Emerging Pollutants: Their Analysis, Occurrence and Removal in Aquatic Environments

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Abstract

The input of emerging pollutants into the natural environment is of considerable concern due to their potential implications for the health and development of humans and wildlife. Knowledge of the occurrence and removal (by sewage treatment) of these chemicals is limited and there is a need for these to be investigated if the transport and fate of these chemicals is to be better understood. To develop our understanding, reliable, accurate and precise measurements of these compounds at the very low (often sub-nanogram) concentrations at which they may be found, and may still be toxic, is crucial. However, as a result of the increasing international concern, increasing research attention has led to a large number of analytical techniques described as being suitable for the analysis of these compounds; this fragmentation and lack of collaborative focus is likely to have resulted in a lack of refinement of the techniques employed. In this research, a number of these proposed analytical and sample pre-treatment techniques have been assessed, both by internal experimentation and through inter-calibration with collaborating laboratories, to identify which techniques are best suited to further development for research in this area, and have subsequently been optimised, to examine the removal efficacy of traditional and novel sewage treatment techniques, and to monitor EDC and Pharmaceutical concentrations in several UK rivers.

Monitoring of the river Ray, Swindon, UK over a period of three years, using spot-sampling and 24-hour and 7-day integrated sampling, combined with solid-phase extraction (SPE) followed mass spectrometric analyses, showed stable EDC and pharmaceutical levels, typical of comparable rivers throughout the EU, but with a significant reduction in concentrations after the installation of a granular activated charcoal plant at the Rodbourne Sewage Treatment Works (STW) of which the river Ray is a conduit. These results were in agreement with results from analyses biological assays, such as yeast estrogen screening performed independently by another laboratory.

List of abbreviations

CEFAS	Centre for ecology, fisheries and aquaculture science
CEH	Centre for ecology and hydrology
CFUF	Cross-flow ultrafiltration
CI	Chemical Ionisation
CRM	Certified Reference Material
Defra	Department for the environment, food and rural affairs (UK)
EEQ	Estrogenecity (in Estradiol Equivalents)
EI	Electron Impact
EPI	Estimation Programs Interface
eV	Electron Volts
GAC	Granular Activated Carbon
GC	Gas Chromatography
GC-MS	Gas Chromatography with mass spectrometry
GC-MS/MS	Gas Chromatography with tandem mass spectrometry
GPC	Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography

IS	Internal Standard
K_{coc}	Colloidal organic carbon normalized partition coefficient
K_{oc}	Organic carbon normalized partition coefficient
K_{ow}	Octanol/water partition coefficient
K_p	Sediment/Water partition coefficient
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave assisted extraction
m/z	Mass-to-charge ratio
MRM	Multiple Reaction Monitoring
NP	Normal Phase
POCIS	Polar organic chemical integrative sampler
PML	Plymouth Marine Laboratory
RF	Response Factor

RRF	Relative Response Factor
RP	Reverse Phase
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
SIM	Selected Ion Monitoring
SPE	Solid phase extraction
US EPA	United States (of America) Environmental Protection Agency

Chemical abbreviations

ACN	Acetonitrile
DCM	Dichloromethane
EDC	Endocrine Disrupting Chemical
E1	Estrone
E2	Estradiol
EE2	Ethinylestradiol
IPA	Isopropyl alcohol

OP	Octylphenol
NP	Nonylphenol
MeOH	Methanol
PAH	Polycyclic Aromatic Hydrocarbon
PPCP	Pharmaceuticals and Personal Care Product
POP	Persistent Organic Pollutant
Pro	Propanolol
Sul	Sulfamethaoxazole
Meb	Mebeverine
Thio	Thioridazine
Carb	Carbamazepine
Tamo	Tamoxifen
Meco	Mecoprop
Indo	Indomethacine
Diclo	Diclofenac

Chapter 1. Introduction

In recent years, with advances in analytical techniques, emerging pollutants have received considerable attention from the scientific and political community [1][2][3][4]. Of these, endocrine disrupting chemicals (EDCs) and residues of pharmaceutical and personal care products (PPCPs) have been shown to have deleterious effects for wildlife and humans and have subsequently been at the forefront of scientific and legislative concern.

Exposure to EDCs can have an effect on organisms, as well as potential implications for progeny. In wildlife, feminisation of fish and other aquatic organisms after exposure to EDCs has been well-documented, with the potential to reduce fertility and impact upon fish populations [5][6]. Additionally, there is an increasing body of evidence to suggest implications for humans, including lower sperm counts, undescended testicles, early puberty, thyroid dysfunction and cancers [7][8]. As well as physiological implications, there is growing concern of the behavioural effects of EDCs and has been linked to neurodevelopment disorders including attention-deficit hyperactivity disorder (ADHD), autism and intellectual impairment [7]. In wildlife, alterations such as in sexual behaviour and aggression have been observed [9].

There are over three-thousand pharmaceutically active compounds in use, not including personal care products (e.g. ingredients used in cosmetics) or illicit drugs [10]. The number of compounds suspected of exhibiting endocrine-disrupting effects is more difficult to quantify, because EDCs transcend typical chemical classes and there is no formal classification or registration system. However, 127 pesticides have been identified as exhibiting such effects [11], on top of some 60 compounds which have similarly been

identified in other chemical classes [12], the EU recently recognised over 546 ‘suspected’ EDCs in a list which notably omits the steroidal estrogens, perhaps due to a lack of understanding of their environmental persistence and the focus of the report on industrial chemicals [13]. Whilst some EDCs and PPCPs degrade rapidly in the environment, where they are continuously emitted, even rapidly degrading substances can exhibit pseudo-persistent behaviour; others may persist in the environment due to their chemical properties and may be classed as persistent organic pollutants (POPs). Among EDCs, estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) exhibit the most potent estrogenic activity and are of greatest concern [14].

Although some EDCs may be naturally occurring, the majority are anthropogenic in origin and the occurrence of these and pharmaceutical residues in the environment, in particular aquatic environs has increased proportionally to the growth in their use. Potential inputs of EDCs and PPCPs to the environment are numerous, including agricultural run-off [15], hospital and pharmaceutical waste [16], industrial waste and spillage incidents [17]. However, sewage effluent is widely accepted to be the primary source of the majority of EDCs and PPCPs [18].

Due to the potential risk to the health of both humans and wildlife, there is widespread acknowledgement of the need to better understand the occurrence of emerging pollutants, to ascertain whether they are present at levels which are sufficient to incite deleterious effects, and subsequently if the risk they pose is of concern. Risk assessment of this kind requires the ability to robustly monitor these compounds, which to date has proved a major scientific challenge, particularly where these compounds are present in the environment at very low, yet still potentially toxic, concentrations.

In light of the potential risk, however, national and international authorities are keen to prevent the release of EDCs as well as other emerging pollutants into the environment as a precaution. As such, granular activated carbon (GAC) has been shown, through laboratory-based experimentation [19][20], to be suitable for the removal of a range of synthetic organic chemicals and dissolved naturally-occurring chemicals from wastewater, including several EDCs and PPCPs, and was commissioned at a UK sewage treatment works (STP) approximately midway through the experimentation stage of this work to enhance the removal of organic pollutants.

1.1 Aims and objectives

1.1.1 Research Aims

The broad aim of this thesis is to determine the occurrence of selected EDCs and pharmaceuticals in water and wastewater and to understand their removal in wastewater by novel post-tertiary techniques.

1.1.2 Objectives

I. To develop robust and optimised analytical methods for the selected compounds

II. To assess the spatial and temporal variability of the selected compounds in wastewater and river water

III. To assess the efficiency of granular activated carbon (GAC) in the removal of the selected compounds in wastewater

1.1.3 Thesis layout

This thesis is comprised of 8 chapters, 4 of which are experimental. Chapter 1 provides a broad introduction to the subject and briefly describes the background to the study. It additionally includes the aims and objectives underpinning the research which this thesis covers. Chapter 2 includes a comprehensive summary of the state of scientific knowledge on the subject of EDCs and pharmaceuticals as environmental pollutants and a review of the literature relevant to this research. In chapter 3, the materials and methods used throughout the experimental work are described and discussed. In chapter 4, three popular techniques for the analysis of the most potent EDCs - the estrogenic steroids E1, E2 and EE2 in environmental water are examined to determine which is most suitable. Chapter 5 discusses a number of techniques to improve the analysis of environmental estrogenic steroids in environmental matrices, including the experimental comparison of numerous popular stabilisation techniques and the use of gel permeation chromatography (GPC) to enhance spectrometric analyses. Chapters 6 and 7 discuss the occurrence of EDCs and pharmaceuticals respectively in environmental matrices, and their removal by sewage treatment processes. Chapter 8 provides a summary of the data presented throughout this thesis and reiterates the conclusions drawn. It additionally contains suggestions for further work, the need for which have become apparent from the data presented in this thesis.

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Chapter 2. Review of the relevant literature

2.1. Introduction

Water pollution from emerging pollutants, which are those chemicals that have recently been shown to occur in water resources and are identified as being a potential environmental or health risk, and yet adequate data do not exist to determine their risk.

Although many of these pollutants have half-lives (t½) significantly lower than more conventional pollutants such as polycyclic aromatic hydrocarbons (PAHs)[1], their continual release into the environment results in their 'psuedo-persistance'. Their research has become an important aspect of current environmental research due to the largely unknown risk posed by their potential toxic effects on wildlife and humans [2-4] and the increasing importance of freshwater resources as a result of an increasing human population and the effects of climate change. As can be seen in table 2.1 there are a wide-range of chemicals which are currently described as 'emerging pollutants', indeed, it has been estimated that some 55,000 commercial chemicals have the potential to enter the environment [5], the majority of which, until 2007 were poorly regulated, due to a lack of comprehensive legislation which required testing only of 'new' chemical substances. In 2007, the European Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) directive was introduced. This directive places greater emphasis on the manufacturers, traders and users of *all* chemical substances which are produced or imported above 1 tonne/year to discern the properties of chemical substances and subsequently register this information on a Europe-wide central database, with exemptions for substances that are already adequately regulated by other legislation, such as medicines, water, oxygen,

some noble gases, most polymers (although monomers may need to be registered) and natural substances which have not been chemically altered, such as cement and ore.

Due to the increasing importance of freshwater resources, as a result of increasing populations and climate change, it becomes critical that we understand the input, transport and fate of micropollutant chemicals, which, together with toxicological information, afford the assessment of risk that they pose to human and wildlife health. Such substances include endocrine disrupting chemicals (EDCs), pharmaceuticals and personal care products (PPCPs), the focus of this work, which are acknowledged to be continuously introduced to the environment, primarily as a result of their incomplete removal in sewage treatment works (STWs). Consequently, implications for wildlife have been observed – EDCs in the environment have been linked to the feminisation of fish; commonly evidenced by the presence of the egg-yolk protein, vitellogenin (Vg) in male fish, and diclofenac, a commonly-used pharmaceutical has been shown to accumulate, with a concentration factor of up to 2732, in the liver of rainbow trout and causes histopathological alterations in both the kidneys and gills [6] In vulture populations this drug has been shown to cause renal failure and has resulted in a significant population decline in Pakistan [7].

Table 2.1 Examples of emerging pollutants

Class	Sub-class	Example compounds
Algal toxins	Cyanotoxics	Microcystin-LR
Antifoaming agents		Surfinol-104
Antioxidants		2,6-Di-tert-butylphenol
		4-tert-Butylphenol
Antifouling compounds	Antifouling compounds	Irgarol
	Organotin compounds	Dibutyl Tin ion
		Triphenyltin ion
Biocides		Triclosan
		Methyltriclosan
		Chlorophene
Bio-terrorism agents		Chloropicrin
Detergents	Ethoxylates/carboxylates of octyl/nonyl phenols	Octylphenols
		Nonylphenols
Disinfection by-products		Cyanoformaldehyde,
		Decabromodiphenyl ethane
		N-Nitrosodimethylamine
Flame retardants	Polybrominated diphenylethers	Heptabromodiphenyl ethers
		Pentabromodiphenyl ethers
	Brominated flame retardants	Bisphenol A
		Decabromodiphenyl ethane
Fragrances	Fragrances	Camphor
		d-limonene
		Terpineol
	Polycyclic musks	Galaxolide
Drugs of abuse	Illicit and illegal drugs	Cocaine
		Heroin
		Morphine
		Codeine
Nanoparticles	Silicon-based	Silicon Carbide
	Fullerenes	Carbon-60 (Buckyballs)
	Metal-based	Titanium Dioxide
Personal care products	Sun-screen agents	Benzophenone,
	Insect repellants	N,N-diethyl-m-toluamide
	Parabens	Methyl-paraben
Pharmaceuticals		Carbamazepine
		Diclofenac
		Caffeine
		Ethinylestradiol
Plasticizers	Phthalates	Benzylbutylphthalate
		Diethylphthalate
	Other	Bisphenol A
		Triphenyl phosphate

2.2 Routes of entry of emerging pollutants into the aquatic environment

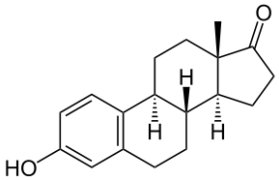
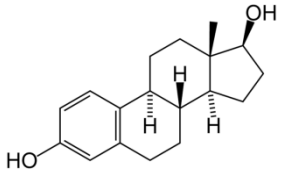
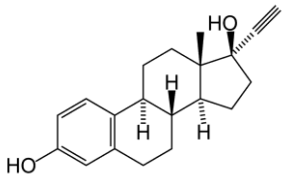
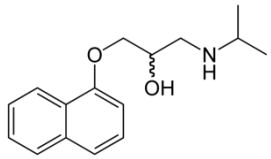
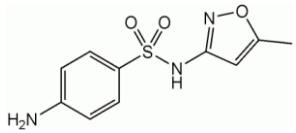
Human activity is the majority source of the emerging pollutants that enter the environment. The wastewaters from our homes and places of work contain not only a multitude of excreted chemicals of both natural and synthetic origin, but also the chemicals we use for our personal care, cosmetic and cleaning purposes. Most STWs are not designed for the removal of these chemicals, and as a result, many pass through the sedimentation and biological processes that these plants employ, into waterways via effluent. Thus, the primary route of entry to the aquatic environment is from household and industrial waste waters via the outfall from STWs. There are, however, other routes of entry, such as: run-off from pavements, roads and agricultural land; atmospheric deposition, bathing, residues from hospitals and pharmaceutical manufacturing, illicit drug manufacturing, fish farming, leaking landfills and the deliberate disposal of unwanted medications into waste waters or directly into waterways.

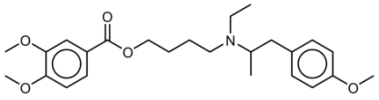
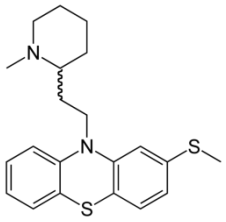
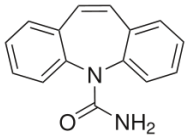
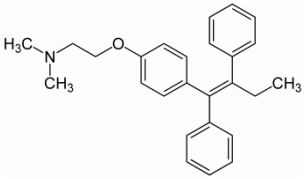
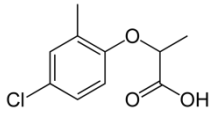
Livestock excreta run-off, directly from fields into the river systems, is another viable route of entry for steroidal hormones and veterinary drugs. However, animal waste is not processed at STWs and the final effluent pumped out into rivers. Instead, it is directly or indirectly excreted straight onto the soil. In the UK, where large numbers of farm animals, such as cows and sheep, vastly outnumber the human population, and so represent a major possible source of emerging pollutants. However, the half-lives ($t_{1/2}$) of estrone (E1) and 17β-estradiol (E2) have been shown to be significantly reduced in soil, under certain environmental and climatic conditions [8]. It has been shown however that dairy farm effluents have the potential to deliver high levels of E1 to water systems [9] due to the population density of cows and the amount of steroid estrogens that a cow excretes – 2

orders of magnitude higher than a human [10]. The degradation of two growth-promoting steroids in cattle have been previously studied [11] by implanting trenbolone acetate, an androgen, into the ears of male and female cattle and feeding them with feed laced with melengestrol acetate, a progestin. It was found that 10 % of both steroids were excreted, and, when the manure was stored, the $t_{1/2}$ was 260 d. When the liquefied manure was spread onto fields the $t_{1/2}$ decreased to 7 d for trenbolone acetate and 2 months for melengestrol acetate. However, the authors were unable to determine what share of the chemicals' disappearances could be explained by microbial degradation or by run-off. The $t_{1/2}$ of E1 and E2 have also been examined [12] and it has been shown that in unamended soils the $t_{1/2}$ of E1 and E2 was 5 – 25 d, depending on soil type. In soils amended with animal manure the rate of degradation of these two chemicals was more rapid (1 – 9 d). They concluded that "*the risk of freshwater contamination by estrogens due to normal landspreading rates and methods was low*". Studies into the run-off of nine PPCPs (Atenolol, Carbamazepine, Cotinine, Gemfibrozil, Naproxen, Ibuprofen, Acetaminophen, Sulfamethoxazole and Triclosan) applied to field plots via biosolid slurry, using both subsurface injection and broadcast application, with simulated precipitation [13]. PPCPs in run-off from the injected plots were found at levels < LOD, whereas 7 of the compounds were detected in levels ranging from 70 -1477 ηg/L in run-off from the broadcast application. This suggests that subsurface injection or ploughing of fields directly after slurry treatment could eliminate surface run-off of PPCPs.

The transport and fate of organic compounds in the aquatic environment are determined by chemical, physical, and biological processes. These processes include: volatilization, absorption, wet and dry deposition, microbial degradation, sorption, hydrolysis, aquatic

photolysis, oxidation, chemical reaction, bio-concentration, advection, and dispersion. The relative importance of each of these processes depends on the characteristics of the organic compound and also of the aquatic system itself.

Compound Name and molecular formula	**Structure**	**Molecular Mass**	**Log Kow**	**Kcoc (L/kg)**	**LogBCF**	**Aqueous solubility (mg/L)**
Estrone $C_{18}H_{22}O_2$		270	3.43	$2.97x10^3$- $2.22x10^5$	1.77- 4.15	13-146.8
Estradiol $C_{18}H_{24}O_2$		272	3.94	$6.40x10^2$- $1.91x10^4$	2.23- 3.97	3.9-81.9
Ethinylestradiol $C_{20}H_{24}O_2$		296	3.67- 4.15	$0.72x10^3$- $5.6x10^4$	1.15- 3.92	4.8- 116.4
Propranolol $C_{16}H_{21}NO_2$		259	3.48	$2.82\text{-}9.01x10^2$	1.71	61-228
Sulfamethoxazole		253	0.89	$2.58x10^2$	0.168- 0.5	610- 3942

$C_{10}H_{11}N_3O_3S$						
Mebeverine $C_{25}H_{35}NO_5$		466	5.12	$5.4x10^3$-$1.3x10^5$	1.81- 3.04	0.05- 0.80
Thioridazine $C_{21}H_{26}N_2S_2$		370	5.90- 6.45	$1.14x10^4$- $1.24x10^5$	1.97- 3.56	0.03- 0.14
Carbamazepine $C_{15}H_{12}N_2O$		236	2.25- 2.45	$1.68x10^2$- $1.33x10^3$	1.28	17.66- 12.0
Tamoxifen $C_{26}H_{29}NO$		371	6.30	$2.51x10^4$- $2.61x10^6$	3.09- 3.83	0.02- 0.10
Mecoprop $C_{10}H_{11}ClO_3$		214	2.94- 3.13	4.85x10- 9.58x10	0.50- 2.11	168-860

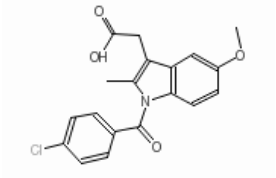
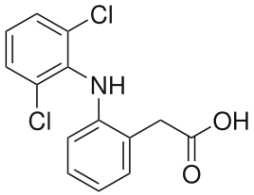
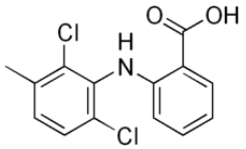
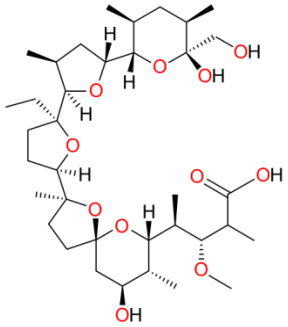
Indomethacine $C_{19}H_{16}ClNO_4$		357	4.23- 4.27	2.20 $8.01x10^2$	$x10^2$-	0.50- 2.93	0.81- 3.11
Diclofenac $C_{14}H_{11}Cl_2NO_2$		296	4.02- 4.15	4.04 $4.57x10^2$	$x10^2$-	0.50- 3.15	2.37- 10.89
meclofenamic acid $C_{14}H_{11}Cl_2NO_2$		296	6.02	$4.19x10^2$-2769 $x10^3$		1.75- 3.35	0.09- 0.11
Monensin $C_{36}H_{62}O_{11}$		670	5.43	6.21x10- $3.65x10^2$		1.00- 3.12	0.01- 356.9

Table 2.2 Chemical properties of steroidal estrogens and selected pharmaceutical compounds

2.2.1 Entry of Endocrine Disrupting Chemicals and Pharmaceutically-active compounds to the aquatic environment

EDCs and PPCPs, as with many other emerging pollutants can be discharged to the aquatic environment from both point and non-point or diffuse sources. A point source has a clearly definable point of entry into the aquatic systems, while a non-point or diffuse source has an unknown point of entry. As far as EDCs and PPCPs are concerned, the major point sources are effluent discharges from STW, industrial wastewater, and landfill leachate, while diffuse sources are diverse including surface runoff from agricultural land and atmospheric deposition[1]. Of all the sources, STW has received the greatest attention worldwide. This emphasis has several reasons, first the estrogenic effects of EDCs (fish feminisation) was first discovered in fish population downstream of STW effluents, a trend since confirmed globally. Secondly, levels of EDCs and PPCPs in wastewater tend to be higher than those in surface waters, and are hence more easily detected. Moreover, water companies are under increasing pressure to improve the efficiency of EDC removal, to meet national and global regulations for EDCs, hence it is in their interest to monitor regularly the EDC concentrations in wastewater samples.

EDCs in STWs have been previously [14], it was found that the removal efficiency of EDCs is highly dependent on the properties of individual compounds, and the technology being used. For example, 90% of PCBs could be removed by biofiltration, whilst 70% of E2 and EE2 could be removed by filtration. Most STW have two stages of treatment, primary sedimentation followed by secondary treatment. Some EDCs such as alkylphenols could be removed significantly in primary treatment, by adsorption to sediments. But for the most potent EDCs such as E1, E2 and EE2, their removal in primary

treatment is very limited as they do not interact strongly with sediments. Hence secondary treatment plays a critical role in removing them from wastewater. Of the different secondary treatment technologies, activated sludge process is the most versatile and most widely used. The performance of activated sludge process in the removal of EDCs has been reviewed by Johnson and Sumpter (2001), who suggested the process can consistently remove over 85% of E2 and EE2, although the removal of E1 is more limited and variable.

Both EDCs and pharmaceutical residues have been monitored previously through the different stages of treatment in a STW in the UK [15-16], these studies found that the most abundant EDC was bisphenol A which ranged from 298 to 1010 ng L^{-1} in the influent. This is expected due to the wide use of bisphenol A as a monomer for the production of polycarbonate and epoxy resins, unsaturated polyester-styrene resins and flame retardants[41,42] The concentrations of other compounds in the influent varied from 14-78 ng L^{-1} for 4-*tert*-octylphenol, 87-321 ng L^{-1} for 4-nonylphenol, 20-60 ng L^{-1} for E1, 26-51 ng L^{-1} for E2, < 0.8-10 ng L^{-1} for EE2, and 24-33 ng L^{-1} for hydroxyestrone, respectively. EDCs were not significantly removed by primary sedimentation, especially natural estrogens, indicating that EDCs were weakly binding to particles contained in wastewater. This is expected, as they are weakly hydrophobic organic pollutants[43], as demonstrated by their small K_{ow} values (Table 2.2). When extracting the coarse particles collected by filtration through 20-μm filter discs in situ, it has been found that the particle fraction contained less than 0.1 ng L^{-1} of E2 [17]. Similarly, other another study found no evidence that estrogenic compounds were adsorbed on the particles collected by filtration through 0.2-μm filter [18]. Not surprisingly, the concentrations of selected EDCs were reduced substantially after biological filtration in an STW in Horsham, UK. Natural estrogens were

reduced to an average concentration of 17 ng L^{-1} for E1 and 16 ng L^{-1} for E2, respectively. For the other compounds, more than 50% reduction was achieved after the secondary treatment. From these data it clear that STW treatment technology is a significant factor in understanding the likely release of endocrine disrupting and pharmaceutical compounds into the aquatic environment. The work in this thesis examines further the role of new tertiary treatment technologies (such as activated carbon) compared to existing wastewater clean-up processes.

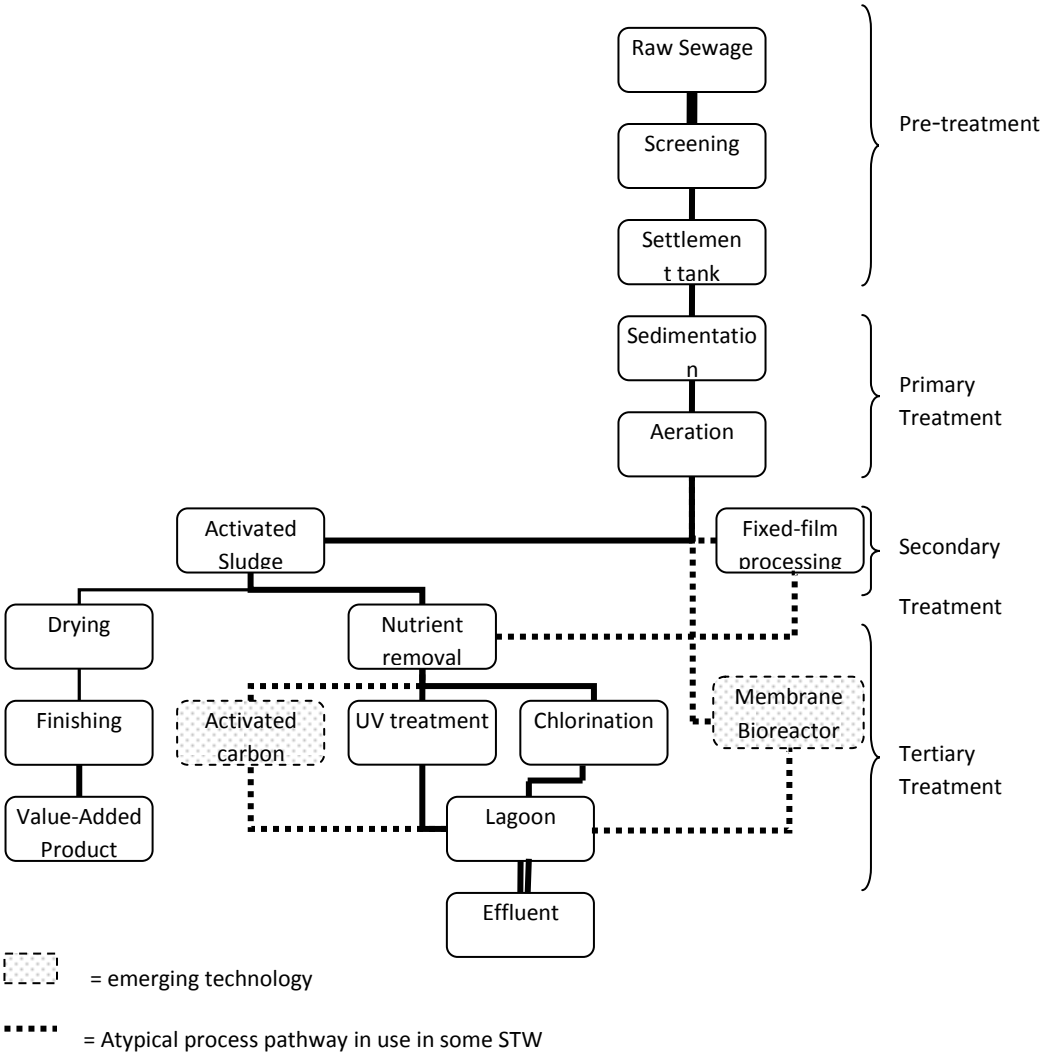


Figure 2.1 **Flow diagram of a typical STW**

The fact that all of the target EDCs were found in the effluent, e.g. 4-nonylphenol (6-36 ng L^{-1}) and E1 (6-10 ng L^{-1}), indicated that EDCs were not completely removed during the treatment processes. Based on the field data, removal efficiency defined as the net change of chemical concentrations between influent and effluent was calculated, which ranged from 59% for 4-nonylphenol to 100% for 4-*tert*-octylphenol. Overall, bisphenol A showed the highest removal efficiency (90-96%), as it is readily biodegraded. It has been suggested that technologies to remove NP are both expensive and to date, not 100% efficient [19]. With a range of chemical classes suspected to have endocrine-disrupting effects, each with a vast range of chemical properties and behaviors, the development of efficient removal technologies for all EDCs is a major challenge, which needs to be balanced with other costs, both financial and environmental. The disposal of pollutant-containing sludge remains a major issue, and where removal technologies are 'CO_2 intensive' they may contribute to other environmental degradation, such as the so-called anthropogenic climate change effect.

In addition to STW, other sources of inputs include animal farming, aquaculture and spawning. It has been proposed [20] that the input of EDCs to aquatic systems by agriculture is likely to increase due to the increasing use of sludge and sludge-derived products as so-called value-added products (VAPs), such as pesticides and fertilizers.

2.3 Endocrine Disrupting Chemicals (EDCs)

2.3.1 Introduction

Over the last two decades a variety of effects that are attributable to EDCs have been observed including changes in sperm counts, genital tract malformations, infertility and an

increased frequency of mammary, prostate and testicular tumours [21-24]. EDCs are a very wide and diverse group having a multitude of action mechanisms on the affected organisms, including substances that mimic and/or antagonise the effects of hormones, alter the pattern of synthesis and metabolism of hormones, and modify hormone receptor levels [25-27]. The UK Environment Agency (EA) has highlighted the seriousness of the problem as the range of substances that have been reported to cause endocrine disruption is extremely diverse and the list of potential suspects is rapidly growing[9]. Among the potential EDCs including steroids, alkylphenols, organochlorine pesticides, triazine herbicides, polychlorinated biphenyls and phthalate esters, three compounds, i.e. estrone (E1) and 17β-estradiol (E2) both being natural hormone, and 17α-ethynylestradiol (EE2, synthetic hormone) have been widely studied and shown to cause feminisation of fish [28-29]. Many of such compounds are classified as priority substances in the EU's Water Framework Directive (2000/60/EC). In terms of estrogenic activity, however, the most important EDCs are E1, E2 and EE2, as they are far more potent than other compounds such as bisphenol A or alkylphenols, and can cause fish feminisation at approximately the ng L^{-1} level[10,12]. Due to uncertainty in their impacts on terrestrial and aerial organisms as a result of lack of data, E1, E2 and EE2 are not yet included in the list of 146 substances with endocrine disruption classification; nevertheless, their feminisation effects in invertebrates and fish have been confirmed worldwide.

2.3.2 The Endocrine System

The endocrine system is essential to both plants and animals as it is responsible for the regulation of growth, reproduction, maintenance, homeostasis and metabolism, through the production of hormones with different functions[1]. Hormones are chemical messengers that

are secreted by endocrine cells into the bloodstream and other extracellular fluids, which go on to bind with specific receptors on a cell and thus communicate instructions.

The endocrine system consists of a number of glands that produce hormones. These include: the pituitary gland, adrenal glands, thyroid glands, parathyroid glands, pancreas, ovaries, and testes. A number of other cells are also capable of producing hormones such as myocytes in the heart and epithelial cells in the stomach and intestines. These cells are part of what is often referred to as the 'diffuse endocrine system'.

The normal function of endocrine system can be disrupted by the so called endocrine disrupting chemicals (EDCs), which interact with the hormone receptors, hence producing unnatural responses.

2.3.3 Definition of EDCs

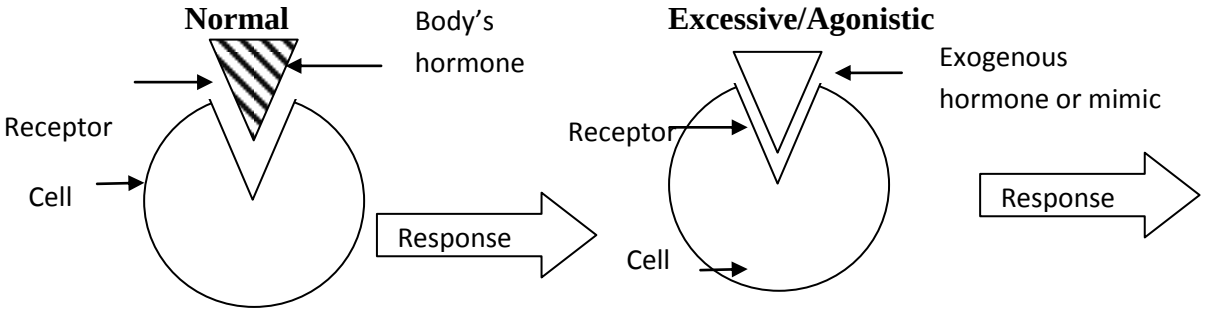
According to the EU, an EDC are defined as "an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny, or (sub)populations". A similar but more detailed definition was proposed by the US Environmental Protection Agency (EPA) as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour".

2.3.4 Implications of EDCs for human health and wildlife

EDCs can operate as hormone mimics (agonistic response) and use the cell's receptors to inhibit or stimulate the normal cell response. This is exactly how a normal hormone works, therefore all hormones are agonists. They can also act as hormone blockers (antagonistic response). EDCs can also affect normal hormone synthesis as well as bring about the

removal of the hormone from the circulation. The processes of endocrine disruption are more succinctly demonstrated diagrammatically, as in figure 2.2.

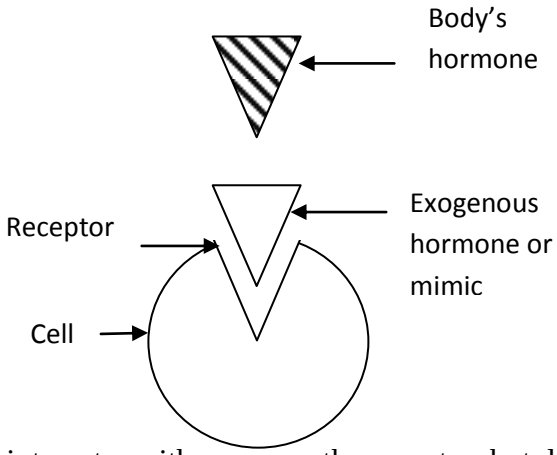
Figure 2.2 Principles of the action of endocrine disruption



Normal hormone activates its receptor at an appropriate level and/or time

Exogenous substance activates the receptor at an excessive level and/or inappropriate time

Blocked/Antagonistic



Exogenous substance interacts with the receptor, but does not illicit a response, thus inhibiting normal activation and response

There is evidence that EDCs are capable of causing feminisation, polymorphism, as well as causing problems with gametogenesis and gonadogenesis in fish [30][31][32][33]. It has

also been shown that they can cause similar problems in insects [34], amphibians [35], reptiles [36] molluscs [37], [38] and even sponges [39].

Birds are also at risk as was highlighted in the book *Silent spring* [40], which discussed the risk to the bird population by the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT). This chemical caused an interference with calcium metabolism and so the birds' eggs became too thin to carry out reproduction.

As for health risks to humans, there is considerable evidence that they have the potential to disrupt the endocrine system. For example, the use of diethylstilbestrol (DES) between 1938 and 1971 as a treatment to help with pregnancy complications such as miscarriage and premature delivery, caused clear-cell adenocarcinoma of the vagina and cervix in daughters of the treated women [41]. The treated women also showed a slightly increased risk of developing breast cancer.

Studies on male hamsters have shown that disruption to spermatogenesis can be caused by neonatal exposure to DES [42]. This is made more alarming with the realisation that DES is also capable of being passed to neonates via breast milk [43].

An hypothesis known as testicular dysgenesis syndrome (TDS) purports that cryptorchidism, hypospadias, impaired spermatogenesis and testicular cancer can all be attributed to disturbed prenatal testicular development [44]. TDS is thought to be related to either genetic or environmental factors. Although there have been studies of children whose parents were accidentally exposed to EDCs, human studies of these problems with regard to EDCs are impossible to undertake for ethical reasons. One study in particular [45]

examined the anogenital distance (AGD) in male children compared to nine phthalate monoester metabolites in prenatal urine samples. The results showed that exposure to phthalates at a concentration below that found in a quarter of American women, was enough to cause genital development irregularities that were consistent with phthalate induced incomplete virilization in rats.

Although a causal relationship between human reproductive disorders and EDCs has not been established, there are some concerning trends in the ratio of male to female children being born and in the general sexual health of humans. A study of birth ratios in Denmark, the Netherlands, Canada and the United States between 1950 and 1998 [46], found that from the normal male proportion of births had declined. The change was very small but was statistically significantly and was repeated in all of the countries under study. For example, in Denmark it had dropped from 0.515 in 1950 to 0.513 in 1994 and in the Netherlands it had dropped from 0.516 to 0.513. They concluded that “reduced male proportion at birth should be viewed as a sentinel health event that may be linked to environmental factors".

A study of the links between testicular cancer, low fertility and an excess of females compared to males among offspring [47] concluded that “specific agents that act prenatally to disrupt normal development and differentiation of the male reproductive organs may be particularly relevant, and that current hypotheses link testicular cancer risk to exposure of the male embryo to maternal or environmental estrogens".

Wier *et al* [48] conducted a population based, case control study that looked at men from Ontario, Canada with confirmed primary malignant germ-cell testicular cancer. Questionnaires completed by the men’s mothers concerning their use of prescription

hormones around the time of conception, gave evidence that exposure to maternal estrogenic hormones was associated with testicular germ-cell cancer risk. They also found that exposure to lower levels of maternal hormones appeared to decrease the cancer risk.

2.3.5 Removal of EDCs by STWs

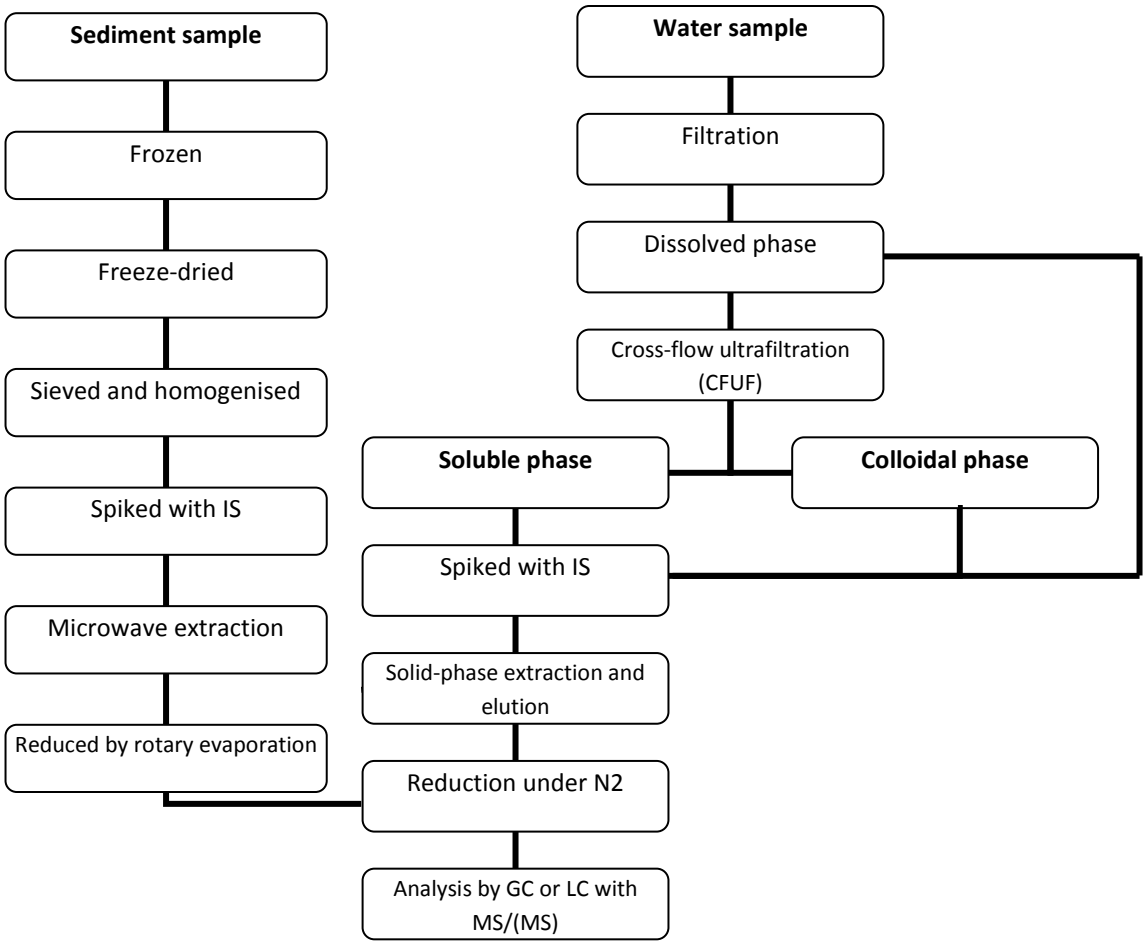
It is generally accepted the most EDCs are discharged into the aquatic environment from the effluents of sewage treatment works (STW). Studies have found that the majority of cases of endocrine disruption occur at or near STW outfall sites [49]. Other sources include animal agriculture, aquaculture and spawning fish [50]. It has also been shown that EDCs can have additive effects when more than one chemical is present [8][15][16]. Estrogenic hormones have been detected in influents and effluents of STW in many countries [10][17-19] surface water [20-21], as well as drinking water [22]. Considering the widespread occurrence and potential impacts of EDCs, it is highly important to remove them before discharge. The current data suggest that wastewater treatment processes (e.g. activated sludge) have variable performance in removing EDCs. By comparing the influent and effluent estrogen concentrations, one study [19] concluded that the removal rates for E1 and E2 were 87% and 61%, respectively. In a Brazilian STW, the observed removal rate for EE2 ranged from 64-78%. In Swedish STW, the average removal rate was 81% by activated sludge treatment and only 28% by solid supported bacterial treatment. It is therefore essential to install additional treatment processes after secondary treatment step, to ensure a more complete removal of such compounds. A typical STW involving primary, secondary and tertiary treatment is shown in Figure 2.1.

2.3.6 Analysis of EDCs in water and wastewater samples

In order to minimise EDC impacts on human and wildlife health, an understanding of occurrence and behaviour their necessary. Accurate, reliable and sensitive analysis of suspected EDCs in environmental water samples is a critical prerequisite for the reliable and routine monitoring of EDCs and EDC-fate studies. The determination of EDCs in environmental water samples can be performed by different techniques, among which GC-MS and LC-MS are the most widely used due to unrivalled sensitivity and selectivity and increasingly wide access. There have been previous reviews on the analysis of EDCs by MS (e.g. [51]). EDC analysis involves many interrelated steps, as shown in Figure 2.2. During sampling, typically 1-2 L of water or wastewater samples is taken and filtered as soon as possible to remove particles. The filtered water samples are then spiked with appropriate internal standards (typically isotope-labelled analogues of the target compounds), followed by concentration using solid-phase extraction (SPE) cartridges or discs. The EDCs adsorbed on SPE are then eluted by using a small volume of organic solvent (e.g. methanol), which are purified (especially important for sewage influent samples) and further concentrated under N_2 blow down. The final sample extracts are analysed by GC-MS or GC-MS/MS after derivatisation, or analysed by LC-MS or LC-MS/MS without further treatment. There have been many studies comparing the performance of different SPE products in concentrating EDCs from water and wastewater samples. Liu et al. (2004) compared the recovery of EDCs in nine different SPE cartridges, and concluded that the Oasis SPE cartridges from Waters generated the highest recovery for E1, E2, EE2, 16α-hydroxyestrone, bisphenol A, 4-nonylphenol, and 4-*tert*-octylphenol. The finding was later confirmed by Zhang et al. (2006). For solid samples such as sediments and sewage sludge, they tend to be freeze-dried first before extraction by various

techniques, one of which is the microwave-assisted extraction. Sample extracts are then analysed in the same way as for water samples.

Figure 2.2 Typical sample processing steps for chemical analysis



The analysis of sample extracts was until recently dominated by GC-MS, which is still widely used. As many EDCs are polar in nature, they need to be derivatised to minimise tailing in GC separation. Two popular reagents used to derivatise compounds bearing hydroxyl groups are *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-(*tert*-butyldimethylsilyl)–*N*–methyltrifluoroacetamide (MTBSTFA), which lead to the formation

of trimethylsilyl (TMS) and *t*-butyldimethylsily (TBS) derivatives, respectively. Catalysts such as trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI) and *tert*-butyldimethylsilylchlorosilane (TBCS) are widely used to enhance derivatisation [28,29]. From the evaluation of a number of similar reagents for detecting both natural and synthetic estrogens by GC-MS, Ding and Chiang (2003) concluded that BSTFA with 1% TMCS operating at 70^{o}C for 30 min was the best derivatisation reagent. However, other researchers suggest a problem with the use of BSTFA or MTBSTFA to derivatise E1 and EE2, as the resulting TMS and TBS derivatives of EE2 could partially be converted to their respective E1 derivatives [28][29]. Zhang et al. (2006) also confirmed that in the absence of pyridine, EE2 can be converted to three different products including TMS-E1, mono-TMS-EE2 and di-TMS-EE2, hence producing erroneous results. It is therefore critical that both pyridine and BSTFA are used simultaneously during derivatisation of EDCs and compounds of similar structures.

More recently, the bench-top tandem MS has become more widely available and hence widely used, due to the advantages of tandem MS being more selective. It has been shown that GC-MS/MS produces increased selectivity and sensitivity compared to GC-MS [30], with limits of detection ranging from 0.01 to 0.49 ng L^{-1} in water samples. A more recent and increasingly popular technique has been the LC-MS/MS, which does not require sample derivatisation. In chapter 4, the performance of GC-MS, GC-MS/MS and LC-MS/MS in the analysis of steroidal estrogens, and it is concluded that the GC-MS technique is the simplest to operate, but fails to detect the estrogens at the lower-end of environmentally relevant concentrations. The tandem MS techniques are more selective than MS, and therefore able to detect lower concentration levels of the three steroidal

estrogens of interest. However, it was observed that the LC-MS/MS technique is more susceptible to matrix interferences for the analysis of samples, resulting in a reduction of the signal-to-noise ratio and a subsequent reduction in reliability and stability compared to GC-MS/MS. With the GC-MS-MS technique offering increased selectivity, the lowest limits of detection, and no false positive identification, it is recommended to be the preferred analytical technique for routine analysis of estrogens in environmental water samples. However, the need for sample derivatisation is a major disadvantage for GC-MS/MS, so overcoming the matrix effect will make LC-MS/MS more reliable in compound identification and hence more attractive, particularly with recent developments such as ultra-performance liquid chromatography (UPLC) and improved mass spectrometric techniques, such as time of flight (TOF) which provide better selectivity than MS/MS, but its sensitivity can be inferior to the latter [52].

As far as water sampling is concerned, two approaches have been used, spot and passive sampling. Spot sampling has long been the preferred method for obtaining environmental water samples for chemical analysis, due to the ease in which samples can be collected, however such samples only yield an instantaneous measurement, and thus suffer from high uncertainty from short- and long-term concentration variability. Subsequently, there has been recent growth in the use of passive sampling techniques allowing for composite sample collection, which can be time and flow weighted. These devices typically employ a sorbent phase encased between two microporous membranes, and can easily be deployed for a range of time periods as required, and may be used to simulate the bioconcentration of pollutants by aquatic organisms. By choosing appropriate sorbent material, passive sampling can be engineered to target for the measurement of

specific classes of pollutants, e.g. Oasis sorbent (Waters) is particularly suited for EDCs while C18 sorbent for non-polar compounds such as triazine herbicides and PCBs. One of the limitations, though, is the need to obtain sampling rates of the target pollutants in the field, which can vary due to different hydrodynamics, sediment load, pollution source strength and biofouling.

In addition, biological-based essays are also widely employed for the measurement of EDCs in environmental samples, on which an extensive review was recently published [33]. These biologically directed methods include whole organism assays, cellular bioassays, and non-cellular assays, among which cellular bioassays such as yeast estrogen screen (YES) and yeast anti-androgen assay (YAS) are perhaps the most widely used. YES was developed by Routledge and Sumpter (1996)[34], by engineering YES cells with a human estrogen receptor gene which then binds to an estrogen response element regulated-expression plasmid (lac-Z) coded to express β–galactosidase. YES provides a measure of the so-called E2 equivalent (EEQ) which equates to the combined additive estrogenicity in the presence of various estrogenic chemicals. Whether a correlation exists between YES-generated value and EEQ derived from chemical measurement is yet to be established.

Another widely used bioassay is the enzyme-linked immunosorbent assay (ELISA). Immunoassays show attractive features for organic trace analysis due to the fact that they require little sample pre-treatment, exhibit high sensitivity, and are inexpensive in comparison to the instrumental analysis such as GC-MS and LC-MS. A considerable number of ELISA kits have been developed, which are commercially available and used for the analysis of a range of contaminants in water samples, such as estrogenic compounds, surfactants and pesticides, and more recently pharmaceuticals, and personal care products.

2.3.7 Occurrence, transport and transformation of EDCs in rivers

EDCs from different sources eventually find their way into natural waters such as rivers, streams, lakes, groundwater, estuaries and sea, in the form of dissolved compounds as well as in association with sediments. EDC monitoring tends to be focused in the dissolved phase due to more frequent detection and direct impact of EDCs on fish.

In the last two decades, numerous publications have reported results of surveys of the most potent EDCs; the natural steroidal estrogens E1 and E2, and the synthetic EE2. These three compounds have been found in natural river systems at concentrations ranging from below the limit of detection to 112 ng L^{-1}, below the limit of detection to 200 ng L^{-1} and below the limit of detection to 50 ng L^{-1} for E1, E2, EE2 respectively. It is suspected that the highest concentrations occur where raw or poorly treated sewage is discharged directly into water streams and rivers. A more complete distribution dataset is shown in Table 2, which shows the focus of environmental monitoring has been in river environments, with less attention to other environmental waters, hence the sparse data for lakes, seawater, estuarine and groundwater environments. Globally, whilst there is a large variability in the ranges of concentrations being reported in each country, the variability between countries appears to be less significant. There does however appear to be greater variability in reported concentrations in Chinese rivers, potentially due to the rapid industrial growth in some regions, although this may also be at least partly attributable to the larger number of separate studies undertaken in comparison to elsewhere.

Natural processes in river environments which may remove steroidal estrogens from the aquatic phase include volatilisation, degradation and sorption to solid phases. It has been suggested that degradation is primarily through the biotic route and that under abiotic

conditions estrogen levels remained relatively constant [47]. In the presence of common bacteria and algae, however, a rapid reduction of estrogenicity is observed, where the biotransformation of E2 to the less potent E1 has been shown to follow first-order kinetics with a half-life of 0.2-9 days compared to >10 days under ideal-laboratory conditions.

Several publications have attempted to estimate the partitioning of these steroidal estrogens in different environmental phases. Predominately, these have focussed on the dissolved aqueous phase and the sediment phase. In laboratory conditions, it has was found that sorption E1 and E2 to sediment was slow and small, reaching equilibrium in 50 days, compared to a half life of 5 days in ideal laboratory conditions and found that the presence of surfactants in effluents reduced sorption [48]. Another study [50] found only a few sediment samples contained EDC concentrations exceeding their quantification limits. Nevertheless, several publications have reported significant concentrations of these steroidal estrogens in river sediments. One study [51] consistently detected E1 is sediments collected from Tokyo Bay in the range of 0.08-3.60 ng g^{-1}, whilst E2 was detected in only 7 of 20 sampling stations, and EE2 was not detected at any of the stations. In the UK, it has been shown that of these three steroidal estrogens, E1 was the predominantly detected compound in surface sediments and was found at peak concentrations in sediments at approximately 15 cm depth, at the alluvium/clay interface [52], suggesting vertical transport of these compounds should be further explored, owing to the risk of release in storm or high-flow events. A significant correlation between the sorption and retention of steroidal estrogens by sediment and its organic carbon content has also been observed, where sorption increases with increasing organic carbon content [46][53]. It has further been observed [54] that in sediments downstream of three sewage effluents in the USA that

E2 was more readily degraded by microbial activity in sediments than E1, and that the degradation of E2 in sediments did not involve conversion to E1, and that microbial degradation was significantly stimulated for E2, but unchanged for E1, downstream of effluent, compared to upstream sediment samples, indicating that sewage effluents may enhance microbial degradation of E2 in receiving waters.

In addition to the dissolved and sediment phases, natural colloids have been suggested as a potential sink of steroidal estrogens, where these compounds readily adsorb to abundant natural colloids, a process enhanced by the presence of surfactants [55][56], which may alter the persistence of these compounds in the environment.

Alkylphenols, such as NP, OP and BPA have also been reported in natural rivers, albeit at concentrations significantly lower recently, than in the past, due to the phasing out of these compounds following EU legislation introduced in 2003 (EU directive 2003/53/EG) restricting their use, with other countries including Japan, and Canada subsequently introducing similar legislation. Reported concentrations of these three alkylphenols in the Glatt River, a tributary of the Reine, since the introduction of the EU legislation, to be an order of magnitude lower than those measured in 1984 [57]. Indeed, other studies in Germany have found that significant reductions of NP occurred between 2003 and 2005, but observed that concentrations in rivers exceeded those attributable to sewage effluent, suggesting additional sources [58]. However, other studies suggest that alkylphenols tend to associate with sediments rather than remain in the aqueous phase [59]. Furthermore, concentrations of NP isomers as high as 4.1 µg L^{-1} in the aqueous phase and 1mg kg^{-1} in sediments still occur in the natural environment despite the introduction of legislation to restrict their use, have been reported [60]. More positively, concentrations of

BPA are generally below predicted-no effect concentrations and concentrations of OP are also generally much lower than concentrations of NP. BPA, NP and OP are readily degraded by biological and photo-oxidation pathways [59].

There are some 127 pesticides recognised to have endocrine-disrupting effects. Of these, several are believed to be particularly pertinent due to their presence at high concentrations, despite their low estrogenic potency. The use of pesticides for agricultural purposes in the UK has grown from ~22,000 tonnes to more than 35,000 tonnes in 1998, although usage has declined slightly since, this still reflects a usage of 2-2.5 kg ha^{-1} of agricultural land. Pesticide use in central and eastern Europe was historically much higher, but reduced significantly (from ~2kg Ha^{-1} in 1989 to <1 kg Ha^{-1} in 1997) following the collapse of the Soviet Union, although in these countries, use is now increasing. Of these volumes, approximately 10% is estimated to be those which have known endocrine disrupting properties. Of these, use has generally declined, as for example DDT and lindane have been phased out. However, use of the 10 most popular endocrine disrupting pesticides has maintained relatively constant. Globally, pesticide-use for agriculture is widely documented in developed countries, as it is usually a legal requirement. However, outside of agriculture, there is no such requirement, and so data concerning non-agricultural use is scarce, with Local Authorities believed to be the predominant non-agricultural user of pesticides. Despite the high volume of use of ED pesticides, little research has been undertaken to quantify the impact of these compounds on the estrogenicity of receiving waters, as such, little is known about the pathways of these compounds into the aquatic environment, with the exception of OP and NP which are discussed previously. Of widespread concern is the risk of human exposure to pesticides, although their endocrine

disrupting properties are seldom taken into account in legislative matters, despite a plethora of research highlighting the potential for deleterious ED effects of pesticides in humans and aquatic organisms [61].

Phytoestrogens are a relatively under-researched area of environmental endocrine disruption. Although phytoestrogens are naturally-occuring in the environment, their excretion in human urine at concentrations as much as 1000-fold higher than E1 or E2, and with only partial removal of these compounds by sewage treatment causes inflated concentrations in receiving river waters. As a result of their presence at high concentrations, despite their low potency, it is believed that phytoestrogens may contribute significantly to their estrogenic activity of river waters – and may contribute as much as 5% of the estrogenic activity of sewage effluent [62].

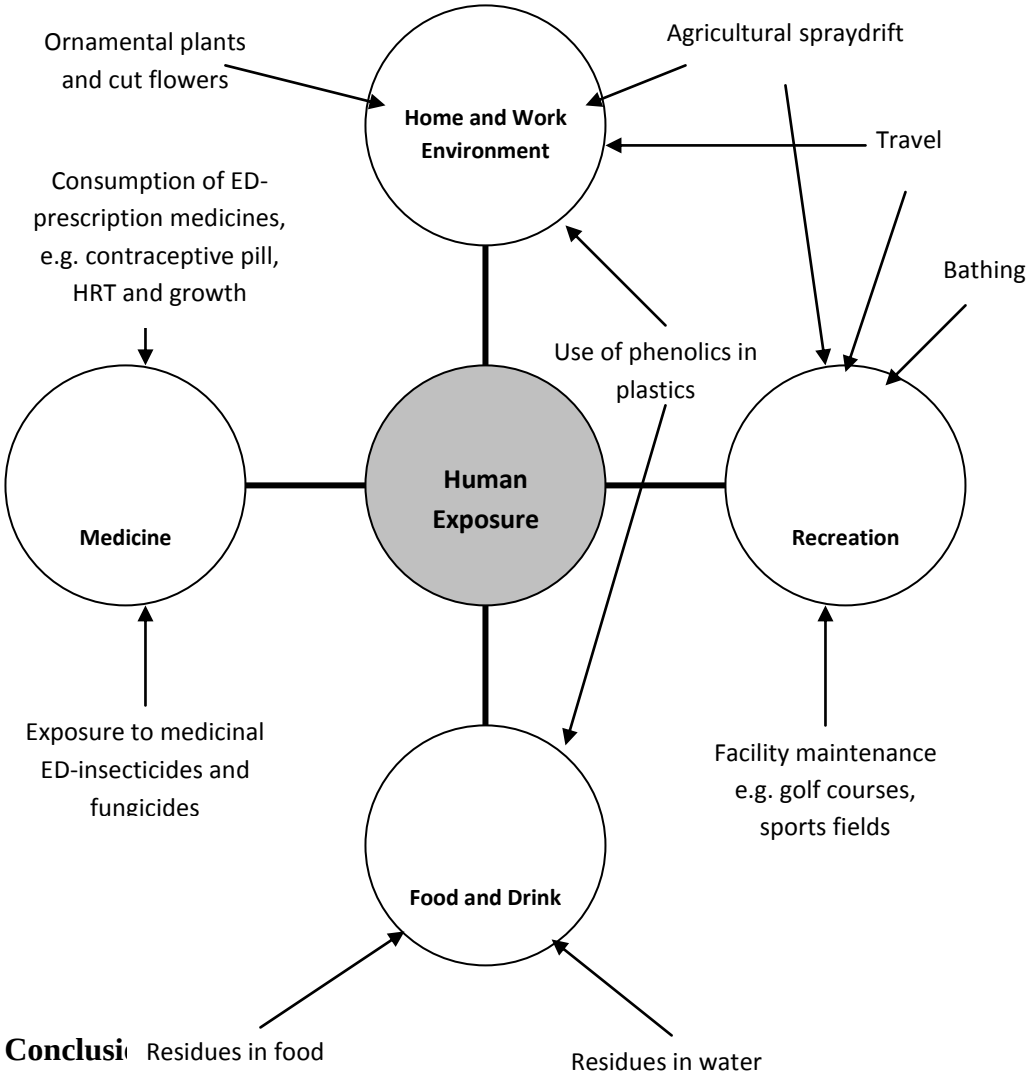
2.3.8 Biological implications and risk assessment

Although EDCs have been shown to have deleterious effects on aquatic organisms in laboratory experiments, whether or not they occur in the environment at concentrations sufficient to cause harm to fish populations remains unclear. Indeed, a recent review [60] concluded that whilst EDCs in the aquatic environment have the potential to impact on the reproductive health of various fish species, there is little evidence to suggest that there is any subsequent implications for fish populations, and suggest that there is a need for a reliable, in-situ indicator of such population-level impacts, and as such, the use of vitollogenin induction in males is insufficient, which has been found not to be a reliable indicator of endocrine disruption in some fish species [62]

Concentrations of the most potent EDCs in rivers, are typically at very low concentrations (up to 66.2 ngL^{-1} for E1, 33.9 ngL^{-1} for E2, and 30.8 ngL^{-1} for EE2), nevertheless, it has been shown that E2 and EE2 can have implications for the reproductive health of fish species at approximately 1 ngL^{-1}, excluding the potential for synergistic modes of action[10,12]. Although far less potent, phenolic and pesticide endocrine-disruptors are often found at much higher concentrations (e.g. up to 14662ngL^{-1} for NP) and with 127 widely-used endocrine disrupting pesticides there is potential that the combined implications of these mixtures may pose a greater risk to aquatic, and indeed, human health[61]. Such a risk may be enhanced by the presence of other stress-causing conditions, such as the presence of other pollutants, climate change or the presence of other diseases amongst a population.

Although the risks associated with EDCs primarily focus on aquatic wildlife, there may also be a significant risk to humans, where endocrine-disruption has been linked to human cancers [63], and numerous exposure pathways have been identified [64], some of which are highlighted in Figure 2.3. It would appear that the risk to humans from EDCs is exacerbated for those most likely to be exposed to such chemicals, such as those working in an agricultural or factory environment, where the use of EDCs may be commonplace. The risk is further enhanced in more vulnerable groups, which includes unborn children, young children, and those who are genetically susceptible to endocrine disrupting effects.

Figure 2.3 Routes of human exposure to EDCs



Conclusi

EDCs have been shown to be present in a variety of aquatic environments globally, primarily due to their incomplete removal from wastewaters, with the effluents of STW being the predominant source. Research has focussed on river environments, because these are often the receiving waters of sewage effluents, and because of the lower dilution-factor compared to seawater. Nevertheless, it is apparent from the limited data that in enclosed seas and harbours, there is the potential for EDC compounds to accumulate, and further attention should be paid to these, and sensitive estuarine environments. The impact of

sorption of EDCs to sediments may potentially play an important role in their transport, and it is suggested [65] that monitoring programmes include analyses of sediment. Despite their widespread use and presence in the environment, the impact of less potent EDCs, such as pesticides and phytoestrogens is poorly understood, as monitoring programmes tend to focus on the more-potent steroidal estrogens and less frequently, phenolic EDCs.

The overall risk to the population of aquatic species remains unclear. Although laboratory investigations have shown the potential for EDCs to pose a significant threat to fish reproductive health [66], there continues to be a lack of conclusive evidence from field observations. Similarly, the risk to human health remains un-quantified, despite associations with deleterious effects.

2.4 Pharmaceutical and Personal Care Products (PPCPs)

The term PPCP refers to any product used for personal health or cosmetic purposes, or by farmers to improve the health and growth of their livestock. They include: prescription and over the counter pharmaceuticals, illicit drugs, fragrances, cosmetics, veterinary drugs, diagnostic agents, sun-screen products and vitamins [67]. These drugs and products are designed with specific purposes in mind and in many cases are aimed at bringing about specific cellular responses. Although the levels of these chemicals found in the environment are generally very low, much lower than the prescribed dose, their continued introduction into the environment confers upon river biota continual multi-generational exposure. The health risks associated with the continual, low dose exposure to a large mixture of chemicals are still not completely understood. It may be that there are low dose effects occurring to river wildlife that as yet remain undetected.

The nine PPCPs used in this work are amongst the most commonly studied of this group of chemicals [68] and with an ‘in-house’ LC-ESI-MS/MS analytical method [69], internal standards and the pure chemicals all readily available in our laboratory, the choice of analytes was relatively simple.

Literature regarding the fate and transport of some of these chemicals is very limited. The physico-chemical properties of the nine PPCPs used in this study can be seen in Table 2.2.

2.4.1 Propranolol

Until recently, this beta-blocker was a first-line treatment for hypertension in the UK. Since then it has been downgraded to fourth-line, partly because it performs less well than other drugs but also due to increasing evidence that commonly used beta-blockers have an unacceptable risk of provoking type 2 diabetes. Propranolol is also used to treat angina and essential tremors where it is prescribed at 120 - 320 mg daily in divided doses. The drug is also used to treat tachyarrhthmia, anxiety and hypertension at the dosage of 10 - 40 mg, 3 - 4 times daily. Propranolol hydrochloride is a white, odourless crystalline powder

Robinson *et al* [70] found that 11 – 68 % of propranolol was removed by photodegradation in US rivers and predicted that up to 27 % could be removed in the River Aire, UK, during the summer. They concluded that phototransformation was the most important process in the attenuation of propranolol (and other pharmaceuticals that partition to the water phase) and that the effect of this process would be most effective in long, shallow rivers with low flow-rate and low turbidity.

Robinson *et al* [70] showed that 80 % removal of propranolol could be brought about by activated sludge in 30 days, although they were not able to say whether this was caused by adsorption to the sludge, or by biodegradation.

2.4.2 Sulfamethoxazole

Sulfamethoxazole is a bacteriostatic antibiotic most often used in combination with the drug trimethoprim, also a bacteriostatic antibiotic, in a 5:1 ratio. It prevents the formation of dihydrofolic acid (Vitamin B9), which is a necessary requisite for bacterial cell division. The drug is used against susceptible forms of *Streptococcus*, *Staphylococcus aureus* and *Escherichia col* and urinary tract infections [71]. Sulfamethoxazole enters the STW in metabolized form as N_4-acetyl-sulfamethoxazole, which can be cleaved by bacteria, converted back to the original compound, and passed into the river system via the sewage outfall [147]. Zhou *et al* [72] found that the removal rates (RR) of this chemical varied between 52.7 and 82.3 % in three UK STWs (Scaynes Hill, East Sussex, Manor Farm Road, Berkshire and Basingstoke, Hampshire).

Due to its Log K_{ow} of 0.89 [1], and an estimated K_{oc} of 72 [74], sulfamethoxazole is expected to have high mobility in soils. With a pKa of 5.5 it should also partially exist in the environment in the anionic form. Anions do not adsorb any more strongly to soils containing organic carbon and clay than their neutral counterparts [75], which again indicates the likelihood of high mobility in soil.

In general, sulfonamide antimicrobials are not readily biodegraded and persist in soils [76] and even STWs. In a closed bottle experiment containing a sewage inoculum from a German STW, Al-Ahmad *et al* found that sulfamethoxazole was not degraded at all [77].

Another closed bottle experiment found a theoretical oxygen demand of 2 and 4 % after 14 and 28 days, respectively. The cut-off for indicating biodegradability is 60 %, so this again categorizes sulfamethoxazole as non-biodegradable [78]. Boxall *et al*, using the OECD 301 D test (closed bottle test) observed no biodegradation at all after 40 days [79]. Lam *et al* [80] found no difference in the t½ of sulfamethoxazole in sunlight-exposed pond water and autoclaved pond water. This is evidence that photodegradation is an important environmental fate process for sulfamethoxazole. The same group also found that in aquatic outdoor field microcosms, with concentrations of: 0.31, 6.31, 19, and 182.78 ug/L the resulting average field half-lives were: 18.7, 19.4, 17.5 and 20.3 days, respectively.

2.4.3 Mebeverine

Often sold under the brand name of Fybogel this compound is an antispasmodic used to relieve cramps or spasms of the stomach and intestines which is particularly useful in treating irritable bowel syndrome [81]. Mebeverine is available as mebeverine hydrochloride in 100 mg and 135 mg tablets, in 10 mg/mL oral liquid form and as a 200 mg slow release preparation. Reported side effects are generally limited to inflammation or reddening of the skin or an itchy rash.

2.4.4 Carbamazepine

This is a mood stabiliser and anti-convulsant used to treat bipolar disorder, schizophrenia, trigeminal neuralgia and epilepsy. It was first marketed in 1962 and has been used in the UK since 1965. Carbamazepine can render certain birth control pills ineffective because it is an enzyme inducer of the cytochrome P450 system, which is instrumental in metabolising the contraceptive. Reported side effects can include: blurred vision, temporary loss of blood platelets and even cardiac arrhythmias.

Only 1 – 2 % of carbamazapine is excreted by the body, but the excreted glucuronide conjugates of this chemical can be cleaved by sewage bacteria, thus increasing its concentration in sewage outfall [82]. Carbamazapine has shown poor RR from STWs [83] [84] [85] and does not appear to adsorb onto sludge [86]. This seems to be confirmed by the high concentrations of this chemical, often in the range of several hundred ηg/L, regularly found in surface waters [87] [69].

Castiglioni *et al* [88] studied the seasonal variability in RR of pharmaceuticals through STWs and found that there was no difference between winter and summer RR for carbamazapine, indicating that this chemical is not removed by photodegradation and that temperature has no effect on removal. This is in contrast to the NSAID ibuprofen (also studied in that work), which showed a median winter RR of 38 % and a summer RR of 93 %.

Nakada *et al* [89] looked at the removal of carbamazapine during activated sludge treatment and found that only 50 % was removed, which again, indicates the high persistence of this chemical. This result is very similar to those found by Zhou *et al* [73], who found that the RR for carbamazepine in three STWs varied between 43 and 54 %, no matter which treatment process was used.

Treibskorm *et al* [81] looked at the cytological effects of pharmaceuticals on freshwater fish (Rainbow Trout (*Oncorhynchus mykiss*) and Common Carp (*Cyprinus carpio*)) and determined that the lowest observed effect concentration (LOEC) of carbamazepine was 1 ug/L. This LOEC is still far higher than the highest concentration found in the River Ouse, UK (554 ηg/L) during this study.

2.4.5 Indomethacine

A non-steroidal anti-inflammatory drug (NSAID) which by the inhibition of prostglandins is able to reduce fever, pain, swelling and stiffness. The compound was discovered in 1963 and was first approved for use in the USA in 1965. Adverse effects can include: peptic ulcers, dyspepsia, diarrhoea and also lithium retention in patients prescribed lithium.

Zhou *et al* [73] found that the RR of indomethacine varied between 60.8 and 88.7 % in three UK STWs. At Scaynes Hill STW, 24 % was removed by primary sedimentation (humus tank) and a further 26 % was removed when passed through a lagoon. In total, the STW at Scaynes Hill, East Sussex, removed 61 % of the indomethacine present in the raw influent.

2.4.6 Diclofenac

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation, and, as an analgesic, is used to reduce pain in conditions such as arthritis. Diclofenac use in cattle has led to a huge decline in the vulture population on the Indian subcontinent, with > 95 % of birds succumbing in some areas. That equates to 10's of millions of birds. Vultures eat the carcasses of cattle and other domesticated animals that have been administered with veterinary diclofenac and are poisoned by the accumulated chemical. Oaks *et al* showed that death is due to renal failure, a known side-effect of diclofenac, which leads to high levels of uric acid in the blood (hyperuricaemia) and visceral gout [90].

Contradictory results for diclofenac removal during conventional wastewater treatment have been reported in the literature. In some STWs, attenuation of 50 – 70 % has been reported [82] [84] [88] [91]. By contrast, many studies showed conventional treatment had extremely low efficiency (10 – 30 % removal) [87] [92] [93] [94]. Radjenovic *et al* [158] found that a membrane bioreactor used during the sewage treatment process was able to remove up to 90 % of diclofenac, while EPISUITE predicts an RR from STWs of 56 %. Gröning *et al* [95] investigated the ability of sediments in the water column to remove this chemical and found that they were capable of removing up to 94 %. which, in addition to a very low BCF of 3.162 L/kg wet-wt predicted by EPISUITE, would seem to indicate that diclofenac has low persistency in the aquatic environment.

2.4.7 Meclofenamic acid

A white, crystalline powder that is practically insoluble in water, meclofenamic acid is another potent inhibitor of prostglandins that is generally used in veterinary medicine for the relief of pain and inflammation, especially that associated with arthritis.

There is very little in the scientific literature regarding the fate and transport of this chemical. However, the United States Geological Survey (USGS) EPISUITE software predicts that this chemical will have an RR in STWs of 92 %, with 91 % of this removed via sorption to sludge. It also predicts a low potential BCF of 56.23 L/kg wet-wt.

2.4.8 Tamoxifen

A selective estrogen receptor modulator that is used in the treatment of estrogen receptor (ER) positive breast cancer in pre and post menopausal women [96]. Dose is 10 - 40 mg/d

taken orally during days 3 - 7 of the women's menstrual cycle. The major excretory route is via the bile, with less than 1 % of the dose excreted in the urine.

Common side effects include: fatigue, nausea, hot flushes, change in menstruation regularity or even cessation, possible harmful effects to a developing foetus and blood clots. There also seems to be correlation between long-term tamoxifen administration and endometrical proliferation [97]. The drug was first approved by the FDA in 1977, but in April 2006 it was announced that the drug raloxifene was just as effective but with fewer side effects.

Retinal damage and keratitis have been reported in patients after large cumulative doses of tamoxifen, generally over 180 mg/d for more than 1 year [97], though sometimes with smaller doses [98].

There is very little in the literature regarding the environmental fate of this chemical. Limited data suggests that tamoxifen has an elimination t½ of about 5 - 7 days (range 3 - 21 days), with the elimination t½ of N-desmethyltamoxifen, the major metabolite estimated to be 9 - 14 days [99].

2.4.9 Thioridazine

An anti-psychotic prescription drug widely used for 30 years to treat schizophrenia, anxiety and psychosis. It has some serious potential side effects, which include: cardiotoxicity, retinopathy, akathisia and a potentially fatal neurological disorder, neuroleptic malignant syndrome.

There is very little in the literature regarding the fate and transport of this chemical in the aquatic environment and as of 2001 there had not been any concerted environmental surveys of this drug undertaken [100]. The EPISUITE software estimates that thioridazine will have a high potential BCF of 3629 L/kg wet-wt, a Log K_{oc} of 5.094 and t½ in a model river of 3.13 d [74]. The software also predicts the RR from an STW to be 91 %.

Like meclofenamic acid, the only experimentally-based information regarding the fate of this chemical in the aquatic environment will be found in this thesis.

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Chapter 3. Materials and Methods

3.1 Introduction

All of the techniques and methods used in this work are described in the following chapter. Section 3.2 describes the glassware preparation and the general quality-control procedures used in the laboratory, including a description the preparation of stock solutions and internal standards, as well as the grade of all solvents used.

Sections 3.4 and 3.5 describe the methods used for the sampling and extraction of EDCs and PPCPs from environmental aqueous and sediment samples, respectively.

The chromatographic techniques and methods employed in this work are described in section 3.6, while section 3.6 outlines all of the methods used to characterise the water and sediment samples.

Section 3.7 describes the CFUF system and the methods used for: isolating aquatic colloids, and, partitioning EDCs and PPCPs between the colloidal and dissolved phases. Section 3.8 explains the methods used in the characterisation of colloids

3.2 Quality control, Chemicals and standard solution

3.2.1 *Ultra-pure water*

The ultra-pure water used in the laboratory was supplied from a Maxima HPLC/LS water purification system (Elga Process Water, UK). This machine was custom-constructed as a hybrid of the Life Science and HPLC models to provide high purity water with low organic

carbon content. The ultra-pure water used in experiments had a quality of 18 MΩ/cm and an organic carbon content of < 3 ppb. The unit consisted of two replaceable filter packs used to remove ions via ion exchange resins, a hygienic, microfibre, 0.05 μm ultra-filter pack, used to remove bacteria and small particulates, and a short-wavelength, UV, photochemical reactor to eliminate all remaining bacteria. Ultra-pure water was dispensed immediately prior to use, to avoid the possibility of contamination.

3.2.2 *Cleaning of glassware*

To reduce the risk of contamination of samples and standards, all glassware used in experimental work was cleaned; by soaking for a period of 24 hours in a solution of 5% Decon-90 (Fisher, UK), and subsequently, thoroughly rinsed with ultra-pure water. Immediately prior to use, all glassware was rinsed a minimum of three times with dichloromethane (DCM) to remove any residual organics, and allowed to air dry in a fume cupboard. Prior to use, glassware and other equipment where appropriate, was further cleaned by combustion in a furnace at 400ºC for 4 hours.

3.2.3 *Preparation of chemical standards*

For all mass spectrometric analyses, internal standards (IS) of the highest purity available were used to allow quantification. For analysis of steroidal estrogens, deuterated E1-d_4, E2-d_4, EE2-d_4, (Qmx Laboratories Ltd., UK) and E2-d_2 (Sigma Aldrich, UK) were prepared in a stock solution of 10 ηg/μL in methanol. For analysis of pharmaceutical compounds, diuron-d_6 and ^{13}C-phenacetin (Sigma Aldrich, UK) were again prepared in a stock solution of 10 ηg/μL in methanol. IS are expected to behave similarly to their non-deuterated counterparts, but are distinguishable in mass spectrometric analyses. A known

volume of IS was added to a sample (at a concentration in the same order of magnitude expected for the analytes), and therefore any loss of analyte is corrected for by quantification relative to the appropriate IS.

Additionally, compound mixes for both steroidal estrogens (E1, E2 and EE2) and pharmaceuticals (propranolol, sulfamethoxazole, meberverine, carbamazepine, indomethacine, diclofenac, and meclofenamic acid) were used by preparing stock solutions of 1000mg/L in methanol, which were further prepared into working solutions of 10ηg/μL. These standards were used for identification purposes in spectrometric analyses, in quantification of analytes, to quantify instrument stability, and method recoveries, and were of the highest purity commercially available.

All standards and IS were stored in darkness at -18ºC, were weighed before and after use to quantify any losses due to evaporation and were regularly analyzed to ensure the compounds had not degraded. A small reduction in mass was presumed to be the result of evaporation of the methanol, and so additional methanol was added. Where a significant reduction of mass (>5%) was observed, the solution was discarded and replacement prepared.

All solvents used throughout were of glass-distilled grade and were purchased from Rathburns, UK. Wherever samples were transferred between glass containers during experimentation, the surfaces were solvent rinsed three times and the rinsed solvent added to the sample extract to minimize losses due to sorption to the glass surfaces.

3.3 The sampling and sample preparation of aqueous samples for the analysis of steroidal estrogens and selected pharmaceutical compounds

3.3.1 *Collection of aqueous samples*

The aqueous 'spot' samples were taken by an auto-sampler that opened at a depth of ~ 0.5m so as to avoid the complex surface micro-layer. This device consisted of a weighted, steel cage that housed a Winchester bottle. The neck of the bottle was fitted with a spring-loaded stopper that could be opened by pulling a rope when the bottle was submerged. The whole device was suspended on another rope and could be lowered from bridges or riverbanks. The samples (2.5 L) were collected in pre-cleaned, 2.5-L amber bottles, to which, 5 mL/L of 2 M sodium azide (Fisher, UK) was added as a broad-spectrum biocide. The bottles were capped, labelled and brought back to the laboratory where they were stored in the dark at 4^{o}C until further preparation.

Composite aqueous samples were collected into pre-cleaned 50L stainless-steel barrels. An automated pumping system was developed which employed a series of mechanical vacuum pumps each attached to a mains electricity supply via a timer switch. When activated, these pumps drew water through PTFE tubing, through an 'overflow' bottle into the 50L barrel. The flow was calibrated to ensure the required volume of water was collected in the correct period of time, by altering the vacuum output of each pump.

Samples were additionally collected using a passive sampling device. The design of the passive sampling device was similar to POCIS except that the holder which supports both the diffusion-limiting membrane and sorbent and seals them in place was made of PTFE rather than stainless steel. The Oasis HLB sorbent (100 mg) from Waters Ltd, UK was

chosen because it sorbs a wider range of polar compounds than C_{18}. The polyethersulfone (PES) membrane (0.1 μm pore size) and polysulfone (PS) membrane (0.2 μm pore size) were provided by Pall Gelman Sciences (VMR International, UK). PTFE used to construct the sampler was from Aquarius Plastics Ltd, UK. Peristaltic pumps (Watson Marlow 401U/DM2) and tubes (0.5 mm x 1.6 mm and 3.2 mm x 1.6 mm) for controlling the flow-through system were from Fisher, UK.

3.3.2 *Preparation of aqueous samples for extraction*

Upon returning to the laboratory, aqueous samples were filtered to remove suspended particulates through pre-ashed (400 ^{o}C for 4 h) Whatman GF/F filter papers (0.7 μm pore size), using a Büchner apparatus. The filtrates of samples were spiked with 100 ng of internal standards by micro-syringe, and were left for 1 h to allow mixing processes to take place, as in nature.

3.3.3 *Solid Phase Extraction*

SPE is a concentrative and purification step which implements a stationary solid phase, through which the analyte-containing aqueous sample is passed. The technique allows the analytes to be separated from their matrix, and such that the analytes are retained onto the stationary phase and the matrix passes through, or vice-versa. Typically, SPE is operated such that the analytes are retained on the stationary phase, in which case they are subsequently eluted and collected using an appropriate organic solvent.

A wide range of SPE devices are available including cartridges, disks and well-plates, in choosing which of these devices to use, the sample volume, matrix complexity and analyte characteristics are fundamental considerations. Typically, cartridges are used in

the chemical analysis of environmental pollutants as they allow for a high throughput of large-volume samples. Additionally, SPE devices may be normal phase (NP), reverse phase (RP) or based on ion-exchange sorbent (Cation or Anion). NP sorbents are typically suitable for polar compounds, RP sorbents non-polar compounds, and cation-exchange and anion-exchange sorbents, bases and acids respectively. However, with the increasing use of SPE for multi-analyte analysis, a range of mixed mode, and balanced sorbents with both hydrophilic and lipophilic functional groups (retaining polar and non-polar compounds respectively) have become popular.

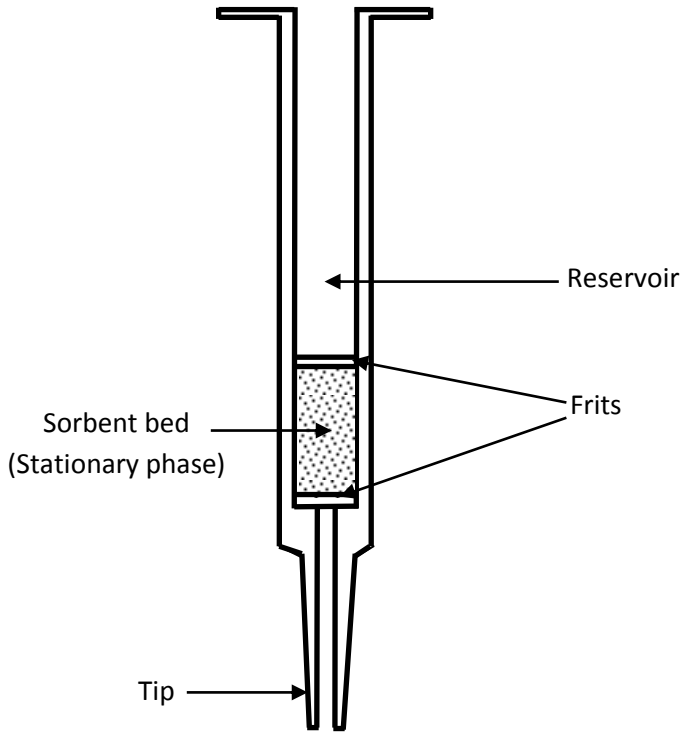


Figure 3.1 An SPE cartridge

Liu et al. (2003) and Zhang and Zhou (2007) compared a range of SPE cartridges for the extraction of EDCs and pharmaceutical compounds, and concluded that Oasis® HLB SPE cartridges offered the best recovery. Liu et al. (2003) and Zhang and Zhou

(2007) also investigated the efficacy of a range of solvents in the extraction of pharmaceutical and Endocrine-disrupting compounds (figure 3.2). For EDCs, Liu et al. (2003) concluded that Ethyl Acetate offered optimal recoveries, and thus opted to use that over the alternatives. Zhang and Zhou (2007) however, concluded that methanol offered optimal recoveries, and is shown in figure 3.2, ethyl acetate gave particularly poor extractions, reporting 0% for some compounds. To allow simultaneous extraction of both EDCs and Pharmaceutical compounds, it was necessary to assess which solvent would give optimal recoveries across the range of compounds. As can be seen in figure 3.2, methanol clearly offers the most efficient recoveries for pharmaceutical compounds, and recoveries comparable to ethyl acetate for steroidal estrogens.

Thus, Oasis® HLB SPE cartridges (Waters, UK) containing 200mg of Poly(divinylbenzene-co-*N*-vinylpyrrolidone) sorbent with a 6mL sample reservoir have been used in this work. The cartridges were conditioned with 2×5mL (10mL) Ethyl Acetate followed by 2×5mL (10mL) of methanol, followed by ultrapure water (3×5mL) at a rate of 1–2mLmin−1. Subsequently, water samples were passed through at a flow rate of 5–10mLmin−1. Afterwards the cartridges were dried for 30 min under full vacuum, to remove any residual water. The analytes were then eluted to pre-cleaned 20mL glass vials from the sorbents with 10mL of methanol. The solvent was reduced to 0.1mL (for LC-MS/MS analyses) or to dryness under gentle N2 flow, and reconstituted according the derivitisation procedure for GC-MS(/MS) analyses. Reduced extracts were subsequently transferred to 350µL fused insert vials and stored at -15ºC until analysis.

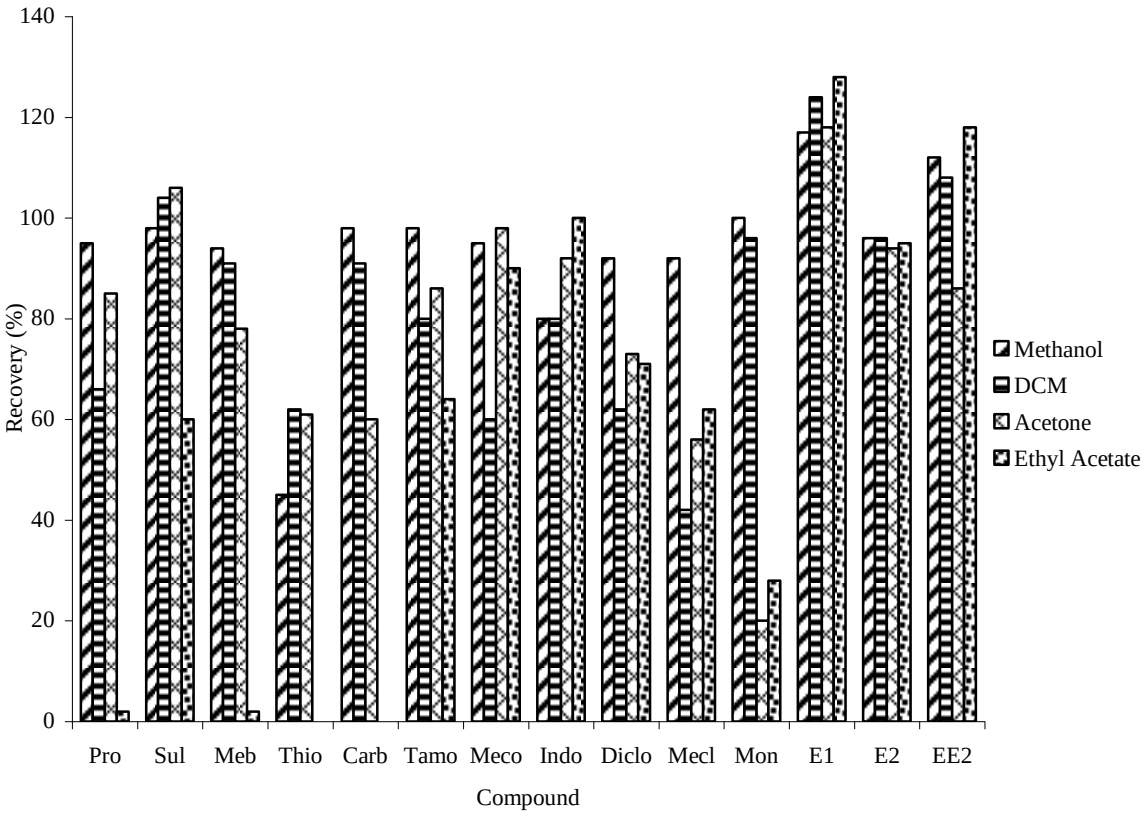


Figure 3.2 The effect of elution solvent on the SPE recoveries of each compound (adapted from Zhang and Zhou, 2007; and Liu et al., 2003)

3.4 The sampling and sample preparation of sediment samples for the analysis of steroidal estrogens and selected pharmaceutical compounds

3.4.1 *Collection of environmental sediment samples*

Environmental sediment samples were collected using a stainless-steel Van Veen grab. Upon collection, the oxic fraction (the ~2cm at the top) was removed and discarded, as it is not representative of river sediment due to it being highly mobile and continuously undergoing disturbance and degradation by river fauna. A sample of the sub-2cm fraction

was transferred into pre-cleaned 250ml glass jars, with a solvent-rinsed stainless-steel spoon.

3.4.2 *Preparation of sediment samples for extraction*

On return to the laboratory, the sediment samples were frozen, and subsequently lyophilised (by a Heto Powerdry PL3000 instrument for 4 days) to ensure only sediment-bound residues are extracted, and because the presence of water can reduce the efficiency of solvent-based extraction, particularly where solvents that are immiscible with water are used. After lypophilisation the samples were ground, homogenised and sieved to <500µm. 3g Sub-samples were weighed into Teflon-lined extraction vessels and spiked with 100 ng of internal standards, were mixed with a pre-cleaned stainless-steel spatula and left for approximately 1.5 hours to allow sorption processes to occur. 2 g of copper granules (Sigma-Aldrich) were then added, to remove sulphurous compounds, followed by 25mL of methanol.

A variety of techniques are appropriate for the extraction of organic contaminants in environmental sediments. Some of these techniques, such as soxhlet extraction have fallen out of favour due to the large volume of solvents required, which may pose a health risk to the operator and the significant amount of time required to process each sample. Moreover, soxhlet extraction at high temperatures is not suitable for the extraction of thermally unstable compounds, which may degrade during the extraction process and is also susceptible to co-extraction of matrix substances which may cause interference in subsequent analyses. Ultrasound-sonication (‘ultrasonication’) has also been shown to be applicable for the extraction of a wide range of organic environmental pollutants with

recoveries comparable to soxhlet extraction whilst using a greatly-reduced volume of solvent and without the need for significant heating. However, ultrasonication and soxhlet extraction techniques typically have relatively poor reproducibility compared microwave-assisted extraction. Ultrasonication baths in particular pose the additional issue of incomplete extraction where the bubbles caused by the ultrasound are unable to penetrate to the centre of samples, as is highlighted in figure 3.3, only the surface of the conical flask is in direct contact with the water bath, thus the central area of solvent undergoes reduced sonicative action. Ultrasonication probes (sonicators) alleviate these 'sonication weakness' issues but are significantly more expensive to purchase and maintain, and can process only one sample at a time. Another risk with ultrasonication in baths arises where conical flasks are uncapped during the process, is that solvent (and with it the analyte) may evaporate. The alternative is to cap flasks which may violently de-cap due to the build up of pressure in the flask, causing a potential health risk to the operator.

3.4.1 *Microwave-assisted Solvent Extraction*

The rate of solvent-based extraction, as with any reaction, is dependent on the proportion of reactants that collide with sufficient energy; the so-called activation energy. Increasing temperature has long been used as a technique to enhance reaction rates, by both increasing the rate of such collisions, and also, and most importantly, increasing the energy of such collisions. Traditional heating techniques, such as conventional ovens, are inefficient as they rely on the heating of an entire surface and so require a large amount of energy and time. Moreover, they require a greater cooling time and less control over heating resulting in a lower sample throughput.

Microwaves act as high frequency electric fields and will generally heat any material containing mobile electric charges, such as polar molecules in a solvent or conducting ions in a solid. Polar solvents are heated as their component molecules are forced to rotate with the field and lose energy in collisions. Thus microwave-assisted extraction offers significant efficiency improvements over alternative techniques and was employed for the extraction of steroidal estrogens and pharmaceuticals from sediment samples.

Thus, a microwave-accelerated solvent extraction method was optimised based upon work previously undertaken by Liu et al. (2004). The PTFE-lined, microwave extraction vessel and its contents were put into a tight-fitting pressure jacket designed to contain the vessel if it should explode under pressure. The jacket and vessel were then placed in a plastic frame and the lid of the vessel was held in place by a large screw (integral to the plastic frame) that was tightened down on top of it by spanner.

The samples were then loaded into the MARS-X laboratory microwave accelerated extraction system (CEM Corp, USA) and heated to 110 ^{o}C for 15 minutes, with a 7 min ramp, at 200 psi. After cooling to room temperature for 1 h, the supernatant was decanted into 250 mL round bottomed flasks along with 3 x 15 mL sample rinses of MeOH. It was very important to make sure that the sediment had completely settled before the supernatants were decanted, otherwise SPE extraction would be slowed down considerably due to sediment particles blocking the SPE cartridge.

The combined supernatants were reduced in volume to ~ 0.5 mL using a Büchi Rotavapor R-205 rotary evaporator. This used a Büchi Vac V-500, 230 v, 50 Hz pump, with a

pumping volume of 1.6 m^3/h. The water bath was set at 40 ^oC and the sample flask was spun at 50 rpm.

3.4.2 *Clean-up of sediment samples*

Attempts at cleaning particularly 'dirty' extracts using alumina/silica columns met with limited success. These extracts were often not cleaned suffciently and when the samples were blown down after extraction they remained very dark green in colour and would not dry completely. Analysis of these 'dirty' extracts by GC-MS, GC-MS/MS and LC-MS/MS resulted in very noisy chromatograms with a high-level of background 'noise' that were impossible to integrate.

The clean-up process devised for this work entailed the addition of the 0.5 mL extracts to 500 mL of ultra-pure water with 3 ultra-pure water rinses. The non-soluble components of the extracts, such as sand, clay and grit, settled to the bottom of the jar. The 500 mL samples were then passed through pre-conditioned Waters Oasis HLB cartridges at < 20 mL/min, as described in section *3.3.3*, The cartridge packing was then rinsed with 3 x 10 mL of 5 % MeOH in pure water. This procedure result in much clearer extracts, removing much of the interfering organic matter. This afforded the integration of the resulting chromatograms.

3.5 Mass Spectrometric analysis of sample extracts

Sample extracts were analysed using a range of mass spectrometric techniques, including LC-MS/MS, GC-MS/MS and GC-MS. Primarily, analyses were performed on the tandem-mass spectrometer instruments; GC-MS/MS and LC-MS/MS.

3.5.1 The principles of gas and liquid chromatography

Gas chromatography is a widely used technique that is able to determine the chemical make-up of a complex volatile mixture by separating its constituents on the basis of their molecular mass and polarity. This separation occurs in the capillary column, which consists of a long (10 – 100 m) thin (100 – 320 µm), hollow coiled tube made from fused silica. The column consists of a lining known as the stationary phase and a mobile phase or carrier gas such as helium that passes through the column. The sample constituents will all have a unique affinity to the stationary phase and the mobile phase. If the chemical has an affinity for the stationary phase then it will pass through the column slowly, if the affinity is for the mobile phase then it will pass through the column more quickly. The time that the individual chemical spends in the column is known as the retention time (RT) and if this is compared to the RT of a pure sample of the chemical, the identification of the chemical can be inferred.

To analyse a sample by GC it must first be dissolved in a volatile solvent. A small amount (1 µL) is injected into a heated injection port where the sample is vaporised. The temperature of the injection port must therefore be above the boiling point of the least volatile component of the sample. The mobile phase then carries the vaporised sample through the column, which is itself contained in a thermostatically controlled oven. The partition of the different components is dependent on temperature, so the temperature of the oven starts off low and gradually increases so that all of the sample components are eluted. When it passes out of the end of the column the chemical passes through a detector (in this

case a mass spectrometer) and the data from this is plotted as a peak on a chromatogram via a computer.

Sample injection can be carried out in two different modes; split or splitless. In split mode a vent is opened which carries away part of the sample to waste. The remaining fraction is transferred to the capillary column. This method prevents overloading of the column. In splitless mode the entire sample is transferred to the column and this is the preferred method for trace-level and quantitative analysis.

Liquid chromatography works on a similar principle to gas chromatography, in that the basis for separation is on the individual chemical's affinity for either the stationary or the mobile phase. However, where as in gas chromatography, where vaporised extracts are carried by a gaseous mobile phase, extracts remain in the liquid phase and are carried by liquid solvents as the mobile phase, at high pressure (via a pump), through a densely-packed separation column, which tend to be much shorter (50-150mm). In liquid chromatography, the selection of a suitable separation column and mobile phase solvents is vital, based on their polarity and the polarity of the chemicals to be analyzed.

3.5.2 The principles of mass spectrometry and tandem mass spectrometry

Separated components pass from the chromatograph and into the source chamber of the mass spectrometer via a transfer line that is heated to a high temperature. Inside the source chamber, a source of electrons is created by heating tungsten or rhenium wire until it is red hot. The negatively charged electrons released from the wire are attracted across a small gap to an ion trap which has a positive charge. The effluent from the chromatograph

passes through this electron beam where the neutral analytes are hit by electrons with enough energy to remove an electron (or electrons) and thus cause ionisation and fragmentation.

$M + e \rightarrow M^+ + 2e$ (ionisation)

$Mm + e^- \rightarrow M^+ + m + 2e^-$ (fragmentation)

The charged particles are next repelled and attracted by charged lenses into a mass analyser (MS1) where the ionic species are separated according to their mass to charge ratio (*m/z*) by magnetic fields. In single-MS, these species would next pass onto a detector, which would be in the form of an electron multiplier. However, the fact that a sample containing compound A gives (for example) two characteristic ions $(M-1)^+$ and $(M-2)^+$ does not mean that the ions are definitely from A, as other compounds in the sample could quite possibly give $(M-1)^+$ and $(M-2)^+$. Tandem Mass Spectrometry goes further and queries whether $(M-2)^+$ comes from the decomposition of M^+ in the mass spectrometer. It does this by separating out $(M-1)^+$ and passing it from MS1 into the collision cell where an inert collision gas (in this case argon) is present. The precursor (or parent) ion interacts with this gas and the kinetic energy of the ion is transformed into internal energy which leads to the fragmentation of the parent into daughter ions. These daughters then pass into the second mass spectrometer (MS2) where they and further fragments are separated and detected. In short, MS1 can be considered as an ion source for MS2, as shown in figure 3.3.

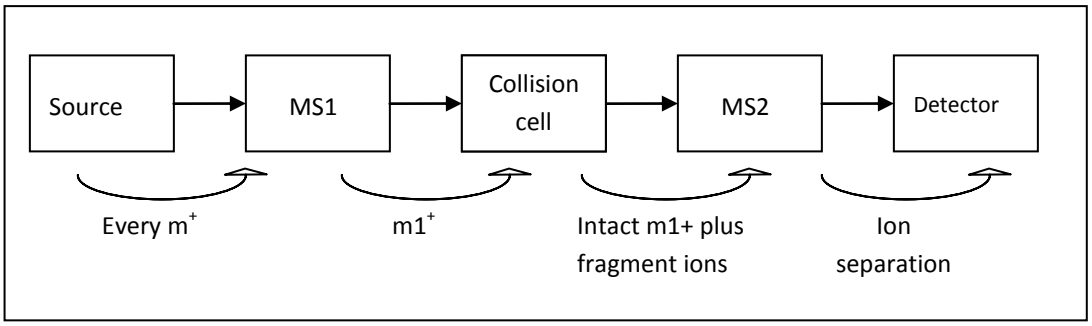


Figure 0.1 Diagram of the tandem mass spectrometer

If this filial relationship is observed in the spectrum of a pure sample of A and also from a mixture containing A then we can be confident that A is present in the mixture.

3.5.3 Mode of LC-MS/MS used for EDC and PPCP analyses

Liquid chromatography – electrospray interface - tandem mass spectrometry (LC-ESI-MS/MS) was used for the analysis of selected pharmaceutical and endocrine disrupting chemicals in water and sediment samples. This method employs a solvent mobile phase for the separation of compounds and is the preferred method for trace analysis of compounds that are thermolabile and have high polarities. The LC-ESI-MS/MS method used in this work was developed from work by Zhang and Zhou (2007).

The LC separation was carried out on a Waters 2695 HPLC separations module (Milford, MA, USA) equipped with a Waters symmetry C_{18} column (4.6 x 75 mm, particle size 3.5 µm). Three eluents were used: Eluent A consisted of 0.1 % formic acid in pure water, eluent B was acetonitrile only and eluent C was MeOH. The gradient elution was operated with 10 % of eluent B, followed by a 25 minute gradient to 80 % of eluent B and a 3 minute gradient to 100 % of eluent B. It was then changed to 100 % of eluent C within 8 minutes and held there for 10 minutes. The system was re-equilibrated for 10 minutes between runs. The injection volume was 10 µL.

The tandem MS analyses were carried out on a Micromass Quattro triple-quadrupole mass spectrometer equipped with a Z-spray electrospray interface. The analyses were done in PI mode for the other compounds. The temperatures of the electrospray source block and desolvation were 100 and 300 ^{o}C, respectively. The capillary and cone voltages were 3.0 kV and 30 V, respectively. Nitrogen as both nebulising gas and desolvation gas was set at

25 and 550 L/h, respectively. Following the selection of precursor ions by the first quadrupole mass analyser, the collision-induced dissociation was performed by argon at 3.6 × 10^{-3} mbar. Product ions were obtained at a series of collision energies and selected according to the fragmentation that produced a useful abundance of product ions. The optimal collision energy, cone voltage and transitions chosen for the MRM experiment were optimised. A dwell time of 100 ms per ion pair was adopted. The mass spectrometer was operated in MRM mode with unit mass resolution on both mass analysers.

Table 3.1 LC-MS/MS MRM Parameters for Pharmaceutical and EDC analyses

Compound	**RT (min)**	**Molecular weight**	**Collision energy (ev)**	**Precursor ion (m/z)**	**Product Ion (m/z)**	**LOD (ηg/L)**	**LOQ (ηg/L)**
Estradiol	17.7	272.4	30	271	145	1.2	3.6
Estrone	19.5	270.4	30	269	183	0.60	1.8
Ethinylestradiol	19.0	296.4	30	295	145	0.40	1.2
Propranolol	9.9	259.15	20	260	116 183	0.0060	0.019
Sulfamethoxazole	11.2	253.05	15	254	92 108	0.043	0.14

Mebeverine	12.2	429.00	25	430	156 101 135	0.045	0.15
Thioridazine	14.3	370.15	25	371	126 98	0.0010	0.004
Carbamazepine	14.8	236.09	20	237	194	0.0028	0.009
Tamoxifen	16.8	371.22	25	372	72 208	0.0030	0.009
Indomethacine	22.0	357.09	25	358	139 141	0.015	0.05
Diclofenac	22.1	295.01	30	296	214	0.016	0.058
Meclofenamic acid	24.1	295.01	25	296	243	0.079	0.27

3.5.4Mode of GC-MS/MS used for EDC analyses

GC-MS/MS analysis was performed using a 6890N network gas chromatograph (Agilent Technologies, USA) interfaced with a mass spectrometer (Quattro Micro, Micromass, USA) with tandem quadrapole. An Agilent 30 m HP-5 capillary column with a 0.25 mm internal diameter and a 0.25 µm film thickness was used. The carrier gas was helium, which was maintained at a constant flow rate of 1.0 mL/min. The GC column temperature was programmed from 100 (initial equilibrium time 1 min) to 200 ^{o}C via a ramp of 10

^{o}C/min, 200 - 260 ^{o}C via a ramp of 15 ^{o}C/min, 260 - 300 ^{o}C via a ramp of 3 ^{o}C/min and maintained at 300 ^{o}C for 2 min, with a total run time of 30.33 minutes.

The MS was by positive mode electron impact ionisation and was operated in full scan mode from m/z 50 - 600 for qualitative analysis. For quantitative analysis multiple reaction monitoring (MRM) mode was used. The inlet and MS transfer line temperatures were maintained at 280 ^{o}C, and the ion source temperature was 250 ^{o}C. Auto-sampler injection (1 μL) was in splitless mode. The electron impact energy used was 70 eV. Calibration of the GC-MS/MS equipment was carried out by analysing the gas Heptacos using the calibration software that is installed on the machine. Likewise, the LC-MS/MS was calibrated using sodium iodide.

3.5.5 Quantification of target compounds using GC-MS/MS and LC-MS/MS

The quantification of the analytes from the water and sediment samples was carried out by comparison between analyte response (peak area) and the response from a standard solution containing all of the targeted compounds and two internal standards [213]. A response factor (RF) was calculated for each compound of interest and also for the two internal standards.

$$RF = \frac{\text{Response analyte}}{\text{Concentration injected}} \qquad (0\text{-}1)$$

Next, a relative response factor (RRF) was calculated by dividing the RF of each compound by the RF of its relevant internal standard.

$$RRF = \frac{\text{RF compound}}{\text{RF internal standard}} \qquad (0\text{-}2)$$

By using the RRF, the amount of the targeted compound in each sample injected could be calculated:

$$\text{Amount of chemical } (\eta g) = \left(\frac{\text{Response}_{\text{compound}}}{\text{Response}_{\text{IS}}}\right)\left(\frac{\text{Amount IS}(\eta g)}{\text{RRF}}\right) \qquad (0\text{-}3)$$

Using the found amount, the concentration of each analyte in the sample could be calculated:

$$\text{Concentration of chemical} = \frac{\text{Amount}(\eta g)\text{ of chemical}}{\text{Amount of sample (W or V)}} \qquad (0\text{-}4)$$

where W is the dry weight of the sediment sample (g) and V is the volume of the water sample (L).

3.6 Characterisation of the river water samples

3.6.1 Physical properties

A Multiline P4 Universal Meter (Semat Ltd, UK) was used to measure salinity, conductivity, pH, dissolved oxygen and the temperature *in situ* from all of the water samples. The meter was calibrated prior to each sampling trip by following the manufacturer's instructions. The dissolved oxygen sensor was also regularly regenerated as per manufacturer's instructions to keep it in good working condition.

3.6.2 Determination of the river sediment water content (%)

The river sediment was homogenised by mixing with a stainless-steel spatula. Triplicate wet samples of ~10 g (W1) were transferred to pre-weighed crucibles. The samples were then dried in an oven at 50 ^{o}C for 24 h until a constant weight was achieved. The crucibles

and their contents were then left to cool and reweighed (W2). The water content could then be calculated:

$$\text{Water content (\%)} = 100*(W_1 - W_2) / W_1 \qquad (0\text{-}5)$$

3.7 References

[1] R. Liu, J.L. Zhou & A. Wilding,. *J. Chromatogr. A,* 1022 (2004), pp. 179–189.

[2]] R. Liu, J.L. Zhou & A. Wilding,. *J. Chromatogr. A,* 1021 (2003), pp. 140–151.

[3] Zhang, Z.L. & Zhou, J.L.,. *J. Chromatogr. A,* 1154 (2007), pp. 205–213.

Chapter 4. Experimental comparison of common analytical techniques for the measurement of steroidal estrogens in environmental water samples

4.1. Introduction

In terms of estrogenic activity, the most important EDCs are E1, E2 and EE2 as they are far more potent than other compounds such as bisphenol A or alkylphenols, and can cause fish feminisation at approximately the ng L^{-1} level [1][2]. Due to uncertainty in their impacts on terrestrial and aerial organisms as a result of a lack of data, E1, E2 and EE2 are not yet included in the list of 146 substances with endocrine disruption classification [3], nevertheless, their feminisation effects in invertebrates and fish have been confirmed worldwide. In addition, it is widely recognised that effluent discharges from sewage treatment works (STW) are the main source of EDC inputs to the aquatic environment such as rivers and streams [4][5]. Other sources include animal agriculture, aquaculture and spawning fish [14].

In order to minimise EDC impacts on fish populations, reliable and sensitive analytical methods are needed to detect EDCs in the aquatic environment. The concentrations of EDCs are generally low in aquatic systems, up to 19.4 ng L^{-1} in surface water, although levels as high as 5400 ng L^{-1} have been found in some STW effluents [7]. As a result, water samples are usually concentrated using solid-phase extraction (SPE). A wide variety of analytical techniques have been developed and subsequently optimised for EDC analyses, among which gas chromatography (GC) coupled with mass spectrometry (MS) and tandem MS is the first developed and still widely used [8][9][10][11][12]. A more recent and increasingly popular technique has been liquid chromatography (LC) coupled with MS or MS-MS which does not require sample derivatisation [13][14][15][16]. As EDCs are being

measured at trace levels, often close to the limit of detection (LOD) of the instruments, there is a need to understand how the different techniques compare in terms of their performance. Only by knowing which technique(s) are most reliable and reproducible, can we appraise relative merits and focus on the optimisation of methodologies.

This study investigates the performance of three analytical techniques including GC-MS, GC-MS-MS and LC-MS-MS, all previously developed and validated for the analysis of emerging contaminants including E1, E2 and EE2 in environmental water samples [10][12][17]. The influence of sample matrix on analytical quality at trace levels is highly important and widely speculated, and is addressed.

4.2. Experimental

4.2.1. Chemicals and standard solution

All solvents used (methanol, ethyl acetate, acetone, dichloromethane, hexane and acetonitrile) were of distilled-in-glass grade (purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland). EDC standards including E1, E2 and EE2, together with their deuterated internal standards E2-d_2 were purchased from Sigma, UK. In addition, other internal standards E1-d_4, E2-d_4 and EE2-d_4 and were obtained from Qmx Laboratories Ltd, UK, all with an isotopic purity >98%. Separate stock solutions of individual standards (1000 mg L^{-1}) were prepared in methanol, from which working standards (10 mg L^{-1}) of individual compounds and mixtures were prepared. All standards were stored at -18 ^{o}C. Ultrapure water was supplied by a Maxima Unit from USF Elga, UK.

4.2.2. Sampling and sample treatment

Water samples (in triplicate) were collected in pre-cleaned Winchester amber-glass bottles (2.5 L) from four sites (sites 1-4) along the River Ray, and at one control site (site 5) on the River Ock, Swindon, UK. Site 1 is approximately 3.5 km upstream from the effluent of Rodbourne STW (adjacent to Site 2). Sites 3 and 4 are 1.7 and 8.3 km downstream of the effluent, respectively. Sodium azide (10 mL, 2M) was added to each sample as a general biocide to eliminate bacteria and thus minimise biodegradation during sample storage and processing. Samples were refrigerated at 4ºC until filtration and extraction. Each sample was filtered under vacuum using pre-ashed glass fibre filters (Whatman, GF/F). The filtrates were subsequently spiked with 100 ng of the internal standards.

4.2.3. SPE

The target compounds were extracted from the filtered water samples using SPE. Oasis® SPE cartridges (0.2 g HLB, Waters) were conditioned with 5 mL of ethyl acetate to remove residual bonding agents, followed by 5 mL of methanol which was drawn through the cartridges under a low vacuum to ensure that the sorbents were soaked in methanol for 5 min. Ultrapure water (3 x 5 mL) was then passed through the cartridges at a rate of approximately 1-2 mL min^{-1}. Water samples (2 L) were then extracted at approximately 10 mL min^{-1}, as this has been shown to be optimal [11]. The SPE cartridges were subsequently dried under vacuum and the extracts eluted from the sorbents into 20 mL vials with 10 mL of methanol at a flow rate of 1 mL min^{-1}. The solvent was then blown down to 100 μL under a gentle N_2 flow, and transferred to 300 μL microvials ready for analysis.

4.2.4. Derivatisation

In the case of GC analyses of EDCs, the target compounds need to be derivatised to produce less polar derivatives. This enhances chromatographic performance by improving peak shape, reduces tailing and provides a better baseline. Briefly, the extracts were transferred into 3 mL reaction vials and were evaporated to dryness under a gentle stream of nitrogen. The dry residues were then derivatised by the addition of 50 µL each of pyridine (dried with KOH solid) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), which were heated in a heating block at 60–70 ^{o}C for 30 min following a previously optimised method [11]. The derivatives were cooled to room temperature, evaporated under a gentle stream of nitrogen to dryness, reconstituted in 100 µL of hexane and transferred to 300 µL microvials ready for analysis by GC-MS and GC-MS-MS.

4.2.5. Sample analyses

4.2.5.1. LC-MS/MS

The untreated extracts in methanol were analysed using a Waters 2695 HPLC separations module (Waters, Milford, MA, USA) fitted with a Waters Symmetry C_{18} column (4.6×75 mm, particle size 3.5 µm). The mobile phase comprised of eluent A (0.1% formic acid in ultrapure water), solvent B (acetonitrile) and eluent C (methanol). The flow rate was 0.2 mL min^{-1} and the elution started with 90% eluent A:10% eluent B, a 25 min gradient to 80% of eluent B, then a 3 min gradient to 100% eluent B, followed by an 8 minute gradient to 100% of eluent C. This was held for 10 min and then returned back to the initial conditions within 4 min. The system re-equilibration time was 10 min and the sample injection volume was 10 µL. The MS-MS analyses were completed with a Micromass Quattro triple-quadrupole mass spectrometer equipped with a Z-spray electrospray

interface. The analyses were in negative ion mode. The parameters for the analyses were: electrospray source block and desolvation temperature 100 and 300ºC, respectively; capillary and cone voltages 3.0 kV and 30V, respectively; argon collision gas 3.6×10^{-3} mbar; cone nitrogen gas flow and desolvation gas: 25 and 550 L h^{-1}, respectively. Following the selection of the precursor ions, product ions were obtained at optimum collision energies and were selected according to the fragmentation that produced a useful abundance of fragment ions. The optimal collision energy, cone voltage and transitions chosen for the multiple reaction monitoring (MRM) experiment were optimised and utilized a dwell time of 100 ms. The mass spectrometer was operated in MRM mode with unit mass resolution on both mass analysers. The precursor and product ions monitored for each compound are shown in Table 4.1.

4.2.5.2. GC-MS/MS

During our method development, silylated EDCs (through BSTFA) are stable for up to 120 h with the exception of TMS-EE2 which has been found to be stable for only 48 h [18]. Others [26] used *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) as the derivatisation agent and obtained stable derivatives for up to 4 weeks. Consequently as a result of the limitations, all GC-MS and GC-MS-MS analyses were performed immediately and definitely within 48 h of derivatisation. To maintain the optimum performance, regular changes were made to the pre-column and injector liner in GC, together with regular cleaning of the ion source.

The GC-MS-MS analyses were performed using a 6890N network gas chromatograph (Agilent Technologies, USA) interfaced with a mass spectrometer (Quattro Micro,

Micromass, USA) with a tandem quadrupole. An Agilent 30 m HP-5 capillary column with a 0.25 mm internal diameter and a 0.25 μm film thickness was used. The carrier gas was helium, which was maintained at a constant flow of 1.0 mL min^{-1}. The GC column temperature was programmed from 100 ^{o}C (initial equilibrium time 1 min) to 200 ^{o}C via a ramp of 10 ^{o}C min^{-1}, 200-260 ^{o}C via a ramp of 15 ^{o}C min^{-1}, 260 - 300 ^{o}C via a ramp of 3 ^{o}C min^{-1} and was maintained at 300 ^{o}C for 2 min, with a total run time of 30.33 min. The MS was set for positive electron impact ionisation (70 eV) and was operated in MRM mode for quantitative analyses, using argon as the collision gas. The inlet and MS transfer line temperatures were both maintained at 280 oC and the ion source temperature was 250 ^{o}C. Sample injection (1 μL) was in splitless mode. The precursor and product ions for each compound are shown in Table 4.1.

4.2.5.3. GC-ion trap-MS

GC–MS analyses were performed using a gas chromatograph (Trace GC 2000, Themoquest CE Instruments, TX, USA) coupled with an ion trap mass spectrometer (Polaris Q, Themoquest CE Instruments, Texas, USA) and an autosampler (AS 2000). A ZB5 (5% diphenyl–95% dimethylpolysiloxane) capillary column of 30 m × 0.25 mm i.d. (0.25 μm film thickness) was used. Helium carrier gas was maintained at a constant flow rate of 1.5 mL min^{-1}, which was found to be the optimum for the separation of target compounds. The GC column temperature was programmed from 100 ^{o}C (initial equilibrium time 1 min) to 200 ^{o}C via a ramp of 10 ^{o}C min^{-1}, 200–260 ^{o}C via a ramp of 15 ^{o}C min^{-1}, 260–300 ^{o}C via a ramp of 3 ^{o}C min^{-1} and maintained at 300 ^{o}C for 2 min. The MS was adjusted for selected ion monitoring mode for quantitative analyses. The inlet and MS transfer line temperatures

were both maintained at 280 ^{o}C, and the ion source temperature was 250 ^{o}C. Sample injection was in splitless mode. The ions monitored are shown in Table 4.1.

Compound	LC-MS-MS			GC-MS-MS			GC-MS		
	RT	Precursor Ion	Product Ion	RT	Precursor Ion	Product Ion	RT	Quantification Ion	Confirmation Ion
E1-d4	-	-	-	-	-	-	14.44	346 (100%)	257 (100%) 285 (30%)
E1	19.50	269	183	18.10	342	257 (100%) 327	14.51	342 (100%)	257 (100%) 218 (20%)
E2-d4	-	-	-	-	-	-	17.72	289 (100%)	420 (100%) 330 (35%)
E2-d2	17.75	273	186	18.50	418	287 (100%) 233	-	-	-
E2	17.70	271	145	18.50	416	285 (100%) 243	18.08	285 (100%)	416 (100%) 326 (40%)
EE2-d4	-	-	-	-	-	-	19.29	289 (100%)	430 (100%) 289 (30%)
EE2	19.05	295	145	19.70	425	193 (100%) 231	19.42	285 (100%)	425 (100%) 232 (30%)

Table 4.1 Retention times (RT) and ions used for the analysis of E1, E2 and EE2 and their respective deuterated internal standards

4.3. Results and discussion

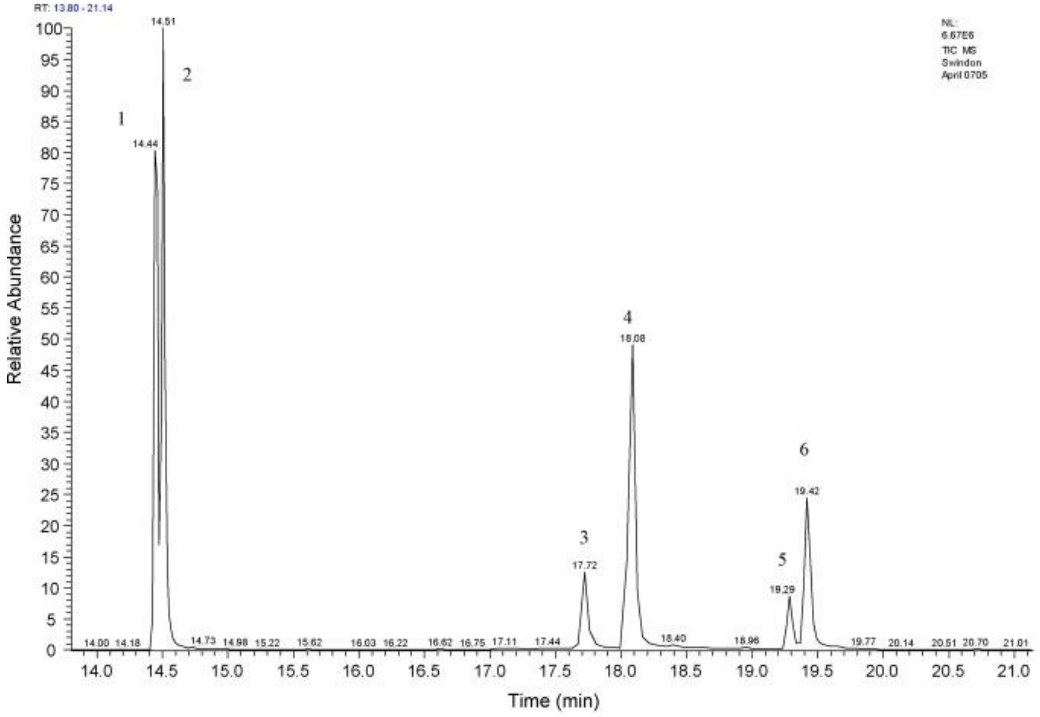
4.3.1. Validation of the analytical methods

Chromatograms for the three steroidal estrogens and their associated internal standards are shown in Fig. 4.1. In GC-MS-MS or LC-MS-MS operation, E2-d_2 was the only deuterated internal standard found to give a satisfactory response with these instruments. A visual comparison of the three chromatograms suggests little difference in the quality of the separation of the standards. Analysis of deuterated internal standards by LC-MS-MS proved, however, to be more difficult than with the GC techniques owing to higher background noise (even with E2-d_2). All three analytical techniques relied upon SPE as the pre-concentration step, which has been assessed extensively for EDC recovery [10][12]. Each method was then thoroughly validated for the linear range of calibration curve, sensitivity, specificity, blanks, precision and bias (through the use of recovery experiments due to lack of certified reference materials). The linear ranges of calibration curve for the GC-MS and GC-MS-MS have previously been determined, from 1 to 500 ng L^{-1} [10][12]. The linear range for the LC-tandem MS was between 15-750 ng L^{-1}. The limit of detection (LOD), defined as the concentration that corresponds to three times the standard deviation of blanks, was measured by integrating blank peak area for each analyte in 10 independent performances with ultrapure water as the blank. As shown in Table 2, the GC-MS-MS method offers improved performance over the two alternatives with regard to LOD. The LOD for the GC-MS technique is relatively poor among the three techniques. The analysis of procedural blanks (blanks being treated as samples) did not detect any of the three compounds in our regular sampling trips, confirming a good quality procedure. Furthermore, extensive recovery experiments were performed regularly by the spiking of

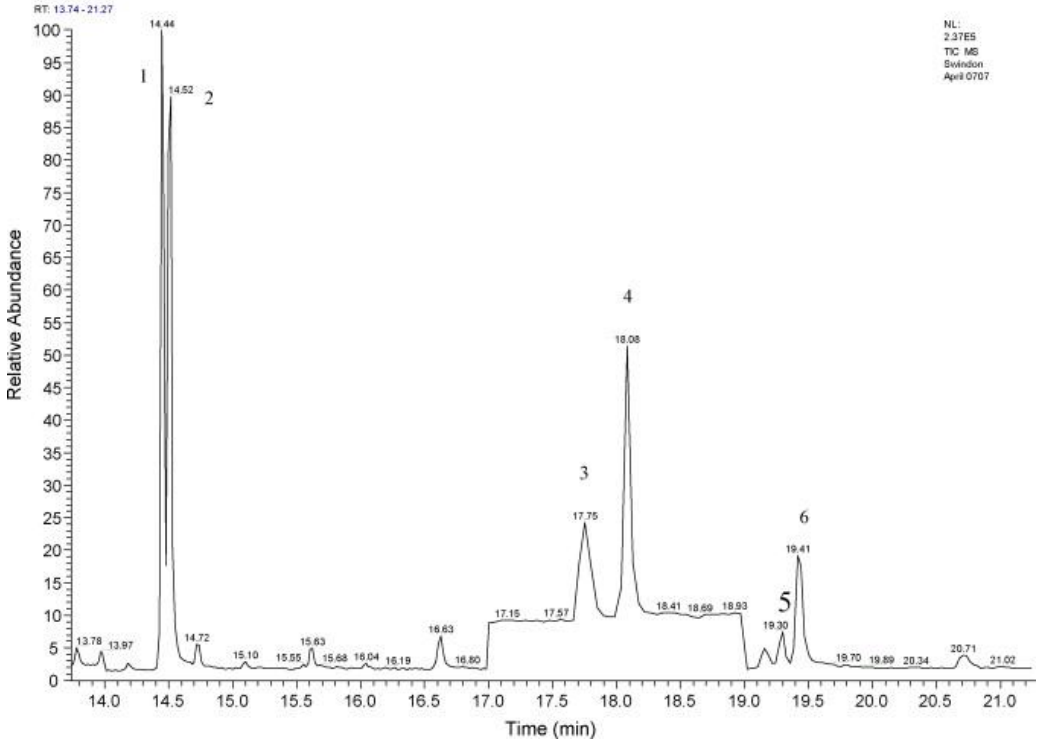
the three estrogens at different levels (1 ng L^{-1}, 20 ng L^{-1}, 50 ng L^{-1}, 100 ng L^{-1}, 200 ng L^{-1}) in different waters (e.g. river water, seawater, groundwater, wastewater), with satisfactory recoveries from 72-119% [10][12]. In addition, an inter-calibration exercise was undertaken recently with three other laboratories in the UK, among which a good agreement was achieved.

(a) GC-Ion Trap-MS

(i) standard

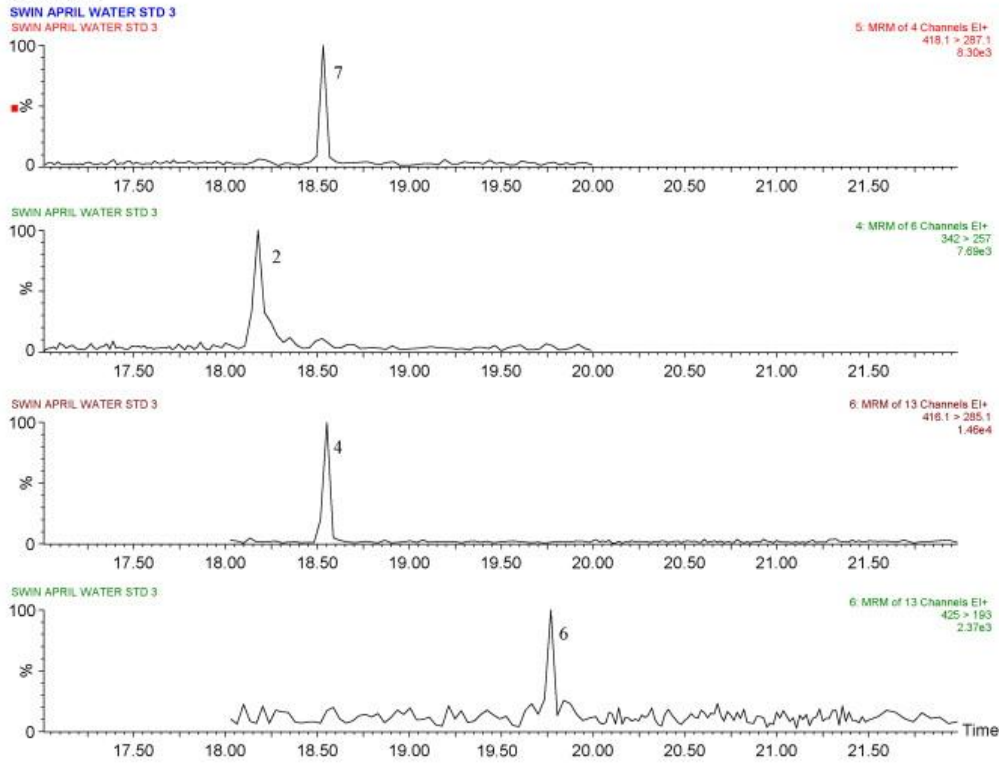


(ii) sample

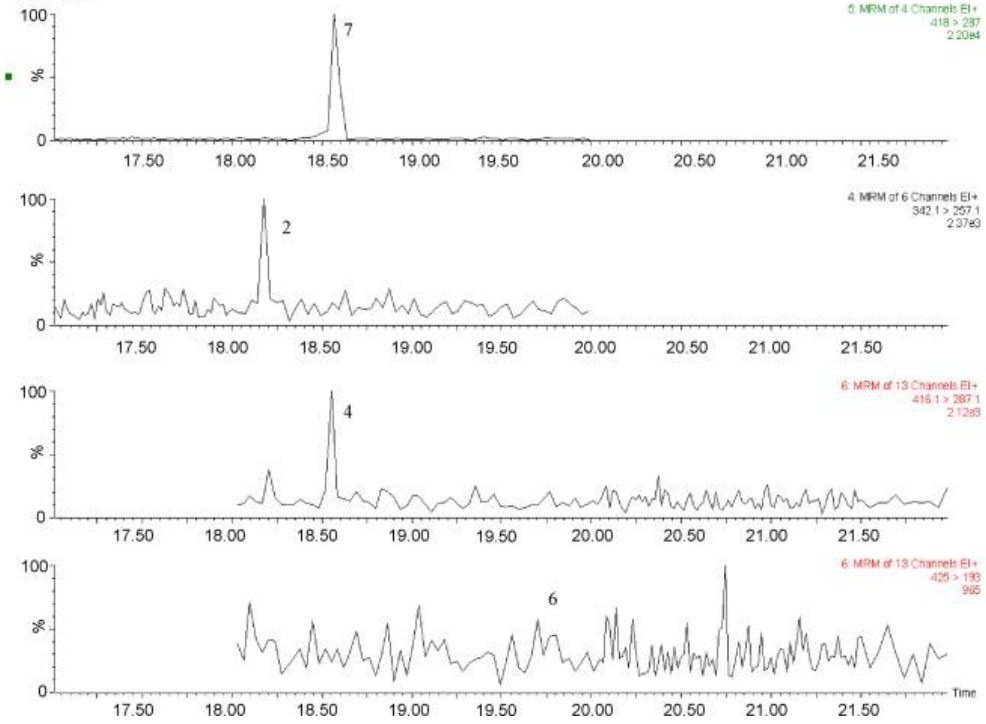


(b) GC-MS-MS

(i) standard



(ii) sample



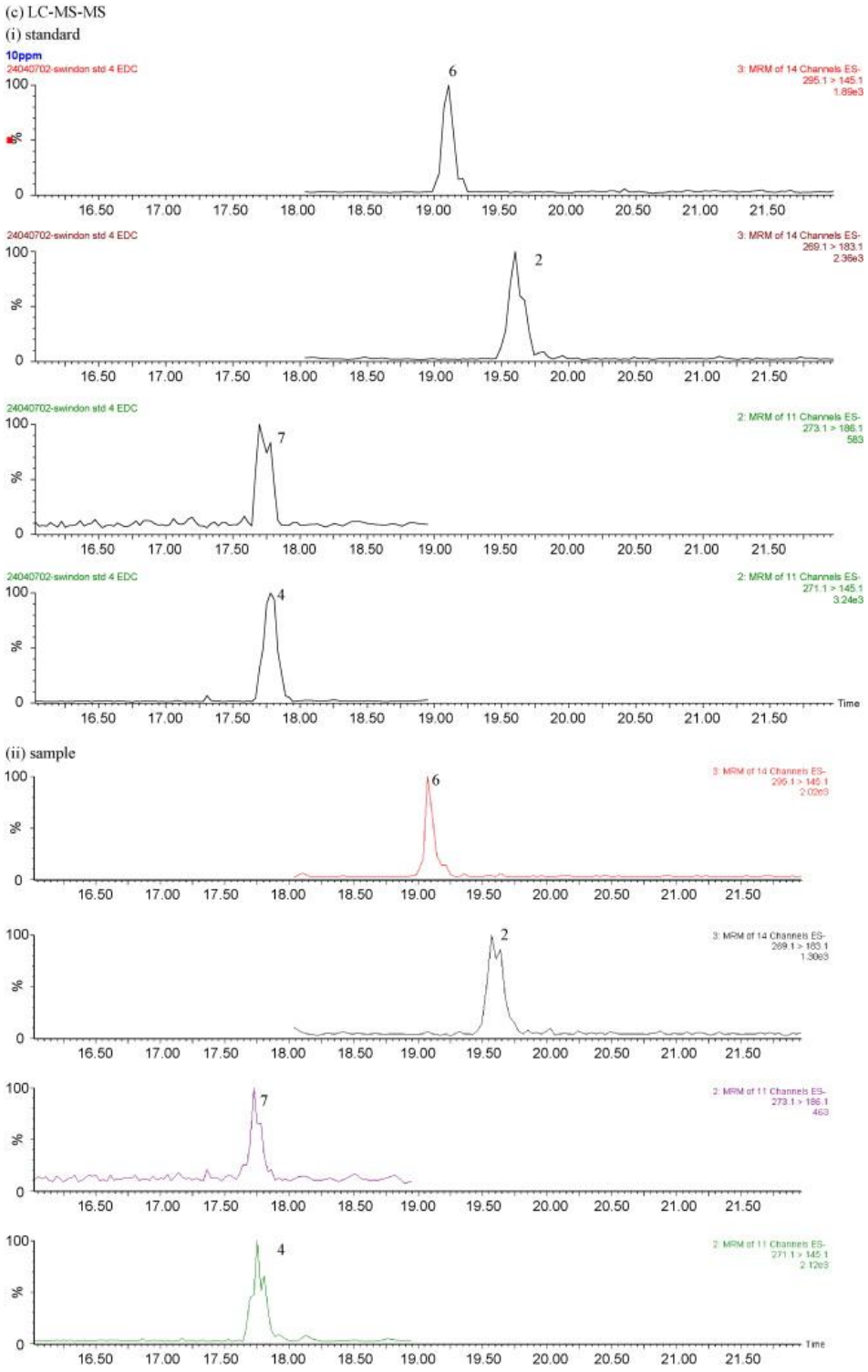


Figure 4.1 Chromatograms of a 10-ng mL^{-1} standard mix (i) and an effluent (site 2) sample (ii) as analysed by (a) GC–ion trap-MS, (b) GC–MS–MS and (c) LC–ESI–MS–MS. Peaks are (1) E1-d_4, (2) E1, (3) E2-d_4, (4) E2, (5) EE2-d_4, (6) EE2 and (7) E2-d_2.

Compound	GC-MS	GC-MS-MS	LC-MS-MS
E1	0.7	0.3	0.6
E2	1.4	0.3	1.2
EE2	0.8	0.3	0.4

Table 4.2 Limits of detection (ng L^{-1}) for three steroidal estrogens by each method

4.3.2. Application of the analytical methods

Once validated, the techniques were applied to the EDC analysis of environmental sample extracts. Overall, the LC-MS-MS was observed to be most heavily affected by matrix interferences in terms of elevated background noise and reduced peak area for the target compounds. Similar matrix interference effect in LC-tandem MS has been reported by Beck et al. [10], who observed a signal suppression of between 80-85% for the three compounds. As a result, the signal-to-noise ratio was reduced such that peaks were less clear than in either of the GC techniques. Surprisingly, a significant interference for the analysis of EE2 was noted for GC-MS-MS of some effluent sample extracts. For both standards and sample extracts, the GC-MS technique appears to offer the best baseline of the three techniques, but it lacks the robustness of the tandem MS techniques, where fragment ions are used to confirm the identity of the analytes.

As the LOD for the GC-MS technique is relatively poor, its signal quality rapidly declines with a reduction in concentration of the analytes, and as can be seen (Fig. 4.2), several of the environmental samples analysed were below LOD for this instrument. However, each of three techniques is operating at or near their detection limits for many of

the samples for at least one of the estrogens due to matrix interferences. The three techniques used here, however, reflect LODs reported by other laboratories using similar extraction and analytical techniques, which range from 0.1-1 ng L^{-1} [15,16,27] for each compound.

As is shown in Fig. 4.2 there is a very good agreement between the three techniques, particularly for E1 (0.1-0.2 ng L^{-1} difference between techinques, RSD = 28%) and E2 (<0.1 to 0.2 ng L^{-1}, RSD = 4%). However, it is evident that the LC-MS-MS measurements of EE2 are significantly higher than measurements by GC techniques, particularly in areas where matrix interferences are likely to be high (e.g. sewage effluents), as a result there is a larger variabililty in measured concentrations between the three techniques for EE2 (0.1-1 ng L^{-1}, RSD = 45%). The precision for each technique which is comparable across the techniques, is the lowest for the GC-MS-MS technique.In addition, all three techniques identify the STW effluent (site 2) as the location at which concentrations of the three estrogens are the highest. This shows that all three techniques are consistent in their identification of pollution hotspots. The results are in agreement with other studies [4][5][10] which identify sewage effluent as the primary source of steroidal estrogens in river waters.

Compound	GC-MS	GC-MS-MS	LC-MS-MS
E1	0.7	0.3	0.6
E2	1.4	0.3	1.2
EE2	0.8	0.3	0.4

Table 4.2 Limits of detection (ng L^{-1}) for three steroidal estrogens by each method

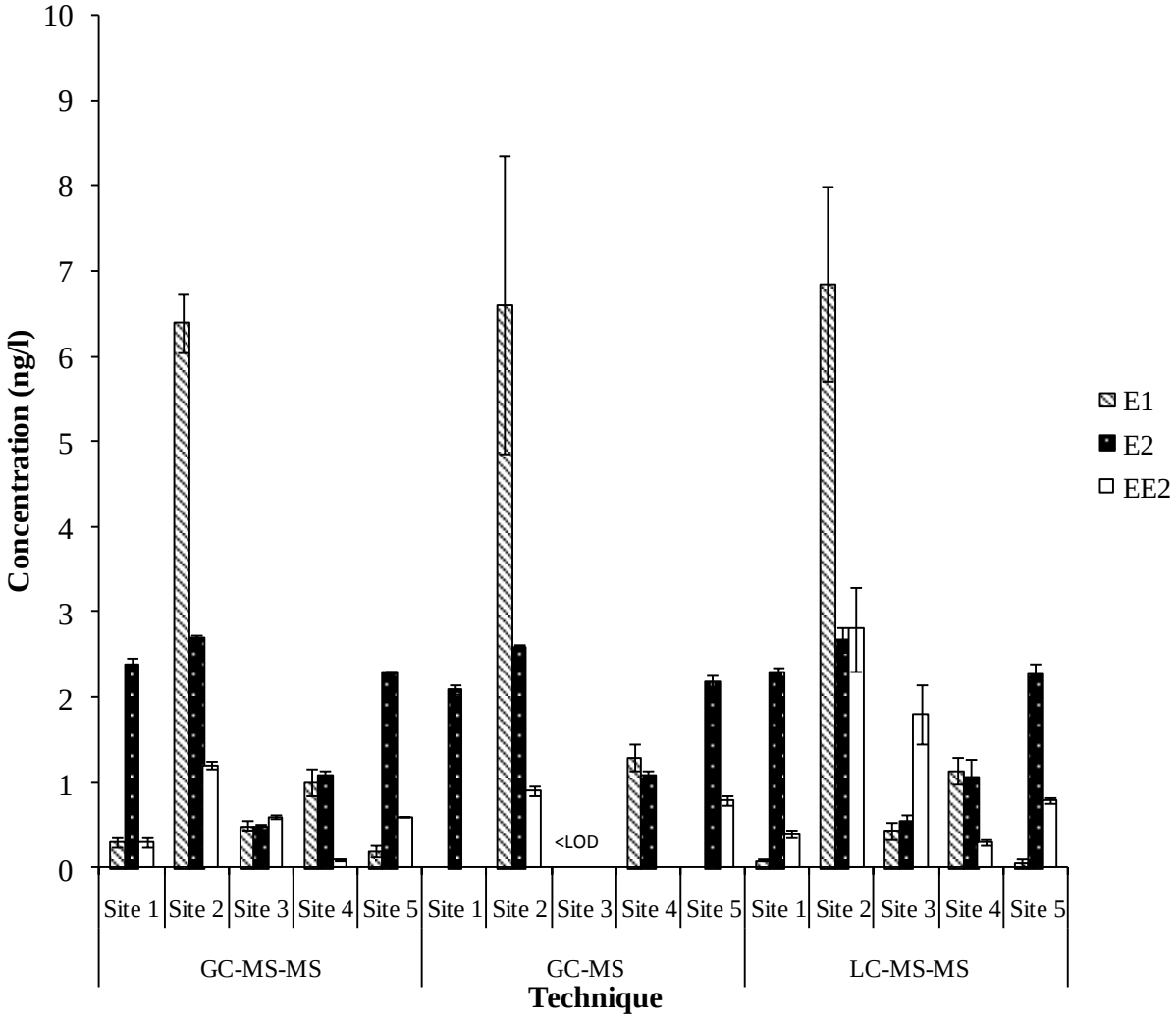


Figure 4.2 Analyses of samples from 5 sampling locations for estrone (E1), estradiol (E2) and ethinylestrodiol (EE2) by GC-MS-MS, GC-MS and LC-MS-MS

4.3.3 Comparisons with other analytical techniques

To ensure the data produced by the participating laboratories as part of the EDCAT project, an intercalibration programme was established with sought to compare the results produced by each laboratory for spiked samples with unknown concentrations of estrogenic compounds, together with an unknown effluent sample.

4.3.3.1 Method of inter-comparison

The inter-comparison was co-ordinated and led by Cefas. Freshwater from a borehole at the Cefas former Burnham Laboratory site, was 'spiked' with the three oestrogens, E1, E2 and EE2 at two different concentrations. Five samples of each concentration along with an unspiked sample and a wastewater effluent were sent to the following laboratories: Centre for Environment Fisheries and Aquaculture Science (Cefas), Sussex University (Sussex), Plymouth Marine Laboratory (PML) and Centre for Ecology and Hydrology Wallingford (CEH).

Each laboratory employed a range of sample processing and analytical techniques. PML employed liquid-liquid extraction (in DCM) with rotary evaporation, with subsequent analysis by GC-MS. CEH employed an SPE-LC-MS technique. CEFAS employed an SPE-disk-GC-ion trap-MS technique. At Sussex, LC-MS/MS was used for the results reported to Cefas and were confirmed using GC-MS/MS.

4.3.3.2 Results of inter-comparison

After submitting data to Cefas, it was revealed that the two spiked sets of samples were spiked at 20ng/L and 1ng/L. As is shown in Table 4.3, there was poor agreement between laboratories for the effluent samples. LC-MS/MS analysis appears to have over-estimated

concentrations of E2 and EE2 compared to analyses by GC-MS(/MS) based techniques employed both at Sussex and elsewhere. Results for E1 however are more encouraging, with good agreement between the LC-MS/MS technique and other laboratories. Table 4.4 shows the results from each laboratory for the analyses of the two sets of samples spiked with standards.

Participant	Analytical instrument	Sample	E1	E2	EE2
Sussex	LC-MS/MS	Effluent	50	43	21
	GC-MS/MS	Effluent	26.7	<0.3	<0.3
	GC-ion trapMS	Effluent	25.6	<1.4	1.5
PML		Effluent	34	11	
Cefas		Effluent	47	8.4	0.6
Cefas		Effluent Preserved	53	19	2.3
		Mean.	**45.9**	**20.3**	**8**
		SD	8.5	15.4	11.0
		CV(%)	18.4	76.1	141.7

Table 4.3 Results for the effluent sample (ng l^{-1})

The results shown in tables 4.3 4.4 are encouraging. At the 20 ng l^{-1} level there is good agreement between the LC-MS/MS technique described in chapter 3 (and compared internally in previous sections of this chapter) compare favourably with the techniques employed by other laboratories, with acceptable levels of error for E2 and EE2 measurements, although E1 is slightly over-estimated. There is a significant over-estimation of concentration measured in samples spiked at 1 ng l^{-1}, more environmentally-

relevant concentrations by the LC-MS/MS technique, possibly as a result of matrix interference. Again, this issue was most severe for E1 analyses, but it should be noted that all laboratories with the exception of PML (which under-estimated) over-estimated concentrations at this level, suggesting the possibility that there may have been some error in the spiking at this level, since there is good agreement in this over-estimation.

		Sample A			Sample B		
Participant	Compound	mean	95% confidence interval	Z	mean	95% confidence interval	Z
CEH	E1	21.0	18.0, 24.0	0.73	3.52	2.98, 4.06*	10.19
	E2	22.5	20.0, 25.1*	2.14	4.96	4.11, 5.81*	10.18
	EE2	19.5	16.2, 22.8	-0.34	1.04	0.79, 1.29	0.34
Sussex	E1	22.5	19.5, 25.5	1.84	2.52	1.98, 3.06*	6.15
	E2	19.9	17.3, 22.4	-0.10	1.34	0.49, 2.19	0.87
	EE2	19.4	16.1, 22.7	-0.39	1.78	1.53, 2.03*	6.69
PML	E1	16.9	13.0, 20.8	-1.75	0.63	-0.06, 1.33	-1.15
	E2	18.3	14.2, 22.4	-1.12	0.90	-0.19, 1.99	-0.20
	EE2	18.4	14.2, 22.7	-0.80	1.03	0.71, 1.36	0.22
Cefas	E1	20.0	16.4, 24.1	0.13	2.20	1.35, 3.05*	3.07
	E2	17.3	13.3, 21.4	-1.76	2.70	1.36, 4.04*	2.76
	EE2	16.3	12.1, 20.6	-1.87	1.70	1.30, 2.10*	3.80

Table 4.4 Results compared against 20 ng l^{-1} spiked samples (sample A) and against 1.0 ng l^{-1} spiked samples (* denotes statistically significantly different at the 5% level)

4.4 Conclusions

Three popular techniques for the analysis of steroidal estrogens in the aquatic environment have been compared. It has been observed that overall, the three techniques appear

comparable, but that tandem-mass spectrometric techniques are able to detect at lower concentration levels of the three steroidal estrogens of interest. In particular, the GC-MS technique fails to detect the pollutants at the lower-end of environmentally relevant concentrations. However, the LC-MS-MS technique is more susceptible to matrix interferences for the analysis of samples resulting in a reduction of the signal-to-noise ratio and a subsequent reduction in reliability and stability. With the GC-MS-MS offering increased selectivity, the lowest LOD, and with a good a signal-to-noise ratio for all compounds in all samples, it is regarded as the preferred analytical method for the reliable identification and analysis of estrogens in environmental water samples. However, it does require derivatisation of samples prior to injection, which can be time consuming and therefore a disadvantage for sample throughput. Additionally, LC-MS-MS affords the sequential analysis of both estrogenic and pharmaceutical compounds, without requiring any additional treatment of samples, allowing for the simultaneous detection of both classes of pollutant.

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Chapter 5. Improvement of sample treatment techniques to reduce uncertainty of analyses of EDCs and Pharmaceuticals in environmental samples

5.1 Introduction

Measurements of contaminant concentration in the environment inevitably have uncertainty. At each stage of an analytical procedure, there are potential sources of uncertainty, as shown in table 5.1.

Table 5.1 Sources of uncertainty in the analysis of contaminants in environmental

Steps of analytical procedure	Sources of Uncertainty
Sampling	Representativeness
	Sample contamination
Sample storage	Analyte transformation
	Loss of analyte
Treatment	Analyte transformation
	Loss of analyte
	Extract purification
Determination	Separation
	Identification
	Calibration
	Detection

In Chapter 4, it was discussed that in order to fully understand the risk posed by emerging pollutants, it is necessary to have precise and accurate analytical procedures. To improve the quality of analytical data, researchers seek to reduce sources of uncertainty optimizing

the analytical procedures used. These efforts often focus on the development of highly optimised techniques for final separation and detection, such as chromatography and mass spectrometric analysis, but little attention is paid to the other factors that may represent sources of uncertainty, including, but not limited to, sample preparation and storage. This chapter seeks to evaluate some of these sources of uncertainty experimentally and suggest optimised strategies to reduce them.

Although it is acknowledged that samples should be prepared and analysed immediately after collection, this is not always practical. It is inevitable that samples are stored prior to analysis, possibly for significant periods of time. Internal standards, due to their similar chemistry are widely assumed to degrade at a rate identical to that of their analyte counterparts, but it is nevertheless preferable to minimise any degradation to reduce any uncertainty that may arise due to any variability in this. There is little understanding of how the the possible loss of analyte compounds during this storage affects the quality of data produced by subsequent analysis, which this chapter seeks to address.

It is common for researchers to employ a preservation technique to prevent degradation of analyte compounds. Photo-degradation has been shown to rapidly degrade both E1 and E2 - Zhang *et al.* (2007) [1] found 94% degradation of both compounds within 1h when exposed to UV radiation. However, due to the widespread storage of samples in dark refrigeration, it is unlikely that photo-degradation nor thermal degradation are a significant cause for concern in most analytical applications. It is, therefore, assumed that the most significant process of degradation is as a result of biotic processes, and as such, many researchers use preservative agents that seek to minimise biotic growth in water samples. However, peer-reviewed research to evaluate the efficacy of these different preservative agents is scarce,

and as such, there is wide variation in the use of different preservatives, with little understanding of the implications this may have for cross-comparison of results, nor for the validity of data in some studies. Fuhrman *et al.* [5] examined the stability of estrogens and estrogen metabolites in human blood and urine samples using acidification treatment, and found no significant benefit of this treatment. However, the concentrations of estrogenic compounds in these samples is typically significantly higher ~2 orders of magnitude in their study. The US EPA [6] conducted a review of their storage and preservation practice of environmental samples for analysis of steroidal estrogens and pharmaceutically-active compounds and concluded that: glass or PTFE amber bottles should be used; acidification should be used to dechlorinate samples; and that samples should be stored in dark refrigeration; samples should be extracted and analysed within 7 days.

In this chapter, the significance of sample storage conditions are investigated, and preservative techniques which offer optimal recovery and allow storage of samples for the longest period of time are identified.

5.2 Experimental

5.2.1 Sample collection and treatment

Water samples were collected from the river Ray, in Wiltshire, UK. It is a tributary of the river Thames and is a conduit for effluent from Rodbourne STW, a large, modern treatment works in Swindon. Samples were collected into ~50 L stainless steel barrels and were subsequently brought back to the laboratory where they were stored in dark refrigeration until further processing. The actual volume of water collected was found by weighing each barrel before and after sampling.

Sub-samples (3x 500mL) were taken from each barrel, prepared for analysis, and subsequently analysed by LC-MS-MS for estrogenic and pharmaceutical compounds within 24 hours, to allow an understanding of background concentrations of these compounds, used in subsequent calculations of concentration. Subsequent to this, each barrel was spiked with 500ngL^{-1} of a standard mixture of both pharmaceutical and estrogenic compounds, prepared as detailed in chapter 3; together with a preservative agent as shown in table 5.2. Although it is recognised that this level of spiking exceeds environmentally relevant concentrations, it was chosen to minimise mass-spectrometric analytical uncertainties and to avoid the possibility of non-detection. The selection of preservatives was based upon the assessment of (1) published analytical procedures used for the determination of estrogens, (2) pharmaceuticals and (3) other organic contaminants. However, it was found that many articles did not report the volume of preservative agent used. As a result of this, the volume of preservative was decided upon based upon (1) existing published techniques (2) understanding of their microbial-growth inhibition and (3) understanding of their potential to interfere with chromatographic separation. pH adjustment was achieved stoichiometrically using 1M sulphuric acid and calcium carbonate (Sigma, UK), and confirmed with an Accumet 925 laboratory pH meter (Fisher, UK). Sub-samples (3x 500mL) were taken daily for 25 days and analysed by LC-MS-MS, as described in chapter 3, for both estrogenic and pharmaceutical compounds.

Table 5.2 Preservative agents examined in this study

Sample ID	Preservative agent
1	Sodium Azide
2	Formaldehyde
3	Dichloromethane
4	pH adjustment – pH 4
5	pH adjustment – pH 2
6	pH adjustment – pH 9
7	Control (no preservative)

5.3. Results and discussion

5.3.1. Initial Concentrations of Estrogens and Pharmaceutical Compounds

By mass, it was found that the barrels contained slightly more than 50L of effluent sample (53.2L-54.1L) and so the addition of standard mixtures was adjusted to account for this. Initial background concentrations were recorded for each barrel and for each compound and deducted from further results accordingly. These initial concentrations ranged from <L.O.D. for EE2 to 144ngL^{-1} for carbemazepine and there was little (<5%) variation between barrels, suggesting that each barrel contained of similar samples of water. After the addition of the standard mixture and preservative agents, sub-samples were immediately prepared and analysed (day 0), to confirm that all samples contained 500ngL^{-1} of analyte

compounds and to allow for the correction of analytical losses and errors that may result from interference caused by the preservative agent, in subsequent analyses

5.3.2. Degradation of Estrogens and Pharmaceutical Compounds over time

Figure 5.1 shows the aggregated mean of all pharmaceutical compounds tested in each barrel over the 25 day test period. There is a reduction in detectable mean concentration of between (2-6.4%) which may be due as a result of the time it takes for homogenisation and stabilisation of the preservatives to occur. Overall, sodium azide represents the most effective degradation-inhibiting agent for the pharmaceuticals tested, with a mean loss of 6.2% over the 25-day period, and 4.1% after the 24 days from day 2 (i.e. after stabilisation). The data suggests that the control sample with no preservative agent gave similar results to sodium azide for the first 7 days, after which there was a rapid loss of pharmaceutical compounds (mean loss after 24 days 12.6%). The barrel which had been adjusted to pH 2 caused the most significant loss in initial detectable pharmaceutical compounds (7.1%) but was more stable after this initial loss than other preservation techniques, with a loss of 5.1% over the 24 days from day 2.

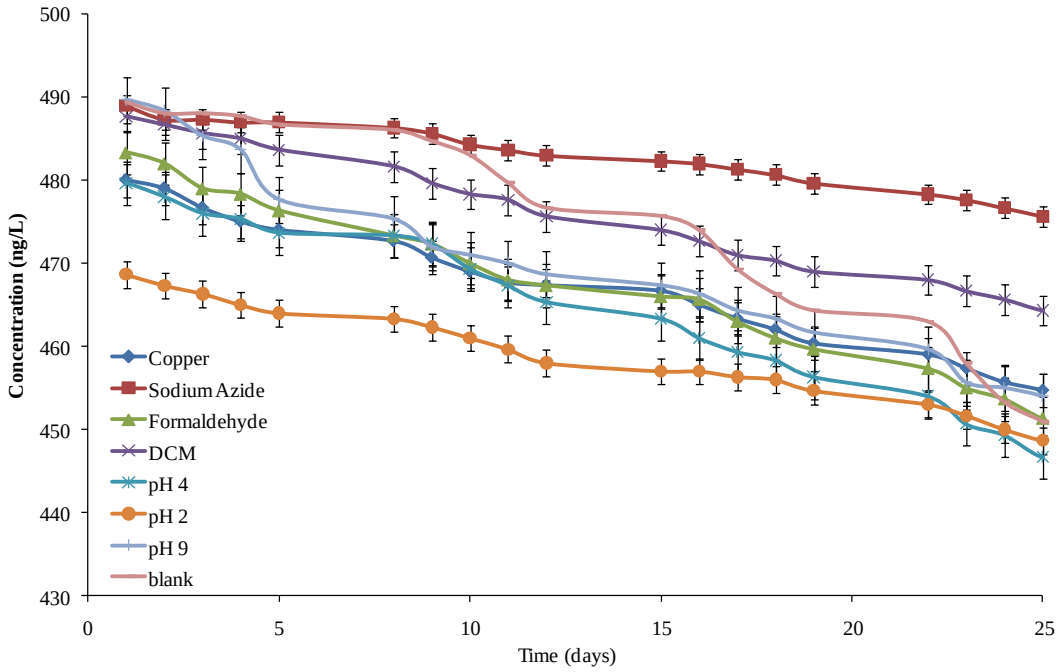

Figure 5.1 Concentrations of pharmaceutical compounds as detected in sub-samples of each barrel up to 25 days after spiking (error bars are standard deviation, $n=3$)

However, for estrogenic substances it is less clear which technique gives optimal results. As is shown in figure 5.2, sodium azide, a commonly used preservative for studies of estrogens in environmental samples, is not effective in stabilizing E1, E2 or EE2 for significant periods of time. There is a rapid decline in concentrations of E2 within 7 days, associated with a period where production of E1 (as a degradation product of E2) almost equals degradation of E1. This is not unexpected, since although the degradation pathways of E1 and E2 are not fully understood, it has been reported [1] that E2 is more rapidly degraded, and has been shown to produce E1 [1] as part of its biodegradation pathway.

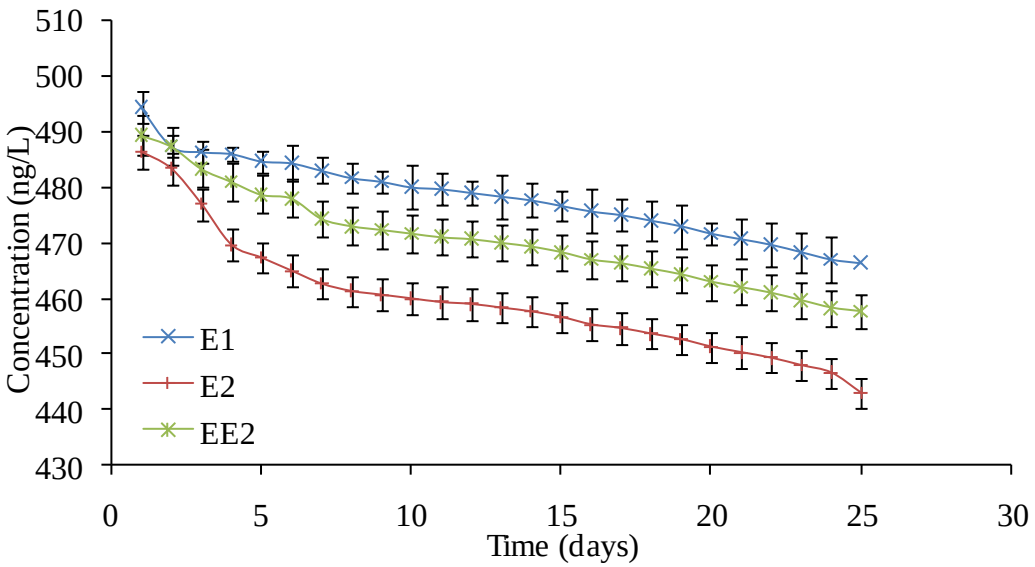

Figure 5.2 Concentrations of E1, E2 and EE2 as detected in sub-samples effluent treated with sodium azide up to 25 days after spiking (error bars are standard deviation, *n=3*)

Figure 5.3 shows the combined mean of E1, E2 and EE2 in each barrel, as detected by LC-MS/MS over the 25 days. As shown, adjustment of pH by sulphuric acid to pH 4 is has been found to offer the greatest stability over the 25 day period with a 7.5% loss after 25 days, compared with a 12.2% loss after 25 days in the control. However, for shorter storage times (< 10 days) sodium azide may be more suitable, since mean estrogen concentrations of effluent treated with it were found to have a percentage loss after 9 days of 5.1%. This is further supported if Estrogenic activity-equivalence (EEQ) is considered. EEQ considers the individual potency of each estrogenic substance, affording a better understanding of the actual estrogenic potential of effluent and comparison with biological analysis techniques.

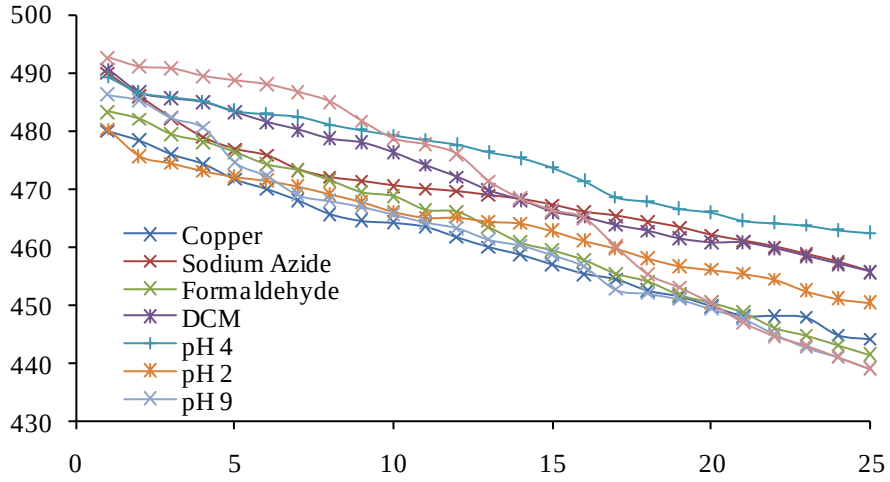

Figure 5.3 Mean concentrations of estrogenic substances as analysed in sub-samples effluent treated with different preservative techniques up to 25 days after spiking (error bars are standard deviation, *n=3*)

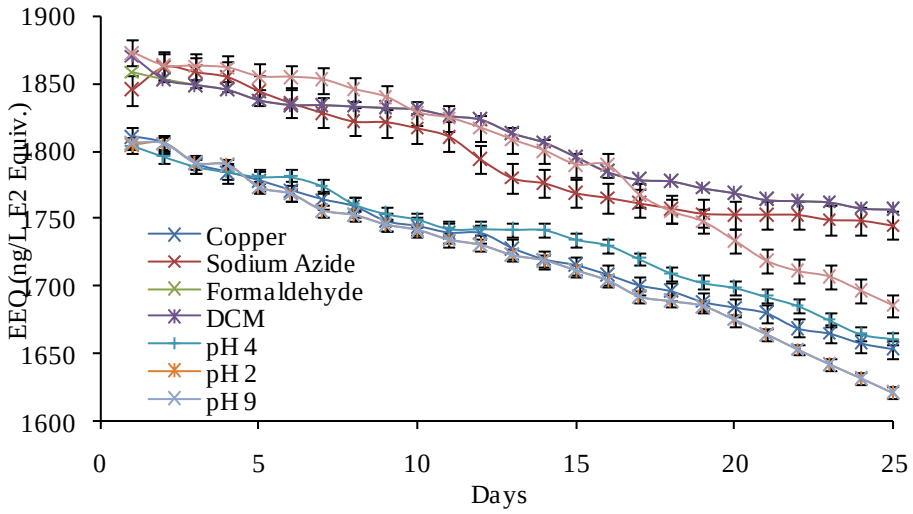

Figure 5.4 EEQ as calculated from analyses of sub-samples effluent treated with different preservative techniques up to 25 days after spiking (error bars are standard deviation, *n=3*)

Figure 5.4 shows Estrogenic activity-equivalence (EEQ) of each barrel over the 25-day period. Since EEQ allows for a better understanding of the actual estrogenic potential of effluent, it is therefore, perhaps more useful than a simple combined mean of E1, E2 and

EE2 concentrations, as it considers the individual potency of each estrogenic substance and allows better comparison with biological analysis techniques. It is directly linked to concentration: $$EEQ=C_{E2}+1/3C_{E1}+10C_{EE2}$$

Where $C_{E2,}C_{E1}$ and C_{EE2} are the concentrations of E2, E1 and EE2 respectively.

As can be seen, there is a clear reduction in the estrogenic potential of samples over time regardless of which preservative is used. This may have significant implications for the risk assessment posed by estrogenic substances in aquatic environments, where analytical data may under-estimate the estrogenic activity of samples. It is therefore suggested that irrespective of preservation technique used, samples should be prepared for analysis as soon as possible after collection, so as to reduce the potential for estrogenic compounds to degrade.

5.3.3. Application to environmental samples

Each preservation technique was subsequently used for environmental samples collected from the influent and effluent of Scaynes Hill STW in West Sussex, UK, 10m downstream and 10m upstream of its effluent in the river Ouse, Sussex. 50L samples were collected (as in chapter 3) at each site and brought back to the laboratory with 1-hour for immediate treatment with preservative and 10µg/L internal standards of both pharmaceutical and estrogenic compounds were added. Triplicate sub-samples (3x2L) were subsequently taken and prepared for analysis. Samples were then stored in dark refrigeration and sub-samples taken every 24 hours for analysis, as previously. Figure 5.5 shows estrogenic equivalence in influent samples as detected by LC-MS/MS over 25 days.

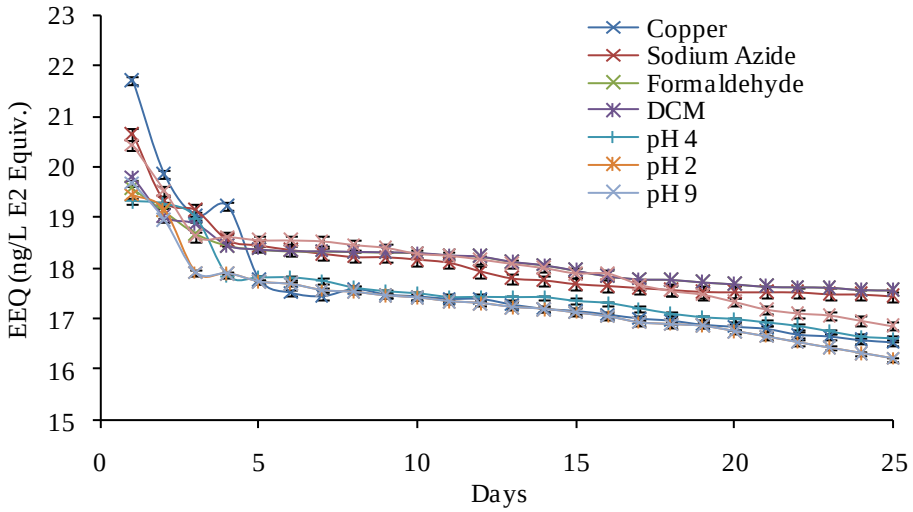

Figure 5.5 EEQ as calculated from analyses of sub-samples of influent from Scaynes Hill STW up to 25 days after sampling (error bars are standard deviation, $n=3$)

As can been seen, initial EEQ was found to be in the 19.7-21.3 range with relatively good agreement between samples treated with the different preservation techniques, although samples containing copper and sodium azide appear to have slightly higher concentrations initially, which may be due to interference caused to chromatography separation or mass spectrometric analyses, or due to slight natural variations between the samples. This relatively high EEQ is likely due to this being untreated raw sewage. However, in samples treated with sodium azide there is a rapid decline in detected estrogens, and albeit after a brief increase in EEQ after 3-4 days, likely due to the production of E1 as a breakdown product of E2, detected estrogens after 5 days are amongst the lowest of all preservation techniques. It is not clear which preservation techniques provides optimum storage conditions for periods over longer periods of time, but it is clear that regardless of the preservation technique used, there is significant reduction in the estrogenic activity detected

in influent samples due to the high content of microbial organisms suspected to be present in STW influent.

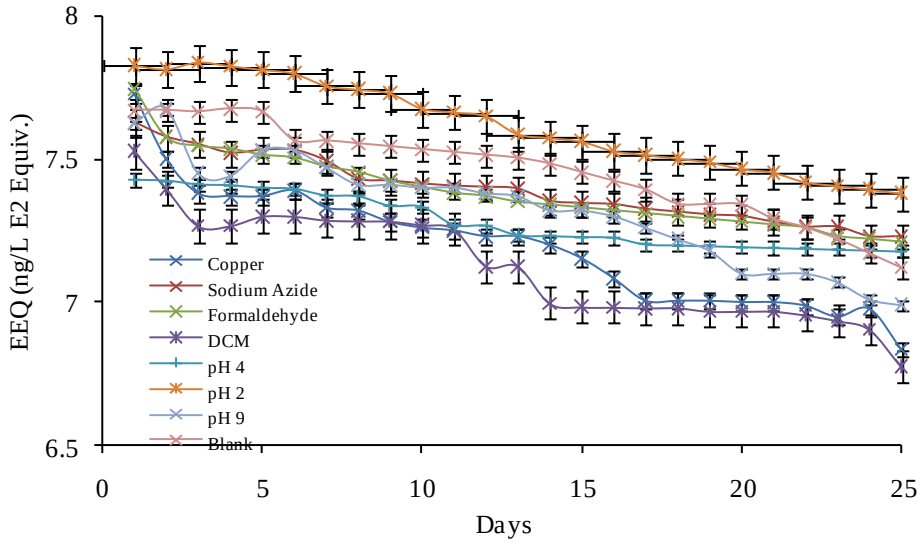

Figure 5.6 EEQ as calculated from analyses of sub-samples of effluent from Scaynes Hill STW up to 25 days after sampling (error bars are standard deviation, *n=3*)

Figure 5.6 shows EEQ in effluent from the Scaynes Hill STW. The reduction in detected estrogenic activity is less marked than in influent, possibly as a result of the reduced number of microbial organisms likely to be present in STW effluent compared to influent. However, with a mean reduction of 7.2% over the 25 days, it is not insignificant.

Concentrations of estrogens were below the limit of detection in all samples. However, as is shown in figure 5.7 estrogenic compounds were detected in samples 10m downstream of effluent. EE2 was no detected in any downstream samples, and as such, the EEQ values include only E1 and E2. However, with time, E2 was degraded to a level at which it was no longer detectable. This serves to highlight the importance of sample storage as a source of uncertainty. Since a laboratory which had stored samples for >10 days using one of the

common preservation techniques, before processing, might incorrectly report no estrogenic activity in those samples. In this regard, pH adjustment to pH4 appears to offer the optimum conditions for sample storage, since it was possible to detect E2 up to 15 days after sample collection, with the least (6.3%) loss of all preservation techniques.

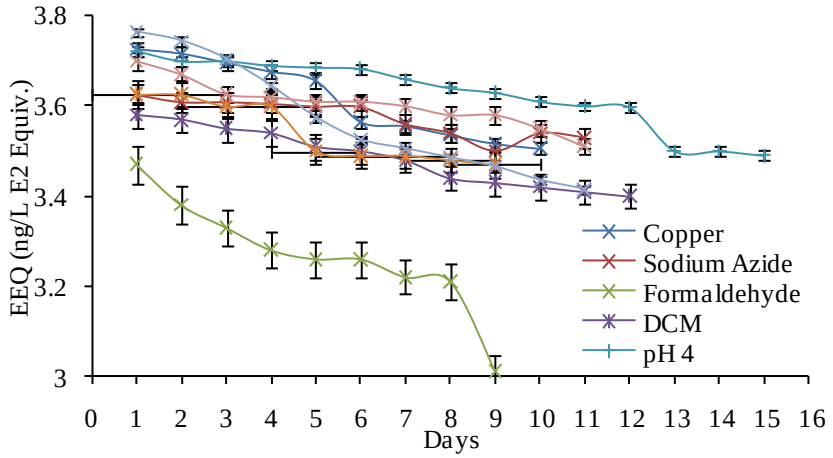

Figure 5.7 EEQ as calculated from analyses of sub-samples of river water collected from the River Ouse, 10m downstream of the Scaynes Hill STW effluent, up to 16 days after sampling (error bars are standard deviation, $n=3$)

5.4. Conclusions

The results of this research suggest that further research is needed to better understand the degradation of samples in storage, and the efficacy of preservation techniques. It suggests that no commonly employed preservation technique prevents degradation of analyte compounds completely. These findings concur with the scarce number of pre-existing publications; Noppe *et al.* [7] found that E1, E2 and EE2 rapidly degraded in storage (up to 90% within 7 days) despite employing a published procedure using acidification. Baronti *et al*. [8] contrastingly, suggest that the use of acidification or formaldehyde may accelerate

the oxidation of E2 to E1, with their analyses detecting concentrations of E1 significantly higher than that they spiked samples with and background levels. Benjits *et al.* [9] however, found that samples extracted by SPE within 2 days of sample collection could be stored for up to 7 days before any loss of analyte was detected.

It is apparent that the mechanisms for the degradation of estrogens and pharmaceutical compounds in samples is unclear – there are many potential pathways – biotic, chemical or adsorptive, and further research is required to determine conclusively which of these mechanisms is the primary cause of sample degradation. However, since the degradation does not appear to be first order, it is possible that atypical processes are occurring. Due to the estrogens' high affinity for organic matter [2] and relatively low water solubility [3], together with EPISUITE behaviour predictions, suggest it is possible that estrogens may have accumulated on organic matter or sediment, or even adsorbed to debris on the barrel surface, which due to its density may have sunk to the bottom of the barrel or been removed during filtration. Quantitative extraction and analysis of estrogens in filtration retentate will afford a greater understanding of losses via this mechanism, although this may not be possible until more robust and sensitive extraction analytical techniques evolve.

This lack of understanding has significant consequences for the analyses of estrogenic and pharmaceutical compounds, as well as in the analysis of wider emerging pollutants. These consequences are exacerbated where researchers do not give full details of the preservation techniques employed in their research, nor the time that samples are stored prior to processing. Where this information is lacking, there will inevitably be uncertainties when trying to compare data between researchers where analyte compounds degrade whilst

samples are in storage. Furthermore, this makes prioritization of risk more challenging where there is inconsistency in data and the potential for under-reporting of concentrations.

5.5 References

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Chapter 6. The Occurrence of Steroidal Estrogens and Pharmaceutical Compounds in Water, Sediment and Wastewater and the implications of Modern STW Treatment Techniques

6.1. Introduction

As discussed in chapter 2, endocrine disrupting chemicals (EDCs) are classed as 'emerging pollutants', which are those chemicals that have recently been shown to occur in water resources and are identified as being a potential environmental or health risk, and yet adequate data do not exist to determine their risk. The majority of EDCs and are man-made, organic chemicals, introduced to the environment by anthropogenic inputs, e.g. 17α-ethinylestradiol (EE2) is the main component of the oral contraceptive pill. In addition, EDCs can be naturally-occurring in the environment, e.g. the female hormones estrone (E1) and 17β-estradiol (E2) are both excreted by females and are hence ubiquitous in the aquatic environment. Such compounds may not be removed by sewage treatment works (STW) and may even be reactivated through deconjugation during these processes [8][9][10]

EDCs are of global concern and are broadly defined as chemicals that may interfere with the function of the endocrine systems in wildlife and humans. Endocrine disruption has been shown to reduce fish fertility, to be linked to human cancers and may also affect human fertility [9][10] A wide range of compounds has been found to possess, or are suspected of possessing, endocrine disrupting properties. Many EDCs are classified as priority substances in the EU Water Framework Directive (2000/60/EC). Due to their potency, the steroidal estrogens, such as the natural E1 and E2, and the synthetic EE2 are of greatest concern and have been found to exhibit feminising affects on fish at very low

concentrations (<1 ng/L) [5][6]. Steroidal estrogens have been found to persist through many sewage treatment processes [9][10][11][12] , and it is widely recognised that effluent discharges STW are the main source of EDC inputs to the aquatic environment, such as rivers and streams [13][14][15][16]. Although the concentrations of steroidal EDCs are generally low in aquatic systems, concentrations of up to 19.4 ng/L have been detected in surface waters, and levels as high as 5400 ng/L have been found in some STW effluents [11].

Due to increasing concerns for the implications for fish populations and human health and the identification of sewage effluents as the major point source, a research focus in recent years has been on the identification of suitable technologies which satisfactorily remove emerging pollutants, from wastewater. Granular activated carbon (GAC), albeit expensive, has been shown to be applicable in the removal of other organic pollutants, and has been proposed as a potential technique to aid in the effective removal of emerging pollutants, particularly EDCs in wastewater treatment [16]. In this chapter, a study to assess the removal efficacy for steroidal estrogens by a recently-commissioned, post-tertiary GAC plant, through the analysis of pre-and post- GAC installation effluent samples over two 24-h periods is described. The concentrations of the selected steroidal compounds have also been analysed at various locations upstream and downstream of the effluent in the receiving river, to evaluate the impact of GAC on the quality on the receiving water and to better understand the long-term temporal and spatial variability of these compounds.

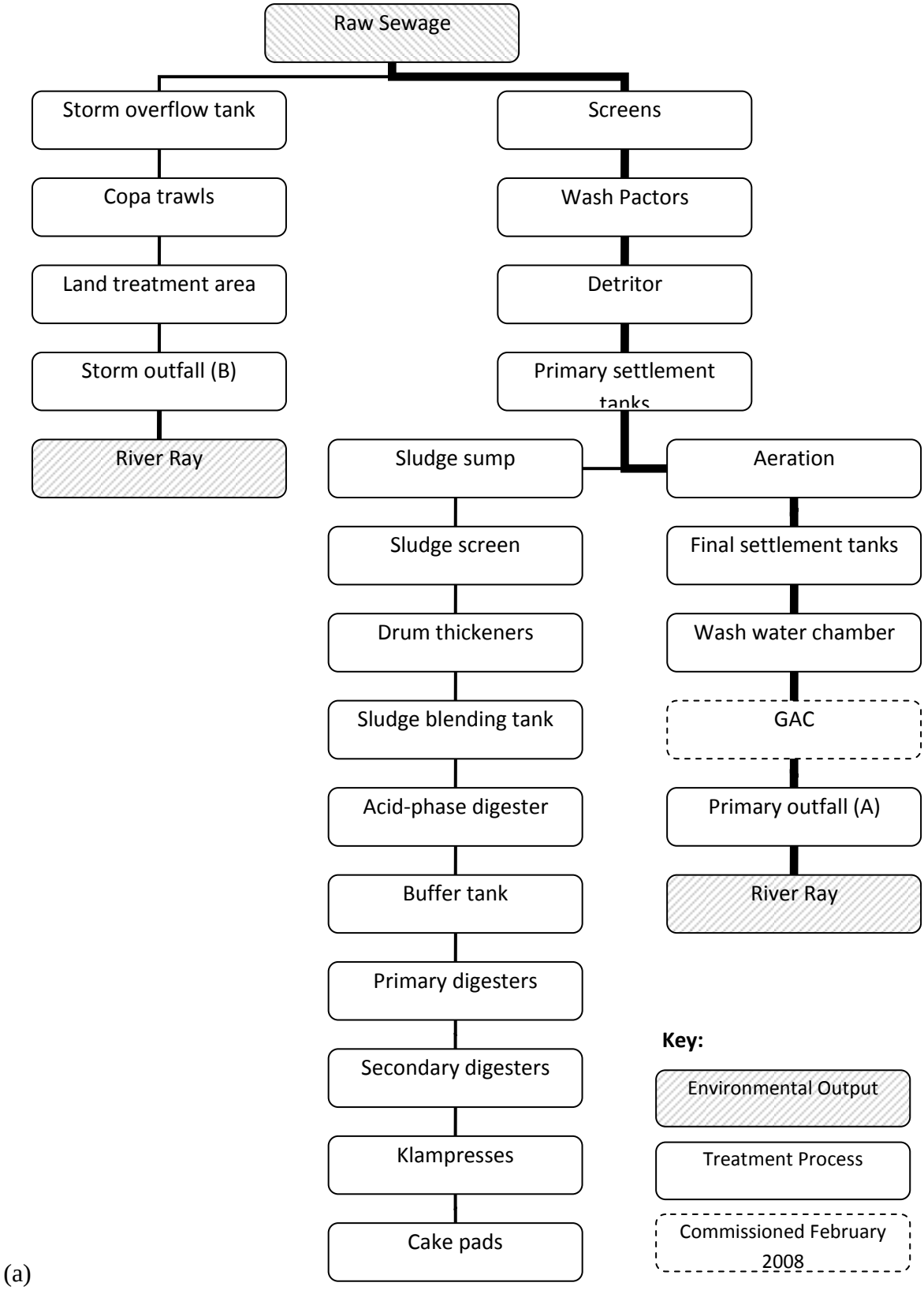
6.2. Experimental

6.2.1. Chemicals and standard solutions

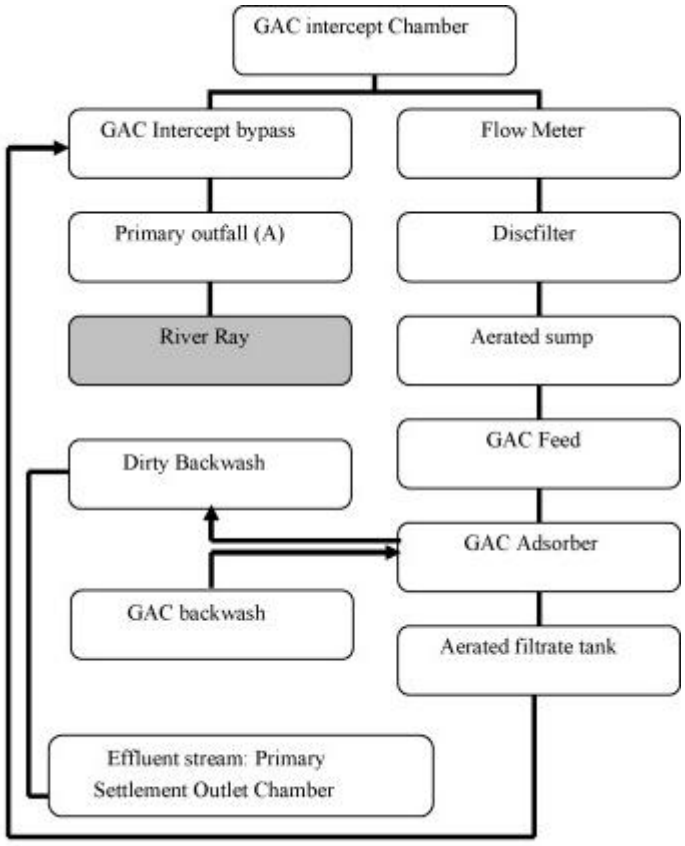
All of the solvents used, including methanol and acetonitrile, were purchased from Rathburns, UK, and were of distilled-in-glass grade. Authentic chemical standards including E1, E2 and EE2, were purchased from Sigma, UK. Deuterated internal standards, E2-d_2, E2-d_4, EE2-d_4 and E1-d_4 were also purchased from Sigma, UK. All standards were prepared in methanol and were stored at -18^{o}C. Ultrapure water was supplied by a Maxima Unit from USF Elga, UK.

6.2.2. Sampling and sample treatment

Effluent samples were collected from the Rodbourne STW in Swindon, Wiltshire, UK, which serves a population of approximately 155000 and its effluent is received by the river Ray, a tributary of the river Thames. The treatment processes and technologies of the studied STW are shown in Fig. 6.1.


Raw Sewage
Storm overflow tank
Copa trawls
Land treatment area
Storm outfall (B)
River Ray
Screens
Wash Pactors
Detritor
Primary settlement tanks
Sludge sump
Sludge screen
Drum thickeners
Sludge blending tank
Acid-phase digester
Buffer tank
Primary digesters
Secondary digesters
Klampresses
Cake pads
Aeration
Final settlement tanks
Wash water chamber
GAC
Primary outfall (A)
River Ray
Key:
Environmental Output
Treatment Process
Commissioned February 2008

(a)



(b)

Figure 6.1 A flow diagram of the (a) STW and (b) GAC Plant under investigation in this study

	Pre-GAC (21/22 February 2008)		Post-GAC (22-23 September 2008)	
Barrel No.	Start time	End time	Start time	End time
barrel 1	10:00	10:28	13:35	14:00
barrel 2	14:15	15:15	17:35	18:05
barrel 3	18:00	18:30	21:35	22:05
barrel 4	22:00	22:30	Pump failure	Pump failure
barrel 5	02:00	02:30	05:35	06:05
barrel 6	06:00	06.:30	09:35	10:15

Table 6.1 Effluent sampling times over the two 24-h periods

Effluent samples were collected into pre-cleaned 50 L stainless steel barrels, by an automated pumping system set to collect samples at 4-h intervals. The sampling schedule is shown in Table 6.1. Triplicate spot samples were also collected 4-times per year (spring, summer, autumn and winter) over 3 years from 2006-2008 from four sites on the River Ray and one site on the River Ock (in Oxfordshire) acting as a reference. Information about these sites is shown in table 6.2. Due to site restrictions from the water company concerned on health and safety grounds, influent samples were not allowed to be taken. It is therefore the limitation of this work that a direct comparison between influent and effluent samples could not be made. Spot samples (2.5 L) taken by a semi-automatic sampler that opened at a depth of ~0.5 m were collected in pre-cleaned amber bottles to which 10 mL of 2 M sodium azide (Fisher, UK) was added as a broad-spectrum biocide. The bottles were

returned to the laboratory where they were stored at 4 °C. In addition, POCIS samplers were deployed at the same sites 1 month before water sampling for around 28 d, and on retrieval, water samples were taken for chemical analyses and additionally vitro assay analyses by CEFAS.

River	Site	Grid Ref.	Description	Distance from Rodbourne STW discharge (km)
Ray	#1	SU138833	Westleaze	3.5 Upstream
Ray	#2	SU127859	Rodbourne STW effluent	~0.1 Downstream
Ray	#3	SU121872	Elborough bridge	2.0 Downstream
Ray	#4	SU119925	Seven bridges bridge	10 Downstream
Ock	#5	SU382943	Charney bassett	Control site

Table 6.2. Site numbers, location and description used in EDCAT sampling.

The river Cole had initially been the preferred control river due to its close proximity to the Ray, but due to it being highly-engineered and the resulting inability to support fish populations, it was decided to be unsuitable. Sodium azide (5mL/L, 2M) was added to all water samples as a general biocide to eliminate bacteria and thus minimise biodegradation during sample storage and processing, although sample processing on return to the laboratory was performed as soon as practicable to further minimise degradation.

Water samples were refrigerated at 4ºC until filtration and extraction. Samples were processed following previously published methods, details of which can be found in chapter 3. Briefly, samples were filtered under vacuum using pre-ashed glass fibre filters (Whatman, GF/F; 0.7 μm pore size). Filtered samples (2 L) in triplicate were spiked with internal standards (100 ng). Sediment samples were frozen and freeze-dried, and subsequently underwent extraction by MAE, the extracts of which were subsequently reduced in volume by rotary evaporation and under a gentle N_2 stream, diluted into 500mL

of ultrapure water and treated as water samples. Further details of these procedures are also available in chapter 3.

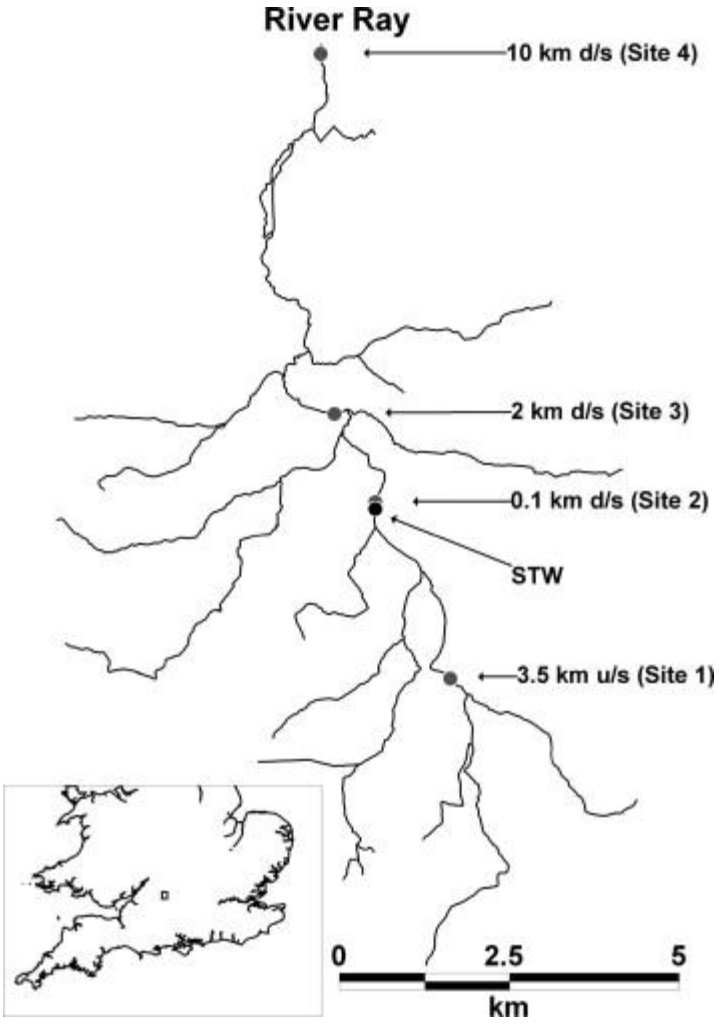


Figure 6.2 A site map to show sampling locations on the River Ray indicating distances from the Rodbourne STW effluent (u/s: upstream, d/s: downstream). Site 5 as a reference in the river Ock is not shown.

6.2.3. Solid phase extraction (SPE)

The target compounds were extracted from the filtered water samples using SPE. The Oasis SPE cartridges (0.2 g HLB, Waters) were conditioned with 5 mL of ethyl acetate to remove

residual bonding agents, followed by 10 mL of methanol which was drawn through the cartridges under a low vacuum to ensure that the sorbents were soaked in methanol for 5 min. Ultrapure water (3 x 5 mL) was then passed through the cartridges at 1-2 mL/min. Water samples were then extracted at approximately 10 mL/min as this has been shown to be the most efficient. The SPE cartridges were subsequently dried under vacuum and the extracts eluted from the sorbents into 20 mL vials with 10 mL of methanol at a flow rate of 1 mL/min. The solvent was then blown down to 100 μL under a gentle N_2 flow and ready for analysis.

6.2.4. Sample analyses by LC-MS/MS

The LC separation was carried out with a Waters 2695 HPLC separations module, manufactured by Waters (Milford, MA, USA), which was fitted with a Waters Symmetry C_{18} column (4.6×75 mm, particle size 3.5 μm). The LC-MS/MS methods used here are discussed briefly and are described fully in chapter 3. The mobile phase comprised eluent A (ultrapure water), eluent B (acetonitrile) and eluent C (methanol). Flow rate was 0.2 mL/min and the elution started with 10% of eluent B, followed by a 25 min gradient to 80% of eluent B and a 3 min gradient to 100% of eluent B, and then changed to 100% of eluent C within 8 min, held for 10 min and then returned back to the initial conditions within 4 min. The system re-equilibration time was 10 min and the sample injection volume was 10 μL. The MS-MS analyses were completed with a Micromass Quattro triple-quadrupole mass spectrometer equipped with a Z-spray electrospray interface. The analyses for steroidal estrogens were done in the negative ion mode. The electrospray source block and desolvation temperature were 100 and 300ºC, respectively; capillary and cone voltages were 3.0 kV and 30V, respectively; argon collision gas 3.6×10^{-3} mbar; cone nitrogen gas flow and desolvation gas: 25 and 550 L/h. Following the selection of the precursor ions,

product ions were obtained at optimum collision energies and were selected according to the fragmentation that produced a useful abundance of fragment ions. The optimal collision energy, cone voltage and transitions chosen for the multiple reaction monitoring (MRM) experiment were optimized and utilized a dwell time of 100 ms. The mass spectrometer was operated in MRM mode with unit mass resolution on both mass analyzer.

6.3. Results and discussion

6.3.1. Concentration of steroidal estrogens in STW effluent

As the source of inputs for EDCs and PPCPs in the river systems, the STW effluent was a focus of this research in terms of sampling and analysis. Initial observations during the filtration of post-GAC effluent water showed a significant reduction in the amount of suspended particulate matter in samples. This was subsequently quantified and a ~10-fold reduction was observed in all effluent samples (mean pre-GAC of 0.05 g L^{-1} compared with a mean post-GAC of 0.006 g L^{-1}).

From the measurement of EDC concentration in the effluent samples, it is clear that a significant reduction in the concentrations of E1, E2 and EE2 in effluent samples was observed. Prior to GAC installation, the measured concentration range for each compound was 0.6–3.1 ng L^{-1}, <1.2–5.4 ng L^{-1} and <0.4–1.7 ng L^{-1} for E1, E2 and EE2, respectively. After GAC installation, the measured concentration range for E1 fell to <0.6–2.0 ng L^{-1}. Concentrations of E2 and EE2 were below the method limit of detection (LOD) of 1.2 ng L^{-1} and 0.4 ng L^{-1}, respectively, in all samples.

Table 6.3. Concentration ranges of steroidal estrogens and pharmaceutical compounds in STW effluent pre- and post-GAC installation

Compound	Pre-GAC effluent (ng/L)	Post-GAC effluent (ng/L)
E1	0.6 – 3.1	<0.6 – 2.0
E2	<1.2 – 5.4	<1.2
EE2	<0.4 - 1.7	<0.4

As is shown in Table 6.3, a significant reduction in the concentrations of E1, E2 and EE2 in effluent samples is observed, this is further highlighted in Figs. 6.3. Accordingly, a significant reduction in the calculated estrogenicity, a key parameter used in assessing the risk posed by estrogenic pollutants, represented by E2 equivalence (EEQ), of effluent is observed, as is shown in Fig. 6.3(b). EEQ, based on the potency of each steroid in reference to E2, is calculated as follows: $EEQ = C_{E2} + \frac{1}{3} C_{E1} + 10\, C_{EE2}$ (6-1)

where C_{E2}, C_{E1}, and C_{EE2} represent the concentrations of E2, E1 and EE2, respectively.

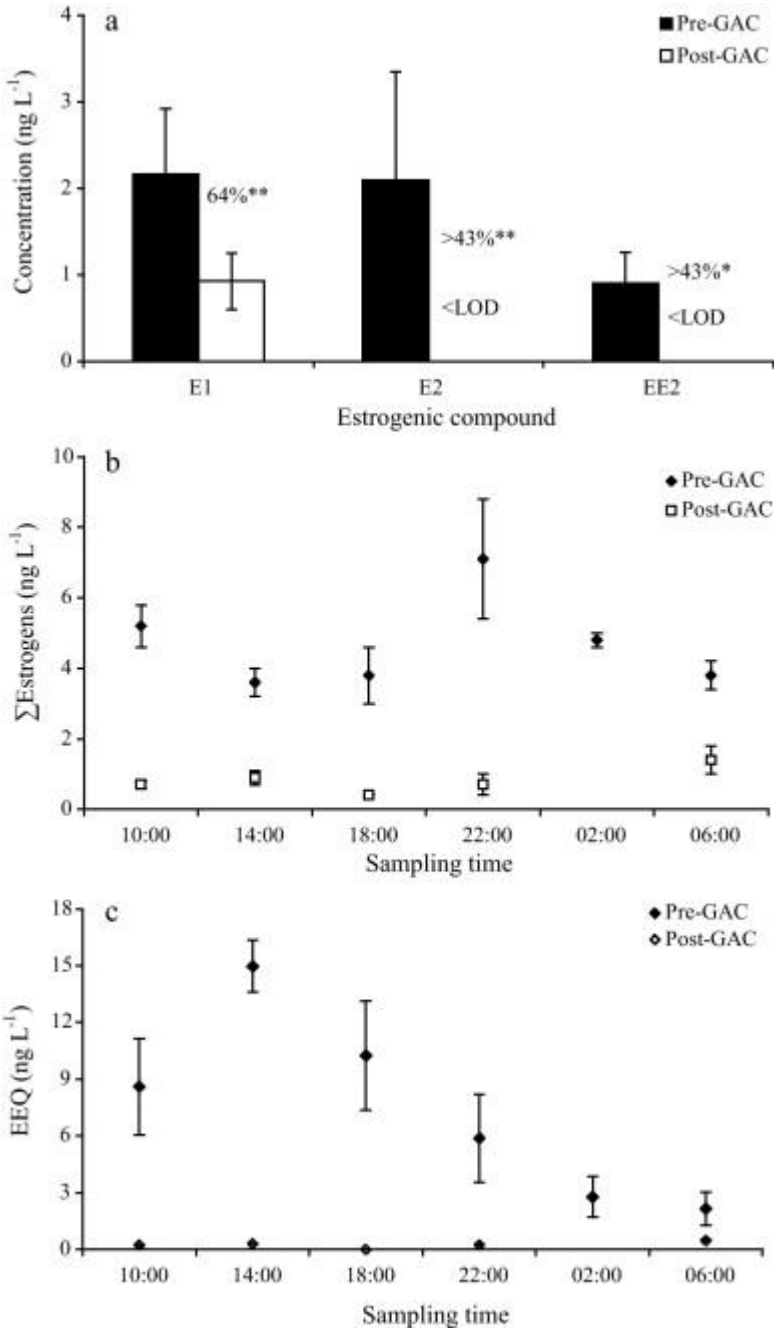


Fig. 6.3 Comparison of the pre- and post-GAC effluent samples for: (a) mean concentrations of steroidal estrogens, (b) temporal variability of measured total steroidal estrogens, and (c) temporal variation of estrogenic activity represented by EEQ. Error bar = one standard deviation. Symbols ** and * represent *P* values of <0.01 and <0.05, respectively.

Prior to GAC installation, the measured concentration range for each compound is 0.6-3.1

ng/L (mean 2.2 ng/L), <1.2-5.4 ng/L (mean 2.1 ng/L) and <0.4-1.7 ng/L (mean 0.7 ng/L)

for E1, E2 and EE2 respectively. The EDC concentrations changed with time during the 24-h periods, as shown in Figure 6.3(b). The total EDC concentrations (E1 + E2 + EE2) ranged from 3.6 ng L^{-1} at 14:00 to 7.1 ng L^{-1} at 22:00, suggesting increased discharge of estrogenic compounds at the end of a working day before GAC was installed. Following GAC installation, the variability of EDC concentrations was less obvious (relative standard deviation (RSD) = 17% for E1, and 0% for both E2 and EE2), primarily because most of the EDCs had been removed by the improved GAC technology.

After GAC installation, the measured concentration range for E1 fell to <0.6-2.0 ng/L (mean 0.8 ng/L). Concentrations of E2 and EE2 were below the method limit of detection (LOD) of 1.2 ng/L and 0.4 ng/L respectively, in all samples. This reflects a mean percentage reduction in concentrations by 63% of E1, and a reduction of at least 25% of E2 and EE2, suggesting a major improvement in the removal of EDCs from sewage effluent with the GAC plant.

As shown in Figure 6.3(b) EEQ concentrations varied from 2.2 to 14.6 ng L^{-1} before GAC, with the maximum value being detected at 14:00 which is primarily due to the highest EE2 concentration (1.3 ng L^{-1}) then. It is expected that there would be a time lag between the use of EE2 (as the main component of oral contraceptive pills) and the time it was found in sewage effluent, due to transport and residence in sewerage and sewage systems. Following GAC treatment, the EEQ values were reduced to ≤0.5 ng L^{-1}. Such major reductions are due primarily to the complete removal of EE2, which contributes most to the EEQ values.

6.3.2 Concentrations of steroidal estrogens downstream of STW effluent

Fig. 6.5 shows that, as expected, since sewage effluent is recognized as the predominant source of environmental estrogens in natural waters, the reduction in the measured concentrations of the steroidal estrogens observed in sewage effluent is reciprocated in the receiving river. Fig 6.5 also shows predicted downstream concentrations for the steroidal estrogens and the pharmaceutical compounds, respectively, using the simple dilution model, as has been discussed in previous publications [12][31]:

$$C_{DW} = \frac{C_{UW} \times V_{UW} + C_{EF} \times V_{EF}}{V_{DW}} \qquad (6\text{-}2)$$

where C_{EF}, C_{UW}, C_{DW} are the pollutant concentrations in the STW effluent, upstream and downstream. V_{EF}, V_{UW} and V_{DW} represent the flow rates in the effluent, upstream and downstream.

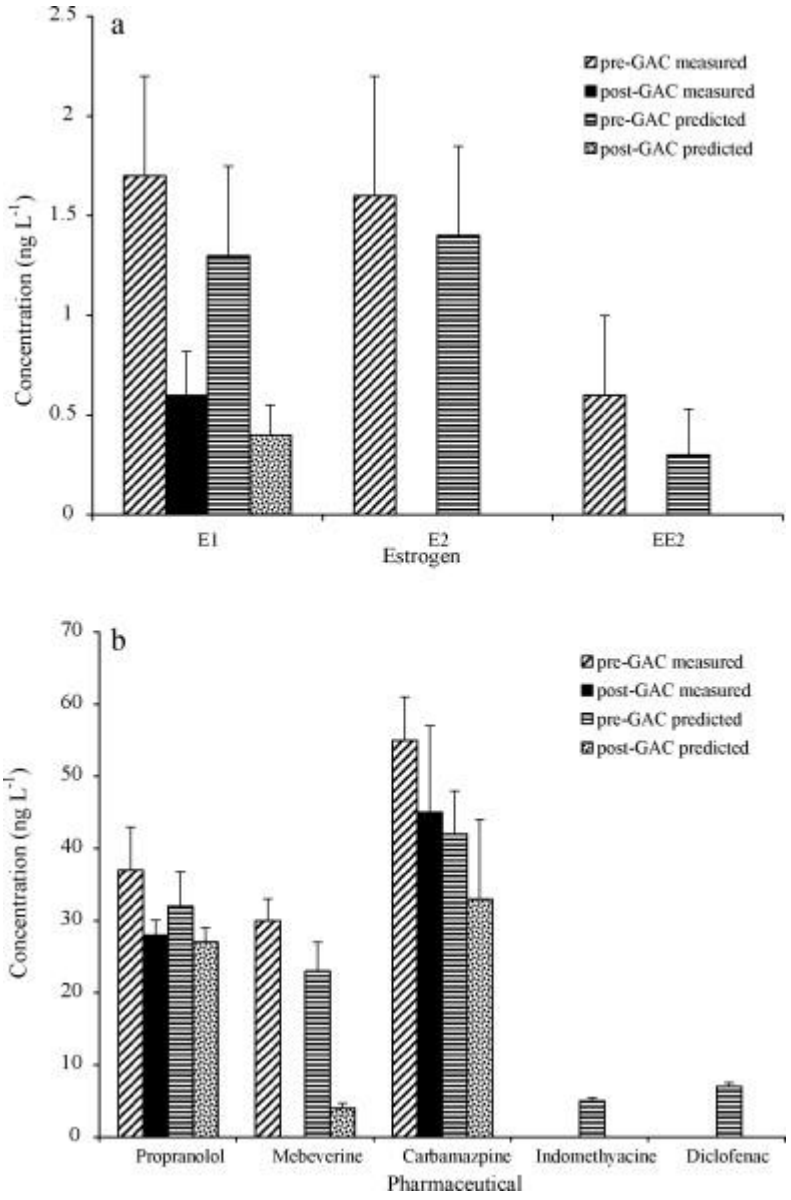


Fig. 6.4. Measured and predicted concentrations of: (a) steroidal estrogens and (b) pharmaceutical compounds 1.7-km downstream of the STW effluent, before and after GAC operation. Error bar = one standard deviation.

Good agreement between measured and predicted concentrations was observed, particularly for the steroidal estrogens. For pharmaceuticals, the model prediction was excellent for propranolol, and still good for carbamazepine. Overall, however, the model tended to underestimate by between 5 and 28% for those compounds that were also detected by

chemical measurement. On the other hand, the model predicted the presence of indomethacine and diclofenac at concentrations significantly above the method LOD in the receiving river water in pre-GAC samples, which differed from the observed non-detectable concentrations through measurement. The model however subsequently predicted these compounds would not be present in post-GAC samples, in agreement with the measured results. The model also predicted low concentrations (ng L^{-1}) of mebeverine in the receiving river waters after GAC commission, which is an over-estimate in comparison to the measured results. Overall, the dilution model has provided a reasonable prediction of the measured EDC and pharmaceutical concentrations, demonstrating the importance of STW effluents as a point source of such compounds into the aquatic environment.

6.3.3. Concentration of PPCPs in STW effluent

It has been widely reported that the removal of pharmaceuticals during conventional sewage treatment is incomplete, sometimes as low as close to zero [13], [27] and [28]. Of the 11 pharmaceutical compounds analysed, only five (propranolol, mebeverine, carbamazepine, indomethacine and diclofenac) were detected in pre-GAC effluent samples, and only three (propranolol, mebeverine, carbamazepine) were detected in post-GAC effluent samples. The concentrations of pharmaceuticals varied from 7.6 ng L^{-1} for indomethacine, to 79.7 ng L^{-1} carbamazepine, in pre-GAC effluents. In a study of three STWs in Ohio, USA, Spongberg and Witter [29] detected 34–111 ng L^{-1} of carbamazepine in sewage effluents, which is comparable to the pre-GAC concentrations found in this study. In addition, the concentrations of carbamazepine in the effluent samples are significantly lower than those found elsewhere, with Kasprzyk-Hordern et al. [28] reporting concentrations up to 3117 ng L^{-1} in the effluent of a STW in Cilfynydd, Wales and Zhou et

al. [12] detecting 233–1061 ng L^{-1} in the effluent of a STW in West Sussex, UK. For other pharmaceutical compounds, pre-GAC concentrations are broadly comparable to other studies, albeit at the lower-end of reported concentrations, such as 6–35 ng L^{-1} for indomethacine [12] [30].

The pharmaceutical concentrations were substantially reduced in post-GAC effluents, varying from <LOD for indomethacine and diclofenac to 47.6–58.4 ng L^{-1} for carbamazepine. On average, the additional removal of pharmaceuticals by GAC was between 17% for propranolol to >98% for indomethacine (Fig. 6.6a). These reductions are broadly comparable to results derived from laboratory testing using activated carbon. The concentrations of pharmaceuticals also varied diurnally, as shown during the 24-h sampling (Fig. 6.6b). Mebeverine varied from 33.39 to 41.5 ng L^{-1} in pre-GAC effluents and 5.0–7.4 ng L^{-1} in post-GAC effluents. The total concentrations of all measured pharmaceuticals did not vary as significantly as EDCs during either 24-h period, but similarly a peak of the total measured pharmaceutical compounds of 201.8 ng L^{-1} at 22:00 in pre-GAC samples was observed, with even less variability in post-GAC effluents (RSD = 3.9%).

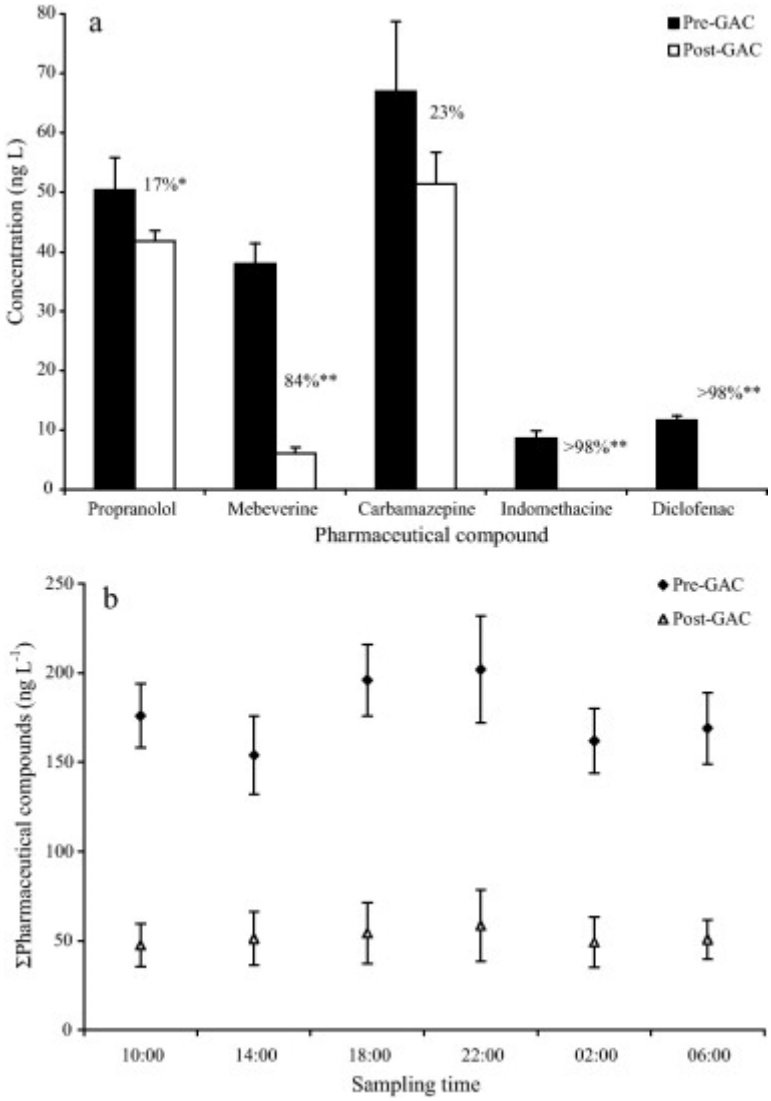


Fig. 6.5. Comparison of the pre- and post-GAC effluent samples for: (a) mean concentrations of pharmaceutical compounds and (b) temporal variability of measured total pharmaceutical concentrations. Error bar = one standard deviation. Symbols ** and * represent *P* values of <0.01 and <0.05, respectively.

6.3.4 Variability of steroidal estrogens throughout the catchment

To fully appreciate the environmental occurrence and persistence of emerging contaminants in river water, as well as the added benefit of GAC installation, a systematic sampling and analysis of STW effluent and river water were undertaken. Results (Fig. 6.6) show the concentrations of E1, E2 and EE2 at four sites in the receiving river, including one upstream and three downstream sites, as well as at a control site over a three-year period. The results demonstrate a clear reduction in the average concentrations from 3.2 ng L^{-1} (pre-GAC) to <0.6 ng L^{-1} (post-GAC, 81% reduction) for E1, 3.8 ng L^{-1} to <1.2 ng L^{-1} (at least 68% reduction) for E2, and 0.8 to <0.4 ng L^{-1} (at least 50% reduction) for EE2 downstream of the effluent, after the installation of the GAC plant. However, there appears to be a lag between the reduction in effluent concentrations and the apparent non-presence of E1 and E2 in the receiving river water, with a small amount of these compounds present in downstream samples some months after installation of the GAC. This is likely due to pre-existing amounts of these compounds from pre-GAC effluent taking some time to degrade. There appears to be no such lag for EE2, which was not detectable in any sample from the catchment after installation of the GAC.

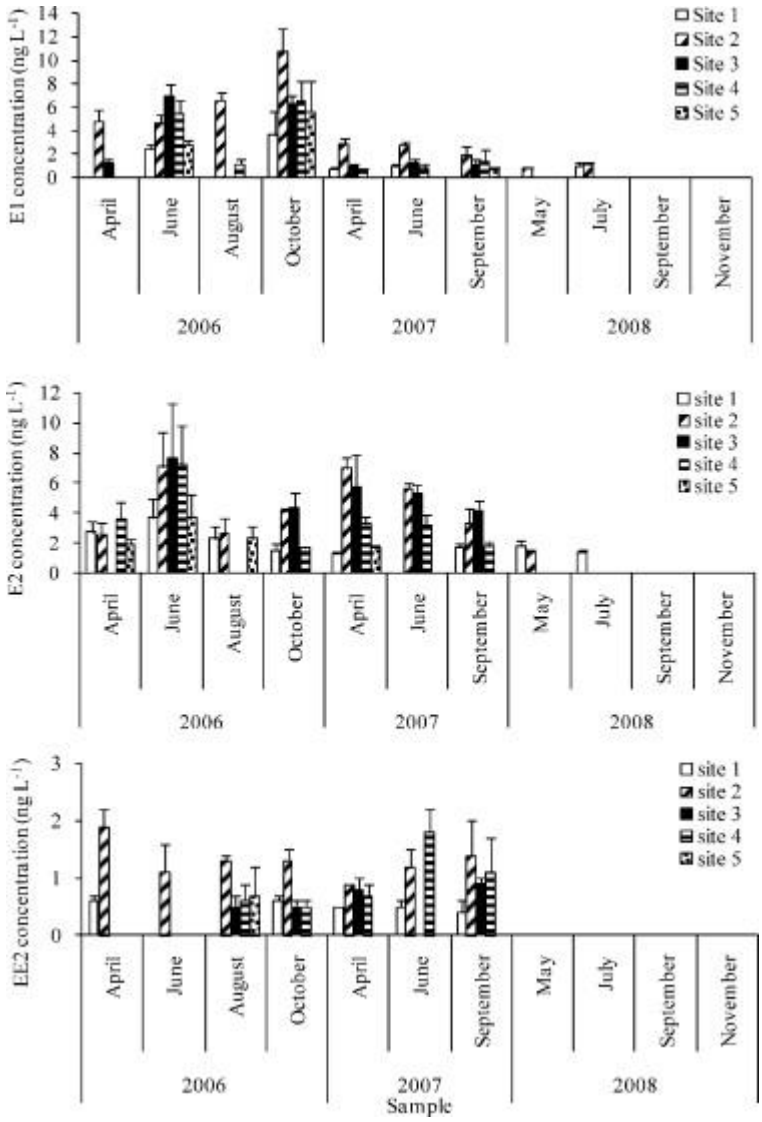


Fig. 6.6. Concentrations of E1, E2 and EE2 across the catchment over the three year period. Site 1 = 3.5 km upstream, site 2 = 10 m downstream, site 3 = 1.7 km downstream, site 4 = 8.3 km downstream, site 5 = control site. Error bar = one standard deviation.

6.4 Comparisons with in-vitro analytical and computer modeling techniques

As part of the Defra-sponsored EDCAT project, the results of the chemical analysis of spot water sample as discussed previously were compared with those obtained by Yeast Estrogen-Screening (YES) assays as developed by Routledge & Sumpter (1996) [32], of spot samples and passive, integrated samples (*T=28 days)* collected using Polar Organic

Contaminants Integrated Sampling (POCIS) devices as developed by Alvarez *et. Al.* (2004) [334], performed by colleagues at Cefas, as well as with predictions made using the Exposure Analysis Modeling System (EXAMS) model, developed by the US EPA [34][35] but adapted and used by colleagues at the CEH [36].

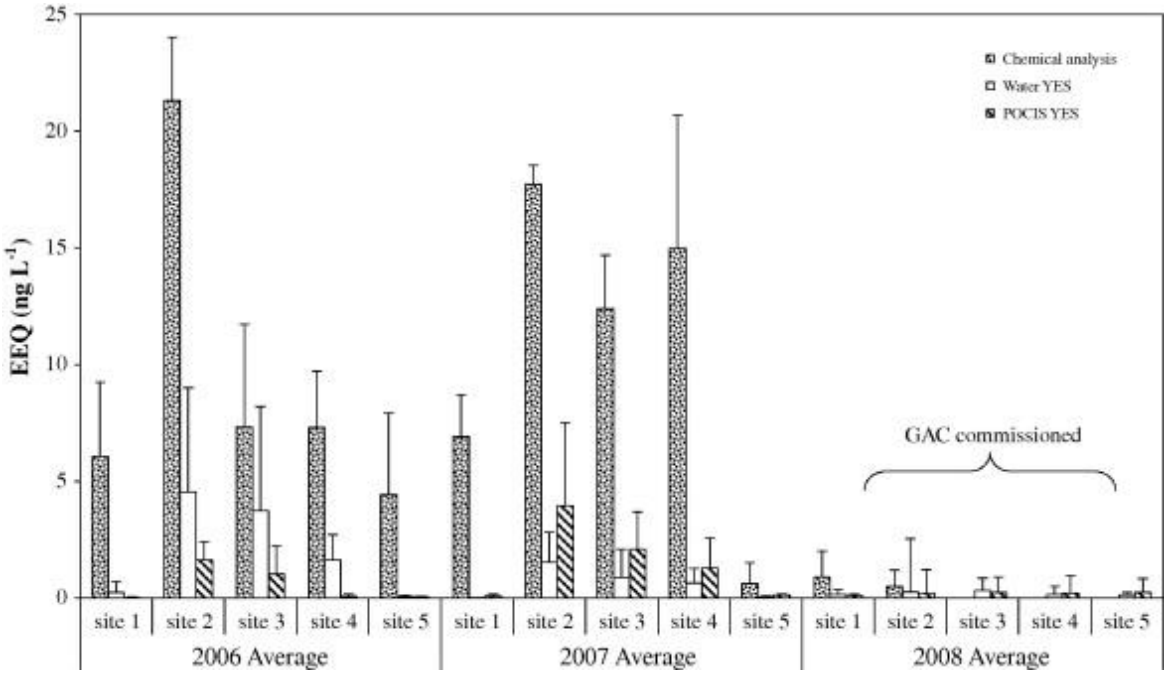


Figure 6.7 Comparison of average EEQ concentrations determined from chemical analysis of water extracts followed by calculation using Eq. (2), YES measurement of water extracts, and YES measurement of POCIS extracts. *N* = 165. Error bars represent standard deviations.

Annual average data for estrogenic activity calculated from the chemical analysis of water samples are shown in Fig. 6.7, together with estrogenic activity determined by YES analysis of spot water and POCIS extracts. It is apparent that although there is generally good agreement of the three techniques with regard to them all showing a clear reduction in detected estrogenic activity after the GAC was commissioned, EEQ values from chemical analysis of spot samples tend to be greater than those determined by YES of either spot or

passive water samples. Such differences between YES and chemical analysis may be ascribed to the following factors:

- The YES assay is recognized to be only semi-quantitative, with Huggett et al. (2003) [37] suggesting it may underestimate in vivo estrogenic activity by 10-fold.
- The limit of detection by chemical analysis (between 0.4 and 1.2 ng L^{-1}) is higher than that by YES assay (0.02 ng L^{-1}). Thus, during the analysis of very low levels of EDCs (i.e. <0.1 ng L^{-1}), chemical analysis will be less sensitive to small differences between samples than YES assay. As a result, the measurement errors from chemical analysis will be potentially greater than from YES.
- For the analysis of EE2, Young et al. (2004) recommend a confidence interval of 0.01 ng L^{-1} which is not achievable with current analytical techniques. By definition, the EEQ formula (Eq. (2)) will inflate errors in the analysis of EE2, where errors of 1 ng L^{-1} or even 0.1 ng L^{-1} may at first seem insignificant, these can cause large overestimates of EEQ (i.e. of 10 or 1 ng L^{-1} respectively).
- Young et al. (2004) recognised intrinsic uncertainty in the EEQ formula due to the precautionary principle, as the derived PNEC values (upon which Eq. (2) is based) are only a conservative estimate based on toxicological data available at the time. For example, for E1, they reported a scarcity of toxicological results but suggested a PNEC of 3–5 ng L^{-1}. The contributions of each compound to overall EEQ are also based on in vivo rather than in vitro data
- There may be contributions in the YES assay from antagonists, which would reduce the overall EEQ values measured.

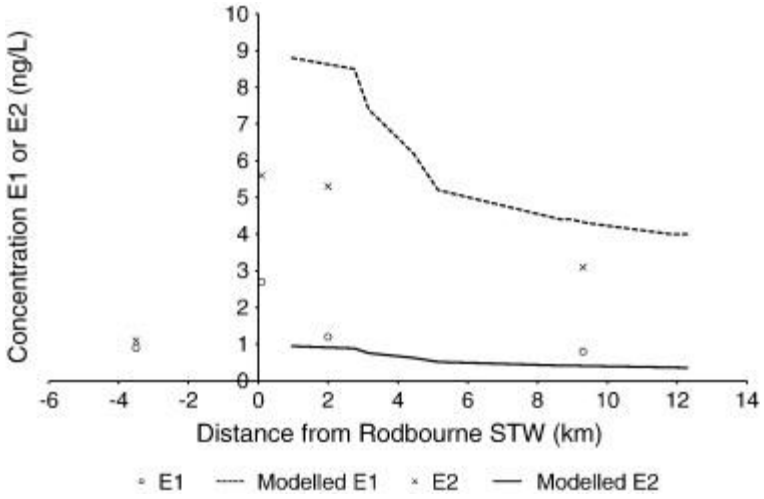


Figure 6.8 Comparison of modelled and measured E1 and E2 concentrations along the River Ray using effluent data calculated from Johnson and Williams (2004) [39]. The dots are the measured data and the lines are the modelled data for June 2007.

Figure 6.8 shows a comparison of measured concentrations of E1 and E2 with those calculated uising the EXAMs model using as its starting point a predicted estrogen discharge for the Rodbourne STW. This prediction was based on the population equivalents and the removal in sewage treatment typical of a secondary treatment works, calculated using the method of Johnson and Williams (2004) [39]. Simulations were compared with measured data for the months of April, June and September. The figure shows the data generated for June compared to measured E1 and E2 concentrations. Starting from these STW input the model predicted higher than observed values for E1 and lower than observed values for E2. Thus, the performance of Rodbourne STW was different from that of many STWs reviewed previously [39][40][41]. These previous observations have shown that E2 removal usually exceeds that of E1, and one would therefore expect to see relatively higher effluent concentrations of E1 compared to E2.

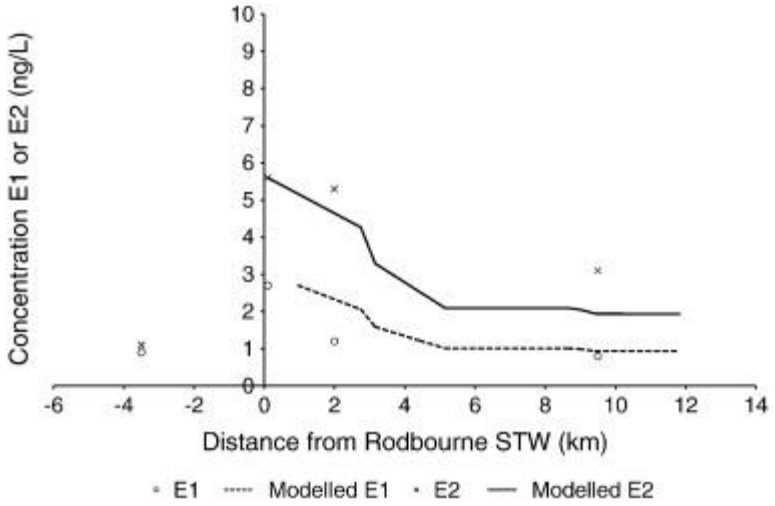


Figure 6.9 Comparison of modelled and measured E1 and E2 concentrations along the River Ray using effluent data calculated from Johnson and Williams (2004). The dots are the measured data and the lines are the modelled data for June 2007.

The EXAMS model was subsequently updated to use the effluent values as shown in figure 6.5 and compared with modelled predicted values in table 6.4. These data fit closely with those derived from the model of Johnson and Williams (2004) [39] for E2 and for EE2, although the E1 measured concentrations were still lower than the modelled predictions Values were also similar to those obtained using POCIS samplers and testing using the yeast estrogen screen. The data suggest, as discussed, that the removal from the STW was around 93% for E1 (compared to a value of 65% used in the model) based on the predicted input from the population served. The predicted estrogen input relies on the known human population served by the STW, its assumed excretion and the flow [39]. This unexpectedly high performance could be due to the biological nutrient removal (BNR) facility at the STW, which may be particularly effective at removing E1. Figure 6.9 shows predicted concentrations of E1 and E2 using the updated model, compared with concentrations determined by chemical analyses. This simulation of downstream concentrations provided a better fit with the observed values for both E1 and E2. For modelling this demonstrates the importance of calibrating the initial discharge with measured values.

Compound	Predicted concentration (ng/L)	Predicted overall estrogenicity (E2 equiv ng/L)	Range of measured values (ng/L)	Overall calculated estrogenicity (E2 equiv ng/L)
E1	19.0	15.8[a]	1.0 to 3.0	7.3–9.3[b]
E2	2.5		1.3 to 4.6	
EE2	0.7		< 0.4 to 1.3	

[a]Calculated using E2 equivalents [38]
[b] Assuming either 0 (lower end) or 0.4 (higher end) for the non-detected EE2 values.

Table 6.4 Comparison between concentrations in the Swindon STW measured in February 2008 and predictions by the model

6.5. Conclusions

A significant reduction in the concentrations of three key steroidal estrogens in sewage effluents as measured by chemical analysis, and subsequently in the receiving river waters, has been observed as a result of the installation of GAC as a post-tertiary sewage treatment process. This concurs with the findings of analysis of spot and integrated samples analysed independently using YES screens. However, both chemical analysis and analysis by YES screening suggest the overall mix and concentrations were significantly below those predicted by the EXAMS model and below those of studies of other STWs. It is plausible that the BNR process, believed to refer to biological phosphate removal in this case, which involves a prolonged biological treatment [42] may have led to this better than expected performance of the Rodbourne STW.

Whether these reductions are sufficient as to prevent endocrine disrupting effects in aquatic organisms on the longer term, however, remains to be confirmed. The reductions in the concentrations of pharmaceutical compounds are less clear, though some compounds are shown to be significantly removed, others are not, and removal appears to vary between

classes of pharmaceuticals. Moreover, GAC-based removal technology has been shown to be less efficient over time for some classes of organic pollutant, including phenolic endocrine disrupting chemicals. As of yet, the long-term efficacy of GAC for the removal of steroidal estrogens is not understood, and existing analytical procedures are unable to detect and resolve concentrations of E2 or EE2 at concentrations likely to be present in post-GAC effluents. Analytical research should focus on the reduction of detection limits so that the behaviour of steroidal estrogens at these concentrations can be better understood. Cost implications will also feature as a major driver in future investigations.

6.6. References

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Chapter 7. A The occurrence of pharmaceutical residues in water, sediment, wastewater and colloids and their removal by sewage treatment processes

7.1 Introduction

Pharmaceuticals are a class of emerging environmental pollutants that are widely used both in human and veterinary medicine. They are known to be ubiquitous in the environment, as many pharmaceuticals have been detected in wastewater treatment works (STW) effluents, surface water and groundwater worldwide [1], [2], [3] and [4]. There is limited data available on bioaccumulation of drug residues in organisms [3] and only a few specific cases have emerged to date showing the serious impact pharmaceuticals can have on wildlife. In India and Pakistan, a common vulture species suffered a severe population collapse, which was suggested to be caused by an analgesic and anti-inflammatory drug, diclofenac. The drug was regularly used for veterinary medication and residues entered the vultures as they fed on dead domestic livestock, causing renal failure and resulting in an over 95% decline in some populations since early 1990s [5]. Another study found the same drug to cause vitellogenin induction in male Japanese medaka (fish) at environmentally relevant concentrations of just 1 $\mu g\, L^{-1}$ [6]. Although evidence is limited, it is clear that pharmaceuticals have the potential to cause serious harm to wildlife and also to humans.

The main route to the environment for pharmaceuticals is through discharged effluent from STW as a result of excretion from humans and animals, as well as from domestic disposal of medicinal products [3]. The concerning issue with pharmaceuticals is not their acute toxic effects but their chronic toxicity. These compounds are commonly present at low levels throughout the lifecycle of many aquatic organisms and are particularly important for

those living in waters receiving sewage effluent (e.g. rivers). These chemicals are persistent and/or biologically active and designed to target a specific metabolic or molecular pathway. Pharmaceuticals generally are biologically active compounds that are intended not to be easily biodegradable and are often water soluble and therefore can be found in wastewaters and can easily end up in natural waters [7]. Potentially, they could have a similar function or cause side effects in non-target organisms as they do in their intended users. Thus, a good understanding of the pharmaceutical concentrations present in treated sewage effluents and their receiving river water and the rate of removal of these compounds during STW is a necessity for improving the knowledge of their fate in the environment.

The work discussed in this chapter aims to determine the concentrations of a range of pharmaceuticals in the different stages of treatment in two STWs in the UK (Table 7.1), to identify the most effective treatment technology for degrading pharmaceutical residues. Secondly the concentrations of pharmaceuticals in their receiving rivers will be compared against their concentrations in effluent, so as to assess the importance of STW as a source of pharmaceuticals in rivers. Finally, the potential toxicological impacts of pharmaceuticals on the aquatic organisms in these rivers will be evaluated.

Table 7.1. Operational characteristics of the UK STWs used in this study

STW	**County**	**Treatment technology**	**Population covered**	**Population equivalent (PE)**	**Typical flow rate ($L\ s^{-1}$)**
Scaynes	West	Primary	22 000	162 619	230

Hill	Sussex	sedimentation, then lagoon. Secondary treatment only.			
Rodbourne	Swindon	Primary sedimentation, aeration, secondary sedimentation, GAC*.	155 000	18 320	570

*Commissioned February 2008

7.2. Experimental

7.2.1. Chemicals and materials

All the solvents used including methanol and acetonitrile, purchased from Rathburn, were of distilled-in-glass grade. Formic acid was of high performance liquid chromatography (HPLC) grade. Propranolol, sulfamethoxazole, meberverine, thioridazine, carbamazepine, tamoxifen, mecoprop, indomethacine, diclofenac, meclofenamic acid and monensin were purchased from Sigma, UK. These target compounds were chosen based on their high risk characterisation ratio [8], quantity of chemicals used per year, reported occurrence worldwide, and availability of an analytical method. The pharmaceutical internal standards (diuron-d_6 and ^{13}C-phenacetin) were supplied by Cambridge Isotope Laboratories, USA. Stock solutions of all standards (1000 mg L^{-1}) were prepared from which working standards solutions (10 mg L^{-1}) were made. All standards and internal standards were

prepared in methanol and stored in a freezer at −18 °C. Ultrapure water was from a Maxima Unit supplied by USF Elga, UK. Sodium azide, silica gel (0.063–0.2 mm) and aluminium oxide (0.05–0.15 mm, neutral) were purchased from Sigma–Aldrich Company Ltd., UK. The Oasis® HLB solid-phase extraction (SPE) cartridges (6 mL/200 mg) were obtained from Waters Ltd., UK.

7.2.2. Sampling and sample treatment

Samples were taken at the three STWs used for this study. For the Scaynes Hill STW, samples were taken at the influent inlet (influent, IN), after primary treatment (humus tank, HU), after secondary treatment (lagoon, LA), and at the effluent pipe (effluent, EF) in November 2006. For the Manor Farm Road STW, samples taken included raw wastewater, and effluents of primary sedimentation and humus tanks. For the Basingstoke STW, samples were taken at the influent, after sedimentation, after activated sludge process and after sand filtration. In addition, water samples were taken from the river Ouse upstream of the Scaynes Hill STW (UW), and downstream from the STW (DW) to assess the impact of STW effluent on downstream water quality. Sodium azide (10 mL, 2 M) was added to each sample on site as a general biocide to eliminate bacteria and prevent sample degradation during storage and processing. The samples were stored in a refrigerator below 4 °C until filtration and extraction. Along with the water samples, a series of measurements were taken for the water quality including pH (7.0–8.2), conductivity (328–1042 μS), dissolved oxygen (1.0–10.9 mg L^{-1}), temperature (8.2–15.2 °C) and redox potential (−280–134 mV). The samples (1 L) were filtered under vacuum through pre-ashed glassfibre filters (Whatman, GF/F). The filtrates were spiked with 100 ng of internal standards.

The discharge flow rates of effluent from the Scaynes Hill STW for the period when sampling was taking place, were obtained from the operator (Southern Water), which varied between 73 and 76 L s^{-1}. The flow rates for the River Ouse were obtained from the Environment Agency, which were measured at Ardingly Weir, approximately 9 km upstream of the outfall, and at Gold Bridge in Newick, approximately 8 km downstream of the outfall.

7.2.3. Sample extraction and clean-up

Filtered water samples were extracted using a SPE system from Supelco, following an established procedure [9]. The Oasis HLB (Waters) cartridges were conditioned with 10 mL of methanol, followed by ultrapure water (3 mL × 10 mL) at a rate of 1–2 mL min^{-1}. Then, water samples were at a flow rate of 5–10 mL min^{-1}. Afterwards the cartridges were dried for 30 min under full vacuum, with the analytes being eluted to 20 mL glass vials from the sorbents with 10 mL of methanol. The solvent was reduced to 0.1 mL under gentle N_2 flow.

Due to the complex nature of the wastewater samples, an additional clean-up step was required to remove the interfering species and particulate matter that could block and damage the HPLC column, and produce false mass spectrometry (MS) signals in the samples. All wastewater extracts were treated with silica:alumina (1:1) columns after N_2 blow-down. Glass columns (5 mL) were filled with ashed and deactivated silica–alumina (1:1) powder with ashed quartz wool used as stoppers at the top and bottom of the column, to which samples were added and eluted with 10 mL of methanol. N_2 blow-down was used again to reduce the sample size back to 100 μL. All the sample extracts were transferred to

VectaSpin Micro centrifuge filters (0.2 μm, Whatman) and centrifuged at 7000 rpm for 10 min in order to further remove particulate matter. The extracts were further concentrated under N_2 blow-down to 100 μL ready for analysis.

7.2.4. Sample analyses

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) coupled with electrospray ionisation was used for sample analysis, following a method developed by Zhang and Zhou [9]. The LC separation was carried out with a Waters 2695 HPLC separations module, manufactured by Waters Corporation (Milford, MA, USA), which was fitted with a Waters Symmetry C_{18} column (4.6 mm × 75 mm, with particle size 3.5 μm). The mobile phase comprised eluent A (with 0.1% formic acid in ultrapure water), solvent B (acetonitrile) and eluent C (Methanol). Flow rate was 0.2 mL min^{-1} and the elution started with 10% of eluent B, followed by a 25 min gradient to 80% of eluent B and a 3 min gradient to 100% of eluent B, and then changed to 100% of eluent C within 8 min, held for 10 min and then returned back to the initial conditions within 4 min. The system re-equilibration time was 10 min and the sample injection volume was 10 μL.

The MS–MS analyses were completed with a Micromass Quattro triple-quadrupole mass spectrometer equipped with a Z-spray electrospray interface. The analyses were done in the positive ion mode. The parameters for the analysis were: electrospray source block and desolvation temperature were 100 and 300 °C, respectively; capillary and cone voltages were 3.0 kV and 30 V, respectively; argon collision gas 3.6×10^{-3} mbar; cone nitrogen gas flow and desolvation gas: 25 and 550 L h^{-1}. Following the selection of the precursor ions, product ions were obtained at a series of collision energies and were selected according to

the fragmentation that produced the highest abundance of fragment ions. The optimal collision energy, cone voltage and transitions chosen for the multiple reaction monitoring (MRM) experiment were optimised and a dwell time of 100 ms was used. The mass spectrometer was operated in MRM mode with unit mass resolution on both mass analysers.

7.2.5. Analytical quality controls

All data were subject to strict quality control procedures, including the analysis of procedural blanks and spiked samples with each set of samples analysed. None of the target compounds were detected in the procedural blanks. Spiked water samples (100 ng of each target compound) in river (Ouse), influent and effluent matrices (Scaynes Hill STW) were determined with good precision and recoveries. The limit of detection (LOD), mean recovery and relative standard deviation (RSD) of our analytical method for pharmaceuticals in water have been reported [9]. Briefly, the LOD of target compounds ranged from 1 to 288 pg L^{-1} in river water, and between 0.05 and 5 ng L^{-1} in wastewater samples. The recovery of most compounds is high (71–95%) except for tamoxifen (52%) and thioridazine (9%) in river water, and from 73 to 107% (except tamoxifen at 55% and thioridazine at 11%) in effluent, and 66–115% (except tamoxifen at 48% and thioridazine at 15%) in influent samples. The precision is also good, with RSD < 20% for all compounds. The internal standards diuron-d_6 and ^{13}C-phenacetin were used to compensate for losses involved in the sample extraction and work-up, to further improve the analytical quality.

7.3. Results and discussion

7.3.1. Pharmaceuticals in STWs

The target pharmaceutical compounds were analysed in wastewater from Scaynes Hill STW daily. Of the eleven compounds, meberverine, thioridazine, mecoprop and meclofenamic acid were all below their LOD in both wastewater and river water samples, suggesting their limited use in the UK. Tamoxifen was detected in 100% of wastewater samples, at 0.1–1.3 ng L^{-1} in influent and 0.1–0.5 ng L^{-1} in effluent samples, although it was not found in river samples. In comparison, the remaining five compounds (propranolol, sulfamethoxazole, carbamazepine, indomethacine and diclofenac) were detected in all water and wastewater samples (Fig. 7.1), suggesting their widespread and frequent use, and some level of persistence in the environment. According to the National Health Service, the quantity of the five substances dispensed in England in 2006, in primary care (excluding hospitals and retailers), varied from approximately 1 ton for sulfamethoxazole and indomethacine to 40 ton for carbamazepine. Similar to Scaynes Hill STW, the five compounds were detected in all samples in Manor Farm Road STW. Their concentrations varied from 65 to 1237 ng L^{-1} in influent, and from 27 to 345 ng L^{-1} in effluent. Slightly higher concentrations of these compounds were found in Basingstoke STW, with their concentrations ranging from 124 to 1833 ng L^{-1} in influent, although similar concentrations (14–233 ng L^{-1}) were observed in effluent samples.

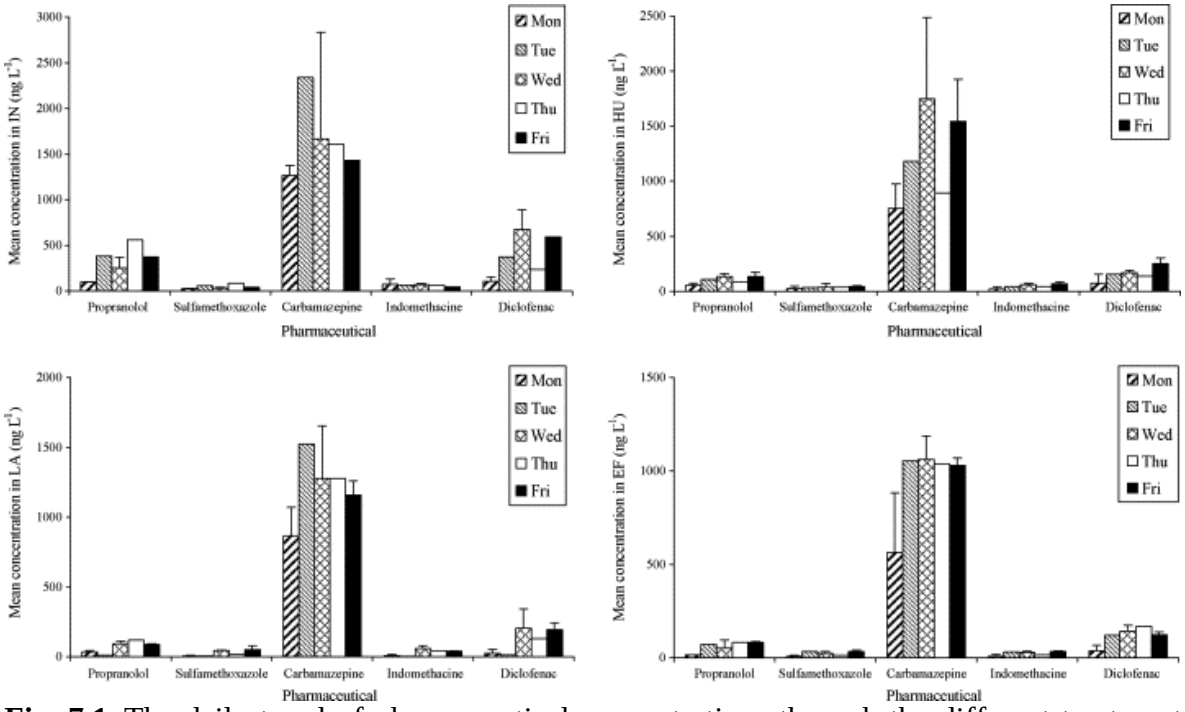

Fig. 7.1. The daily trend of pharmaceutical concentrations through the different treatment stages in Scaynes Hill STW. IN, influent; HU, humus tank; LA, lagoon; EF, effluent.

Table 7.2 shows the concentrations determined by analyis of samples collected in the areas studied in this work, compared to the concentrations determined by other researchers internationally. As can be seen, concentrations propranolol, sulfamethoxazole, carbamazepine, indomethacine and diclofenac varied greatly from 24 to 2336 ng L^{-1} in influent, such a major difference in concentrations between different compounds has also been reported by Bendz et al. [10] in Källby STW in Sweden. The concentration of propranolol (100–1090 ng L^{-1}) is similar to 542 ng L^{-1} found in Cilfynydd STW in Wales [11]. The concentrations of sulfamethoxazole (24–181 ng L^{-1}) and diclofenac (107–981 ng L^{-1}) are also comparable to those reported in STW effluents in Canada [12], Ohio, USA [13] and Soseigawa, Japan [14]. Carbamazepine levels are similar to those found in Källby STW, Sweden and Cilfynydd STW in Wales, but significantly higher than those in Ohio.

Pharmaceuticals concentrations (single value, min–max, or mean ± SD) in wastewater samples worldwide.

Pharmaceutical	STW	Influent	Effluent	Apparent	Reference
Propranolol	5 STWs,		16–284		[8]
	Cilfynydd,	542	388	28%	[11]
	Källby,	50	30	32%	[10]
	7 STWs, New		32–77		[17]
	Sheffield Park	100–1090	20–72	80–90%	[16]
	3 STWs,		16–135		This study
Sulfamethoxazole	8 STWs,		Up to 871		[12]
	5 STWs,		<50–132		[8]
	Källby,	20	70	0%	[10]
	Cilfynydd,	<3	12	0%	[11]
	Ohio, USA	14–261	79–472		[13]
	7 STWs, New		98–2200	53–82%	[17]
	Sheffield Park	24–181	12–25		[16]
	3 STWs,		8–37		This study
Carbamazepine	Källby,	1680	1180	30%	[10]
	Cilfynydd,	2593	3117	0%	[11]
	Ohio, USA	25–51	34–111		[13]

	7STWs, New		70–800	43–54%	[17]
	Sheffield Park	1237–2336	399–652		[16]
	3 STWs,		233–1061		This study
Tamoxifen	5 STWs,		42		[8]
	3 STWs,	0.2–1.5	0.2–0.7	32–45%	This study
Indomethacine	Sheffield Park		6–9		[16]
	3 STWs,	46–124	9–35	61–89%	This study
Diclofenac	49 STWs,		Up to		[1]
	5 STWs,		<20–2349		[8]
	Källby,	160	120	22%	[10]
	Soseigawa	251 ± 100	145 ± 32		[14]
	Cilfynydd,	70	123	0%	[11]
	Ohio, USA	<1–14	8–32		[13]
	Sheffield Park		49–85	70–92%	[16]
	3 STWs,	107–981	37–176		This study

Table 7.2 Influent and Effluent concentrations of selected pharmaceuticals in STW in different countries compared with those in this study.

With each stage of wastewater treatment in Scaynes Hill STW, the concentrations of the 5 compounds showed a gradual decrease. Similar to influent, the concentrations of individual

compounds in the STW effluent also varied significantly during the five day sampling period, with RSD values varying from 23% for carbamazepine to 50% for sulfamethoxazole. However, Jones et al. [15] found that the pharmaceuticals (paracetamol, salbutamol, ibuprofen and mefenamic acid) entering and leaving an activated sludge STW did not show major changes. Zhang et al. [16] also found that the same 5 compounds being studied here did not vary significantly in their concentrations in effluent from Sheffield Park STW, West Sussex, UK.

Overall, the concentrations of propranolol in the effluents from the three STWs (16–135 ng L^{-1}) are comparable to 16–388 ng L^{-1} detected in other UK STWs, 30 ng L^{-1} in Källby STW in Sweden, and 32–77 ng L^{-1} in New Mexico, USA. Sulfamethoxazole was detected in 100% of effluent samples at the concentrations of 8–37 ng L^{-1}. In comparison, sulfamethoxazole was only detected in 9% of effluent samples in other UK STWs [8], albeit at similar concentrations (<50–132 ng L^{-1}). Similar concentrations of sulfamethoxazole (70 ng L^{-1}) were observed at Källby STW in Sweden [10], but significantly higher concentrations (up to 2200 ng L^{-1}) have been determined in New Mexico, USA [17]. Carbamazepine was the dominating compound in terms of abundance in all stages of the wastewater treatment, consistent with similar findings in Sheffield Park STW [16]. Its concentrations in effluent varied between 233 and 1061 ng L^{-1}, similar to 1180 ng L^{-1} found in Källby STW in Sweden [10] and <1–6300 ng L^{-1} being reported in STW effluents worldwide [18]. Tamoxifen were detected at very low level (0.1–0.7 ng L^{-1}), which are significantly lower than those found in other UK STWs at up to 42 ng L^{-1} [8].

7.3.2. Removal of pharmaceuticals during STW processes

As the wastewater was passed through the STWs there was typically a gradual reduction in the concentrations of pharmaceutical compounds being observed, as shown in Figure 7.2 for Scaynes Hill STW. For example, primary sedimentation (humus tank) removed 24% of indomethacine, 26% of carbamazepine, 28% of sulfamethoxazole, 60% of diclofenac and 69% of propranolol. By passing through a lagoon, a further reduction in concentration of between 0% for carbamazepine and 26% for indomethacine was made, suggesting that lagoon is a relatively inefficient secondary treatment method for pharmaceuticals. The overall removal efficiency for the pharmaceuticals was calculated from the following formula:

$$\% \text{ Apparent removal} = \frac{100(C_{IN} - C_{EF})}{C_{IN}} \quad (1)$$

where C_{IN} and C_{EF} are the daily pharmaceutical concentrations in the influent and effluent, respectively.

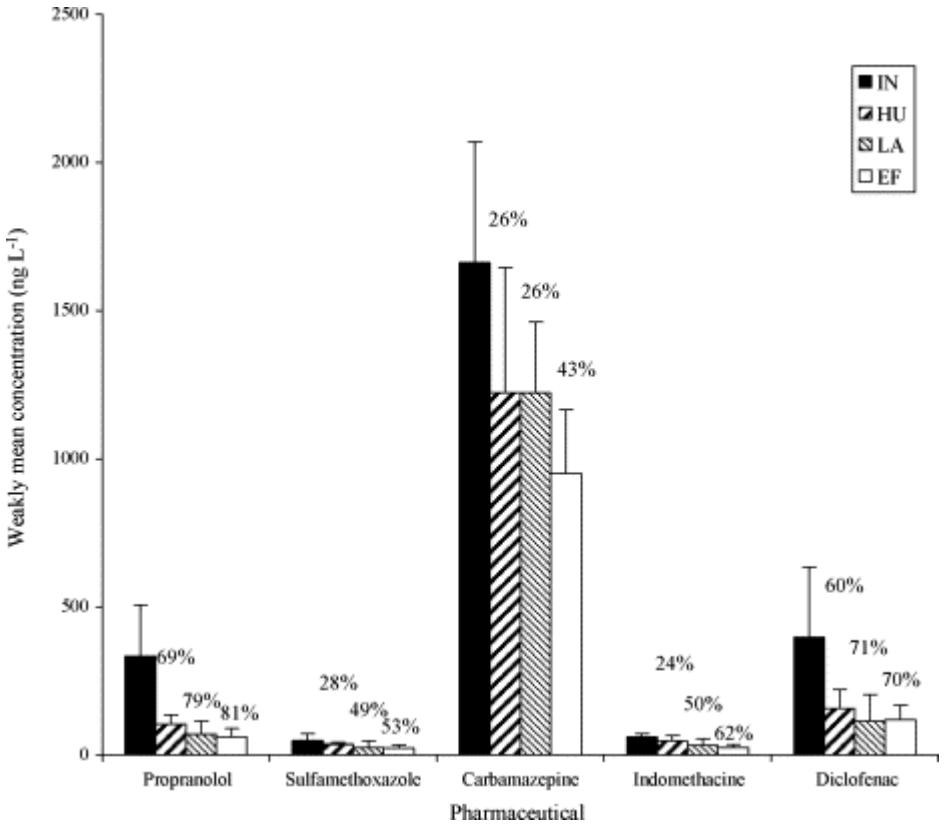

Fig. 7.2. Removal of five pharmaceutical compounds in the different stages of treatment in Scaynes Hill STW. Concentrations shown are the weekly mean in influent (IN), humus tank (HU), lagoon (LA) and effluent (EF).

As shown in Table 7.3, the overall removal efficiency varied highly between compounds and between STWs. A clear feature common to all three plants is that the lowest removal was found for carbamazepine, varying from 43 to 54%, no matter which treatment processes were used. Secondly, the use of tertiary treatment at Basingstoke STW did show an improvement in the removal of all 5 compounds (from 54 to 92%) over the other two plants, suggesting that pharmaceutical residues can be removed more completely by investment in tertiary treatment. At Scaynes Hill STW, the removal efficiency ranged from 43% for carbamazepine to 81% for propranolol. The mean concentration of propranolol was reduced from 334 ng L^{-1} entering the works to 62 ng L^{-1} in the final effluent; a

reduction of 81%. Similarly, the treatment works removed 61% of indomethacine present in the raw influent.

Table 7.3. Weekly mean pharmaceutical concentrations in the influent and effluent of STWs and their apparent removal.

Pharmaceuticals	**Scaynes Hill STW**			**Manor Farm Road STW**			**Basingstoke STW**		
	C_{IN} (ng	C_{EF} (ng	**Remo val**	C_{IN} (ng L^{-}	C_{EF} (ng L^{-1}	**Remo val**	C_{IN} (ng L	C_{EF} (ng L	**Removal (%)**
Propranolol	334	62	81.4	690	135	80.4	1090	110	89.9
Sulfamethox	49	23	52.7	110	37	66.4	181	32	82.3
Carbamazepi	166	950	42.8	1237	637	48.5	1833	837	54.3
Indomethaci	62	24	60.8	65	19	70.8	124	14	88.7
Diclofenac	397	119	70.1	782	176	77.5	981	78	92.0

Lower removal efficiency of 22–32% has been reported for propranolol, carbamazepine and diclofenac at Källby STW in Sweden [10]. Many other previous studies have shown that the reduction of pharmaceutical compounds in STWs is often incomplete. In Brazil, removal for polar pharmaceutical compounds varied from 12 to 90% [19]. In Germany, reported reduction ranged from 10 to 90% [1], depending on the nature of the compounds. These reductions occurred in common tertiary treatment STWs, consisting of preliminary clarification followed by aeration and then finally endpoint clarification. To achieve non-detectable concentrations of pharmaceutical residues, additional advanced treatment by

oxidation (e.g. ozonation at 10–15 mg L^{-1}), activated carbon or membrane filtration is needed [20].

A further complication with pharmaceuticals is that although they may be removed by processes such as sedimentation and sand filtration, they are only temporarily stored in the sand particles by partitioning into the sludge component of the processes, which may be eventually sprayed in landfill sites, incinerated or amended to agricultural soils, posing potential threats to the environment. Only a complete degradation will provide a lasting solution to preventing pharmaceutical exposure to the environment. In addition, as no measurement was made of pharmaceutical concentration in sediments and sludge, the data did not reflect a full mass balance. Further work should also include the determination of pharmaceuticals in the particulate phase.

7.3.3. Pharmaceuticals in the River Ouse

In addition to sampling in Scaynes Hill STW, the concentrations of the pharmaceutical compounds in the river Ouse close to effluent discharge were measured. River water was sampled both upstream and downstream of the STW, to identify a potential source–sink relationship. Six compounds including meberverine, thioridazine, tamoxifen, thioridazine, monensin and meclofenamic acid were on average below the limit of detection at both river sites. The other compounds propranolol, sulfamethoxazole, carbamazepine, indomethacine and diclofenac were found in 100% of river samples (7.3 and b), consistent with their widespread occurrence in the STW. Their concentrations in river water were found to vary daily over the sampling period, with the exception of propranolol in upstream, confirming

the need for regular sampling and analysis in order to monitor pharmaceutical concentrations in rivers.

Similar to wastewater samples, the highest concentrations were obtained for carbamazepine at 46–67 ng L^{-1} in upstream, to 167–334 ng L^{-1} in downstream. Significantly higher concentrations at up to 1100 ng L^{-1} have been detected in surface waters in Germany [2] and [18]. The lowest concentrations were shown by indomethacine at 0.2–0.9 ng L^{-1} in upstream, to 0.1–3 ng L^{-1} in downstream. Overall, a clear elevation in pharmaceutical concentrations is observed in the downstream over upstream, indicating that the Scaynes Hill STW is a source of pharmaceutical inputs to the river Ouse.

To make a more quantitative estimation of the STW as a source of pharmaceuticals in the river Ouse, the concentrations of pharmaceuticals in the downstream of effluent discharge site were estimated using a simple dilution model assuming the mass balance being observed:

$$C_{DW} = \frac{C_{UW} \times V_{UW} + C_{EF} \times V_{EF}}{V_{DW}} \quad (2)$$

where C_{DW} and C_{UW} are pharmaceutical concentrations in downstream and upstream, while V_{UW}, V_{DW} and V_{EF} are the flow rates in upstream, downstream and effluent, respectively.

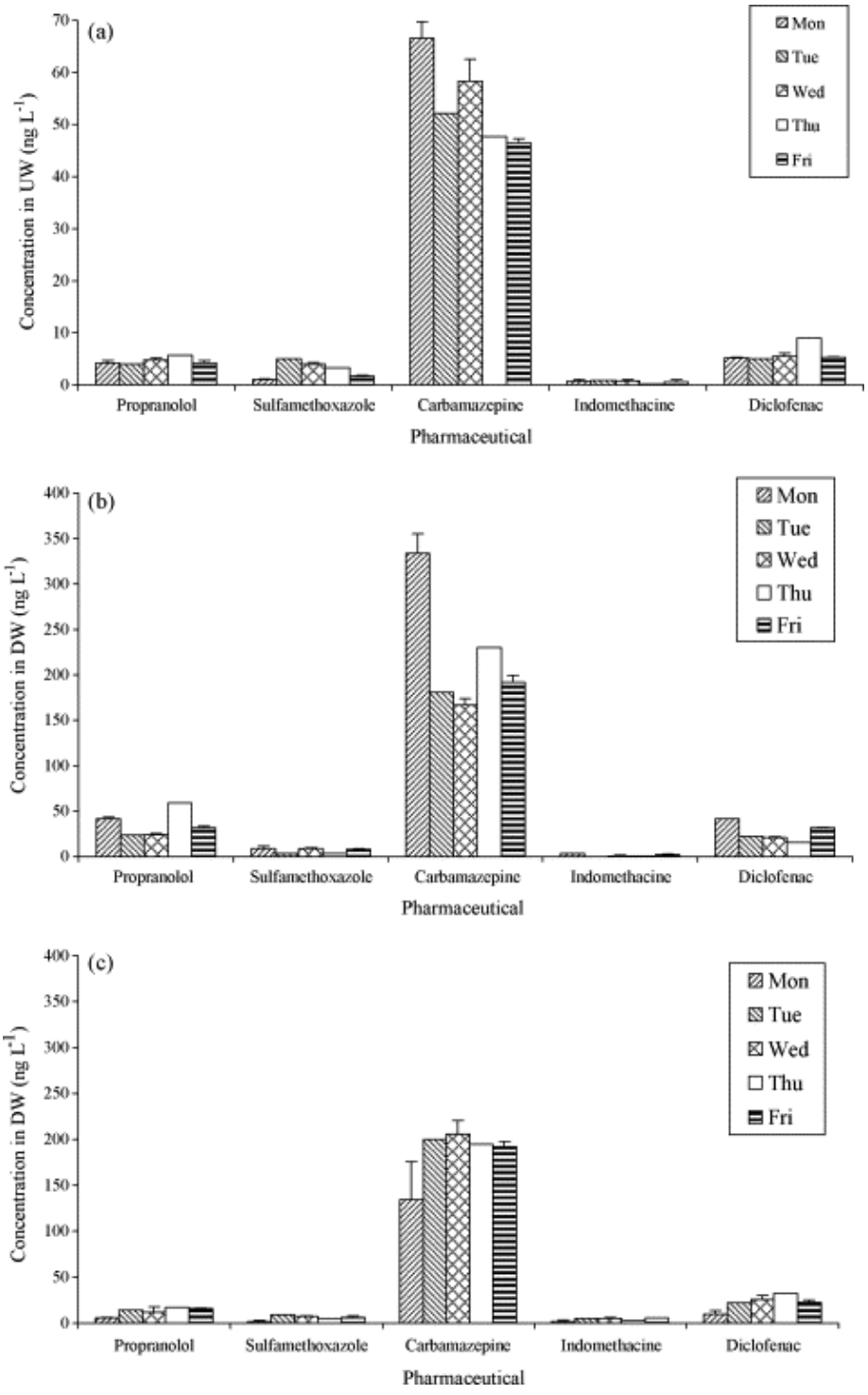

Fig. 7.3. Daily variation of pharmaceutical concentrations in the upstream (a) and downstream (b) of Scaynes Hill STW outfall in the River Ouse. Concentrations in the downstream were also predicted (c) using Eq. (2). UW, upstream; DW, downstream.

As shown in 7.3c, the predicted pharmaceutical concentrations in downstream site closely resembled those being measured (Fig 7.3b). Statistical analysis showed that the prediction underestimated measured values by 26%. But if one of the data points (i.e. carbamazepine on Monday) was excluded in the statistical analysis, then the underestimation from prediction was reduced to only 2.6%, with a r^2 value of 0.932, and a P value <0.001, suggesting a significant relationship.

A further comparison was made between the weekly mean pharmaceutical concentrations in the effluent and the receiving river water. It is clear that for all compounds, their concentrations were higher in the downstream than in the upstream, and the highest concentrations were always found in effluent. For example, the mean concentration of propranolol in the effluent was 62 ng L^{-1}. In comparison, lower concentrations were detected in the receiving river in downstream (36 ng L^{-1}) and in upstream (4 ng L^{-1}). The same trends were observable for other compounds (sulfamethoxazole, carbamazepine, indomethacine and diclofenac) when their concentrations were compared, further confirming the STW as a key source of pharmaceuticals into river Ouse.

7.3.4. Risk assessment of pharmaceutical compounds

Safety threshold values for pharmaceutical compounds are limited and often related to single compound-single organism toxicity studies. Many pharmaceutical compounds have not yet been studied as extensively as others and reliable toxicity data are limited to acute effects only. Cleuvers [21] studied the toxicity of a number of compounds to *Daphnia magna* including diclofenac, carbamazepine and propranolol. The EC_{50} values were found to be 68, 72 and 7.5 mg L^{-1} respectively, which are substantially higher in comparison to

the concentrations measured in this study at ng L^{-1} range. Nevertheless, it must be noted that the impact of a mixture of these chemicals could prove more toxic than the individual compounds alone. For example, Flaherty and Dodson [22] found that pharmaceutical mixtures behaved unpredictably and caused serious side effects such as deformities and increased mortality in *D. magna.*

Due to low pharmaceutical concentrations found in natural waters, their impact in causing chronic toxicity to aquatic populations close to sewage effluents is of more importance. Recently when studying cytological effects of pharmaceuticals in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*), Triebskorn et al. [23] determined that the lowest observed effect concentrations (LOEC) for carbamazepine and diclofenac were 1 μg L^{-1}. Although the highest pharmaceutical concentration (334 ng L^{-1} of carbamazepine) in the river Ouse is still lower than its LOEC, the safety margin becomes relatively constrained. Furthermore, due to the more significant impacts from mixtures of pollutants and potential persistence of such chemicals, it is prudent that these chemicals should be monitored regularly.

7.4. Conclusions

Five pharmaceutical compounds propranolol, sulfamethoxazole, carbamazepine, indomethacine and diclofenac were frequently detected in wastewater and river water samples, suggesting their widespread use and some degree of persistence. Pharmaceuticals were found to vary in concentrations, with carbamazepine being the most abundant. During wastewater treatment, all compounds were found to show concentration decline from influent to effluent, with removal efficiency from 43 to 92%. These compounds were also

found in both the upstream and downstream of the effluent outfall at Scaynes Hill STW, with concentrations elevated at the outfall. Through a simple dilution model, the STW was shown to be a key source of pharmaceuticals in the river Ouse. Further research is needed to assess potential bioaccumulation of pharmaceuticals in aquatic organisms and resulting chronic toxic effects.

7.5 References

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Chapter 8. Conclusions and Further work

8.1 Monitoring of EDCs and PPCPs in UK rivers

The GC-MS/MS and LC-MS/MS techniques described have been shown to be suitable for the determination of EDCs and Pharmaceutical pollutants in aquatic environments, with good agreement between the two techniques as well as with other analytical techniques and modelling approaches. The two techniques have been used to determine the concentrations of both classes of pollutants in two major UK rivers; the Ouse (Sussex) and Ray (Wiltshire). In both rivers, concentration data indicates that STWs are the major source of these compounds into the river waters. Prior to the commissioning of a Granular Activated Carbon treatment plant at the Rodbourne STW, the effluent of which enters the River Ray, the contaminant profile of the River Ray was broadly comparable to those of similar rivers in the UK and internationally. There was a reduction in concentrations of EDCs and pharmaceuticals in the River Ray after the introduction of the GAC plant, suggesting a reduction in the risk posed by these chemicals. However, as some pharmaceutical compounds were found to persist GAC treatment, the risk that these pose remains a cause for concern.

8.2 Analytical Procedures

Analysis of emerging pollutants remains an area of development. To ensure valid risk assessment, reliable techniques for the quantification of these ultra-trace contaminants is a concern, particularly where potentially-toxic concentrations are sufficiently low so as to push instrumental techniques to their detection limits. Since mass-spectrometric instruments are relatively expensive to purchase, an assessment of three common techniques has been completed and has shown that whilst GC-MS/MS offers optimal

sensitivity, GC-MS and LC-MS/MS are suitable alternatives in some cases. All three techniques have been shown to have good agreement with alternative techniques, including yeast assays and modelling approaches, highlighting the potential of these techniques to be used where access to mass spectrometric instrumentation is not available.

A significant source of uncertainty has been identified in the storage of environmental samples. Literature surveys revealed that often, information relating to the preservation and storage of samples is limited, and the implications that this may have for risk assessment and the comparison of results from different studies has been revealed. It has been suggested in this work that as a result, further work is required to identify more effective preservation techniques, as well as in focussing efforts to streamline sampling processing techniques to reduce the time samples need to be stored.

8.3 Removal of emerging pollutants by STWs

It has been shown that STWs do not entirely remove pharmaceuticals or estrogenic compounds present in sewage. As a result effluents released in to receiving river water is contaminated with potentially significant concentrations of these compounds. Results from a three-year study on the River Ray has shown that GAC treatment significantly reduces concentrations of three potent estrogenic compounds, but is less effective at removing some pharmaceutical substances. In light of this, it is suggested that further work to investigate the efficacy of GAC in the removal of pharmaceutical compounds is conducted, specifically to investigate how the conditions of GAC operation might improve removal. However, with GAC being an expensive treatment technique to implement, both in regard to initial set-up as well as maintenance, research into alternatives which are more effective and cost-efficient, such as UV-treatment could be pursued.

Publications and presentations arising from this work

Articles in Peer-reviewed Journals

(1) **Grover, D. P.**, Zhang, Z.L., Readman, J.W., & Zhou, J.L. (2009) A comparison of three analytical techniques for the measurement of steroidal estrogens in environmental water samples, *Talanta*, 78 1204-1210.

(2) Zhou, J.L., Zhang, Z.L., Banks, E., **Grover, D.P.,** & Jiang, J.Q. (2009) Pharmaceutical residues in wastewater treatment works effluents and their impact on receiving river water, *Journal of Hazardous Materials*, 166 655-661.

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(4) **Grover**, **D.P. ,** Balaam, J., Pacitto, S., Readman, J.W., White, S. & Zhou, J.L. (2011) Endocrine disrupting activities in sewage effluent and river water determined by chemical analysis and in vitro assay in the context of granular activated carbon upgrade. *Chemosphere*, 84 1512-1520.

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(11) **Grover, D.P.** (2009) Emerging Pollutants in the Aquatic Environment: Pharmaceuticals and Steroids in rivers. *Environmental Systems and Processes Research Seminars*, University of Sussex, UK. **Oral presentation**

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