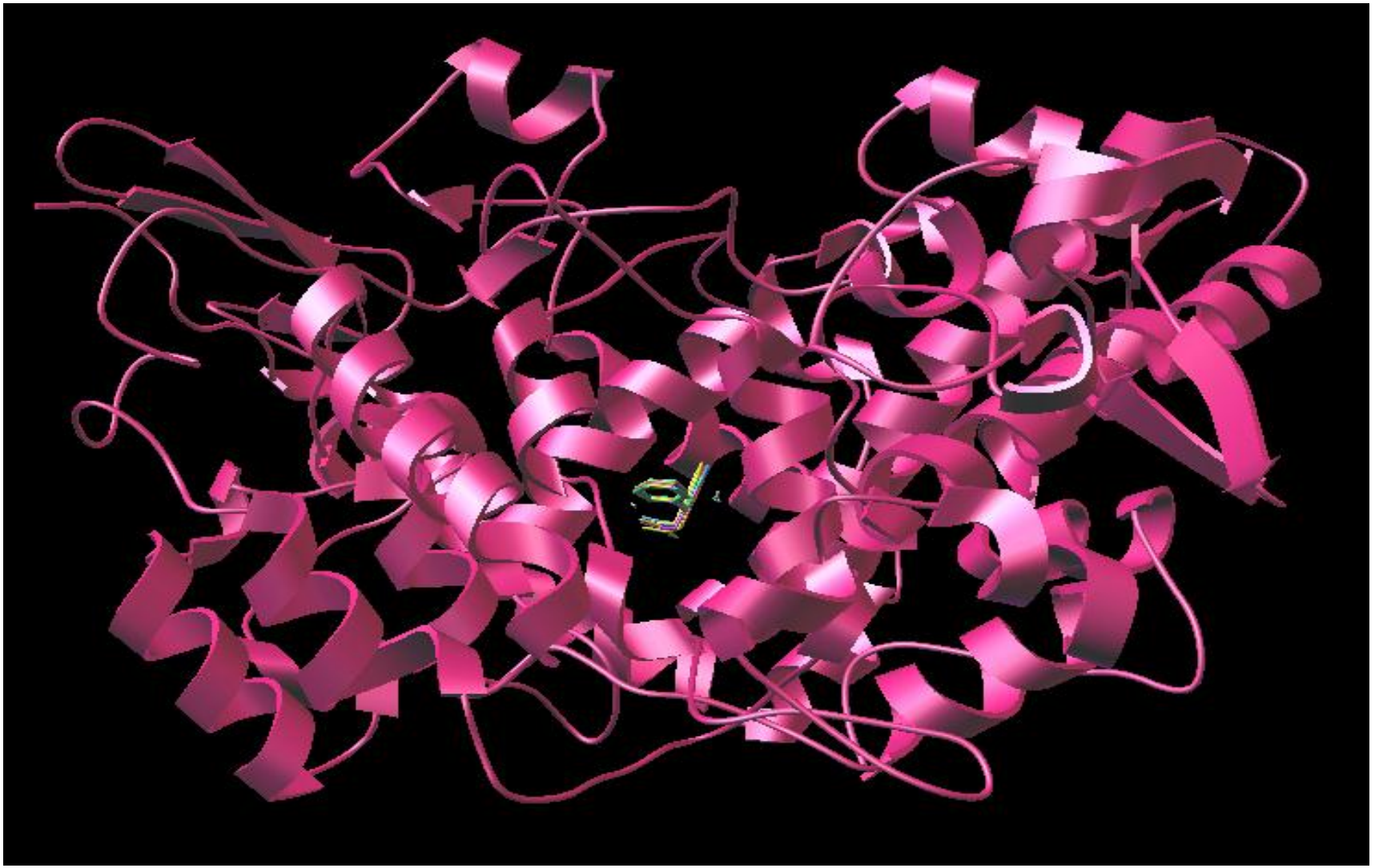


Fig 4.3 Results of the ten lowest energy dockings of *human* COX 2 and diclofenac (COX 2 shown in pink)

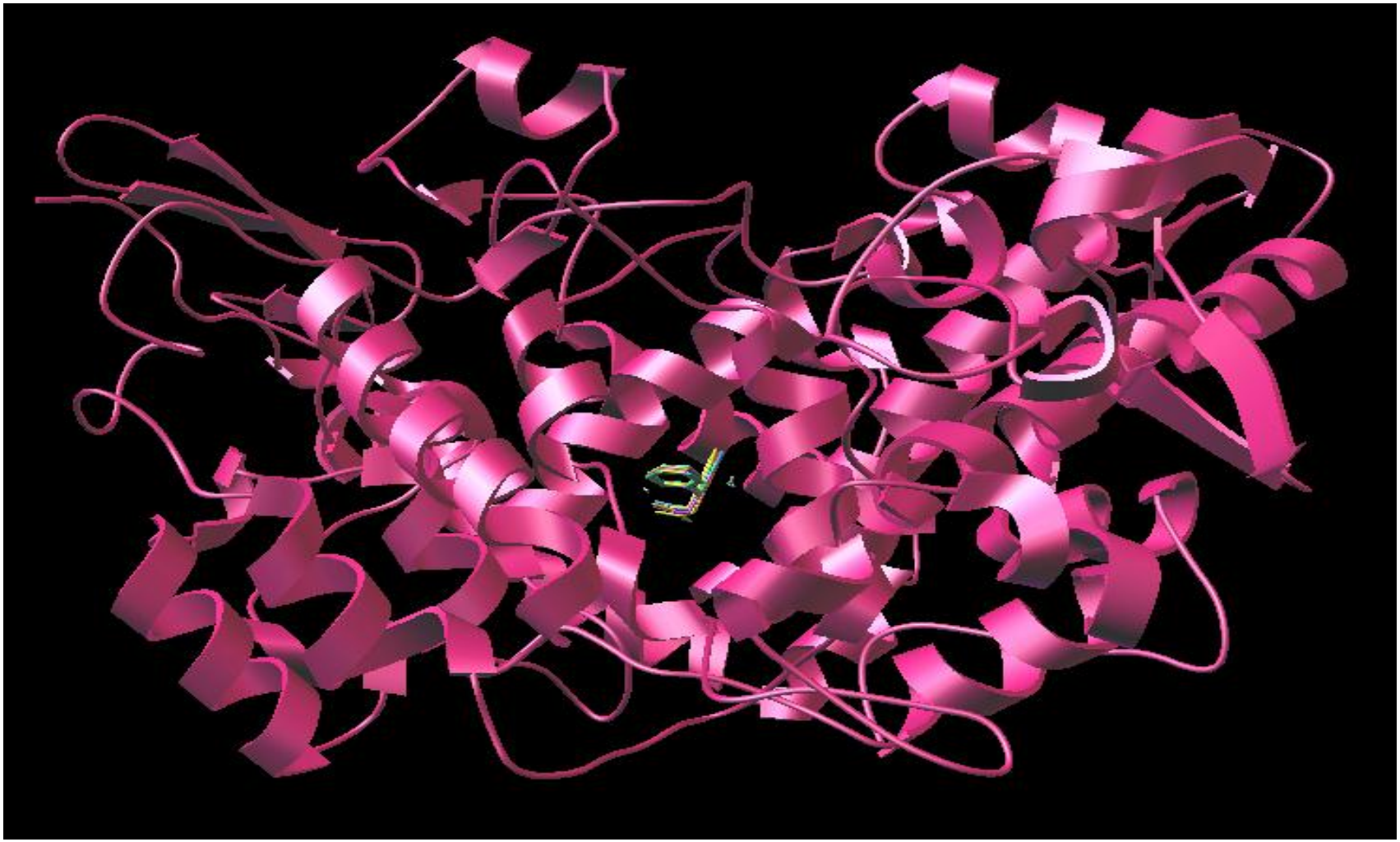


Fig 4.4 Results of the ten best lowest energy dockings of *O.mykiss* COX 2 and diclofenac (COX 2 shown in pink)

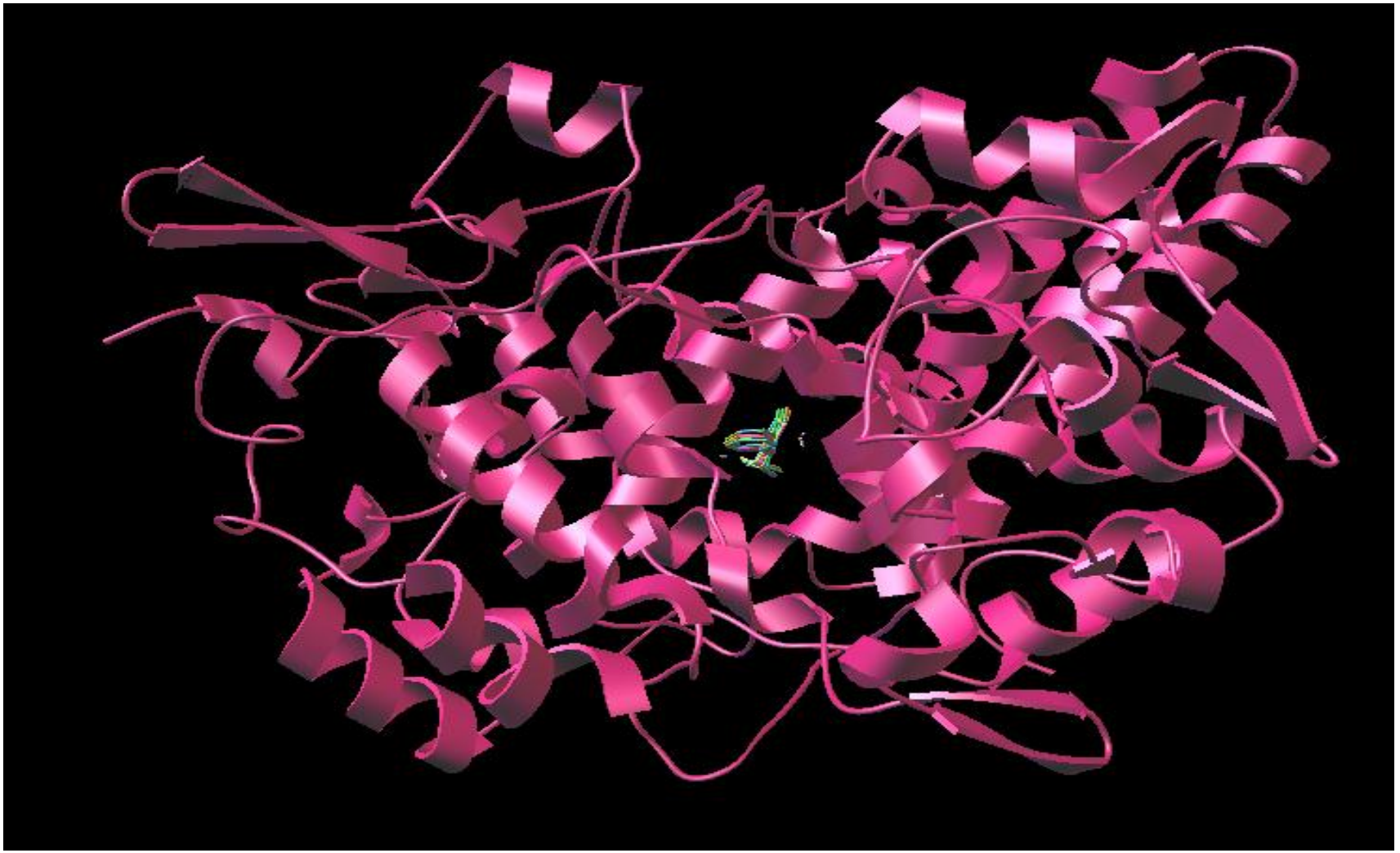


Fig 4.5 Results of the ten lowest energy dockings of *S.salar* COX 2 and diclofenac (COX 2 shown in pink)

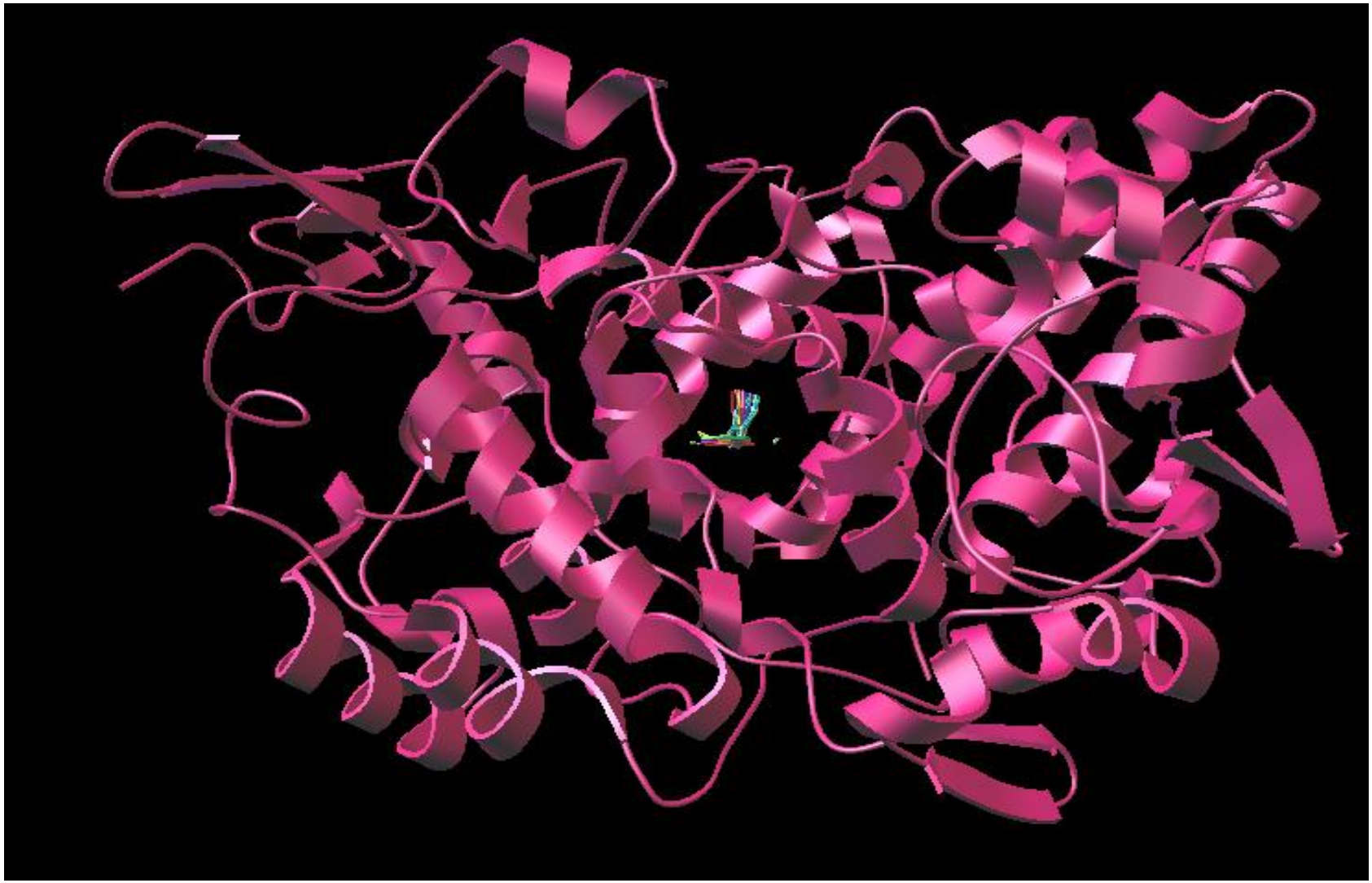


Fig 4.6 Results of the ten lowest energy dockings of *D.rerio* COX 2 and diclofenac (COX 2 shown in pink)

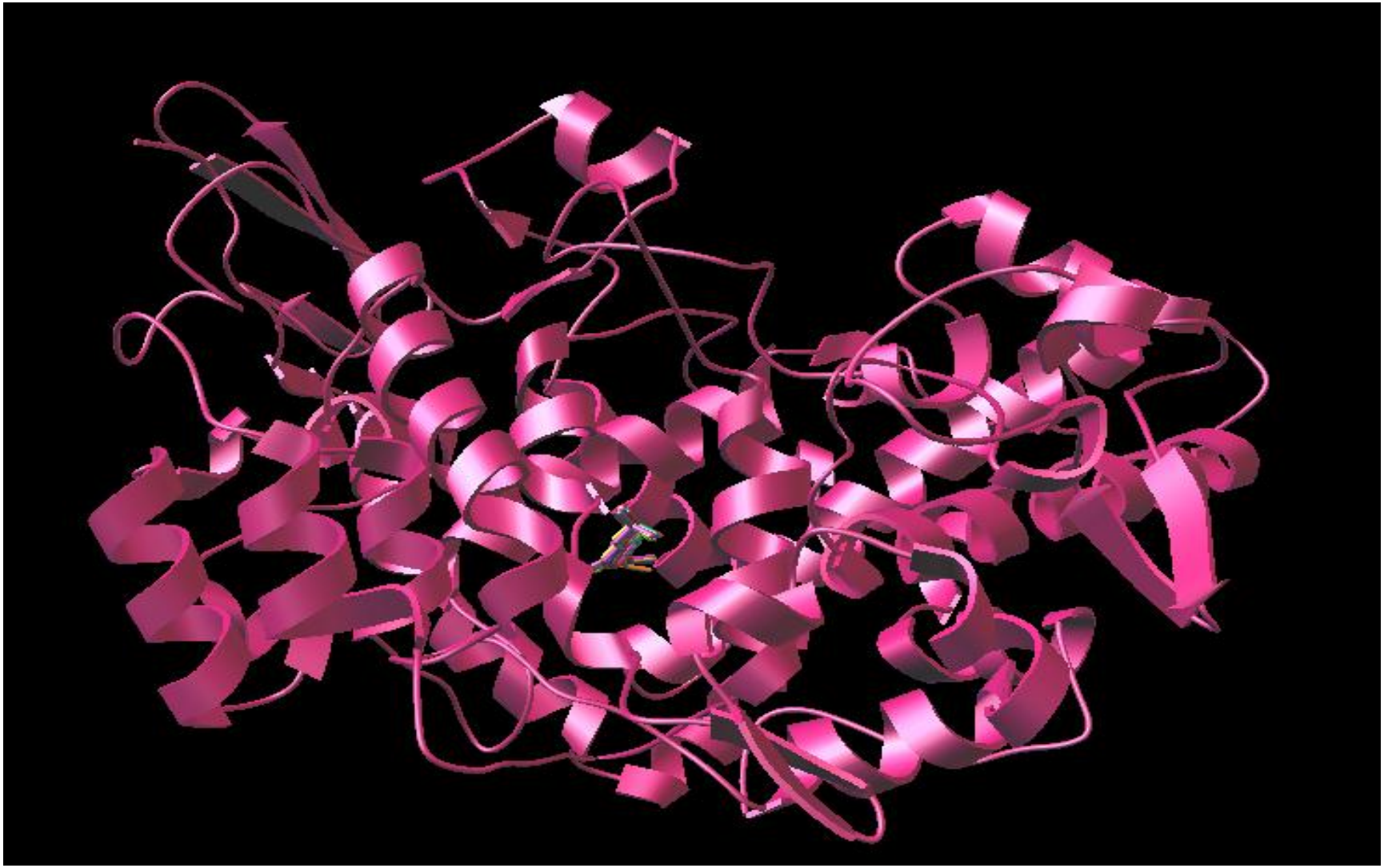


Fig 4.7 Results of the ten lowest energy dockings for *human* COX 2 and ibuprofen (COX2 shown in pink)

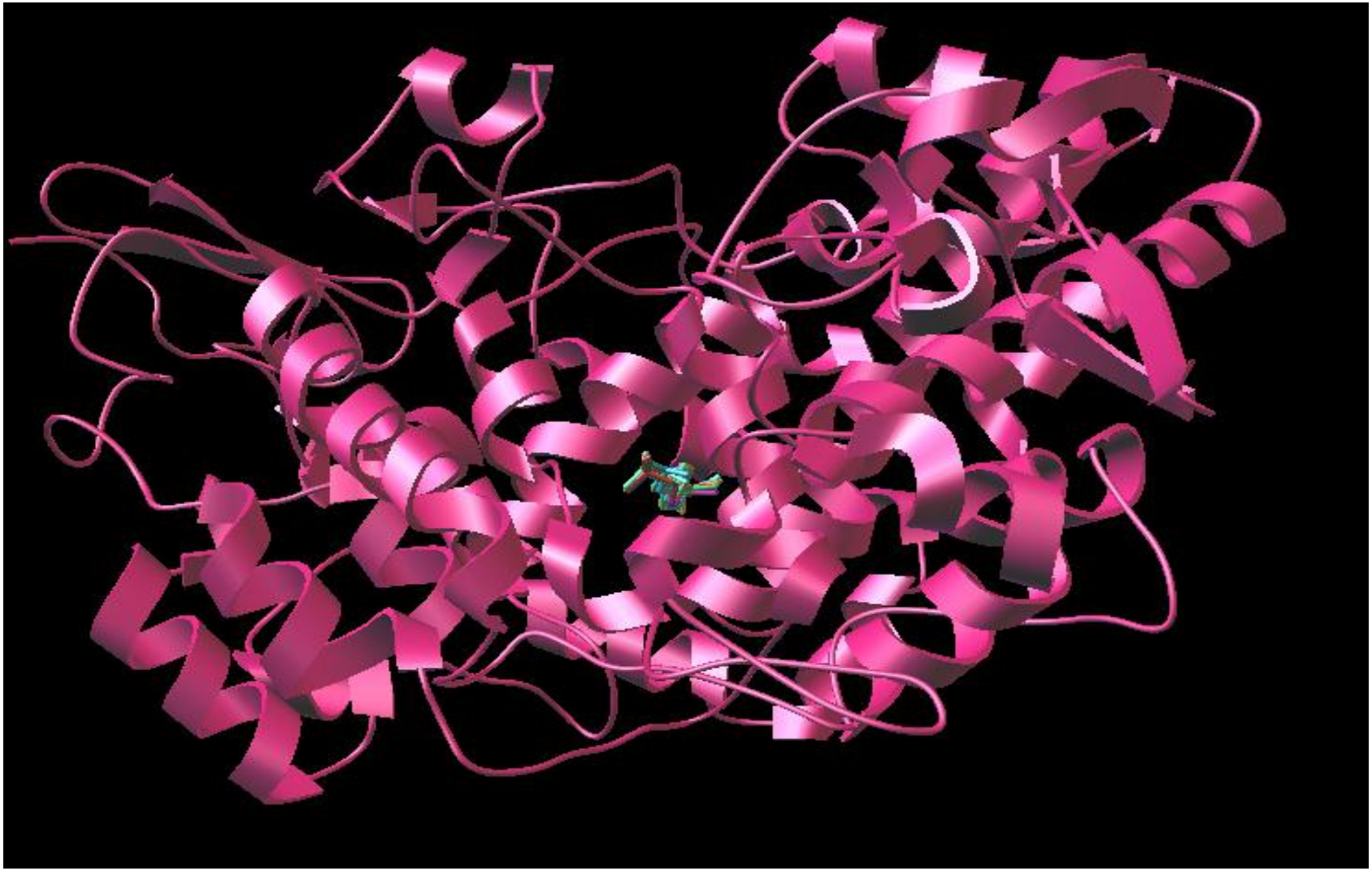


Fig 4.8 Results of the ten lowest energy dockings for *O.mykiss* COX 2 and ibuprofen (COX 2 shown in pink)

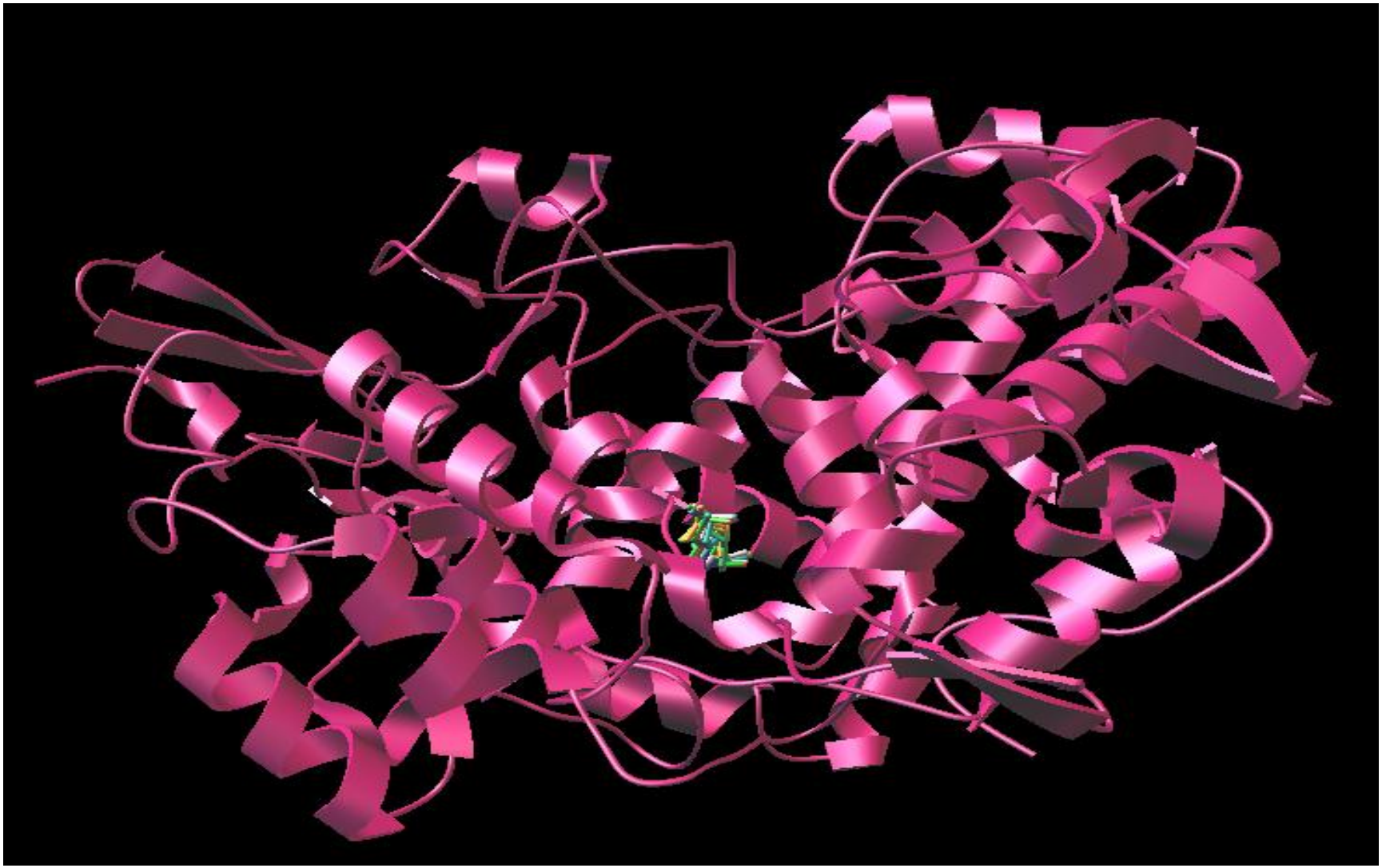


Fig 4.9 Results of the ten lowest energy dockings for *S.salar* COX 2 and ibuprofen (COX 2 shown in pink)

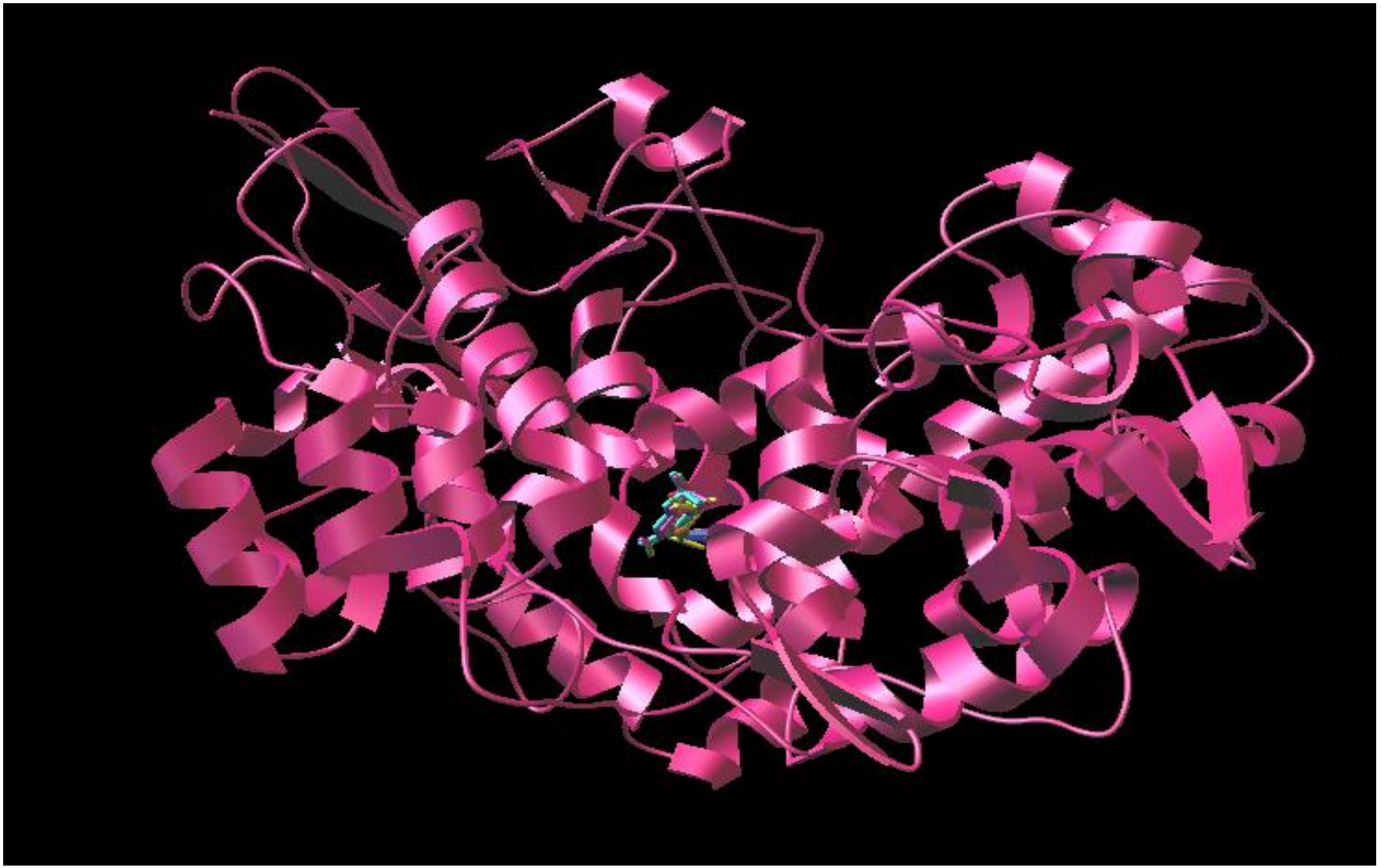


Fig 4.10 Results of the ten lowest energy dockings for *D.rerio* COX 2 and ibuprofen (COX 2 shown in pink)

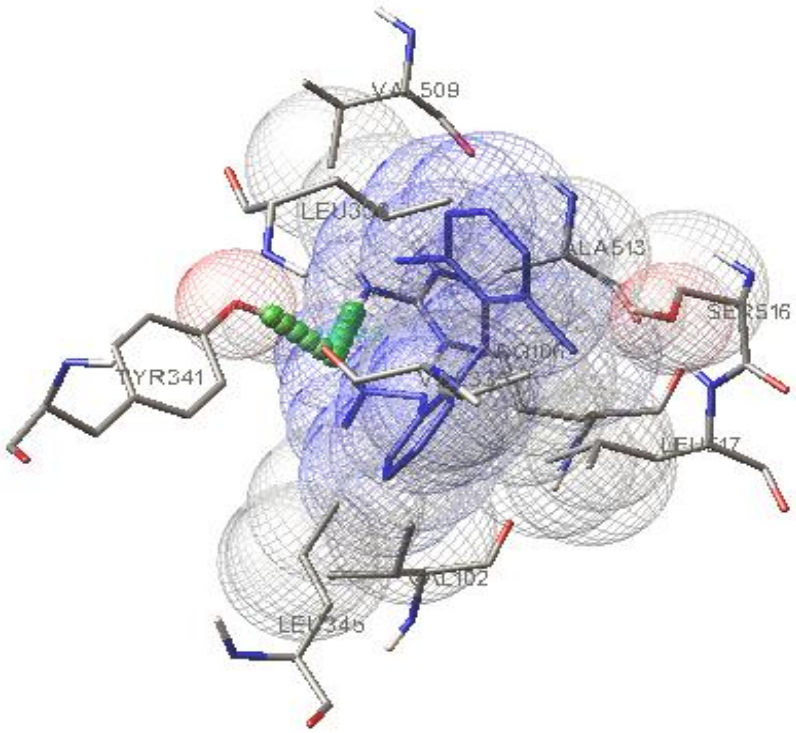


Fig 4.11 Hydrogen bonding of *human* COX 2 and diclofenac
(hydrogen bonds shown in green, diclofenac shown in blue)

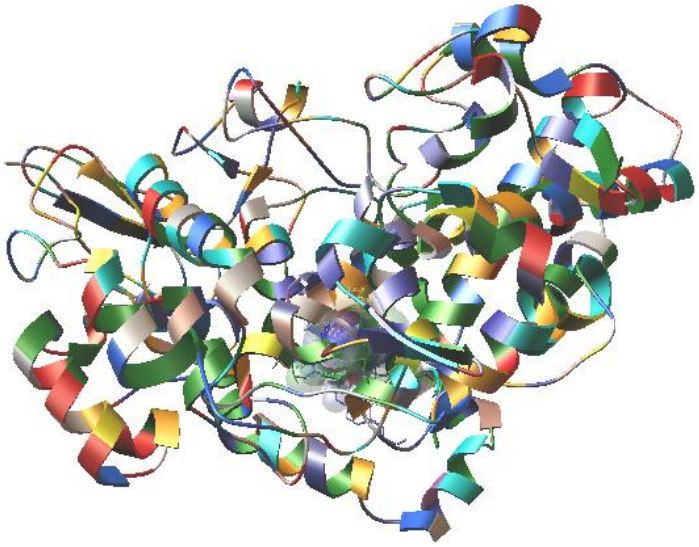


Fig 4.12 Positioning of diclofenac and *human* COX 2
(*human* COX 2 coloured by residue)

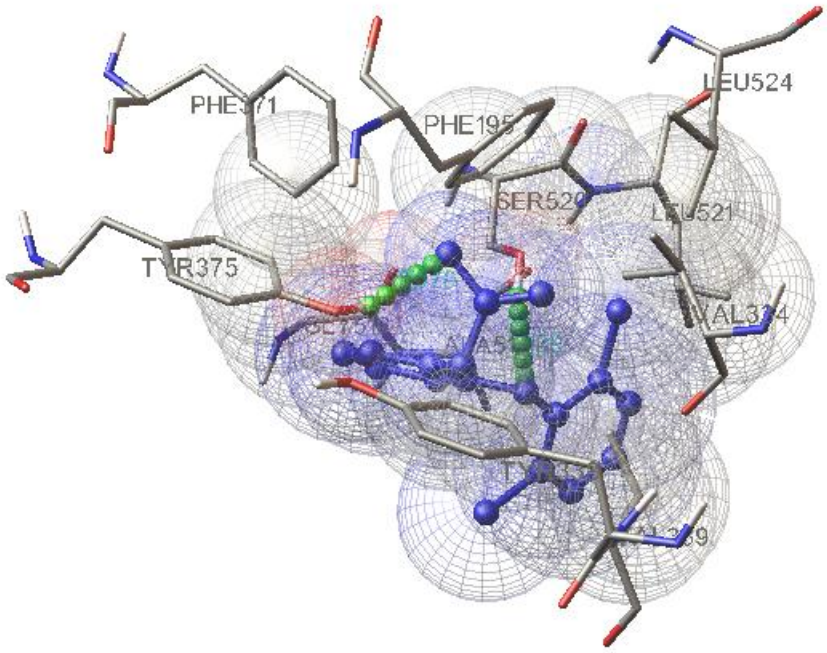


Fig 4.13 hydrogen bonding of O.*mykiss* COX 2 and diclofenac
(hydrogen bonds shown in green, diclofenac shown in blue)

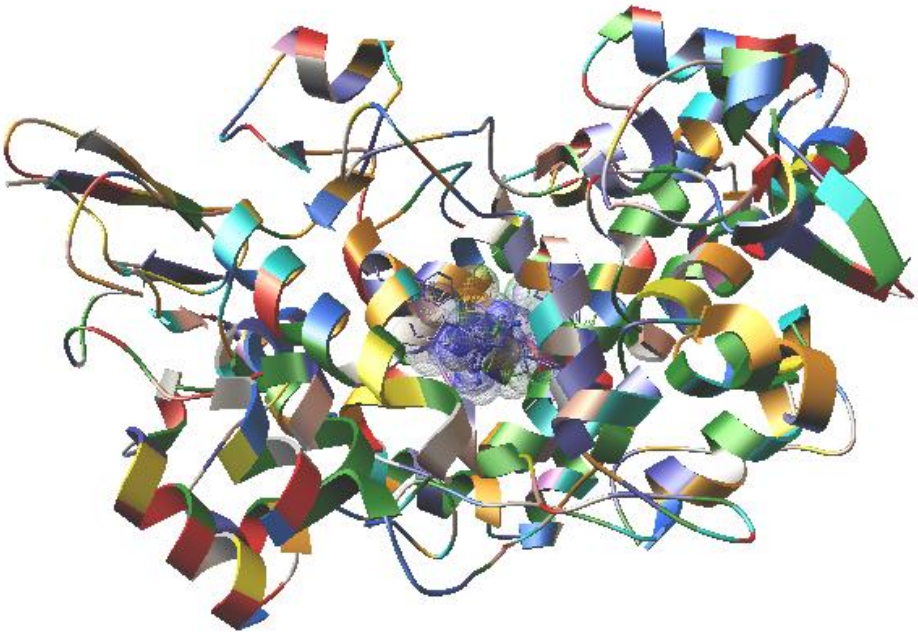


Fig 4.14 Positioning of diclofenac and *O.mykiss* COX 2
(*O.mykiss*COX 2 coloured by residue)

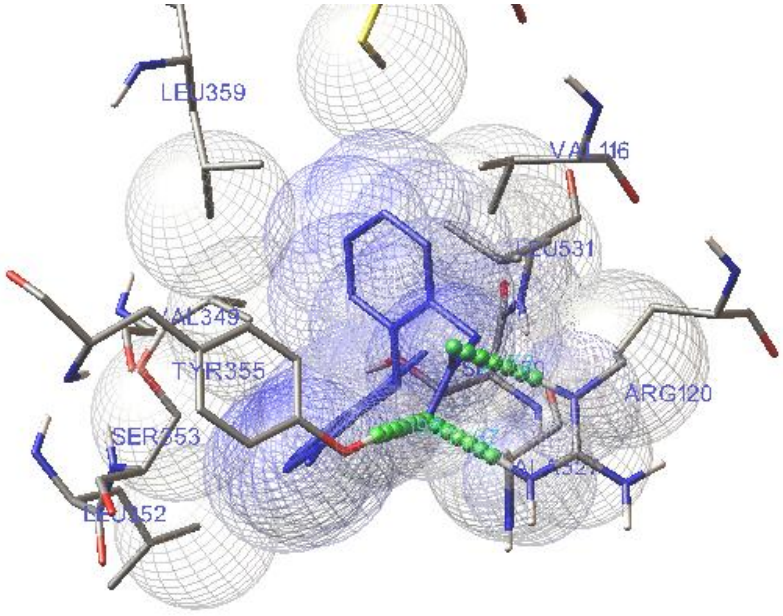


Fig4.15 Hydrogen bonding of *S.salar* COX 2 and diclofenac (hydrogen bonds shown in green; diclofenac shown in blue)

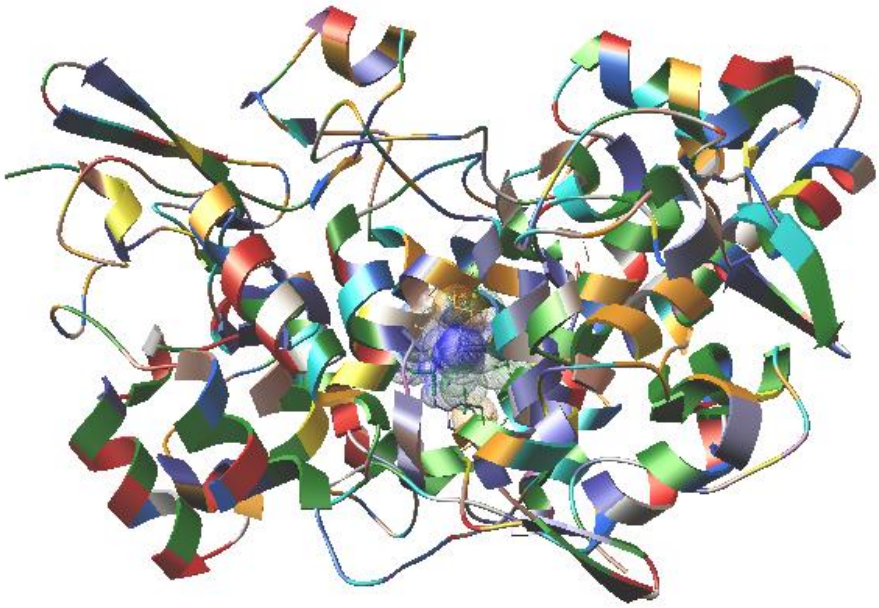


Fig 4.16 Positioning of diclofenac and *S.salar* COX 2 (COX 2 coloured by residue)

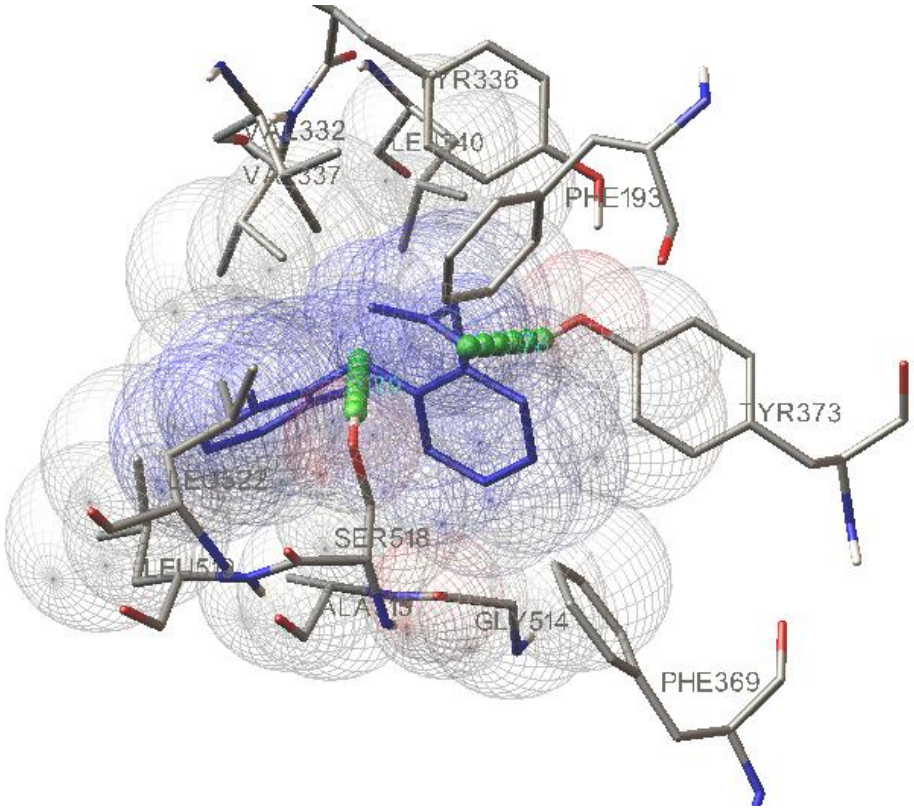


Fig4.17 Hydrogen bonding of *D.rerio* COX 2 and diclofenac (Hydrogen bonds shown in green; diclofenac shown in blue)

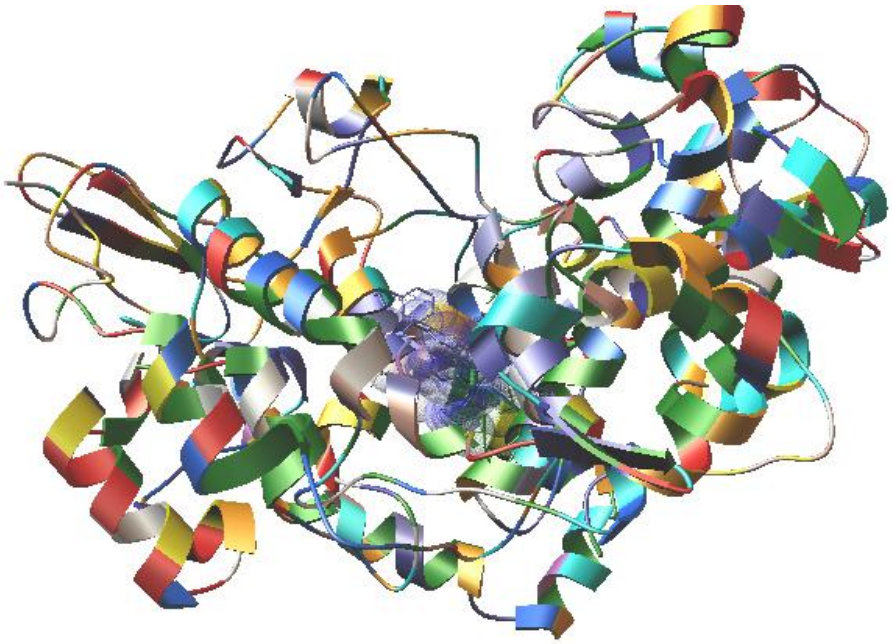


Fig 4.18 Positioning of diclofenac and *D.rerio* COX 2

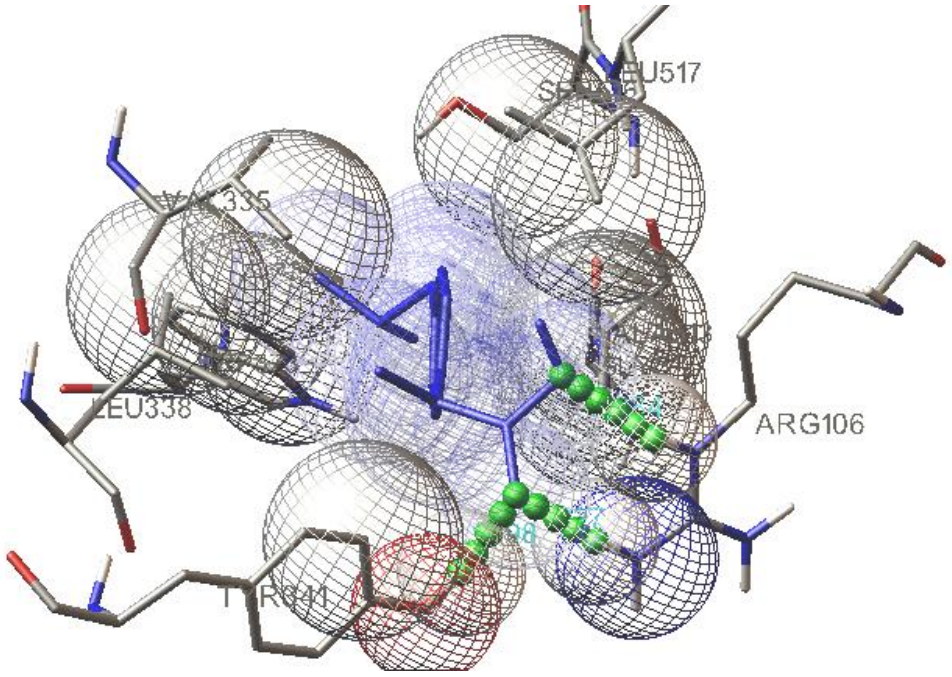


Fig 4.19 Hydrogen bonding of *human* COX 2 and ibuprofen (Hydrogen bonds shown in green; ibuprofen shown in blue)

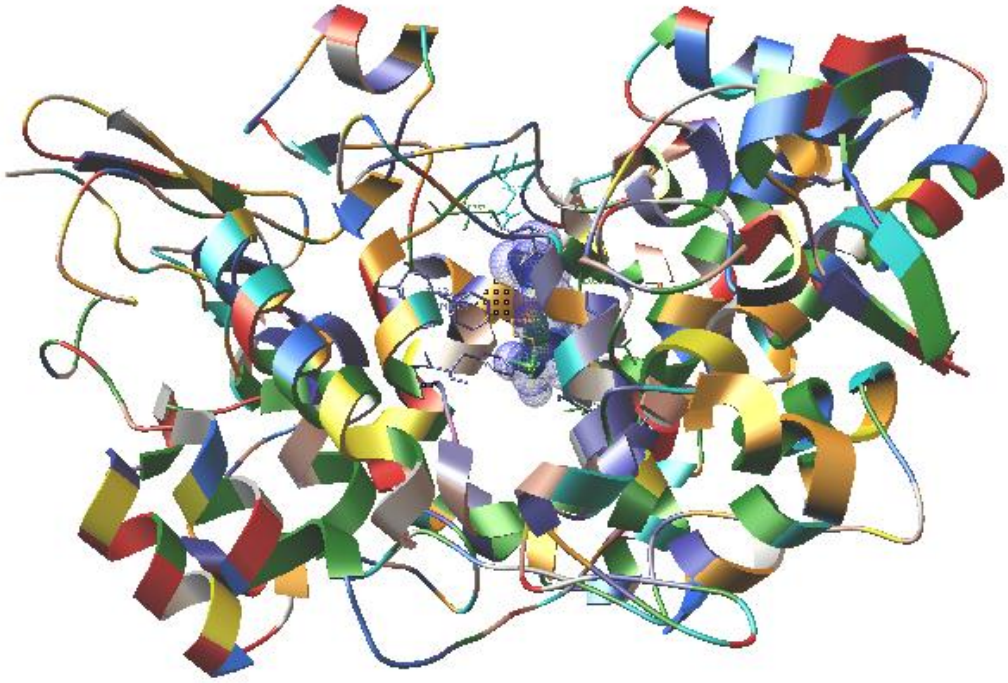


Fig 4.20 Positioning of ibuprofen and *human* COX 2

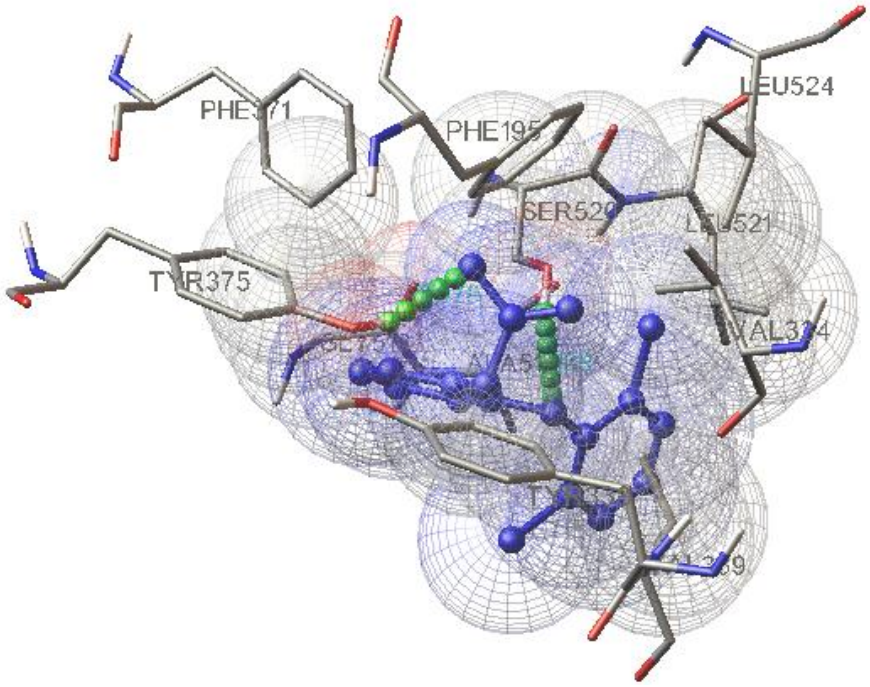


Fig 4.21 Hydrogen bonding of *D.rerio* COX 2 and ibuprofen (Hydrogen bonds shown in green; ibuprofen shown in blue)

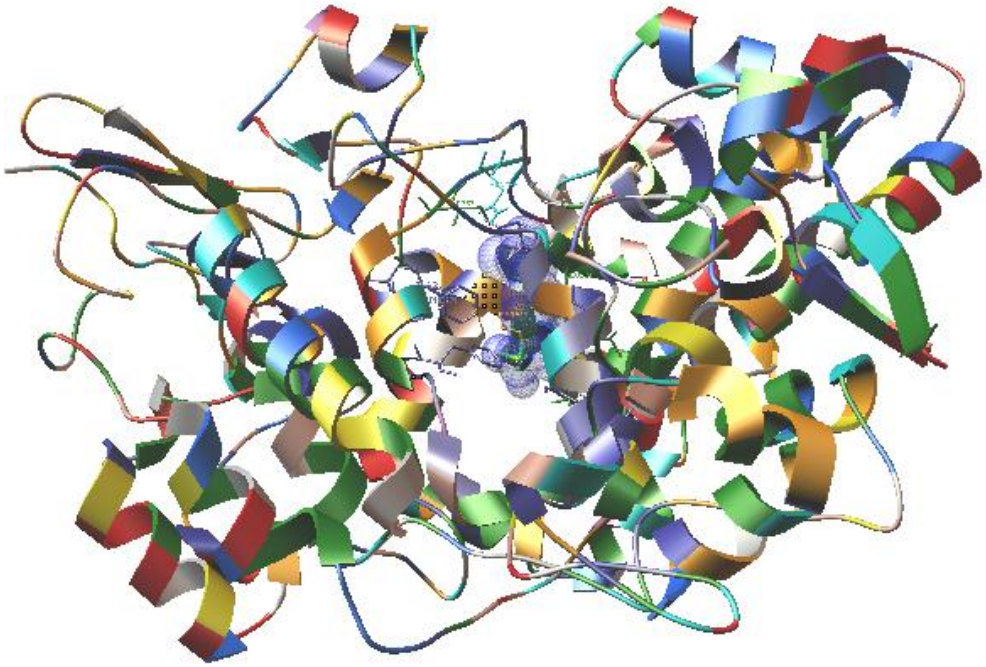


Fig 4.22 Positioning of ibuprofen and *D.rerio* COX 2

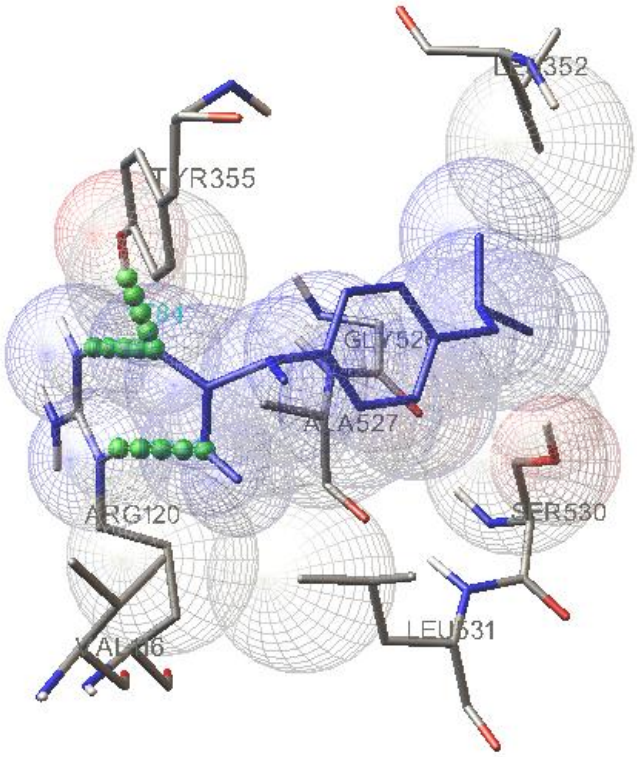


Fig 4.23 Hydrogen bonding of *S.salar* COX 2 and ibuprofen (Hydrogen bonds shown in green; ibuprofen shown in blue)

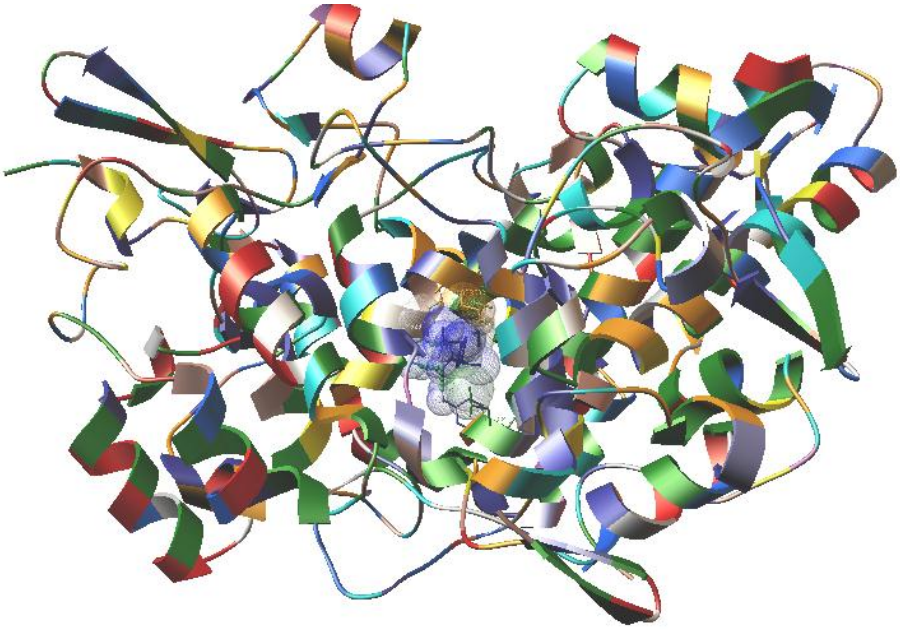


Fig 4.24 Positioning of ibuprofen and *S.salar* COX 2

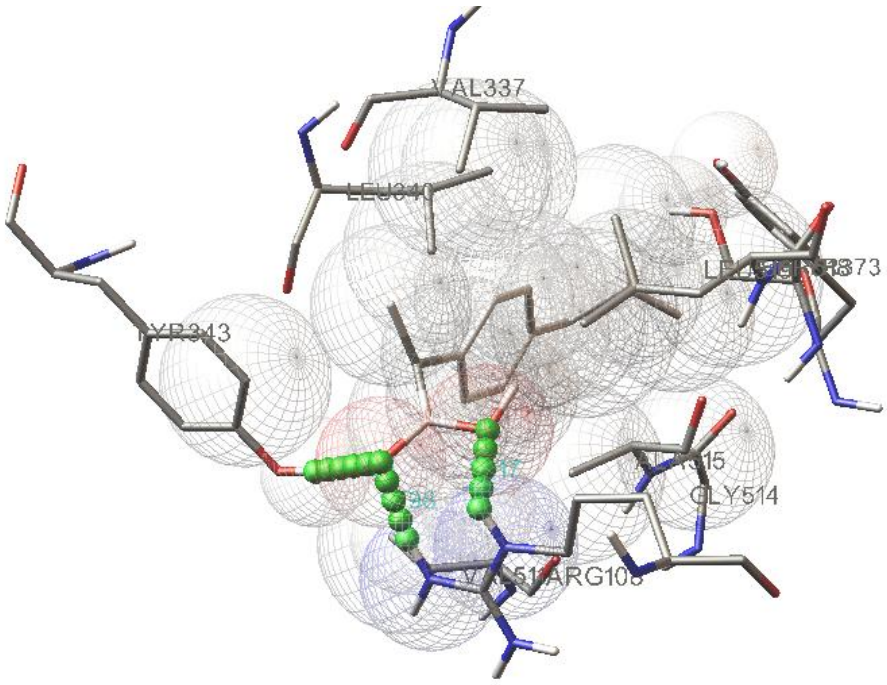


Fig 4.25 Hydrogen bonding of *D.rerio* COX 2 and ibuprofen (Hydrogen bonds shown in green; ibuprofen shown in blue)

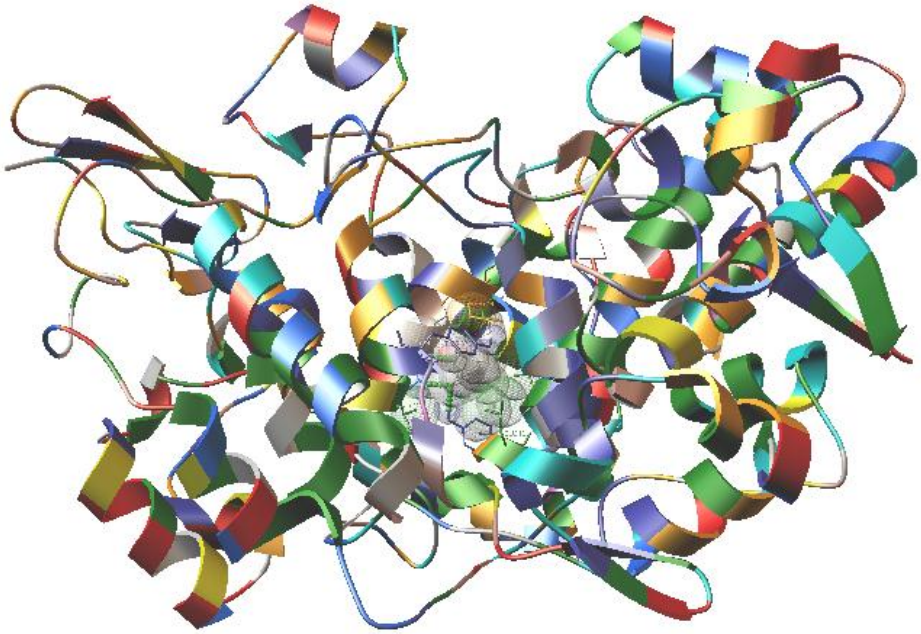


Fig 4.26 Positioning of ibuprofen and *D.rerio* COX 2

4.5 Discussion

Aquatic organisms have a high degree of human drug target conservation and may be susceptible to a similar MoA occurring when exposed to pharmaceuticals.

4.5.1 Drug target conservation in aquatic wildlife

The BLAST search revealed that aquatic vertebrates have the highest degree of sequence homology with the human drug targets investigated. Fish and frogs were the organisms found to have the most homologues with the highest percentage of similarity (Table 4.3-4.6). It is, therefore, probable that human drugs would exhibit a similar mode of action in these organisms. Organisms such as aquatic invertebrates and algae were found to lack similar drug target receptors. This could be because the genomes for these organisms have yet to be sequenced; however, if this is not the case, it is therefore unlikely that they would show a similar effect to humans when exposed to the selected drugs. Ankley *et al*., (2007) suggest that ecotoxicity testing should be focused in two ways (1) identification of drugs with the most potential to elicit adverse effects and (2) determination of which species and end points should be used for testing. The results of the homology BLAST search and molecular docking experiments performed here can provide important and useful information for both of these tasks.

Fish were found to have a degree of protein sequence conservation of drug targets for all of the nine pharmaceuticals investigated. *D.rerio*, the only fish so far to have its whole genome sequenced was found to have some protein sequence conservation of the target proteins for all the drugs investigated. *D.rerio* drug target homologues regularly had over 60% identity (Table 4.3-4.6). This indicates a high potential for *D.rerio* to exhibit similar metabolic responses to humans when exposed to these pharmaceuticals. This is significant when considering what end points should be used in sub chronic early life stage tests performed as part of an ERA. Intelligent testing would incorporate the mode of action of these compounds which is likely to occur in fish.

The frogs *Xenopus laevis* and *Xenopus tropicalis* regularly appeared in the homology search (Table 4.3-4.6). They often had an even higher percentage sequence homology than that of the fish homologues. This could be an important finding with regards to ERA of human pharmaceuticals because amphibians are not covered in the current guidelines for ecotoxicity testing. It is known that exposure to EE2 can cause persistent sex reversal in *X.tropicalis*

(Petterson *et al*., 2006; Gyllenhammar *et al*., 2009). Exposure of frogs to sewage effluent can disrupt the production and bioactivity of protective peptides which are a critical part of amphibian resistance to pathogens such as the chytrid fungus (Gibble & Baer, 2011). The antidepressant sertraline has been shown to disrupt the neuroendocrine system in tadpoles causing developmental toxicity at environmentally relevant concentrations (Conners *et al*., 2009). Amphibians have declined dramatically in many areas of the world. These declines seem to have worsened over the past 25 years and amphibians are now more threatened than either mammals or birds (Beebee & Griffiths, 2005). Currently, the highest trophic level used for ecotoxicity tests is a fish. Although not an issue in the UK, crocodiles and alligators were also identified as having high conservation of drug target proteins for tamoxifen, EE2 and gemfibrozil. This could be an environmental problem for other countries outside the UK such as the USA and Australia.

Two species of birds appeared several times in the homology BLAST results. The zebra finch (*Taeniopygia guttata*) had similar targets for the drugs diclofenac, paracetamol, ibuprofen, propranolol, gemfibrozil, tamoxifen and EE2 (Table 4.3-4.6). The mallard duck (*Anas platyrhynchos*) had homologues for targets of diclofenac, EE2, tamoxifen and gemfibrozil. Although it is not immediately thought of as an aquatic species it does spend the majority of its life associated with the water and feeds on aquatic plants. Aquatic birds such as ducks that feed and drink from rivers could represent an exposure pathway that has not been considered with regards to ERA of human pharmaceuticals but potentially should be included. Although it may seem unlikely, the massive decline of vultures in India and Pakistan was not an exposure pathway that had been considered (Oaks *et al*., 2004). The exposure pathway is more likely to occur if the pharmaceutical compound has a tendency to bioaccumulate in fish or aquatic invertebrates, which is the case with diclofenac and fluoxetine (Mennigen *et al*., 2011; Kallio *et al*., 2010). All new medicines that have an octanol partition coefficient ($K_{ow)}$ >4.5 must undergo a test for persistence, bioaccumulation and toxicity during the ERA. However K_{ow} has been shown to be a poor indicator of bioaccumulation for some compounds such as diclofenac and ibuprofen (Schnell *et al*., 2009).

Molluscs and mussels have been highlighted as a species that should be covered by ecotoxicity testing of chemicals and pharmaceuticals but which are not currently (Rittschof & McClellan-Green, 2005). The homology research results revealed some conservation of drug targets in molluscs. The Pond snail (*Lymnaea stagnalis)* has a protein with 32% identity to the

carbamazepine drug target and the scallop *(Mizuhopecten yessoensis)* had 39% sequence identity with the propranolol drug target (Table 4.5 & 4.6). It is worth noting that although the sequence homology is quite low, generally speaking any protein with above 30% sequence identity probably has the same function because it is the binding motifs that are particularly important in conservation (Palowski *et al*., 2000). It is possible, therefore that these proteins may bind the drugs and react in the same way. Mollusks may be very sensitive to propranolol and fluoxetine (Lazzara *et al*., 2012; Bringolf *et al*., 2010; Ericson *et al*., 2010).

Daphnia showed some sequence homology with the COX enzyme targets for paracetamol, diclofenac and ibuprofen. This organism showed no significant sequence homology with any of the other drug targets, making it is less likely that these drugs would produce a similar effect in *Daphnia* species as in humans. Again this could be because the whole genome has yet to be sequenced. The same was true for algae which also showed little sequence homology with the drug targets. So far, little is known about their potential detrimental effects of pharmaceuticals on algae. In a recent study on the effects of a mixture of 13 pharmaceuticals on the microalga, *Pseudokirchneriella subcapitata* the findings were that the cells could absorb the drugs and whilst no genotoxic effect was detected, proteomic analysis showed that algae are sensitive to the presence of drugs and that, in particular, the chloroplast is affected.

These organisms represent two of the three trophic levels required in the toxicological assessment of pharmaceuticals for an ERA. If human drug targets are not conserved in these species then any effect produced from chronic pharmaceutical exposure is more likely to be general toxic effect rather than that related to a specific mode of action. A search of the literature revealed that adverse effects of pharmaceuticals on *daphnids* are unlikely at concentrations below 1 mgl^{-1} (Santos *et al*., 2009). This lack of likely toxicological effects on the *Daphnia* genus at environmentally relevant concentrations brings into question its use in ERAs. However, another crustacean, *Gammurus pulex* has been shown to exhibit changes in behaviour with exposure to concentrations of fluoxetine, ibuprofen and carbamazepine at concentrations as low as 10 ngl^{-1} (De-Lange *et al*., 2005). Behavioral changes in the marine amphipod, *Echinogammarus* at concentrations of 100 ngl^{-1} have also been reported (Guler & Ford, 2010). It has also been shown that *G.pulex* can bioconcentrate the pharmaceuticals fluoxetine and carbamazepine, which may have implications for fish that use amphipods as their food source (Meredith-Williams *et al*., 2012). These findings suggest that subtle chronic toxicological effects

may be occurring in these species when exposed to pharmaceuticals. It is unknown whether these toxicological effects are related to evolutionary conservation of drug targets because at present, the entire genomes of these organisms have yet to be sequenced. In order to increase the amount of information that ecotoxicology tests reveal, they need to be made more relevant to the actual environmental situation. The choice of end points for chronic tests needs to be connected to the mode of action of the drug and the presence of a functional gene target in the organism you are testing. In order to achieve this more intelligent approach it is suggested that sequencing the genomes of species regularly used in ecotoxicology tests is made a priority.

4.5.2 Lack of protein target homology

If a protein homologue of the drug target does not exist in a particular species it does not mean that toxicity effects from human drugs does not occur. They may exhibit a different mode of action and affect differing pathways to humans through acting upon target proteins that are present in other organisms but not humans. Functional interactions between a drug and non-orthologous proteins are also possible (Gunnarsson *et al*., 2008). For example EE2 can induce reproductive responses in molluscs (Jobling *et al*., 2004) although the oestrogen receptor described in molluscs is not activated by this synthetic hormone (Thornton *et al*., 2003). Although impossible at the moment, in the future molecular docking packages may be developed which could screen a vast number of proteins (entire genomes) for potential interactions with pharmaceuticals or in fact any small molecule.

A COX enzyme homologue was not found in the BLAST search for *P.promelas* although COX or COX like proteins with significant sequence homology were found for several other fish including *O.mykiss*, *Salmo salar*, Atlantic croaker (*Micropogonias undulates*) and *Danio rerio* (Table 4.4). Another example of inconsistencies in the drug target homologue results was found for carbamazepine. The drug target for carbamazepine, a sodium transport protein also showed no sequence homology with *P.promelas* or *S.salar*, but did with several other fish. A similar protein with 72% identity was found for *O.mykiss* (Table 4.6) which is an evolutionary similar fish to *S.salar*. This is a surprising finding because it would seem logical that these fish would have very similar molecular pathways and processes and therefore express similar enzymes. The reason for this could be that the proteins have yet to be sequenced because the genome for these organisms is not yet complete, however, a great number of the proteins have been characterised

and a substantial quantity of genomic information has been assembled for *P.promelas*; probably because it is frequently used in ecotoxicity testing. If they do not posses the protein target, then this is very important information from an ecotoxicological and ERA stance. If an organism does not possess the drug target protein then the organism cannot react to a drug by a similar mode of action and would only respond through any non-specific general toxicity effects or through possible binding to non-target proteins. Thus choice of test species is particularly important for drugs that have functional receptor drug targets in other organisms (Gunnarsson *et al*., 2008). Bioinformatics databases can provide this kind of information which may be crucial for identifying and selecting vulnerable species for ecotoxicology testing.

4.5.3 Presence of homologous drug receptors and present ecotoxicology knowledge

One of the findings of this study was that human drugs may have multiple targets. Tamoxifen, a drug used in the treatment of breast cancer had several drug target proteins the first of which was listed as the oestrogen receptor (Table 4.3). The order of targets listed in drug bank reflects their importance regarding therapeutic indication or physiological effect (Wishart *et al*., 2008). This drug target is the same as the highly potent EE2 known to cause intersex changes in fish and frogs downstream of sewage outfalls (Jobling et al., 2004). The BLAST results revealed a high sequence homology of the oestrogen receptor with several aquatic species (Table 4.3). The data analysis results show that tamoxifen has been detected in surface waters in the UK and France at concentrations of up to 210 ngl^{-1} (Roberts and Thomas, 2006), (Section 2) and if it is as potent as EE2 it could potentially be a problem for aquatic species. Tamoxifen has a high estrogenic potential and can inhibit the proliferation of yeast cells (Isidori et al., 2010). Therefore the potential for adverse reproductive effects on fish would seem likely. However a NOEC of 5.1 $mgl^{-1}$for a full lifecycle study on reproductive effects of tamoxifen on *P.promelas* has been reported (Williams *et al*., 2007) which is considerably higher than concentrations reported in surface water, indicating that despite *P.promelas* having a protein with high sequence homology with the human oestrogen receptor (Table 4.3) tamoxifen has a low potential for adverse effects at environmentally relevant concentrations on this fish. However, this was one of the only chronic ecotoxicity studies for this drug. Tamoxifen has a high log K_{ow} (Table 2.1), (unfortunately a pH corrected log K_{ow} value was not available) therefore the potential for

bioaccumulation exists. Tamoxifen also scores highly for PBT potential (Roos *et al*., 2012). Clearly the potential for adverse effects of this pharmaceutical cannot be excluded.

The beta blocker propranolol mainly targets beta adrenoreceptor proteins in humans. The bioinformatics results showed a high degree of conservation of these proteins in lower vertebrates and fish (Table 4.5). Nickerson *et al*., (2001) also found beta adrenoreceptors in *O.mykiss* and a high degree of sequence conservation in lower vertebrates. Propranolol can cause cell membrane stabilization and known side effects include broncho-constriction and disturbed peripheral circulations in humans. It can pass the blood brain barrier and act in the central nervous system (Giltrow *et al*., 2009). If these conserved proteins in aquatic species act in the same way when bound to propranolol, similar effects could be taking place. In a study by Owen *et al*., (2009) it was concluded from chronic ecotoxicology studies that effects of propranolol were unlikely at environmentally relevant concentrations on *O.mykiss* but found that responses of *O.mykiss* supported the use of mammalian toxicology data to determine responses in fish. These findings (Table 4.5) are also supported by a recent study by Giltrow *et al*., (2011) that confirms that the beta-adrenergic homologue found in *P.promelas* (Table 4.5) contains the molecular signatures required for propranolol binding and can alter the expression profile. The authors concluded that characterization of the molecular targets for beta-blockers in fish will aid informed environmental risk assessments of these drugs.

Although no homologues of the human beta adrenergic receptor were found in algae or mussels (Table 4.5), beta blockers such as propranolol may also adversely affect mechanisms that are not present in mammals such as photosynthesis in algae (Escher *et al*., 2006) or immune systems in mussels (Canesi *et al*., 2007). As a consequence further investigation into interactions between non specific receptors and propranolol in non target species are recommended. Molecular docking programs may offer a method for this investigation.

Fluoxetine (Prozac) is an antidepressant belonging to a group of compounds called selective serotonin reuptake inhibitors (SSRIs) it blocks the reuptake of serotonin at the serotonin reuptake pump of the neuronal membrane, enhancing the actions of serotonin on the serotonin receptor. This is a neurotransmitter involved in hormonal and neuronal mechanisms. It is important in food intake and sexual behaviour and mediates endocrine functions in aquatic organisms (Pery *et al*., 2008). The bioinformatics results revealed a high degree of homology between the sodium dependant serotonin transporter protein target in humans with those found in

S.salar, D.rerio, and X.tropicalis (Table 4.6). This is in agreement with Fong, (1998) who found serotonin in lower vertebrates and invertebrates. Several fish species possess serotonin receptors making it possible to predict that SSRIs can modulate serotonin levels in these animals (Brooks *et al*., 2005). There are several reports of ecotoxicity of fluoxetine on aquatic organisms, some of which are clearly related to the mode of action of this drug supported by the high degree of homology in target proteins (Table 4.6). Fluoxetine exposure has been shown to affect the serotonin receptor and cause sub lethal changes in the serotonergic pathway in the sheepshead minnow (*Cyprinodon variegatus*) which may result in behavioral changes that could, in turn, have implications for the ecological response of populations to additional environmental stressors (Winder *et al*., 2009). This supports observations of behavioral changes in non target organisms reported by other authors. For instance, inhibition of innate C start predator avoidance in fathead minnows (Painter *et al*., 2009) and disrupted feeding behaviour goldfish (*Carassius auratus*) (Mennigen *et al*., 2010). Reproductive disturbances have also been reported.These include reduction of the number of neonates and induced spawning of zebra mussels (Fong & Molar, 2008), changes in and reduced and changes in reproductive patterns of the invertebrate *Ceriodaphnia dubia* (Henry *et al*., 2004), and reproductive changes in goldfish (Mennigen *et al*., 2010b). Brooks *et al*., (2005) found fluoxetine and its metabolite norfluoxetine bioaccumulates in all tissues of fish.

At present the human serotonin receptor protein has not been crystallized. This is probably due to the fact that membrane proteins are often difficult to characterise (Celik *et al*., 2008). This poses a problem when creating a reliable model of this protein or the homologues found in this study for molecular docking purposes. It would be beneficial to be able to model interactions of fluoxetine with serotonin receptor homologues in light of the mounting evidence of potential chronic effects on non target organisms (see above). Oakes *et al*., (2010) concluded that fluoxetine may pose an environmental risk to the aquatic compartment and has a risk quotient (RQ) of greater than 1. There is also the distinct possibility of additive and synergistic effects of fluoxetine with other SSRIs (section 4.4.6). Further characterization of the serotonin receptor would aid computational molecular docking simulations which may prove beneficial for ERA by providing evidence of potential interactions between protein homologues and fluoxetine as well as other SSRIs.

Gemfibrozil acts by increasing the synthesis of lipoprotein lipase, thereby increasing the clearance of lipoproteins (DrugBank, 2012). A high sequence homology (73%) was found between the human peroxisome proliferator-activated receptor alpha drug target for gemfibrozil, with the protein in *O.mykiss* (Table 4.5). This indicates the potential that this pharmaceutical may act similarly in fish as it does in humans. This theory is supported by a study by Prindiville *et al*., (2011) in which it was demonstrated that gemfibrozil reduced lipoprotein concentration as dramatically in trout as it does in mammals.

Carbamazepine targets the human sodium channel receptor protein SCN5A, its mode of action is still not fully understood. Significant homology was found with the proteins of *D.rerio* and *O.mykiss* (Table 4.6) indicating that a similar mode of action may be occurring in aquatic species. Chronic ecotoxicity studies involving the MoA of carbamazepine were not found in the literature representing a research need. However ecotoxicity testing of carbamazepine has shown that it can have a negative effect at environmentally significant concentrations. Long term exposure to carbamazepine could cause low level oxidative stress inducing adaptive responses in antioxidant enzymes and serious oxidative damage in fish brain (Li *et al*., 2010) and carbamazepine is able to alter expression of fish genes associated with development, regulation and differentiation of synapses, neurons and neurotransmitters (Thomas *et al*., 2012).

4.5.4 Evaluation of BLAST search against available ecotoxicity data

Unfortunately the evidence for an organism having a functional drug receptor being more sensitive to exposure than an organism which does is very limited. Chronic ecotoxicity data is quite limited for human pharmaceuticals and even scarcer for chronic mode of action related toxicity tests (Table 4.3-4.6). A study investigating chronic MoA toxicity in organisms containing a functional drug receptor and organisms without is a research need. This type of information would provide valuable support for inclusion of identification of gene conservation of drug targets in ecotoxicity tests as well as molecular modelling and docking. It may of course transpire that the opposite is true; nevertheless it is important to establish whether in *silico* investigations might provide a valuable tool in ecotoxicity testing.

One of the key problems in identification of organisms that lack the relevant drug target gene is that as yet limited organisms have been fully sequenced. This means that if the gene is not found during a BLAST homology search for a particular organism it is not known whether

the organism actually lacks that gene or it has simply not been sequenced yet. Sequencing of the genomes of organisms regularly used in ecotoxicity tests in particular needs to be made a priority.

4.5.5 Molecular docking of drugs with proteins in non-target species

The presence of a drug target homologue in a species does not guarantee that a functional interaction with the drug will occur (Gunnarsson *et al*., 2008). A more precise prediction of potential drug target interaction might be possible with better knowledge about drug binding domains and three dimensional structures of the target proteins (Gunnarsson *et al*., 2008). Molecular modelling programmes and databases such as Swiss Model and RCSB can help provide this type of information. The molecular docking experiments using ibuprofen and diclofenac with the COX2 enzyme performed here show that this can be done.

The analgesics diclofenac and ibuprofen act by inhibiting (reversibly or irreversibly) the COX enzyme which catalyze the synthesis of prostaglandins (Vane & Botting, 1998). Prostaglandins are involved in inflammation, pain regulation, and regulation of blood circulation especially in the kidney, coagulation processes, and synthesis of gastric mucosa, vascular permeability and kidney function including ion retention (Sali, 2005; Mutschler, 1996; Fent *et al*., 2003). Nephropathy is thought to be directly related to inhibition of prostaglandin production (Sanchez *et al*., 2002). COX mediated production of prostaglandins is important for ovulation in mammals (Gaytan *et al*., 2006) and fishes (Mercure and Van Der Kraak, 1996; Sorbera et al., 2001). COX like proteins with significant similarity to the human forms were identified in several aquatic species including: *O.mykiss*, brook trout (*Salvelinus fontinalis*), *S.salar*, *M. undulates*, *D.rerio*, puffer fish (*Tetraodon nigroviridis*), platypus (*Ornithorhynchus anatinus*), *X.tropicalis*, and *D. pulex* (Table 4.4). The COX enzymes are part of the cytochrome P450 (CYP) family of enzymes particularly important in most drug metabolism (Dorne *et al*., 2007). The amino acids Ser-530 and Tyr-385 in COX 2 enzymes have been identified as important in the binding diclofenac in sheep (Rowlinson *et al*., 2003). The amino acid residue Arg-120 in human COX2 has also been identified as important for hydrogen bonding of NSADs (Pouplana *et al*., 2002). All three of these residues were present on the COX enzymes identified in these aquatic organisms (Fig 4.2). This indicates a high probability that the COX enzymes in fish and other aquatic organisms will bind and react to diclofenac in the same or a similar way as humans. These findings are supported Wallace *et al*., (2000) who showed that rat COX enzymes will bind

and be inhibited by diclofenac, ibuprofen and paracetamol and Zou *et al.*, (1999) who found a fish inducible COX 2 homologue in rainbow trout, the translation product of which had a high homology of 83-84% to its human counterpart.

Ecotoxicity data indicates a low risk of acute toxicity on aquatic organisms for diclofenac and ibuprofen (Ferrari *et al*., 2003; Schwaiger *et al*., 2004). Chronic toxicity data is generally lacking for most pharmaceuticals but there is a growing body of knowledge for diclofenac. Diclofenac has been found to cause serious adverse effects in vertebrate species at much lower concentrations than suggested by acute or sub chronic toxicity tests with invertebrate species (Hoeger *et al*., 2005). This indicates that traditional ecotoxicity studies and sub chronic fish studies recommended in the EMEA, 2006 guidelines for ERA of pharmaceuticals may miss whole lifecycle chronic effects and underestimate potential adverse effects in the environment.

The molecular docking results in this study show that COX2 homologues in *O.mykiss, S.salar, and D.rerio* would all bind diclofenac and ibuprofen (Fig 4.3-4.26). The docking results show that the free energy of binding for diclofenac and ibuprofen is similar in these fish homologues as the human form (Table 4.7). The hydrogen bonding highlighted as critical for diclofenac binding to residues Ser-530 and Tyr-385 for the sheep COX 2 crystalline structure (Rowlinson *et al*., 2003) and residue Arg-120 for the binding of NSAIDs generally (Pouplana *et al*., 2002) also occurred in the fish docking simulations (Fig 4.11, 4.13, 4.15, 4.17, 4.19, 4.21, 4.23, 4.25). The fact that binding occurs suggests that fish would react in a similar way when exposed to these pharmaceuticals as humans, and in all probability that prostaglandin production in these fish would be inhibited. This finding is supported by Hoeger *et al*., (2005) which reported that diclofenac provokes the same mechanism of action in brown trout (*Salmo trutta*) (i.e. inhibition of prostaglandin synthesis) as mammalian species at environmentally relevant concentrations of 500 ngl^{-1}. Lack of prostaglandin production may lead to renal failure in fish, the established cause of the death of exposed vultures (Taggart *et al*., 2007). Renal complications are a known side effect of NSAIDs in humans (Banks *et al*., 1995). This effect may be further reinforced by the accumulation of diclofenac in the liver and kidneys of fish by factors of up to 2700 (Schwaiger *et al*., 2004) and in the bile by a factor of over 600 (Mehinto *et al*., 2010). Cytological alterations in liver, kidneys and gills have been observed in rainbow trout following exposure to diclofenac for 28 days at a concentration of 1 μgl^{-1}. Other toxicological effects include reduced haematocrit levels, increased monocyte concentration in the liver, telangiectasis

in gills and mild tubular necrosis in trunk kidney (Schwaiger *et al*., 2004 Mehinto *et al*., 2010; Hoeger *et al*., 2004). Further evidence to support accuracy of the docking results is that expression levels of both COX1 and COX2 in the liver gills and kidney of rainbow trout were significantly reduced by diclofenac exposure of 1 μgl^{-1} (Mehinto *et al*., 2010). This study concluded that sub-chronic exposure of environmental concentrations of diclofenac can interfere with the biochemical functions of fish and lead to tissue damage.

Daphnia is a common invertebrate model organism for freshwater systems and plays a central role as an algal grazer and important food source for fish (Dietrich *et al*., 2010). High sequence homology was found for COX2 proteins in *Daphnia pulex*. A percent identity of 46% was found with the human form (Table 4.4). The CLUSTAL W multiple alignment also shows a high sequence homology especially in the regions where the amino acid residues, Ser530, Tyr385 and Arg-120 identified as important in binding diclofenac, were present (Fig 4.2). However molecular docking experiments with the *D.pulex* homologue were unsuccessful. This could be due to the presence of mutations or differences in the amino acid sequence in the homologue protein leading to a different secondary and tertiary protein structure preventing binding of diclofenac. The *Daphnia* homologue has a different amino acid at position 525 (Fig 4.2). In *Daphnia* this residue is an isoleucine but in the human, *D.rerio, O.mykiss, S.salar* and sheep homologues, this residue is a leucine. Pouplana *et al*., (2002) found that an isoleucine in this position could severely restrict access of the active site of cyclooxygenase. Further docking experiments should be run using a mutant *D. pulex* model with the isoleucine changed to a leucine and re modelled in Swiss model to test that there is a similar effect in *Daphnia*. The molecular docking results then suggest that diclofenac would not have an impact through its mode of action on *Daphnia*; this is supported by ecotoxicological studies. Chronic multigenerational toxicity of diclofenac on *D.magna* was observed at concentrations of 40 mgl^{-1} (Dietrich *et al*., 2010), it did not appear to be due to any obvious mode of action related cause. This concentration is considerably higher than concentrations detected in the environment (Section 2).

Ibuprofen is a nonspecific COX inhibitor and is shown to inhibit both COX 1 and COX 2 (Van Hecken *et al*., 2000). Ecotoxicological assessment of ibuprofen is somewhat lacking in the scientific literature. Effect concentrations have been reported for *Daphnia* species at between 10 and 108 mgl^{-1} for immobilization, 13.4 mgl^{-1} for reproduction, a NOEC for survival of 20 mgl^{-1}

and a LOEC for population growth of 20 mgl^{-1} (Heckamann *et al*., 2007; Cleuvers, 2003). These concentrations are far greater than that detected in raw sewage let alone surface waters (section 2). However, behavioural changes in *Gammurus pulex* have been reported at 10 ngl^{-1} (De Lange *et al*., 2006). Toxicological studies of ibuprofen on fish are also sparse. Acute assessment indicates a LC_{50} (50% lethal dose) on *Oryzias latipes* (Japanese medaka) of >100 mgl^{-1} (Pounds *et al*., 2008). Kim *et al*., (2009) also reported a lack of acute affects of ibuprofen on *O.latipes* and also the crustacean *Thamnocephalus platyurus.* Further investigation on toxicological effects on *O.latipes* with ibuprofen exposure found no acute effects but did highlight changes in timing of reproduction, less frequent reproduction and more eggs per reproductive event (Flippin *et al*., 2007). This is not surprising since COX 1 activity is important for ovulation in fish. However although some stabilization in COX activity in female fish was recorded no overall reduction was found. Overturf *et al*., (2011) found no chronic effects of ibuprofen on survival or growth of *P.promelas*. The reasons for this are unknown but it could be because the organism lacks the COX protein as this enzyme was not detected in the BLAST results (Table 4.4).

These docking experiments could help direct ecotoxicity tests by selecting chronic test endpoints that are related to the MoA of the drug. The selection of organisms is also important. *Daphnia* is regularly used in ecotoxicity tests but in this case it appears that this organism does not have a functional drug target receptor for ibuprofen or diclofenac. This sort of information would be highly relevant to the ERA procedure.

4.5.6 Additive effects

There is clearly potential for synergistic and additive effects for ibuprofen, diclofenac and paracetamol on aquatic species as our findings show these three pharmaceuticals commonly occur as a mixture in surface waters (Section 2) and act upon the same drug target (this Section). The results of the molecular docking experiments show that ibuprofen and diclofenac both bind to the same active site on the COX2 enzyme (Figs 4.3-26). Ecotoxicological effects may not be invoked when a single pharmaceutical concentration is too low (Pomati *et al*., 2008). However, the combined effect may be significant. In toxicity studies mixtures of diclofenac and ibuprofen have been shown to exhibit combined toxicity following a concentration addition concept (Schneel *et al*., 2009). Considerable mixture toxicity for NSAIDs has been reported at concentrations where a single compound showed no effect (Cleuvers, 2003). Significant sub

lethal effects have been found for the zebra mussel (*Dreissena polymorpha*) when exposed to a mixture of diclofenac, ibuprofen and paracetamol (Parolini & Binelli, 2011). This has also been shown for other pharmaceuticals when they are in the same therapeutic class (Schnell *et al*., 2009). Fluoxetine is one of five selective serotonin reuptake inhibitors (SSRI) available on the market (Johnson *et al*., 2007). The drug target protein for fluoxetine SL6A4, for example, is also the drug target receptor for 3 other SSRIs; citalopram, escitalopram and zimelidine (Table 4.6). Thus synergistic, additive or antagonistic effects are also likely to occur because these compounds are probably present in mixtures in surface waters (Styrishave *et al*., 2011).

Accurate prediction of mixture toxicity is indispensible for ERA (Cleuvers, 2004). The current ERA does not include additive, synergistic or antagonistic effects of mixtures of pharmaceuticals when calculating PECs, PNECs and risk quotients, some revision of ERAs is needed in this respect to reduce scientific uncertainties related to pharmaceutical effects on aquatic species. In addition the retrospective toxicology assessment used to set the 10 ngl^{-1} trigger limit used in the EMEA, (2006) ERA did not account for mixtures of pharmaceuticals bringing into question sound ecotoxicological evidence in its calculation (Montforts, 2003). Molecular docking may offer a way of establishing synergistic/additive effects.

4.5.7 Choice of chronic toxicology end points

Bioinformatics and molecular docking might be a valuable aid in ecotoxicity tests directing the choice of suitable endpoints in chronic ecotoxicity testing. These tests could be made relevant to organisms that are likely to be exposed to a pharmaceutical and have been shown in molecular docking experiments to have target proteins that bind a pharmaceutical. Ecotoxicity tests have shown that the mode of action for diclofenac is similar in fish species and that a reduction of prostaglandin synthesis occurs. The molecular docking experiments for *D.rerio, O.mykiss* and *S.salar* clearly showed that the drugs ibuprofen and diclofenac bound to COX2 proteins in these organisms suggesting the inhibition of the enzyme and possible end points for ecotoxicity testing. Chronic test end points, therefore, could be selected on the basis of the function of that protein and the known mode of action of the drug.

4.5.8 Increasing the number of species used in ERA

Molecular docking provides a means of increasing the number of trophic levels and quantity of species tested without doing any further testing on animals. AutoDock or other

molecular docking software could guide the ecotoxicity tests performed by selecting organisms which could be exposed and identifying organisms with conserved proteins that would probably be affected through the mode of action of the drug. *In-silico* studies would effectively extend the range of test organisms with no 'wet' laboratory work and no need to sacrifice organisms.

4.5.9 Choice of sensitive organisms

Choice of sensitive organisms is important for risk assessment. Some organisms may be more sensitive that others. The dramatic decline of *Gyps* vultures in India, Nepal and Pakistan caused by the veterinary drug diclofenac is one example of this. Sensitivity varies substantially with species. Birds such as the pied crow (*Corvus albus*), an organism recently evaluated for toxicity testing of NSAIDS, was found to be unaffected by concentrations of 10 $mgKg^{-1}$ of diclofenac. Sensitivity varies considerably even across vultures, the *Gyps* showing very high sensitivity to diclofenac and other species showing no toxicity at all (Rattner *et al*., 2008). It is not yet known why the *Gyps* vultures are more sensitive to diclofenac as yet the full genome sequences are not available for all these birds, but eventually may give the clue through bioinformatics and molecular docking to the mechanisms for these differences.

4.5.10 Bioinformatics as a potential tool in environmental risk assessment

As the data on genomes increases, so too does its potential application in ecotoxicological assessments. The potential for use in bioinformatics and molecular docking as a tool in ERA is clear from the work described above. As genome sequencing becomes faster, however, it is conceivable that full genome sequences may become available for all UK fish species. New drug targets are continually being added to the DrugBank database as the mechanisms and modes of actions of pharmaceuticals are elucidated. This is true even for some older long established pharmaceuticals where the mode of action was often not well understood. It is important that the potential of this new knowledge forms a part of ecotoxicology data for ERA. Gunnarsson *et al*., (2008) concluded in a similar study on homologue drug targets in aquatic organisms' that a more comprehensive understanding of the mechanisms of actions of drugs in wildlife was required. *In-silico* molecular docking experiments performed here may provide an insight into this, and will certainly provide a tool to inform and direct chronic ecotoxicological experiments.

The findings of the homology search suggest that some lower vertebrates and invertebrates have a degree of conservation in proteins that are drug target receptors in humans.

Organisms with these similar or conserved proteins may bind pharmaceuticals with possible physiological effects. Bioinformatics databases and analysis tools could indeed potentially be useful in identifying species that may be more susceptible to a pharmaceutical for ecotoxicological tests. This could help make chronic ecotoxicology tests more appropriate by improving the selection of species used and choosing more realistic end points better fitted to the drug being tested. It is not possible to test all the species likely to be exposed to a compound including all potential toxicological endpoints as well as taking into account all routes of exposure such as aquatic, terrestrial and food chain pathways. It is imperative that toxicologists use all of the available data including that from *in vivo* experiments (Winter *et al*., 2009) and in the case of bioinformatics *in silico* work.

Increasing life expectancy is likely to lead to a greater consumption of pharmaceuticals and hence increased presence in the environment (van den Brandhof & Montforts, 2010). Due to the low level chronic nature of the exposure to pharmaceuticals there is a possibility that adverse effects may go undetected and that these effects could accumulate slowly leading to an irreversible change (Schnell *et al*., 2009) reflected at species, population and ultimately community level. The idea of intelligent testing in environmental toxicology has been suggested as a way to address this problem (Lange & Dietrich, 2002). This includes the use of information on the mode of action of a substance to predict or anticipate effects in a range of species and based on this tailor the tests and select species as part of ERA (Fent *et al*., 2006; Sumpter, 2007; Montforts *et al.*, 2007). There are clear legislative, economic and ethical advantages to maximising the use of existing data and minimising inappropriate testing. However data to support this approach is lacking with few published studies testing theses concepts experimentally (Winter *et al*., 2009).

Molecular docking software such as AutoDock could also be used to select organisms that may be more susceptible to a particular mode of action of the drug. The experiments performed here show that *S.salar, D.rerio* and *O.mykiss* are more sensitive to diclofenac than *Daphnia* and this is supported by actual ecotoxicology results. This information could be invaluable for toxicology tests.

The work carried out in this section has led to the development of a flow chart which indicates how drug target information, molecular modelling and molecular docking could be used as part of the ERA (Fig 4.27). The proposed revisions to the ERA include early

identification of drug target conservation in aquatic species. This should be done using the NCBI BLAST database. The results of this search can then be used to perform molecular modelling and docking studies to ascertain the likelihood of non target organisms producing a functional drug target receptor protein. It is proposed that organisms which have potentially functional target receptors should then be selected for chronic ecotoxicology tests incorporating the MoA of the pharmaceutical. Standard ecotoxicological tests would still be required. The lowest PNEC should then be selected from both the chronic MoA tests and standard tests and the ERA continues as in the 2006 EMEA guidelines.

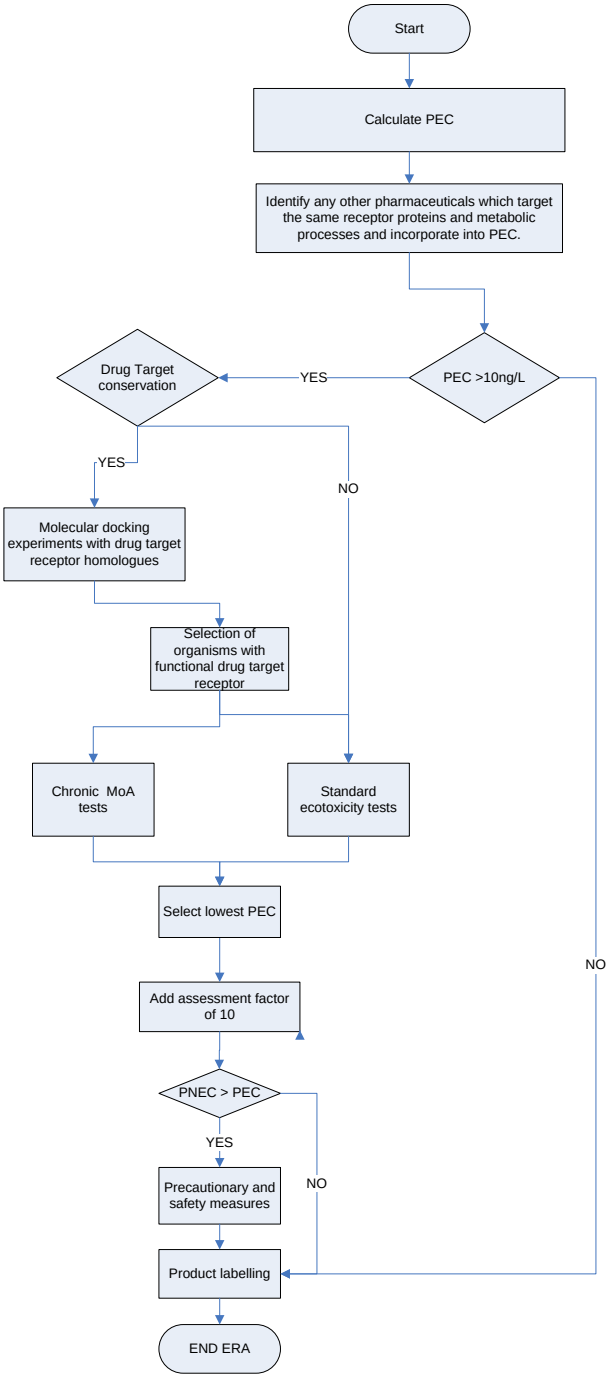


Fig 4.27 Proposed environmental risk assessment flow chart

4.5 Conclusion

Although bioinformatics databases at present are not capable of replacing ecotoxicology tests they may be a useful tool in ERAs. There is a need to focus on long term exposure assessment regarding specific modes of action of pharmaceuticals to better judge the implications of pharmaceutical residues in aquatic systems (Fent *et al*., 2003). Some of these specific modes of action can be identified using bioinformatics databases. If a similar mode of action occurs in other organisms, existing human toxicology data which is held by the pharmaceutical companies may be useful (Winter *et al*., 2009). This may provide information on the MoA, therapeutic plasma concentrations or potential adverse side effects which could aid a more thorough ecotoxicological assessment.

Bioinformatics data reveals that a drug target protein sequence may not occur in all species of fish suggesting that selection of species known to carry a target gene or protein for testing sequence could be beneficial. Bioinformatics databases may provide useful indications of vulnerable species for ecotoxicological tests or environmental monitoring. Knowledge about what affects a drug is likely to have on an organism could help identify which effects to monitor. The advantages of using computer models include the reduction of animal testing and costs. In the current regulatory environment, there is a clear need to develop and validate alternatives to existing *in vivo* ecotoxicity test methods, without increasing the uncertainty in risk assessment (Embry *et al*., 2010).

Some of the problems with the current ERA, e.g. that the range of species used to support the action limit is inadequate and that acute toxicity data are predominantly used (see Section 3), might be addressed by the use of bioinformatics. The toxicity data sets used to calculate the action limit should include chronic toxicity data for the entire lifecycle exposure of a large range of species to make more accurate predictions about environmental effects of pharmaceuticals on aquatic organisms. Bioinformatics databases could help identify vulnerable species which should be included and the types of chronic toxicity tests and endpoints which should be employed. The experiments undertaken in this work show that molecular docking could be used as a tool to aid and direct ERA by predicting potential reactions between human drugs and conserved drug targets in other organisms. The advantages of this are that ERA could be made more precautionary by incorporating predictions of sensitive species. There is a strong societal and

regulatory pressure that alternatives to vertebrate testing are developed (van den Brandhof & Montforts, 2010) and bioinformatics represents a means by which this could possibly be achieved.

The limitations of using molecular docking for toxicity testing are that a limited number of genomes have been sequenced. Often proteins that have been sequenced have not been crystallized and there is limited information about the three dimensional structure and folding of proteins. This is especially true for membrane proteins which are difficult to isolate. For instance it was found that there is no human crystal structure known for the serotonin receptor protein and currently modelling of this protein is based on a microbial homologue with only 20% homology (Celik *et al*., 2008). This means that a reliable or similar enough template is not yet available on which to base a protein model. Another limitation is that sometimes the mode or mechanism of action is not well known for a drug, with insufficient information available on which proteins interact with or where a drug binds when a protein receptor is identified. New drugs however are usually quite well characterised before release. Drug discovery uses molecular modelling and docking much more frequently today than in the past. The modes of action are usually well understood for new pharmaceuticals for human health and safety reasons. The new AutoDock Vina package is able to perform docking experiments for hundreds of small molecules on a whole protein. There is the potential to identify other mode of actions or affects on other non-target pathways. Blind docking, where the area of binding is not defined, has proven difficult in the past, but as technology advances it is becoming easier to do and produces more successful and reliable results. This not only provides information on whether the drug will bind but also where it may bind, which sometimes this can happen in more than one location on the protein. The potential now exists to test chemicals other than pharmaceuticals if their mode of action is known for example endocrine disrupting chemicals.

According to the principles of green chemistry the functionality of a chemical should include the properties necessary for its application for fast, easy degradability after use (Kummerer, 2010; Khetan & Collins, 2007). One way to do this may be to develop tools which can predict a compounds degradation potential, lipophilicity, hydrolysis, persistence, bioaccumulation and toxicity properties before it is synthesized. This would mean a potential detrimental effect on the environment could be examined before extensive mammalian tests and manufacturing considerations begin. Boxall, (2004) suggests that environmental side effects

should be considered alongside mammalian toxicological side effects. Many pharmaceutical compounds never reach the market for reasons such as safety, efficacy, lack of market potential, difficulty in sourcing raw materials or lack of perceived profit. It is suggested that environmental considerations should also come into play. Low toxicity in humans is crucial for registration of pharmaceuticals, so could low toxicity towards non target organisms be considered as important as well? The bioinformatics and molecular docking work show that these tools could be useful in this respect by providing a first screen for predicting chronic toxicity effects on non target organisms.

The advent of genomics in the drug development process with specific target definition means that bioinformatics and molecular docking packages are an important tool in the drug development process (Schoichet *et al*., 2002). For example the recent development of the anti viral drug for bird flu used this technology (Nguyen *et al*., 2009). The development of anticancer drugs is also an example of where molecular docking packages are being utilized (Mukherjee & Majumder, 2009). The bioinformatics work shows that these packages could also be used to predict toxicity of pharmaceuticals on aquatic organisms and aid and direct laboratory ecotoxicity tests.

Section 5

Conclusion

This study identified several flaws in the current environmental risk assessment (see Sections 2, 3, 4). ERA is a key stage in the life cycle of the drug because it quantifies the amount of pharmaceutical that will be released into the environment (i.e. the exposure) and the effects that that pharmaceutical may have on the environment. In order to increase the 'greenness' of a pharmaceutical some of the limitations and gaps in the ERA need to be addressed.

5.1 PECs

One finding from the interviews (Section 3) indicates that post authorisation monitoring sales of a pharmaceutical should be addressed in the risk assessment. It is not precautionary to predict a compounds use and therefore likely environmental exposure concentrations prior to marketing and not establish the accuracy of the prediction subsequently. There needs to be a reassessment of exposure once actual consumption data is available say two or three years post market authorisation. The results of section 2 show that a lack of accurate data for consumption and clarity in the PEC calculation in the EMEA guidelines is hampering accurate assessment of exposure concentrations. The data that is collected currently on sales of pharmaceuticals should be more accurate and detailed to make PECs more realistic. Drug companies, pharmacies and the NHS should be encouraged to keep more detailed records of sales of drugs and the locations in which they are sold and prescribed. This data could then be fed into geographical information for exposure assessment and give indications of where 'hot spots' for specified drugs might occur. The results of the interview analysis support this recommendation (Section 4). At present there is no accurate indication of over the counter sales which must be taken into account when predicting exposure. This reassessment of exposure concentrations is also important when a drug comes off patent (see results of interview analysis section 3). This is because the market penetration factor of a drug may increase markedly when generics are produced because the drug price is likely to fall. Again an estimate of market increase because of loss of patent may not be entirely reliable, so it would be advisable to obtain accurate sales data and calculate exposure perhaps two or three years later. It may be necessary to recalculate the exposure of a drug every three years or so. This could be particularly important when new drugs that may have the same or similar MoA come onto the market because of changing markets affecting the use of older pharmaceuticals. New compounds that target similar metabolic pathways, however, may have additive or synergistic effects. This has been indicated in the bioinformatics and molecular

docking work for diclofenac and ibuprofen (Section 4). Bioinformatics databases and molecular docking could be useful tools when assessing likely additive and synergistic effects especially for older drugs where the MoA and target protein were not fully understood at the time of their registration. Bioinformatics and molecular docking package also may be useful when considering replacing or substituting a drug that has the same MoA or therapeutic action with one that has a better environmental profile.

5.1.1 PEC Refinements

One route to protecting the environment and considered important in green pharmacy is degradation during sewage treatment in order to reduce environmental exposure. The results from Section 2 show that not only is there is a large disparity in the effectiveness of different sewage treatment technologies in removing pharmaceuticals, but that the effectiveness of the same STP can vary substantially. It was also found (Section 2) that some drugs are highly resistant to most sewage treatment technologies e.g. carbamazepine. The interview analysis (Section 3) revealed that many pharmaceuticals do not pass the ready biodegradation study and therefore a rate constant cannot be calculated for STP removal. It is also known that log K_{ow} is a poor indicator of sewage sludge adsorption because pharmaceuticals are generally ionisable compounds (Wells, 2006). This means that packages such as SimpleTreat recommended by the EMEA, which substantially rely on this data, are flawed. Another problem is that combined sewage overflows, storm events and misconnections mean that sewage can regularly enter water courses untreated. Given these factors and the lack of accurate figures it is not precautionary to refine a PEC for sewage treatment removal. The factors that increase sewage treatment degradation of compounds need to be examined in order to increase removal and reduce the exposure in the environment and promote green pharmacy. This, however, is not solely possible through 'benign by design', the sewage treatment process is also important.

The results of Section 2 show that although concentrations of some pharmaceuticals are reduced after sewage treatment this does not occur consistently. The PEC and MEC analysis shows that when refinements are made to PECs for sewage treatment removal they may no longer provide conservative predictions.

It would seem reasonable to take metabolism by the body into account when estimating exposure. However the results of this study indicate that when this type of data is used to refine a

PEC it is more likely to be an underestimate. Patients are wide ranging in their ability to metabolise some drugs (Carballa *et al*., 2008). It has been estimated that up to fifty percent of medicine is not consumed (Grass & Lalande, 2005) and may also enter sewage treatment without any removal or transformation by metabolism. These factors make refining PECs for excretion unprecautionary. Decreasing excretion rates of drugs in patients is seen as a potential route to green pharmacy.

Due to the assumptions made in the PEC calculation for dilution, market penetration, wastewater production, even usage and distribution, PECs may not be precautionary especially in areas of high drug usage or low dilution (see Section 2). Refinements made to PECs for excretion and sewage treatment amplify the risks of obtaining an under estimate of environmental exposure.

5.1.2 Exposure Modelling

The findings of the interview engagement (Section 3) performed during this research indicated that modelling of environmental concentrations using computer packages and GIS was generally viewed favourably. The results of Section 2 also show that pharmaceutical concentrations can vary substantially and that a single blanket prediction of environmental concentration does not adequately reflect the real environmental situation. It was thought that with the right training and understanding of how exposure models work and acknowledgement of their limitations, that the accuracy of PECs could be improved substantially. The potential of producing a range of concentration data and a worst case scenario makes the applicability of exposure concentrations more useful for risk assessment. It was considered that the analytical chemistry shortfalls, for example difficult matrices such as sewage and the extremely low detection limits required could be overcome by using models such as *Ph*ATE and GREAT-ER in conjunction with some environmental monitoring. Studies on the accuracy and potential of using these exposure models to predict environmental concentrations of pharmaceuticals have also been viewed very favourable in the literature (Johnson *et al*., 2007; Robinson *et al*., 2007; Cunningham *et al*., 2011). The utility of using computer models to estimate exposure could be beneficial in risk assessment. The incorporation of low flow data for geographical locations could help identify the ‘hot spot’ areas that perhaps are not fully protected by the current PEC calculation. Overall the limitations of the PEC calculation identified in Section 2 such as dilution

factor of 10 and assumption that drug usage is equal across time frames and geographical area could be reduced. The evidence gathered in the in depth expert knowledge holder interviews suggests that these models used in conjunction with measured concentrations are likely to help ensure validity of predicted environmental concentrations and better protect the environment.

5.2 Metabolites

The in depth knowledge holder interviews indicated that it would be more precautionary for pharmaceutical companies to consider pharmaceutical metabolites in more detail (Section 3). It is known that for some drugs active metabolites can be toxic. In some cases the parent compound undergoes extensive hydrolysis. This means that the parent compound may not be the compound that reaches the environment (see interview analysis Section 3) and that in some cases the metabolites are of paramount importance. For example the anti-viral pro-drug Tamiflu® shows increased toxicity in combination its active metabolite (Escher *et al*., 2010). Norfluoxetine, a key metabolite of fluoxetine is known to be as toxic as the parent compound (Nałecz-Jawecki, 2007). Active metabolites with the same MoA as the parent compound should be considered during an ERA (Besse & Garric, 2010). The results of the data analysis performed in Section 2 shows that there is a distinct data gap in the identification and ecotoxicological analysis of metabolites and transformation products of pharmaceuticals. The PEC analysis (Section 2) also highlights the lack of incorporation of metabolite data when considering exposure, especially if PECs are refined for human metabolism and excretion rates.

In order to be precautionary pharmaceutical metabolites should not be released into the environment without any identification or ecotoxicity analysis. There is a potential that the transformation products formed after metabolism in the body and biodegradation during sewage treatment or in the environment may be more stable and therefore more persistent than the parent compound (Kummerer, 2009). At present a final stage of the ERA involves the identification of metabolites that form at levels of 10% or greater of the parent compound. It was suggested that key metabolites of a pharmaceutical, should be identified earlier on in risk assessment (see interview analysis Section 3) in order to fit into the paradigm of green pharmacy. It is potentially unrealistic to identify all the metabolites if the number is substantial or they are formed in such small quantities that they are insignificant.

The results of the molecular docking (Section 4) show that is possible to predict interactions between small molecules and proteins. This may be a way forward for screening metabolites for potential activity. Although the target proteins that a metabolite may interact with are unknown, it may be possible to do a more general screening of molecular docking to a range of proteins as the *in silico* capability increases. The interview analysis also found that QSARs may be useful to predict an increase in PBT through changes in structure in relation to the parent compound. Structural changes in pesticide metabolites/transformation products that increase toxicity and persistence have been identified (Neuwoehner *et al*., 2010). This knowledge could be applied to analysis of pharmaceutical metabolites. One of the potential problems with this solution is that if a new pharmaceutical is unlikely to have a PEC greater than 10 ngl^{-1} (Section 3) then it is also likely that an associated metabolite will be formed at concentrations lower than the parent compound and inevitably, will also have a PEC under 10 ngl^{-1}. Metabolites, therefore, would not undergo the second phase of the risk assessment.

5.3 The 10 ngl^{-1} action limit

The findings of this research indicate that the 10 ngl^{-1} action limit may not be an appropriate trigger action limit for risk assessment of pharmaceuticals (Section 2 & 3). Pharmaceuticals differ from other chemical pollutants in that they are designed to have a specific biological effect. This fact means that having an arbitrary cut off at 10 ngl^{-1} could miss potentially toxic compounds. In order to be precautionary, the environmental impacts of a pharmaceutical should be fully investigated and this is not the case when no effects assessment is performed. Ideally each pharmaceutical should be considered in terms of action limit on a case by case basis. Pharmaceuticals that are carcinogenic, mutagenic or reproductively toxic may be highly potent at low concentrations. Given that the exposure prediction is simply an estimate (Section 2), actual measured concentrations could be above or below 10 ngl^{-1}. The actual concentrations may vary depending on real market penetration and post patent protection. 'Hot spot' areas where the population of a specific location may have higher usage of drug e.g. more elderly populated areas are not reflected in PEC calculations and may have MECs above the trigger value. 'Hot spots' could also occur at certain times of year due to seasonal effects such as

increased usage of some drugs, or low flow surface water conditions. The use of the 10 ngl^{-1} action limit has substantial limitations.

5.4 Intelligent ecotoxicology

Intelligent testing has been highlighted as the forward for ecotoxicity testing of pharmaceuticals by several authors (Winter *et al*., 2009; Boxall & Greenwood, 2010; Gunnarsson *et al*., 2008; Christen *et al*., 2010). The results of the interview analysis also echo this view (Section 3). The results of this research show that bioinformatics databases could be used to aid and direct intelligent testing (Section 4). The results of the interview analysis also indicate that the unwanted side effects in humans of pharmaceuticals should also be assessed as potential modes of action in non target organisms (Section 3). Bioinformatics databases could also be used in this context. It would be interesting to examine the gene sequence differences that led to the surprising sensitivity of *Gyps* vultures in comparison to other avian species. It is not impossible that such effects may be replicated in other systems.

5.4.1 Chronic ecotoxicity tests end point and species selection

The effects assessment and engagement exercise with key players involved in the ERA of pharmaceuticals identified a distinct need for a more intelligent approach to ecotoxicology testing (Section 3). It is impossible to test all the species that might be exposed to a pharmaceutical using all conceivable chronic test end points. It is important to be intelligent in the approach for ERA. The bioinformatics work indicates that bioinformatics and molecular docking be a useful aid to ecotoxicology tests providing a technique to select potentially vulnerable species and direct choice chronic test end points. Molecular docking exercises here provide an inexpensive tool to predict drug interactions with non target organisms. Simple homologue mining exercises such as the ones performed as part of this research using databases such as BLAST and drug bank can be used to identify protein targets for MoA assessment and incorporation of potential drug mixture effects (Section 4).

As the area of genomics increases the usefulness of bioinformatics to ecotoxicity work is also likely to increase. If you consider that there are a finite number of drug targets, and a limited number of species of fish that will be exposed (in UK rivers) it may ultimately be possible to

actually consider all the species that may be exposed and potentially select the most sensitive one. This avoids unnecessarily testing drugs on species that do not have the target receptor. Molecular docking exercises can also predict whether similar drug target receptors would actually interact with the drug. The AutoDock results in this study show that although *D.pulex* produces a similar COX 2 protein, important differences in the amino acid sequence produce differences in the 3D structure of the binding site. These differences prevent interactions with diclofenac and ibuprofen and may explain the lack of chronic toxicity reported in the literature.

This kind of experiment may be more limited in respect to some organisms. The number of species of invertebrates that may be exposed could be substantial. The likelihood of these organisms having complete genomes sequenced is quite slim. However one can never obtain total scientific certainty but it is possible to reduce the uncertainty somewhat using bioinformatics and molecular docking techniques.

5.4.2 Mixtures of pharmaceuticals

One of the potentially serious failings of the current ERA is the lack of inclusion of mixture effects of pharmaceuticals which is the norm in aquatic systems (Section 2). The additive, synergistic or antagonistic effects of other pharmaceuticals that will be present in the environment need to be considered when calculating a PEC for a drug and when performing ecotoxicological tests. The MoA of each pharmaceutical should be considered in respect to other drugs already available that share that same MoA. Bioinformatics databases and molecular modelling such as these performed during this study (Section 4) could significantly increase the depth and breadth of knowledge in this area. Bioinformatics could help reduce some of the scientific uncertainty related to these issues without increasing animal tests or incurring enormous financial costs. Human medicine identifies the effects of combinations of drugs and this information could be important and useful for risk assessment. Pharmacovigilance monitors these effects and could be extended to environmental effects as well and termed pharmaecovigilance (Daughton & Ruhoy, 2008).

5.4.3 Biomarkers and bioassays

One of the findings of the key player engagement interviews was that a potential shortfall of ecotoxicity tests in general was the inherent differences between the laboratory and the field. The laboratory cannot replicate the vast amount of pressures and variables that are present in the

environment. One suggested solution for this was to monitor effects in the environment using bioassays and biomarkers (Section 3). Environmental toxicologists and regulators felt that sharing of test methods including bioassays from pharmaceutical companies would be extremely beneficial. The Environment Agency argues for the EMEA and MHRA to put in place a suitable system to make environmental information on human pharmaceuticals easily available and accessible to facilitate ERA (EA, 2003). More communication and transparency between environmental regulators and pharmaceutical companies is a key aspect of moving towards green pharmacy. Increasing communication between organisations can also help environmental regulators become aware of potential limitations.

5.5 Retrospective environmental risk assessment

The effects of pharmaceuticals which are not new to the market i.e. licensed prior to the current ERA and authorisation requirements must also be examined for effective environmental protection. The findings from the MEC data (Section 2) indicate that many 'old' medicines are present in the environmental at concentrations that require an environmental risk assessment and that chronic effects data for most of these pharmaceuticals is missing. The interviews revealed that regulators and academics believed that this was something that needed to be addressed (Section 3). A prioritisation method was seen as the best way forward with regard to this matter. The example put forward in Sweden (Agerstrand & Ruden, 2009) and suggestions by other authors (Besse & Garric, 2008; Kumar & Xagoraraki, 2010; Roos *et al*., 2012; Cooper *et al*., 2008) for prioritisation procedures should be fully examined. The Water Framework Directive may provide a mechanism by which retrospective risk assessment and environmental monitoring of priority pharmaceuticals can be achieved (von der Ohe *et al*., 2011). The Water Framework Directive 2000/60/EC (WFD) requires "good chemical status" of water bodies in the EU by 2015. WFD contains a defined list of priority substances that can adversely affect the ecosystem. It puts emphasis on the precautionary principle stating that especially in identifying priority hazardous substances, any potential adverse effects of the product should be taken into account and should lead to scientific assessment of the risk (recital 11 & 44, WFD). Inclusion on the priority list is governed by toxic, persistent and bio accumulative characteristics. On this basis three pharmaceuticals have been suggested for inclusion by the German Environment Agency, carbamazepine, diclofenac and ibuprofen (Kampa *et al*., 2010) and recently it was announced

that diclofenac and EE2, two of the pharmaceuticals investigated in this study are to be added to this list (Europa, 2012).

5.6 Sound science and reporting standards

A crucial finding during this research was that there were substantial inadequacies in the literature reporting pharmaceutical concentrations in water bodies and effluent. These were sufficient to limit any contribution to monitoring and risk assessment. It was frequently the case that much environmental data e.g. flow rate etc needed to make PECs environmentally relevant were not included in papers (Section 2). Improvements in standards of reporting of environmental concentrations of pharmaceuticals are needed to enable regulators to use data for risk assessment and this falls to standards set by scientific journals. Monitoring campaigns are expensive and availability of good quality data from scientific publications could effectively feed into monitoring. Work needs to be undertaken to establish guidelines and standards for reporting exposure data in scientific journals. Communication between risk assessors, regulators, ecotoxicologists and academics is needed to determine the requirements and the information to be included when reporting environmental concentrations. Statistical analysis of data should be uniform across publications in order to be able to make comparisons. The data analysis undertaken here (Section 2) was significantly hampered by the lack of uniform statistical analysis or lack of key parameters such as flow rates, population sizes, sampling dates and sewage treatment employed.

The interviews revealed that a lack of sound science and poor reporting standards were also a major problem with toxicological effects literature as well as concentration data related literature (Section 3). The pharmaceutical company interviewee said that as a company risk assessments (PECs, MECs & PNECs) were continually reviewed in the light of new data. However this data was often not of a standard that would lead to a revision in the figures.

One of the limitations in presenting all the information relevant to monitoring and risk assessment in a publication are the journals content and word limits. As a consequence it would help to make access to raw data in supplementary sections or on line access permanently available. It was often impossible to retrieve raw data from authors if they had changed job or if the work was undertaken some time ago.

A standardisation in reporting standards and scientific procedures for effects and exposure data for pharmaceuticals is required to fulfil a green agenda for the whole life cycle of a drug. This would ensure an increase in knowledge on the exposure and effects of pharmaceuticals in the environment. A reduction in scientific uncertainty is an important aspect of risk management. There is also an opportunity to reduce animal testing of pharmaceuticals if ecotoxicological studies are only performed once in a correct and meaningful way with a full sharing of data.

An improvement in the quality of ecotoxicological data and exposure concentration data would also be beneficial when assessing the successes of any mitigation practices that may be put into place to reduce environmental damage by pharmaceuticals.

5.7 Mitigation

Ultimately there has to be a level at which the potential for serious environmental damage from a pharmaceutical outweighs the benefit to human health. If this not the case then what is the point of having an environmental risk assessment? Time, money and effort are wasted in conducting an environmental risk assessment if a medicine cannot be turned down in the event of a predicted high risk.

A key finding from the interviews was the need for some sort of cost to the environment versus benefit to human health analysis to be brought into the risk assessment process if an environmental risk was perceived (Section 3). If the cost was considered too high then mitigation and risk management processes could be put in place. This could involve elements such as potential substitutions to more environmentally friendly drugs, some limitation to the amount of product that could be sold in each country or limitations on where drugs could be taken, e.g. hospitals.

The problem with limiting sales of a drug that is required for human health is that it would be unethical and unfair to start making judgements about who should receive the medication and who should not. If a medication is authorised for market then everybody should be entitled to take it. This consideration leads to more pragmatic management approaches such as trying to reduce the therapeutic dose, the frequency of dose or the administration method (e.g. intravenous as opposed to tablet form). The question is have any pharmaceuticals failed the risk assessment since the 2006 EMEA guidelines were introduced? If not then potentially these

mitigation strategies in reality, may not be needed. Although perhaps unlikely it still does not preclude a new drug posing a high environmental risk that requires risk management and mitigation strategies.

5.7.1 Substitutions

One of the key failings or the ERA is the lack of mitigation measures that are available to reduce the exposure to the environment in light of a real or perceived risk. One solution to this may be the approach taken in Sweden which is to substitute medicines that do the same job with more environmentally friendly ones where they exist (Agerstrand *et al*., 2009). This could also incorporate incentives for producing new drugs that have the same or improved therapeutic efficacy but have a better environmental profile.

The other side of this approach is to make doctors and physicians and the public aware of the environmental impact of pharmaceuticals on labels, websites, leaflets and potentially advertisements and the media. Information could also be supplied on more environmentally sound drug choices. This is one of the more practical approaches to managing risk and green pharmacy that does not prevent medicine being available to those who need it but reduces environmental impacts. It is clearly an achievable goal as demonstrated in Sweden where a system for assessing the environmental effects of drugs as well as clinical needs has been successful (Vidaurre *et al*., 2010).

5.7.2 Pharmaceutical return schemes

Pharmaceutical return schemes are one of the most deliverable options to limit exposure in the environment. The system in place at present for advising and educating the public on the return of unused medicines to the pharmacy for disposal should be given higher prioritisation (Section 3). All of the key players, pharmaceutical companies, doctors, dentists, other prescribing physicians, pharmacies, regulators, water companies and the government should collaborate in addressing public awareness. Pharmaceutical labels and package inserts need to be clear and specific about the return of unused pharmaceuticals. The message needs to be unambiguous. Campaigns on television and newspapers would be beneficial to raise public awareness. Investigation into practical and safe pharmaceutical returns bins for example in GP surgeries and at pharmacies should be undertaken. There is no need at all for unnecessary pollution of the environment from unused medicines. It is unknown what proportion of

environmental burden comes from unused and expired drugs but if estimates that 50% of medicines are not taken are accurate then it could be substantial. Warnings to the pubic about the potential dangers to children and pets from accidental poisonings in the home could also remind people of the importance of returning them to a pharmacy for destruction.

5.7.3 Prescription habits

The results of the interviews (Section 3) indicated that improvements in prescription habits could be one way to reduce the environmental exposure to pharmaceuticals. New methods are beginning to be developed for a more intelligent approach to prescribing. Doctors and prescribing practitioners need to inform patients about potential side effects of medication before the patient picks up the prescription as this can account for a proportion of non compliance. The medical profession should ideally inform patients about returning unused medicines to pharmacies when changes are made in therapy.

Overall the findings (Section 3) and the literature indicate that more could be done to incorporate the environmental significance of pharmaceuticals into training in the medical professions and that there should be dialogue between these professionals and other stakeholders.

5.7.4 Sewage treatment plant improvements

Ultimately regardless of whether pharmaceuticals are consumed or disposed of via the drain, STPs are a key stage in the life cycle of pharmaceuticals. The findings of the data analysis on sewage treatment removal indicated wide ranging performances of STPs across different plants and within the same facility (Section 2). One obvious way to reduce environmental exposure to pharmaceuticals is to increase the capacity of STPs to remove these chemicals. The results of the interviews, however, suggested that this was one of the less favourable options for mitigation of the risk posed to the environment (Section 3). There is a key problem with improving STPs by increasing residence times or adding extra steps such as tertiary treatments e.g. granular activated carbon, ozone or membrane filters, in order to optimize removal of drugs. This is that different drugs achieve better removal with different treatments and that some drugs are resistant to most forms of sewage treatment such as carbamazepine (Section 2). This means that no matter what extra treatment is put in place there will always be some compounds that enter the environment unchanged.

One of the findings during this research was that water companies are wary of any extra burden from upgrading STPs in order to remove greater amounts of pharmaceuticals (Section 3). This was especially true in the light of the fact that apart from EE2, no actual or real detrimental effects of pharmaceuticals have been identified. The precipitous decline of amphibians for example has not been linked to drug residues. The financial implications of upgrading STPs are substantial and it was considered by the majority of interviewees that this was unacceptable without an actual problem being identified. Simply taking a precautionary approach was not justified (Section 3). The other highly relevant consideration aside from financial burden of sewage treatment improvements is that STP upgrades have substantial impact on energy use and emissions i.e. greenhouse gas emissions. Increases in energy use and carbon emissions outweigh any environmental impacts from pharmaceuticals.

5. 8 Green pharmacy

The results of this study highlight some of the scientific uncertainty surrounding the issue of human pharmaceuticals as pollutants of the aquatic environment. The concept of green pharmacy may be a potential way forward to reduce some of the scientific uncertainty and the risks to the environment.

Green pharmacy should cover the entire life cycle of the drug from initial design and lead candidate selection to final monitoring in the environment post market authorisation (Kummerer *et al*., 2007; Daughton & Ruhoy, 2008; Daughton, 2003a & b). The consequences for the environment should be considered at each stage of the lifecycle of a pharmaceutical including the following: 'benign by design' approaches, PBT considerations at lead candidate stage; manufacture, risk assessment; excretion rates; and the pre existence of more or less environmentally sound drugs that already fill the therapeutic role, packaging, marketing and sales, prescription, dispensing, compliance, return schemes, disposal, sewage treatment, degradation during sewage treatment, degradation in the environment, potential remediation strategies.

5.8.1 'Benign by Design'

One way to reduce the exposure and risks to the environment is the benign by design approach. The idea is to consider the potential for PBT and ultimate degradation at the design stage of the drug development process. This is being considered for commercially available

chemicals (Boethling *et al*., 2007).The results of the data analysis in this study (Section 2) show that many pharmaceuticals are pollutants in many aquatic environments, have known detrimental effects but may also have potential unknown subtle chronic effects. The interview analysis (Section 3) revealed that many key players in the pharmaceutical industry and regulation believe that the risk assessment process needs to be moved to a much earlier stage in drug development. Pharmaceutical companies need to think about environmental stability earlier on in the process (Sumpter, 2010). Large pharmaceutical companies are now thinking along these lines (Lubick, 2008).

5.9 Hypothesis revisited

1) The environmental risk assessment (ERA) for human pharmaceuticals and use of predicted environmental concentrations is probably inadequate to protect the aquatic environment. The availability of good quality environmental monitoring data, however, is needed to fully address this hypothesis.

The work carried out on environmental concentrations and PECs of human pharmaceuticals (Section 2) and the expert knowledge holder interviews (Sections 3) suggest that the ERA for human pharmaceuticals could be improved to better protect the aquatic environment. This work has led to a set of recommendations (Section 5.10) for improvement in the ERA.

2) Bioinformatics and molecular docking may be a potential tool to aid and direct the ERA of human pharmaceuticals through a focus on mode of action.

Bioinformatics and molecular docking was shown to be effective in predicting a toxicological effect by diclofenac and ibuprofen on non target aquatic organisms. This was substantiated by ecotoxicology data available in the literature (Section 4). The hypothesis that bioinformatics and molecular docking may be a valuable tool in ERA of human pharmaceuticals is accepted.

5.10 Recommendations and suggestions for future research

In light of the findings in this work on the limitations of the current environmental risk assessment, the following recommendations are made to reduce the risks to the aquatic environment from human pharmaceuticals:

1) **ERA should be performed earlier than currently in the drug development process with consideration of environmental impacts made at the lead candidate stage in the development of new pharmaceuticals.**

One of the major shortfalls identified with the current ERA is that a pharmaceutical cannot be refused for market authorisation even if an adverse environmental risk is identified. The results of the interview analysis (Section 3) lead to the conclusion that mitigation and risk limitation or management action is currently very limited for the reduction of risks to the environment. It would be prudent, therefore, to consider the environmental impact of pharmaceuticals at a much earlier stage in drug development, i.e. the drug candidate stage. The interview with the Environment Agency representatives revealed strong agreement for this and the pharmaceutical company manager revealed that this was a possibility for the future.

2) **ERAs should be performed post marketing and post patent expiry.**

The results of the data analysis (Section 2) show that environmental exposure to pharmaceuticals can fluctuate substantially in time and space. Environmental concentrations of pharmaceuticals are linked to usage which is variable over the life of a drug. The results of the PEC & MEC analysis show that pharmaceuticals can regularly exceed predictions. Currently, when the ERA is performed a default Fpen is used as an estimate of usage as the drug has yet to be marketed. If the drug is successful it is possible that consumption may be above original estimates. If an ERA were performed this would increase the PEC making post marketing ERAs important in environmental protection. The interview analysis revealed that drug sales may also increase once the patent has expired because of the development of generic drugs (Section 3). The bioinformatics work (Section 4) shows that many drugs such as NSAIDs target the same protein receptors potentially producing additive effects. When new drugs reach the market, it is recommended that consumption of existing drugs which target the same receptors should be incorporated into the PEC (See flow chart Section 4.4).

3) **Accurate and reliable data collection schemes should be established to determine consumption of pharmaceuticals for exposure assessments.**

Expert knowledge holders of environmental risks of human pharmaceuticals (Section 3) believed that environmental exposure too pharmaceuticals might be underestimated in regional

'hot spots'. Presently the ERA assumes even consumption of a pharmaceutical over the whole country over the course of the year. It would be beneficial to identify where and at what times of year 'hot spots' may occur. Currently data for regional prescriptions of pharmaceuticals are not available. Accurate data for regional sales of over the counter medicines would also be valuable for exposure assessments because these drugs are often consumed in large quantities which can vary with the time of year e.g. antihistamines and analgesics. One reason for the sometimes orders of magnitude fluctuations in surface water concentrations of pharmaceuticals (Section 2) might be variations in usage. The inclusion of accurate sales data in the calculation of PECs would make them a more reliable prediction of environmental exposure.

4) **Refinements to PEC calculations should not be made without substantial, validated and accurate data.**

The results of the work carried out in Section 2 show that removal efficiencies of pharmaceuticals in STPs are highly varied. The results of the data analysis show that increases in concentration of pharmaceuticals after sewage treatment can occur as well as reducing concentration. Some pharmaceuticals such as carbamazepine are highly resistant to sewage treatment. The PEC and MEC analysis (Section 2) revealed that PECs were much more likely to be an underestimate when refinements were made for removal by STPs. The interview analysis also revealed that estimation of removal of pharmaceuticals by STPs was very difficult because they are often highly polar ionisable compounds (Section 3).

The default values in the crude PEC calculation seem to be precautionary only when refinements for excretion and STP removal are not made. If allowances for these are applied then discrepancies, which are probably caused by underestimates of dilution and wastewater production, become apparent.

5) **Modelling of environmental exposures could be more informative than the current PEC calculation.**

The interviews performed in Section 3 show that expert knowledge holders believe that analytical chemistry techniques for measuring pharmaceuticals have some failings. The analysis also showed that there was scope for improving predictions of pharmaceutical exposure by including some computer modelling (Section 3). It was revealed that this type of tool can

increase knowledge about 'hot spots' due to higher consumption or low dilution may occur. This information could identify areas where monitoring campaigns may be required. Monitoring of pharmaceuticals is expensive and time consuming and making targeted monitoring essential.

6) **All new medicines should undergo ecotoxicological assessment prior to market authorisation regardless of the PEC.**

The work carried out in Section 3 revealed that expert knowledge holders were sceptical about the 10 ngl^{-1} action limit for ecotoxicological assessment. The reason for this primarily was that EE2 has effects at less than 10ngl^{-1}. The data analysis performed in section 2 shows that many pharmaceuticals reach concentrations above this action limit. The work carried out in Sections 2 & 3 reveals that concentrations regularly fluctuate and that PECs can be inaccurate predictors of environmental loads especially if refinements are made to the PEC. It is recommended that some ecotoxicological assessment should be performed prior to market authorisation. This would help reduce uncertainties around unanticipated effects and better protect the environment.

7) **Ecotoxicological assessment of human medicines should include MoA information.**

The work carried out in Section 4 shows that human drug target receptors can be highly conserved in non target species and that there can be a similar MoA. The interviews with expert knowledge holders (Section 3) also revealed that traditional ecotoxicology tests may underestimate effect concentrations and should be revised to include MoA tests.

8) **Bioinformatics and molecular docking packages should be examined for their potential to aid and direct ecotoxicological tests for ERA of human pharmaceuticals. Including use of MoA information and selection of sensitive species.**

The work in Section 4 shows that bioinformatics databases and molecular docking tools can provide valuable information to help predict the effects of human pharmaceuticals in aquatic organisms. The flow chart in Section 4 indicates how these tools could be used to improve the ERA. These tools could be used cheaply to guide selection of sensitive organisms and chronic test end points in ecotoxicological tests.

9) **The effects of mixtures of pharmaceuticals including those that may cause synergistic, antagonistic or additive effects need to be listed in ERAs and included in PEC and PNEC calculations.**

The results of the work carried out in Section 4 show how drugs of the same therapeutic class may target the same receptor protein and produce additive effects. The data analysis in section 2 shows that the pharmaceuticals investigated occur as mixtures in surface waters in order to address these problems. PECs should be revised to include additive, synergistic or antagonistic effects of other drugs currently marketed (see proposed flow chart for ERA Section 4.4.10).

10) **A prioritisation strategy should be developed in order to conduct retrospective risk assessment for existing medicines.**

The interviews performed in Section 3 show that retrospective risk assessments of pharmaceuticals licensed prior to 2006 is thought to be necessary by many expert knowledge holders. The data analysis in section 2 also shows that the selected pharmaceuticals frequently occur in concentrations far exceeding 10 ngl^{-1} in surface waters. It is clearly not possible to conduct an ERA for all licensed medicines and it is proposed that a system for prioritisation such as that employed in Sweden would be the most pragmatic approach for retrospective risk assessment of existing medicines.

11) **There should be a public education campaign in order to improve understanding of the correct disposal of medicines. Public education is crucial for the reduction of pollution by human pharmaceuticals.**

The work carried out in Section 3 revealed that expert knowledge holders believed that informing the public on the correct disposal of medicines would be one of the easiest ways to reduce environmental concentrations. Inappropriate disposal down sinks and toilets is a particular problem, especially in the context of the variable performance of STPs in pharmaceutical removal. It was found that this was something that was currently not being performed successfully in the UK and that doctors, pharmacists and the media needed to take action. Government information campaigns might be valuable in this regard.

12) **Mitigation strategies for pharmaceuticals that may pose a significant risk to the environment should be developed.**

The work carried out in this thesis leads to the conclusion that one of the major flaws in the ERA is a lack of any strategy for mitigation of environmental risk. Currently pharmaceuticals cannot be refused market authorisation even if they pose a substantial environmental risk. This results in a lack of incentive to investigate precautionary management and renders the identification of risks somewhat pointless. Human health must take priority but in a world where population and age expectancy is increasing so will the environmental burden caused by pharmaceuticals. It may be useful to build in, as a significant stage in drug design, consideration of ways to limit environmental risk.

13) **Research is needed to develop a set of reporting standards for environmental exposure data articles in peer reviewed scientific journals useful to regulators, risk assessors and policy makers.**

The work carried out in Section 2 shows that currently reporting standards in the peer reviewed literature are too poor to perform meaningful meta analysis of pharmaceutical concentrations. The increasing need for science to help inform policy and the current fiscal climate mean that it is paramount that expensive and time consuming research is made usable to environmental regulators and risk assessors. This has led to the development of a list of proposed reporting standards that could be used by researchers and peer reviewed journals to improve the quality of the publication of environmental concentration data (Section 2.4.7).

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