Cranfield University

Cranfield Water Science Institute
School of Applied Sciences

PhD

2014

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Enhancing the removal of a diverse range of hazardous chemicals from wastewaters

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January 2014

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Due to increasingly stringent legislation covering the discharge of hazardous chemicals into the environment, existing wastewater treatment processes need to be upgraded for their removal. This thesis explores the removal of a diverse range of hazardous chemicals during secondary wastewater treatment with the overall aim of enhancing their removal simultaneously by activated sludge.

Previous research in this field has made the broad comparison of full-scale activated sludge plants (ASPs) which receive varying influent sewage compositions and flow. Consequently, assessing the direct impact of process operation to hazardous chemical removal has been difficult. In this study, the independent impact of the process variables solids retention time (SRT) and hydraulic retention time (HRT) were examined using a pilot-scale ASP. To measure ASP resilience for the removal of a wide range of hazardous chemicals of varied chemistry and preferred removal pathways steroid estrogens, nonylphenolic surfactants and metals were monitored.

In pilot plant trials where SRT was varied and HRT fixed, the highest SRT studied (27 days) achieved the greatest removal of all hazardous chemicals collectively. Interestingly, significantly greater estrogen biodegradation per viable bacterial cell was observed as SRT increased. To demonstrate, estrogen biodegradations were 499, 1,361 and 1,750 ng 1 x 10^{12} viable $cells^{-1}$ d^{-1} for 3, 10 and 27 day SRTs respectively, suggesting a more responsive bacteria or an improved efficiency of the same bacteria at higher SRTs. Successful implementation of higher SRT at full-scale ASPs requires more control over process operation. The use of *in-situ* suspended solids probes and real-time flow measurements could achieve this. In trials investigating the impact of altering HRT (whilst at a fixed SRT of 27 days), lengthening HRT was beneficial to the biodegradation of the organic hazardous chemicals. To demonstrate, estrogen biodegradations were 90 ±2 %, 93 ±1 % and 96 ±2 % for 8, 16 and 24 hour HRTs, respectively. Most significantly, 17α-ethinylestradiol exhibited removals of 65 ±19 % at the 24 hour HRT condition, the highest observed in these studies. Furthermore, nonylphenol removals were greater (63-70 %) at longer HRTs. However, longer HRT resulted in increased solubilisation of particulate metals. This is of concern as only dissolved concentrations are considered for environmental compliance. Consequently, any operational change to lengthen HRT for organic hazardous chemicals removal requires suitable provisions to enhance the removal of particle bound metals during primary treatment. It has been demonstrated that ASP operation can be modified to specifically target the removal of a diverse range of hazardous chemicals.

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ACKNOWLEDGEMENTS

Firstly, a massive thanks to Elise Cartmell for being a fantastic supervisor throughout these three years. Thanks also to Ewan McAdam for pushing me the extra mile – you're definitely smarter than the average bear although your attempts at writing emails in French need a bit of work! Thanks to John Lester.

I am also thankful to EPSRC, Anglian Water, Northumbrian Water, Severn Trent Water, Unitied Utilities and Yorkshire Water who funded the research.

Thanks to everyone at Cranfield Water Science Institute for their help and guidance. Thanks also to all my family and friends for your support, especially Andrew James McLeod - I acknowledge you!

Last but not least, Yolanda. I'm not going to write anything mushy but thanks for everything, but please keep practising those culinary skills!

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ABBREVIATIONS

ACN	Acetontrile
AMO	Ammonia mono-oxygenase
ASE	Accelerated solvent extraction
ASP	Activated sludge plant
ATU	Allylthiourea
BOD	Biological oxygen demand
CIP	Chemicals Investigation Programme
CLSM	Confocal laser scanning microscope
COD	Chemical oxygen demand
DCM	Dichloromethane
DF	Dilution factor
DP	Degradation product
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE2	17α-ethinylestradiol
E1-3S	Sulfate conjugate of estrone
EPS	Extracellular polymeric substance
EPSRC	Engineering and Physical Sciences Research Council
EQS	Environmental Quality Standard
ESI	Electro-spray ionisation
EtOAc	Ethylacetate
FE	Final effluent
FOV	Field of view
GAC	Granular activated carbon
GC	Gas chromatography
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatogarphy
HRT	Hydraulic retention time
IDL	Instrument detection limit
LAS	Linear alkylbenzene sulphonate
LC	Liquid chromatography
LIT	Linear ion trap
MDL	Method detection limit
MeOH	Methanol
MLSS	Mixed liquor suspended solids

MLVSS	Mixed liquor volatile suspended solids
MQL	Method quantitation limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NP	Nonylphenol
NPEC	Nonylphenol carboxylate
NPEO	Nonylphenol ethoxylate
NSAID	Non-steroidal anti-inflammatory drug
OD	Oxidation ditch
PE	Population equivalent
PNEC	Predicted no effect concentration
QqQ	Triple quadrupole
RAS	Return activated sludge
RSD	Relative standard deviation
SE	Secondary effluent
SIM	Selected ion monitoring
SMP	Soluble micro-products
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SRT	Solids retention time
SS	Settled sewage
SSVI	Stirred sludge volume index
STWs	Sewage treatment works
SW	Surface water
TE	Tertiary effluent
TIC	Total ion chromatogram
TOF	Time of flight
UP	Ultra-pure
UPLC	Ultra-performance liquid chromatography
USE	Ultra-sonic solvent extraction
VP	Vapour pressure
WAS	Waste activated sludge
WW	Wastewater
WwTWs	Wastewater treatment works

All abbreviations are introduced within the text and re-introduced for each individual chapter.

NOTATIONS

τ	Flow time
Q	Discharge
d_{10}, d_{50}, d_{90}	Particle diameter below which lies a % of the total volume of all particles in the sample
F: M	Food: microorganism ratio
g	Gravity
H_2O	Water
k	Degradation rate
K_d	Distribution coefficient
K_{oc}	Organic carbon-water partitioning coefficient
K_{ow}	Octanol-water partitioning coefficient
MΩ	Megaohm
NaCl	Sodium chloride
Na_2EDTA	Ethylenediaminetetraacetic acid disodium salt
NH_4^+	Ammonium
NH_4OH	Ammonium hydroxide
NO_2^-	Nitrite
NO_3^-	Nitrate
p*Ka*	Negative loss to the base ten of the acid dissociating/acidity constant *Ka*
Q	Volumetric flow rate
v	Velocity
V	Volume

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

1.1. BACKGROUND

The most well-known impact of hazardous chemicals presence in the aquatic environment is the feminisation of fish. Evidence of this has been observed in surface waters in the UK (Jobling *et al*., 1998) and globally (Hashimoto *et al*., 2000; McArdle *et al*., 2000; Solé *et al*., 2003; Barnhoorn *et al*., 2004). Further concerns include the possible adverse implications for water re-use (Martin *et al*., 2008). Due to the risks posed, the European Union has established environmental quality standards (EQS) and proposed legislative targets for a wide range of hazardous chemicals present in surface waters (European Commission, 2008; European Commission, 2012). Their presence in the aquatic environment is attributed to their incomplete removal during wastewater treatment (Kirk *et al*., 2002). Therefore it is essential to monitor removal performance of wastewater treatment works (WwTWs) and to improve process operation to enhance hazardous chemical removal. This relies on the application of robust analytical methodologies to measure hazardous chemical concentrations. However, hazardous chemicals tend to be in the ng l^{-1} to sub ng l^{-1} range and the high complexity of environmental matrices makes their analysis difficult. This can result in a number of analysis being below the method reporting limit (Braga *et al*., 2005; Chimchirian *et al*., 2007; Stanford and Weinberg, 2007; Koh *et al*., 2009; Kumar *et al*., 2009) leading to a poor understanding of hazardous chemical fate and removal. Furthermore, significant concentrations of hazardous chemicals can be associated with the particulate fraction of wastewaters (Koh *et al*., 2008; Baker and Kasprzyk-Hordern, 2011a). Therefore, to better understand fate, monitoring here is essential. However, there is a lack of analysis undertaken in the particulate fraction of wastewater due to the greater labour requirements and the complex nature of the matrix.

The current operations of widely implemented secondary WwTWs do not enable EQS and proposed EQS compliance despite accounting for typical riverine dilution ratios (Gardner *et al*., 2012). A possible solution is to upgrade WwTWs with advanced treatment processes (e.g., granular activated carbon) to improve hazardous chemical removals. However, such processes require significant capital and operational costs, and with increased energy consumptions required, make existing secondary WwTWs improvement desirable (Jones *et al*., 2007a). Activated sludge is a widely implemented secondary process with a proven ability to remove hazardous chemicals (Kreuzinger *et al*., 2004; Clara *et al*., 2005; McAdam *et al*., 2010; McAdam *et al*., 2011). Hazardous chemical removal is mainly attributed to activated sludge sorption and biodegradation (Andersen *et al*., 2005; Langford *et al*., 2005). Therefore improved process operation is needed to facilitate the removal of hazardous chemicals exhibiting extremities of propensity to these mechanisms. Natural and synthetic steroid estrogens, pharmaceuticals, nonylphenolic surfactants and metals encompass such

diversity. These can be described as biodegradable and refractory organics and non-biodegradable inorganics.

Hazardous chemical removal is considered to be influenced by the activated sludge process variables, solids retention time (SRT) and hydraulic retention time (HRT) (Johnson *et al*., 2005). However, it remains unclear whether operation can be modified to achieve maximum removals of all hazardous chemicals simultaneously. To illustrate, an extended SRT (≥10 days) is considered necessary to augment removal of those biodegradable chemicals (Kreuzinger *et al*., 2004; Clara *et al*., 2005; McAdam *et al*., 2010), whilst these conditions are hypothesised to be detrimental to metals removal (Santos *et al*., 2010). Also, previous research in this area has focussed on the comparison of different full-scale WwTWs (Kreuzinger *et al*., 2004; Clara *et al*., 2005; Johnson *et al*., 2005; McAdam *et al*., 2010; McAdam *et al*., 2011). Full-scale processes tend to suffer from poor process control and significant variations in receiving wastewater flow and composition. This results in a dynamic system with substantial variations in both SRT and HRT. Therefore, understanding the impact of SRT and HRT to hazardous chemical removal has been difficult.

1.2. AIMS AND OBJECTIVES

The thesis reports research funded by the Engineering and Physical Sciences Research Council (EPSRC), Anglian Water, Northumbrian Water, Severn Trent Water, United Utilities and Yorkshire Water. The research aims to understand the fate and behaviour of a diverse range of hazardous chemicals during secondary wastewater treatment and to enhance their removal simultaneously during activated sludge treatment. Consequently, the following objectives were identified:

1. To review analytical methodologies used for the determination of organic hazardous chemicals in complex environmental matrices.
2. To determine the benefits of using ultra-performance liquid chromatography (UPLC) for environmental analysis over conventional high-performance liquid chromatography (HPLC).
3. To assess EQS compliance and the impact of hazardous chemical precursors present in wastewaters.
4. To address the importance of undertaking particulate phase analysis of wastewaters to better understand hazardous chemical fate and pathways of removal.
5. To evaluate the impact of changing process operation to the mechanisms of hazardous chemicals removal.

6. To examine the prospect of enhancing the removal of a diverse range of hazardous chemicals simultaneously by modifying activated sludge operation using a pilot-scale plant to enable close control of SRT and HRT.

1.3. THESIS PLAN

This thesis is presented in paper format. All papers were written by the first author Bruce Petrie and edited by Professor Elise Cartmell, Dr Ewan J. McAdam and Professor John N. Lester. All laboratory work was undertaken by Bruce Petrie with exception to Chapter 6 where the bacterial viability determinations were conducted by another PhD student (the third author).

The linkage between various chapters of the thesis is presented in Figure 1.1. The thesis begins with a review of the literature on methods of analysis used to determine the fate of organic hazardous chemicals (pharmaceutical drugs) in environmental matrices (Chapter 2, published in Trends in Analytical Chemistry 49 (2013) pages 145-159 - *Bruce Petrie, Ewan J. McAdam, Mark D. Scrimshaw, John N. Lester, Elise Cartmell. Fate of drugs during wastewater treatment*).

Chapter 3 compares the use of UPLC over HPLC for the determination of hazardous chemicals in environmental matrices (Chapter 3, in press in International Journal of Environmental Analytical Chemistry - *Bruce Petrie, Ewan J. McAdam, Keith H. Richards, John N. Lester, Elise Cartmell. Application of ultra-performance liquid chromatography-tandem mass spectrometry for the determination of steroid estrogens in wastewater*).

Chapter 4 investigates the impact of hazardous chemicals precursors in wastewaters to EQS compliance. This includes the determination of nonylphenol and its precursors in wastewater matrices and modelling the possible impact of typically observed precursor concentrations in final effluents (Chapter 4, published in Analytical and Bioanalytical Chemistry 405 (2013) pages 3243-3253 - *Bruce Petrie, Ewan J. McAdam, Mick J. Whelan, John N. Lester, Elise Cartmell. The determination of nonylphenol and its precursors in a trickling filter wastewater treatment process*).

Chapter 5 determines the importance of undertaking particulate phase analysis for understanding hazardous chemical fate. This includes the development of an analytical methodology for the determination of pharmaceuticals in the aqueous and particulate fractions of wastewaters (Chapter 5, in preparation for Environmental Science and Pollution Research - *Bruce Petrie, Markus Lutz, Ewan J. McAdam, John N. Lester, Elise Cartmell. A multi-residue method to determine the fate and behaviour of pharmaceuticals during secondary wastewater treatment).*

Chapter 6 is a study on the mechanisms of hazardous chemical removal during activated sludge treatment, focussed on the impact of SRT to steroid estrogen sorption and biodegradation (Chapter 6, submitted to Chemosphere - *Bruce Petrie, Ewan J. McAdam, Francis Hassard, Tom Stephenson, John N. Lester, Elise Cartmell. Diagnostic investigation of steroid estrogen removal by activated sludge at varying solids retention time*).

Chapter 7 assesses the impact of activated sludge operational variables (SRT and HRT) to the simultaneous removal of steroid estrogens, nonylphenolic surfactants and metals (Chapter 7, in preparation for Water Research - *Bruce Petrie, Ewan J. McAdam, John N. Lester, Elise Cartmell. Simultaneous removal of a diverse range of hazardous chemicals from wastewater by activated sludge*).

Chapter 8 is the overall discussion of the research.

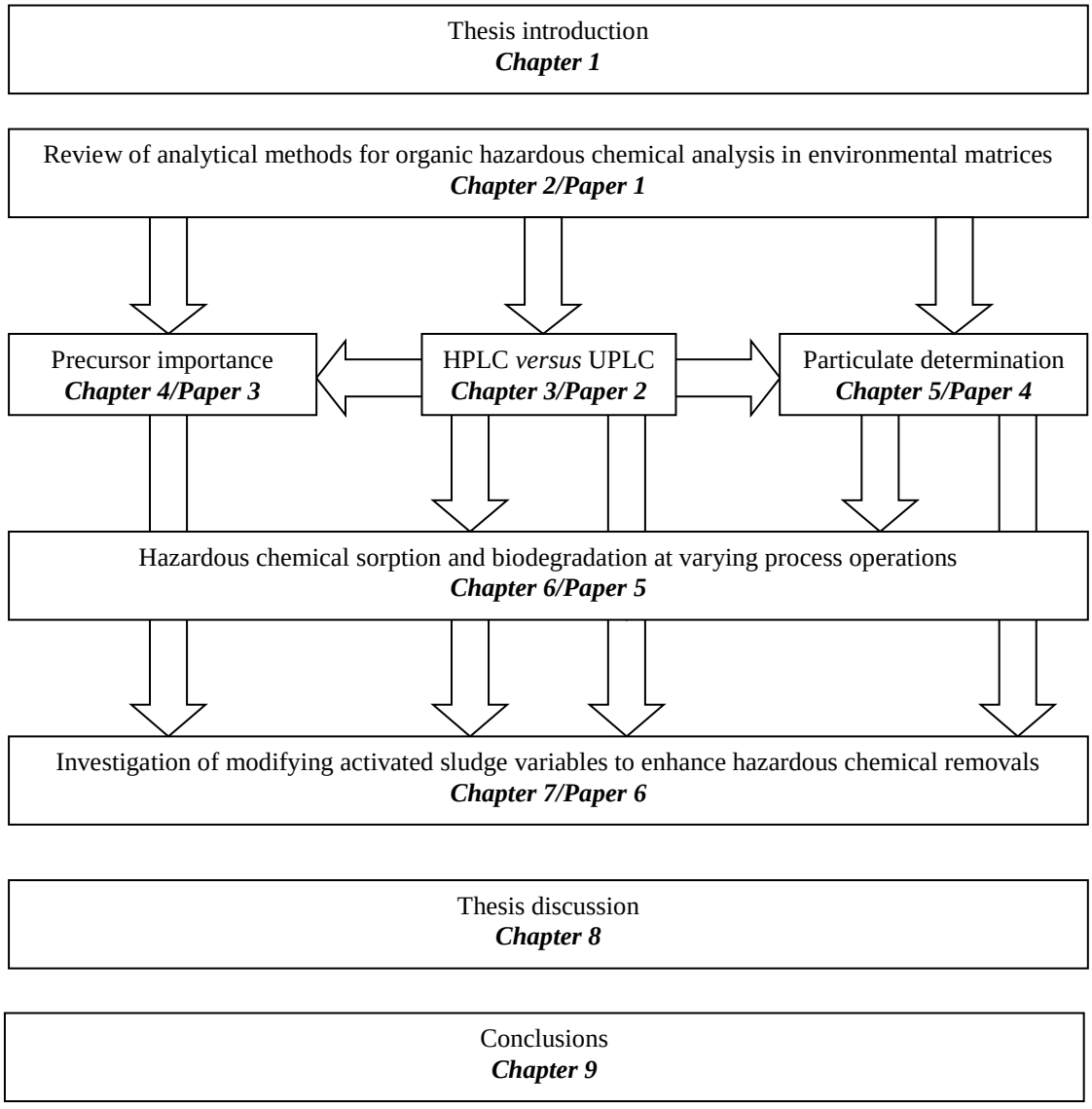

Figure 1.1 The thesis structure as a flow chart

CHAPTER 2

LITERATURE REVIEW – FATE OF DRUGS DURING WASTEWATER TREATMENT

PUBLISHED: *Trends in Analytical Chemistry 45 (2013) 145-159*

2. FATE OF DRUGS DURING WASTEWATER TREATMENT

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ABSTRACT

Recent trends for the determination of pharmaceutical drugs in wastewaters focus on the development of rapid multi-residue methods. These encompass a large number of drugs (up to 90) of varying physico-chemical composition (hydrophobicity, molecular weight etc) at ng l^{-1} concentrations in the aqueous phase of complex heterogeneous matrices. These are well suited for drug determination in secondary effluents which contain notable concentrations of pharmaceuticals. Drugs are not routinely monitored for in particulate phases of wastewaters despite being essential for fate determination, particularly in secondary processes receiving relatively high concentrations of suspended solids. Secondary effluents tend to contain multiple drugs, often above their proposed legislative targets for consent. Thus, tertiary processing may be considered to enhance drug removal and provide additional environmental protection. However, current analytical methods do not enable the efficacy of tertiary processes to be fully ascertained due to the inherently lower drug concentrations achieved. Existing method improvement is required to lower detection capabilities for tertiary process monitoring. This would aid the understanding of breakdown reaction completeness and the criticality between parent drug and degradation product concentrations. Numerous degradation products are formed by biological and chemical processes which can exhibit toxicity. The complimentary use of biological assays here would improve understanding of the synergistic toxicological effect of multiple drugs and their degradation products at low concentration. Additionally, tertiary processes receive secondary effluents comprising comparatively high concentrations of dissolved organics (e.g., colloids). However, knowledge of drug behaviour in the charged colloidal fraction of wastewater and its impact to treatment is limited. Monitoring and understanding here is needed to develop tertiary process diagnosis and optimisation.

2.1. INTRODUCTION

It is well established that wastewater contains a diverse range of anthropogenic chemicals (Bedding *et al*., 1982) and that their quantitative analysis poses numerous problems analytically, both in terms of extraction efficiency (Buisson *et al*., 1984) and interference from co-extractives (Robertson and Lester, 1994). Through medical use large numbers of drugs are now included in this group of determinants (Jones *et al*., 2001; Jones *et al*., 2003; Kreuzinger *et al*., 2004; Clara *et al*., 2005). A clear trend in legislation has been a lowering of acceptable environmental concentrations (e.g., 17α-ethinylestradiol (EE2)) and analytical method development has become an iterative process to achieve ever lower detection limits (Gomes *et al*., 2003; Gomes *et al*., 2004a) as further potential environmental health issues emerge (Gomes *et al*., 2004b). A growing understanding of the possible environmental impact to aquatic ecosystems has led to the proposal of diclofenac and the steroid estrogen EE2 as priority hazardous chemicals (European Commission, 2008; European Commission, 2012). These drugs have proposed environmental quality standards (EQS) of 100 ng l^{-1} and 0.035 ng l^{-1} respectively, and require monitoring to ensure compliance to 'good' water status (European Commission, 2008; European Commission, 2012). The effects of EE2, which results in intersex in male fish, are the most studied of the drugs in the environment (Sumpter and Jobling, 2013). Concentrations of EE2 below 1 ng l^{-1} are known to cause intersex and vitellogenin induction in male fish during laboratory studies. The environmental impact of other drugs and mixtures is less clear. However, it has been demonstrated that a mixture of acetaminophen, carbamazepine, gemfibrozil and venlaflaxine (in the low µg l^{-1} range) had a significant impact to fish embryo development in the short term (Galus *et al*., 2013). The chronic impact of drugs (i.e., ecological and evolutionary), either individually or as mixtures, remains unknown (Brodin *et al*., 2013). Without such information it is desirable to limit entry of these chemicals into the aquatic environment. A total of 12 drugs of varying therapeutic class (ibuprofen, diclofenac, naproxen, ketoprofen, carbamazepine, bezafibrate, propranolol, fluoxetine, EE2, ofloxacin, erythromycin and oxytetracycline) are highlighted in this review to represent a variety of physico-chemical compositions (e.g., molecular size, hydrophobicity) (Table 2.1). This includes those most studied in the literature (Clara *et al*., 2005; López-Serna *et al*., 2011; Gros *et al*., 2012) and those in national studies (i.e., the UK Chemical Investigations Programme (CIP)) (Gardner *et al*., 2012). The CIP found diclofenac, propranolol, fluoxetine, EE2, erythromycin and oxytetracycline above their indicative legislative targets for consent (10 ng l^{-1} for those not listed as priority hazardous substances) in over 50 % of effluents studied (Gardner *et al*., 2012). Consequently, a variety of drugs have been observed in surface waters in the ng l^{-1} range (Table 2.2). This underpins the need to understand drug fate during wastewater treatment for process/strategy improvement.

Table 2.1. Physico-chemical properties of pharmaceutical drugs present in crude wastewaters and secondary effluents (EPI Suite, 2013)

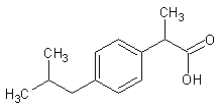
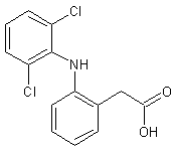
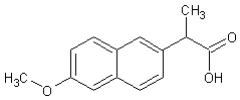
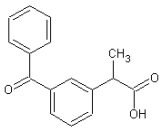
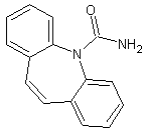
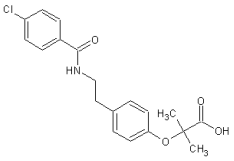
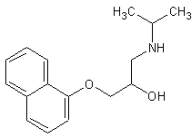
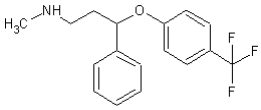
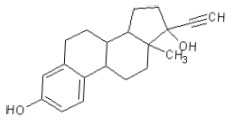
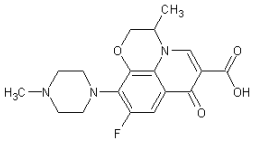
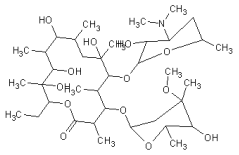
Drug	MW ($g\ mol^{-1}$)	Water solubility ($mg\ l^{-1}$)	Henry's law ($atm\ m^3\ mol^{-1}$)	VP (mm Hg)	pKa	Log K_{ow}	Log K_{oc}
Ibuprofen	206.30	21.0	$1.52.10^{-7}$	$1.86.10^{-4}$	4.91	3.79-3.97	2.35
Diclofenac	296.15	2.4	$4.73.10^{-12}$	$2.18.10^{-6}$	4.15	4.02-4.51	2.61
Naproxen	230.26	15.9	$3.39.10^{-10}$	$1.27.10^{-6}$	4.15	3.10-3.18	1.97
Ketoprofen	254.28	51.0	$2.12.10^{-11}$	$6.81.10^{-6}$	4.45	3.00-3.12	2.08
Carbamazepine	236.27	112	$1.08.10^{-10}$	$8.80.10^{-8}$	-	2.25-2.45	2.23
Bezafibrate	361.82	7.9	$2.12.10^{-15}$	$6.12.10^{-11}$	-	4.25	2.31
Propranolol	259.35	61.7	$7.98.10^{-13}$	$9.44.10^{-8}$	9.42	2.60-3.48	2.45
Fluoxetine	309.33	60.3	$8.90.10^{-8}$	$2.52.10^{-5}$	-	4.05-4.65	4.97
EE2	296.40	11.3	$7.94.10^{-12}$	$1.95.10^{-9}$	-	3.67-4.12	4.65
Ofloxacin	361.37	$2.8.10^{4}$	$4.98.10^{-20}$	$9.84.10^{-13}$	-	<0	1.09
Erythromycin	733.94	0.5	$5.42.10^{-29}$	$2.12.10^{-25}$	8.88	2.48-3.06	2.75
Oxytetracycline	460.43	313	$1.70.10^{-25}$	$9.05.10^{-23}$	3.27	<0	1.87

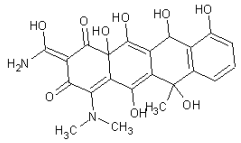
Key: MW, molecular weight; VP, vapour pressure; pKa, acid dissociation constant; log K_{ow}, octanol-water coefficient; log K_{oc}, organic carbon-water coefficient

Determining drug fate during wastewater treatment relies on the application of robust analytical methodologies. These require the ability to simultaneously determine a number of drugs, of differing physico-chemical properties, within environmental matrices comprised of comparatively high concentrations of complex bulk organics (Petrie *et al*., in press). The high complexity of environmental matrices underlines the analytical challenge posed. Drugs and their metabolites were first reported in wastewater effluents in the 1970's (Hignite *et al*., 1977). Hignite *et al* (1977) measured chlorophenoxyisobutyrate and salicylic acid in wastewater effluents at relatively high mean concentrations of 7 $\mu g\ l^{-1}$ and 29 $\mu g\ l^{-1}$, respectively. Ion exchange chromatography was used for extraction followed by gas chromatography-mass spectrometry (GC-MS). Numerous other drugs have been given significant attention recently due to the development of sophisticated analytical methodologies (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Gros *et al*., 2012). Extraction media enabling simultaneous retention of acidic, basic and neutral species of varying polarities has aided this. Furthermore, the coupling of liquid chromatography (LC), and particularly ultra-performance liquid chromatography (UPLC) to MS has seen significant reductions in sample pre-treatment requirements and instrument analysis time (Petrie *et al*., in press). Other than analytical difficulties, the variety of physico-chemical behaviour (e.g., hydrophobicity) exhibited by drugs results in significant differences in their fate and removal during wastewater treatment (Kreuzinger *et al.,* 2004; Lishman *et al*., 2006). Tertiary treatment processes are being considered to enhance hazardous chemical removal to levels which comply with proposed EQS's (McAdam *et al.,* 2011) as are the analytical methods

capable of supporting their diagnosis and optimisation for this critical group of emerging chemicals. This review addresses recent analytical trends for drug determination in environmental matrices used to facilitate fate studies. Analytical requirements for further fate evaluation and tertiary process selection/optimisation are also discussed.

Table 2.2. Recently reported occurrence of pharmaceutical drugs in surface waters

Drug of interest	Class	Chemical structure	Proposed legislative target (ng l^{-1})	Surface water[a] (ng l^{-1})	Location
Ibuprofen	NSAID		10[b]	<0.3-56 <6.4-542 21-2,796	UK Mainland Europe North America
Diclofenac	NSAID		100[c]	<0.5-261 <12-154 17-42	UK Mainland Europe North America
Naproxen	NSAID		-	<0.3-55 <3.1-109 22	UK Mainland Europe North America
Ketoprofen	NSAID		-	<0.5-4 <15-517 -	UK Mainland Europe North America
Carbamazepine	Anti-epileptic		-	0.5-495 <1.5-54 1-1,238	UK Mainland Europe North America
Bezafibrate	Lipid regulator		-	<10-66 <2.0-26 -	UK Mainland Europe North America
Propranolol	Beta blocker		10[b]	<0.5-27 <0.4-39 53	UK Mainland Europe North America
Fluoxetine	Anti-depressant		10[b]	- <7.4-24 <1.3-65	UK Mainland Europe North America
EE2	Contraceptive		0.035[c]	- - -	UK Mainland Europe North America
Ofloxacin	Antibiotic		10[b]	- 4.8-105 -	UK Mainland Europe North America
Erythromycin	Antibiotic		10[b]	<0.5-20 <28-52 2-438	UK Mainland Europe North America

Oxytetracycline	Antibiotic		10[b]	- <12-37 -	UK Mainland Europe North America

[a]occurence data taken from: UK-(Kasprzyk-Hordern *et al.,* 2009) Mainland Europe-(López-Serna *et al*., 2011) North America-(Wu *et al*., 2009; Ferrer *et al*., 2012), [b]UK Chemicals Investigation Programme (Gardner *et al*., 2012), [c](European Commission, 2012)
Key: NSAID, non-steroidal anti-inflammatory drug

2.2. ANALYTICAL STRATEGIES FOR FATE EVALUATION

Prior to laboratory work, the first step for drug determination in wastewaters is sampling. This process is fundamental to any strategy for monitoring, and careful consideration of sampling equipment, sample handling and types of samples is needed. It is beyond the scope of this review to examine further; however an excellent overview of sampling strategies has been given by Ort *et al* (2010a; 2010b). Nevertheless, it is important to highlight that to fully understand fate during wastewater treatment, determination in both aqueous and particulate phases of wastewaters is essential (Gomes *et al*., 2004a; Baker and Kasprzyk-Hordern, 2011a).

Trace determination of drugs in aqueous wastewater fractions (typically 0.45 µm filtered) requires an enrichment step followed by chromatographic separation and mass spectrometry (MS) detection. Sample pre-concentration and clean up commonly involves solid phase extraction (SPE), and can be undertaken off-line (using extraction systems not linked to analytical equipment) or on-line, where extraction and quantification are automated and linked together. Off-line analysis tends to use up to 1 litre of sample, and Gros *et al* (2006) investigated the efficacy of various SPE sorbents (Oasis HLB, Oasis MCX, Isolute C18 and Isolute ENV+) for the simultaneous extraction of 13 pharmaceutical drugs of varying physico-chemical composition. The Oasis HLB sorbent (polystyrene-devinylbenzene-N-vinylpyrrolidone terpolymer) (Radjenović *et al*., 2007a) (without pH adjustment) exhibited superior performance, utilising both hydrophilic and lipophilic retention mechanisms. Consequently, a full suite of drugs (up to 90) can be extracted simultaneously off-line using this sorbent (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Grabic *et al*., 2012; Gros *et al*., 2012). The introduction of fully automated methods further reduces sample processing restrictions (López-Serna *et al*., 2010; Huntscha *et al*., 2012). At present these are emerging techniques whose use are not widespread. Their reproducibility and ability to use smaller total sample volumes mean they will supersede traditional labour intensive SPE protocols in commercial laboratories in the future. Drug determination in the particulate phase of wastewaters is not routinely monitored but those methods which do, use ultrasonic solvent extraction (USE) (Ternes *et al*., 2005) or most commonly, accelerated solvent extraction

(ASE) (Nieto *et al*., 2010; Baker and Kasprzyk-Hordern, 2011a; Jelic *et al*., 2011; Silva *et al*., 2011; Chen *et al*., 2013). The application of pressure enables the use of extraction solvents (e.g., methanol) at temperatures much higher than their boiling point, increasing solubility and mass transfer (Zuloaga *et al*., 2012). Following extraction the solvent can be diluted in water to <5 % (v/v) and subjected to SPE as an aqueous fraction (Jelic *et al*., 2011; Silva *et al*., 2011).

Gas chromatography-mass spectrometry is well established for quantification of chemicals in environmental samples, achieving method quantitation limits (MQLs) ≤10 ng l^{-1} for some drugs (Zhao *et al*., 2009; Samaras *et al*., 2010). However, derivatization of more polar drugs with toxic chemicals is required prior to analysis to improve volatility, thermal stability and sensitivity of detection, increasing cost and time of sample preparation. A further disadvantage is the run time required for analysis; often up to 1 hour per sample (Zhao *et al*., 2009; Samaras *et al*., 2010). This has been a rate limiting step of such research in the past, severely restricting sample numbers which can be analysed. Despite these limitations, methods report the ability to simultaneously measure ≥63 drugs of varying therapeutic class from a single injection by GC-MS following derivatization (Sebok *et al*., 2009; Andrási *et al*., 2011). The use of LC coupled to tandem MS detection (MS/MS) improves sample throughput without the need for additional sample preparation (Barceló and Petrovic, 2007). Furthermore, the introduction of UPLC offers additional reductions in run times, whilst improving sensitivity over conventional LC (Petrie *et al*., in press). For UPLC, run times are generally less than 10 minutes with MQLs <100 ng l^{-1} for most drugs (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Gros *et al*., 2012). However, a well-known problem of environmental sample analysis by LC-MS, with electrospray ionisation (ESI) source particularly, is the quenching influence (ionisation suppression) of sample matrix on analyte signal strength (Petrie *et al*., 2013a; Petrie *et al*., in press). The commercial availability of deuterated surrogates now offers substantial improvements in minimising the impact of matrix interferences, improving accuracy of analysis and up to 50 isotopic labelled standards are used in some multi-residue methods (Huntscha *et al*., 2012).

The selection of MS/MS detector type is critical for analysis type (i.e., qualitative or quantitative). Quantitative analysis commonly employs a triple quadrupole (QqQ) due to its high sensitivity. The use of hybrid detectors such as quadrupole time of flight (QqTOF) offers the ability to screen and identify unknown degradation products/metabolites. Its full scan sensitivity, high selectivity and specificity enable structural elucidation of non-target species (Radjenović *et al*., 2007a). This is a significant advance in the determination of drug fate where degradation products in both biological and chemical processes are formed; enabling degradation pathways to be identified. However, data processing can be time

consuming due to the lack of searchable libraries, often requiring manual spectral interpretation (Barceló and Petrovic, 2007; Radjenović *et al*., 2007a). A further disadvantage of TOF detection is it generally offers lower sensitivity (3 to 5 times) than conventional MS/MS such as QqQ (Marchese *et al*., 2003). Thus, the ever increasing requirement to reduce MQLs for trace analysis confines its use to qualitative fate evaluation. Alternatively, hybrid linear ion trap (LIT) Orbitrap instrumentation offers high sensitivity for environmental quantitation (as offered by QqQ) and the ability to perform accurate mass determinations for drug identification (as offered by TOF) (Barceló and Petrovic, 2007). Although Orbitrap technology has been used to screen environmental samples for unknowns (Chiaia-Hernandez *et al*., 2013), unequivocal confirmation of degradation products observed by non-target MS/MS screening requires a complementary analytical technique or use of analytical reference standards (Helbling *et al*., 2010). However, as degradation products are often not known, there is a subsequent lack of standards for these. Further opportunities are offered by Fourier transform-MS which demonstrates unrivalled mass accuracy, rapid data collection, good dynamic range and, high sensitivity and resolution. However, the high cost of such instrumentation limits its widespread application.

Overall, recent trends for drug determination in wastewaters tend to use a single stage Oasis HLB off-line SPE followed by UPLC with detection by QqQ (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Grabic *et al*., 2012) or LIT (Gros *et al*., 2012) (Table 2.3). These methods are well suited for the determination of multiple drugs in the aqueous phase of crude wastewaters and secondary effluents where their concentrations are relatively high. However, monitoring EE2 at environmentally relevant levels requires devoted clean up protocols which can be laborious due to its inherently lower concentrations (Petrie *et al*., in press). Nevertheless, there is a lack of particulate phase analysis undertaken and the concentrations present in tertiary effluents continue to pose an analytical challenge.

Table 2.3. Recently validated LC-MS/MS methods applied for the quantitation of drugs in environmental matrices

No. of drugs	Sample	SPE	Chromatography	Detector	Run time (min^{-1})	SE recovery (%)	SE MQL ($ng\ l^{-1}$)	No. of drugs quantified in methods application				Method benefits	Method limitations	Reference
								Crude WW	SE	TE	SW			
81	Aq.	Oasis HLB	UPLC	QqLIT	4[a] 7[b]	22-146	0.6-51	57[c]	59	28	45	√	XX	Gros *et al*., 2012
47	Aq.	Oasis HLB	UPLC	QqQ	10	49-127	0.8-170	-	37	-	31	√	X	Gracia-Lor *et al*., 2011
74	Aq.	Oasis HLB	UPLC	QqQ	8[a] 5[b]	0-174	0.1-378	-	-	-	73	√	X XX	López-Serna *et al*., 2011
90	Aq.	Oasis HLB	HPLC	QqQ	25	5-246	0.1-78	-	63	-	-	√	X	Grabic *et al*.,2012
33[d]	Aq.	Oasis HLB + 3 sorbents	HPLC	QqQ	36[e]	64-166	2.3-186	-	-	-	16	√√ √√√	X	Huntscha *et al*., 2012
87	Aq.	TurboFlow column	HPLC	QqQ	22[e]	12-345	0.1-164	-	-	-	44	√ √√ √√√	X XX	López-Serna *et al*., 2010
14	Part.	Oasis HLB	HPLC	QqQ	-	70-120	0.6-19[f,g]	-	11[g]	-	-	√√√√	X	Chen *et al*., 2013
60	Part.	Oasis MCX	UPLC	QqQ	20	7-142[e]	0.1-20[f,h]	30[f]	-	-	-	√	X	Baker and Kasprzyk-Hordern, 2011a
5	Aq. Part.	MIP	HPLC	QqQ	25	95-105 77-91	4-12 4-10[f,i]	-	5[j]	-	4[f,i]	√√√√	X XXX	Duan *et al*., 2013
1[k] (EE2)	Aq. Part.	C18, NH_2 Silica, NH_2	UPLC	QqQ	9	96 97	0.06 2.96[f]	1	1	-	-	√√√√ √√√√√	X XXXX	Petrie *et al*., in press

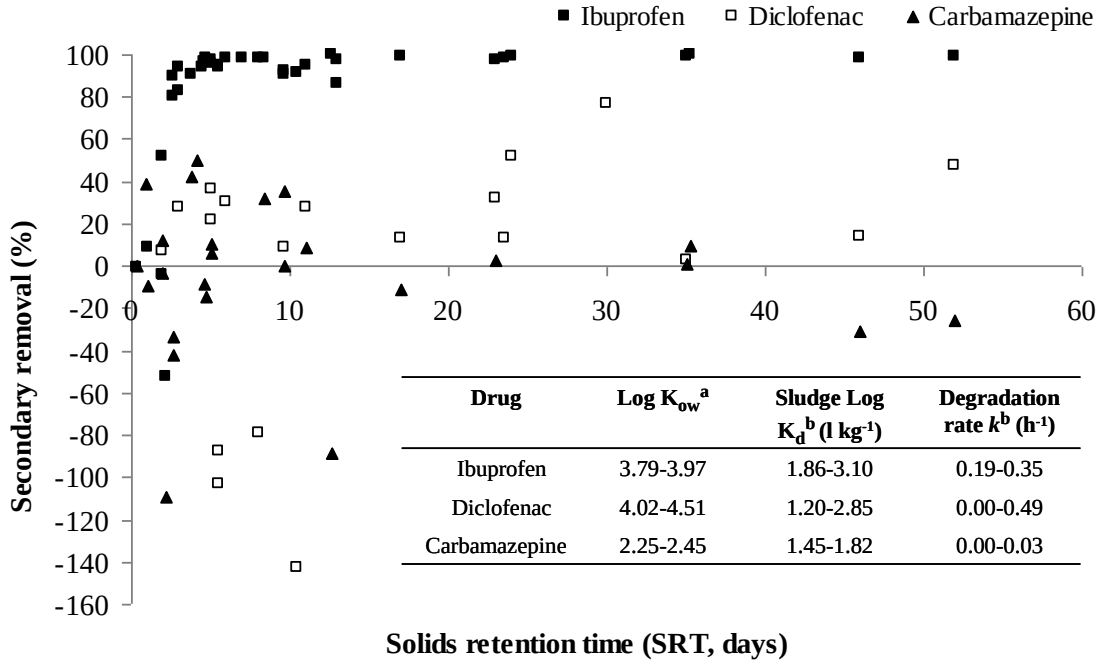
[a]positive ionisation mode [b]negative ionisation mode [c]application is not a sequential train of processes [d]includes additional 55 chemicals in method [e]includes on-line SPE time [f]ng g^{-1} [g]de-watered sludge [h]settled sewage [i]sediment [j]aqueous only [k]includes 4 natural estrogens in method

Key: MQL, method quantitation limit; SPE, solid phase extraction; SE, secondary effluent, WW, wastewater; TE, tertiary effluent; SW, surface water; Aq., aqueous; Part., particulate; QqLIT, linear ion trap; QqQ, triple quadrupole; MIP, molecularly imprinted polymer; √, high number of drugs varying in physicochemical composition; √√, includes some known degradation products; √√√, fully automated, small sample requirements; √√√√, high recoveries for all drugs measured; √√√√√, very low MQL; X, application to real samples limited; XX, numerous drugs reported <MQL; XXX, limited to drugs of specific physicochemical composition; XXXX, extensive sample pre-treatment required

2.3. DRUG REMOVAL BY CONVENTIONAL WASTEWATER TREATMENT; THE CURRENT PROBLEM

Activated sludge is an extensively implemented secondary wastewater treatment process, effective for carbonaceous material removal and can be adapted for nutrient removal. Removals of many drugs (and other chemicals of anthropogenic origin) are also observed (Kreuzinger *et al*., 2004; Clara *et al*., 2005). Removal of drugs and other hazardous chemicals during treatment is attributed to biological degradation and sorption onto biomass (Langford *et al*., 2005; Urase *et al*., 2005; Khunjar and Love, 2011). Pharmaceutical drugs have low vapour pressures and pKa's ranging from 3 to 10 (Table 2.1) restricting any removal by volatilisation. The relative resistance to biodegradation and/or sorption of some drugs makes enhancing removal by such processes difficult. Ibuprofen, diclofenac and carbamazepine encompass extremities in susceptibility to biodegradation and sorption. These represent drugs reasonably amenable to sorption and biodegradation (ibuprofen), sorption only (diclofenac) and neither sorption or biodegradation (carbamazepine), respectively. Consequently, removal differs between one another during activated sludge treatment (Kreuzinger *et al*., 2004; Clara *et al*., 2005). Solids retention time (SRT) (which is proportional to the food: micro-organism (F: M) ratio) is the simplest way of manipulating existing activated sludge operation in the short term and, is considered critical to the removal of non-drug derived hazardous chemicals (Kreuzinger *et al*., 2004; Clara *et al*., 2005; McAdam *et al*., 2011). An increased SRT (>10 days) is often cited as the condition required to achieve greatest hazardous chemical removal (Kreuzinger *et al*., 2004; Clara *et al*., 2005) but has little effect to removals of these drugs (Figure 2.1, Table 2.4). Generally, diclofenac is removed by ≤50 % and any carbamazepine removal is negligible. Negative drug removals are also observed during treatment and are considered attributable to the deconjugation of metabolites present in the crude stream (Lishman *et al*., 2006). Conjugates and intermediate chemicals tend to go undetermined by current analytical approaches. Parent drugs are often above their legislative targets for consent in secondary effluents despite accounting for typical dilution ratios in the environment (Gardner *et al*., 2012). Source control would limit drug entry into wastewater, similar to what has been achieved with nonylphenol (Petrie *et al*., 2013a). Without the availability of substitute drugs, less persistent and with a less burdensome environmental impact, this will not be achievable. However, the possibility of separate treatment of urine streams may be an effective solution in some circumstances (Maurer *et al*., 2006). Additionally, membrane bioreactor systems generally achieve greater drug removals than conventional secondary processes (Kimura *et al*., 2007; Radjenović *et al*., 2007a; Radjenović *et al*., 2007b). Without their application, the remaining alternative to enhance drug removal is the addition of a tertiary process or processes to an existing

conventional secondary treatment asset (e.g., activated sludge). This requires a process not excessively space consuming and can treat secondary effluents at relatively short contact times. Some available options suiting these criteria include; biofiltration (sand or trickling filters), chemical (ozone) and adsorption (granular activated carbon) processes. The fate and removal of drugs differ substantially between these systems.



Drug	Log K_{ow} [a]	Sludge Log K_d [b] (l kg^{-1})	Degradation rate k [b] (h^{-1})
Ibuprofen	3.79-3.97	1.86-3.10	0.19-0.35
Diclofenac	4.02-4.51	1.20-2.85	0.00-0.49
Carbamazepine	2.25-2.45	1.45-1.82	0.00-0.03

Figure 2.1. Removals of ibuprofen (■, n=36), diclofenac (□, n=19) and carbamazepine (▲, n=25) by activated sludge operating at varying solids retention time. Ibuprofen, diclofenac and carbamazepine represent drugs reasonably amenable to sorption and biodegradation, sorption only and neither sorption or biodegradation, respectively. Inset, octanol-water coefficients, partition coefficients and degradation rate constants – [a]EPI suite, 2013 [b]Urase *et al*., 2005 (removal data obtained from: Metcalfe *et al*., 2003; Kreuzinger *et al*., 2004; Clara *et al*., 2005; Joss *et al*., 2005; Bernhard *et al*., 2006; Lishman *et al*., 2006; Nakada *et al*., 2006; Jones *et al*., 2007b; Kimura *et al*., 2007; Radjenović *et al*., 2007b; Zorita *et al*., 2009 – Table 2.4, no data reported as <MDL)

Table 2.4. Removal of ibuprofen, diclofenac and carbamazepine in activated sludge at varying SRT

Drug	Flow rate (m^3 d^{-1})	SRT (d^{-1})	HRT(h^{-1})	MLSS (mg l^{-1})	COD (mg l^{-1})	SS conc. (ng l^{-1})	SE conc. (ng l^{-1})	Removal (%)	Reference
Ibuprofen	-	0.3	-	-	-	-	-	-1.0	Kreuzinger *et al*., 2004
	-	1	-	-	-	-	-	9.0	Kreuzinger *et al*., 2004
	626,000	1.9	7	-	-	50,700	24,600	51.5	Metcalfe *et al*., 2003
	-	2	1.9	4,000	-	2,300	2,400	-4.2	Clara *et al*., 2005
	47,860	2.2	13	-	-	14,200	21,700	-52.8	Metcalfe *et al*., 2003
	185,000	2.7	12	-	-	27,900	5,400	80.6	Metcalfe *et al*., 2003
	585,667	2.7	14	-	-	58,200	6,200	89.3	Metcalfe *et al*., 2003
	3,967	3	10	3,030	113*	-	-	94.0	Lishman *et al*., 2006
	22,000	3	12	-	508.2	13,355-17,585	1,420-6,056	82.5	Radjenović *et al*., 2007b
	645,000	3.8	8.6	-	-	909	86.7	90.5	Nakada *et al*., 2006
	1,984	4.5	15	2,482	113*	-	-	94.0	Lishman *et al*., 2006
	409,000	4.6	8.0	-	-	593	21.0	96.4	Nakada *et al*., 2006
	125,248	4.7	13	-	-	21,800	300	98.6	Metcalfe *et al*., 2003
	1,199,000	5.0	7.1	-	-	578	14.3	97.5	Nakada *et al*., 2006
	-	5.0	-	-	-	-	-	96.0	Kreuzinger *et al*., 2004
	5,506	5.5	15	2,836	205*	-	-	95.0	Lishman *et al*., 2006
	15,300	5.5	15	1,743	154*	-	-	94.0	Lishman *et al*., 2006
	19,260	6	22	2,084	128*	-	-	98.0	Lishman *et al*., 2006
	125,000	7	12	2,000	-	1,966	40.0	98.0	Kimura *et al*., 2007
	20,000	6-10	35	-	510-680	5,700	88.5	98.4	Zorita *et al*., 2009
	210,000	8.4	8.9	-	-	595	8.0	98.7	Nakada *et al*., 2006
	68,498	9.6	15	-	-	27,300	2,700	90.1	Metcalfe *et al*., 2003
	-	9.6	-	-	-	-	-	92.0	Kreuzinger *et al*., 2004
	17,994	10.5	13	2,105	51*	-	-	91.0	Lishman *et al*., 2006
	-	10-12	7.3	-	-	-	-	91-99	Joss *et al*., 2005
	366,898	12.6	23	-	-	39,100	50.0	>99.9	Metcalfe *et al*., 2003
	60,000	12-14	22	5,000-6,000	-	6,242	194	97.0	Bernhard *et al*., 2006
	11,783	13	13.5	2,740	143-160	-	-	86.0	Jones *et al*., 2007b
	-	17.0	-	-	-	-	-	99.0	Kreuzinger *et al*., 2004
	-	22-24	16.8	-	-	-	-	96-98	Joss *et al*., 2005
	-	23.6	-	-	-	-	-	98.0	Kreuzinger *et al*., 2004
	-	24.0	-	-	-	-	-	99.0	Kreuzinger *et al*., 2004

	-	35.0	-	-	-	-	-	99.0	Kreuzinger *et al*., 2004
	5,074	35.3	27	-	-	58,900	50.0	>99.9	Metcalfe *et al*., 2003
	-	46	28.8	3,100	-	1,200	24.0	98.0	Clara *et al*., 2005
	-	52	326	4,000	-	2,448	20.0	99.2	Clara *et al*., 2005
	-	1.0	-	-	-	-	-	0.0	Kreuzinger *et al*., 2004
	-	2	1.9	4,000	-	1,400	1,300	7.1	Clara *et al*., 2005
	3,967	3	10	3,030	113*	-	-	28.0	Lishman *et al*., 2006
	105,300	5	16	2,450	127*	-	-	22.0	Lishman *et al*., 2006
	-	5.0	-	-	-	-	-	36.0	Kreuzinger *et al*., 2004
	5,506	5.5	15	2,836	205*	-	-	-88.0	Lishman *et al*., 2006
	15,300	5.5	15	1,743	154*	-	-	-103	Lishman *et al*., 2006
	19,260	6	22	2,084	128*	-	-	30.0	Lishman *et al*., 2006
	20,000	6-10	35	-	510-680	100	485	-79.4	Zorita *et al*., 2009
Diclofenac	-	9.6	-	-	-	-	-	9.0	Kreuzinger *et al*., 2004
	17,994	10.5	13	2,105	51*	-	-	-143.0	Lishman *et al*., 2006
	-	10-12	7.3	-	-	-	-	22-33	Joss *et al*., 2005
	-	17.0	-	-	-	-	-	13.0	Kreuzinger *et al*., 2004
	-	22-24	16.8	-	-	-	-	30-34	Joss *et al*., 2005
	-	23.6	-	-	-	-	-	13.0	Kreuzinger *et al*., 2004
	-	24.0	-	-	-	-	-	52.0	Kreuzinger *et al*., 2004
	4,554	30	23	4,554	178*	-	-	77.0	Lishman *et al*., 2006
	-	35.0	-	-	-	-	-	3.0	Kreuzinger *et al*., 2004
	-	46	28.8	3,100	-	905	780	13.8	Clara *et al*., 2005
	-	52	326	4,000	-	3,190	1,680	47.3	Clara *et al*., 2005
	-	0.3	-	-	-	-	-	0.0	Kreuzinger *et al*., 2004
	17,364	0.9	12	-	-	1,300	800	38.5	Metcalfe *et al*., 2003
	-	1.0	-	-	-	-	-	-9.0	Kreuzinger *et al*., 2004
	626,000	1.9	7	-	-	800	700	12.5	Metcalfe *et al*., 2003
	-	2	1.9	4,000	-	670	690	-3.0	Clara *et al*., 2005
Carbamazepine	47,860	2.2	13	-	-	1,100	2,300	-109.1	Metcalfe *et al*., 2003
	185,000	2.7	12	-	-	1,200	1,700	-41.7	Metcalfe *et al*., 2003
	585,667	2.7	14	-	-	600	800	-33.3	Metcalfe *et al*., 2003
	645,000	3.8	8.6	-	-	55.9	32.1	42.6	Nakada *et al*., 2006
	6,243	4.1	14	-	-	1,000	500	50.0	Metcalfe *et al*., 2003
	409,000	4.6	8.0	-	-	43.1	46.9	-8.6	Nakada *et al*., 2006

125,248	4.7	13	-	-	700	800	-14.3	Metcalfe *et al*., 2003
1,199,000	5.0	7.1	-	-	50.5	45.4	10.2	Nakada *et al*., 2006
-	5.0	-	-	-	-	-	6.0	Kreuzinger *et al*., 2004
210,000	8.4	8.9	-	-	173	117	32.4	Nakada *et al*., 2006
68,498	9.6	15	-	-	700	700	0.0	Metcalfe *et al*., 2003
-	9.6	-	-	-	-	-	9.0	Kreuzinger *et al*., 2004
-	10-12	7.3	-	-	-	-	0-18	Joss *et al*., 2005
366,898	12.6	23	-	-	900	1,700	-88.9	Metcalfe *et al*., 2003
-	17.0	-	-	-	-	-	-11.0	Kreuzinger *et al*., 2004
-	22-24	16.8	-	-	-	-	0-5	Joss *et al*., 2005
-	35.0	-	-	-	-	-	1.0	Kreuzinger *et al*., 2004
5,074	35.3	27	-	-	1,000	900	10.0	Metcalfe *et al*., 2003
-	46	28.8	3,100	-	325	426	-31.1	Clara *et al*., 2005
-	52	326	4,000	-	704	952	-26.1	Clara *et al*., 2005

*Biological oxygen demand

Key: SRT, solids retention time; HRT, hydraulic retention time; MLSS, mixed liquor suspended solids; COD, chemical oxygen demand; SS, settled sewage; SE, secondary effluent

2.4. DRUG FATE AND REMOVAL IN TERTIARY PROCESSES

2.4.1. Biologically active sand filters

Drug removal by biofiltration processes (tertiary sand or trickling filters) is by physical and biological mechanisms. Total removals by sand filters vary from 2 % for carbamazepine to >95 % for ibuprofen (Zearley and Summers, 2012) (Figure 2.2, Table 2.5), similar to those achieved by activated sludge. Despite treating different wastewaters of differing composition, activated sludge and tertiary sand filters essentially rely on the same mechanisms. Sand filters depend on a fixed biofilm comprised of a diverse community of micro-organisms embedded within a matrix of extracellular polymeric substances (EPS) consisting of proteins, nucleic acids, polysaccharides and amphiphilic polymeric compounds (Wagner *et al*., 2009). The composition of EPS shifts with biofilm age (Wagner *et al*., 2009), and is known to influence EE2 uptake (Khunjar and Love, 2011). The high tendency of some drugs to partition to solid organic matter, similar to biofilms has been confirmed by ASE followed by LC-MS/MS analysis (Baker and Kasprzyk-Hordern, 2011; Jelic *et al*., 2011; Silva *et al*., 2011; Chen *et al*., 2013). Those hydrophobic drugs with a comparatively high log K_{ow} (>4) (e.g., diclofenac and fluoxetine) are considered to have a tendancy to sorb to solid organic surfaces such as biofilms (Jones *et al*., 2005; Wunder *et al*., 2011). However, sorption cannot be predicted by hydrophobicity alone (Hörsing *et al*., 2011; Wunder *et al*., 2011) other properties such as molecular weight and ionic speciation are of known importance (Wunder *et al*., 2011), as is the nature of other dissolved species with which they may interact (Gong *et al*., 2012). Extracellular polymeric substances offer both anionic and cationic functional groups for the exchange of charged species (Wunder *et al*., 2011). At a pH typical of municipal wastewaters (e.g., 7-8), EPS is negatively charged (Wunder *et al*., 2011) with pKa's generally ranging from 6.2 to 10.1 (Guibaud *et al*., 2005). Those drugs whose pKa is <7 (e.g., ibuprofen, diclofenac, naproxen, ketoprofen and oxytetracyline) (Table 2.1) may themselves be negatively charged and repulsion with the biofilm could restrict sorption. Removal will also be influenced by biofilm porosity. Drugs whose molecular size is comparatively large (e.g., erythromycin), will have a reduced rate of mass transfer between the liquid medium and the biofilm, limiting partitioning (Wunder *et al*., 2011). Drugs which are comparatively smaller (<300 g mol^{-1}) and relatively hydrophobic in nature (log K_{ow}'s >3) (e.g., ibuprofen, diclofenac, naproxen, ketoprofen, propranolol and EE2, Table 2.1) are expected to partition well within the biofilm matrix.

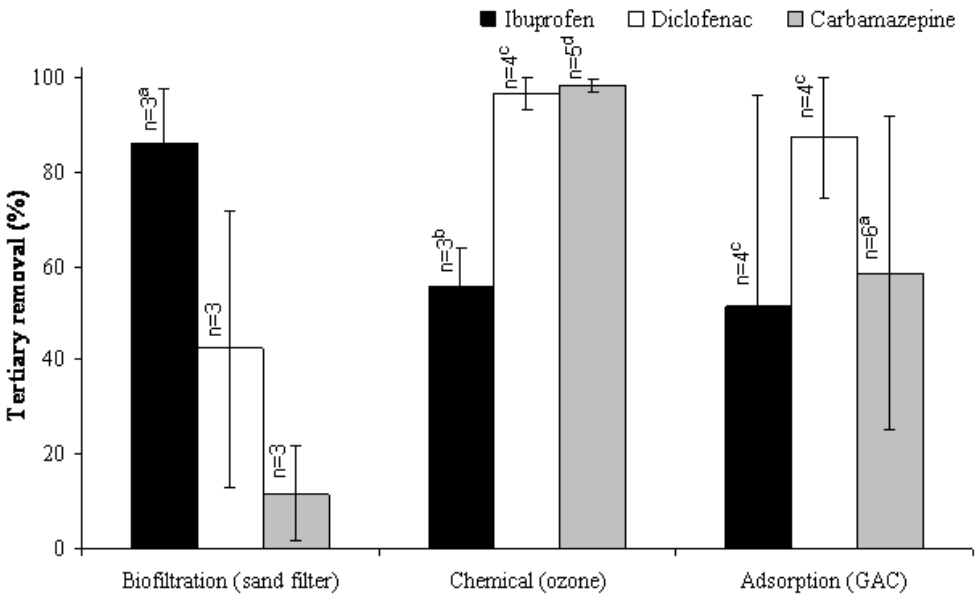


Figure 2.2. Removals of ibuprofen, diclofenac and carbamazepine reported in the literature for biofiltration, chemical and adsorption processes. Ibuprofen, diclofenac and carbamazepine represent drugs reasonably amenable to sorption and biodegradation, sorption only and neither sorption or biodegradation, respectively (removal data obtained from: (Huber *et al*., 2003; Ternes *et al*., 2003; Kim *et al*., 2007; Matamaros *et al*., 2007; Nakada *et al*., 2007; Snyder *et al*., 2007; Rosal *et al*., 2010; Schaar *et al*., 2010; Grover *et al*., 2011; Reungoat *et al*., 2011; Yang *et al*., 2011; Zearley and Summers, 2012 - Table 2.5, 2.6 and 2.7) [a]1/3 removed <MQL [b]2/3 removed <MQL [c]3/4 removed <MQL [d]4/5 removed <MQL

Sorption is also considered to be an intermediate step in biodegradation (Langford *et al*., 2005; Khunjar and Love, 2011). Assuming similar behaviour to EE2 and other hazardous chemicals in biological processes, drug biodegradation is likely to be mediated on the surface and/or within intact bacterial cells (Langford *et al*., 2005; Khunjar and Love, 2011; Bagnall *et al*., 2012). Free ammonia mono-oxygenase enzymes released by lysis extracellularly are not likely to be involved in biodegradation (Langford *et al*., 2005; Bagnall *et al*., 2012). Gaulke *et al* (2008) observed that EE2 removal in nitrifying batch studies was not by nitrifying bacteria activity, dismissing the hypothesis that nitrification augments EE2 removals (Vader *et al*., 2000). The synthesis of nitro-EE2 confirmed that EE2 is nitrated at high ammonia feed concentrations caused by the high production of nitrates; EE2 removal here is an artefact of laboratory scale investigation. Biodegradation at environmentally representative conditions is by heterotrophic micro-organisms, capable of scavenging a broad range of organic material (Bagnall *et al*., 2012). Interestingly, differences in biodegradation are observed for drugs which sorb similarly to biomass (e.g., ibuprofen and diclofenac, Figure 2.1) suggesting chemical structure controls susceptibility to biological attack. The structure of ibuprofen is comparatively simpler than that of diclofenac (i.e., single aromatic ring and non-halogenated, Table 2.2) which may aid its biodegradation. Drugs of increasing structural complexity and elemental diversity such as antibiotics (e.g., ofloxacin, erythromycin and oxytetracycline)

may be less favourable to biodegradation especially considering their possible toxicity to bacteria. However, 'biodegradation' here is not indicative of complete mineralisation. Drugs rich in functional groups provide more possible sites for biological attack, inducing a change to the parent structure. Kosjek *et al* (2009) investigated the degradation products of diclofenac, utilising Oasis HLB SPE and UPLC separations to ensure adequate sensitivity required by QqTOF. In full scan mode the total ion chromatogram (TIC) was screened and a protonated compound was then selected for further product ion scans (Kosjek *et al*., 2009). Accurate mass measurements and in-source fragmentation enabled chemical structure elucidation of three biotransformation products (Figure 2.3A). Similarly Helbling *et al* (2010) used LIT-Orbitrap MS to identify five degradation products of bezafibrate (Figure 2.3B). The dehydrogenation product (DP1) is structurally similar to the parent drug indicating it may behave similarly in the environment.

Table 2.5. Removal of drugs from environmental waters by biofiltration processes

Drug	Process	Temperature (°C)	HRT (h^{-1})	Wastewater type	Upfront process	SE. conc. (ng l^{-1})	TE conc. (ng l^{-1})	Removal (%)	Reference
Ibuprofen	SF	-	4-6	Municipal	-	11,700[a]	1,170	90	Matamaros *et al*., 2007
	SF	-	0.3	River	-	276	<14	>95	Zearley and Summers, 2012
	SF	-	1	Municipal	ASP	-	-	73[b]	Nakada *et al*., 2007
Diclofenac	SF	-	4-6	Municipal	-	820[a]	197	76	Matamaros *et al*., 2007
	SF	-	0.3	River	-	252	181	28	Zearley and Summers, 2012
	SF	22	2	Municipal	-	-	-	23	Reungoat *et al*., 2011
Carbamazepine	SF	-	4-6	Municipal	-	2,060[a]	1,833	11	Matamaros *et al*., 2007
	SF	-	0.3	River	-	85	84	2	Zearley and Summers, 2012
	SF	-	1	Municipal	ASP	-	-	22[b]	Nakada *et al*., 2007
Naproxen	SF	-	4-6	Municipal	-	1,570[a]	314	80	Matamaros *et al*., 2007
	SF	-	0.3	River	-	170	24	86	Zearley and Summers, 2012
Ketoprofen	SF	-	1	Municipal	ASP	-	-	32[b]	Nakada *et al*., 2007
	SF	-	1	Municipal	ASP	-	-	16[b]	Nakada *et al*., 2007
EE2	SF	-	0.3	River	-	316	246	22	Zearley and Summers, 2012
	SF	18	-	Municipal	ASP	-	0.2	82	Gunnarsson *et al*., 2009
	SF	-	-	Municipal	ASP	-	-	7	Ifelebuegu, 2011
	SF	-	-	Municipal	OD	-	-	9	Ifelebuegu, 2011
Erythromycin	SF	22-25	-	Municipal	-	109	64	41	Ho *et al*., 2011
	SF	-	0.3	River	-	104	75	28	Zearley and Summers, 2012
	SF	19-22	0.4	Municipal	ASP	-	-	20	Göbel *et al*., 2007
	SF	22	2	Municipal	-	-	-	20	Reungoat *et al*., 2011

Key: HRT, hydraulic retention time; SE, secondary effluent; TE, tertiary effluent; SF, sand filter; ASP, activated sludge plant; OD, oxidation ditch

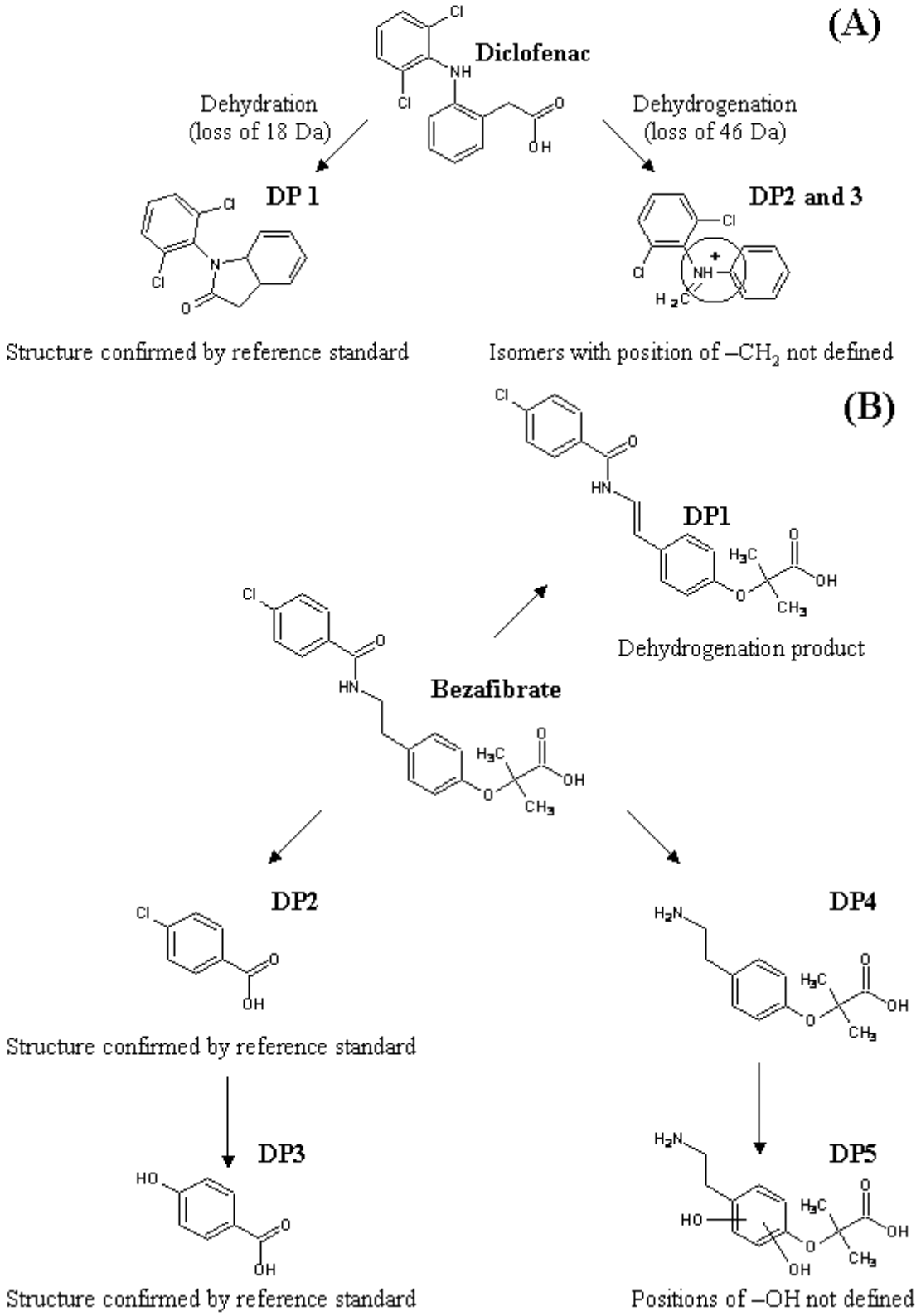


Figure 2.3. Biological degradation products (DP) of diclofenac (A, adapted from Kosjek *et al.*, 2009) and the proposed biotransformation pathway of bezafibrate (B, adapted from Helbling *et al.*, 2010)

2.4.2. Chemical Oxidation

Titanium dioxide photocatalysis (Carbonaro *et al*., 2013) and Fenton chemistry (i.e., catalytic oxidation of hydrogen peroxide) (Chen *et al*., 2012) have been applied to water treatment however ozone is the most well established and studied of the chemical processes for drug removal. Ozone treatment enhances the removal of all drugs including carbamazepine where removals of ≥96 % are observed (Ternes *et al*., 2003; Rosal *et al*., 2010; Schaar *et al*., 2010; Reungoat *et al*., 2011; Yang *et al*., 2011) (Figure 2.2, Table 2.6). However, typical ozone doses applied during water treatment often do not enable full mineralisation of drugs (Huber *et al*., 2004), likely to be caused by the clouding influence of the matrix. Wastewaters contain relatively high concentrations of bulk organics which can shield targeted chemicals from removal, quenching the ozone dose. Furthermore, Huber *et al* (2004) observed that following removal of EE2 from clean water by a high ozone dose, a slow re-appearance of the drug (0.1-0.5 % of the initial concentration) occurred. It is hypothesised that some EE2 is in the form of hydroperoxides which are not readily reactive to ozone. This could be greater in wastewater where clouding will reduce reaction kinetics and this will hinder the complete mineralisation of the parent drug. Degradation by ozonation can occur selectively by direct ozone attack itself and non-selectively by hydroxyl radicals formed upon ozone decay (Huber *et al*., 2004). Ozone reacts rapidly with phenols at neutral or basic pH (Hoigne and Bader, 1983) therefore it will readily attack the phenolate anion of EE2 and oxytetracycline. It also selectively attacks amines and double bonds of aliphatic chemicals (Snyder *et al*., 2003). The chemical structure of all the drugs considered here (except ibuprofen) are highly susceptible to direct ozone attack (Table 2.2). Hydroxyl radicals are less selective and react with a range of chemical functional groups. The non-selective behaviour of the hydroxyl radicals can induce complex reaction pathways (Huber *et al*., 2004). Numerous authors observed high removals of various drugs by ozone treatment, often to concentrations below their MQLs (Ternes *et al*., 2003; Rosal *et al*., 2010; Schaar *et al*., 2010). However, complete removal of the parent drug does not necessarily represent removal of toxicity. Structurally similar degradation products of potential toxicity can be formed and remain undetected using conventional MS/MS (i.e., QqQ). A large number of degradation products for various drugs have been observed (Huber *et al*., 2004; Coelho *et al*., 2009). Non-target screening of ozone treated water enabled determination of 17 degradation products of diclofenac (Coelho *et al*., 2009) (Figure 2.4). The majority of these products are structurally similar to the parent drug indicating similar behaviour in the receiving environment. Again these were identified by Oasis HLB extraction and QqTOF detection. Accurate mass spectra were collected at mass to charge (m/z) ratios >50 encompassing all degradation products of notable size.

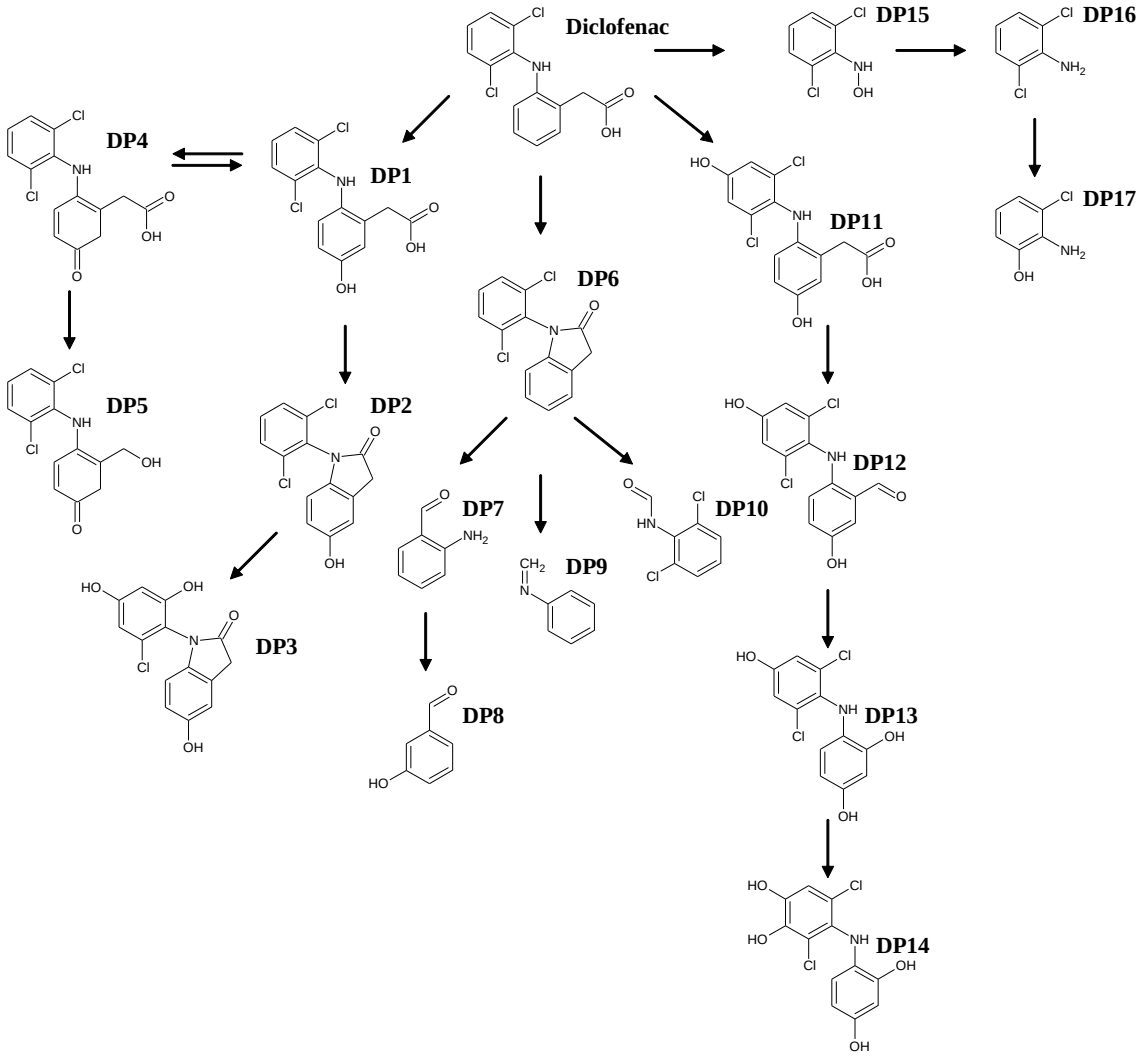


Figure 2.4. Degradation intermediates formed by ozone treatment of diclofenac and proposed degradation pathways (adapted from Coelho *et al*., 2009). Position of some functional groups not defined.

Table 2.6. Removal of drugs from environmental waters by ozone treatment

Drug	Process	Chemical dose	HRT (h^{-1})	Wastewater type	Upfront process	SE. conc. ($ng\ l^{-1}$)	TE conc. ($ng\ l^{-1}$)	Removal (%)	Reference
Ibuprofen	Ozone	10-15 mg l^{-1}	0.3	Municipal	ASP	130	<50	>62	Ternes *et al*., 2003
	Ozone	2 mg l^{-1}	0.2	Surface waters	-	-	-	40-77	Huber *et al*., 2003
	Ozone	3 mg l^{-1}	0.5	Municipal	ASP + SF	-	-	>46[a]	Nakada *et al*., 2007
Diclofenac	Ozone	0.6 g O_3 g DOC_0^{-1}	-	Municipal	ASP	2,000	<10	>99	Schaar *et al*., 2010
	Ozone	3 mg l^{-1}	0.3	Municipal	-	-	-	92	Reungoat *et al*., 2011
	Ozone	50 µM	<0.1	Municipal	-	433	<1	>99	Rosal *et al*., 2010
	Ozone	10-15 mg l^{-1}	0.3	Municipal	ASP	1,300	<50	>96	Ternes *et al*., 2003
	Ozone	0.6 g O_3 g DOC_0^{-1}	-	Municipal	ASP	900	<1	>99	Schaar *et al*., 2010
Carbamazepine	Ozone	3 mg l^{-1}	0.3	Municipal	-	-	-	96	Reungoat *et al*., 2011
	Ozone	130 µM	0.1	Municipal	-	106	<1	>99	Rosal *et al*., 2010
	Ozone	1 mg l^{-1}	-	Municipal	GAC	67	1	99	Yang *et al*., 2011
	Ozone	10-15 mg l^{-1}	0.3	Municipal	ASP	2,100	<50	>98	Ternes *et al*., 2003
Naproxen	Ozone	3 mg l^{-1}	0.5	Municipal	ASP + SF	-	-	>99[a]	Nakada *et al*., 2007
	Ozone	10-15 mg l^{-1}	0.3	Municipal	ASP	100	<50	>50	Ternes *et al*., 2003
Bezafibrate	Ozone	0.6 g O_3 g DOC_0^{-1}	-	Municipal	ASP	1,500	345	77	Schaar *et al*., 2010
	Ozone	340 µM	0.3	Municipal	-	115	4	97	Rosal *et al*., 2010
Fluoxetine	Ozone	2 mg l^{-1}	0.2	Surface waters	-	-	-	>98	Huber *et al*., 2003
	Ozone	50 µM	<0.1	Municipal	-	17	<2	>88	Rosal *et al*., 2010
Ketoprofen	Ozone	3 mg l^{-1}	0.5	Municipal	ASP + SF	-	-	73[a]	Nakada *et al*., 2007
	Ozone	340 µM	0.3	Municipal	-	162	3	98	Rosal *et al*., 2010
EE2	Ozone	7.5 mg l^{-1}	0.3	Municipal	ASP + SF	7.4	<2.1	>72.0	Schaar *et al*., 2010
	Ozone	1 mg l^{-1}	0.3	Municipal	ASP	200[b]	<20.0	>90.0	Hashimoto *et al*., 2006
	Ozone	3 mg l^{-1}	0.3	Municipal	ASP	200[b]	<0.7	>99.6	Hashimoto *et al*., 2006
Ofloxacin	Ozone	15 mg l^{-1}	0.5	Municipal	ASP + SF	0.2	<0.1	>50.0	Gunnarsson *et al*., 2009
	Ozone	340 µM	0.3	Municipal	-	3,594	10	>99	Rosal *et al*., 2010
Erythromycin	Ozone	90 µM	<0.1	Municipal	-	72	<10	>86	Rosal *et al*., 2010
	Ozone	1 mg l^{-1}	-	Municipal	GAC	28	2	93	Yang *et al*., 2011
Propranolol	Ozone	10-15 mg l^{-1}	0.3	Municipal	ASP	620	<50	>92	Ternes *et al*., 2003
	Ozone	10-15 mg l^{-1}	0.3	Municipal	ASP	180	<50	>72	Ternes *et al*., 2003

[a]average of four sampling campaigns [b]spiked concentration

Key: HRT, hydraulic retention time; SE, secondary effluent; TE, tertiary effluent; ASP, activated sludge plant; SF, sand filter; GAC, granular activated carbon

2.4.3. Adsorption by activated carbon

Activated carbon often contained in a packed bed or filter is a highly porous medium offering a large internal surface area for sorption to take place. Performance is dependent on activated carbon properties (e.g., pore size, surface charge) and solute characteristics (e.g., shape, size) (Snyder *et al*., 2007). Preferential attraction to the activated carbon surface is by hydrogen bonding and London forces creating a strong binding affinity. Even moderately hydrophobic chemicals (log K_{ow} >2) have a high propensity to removal (Snyder *et al*., 2003). The availability of some drugs as anions causes them to be attracted to the carbon surface. As a result substantial removals of hydrophobic and hydrophilic drugs have been observed by granular activated carbon (GAC) (Kim *et al*., 2007; Reungoat *et al*., 2011; Yang *et al*., 2011) (Figure 2.2, Table 2.7). Carbamazepine removals up to 97 % have been achieved (Reungoat *et al*., 2011). Despite the very hydrophilic nature of oxytetracycline (Table 2.1), its large molecular size is likely to be entrapped within the highly porous structure of the activated carbon. At full-scale treatment processes low removals have been observed for some drugs; ibuprofen (16 %) (Snyder *et al*., 2007), carbamazepine (16-23 %) (Snyder *et al*., 2007; Grover *et al*., 2011) and propranolol (17 %) (Grover *et al*., 2011) (Table 2.7). The quality of the secondary effluent will have a significant influence on the performance of GAC through the competition for available sorption sites (Snyder *et al*., 2007). The frequency of replacement/regeneration of the activated carbon medium is another controlling factor to its success, especially whilst treating wastewaters comprising relatively high concentrations of bulk organics. This may account for large variations in drug removals observed between processes. Chiu *et al* (2013) demonstrated the possibility of *in-situ* catalytic regeneration of GAC using iron nano-catalysts. This could provide an effective means of regeneration in the future, limiting variations in performance currently observed.

Table 2.7. Removal of drugs from environmental waters by adsorption processes

Drug	Process	HRT (h^{-1})	Wastewater type	Upfront process	SE. conc. ($ng\ l^{-1}$)	TE conc. ($ng\ l^{-1}$)	Removal (%)	Reference
Ibuprofen	GAC	0.3	Municipal	ASP	64	<10	>84	Yang *et al*., 2011
	GAC	-	Raw water	-	23	<1	>96	Kim *et al*., 2007
	GAC	-	Surface water	-	1.1	<1	>10	Snyder *et al*., 2007
	GAC	-	-	SF	8,760	7,325	16	Snyder *et al*., 2007
Diclofenac	GAC	0.3	Municipal	ASP	99	<10	>90	Yang *et al*., 2011
	GAC	-	Municipal	ASP	-	-	>98	Grover *et al*., 2011
	BAC	2.0	Municipal	MBR	-	-	92	Reungoat *et al*., 2011
	GAC	-	-	SF	3.2	<1	>69	Snyder *et al*., 2007
Carbamazepine	GAC	0.3	Municipal	ASP	250	67	73	Yang *et al*., 2011
	GAC	-	Municipal	ASP	-	-	23	Grover *et al*., 2011
	BAC	2.0	Municipal	MBR	-	-	97	Reungoat *et al*., 2011
	GAC	-	Raw water	-	8	<1	>87	Kim *et al*., 2007
	GAC	-	Surface water	-	2.2	<1	>55	Snyder *et al*., 2007
	GAC	-	-	SF	199	168	16	Snyder *et al*., 2007
Propranolol	GAC	-	Municipal	ASP	-	-	17	Grover *et al*., 2011
EE2	GAC	-	Municipal	ASP	-	-	>43	Grover *et al*., 2011
Erythromycin	GAC	0.3	Municipal	ASP	270	28	90	Yang *et al*., 2011
	BAC	2.0	Municipal	MBR	-	-	92	Reungoat *et al*., 2011

Key: HRT, hydraulic retention time; SE, secondary effluent; TE, tertiary effluent; ASP, activated sludge plant; GAC, granular activated carbon; BAC, biologically activated carbon; MBR, membrane bioreactor

2.5. FUTURE TRENDS

Secondary effluents typically demand analytical MQLs in the low ng l^{-1} range to determine most drugs. Reported analytical methods are well suited for determining drugs in the aqueous phase of secondary effluents. To demonstrate, no data in Figure 2.1 (and Table 2.4) was reported <MQL (n=80). Despite proposed legislative targets being applied to the aqueous phase of wastewaters (i.e., a pre-filtered sample); suspended solids can provide a pathway to their release into the environment (Baker and Kasprzyk-Hordern, 2011a). Their determination in the particulate phase is also essential for fate evaluation. Suspended solids are ubiquitous to wastewaters and can vary spatially and temporally. Particulate bound drugs often go undetermined (Table 2.3), owing to the complexity of the matrix and the additional analytical requirements it demands. The proposed requirement to undertake particulate phase analysis to determine drug fate is most pertinent to secondary processes which receive relatively high concentrations of suspended solids. The relatively hydrophobic nature of some drugs can cause them to partition well to solids. For example crude wastewaters can contain >50 % of fluoxetine bound to particulates (Baker and Kasprzyk-Hordern, 2011a). Monitoring here enables complete process mass balances to be determined, aiding fate and performance understanding. Particulate fate understanding may indicate a clouding influence during treatment which limits removal. Activated sludge sorption and biodegradation may be restricted for drugs associated with particulates in the receiving wastewater. This could lead to conventional process optimisation to enhance drug removal. For example, the use of micro-screens in place of conventional primary sedimentation tanks could enhance particulates removal from the crude stream. However, there is a substantial gap between drug concentrations achieved by the current operations of existing secondary assets and proposed legislative targets (Gardner *et al*., 2012) (Table 2.2, Table 2.4). Therefore there is an expectant need for tertiary treatment technologies to target these specific chemicals.

Tertiary treatment processes enhance drug removal, significantly reducing effluent concentrations. To fully ascertain tertiary process performance, analytical methods require MQLs <10 ng l^{-1} (Rosal *et al*., 2010; Schaar *et al*., 2010; Yang *et al*., 2011; Zearley and Summers, 2012), and ideally <1 ng l^{-1} (Kim *et al*., 2007; Snyder *et al*., 2007; Rosal *et al*., 2010; Schaar *et al*., 2010) (Tables 2.5, 2.6 and 2.7). This poses a further analytical challenge as such concentrations cannot be ascertained for the majority of drugs with current MQLs. To illustrate, concentrations of the representative drugs; ibuprofen, diclofenac and carbamazepine in sand filtration, ozone and activated carbon treated effluents were reported below MQL in 49 % of cases (n=35) (Figure 2.2). Despite these being below proposed legislative targets for most drugs (Table 2.2), monitoring at these concentrations is needed as the cumulative toxicological effect of drugs is not known. This could result in a future reduction in

legislative requirements. To illustrate, the previous EE2 predicted no effect concentration (PNEC) in the UK was 0.1 ng l^{-1} (Environment Agency, 2000). The proposed EQS is now 0.035 ng l^{-1} following its proposal as a priority hazardous chemical (European Commission, 2012). This has created a serious analytical burden as such concentrations are now beyond current analytical capabilities (Petrie *et al*., in press). Lowering current MQLs is also needed to assess breakdown reaction completeness. The first stage of this is to determine parent drug removal. Further investigation of specific breakdown mechanisms to understand the criticality between parent drug final concentration, and degradation product production is needed. Methodologies to quantify the full range degradation products will be restricted in the short term by the lack of unique reference standards available for these. However, the identification of numerous degradation products in both biological and chemical processes has brought attention to their presence and created a demand for their commercial availability (Figures 2.3 and 2.4). The complimentary use of biological assays would improve understanding of the synergistic toxicological effect of multiple drugs and their degradation products at low concentration. Process design and operation must integrate the removal of these intermediates which can be of greater concern than the parent chemical due to their subsequent transformation to more toxic chemicals in the environment (Petrie *et al*., 2013a).

Lowering drug MQLs requires existing analytical method optimisation. The low recoveries (<50 %) typically stipulated prior to internal standard correction (Gracia-Lor *et al*., 2011), can be improved to reduce the achievable MQL. Baker and Kasprzyk-Hordern (2011b) gave an excellent account of sample preparations parameters which can influence recovery. For example drugs can be adsorbed onto glassware surfaces during handling and processing. Using silanised SPE extract vials gave recoveries six times higher than non-silanised vials for some drugs. All glassware used during sample collection and processing requires silanisation to ensure maximum recoveries. Silanisation of glassware is not mentioned in the procedures of most reported analytical methods (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Grabic *et al*., 2012; Gros *et al*., 2012). Improving chromatographic separations could also significantly increase detection capabilities. Incorporating a large number of drugs into a single short UPLC run (<10 minutes) results in a number of co-eluting peaks (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Gros *et al*., 2012). Despite the use of mass scanning windows which typically range from 0.3 to 2 minutes in length for UPLC separations (López-Serna *et al*., 2011; Gros *et al*., 2012), sensitivity can be lost whilst simultaneously scanning for a number of transitions registered at the same time (López-Serna *et al*., 2011). To demonstrate, Gros *et al* (2012) reports up to 10 drugs co-eluting within a 0.1 minute time period with scanning windows of 0.5 minutes. Thus, only monitoring for one drug (of most criticality) in this time period could increase sensitivity and notably reduce the MQL.

Tertiary processes receive secondary effluents comprising comparatively high concentrations of dissolved organics (e.g., colloids). However, knowledge of drug behaviour in the charged colloidal fraction of wastewater is limited. Shen *et al* (2012) successfully showed humic acid, a small molecular weight charged species could effectively retain the hormone, estrone in solution. This can restrict sorption in tertiary processes characterised by very short contact times. Furthermore, the complexity of the colloidal fraction and its interaction with the drugs could also lead to incomplete breakdown reactions in both biological and chemical processes. A fractionation step during sample pre-treatment to separate dissolved colloids by molecular weight will aid this. It is postulated that drugs will preferentially be in specific size fractions. This is likely to vary between drugs due to the range of physico-chemical behaviour they exhibit. Understanding drug fate in the colloidal fraction of wastewater is essential for tertiary process selection, diagnosis and optimisation.

2.6. CONCLUSION

Advances in both quantitative and qualitative determinations of pharmaceutical drugs have aided the understanding of their occurrence and fate during wastewater treatment. A robust understanding of tertiary process performance is now needed by improving analytical focus. An appropriate treatment strategy could then be implemented to ensure adequate protection of the aquatic environment is achieved.

CHAPTER 3
APPLICATION OF ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF STEROID ESTROGENS IN WASTEWATERS

ACCEPTED FOR PUBLICATION: *International Journal of Environmental Analytical Chemistry*

3. APPLICATION OF ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE DETERMINATION OF STEROID ESTROGENS IN WASTEWATERS

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ABSTRACT

An ultra-performance liquid chromatography method using a triple quadrupole mass spectrometer was developed and validated for the determination of steroid estrogens in wastewater matrices. To date, analytical methods established in the literature for 17α-ethinylestradiol have been unable to achieve the proposed predicted no effect concentration of 0.1 ng l^{-1}. The extensive sample pre-treatment and analytical methodology proposed herein enables 17α-ethinylestradiol to be determined at very low background concentrations with a theoretical method detection limit of 0.06 ng l^{-1} which has been applied in real environmental matrices. During the validation process, a trickling filter wastewater treatment works was monitored to demonstrate the methods application. Estrogen removal across the filters demonstrated good removals of natural free estrogens (≥62.0 %) with lower removals of the synthetic estrogen 17α-ethinylestradiol (29.2 %) from wastewaters at 10 °C. The methods application illustrates its capability of detecting estrogen concentrations in real wastewater samples comprising complex organics of comparatively high concentration. Furthermore, a complete process mass balance for 17α-ethinylestradiol is now attainable which has previously posed a challenge due to the low environmental concentrations typically exhibited, but more significantly as a result of the lower sensitivity inherent in previously reported analytical methods.

3.1. INTRODUCTION

Steroid estrogens present in the aquatic environment at ng l^{-1} concentrations can act as endocrine disrupting chemicals (Lai *et al*., 2002a; Urbatzka *et al*., 2012) (EDCs) which exert adverse health effects on the sexual characteristics of fish (Solé *et al*., 203; Kidd *et al*., 2007; Woods *et al*., 2011). It is estimated that substantial estrogenic activity in surface waters is attributable to the presence of free steroid estrogens which are discharged in sewage effluents (Chui *et al*., 2009). Estrogens detected in municipal wastewaters are: estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2) and the conjugate, estrone 1-3 sulphate (E1-3S) (Gomes *et al*., 2004a; Gomes *et al*., 2005; Kumar *et al*., 2009). Wastewater treatment works (WwTWs) are critical to controlling the discharge of hazardous substances, including estrogens, to the aquatic environment (Bedding *et al*., 1982). Aerobic biological WwTWs are a major process type used in the UK with a significant portion of these being fixed film processes. Three of the main water utilities in England and Wales use fixed film processes at 70 % of their sites (Xuo and Hickey, 2007; UKWIR, 2009) and this is mirrored internationally (Tilley, 2011). Interestingly, there is a paucity of data in the literature regarding the factors controlling the fate and behaviour of estrogens in such processes. Undoubtedly the analytical difficulties of determining these types of compounds in such complex matrices at environmental concentrations (ng l^{-1}) has been a major constraint on such research (Buisson *et al*., 1984; Robertson and Lester, 1994). Determining the removal of estrogens across WwTWs and the concentrations discharged into the receiving environment is essential for environmental protection and monitoring plant performance.

The analysis of estrogens in environmental matrices requires sophisticated analytical methodologies, involving extraction and clean-up sample pre-treatments. Such extensive clean-up of samples is required due to the complex heterogeneous composition of wastewaters. The synthetic estrogen EE2 is of most environmental concern despite its low wastewater concentrations due to its high estrogenic potency and relative persistence to biodegradation in wastewaters and surface waters. There are continuing requirements to lower the EE2 detecting capabilities of analytical methodologies. The lowest reported method detection limit (MDL) of this compound in aqueous wastewater matrices is 0.2 ng l^{-1} (Koh *et al*., 2007). This is currently insufficient to monitor background concentrations of EE2 at and below the predicted no effect concentration (PNEC) of 0.1 ng l^{-1} proposed by the Environment Agency of England and Wales (Environment Agency, 2000). High performance liquid chromatography (HPLC) with mass spectrometry detection is favoured for analysis due to the low detection limits attainable and further sample pretreatments such as chemical derivatization are not needed (Stanford and Weinberg, 2007). However, the desire to improve analysis time, sensitivity and separation efficiency has led to the introduction of ultra-

performance liquid chromatography (UPLC) (Farré *et al*., 2007; Kasprzyk-Hordern *et al*., 2009). Here column efficiency is inversely proportional to stationary phase particle size as described by the van deemter equation (de Villiers *et al*., 2006). Ultra performance liquid chromatography delivers high pressures which enables columns packed with smaller particle sizes to approach their theoretical performance. This cannot be exploited by HPLC due to the limited operating pressures inherent of such instruments (Swartz, 2005). This study develops a UPLC method for the analysis of steroid estrogens in wastewater matrices (aqueous/particulate) using existing extraction methodology. The advances of utilising UPLC as the analytical separation method were assessed by comparing the performance to an existing HPLC method. The method was applied to determine the performance of a trickling filter for the removal of steroid estrogens during low temperature operation.

3.2. EXPERIMENTAL

3.2.1. Materials

All steroid estrogens (>98 % purity) were purchased from Sigma-Aldrich (Dorset, UK). Deuterated internal standards; estrone-2,4,16,16-d_4 (E1-d_4), 17β-estradiol-2,4,16,16,17-d_5 (E2-d_5), estriol-2,4,17-d_3 (E3-d_3), 17α-ethynylestradiol-2,4,16,16-d_4 (EE2-d_4) and sodium estrone-2,4,16,16-d_4 sulfate (E1-3S-d_4) were obtained from QMX laboratories (Thaxted, UK). Individual stock solutions of 1 mg ml^{-1} were prepared in methanol (MeOH) and stored at -20 °C. Working standard mixtures of estrogen concentrations; 0, 1, 5, 10, 25, 50, 75 and 100 ng ml^{-1}, containing 75 ng ml^{-1} of the deuterated surrogates were prepared daily. Methanol, dichloromethane (DCM), ethyl acetate (EtOAc) and hexane were obtained from Rathburn Chemicals (Walkerburn, UK) and were HPLC grade. Ammonium hydroxide (NH_4OH) of ACS grade was purchased from Sigma Aldrich (Dorset, UK) and UP water of 18.2 MΩ quality (Elga, Marlow, UK) was used in the preparation of mobile phases. Wastewater samples (settled sewage and final effluent) were collected in borosilicate glass vessels with teflon lined caps from a trickling filter site of population equivalent 3,000 in the East of England, UK. Corresponding grab samples of settled sewage and filter effluent were collected over a 3 day period to determine process performance. Samples were filtered and loaded onto solid phase extraction (SPE) cartridges within 30 minutes of collection and once dried, stored at -20 °C prior to further clean up.

3.2.2. Extraction process

Settled sewage and final effluent samples (1 l) were filtered under vacuum through 1.2 µm GF/C filters (VWR, Lutterworth, UK) prior to SPE. Filter papers with retained solids were frozen before extraction. The aqueous phase was loaded onto 500 mg: 6 cc tC18 cartridges (Waters, Elstree, UK) preconditioned with 5 ml MeOH then 5 ml UP water. Cartridges changes were made after 500 ml to avoid cartridge saturation. The flow rate was controlled between 5 ml min^{-1} and 10 ml min^{-1} using a vacuum manifold. The cartridge was then washed with 5 ml UP H_2O and dried for 60 minutes under vacuum. Analytes were eluted using 10 ml MeOH followed by 10 ml DCM. Extracts were concentrated to 1 ml by rotary evaporation (Heidolph Instruments, Schwabach, Germany) then to complete dryness under a gentle stream of nitrogen gas. The dried sample was reconstituted in 0.2 ml DCM: MeOH (90: 10). The sample was then subject to clean up by gel permeation chromatography (GPC) by injection onto a 5 µm, 300 mm x 7.5 mm gel permeation size exclusion column (Varian, Oxford, UK) under isocratic conditions (DCM: MeOH, 90: 10 v/v) at a constant flow rate of 1 ml min^{-1} and the fraction between 5.5 minutes and 11.5 minutes collected. The 6.0 ml fraction was evaporated to approximately 0.2 ml under a nitrogen stream then reconstituted to 2 ml with hexane. This was loaded onto a preconditioned (2 ml hexane) 500 mg: 6 cc NH_2 SPE cartridge (Varian, Oxford, UK), at a flow rate between 5 ml min^{-1} and 10 ml min^{-1}. The cartridge was then washed using 4 ml EtOAc: hexane (10: 90). Non polar steroids (E1, E2 and EE2) were eluted using 6 ml EtOAc. The polar steroids (E1-3S and E3) were eluted separately using 6 ml of 3 % NH_4OH in MeOH. Separate eluants were blown to dryness under a gentle stream of nitrogen gas, then reconstituted with 0.2 ml UP H_2O: MeOH (80: 20) containing 0.1 % NH_4OH and divided between two identical autosampler vials prior to quantification by HPLC-MS/MS and UPLC-MS/MS. Prior to extraction the aqueous phase samples were spiked with 15 ng l^{-1} deuterated estrogens. These were used to monitor the extraction process and to correct for any variability in detector response. To ensure analytical reliability; both low (2 ng l^{-1}) and high (15 ng l^{-1}) spikes of additional mixed estrogens were used to determine recoveries. These spike levels represent estrogen concentrations approximately 1 order of magnitude greater than the MDL, and those concentrations expected to be found in real sample matrices (Koh *et al*., 2007; McAdam *et al*., 2010).

For the settled sewage and final effluent samples, filter papers were freeze-dried, shredded and extracted using 10-20 ml EtOAc whilst being mechanically agitated using a multi-reax system (Heidolph Instruments, Schwabach, Germany) in a 25 ml Teflon tube for 60 minutes. This was followed by centrifugation at 1500 x *g* for 10 minutes. The extraction was repeated twice then the combined supernatants were evaporated to approximately 0.2 ml and reconstituted to 2 ml with hexane. The sample was subjected to SPE by passing through a

500 mg: 3 ml silica cartridge (Waters Ltd., Watford, UK) preconditioned with 2 ml hexane at constant flow rates of between 5 ml min^{-1} and 10 ml min^{-1}. Analytes were eluted using 3 ml EtOAc followed by 2 ml MeOH ensuring the cartridge media remained saturated and then evaporated to dryness on a rotary evaporator prior to reconstitution in 0.2 ml DCM: MeOH (90: 10). The extracts were then subject to the same clean up procedure as the aqueous samples by GPC and normal phase SPE. To ensure analytical reliability all samples prior to freeze drying were spiked with deuterated (75 ng g^{-1}) estrogens and both low (10 ng g^{-1}) and high spikes (75 ng g^{-1}) to determine recoveries. A summarised representation of the extraction procedure for aqueous and particulate phases is outlined (Figure 3.1).

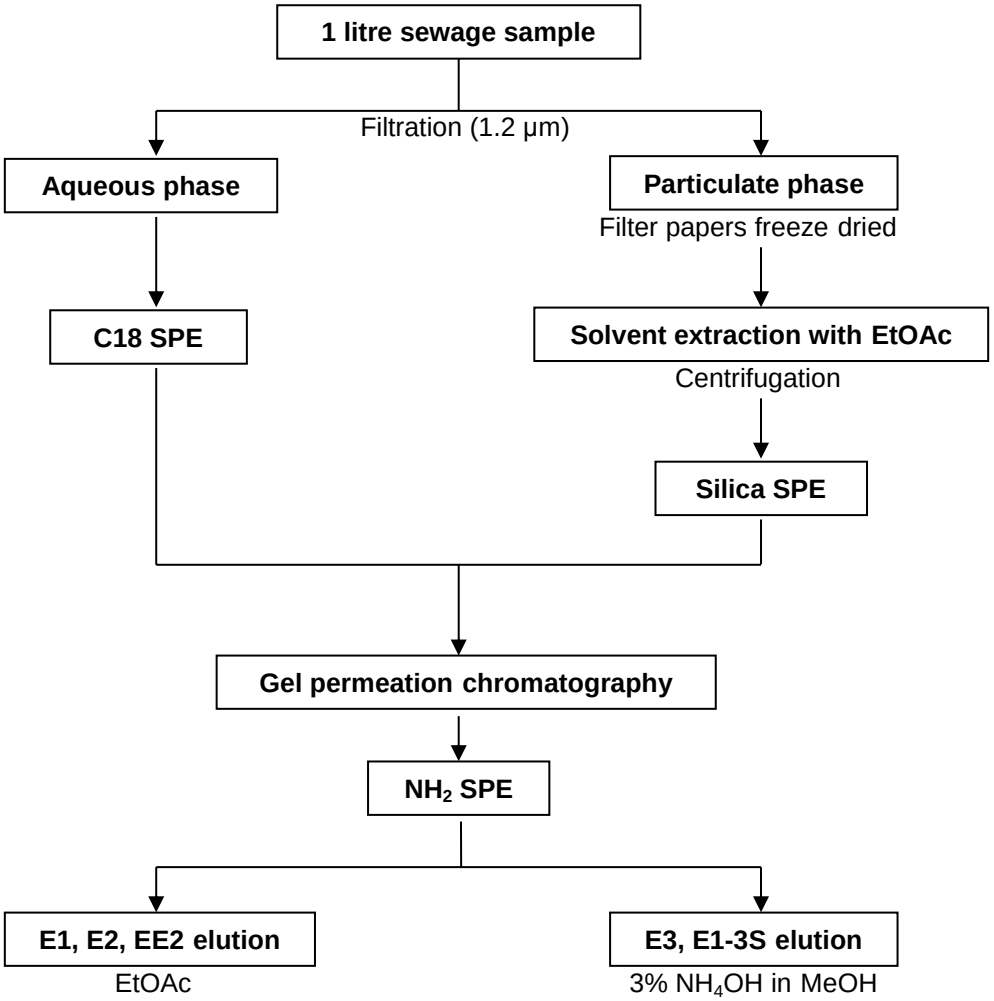

Figure 3.1. Summarised extraction methodology for aqueous and particulate phases

3.2.3. High performance liquid chromatography

High performance liquid chromatography was performed using a Waters Alliance 2695 system (Waters, Manchester, UK). Separations were achieved using a Gemini C18 column (100 mm x 2.0 mm i.d., 3.0 µm particle size; Phenomenex, Macclesfield, UK) maintained at 35 °C. A gradient separation was achieved using water containing 0.1 % NH_4OH (A) and methanol containing 0.1 % NH_4OH (B) at a constant flow rate of 0.2 ml min^{-1}. Initial conditions of 80 % A and 20 % B were increased to 50 % B over 3.5 minutes and then further increased to 60 %. This was maintained for 9 minutes before returning to starting conditions over 3 minutes and held for 2.5 minutes for equilibration. The total run time was 18 minutes and an injection volume of 20 µl was used. This method has been previously validated and applied to quantify the concentration of steroid estrogens in wastewater matrices (Koh *et al*., 2007; Chiu *et al*., 2009; McAdam *et al*., 2010).

3.2.4. Ultra-performance liquid chromatography

Ultra performance liquid chromatography method development was performed using a Waters Acquity UPLC (Waters, Manchester, UK). Separations were achieved using an Acquity UPLC BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm particle size; Waters, Manchester, UK) maintained at 45 ˚C. A gradient separation of two mobile phases was utilised consisting of water containing 0.1 % NH_4OH (A) and methanol containing 0.1 % NH_4OH (B) at a constant flow rate of 0.2 ml min^{-1}. Gradient elutions were tested to determine which would give adequate separation of the analytes and matrix not removed by the extraction and clean up processes. Initial conditions of 80 % A and 20 % B were increased gradually to 80 % B over 6 minutes and maintained for 1 minute. This was then returned to starting conditions for 2 minutes for equilibration. These conditions gave adequate separation of analytes in a reduced run time of 9 minutes. The maximum injection volume which did not result in band broadening and loss of efficiency was 10 µl and was used throughout the investigation.

3.2.5. MS/MS detection

Detection was achieved by a Waters Quattro Premier XE mass spectrometer with a Z-spray ESI interface (Micromass, Watford, UK) for both HPLC and UPLC separations. This was operated in the negative electrospray ionisation mode utilising multiple reaction monitoring (MRM). Two MRM transitions were monitored for each estrogen compound; the most sensitive transition monitored for quantitation and the second for analyte confirmation. The optimised MS/MS conditions for the detection of steroid estrogens are outlined (Table 3.1).

To maximise sensitivity the detection of estrogens was divided into two acquisition periods. Period 1 monitored for E1-3S and E3 and period 2 for those later eluting, less polar estrogens; E1, E2 and EE2. The parameters for detection by the mass spectrometer were as follows: capillary voltage, 3.20 kV; multiplier voltage, 650 V; desolvation gas flow, 1000 l h^{-1}; cone, -55 V; RF lens, 0.2 V; cone gas flow, 49 l h^{-1}; desolvation temperature, 350 °C and source temperature, 120 °C (Koh *et al*., 2007).

Table 3.1. Optimised LC-MS/MS parameters for MRM transitions of steroid estrogens

Compound	Molecular formula	Molecular weight (g mol^{-1})	MRM[a] (m/z)	Dwell time (ms)	Collision energy (V)	Cone (V)
E1[d]	$C_{18}H_{22}O_2$	270.37	269.10 > 144.85	85	40	70
			269.10 > 158.80[b]	85	45	70
E2[d]	$C_{18}H_{24}O_2$	272.38	271.10 > 144.85	85	45	60
			271.10 > 158.80[b]	85	40	60
E3[c]	$C_{18}H_{24}O_3$	288.39	287.10 > 170.85	95	50	55
			287.10 > 144.85[b]	95	50	55
EE2[d]	$C_{20}H_{24}O_3$	296.40	295.15 > 144.85	85	40	60
			295.15 > 158.80[b]	85	40	60
E1-3S[c]	$C_{18}H_{22}O_5S$	350.50	349.05 > 144.85	60	65	50
			349.05 > 269.00[b]	60	40	50
E1-d_4[d]	$C_{18}D_4H_{18}O_2$	274.40	273.10 > 146.85	85	45	60
E2-d_5[d]	$C_{18}D_5H_{19}O_2$	277.42	276.10 > 146.85	85	50	55
E3-d_3[c]	$C_{18}D_3H_{24}O_3$	291.40	290.15 > 146.85	90	65	50
EE2-d_4[d]	$C_{20}D_4H_{20}O_2$	300.43	299.15 > 146.85	85	50	60
E1-3S-d_4[c]	$C_{18}D_4H_{17}SNaO_5$	376.44	353.10 > 146.85	60	65	50

[a][M-H]$^-$ [b]confirmatory transition [c]Transition period 1 [d]Transition period 2

3.3. RESULTS

3.3.1. Instrument performance comparison

Utilising UPLC, in comparison to HPLC, offered reductions in the time required for chromatographic separations, with total run times of 9 minutes versus 18 minutes, respectively (Table 3.2). In terms of chromatographic separations, UPLC allowed efficiencies of 11,500 theoretical plates in comparison to HPLC and 4,500 theoretical plates. Similarly solvent consumption and sample volume requirements were reduced by 50 % with the use of UPLC. A total of 3.6 ml of mobile phase was required for an analysis run by HPLC whereas UPLC required 1.8 ml. At these reduced analysis times, sensitivities were superior to those achieved by HPLC. The signal to noise ratio (S: N) of analyte responses were generally improved by two fold offering improved instrument detection limits (IDLs) and precision

(Table 3.2). Replicate measurements of calibration standards (1 ng ml^{-1} and 10 ng ml^{-1}) gave RSDs of ≤10.2 % for all steroid estrogens by UPLC in comparison to ≤16.4 % for HPLC. Trueness expressed as bias varied from -6.3 % to 7.3 % for HPLC and from -0.5 % to 6.7 % for UPLC. Calibration curves were linear over the range of 1 ng ml^{-1} to 100 ng ml^{-1} with correlation coefficients >0.995 for all analytes for both HPLC and UPLC separations (Table 3.2).

Table 3.2. Instrumental detail for the determination of steroid estrogens by HPLC-MS/MS and UPLC-MS/MS

Method	Steroid estrogen	Retention time (min)	r^2	Precision[a] (%)		Trueness[a] (bias %)		IDL[b] (pg)
				1 ng ml^{-1}	10 ng ml^{-1}	1 ng ml^{-1}	10 ng ml^{-1}	
HPLC	E1	13.1	0.999	12.4	1.0	-6.3	-3.6	10.1
	E2	13.6	0.999	4.2	3.3	4.9	-2.0	16.6
	E3	9.1	0.997	16.4	1.7	7.3	2.3	12.4
	EE2	13.8	0.997	5.6	2.1	6.0	-2.5	11.3
	E1-3S	7.1	0.999	0.1	0.6	-5.3	-1.1	6.4
UPLC	E1	6.7	0.999	5.1	0.1	3.7	-0.5	2.4
	E2	6.8	0.995	4.2	1.2	5.7	1.8	6.4
	E3	5.0	0.999	4.3	0.6	6.7	4.3	4.5
	EE2	6.9	0.999	10.2	3.0	0.1	2.8	2.7
	E1-3S	4.1	0.999	5.1	0.6	1.3	0.1	1.1

[a]Replicate (n=3) inter-day measurements of quality control samples [b]3 times the standard deviation of replicate (n=7) UP water extractions spiked at 1 ng l^{-1}
Note: IDL=instrument detection limit

3.3.2. Environmental matrices

The MRM chromatograms of all estrogens in settled sewage spiked at 2 ng l^{-1} by HPLC-MS/MS and UPLC-MS/MS illustrate the differences in their respective chromatography (Figure 3.2). The improved sensitivity of UPLC offers reduced peak widths and apparent background noise. Those peaks produced using HPLC displayed baseline widths of ≥30 seconds in contrast to UPLC where widths of ≤15 seconds were noted. As a result those peaks produced by UPLC were 'sharper' and presented a Gaussian distribution enabling more reproducible quantitation of peak response in environmental matrices. Mean recoveries determined by UPLC and HPLC were 94.1 (+/- 5.6) % and 93.6 (+/-7.6) %, with RSDs up to 22.8 % and 24.5 % respectively (Table 3.3).

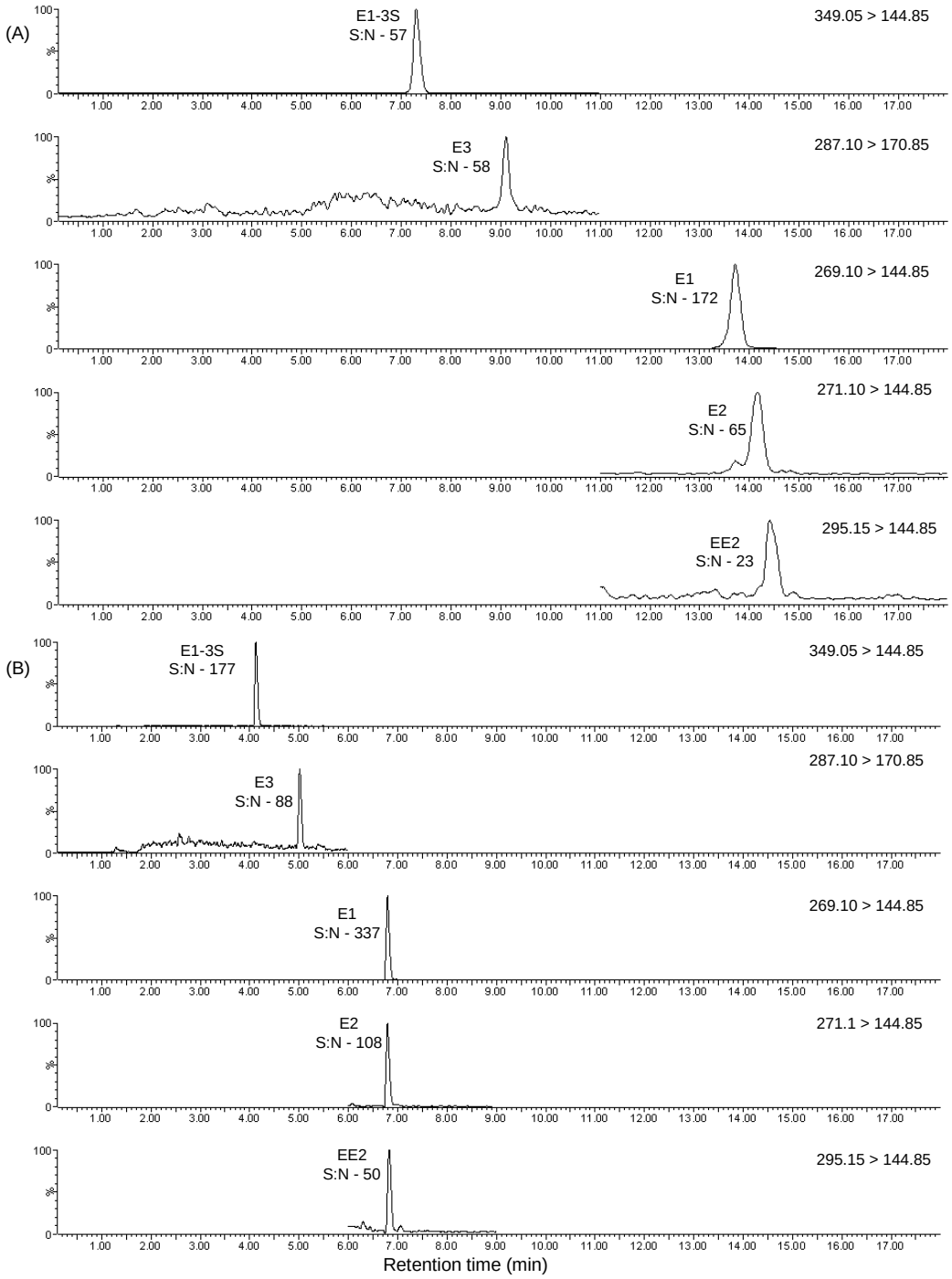

Figure 3.2. MRM chromatograms of estrogens by HPLC-MS/MS (A) and UPLC MS/MS (B) (settled sewage spiked at 2 ng l^{-1}). Note: S: N=signal to noise ratio

Table 3.3. Method recoveries (%) and relative standard deviations (RSD %) from settled sewage and final effluent matrices using high (15 ng l^{-1}) and low (2 ng l^{-1}) spikes

Method	Steroid estrogen	Aqueous phase recoveries (RSD %)		Particulate phase recoveries (RSD %)	
		Settled sewage	Final effluent	Settled sewage	Final effluent
HPLC	E1	100.3 (2.8)	93.9 (13.1)	99.2 (7.3)	103.2 (0.5)
	E2	100.9 (4.3)	97.6 (6.1)	96.8 (11.3)	85.9 (24.5)
	E3	92.2 (11.0)	96.2 (7.5)	80.5 (9.0)	103.6 (0.7)
	EE2	92.2 (5.6)	91.6 (3.7)	90.6 (9.9)	100.2 (2.4)
	E1-3S	71.3 (6.4)	99.4 (16.7)	68.9 (5.0)	107.5 (5.0)
UPLC	E1	100.7 (0.6)	97.6 (0.4)	99.0 (2.3)	101.0 (4.2)
	E2	91.1 (6.5)	96.7 (8.2)	87.9 (2.8)	87.4 (3.8)
	E3	86.8 (6.0)	88.6 (9.1)	97.4 (22.8)	104.6 (5.5)
	EE2	100.8 (6.4)	96.3 (5.4)	103.0 (2.1)	97.2 (5.3)
	E1-3S	81.9 (4.7)	93.9 (6.6)	69.6 (5.0)	101.3 (5.0)

The effect of the sample matrix on analyte signals was determined for both aqueous and particulate extractions using the algorithm reported by Vieno *et al* (2006). A maximum of 7.4 % signal suppression was observed for both polar (E3 and E1-3S) and non-polar (E1, E2, and EE2) analytes in final effluent extracts by UPLC. In contrast HPLC gave signal suppressions of between 6.8 % and 11.7 % for polar analytes and between 4.2 % and 25.9 % for non-polar analytes in final effluent extracts. In settled sewage, analyte signal suppressions of between 2.0 % and 11.4 % for polar analytes and between 5.7 % and 23.4 % for non-polar analytes were observed by UPLC. Signal suppressions of 5.1 % to 9.1 % for polar analytes and 5.2 % to 29.7 % for non-polar analytes were determined by HPLC.

The use of UPLC offered the advantage of reduced MDLs for all analytes in settled sewage and final effluent phases (Table 3.4). Method detection limits were calculated and corrected for matrix interferences using (Vieno *et al*., 2006):

$$MDL = \frac{IDL.100}{(R.C)} . \frac{100}{(100 - M)} \qquad (3.1)$$

Where *IDL* is instrument detection limit (ng l^{-1}), *R* is recovery (%), *C* is concentration factor and *M* is matrix suppression (%). High performance liquid chromatography provided MDLs of 0.08 ng l^{-1} to 0.18 ng l^{-1} for estrogens in aqueous phases and between 3.20 ng g^{-1} and 11.58 ng g^{-1} for particulate phases. In comparison UPLC gave MDLs ranging from 0.02 ng l^{-1} to 0.17 ng l^{-1} in aqueous phases and 1.12 ng g^{-1} to 8.85 ng g^{-1} in particulate phases. The MDLs in the aqueous phase of final effluents are of most significance due to the majority of estrogens being present in this phase. The MDLs achieved in this study superseded those

previously reported (Table 3.5). Considering the MDLs reported for EE2 they follow the order based on the separation and detection method; UPLC-MS/MS<HPLC-MS/MS<GC-MS/MS<GC-MS, although differences in extraction methodology may in part account for this. Importantly, the UPLC MDLs reported in this study are below their PNECs proposed by the Environment Agency of England and Wales (Environment Agency, 2000).

Table 3.4. Method detection limits of steroid estrogens in wastewater matrices

Method	Steroid estrogen	MDL in settled sewage		MDL in final effluent	
		Aqueous (ng l^{-1})	Part. (ng g^{-1})	Aqueous (ng l^{-1})	Part (ng g^{-1})
HPLC	E1	0.11	5.58	0.12	5.12
	E2	0.18	10.93	0.18	11.58
	E3	0.14	8.20	0.14	6.50
	EE2	0.14	8.95	0.14	7.68
	E1-3S	0.10	4.89	0.08	3.20
UPLC	E1	0.05	3.02	0.05	2.48
	E2	0.17	8.85	0.14	8.14
	E3	0.11	5.02	0.10	4.27
	EE2	0.06	3.42	0.06	2.96
	E1-3S	0.03	1.61	0.02	1.12

Part.=particulate

Table 3.5. Comparison of steroid estrogen PNECs and MDLs (ng l^{-1}) in wastewaters (aqueous phase)

Steroid estrogen	PNEC (Environment Agency, 2000)	MDL this study FE	MDL Koh *et al* FE (2007)[a]	MDL Liu *et al* FE (2011)[b]	MDL Belfroid *et al* FE (1999)[c]	MDL Gibson *et al* WW (2007)[d]
E1	3.0	0.05	0.12	0.05	0.3-1.0	1.0
E2	1.0	0.14	0.20	0.29	0.5-2.4	0.5
E3	-	0.10	0.22	-	-	-
EE2	0.1	0.06	0.16	0.49	0.3-1.8	2.5
E1-3S	-	0.02	0.09	-	-	-

[a]Two stage SPE, size exclusion chromatography and HPLC-MS/MS [b]Two stage SPE and rapid resolution LC-MS/MS [c]Three stage SPE, HPLC clean up, silylation and GC-MS/MS [d]SPE, derivatization and GC-MS

Note: PNEC = predicted no effect concentration, FE = final effluent, WW=wastewater

3.3.3. Application of UPLC to evaluate the removal and biodegradation across a trickling filter

The UPLC method developed was applied to the determination of steroid estrogens in wastewaters pre and post trickling filter treatment to examine removal efficiency. Corresponding grab samples were collected for settled sewage and filter effluent to compensate for any intraday variation in receiving concentration. Over the three day period, mean removals of the free natural estrogens were ≥62.0 % whereas 29.2 % of the synthetic estrogen EE2 was removed. No removal of the conjugate E1-3S was observed (Table 3.6). Estrogens were found to be predominantly distributed in the aqueous phase of wastewater (>78 %) with the exception to EE2 which had a greater proportion found in the particulate phase. The distribution of estrogens between solid and aqueous phases (log K_d) of secondary effluent was calculated using:

$$K_d = \frac{P}{A} \qquad (3.2)$$

Where P is the concentration of the analyte in the particulate phase (ng kg^{-1}) and A is the concentration in the aqueous phase (ng l^{-1}). The determined log K_d of estrogens in secondary effluent was found to range from 2.3 to 3.5 for E1-3S and EE2, respectively.

Table 3.6. Determination of steroid estrogens in wastewater at 10 (+/- 0.5) °C from a trickling filter wastewater treatment works over a three day period

Steroid estrogen	Settled sewage (ng l^{-1})		Secondary effluent (ng l^{-1})		Mean removal[a] (%)	Log K_{ow}[b]	Log K_d[c]
	Aqueous	Particulate	Aqueous	Particulate			
E1	106.2	2.3	32.4	1.3	69.0	3.1-3.4	2.5
E2	16.3	1.2	5.5	1.2	62.0	3.9-4.0	3.2
E3	66.5	0.4	16.7	0.5	74.2	2.5-2.8	2.4
EE2	0.6	0.4	0.5	0.2	29.2	3.7-4.2	3.5
E1-3S	59.2	0.9	64.0	1.7	-	NA	2.3

[a] *Removal (%)=($(\sum_{in}-\sum_{out})/\sum_{in}$) x 100 %* [b]Octanol:water partition coefficient (Lai *et al.*, 2000a; Liu *et al.*, 2009) [c]Secondary effluent partition coefficient
Note: NA = not available

3.4. DISCUSSION

3.4.1. Instrument performance

A substantial improvement in analytical performance was achieved by utilising UPLC in comparison to HPLC with the reduction in analysis time enabling a faster sample throughput doubling the number of samples which can be analysed. Ultra-performance liquid chromatography also offered the advantage of lowering solvent consumptions by 50 % which presents significant economic benefits to analytical resources in comparison to conventional HPLC (Koh *et al*., 2007). The UPLC chromatography exhibited considerable improvements in peak shape with reduced peak widths. Superior signal to noise allows greater reproducibility of peak integration particularly at reduced analyte concentrations and consequently a greater reliability in estrogen quantitation (Swartz, 2005; de Villiers *et al*., 2006). This improved sensitivity of UPLC offers reduced IDLs which will aid in the determination of steroid estrogens in wastewater matrices often present at concentrations <5 ng l^{-1} (Gomes *et al*., 2004a; Gomes *et al*., 2005; Chui *et al*., 2009; McAdam *et al*., 2010; Zhou *et al*., 2012).

3.4.2. Method performance

The success of removing background interferences in the sample clean up stages is apparent in all chromatograms. Reported recoveries were similar to those previously reported in the literature (Koh *et al*., 2007). The recoveries of estrogens in aqueous and particulate phases of settled sewage and final effluent matrices determined by UPLC and HPLC were found to be similar. The small discrepancies observed between recoveries determined by HPLC and UPLC are attributed to analytical variability. The MDLs reported for HPLC are comparable to a previous study which utilised the same methodology attaining limits varying from 0.1 ng l^{-1} to 0.2 ng l^{-1} for all estrogens in final effluent and settled sewage aqueous phases (Koh *et al*., 2007). The current HPLC MDL of EE2 in final effluent is 0.14 ng l^{-1} which is above the PNEC of 0.1 ng l^{-1} (Environment Agency, 2000). This estrogen is of greatest environmental concern due to its high estrogenic potency. It is important that these compounds can be monitored at concentrations below their respective PNECs to ensure environmental protection. The UPLC method developed; along with the 3 stage sample extraction process achieved a MDL in final effluent of 0.06 ng l^{-1} for EE2 which offers the opportunity to determine EE2 concentrations at and below the PNEC with greater reliability and confidence. The improved chromatographic separation efficiencies reduced the effect of matrix interference on analyte signal intensity, thus aiding sensitivity and providing the means to monitor and manage estrogens not only in final effluent but also in receiving waters and other exposed environment matrices (Lai *et al*., 2002a; Gomes *et al*., 2004a). The reduction in

matrix interferences by UPLC will be particularly advantageous when estrogen concentrations are close to the MDL and analyte signal suppression is most critical. There are numerous publications which have been restricted by the detection capabilities of their methods in monitoring environmental EE2 concentrations across WwTWs (Braga *et al*., 2005; Chimchirian *et al*., 2007; Stanford and Weinberg, 2007; Kumar *et al*., 2009; Zorita *et al*., 2009; Zhou *et al*., 2012). Although this method required a three stage sample extraction and clean up procedure, the benefits of this sample pretreatment were substantial considering the MDLs achieved.

3.4.3. Removal of estrogens across a trickling filter

Sewage treatment works are critical points in determining the environmental distribution of estrogens and hence their environmental (Bedding *et al*., 1982; Lai *et al*., 2002) and ecological impact (Gomes *et al*., 2003). The trickling filter achieved relatively good removals (≥62.0 %) of the natural free steroid estrogens in wastewater at 10 °C. Trickling filters are widely implemented offering a low energy wastewater treatment technology which has the potential to substantially reduce the concentration of free natural estrogens in wastewaters (Chimchirian *et al*., 2007; Koh *et al*., 2007; Dagnino *et al*., 2009). Removals of the synthetic estrogen EE2, across the trickling filter are typical to those previously reported (Koh *et al*., 2007), with 29.2 % removed reiterating its more recalcitrant nature. The effluent concentrations of E1, E2 and EE2 were greater than their PNECs of 3.0, 1.0 and 0.1 ng l^{-1}, respectively (Environment Agency, 2000), which indicates that wastewater treatment by stand-alone trickling filters is unlikely to reach proposed estrogen PNECs. Although substantial removals of estrogens were achieved using trickling filters, reaching PNEC targets in the future may require additional tertiary treatment to further enhance removals. However, care must be taken in tertiary treatment process selection so as not to cause overall environmental harm or excessive cost (Jones *et al*., 2007a). The presence of E1-3S in both settled sewage and secondary effluent at relatively high concentrations (circa 60 ng l^{-1}) during periods of low ambient temperature (10 ºC) demonstrates the need to monitor this compound considering its possible deconjugation to E1 during wastewater treatment and in the environment. The recalcitrance of E1-3S has been previously observed in trickling filters (Koh *et al*., 2007) and activated sludge (Gomes *et al*., 2009).

The majority of estrogens were found to be distributed in the aqueous phase of wastewaters yet their presence in the particulate phase makes a notable contribution to their total concentration. In settled sewage, 40 % of EE2 was found to be present in the particulate phase in contrast to E3 which had <1 % of the total concentration present in the particulate phase. The high proportion of EE2 bound to particulate matter reiterates its hydrophobicity

and the need to monitor the concentration of estrogens in this phase. Estrogen log K_d coefficients in secondary effluent correspond to their relative polarities and respective log K_{ow} values. The most lipophilic compound (EE2) had the greatest solid/liquid partition coefficient of the compounds studied in contrast to E1-3S which was most likely to be distributed in the aqueous phase of wastewater matrices.

3.5. CONCLUSION

The validated UPLC-MS/MS method was suitable for the determination of steroid estrogens at typical environmental concentrations found in wastewater matrices. The superior sensitivity and selectivity of UPLC was exploited following extensive sample cleanup. Ultra-performance liquid chromatography offers advantages over HPLC in terms of speed, (with a reduction in run time from 18 minutes to 9 minutes) improvements in analyte response and in the reduction of interferences caused by the sample matrix. Method detection limits of steroid estrogens in the aqueous phase of final effluents were determined to range from 0.02 ng l^{-1} to 0.13 ng l^{-1}, lower than their respective proposed PNEC values. The UPLC method was applied to monitor estrogen removal across a trickling filter. Relatively high removals of the natural free estrogens (≥62.0 %) were demonstrated with lower removals observed for EE2 (29.2 %). Secondary effluent concentrations of all estrogens were above their respective PNEC limits.

CHAPTER 4

THE DETERMINATION OF NONYLPHENOL AND ITS PRECURSORS IN A TRICKLING FILTER WASTEWATER TREATMENT PROCESS

PUBLISHED: *Analytical and Bioanalytical Chemistry 405 (2013) 3242-3253.*

4. THE DETERMINATION OF NONYLPHENOL AND ITS PRECURSORS IN A TRICKLING FILTER WASTEWATER TREATMENT PROCESS

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ABSTRACT

An ultra-performance liquid chromatography method coupled to a triple quadrupole mass spectrometer was developed to determine nonylphenol and 15 of its possible precursors (nonylphenol ethoxylates and nonylphenol carboxylates) in aqueous and particulate wastewater matrices. Final effluent method detection limits for all compounds ranged from 1.4 ng l^{-1} to 17.4 ng l^{-1} in aqueous phases and from 1.4 ng g^{-1} to 39.4 ng g^{-1} in particulate phases of samples. The method was used to measure the performance of a trickling filter wastewater treatment works, which are not routinely monitored despite their extensive usage. Relatively good removals of nonylphenol were observed over the biological secondary treatment process, accounting for a 53 % reduction. However, only an 8 % reduction in total nonylphenolic compound load was observed. This was explained by a shortening in ethoxylate chain length which initiated production of shorter polyethoxylates ranging from 1 to 4 ethoxylate units in length in final effluents. Modelling the possible impact of trickling filter discharge demonstrated that the nonylphenol environmental quality standard may be exceeded in receiving waters with low dilution ratios. In addition, there is a possibility that the environmental quality standard can be exceeded several kilometres downstream of the mixing zone due to the biotransformation of readily degradable short chained precursors. This accentuates the need to monitor 'non priority' parent compounds in wastewater treatment works since monitoring nonylphenol alone can give a false indication of process performance. It is thus recommended that future process performance monitoring and optimisation is undertaken using the full suite of nonylphenolic moieties which this method can facilitate.

4.1. INTRODUCTION

Nonylphenol (NP) is a xenoestrogen which exhibits estrogenic activity in aquatic environments. Identification of the possible toxic effects to aquatic organisms (Christiansen *et al*., 1998; Lye *et al*., 1999) prompted NP to be classified as a priority hazardous substance in 2000 (European Commission, 2000) leading to restrictions on NP and nonylphenol ethoxylate (NPEO) use throughout Europe (European Commission, 2003a) and the US (Soares *et al*., 2008; Lara-Martin *et al*., 2012). However, more than a decade later, NP remains detectable in treated wastewater and environmental waters in these regions at concentrations above the environmental quality standard (EQS) of 300 ng l^{-1} (Christiansen *et al*., 1998; Soares *et al*., 2008; Stanford and Weinberg, 2010; McAdam *et al*., 2011; Barber *et al*., 2012). A source of NP occurrence in wastewaters and river waters is attributed to the breakdown of the longer chained NPEOs. Under appropriate environmental conditions these parent compounds biotransform to shorter chained metabolites which exhibit estrogenic activity (Giger *et al*., 1984; McAdam *et al*., 2011; Lara-Martin *et al*., 2012). Compounds comprising 1 to 3 ethoxylate units exhibit estrogenic activity whereas those with greater than 3 units display very little, if any estrogenic activity (White *et al*., 1994). Wastewater treatment works (WwTWs) are critical in controlling the discharge of these hazardous substances to the aquatic environment (Bedding *et al*., 1982). The removal of organic hazardous chemicals during secondary wastewater treatment is mainly attributed to biological and physical processes (Langford *et al*., 2005; Soares *et al*., 2008). There is limited information regarding the factors which control the breakdown mechanism of parent NPEO compounds into NP; information vital for understanding their fate in WwTWs. In activated sludge processing it has been cited that amphiphilic NPEO biodegradation proceeds preferentially, followed by attack of the lipophilic NP moiety (McAdam *et al*., 2011) which can result in their accumulation during secondary biological treatment. Interestingly there is a paucity of information on the fate of NP and its parent compounds in trickling filters even though these processes are utilised extensively. Throughout England and Wales, three of the major water utilities employ fixed film processes at 70 % of their sites (Huo and Hickey, 2007; UKWIR, 2009), and this is reflected internationally (Tilley, 2011). These offer a relatively low energy process type characterised by shorter hydraulic retention times (HRT) than typical activated sludge processes. Considering the widespread usage of trickling filters, a greater understanding of NPEO behaviour here is essential.

Historically, most monitoring has employed analysis solely for NP and not for its precursor compounds, given that NP is listed as a substance of priority concern, due to its higher toxicity, along with the complication of incorporating a number of compounds into the same extraction and analysis method (Chang *et al*., 2004; Ghanem *et al*., 2007; Gomez *et al*., 2007;

Langdon *et al*., 2011; Moreira *et al*., 2011). Furthermore, the analytical difficulty of measuring these determinands at ng l^{-1} concentrations in such complex environmental matrices (Buisson *et al*., 1984; Robertson and Lester, 1994; Gomes *et al*., 2004a) has been a major constraint. As a result large numbers of studies have determined NP independently in WwTWs (Gomez *et al*., 2007; Leusch *et al*., 2006; Ifelebuegu, 2011), sewage sludges (Ghanem *et al*., 2007; Kouloumbos *et al*., 2008) sediments (Chang *et al*., 2004; Yuan *et al*., 2004; Sarmah *et al*., 2008), river waters (Moreira *et al*., 2011; Tao *et al*., 2011) and soils (Brown *et al*., 2009; Langdon *et al*., 2011). Analytical methodologies recently reported in the literature for monitoring trace levels of NP in wastewaters typically utilise high-performance liquid chromatography (HPLC) with mass spectrometry (MS) detection (Jahnke *et al*., 2004; Loos *et al*., 2007; Koh *et al*., 2008). Detection capabilities of such methods are suitable for determining complete NP mass balances at and below the EQS of 300 ng l^{-1} (Jahnke *et al*., 2004; Loos *et al*., 2007; Koh *et al*., 2008). However the desire to improve sensitivity, solvent consumption and sample run time has led to the introduction of ultra-performance chromatography (UPLC) (Lara-Martin *et al*., 2012). A further advantage of utilising UPLC for environmental applications is the improved separation efficiencies obtainable by operation at high pressures and reduced column particle sizes. This aids the reduction of matrix interferences known to suppress analyte signal strength (Vieno *et al*., 2006; Petrie *et al*., in press). Minimising these interferences is vital in matrices containing comparably high concentrations of complex organics. The work herein describes the development of a method for the analysis of NP, short chained carboxylates ($NP_{1-3}EC$) and NPEOs of varying chain length ($NP_{1-12}EO$) in aqueous and particulate phases of wastewater matrices using UPLC. The ability to monitor these precursor compounds in WwTWs will enable future process optimisation. This methodology was applied to determine trickling filter performance under low temperature operation (~10 °C). Finally, the possible impact of trickling filter effluent on compliance with water quality standards in a hypothetical river system was explored using a simple numerical model.

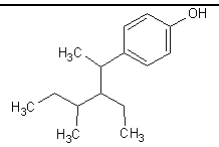
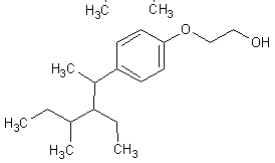
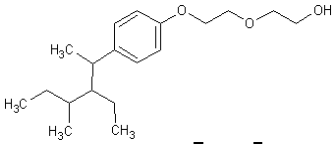
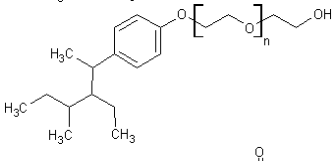
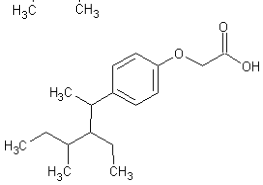
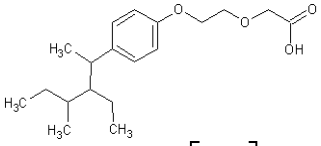
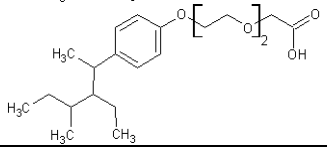
4.2. MATERIALS AND METHODS

4.2.1. Chemicals

Technical 4-nonylphenol, 4-nonylphenol-monoethoxylate (NP_1EO), the diethoxylate compound (NP_2EO) and the long chain NPEOs were purchased from Sigma Aldrich (Dorset, UK). Long chain NPEOs were purchased as the technical mixtures CO210, CO520 and CO720. Nonyl-phenoxy acetic acid (NP_1EC) was obtained from QMX laboratories (Thaxted, UK). All chemicals were quantified externally. As unique standards were not available for NP_2EC and NP_3EC these were quantified assuming a similar response to their respective

NPEO. Names, abbreviations and chemical structures of the nonylphenolic moieties studied are provided (Table 4.1). Methanol (MeOH), dichloromethane (DCM), ethylacetate (EtOAc) and hexane were obtained from Rathburn Chemicals (Walkerburn, UK) and were HPLC grade. Ammonium hydroxide (NH_4OH) and acetic acid of ACS grade was purchased from Sigma Aldrich (Dorset, UK) and ultrapure (UP) water of 18.2 MΩ quality (Elga, Marlow, UK) was used in the preparation of mobile phases.

Table 4.1. Name and structures of the nonylphenolic chemicals

Name	Abbreviation	CAS	Molecular formula	Chemical structure
Nonylphenol	NP	104-40-5	$C_{15}H_{24}O$	
Nonylphenol monoethoxylate	NP_1EO	104-35-8	$C_{17}H_{28}O_2$	
Nonylphenol diethoxylate	NP_2EO	20427-84-3	$C_{19}H_{32}O_3$	
Nonylphenol polyethoxylate	$NP_{3\text{-}12}EO$	68412-54-4	-	
Nonylphenoxy acetic acid	NP_1EC	3115-49-9	$C_{17}H_{26}O_3$	
Nonylphenoxy monoethoxy acetic acid	NP_2EC	106807-78-7	$C_{19}H_{30}O_4$	
Nonylphenoxy diethoxy acetic acid	NP_3EC	108149-59-3	$C_{21}H_{34}O_5$	

4.2.2. Site description

The trickling filter plant is situated in the east of England serving a population equivalent (PE) of 2,800. The site has an average daily flow of 650 m^3 d^{-1} of which <10 % is attributed to a local airfield. The trickling filter consists of a roughing filter for bulk biochemical oxygen demand (BOD) removal followed by two duplex filters for further BOD removals and nitrification. Corresponding grab samples of settled sewage and final effluent were collected in 2.5 l borosilicate glass bottles with Teflon lined caps over a three day period. Collected samples were filtered and loaded onto solid phase extraction (SPE) cartridges within 30 minutes of collection and once dried, stored at -20 °C prior to extraction. Sampling was completed in January and the wastewater temperature was 10 (±0.5) °C during the study.

4.2.3. Extraction procedure

Settled sewage (100 ml) and final effluents (250 ml) were filtered through 1.2 µm GF/C filters (VWR, Lutterworth, UK) prior to solid phase extraction (SPE). The aqueous phase was loaded onto 500 mg: 3 cc tC18 cartridges (Waters, Elstree, UK) preconditioned with 5 ml MeOH then 5 ml UP water. The sample was loaded at a flow rate of between 5 and 10 ml min^{-1} using a vacuum manifold. A 5 ml aliquot of UP water was then used to wash the cartridge. The cartridge was then dried for at least an hour under vacuum prior to elution. Analytes were eluted using 5 ml EtOAc, 5 ml 0.1 % acetic acid in MeOH and then 5 ml DCM. Extracts were then concentrated to approximately 1 ml by rotary evaporation (Heidolph Instruments, Schwabach, Germany) to complete dryness under a gentle stream of nitrogen gas. Once dried the extract was reconstituted in 0.25 ml of UP water: MeOH (80: 20). This was transferred to an autosampler vial prior to quantification by UPLC-MS/MS. To ensure analytical reliability, prior to extraction; both low (100 ng l^{-1}) and high (1,000 ng l^{-1}) spikes of all compounds were used to determine recoveries. These selected spike levels represent concentrations approximately one order of magnitude greater than the method detection limit (MDL), and those concentrations anticipated to be present in real samples (Koh *et al*., 2008; McAdam *et al*., 2011).

Particulate phase analysis was performed by solvent extraction of filter papers containing suspended solids from filtering 1 litre of settled sewage or final effluent. These were freeze dried, shredded and extracted using 10 ml acetone and 10 ml MeOH by mechanically agitating using a multi-reax system (Heidolph Instruments, Schwabach, Germany) in a 25 ml Teflon tube for 30 minutes. The extracts were centrifuged at 1500 x *g* for 10 minutes then decanted off. This process was repeated and the extracts combined and concentrated to 0.2 ml. These were reconstituted to 2 ml with hexane and loaded onto a 500 mg: 3 ml silica cartridge (Waters Ltd., Watford, UK) preconditioned with 2 ml hexane. Analytes were eluted

using 10 ml of 10 % acetic acid in MeOH then dried and reconstituted in 0.25 ml of UP water: methanol (80: 20) prior to quantification by UPLC-MS/MS. Before freeze drying, selected samples received either low (100 ng g^{-1}) or high spikes (1,000 ng g^{-1}) of all compounds to determine recoveries

4.2.4. UPLC-MS/MS

Ultra-performance liquid chromatography was performed using a Waters Acquity UPLC (Waters, Manchester, UK). Chromatographic separations were achieved using an Acquity UPLC BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm particle size; Waters, Manchester, UK) controlled at 30 ˚C. A gradient elution of 0.1 % NH_4OH in UP water (A) and 0.1 % NH_4OH in MeOH (B) at a flow rate of 0.4 ml min^{-1} was used. Initial conditions of 20 % B were maintained for 4 minutes prior to gradually increasing to 80 % over 5 minutes. This was maintained for 12 minutes then returned to starting conditions over 3 minutes. The total run time was 26 minutes and an injection volume of 10 µl was used. Detection was achieved using a Waters Quattro Premier XE mass spectrometer with a Z-spray ESI interface (Micromass, Watford, UK). This was operated in the negative and positive electrospray ionisation mode utilising multiple reaction monitoring (MRM). The optimised MS/MS conditions for detection are detailed (Table 4.2). The parameters for detection by the mass spectrometer were as follows: capillary voltage, 3.20 kV (positive mode) and -2.3 kV (negative mode); extractor lens; 3.0 V, multiplier voltage, 650 V; desolvation gas flow, 1000 l h^{-1}; RF lens, 0.5 V (positive mode) and 1.0 V (negative) mode; cone gas flow, 50 l h^{-1}; desolvation temperature, 350 °C and source temperature, 120 °C (Koh *et al*., 2008).

Table 4.2. Chromatographic detail and MS/MS parameters of NP, NPECs and NPEOs

Compound	Retention time (minutes)	*m/z* precursor	Product ions (collision potential)	Cone (V)	IDL (pg)
NP	11.4	219[a]	132.5(30), 106(20)	30	82
NP_1EC	8.2	263.5[a]	205(20), 106(30)	20	98
NP_2EC	8.4	307[a]	205(20)	20	-
NP_3EC	8.7	351[a]	205(20)	20	-
NP_1EO	11.7	282[b]	127(25), 265(25)	25	118
NP_2EO	12.0	326[b]	183(40), 121(40)	40	27
NP_3EO	12.1	370[b]	353(10), 226.5(20)	40	8
NP_4EO	12.2	414[b]	397(14), 270(20)	30	60
NP_5EO	12.2	458[b]	440(20), 315(25)	30	20
NP_6EO	12.2	502[b]	485(20), 359(30)	30	28
NP_7EO	12.3	546[b]	529(23), 403(25)	30	21
NP_8EO	12.3	590[b]	573(25), 447(27)	30	12
NP_9EO	12.3	634[b]	617(25), 335(30)	30	17
$NP_{10}EO$	12.4	678[b]	661(25), 132.5(40)	30	20
$NP_{11}EO$	12.4	722[b]	705(25), 291(40)	30	23
$NP_{12}EO$	12.4	766[b]	749(30), 291(35)	30	69

[a] $[M\text{-}H]^-$ [b] $[M+NH_4]^+$

Note: IDL; instrument detection limit, calculated by replicate (n=7) extractions of 250 ml of spiked (50 ng l^{-1}) UP water

4.2.5. Modelling

The potential for exceeding the NP EQS in receiving waters was explored by applying a simple mathematical model based on Whelan *et al* (1999) of riverine mixing, in-stream dilution and degradation for a range of feasible scenarios. The potential biodegradability of the range of different compounds in the final effluent were assessed using BIOWIN v4.10 (Howard *et al*., 2005) estimation software, which is part of EPI Suite™ v4.10 (EPI Suite, 2013). BIOWIN uses chemical structure to predict the likelihood of a compound to pass a standardised "ready" biodegradability test (OECD, 1992) (60% complete mineralisation of an organic compound added to a vessel within 28 days). Those compounds which pass a ready test are likely to degrade rapidly in the natural environment. Here, we assume that those compounds which are predicted to pass the ready test will yield NP as an intermediate reaction product in the receiving system. The model considers two substance types A and B. Substance type A represents any organic compound which is readily biodegradable and which can yield NP as an intermediate metabolite in the degradation process. Substance type B represents NP. Both substance types are assumed to degrade according to first order kinetics as follows:

$$\frac{dC_A}{dt} = -k_A.C_A \qquad (4.1)$$

$$\frac{dC_B}{dt} = k_A.C_A - k_B.C_B \qquad (4.2)$$

where C_A and C_B are, respectively, the molar concentrations of substance types A and B, t is time and where k_A and k_B are first order rate constants. This assumes that 100 % of the substance A which degrades is converted into NP. This is unlikely to be the case and the model is, therefore, conservative with respect to C_B. These equations can be solved as follows:

$$C_A(\tau) = \frac{Q_0}{Q(\tau)}.C_A(0).exp(-k_A.\tau) \qquad (4.3)$$

$$C_B = \left(\frac{Q_0}{Q(t)}\right).\left(C_A(0).\frac{k_A}{k_B - k_A}.\left(\exp(-k_A.t) - \exp(-k_B.t)\right) + C_B(0).\exp(-k_B.t)\right) \qquad (4.4)$$

where $Q(\tau)$ is the river discharge at a point corresponding to flow time τ, Q_0 is the river discharge at the point of emission (i.e., at flow time 0), $C_A(\tau)$ and $C_B(\tau)$ are the concentrations of substance types A and B at flow time t and $C_A(0)$ and $C_B(0)$ are the concentrations of substance types A and B at flow time 0 which are, in turn, calculated from the effluent concentrations (C_{EA} and C_{EB}) and a dilution factor (DF) via:

$$C_A(0) = \frac{C_{EA}}{DF} \qquad (4.5)$$

$$C_B(0) = \frac{C_{EB}}{DF} \qquad (4.6)$$

We assume that C_{EA} and C_{EB} are the observed molar concentrations in the trickling filter effluent (see Figure 4.4). All solutes are assumed to move entirely by advection in one dimension (i.e., hydrodynamic dispersion is neglected). This assumption has been shown to hold for low frequency emission variations, such as time-constant emission, which approach a steady state by Gandolfi *et al* (2001). Some of the compounds are relatively hydrophobic which means that they will have a propensity to sorb to sediment solids. The assumption of zero interaction with the sediment is based on an *a priori* assumption of steady state under which it is likely that an equilibrium will have established between chemical in the sediment and chemical in the overlying water along the entire reach considered, such that the net exchange (and thus net removal from the water column) will be zero (Schwarzenbach, 2003). Flow time τ is calculated from the reach distance divided by the river velocity, which was assumed to be constant with distance downstream. River discharge, however, was assumed to increase linearly with distance (0.01 km^{-1} i.e., a 50 % increase in Q over 50 km) to represent increased dilution resulting from additional inputs of water from an increasing contributing

catchment area. The range of half lives considered for substance type A were derived from the range reported for the primary degradation of another readily degradable substance, linear alkyl benzene sulphonate (LAS), in field studies (Whelan *et al*., 1999; Fox *et al*., 2000; Whelan *et al*., 2007) conducted on rivers and streams of different dimensions. To our knowledge no field-based studies of NP precursor die away have been published in the international peer reviewed literature. Scenario 1, 2 and 3 represent river velocities of 0.03, 0.1 and 0.9 m s^{-1}, respectively, typical of the range observed in lowland streams (Richards, 1982). Further model parameters are outlined (Tables 4.3 and 4.4).

Table 4.3. Key model parameters of three hypothetical river scenarios

Model parameter	Hypothetical scenario		
	1	2	3
Readily degradable precursor t½ (h)	3	7	14
NP t½ (h)	240	240	240
River *v* (m/s)	0.03	0.1	0.9
Initial dilution factor	10	10	10
Dilution increase per km	0.01	0.01	0.01

Key: t½, half-life; NP, *v*, velocity

Table 4.4. Reported LAS half lives in river systems

River	Country	Q (m^3 s^{-1})	t½ (h^{-1})	*v* (m s^{-1})	Reference
Red Beck	UK	0.1[a]	2.2-2.7	0.01-0.03[c]	Fox *et al*., 2000
Houay Mak Hiao	Laos	0.8[a]	7.0	0.08[c]	Whelan *et al*., 2007
Lambro	Italy	5.0[b]	13.9	0.9[d]	Whelan *et al*., 1999

[a]Discharge reported for monitoring period, [b]mean annual discharge at one station in monitored reach, [c]velocity calculated from tracer travel time, [d]velocity calculated from empirical velocity-discharge relationship at one station at mean annual discharge
Key: Q, discharge; t½, half-life; *v*, velocity

4.3. RESULTS

4.3.1. Method performance

The extraction process gave mean recoveries of all compounds collectively from aqueous phases of 54-99 % and 56-99 % for settled sewage and final effluents, respectively. Similarly particulate phase recoveries were 37-89 % and 35-102 % for settled sewage and final effluents. Generally recoveries were poorer for the long chained ethoxylates and NP_1EC, compared to NP and the short chained ethoxylates (Table 4.5). Relative standard deviations (RSD) of recoveries were below 20 % for all compounds in all matrices. Chromatography from the extracted samples showed Gaussian distribution for all compounds as exhibited for NP, the short chained ethoxylates (Figure 4.1) and the NPECs (Figure 4.2).

Table 4.5. Method recoveries (%) and relative standard deviations (RSD %) of NP, NPEC and NPEOs in wastewater matrices (n=6)

Compound	Aqueous recoveries (RSD %)		Particulate recoveries (RSD %)	
	Settled sewage	Final effluent	Settled sewage	Final effluent
NP	99.3 (4.3)	98.8 (3.2)	85.8 (10.5)	73.2 (15.3)
NP_1EC	53.8 (10.3)	80.0 (4.8)	36.8 (3.4)	34.7 (4.2)
NP_1EO	96.0 (4.3)	84.1 (7.3)	83.2 (8.7)	90.8 (6.3)
NP_2EO	67.2 (9.3)	93.0 (1.2)	89.0 (3.2)	89.7 (2.3)
NP_3EO	61.0 (12.2)	62.8 (5.7)	53.5 (4.3)	69.8 (7.8)
NP_4EO	54.5 (9.9)	56.0 (13.1)	56.5 (14.8)	80.5 (5.3)
NP_5EO	57.2 (12.4)	75.2 (8.9)	62.0 (8.8)	88.0 (3.7)
NP_6EO	58.2 (6.2)	72.7 (4.9)	69.5 (12.0)	101.5 (7.6)
NP_7EO	66.3 (5.3)	73.2 (3.2)	73.7 (6.9)	88.2 (4.3)
NP_8EO	65.3 (8.3)	81.3 (4.5)	75.7 (9.5)	93.3 (8.8)
NP_9EO	62.2 (5.5)	75.8 (3.1)	75.0 (16.3)	83.2 (6.9)
$NP_{10}EO$	70.3 (7.1)	76.2 (1.9)	76.3 (8.8)	92.2 (7.6)
$NP_{11}EO$	56.3 (13.5)	62.7 (8.3)	81.0 (19.4)	86.0 (8.1)
$NP_{12}EO$	57.8 (7.3)	64.5 (5.4)	86.5 (9.9)	81.5 (2.0)

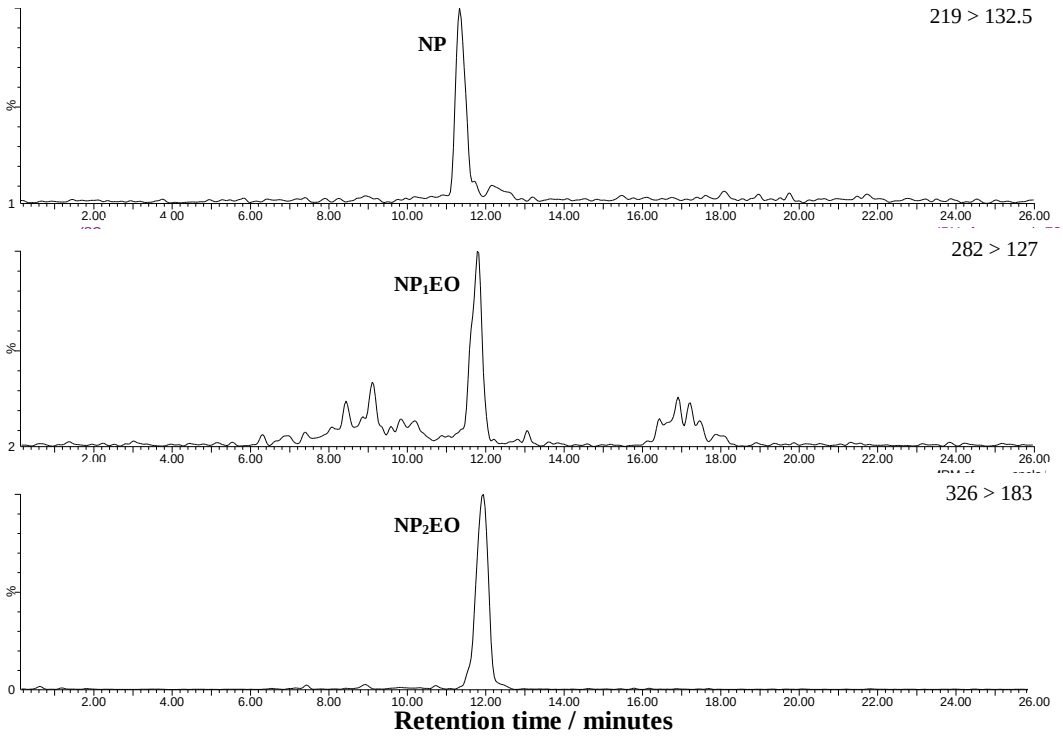


Figure 4.1. Multiple reaction monitoring chromatograms of NP, NP_1EO and NP_2EO in final effluent

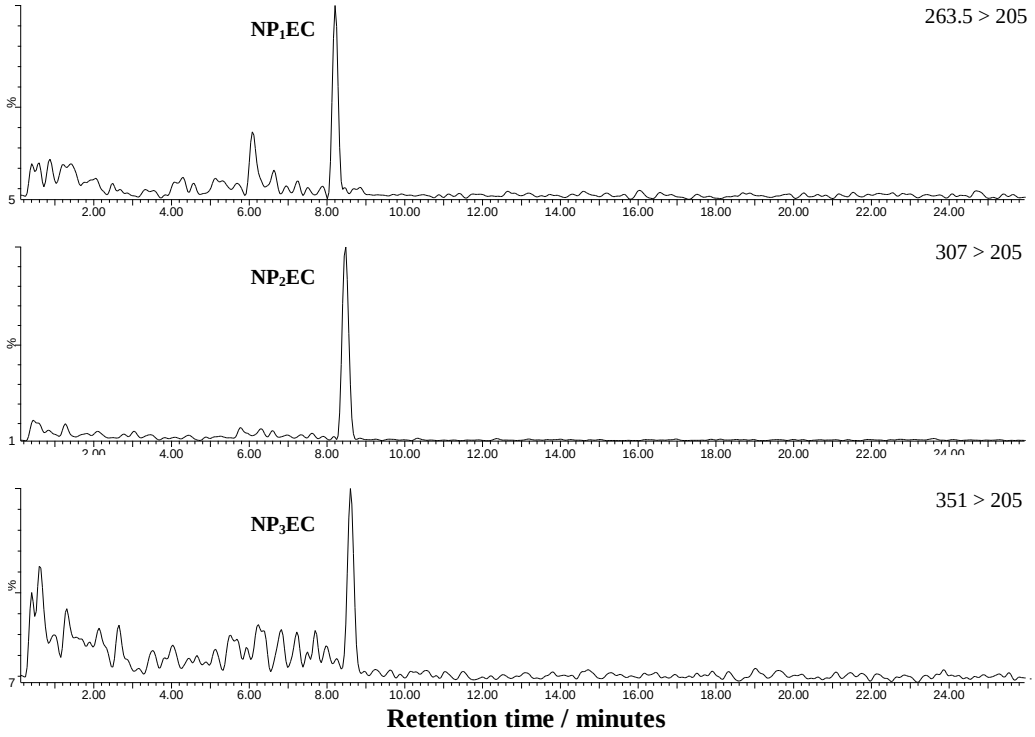


Figure 4.2. Multiple reaction monitoring chromatograms of NP_1EC, NP_2EC and NP_3EC in final effluent

Method detection limits were calculated using (Vieno *et al*., 2006; Petrie *et al*., in press):

$$MDL = \frac{IDL.100}{(R.C)} . \frac{100}{(100 - M)} \qquad (4.7)$$

where *IDL* is the instrument detection limit (ng l^{-1}), *R* is the recovery (%), *C* is the concentration factor and *M* is the matrix suppression (%). Final effluent MDLs of the short chained ethoxylates ($NP_{1-2}EO$) ranged from 3.2 to 14.7 ng l^{-1} and the longer chained NPEOs were between 1.4 and 11.2 ng l^{-1}. Greater MDLs in settled sewage were observed; up to a maximum of 32.0 ng l^{-1} and 33.0 ng l^{-1} for short and long chained ethoxylates respectively. The majority of compounds have MDLs below 15 ng g^{-1} in the particulate phase of both settled sewage and final effluent (Table 4.6). Method detection limits were greatest for NP_1EC; a consequence of lower recovery and greater matrix suppression.

Table 4.6. Method detection limits of NP, NPEC and NPEOs in wastewater matrices

Compound	Aqueous MDL / ng l^{-1}		Particulate MDL / ng g^{-1}	
	Settled sewage	Final effluent	Settled sewage	Final effluent
NP	24.6	9.0	11.8	13.4
NP_1EC	45.3	17.4	38.5	39.4
NP_1EO	32.0	14.7	18.8	13.9
NP_2EO	12.0	3.2	3.9	3.8
NP_3EO	4.0	1.4	2.1	1.4
NP_4EO	28.7	11.2	13.3	8.3
NP_5EO	11.0	2.9	4.1	4.1
NP_6EO	13.4	3.9	5.2	3.6
NP_7EO	8.0	3.0	3.2	2.9
NP_8EO	5.3	1.6	1.8	1.4
NP_9EO	8.3	2.3	2.8	2.8
$NP_{10}EO$	8.4	2.9	3.1	2.7
$NP_{11}EO$	11.2	3.8	3.5	3.1
$NP_{12}EO$	33.0	11.1	9.6	11.0

Note: MDL, method detection limit.

4.3.2. Matrix suppression

The full scan analysis of an extracted wastewater sample provides a subjective insight into the possible influence of remaining matrix in the sample extracts on analyte response. The full scan profile of extracted settled sewage showed a relatively large quantity of unwanted material remaining in the sample extract (Figure 4.3). In the negative ion monitoring mode, the majority of the extracted material eluted between 5 and 10 minutes. Nonylphenol and the NPEOs elute after this, however the NPECs do so between 8 and 9 minutes. Matrix

suppressions were quantified using the algorithm reported by Vieno *et al*. (2006). Signal suppressions of NP_1EC were between 29 % and 31 % in particulate matrices. In the positive monitoring mode $NP_{1\text{-}12}EO$ elutes around 12 minutes and after the majority of the matrix. In aqueous samples, signal suppressions of the ethoxylates were between 0 % and 10 %, and 2 % and 21 % for final effluent and settled sewage, respectively. Greater matrix effects were observed in particulate phases ranging from 9 % to 27 % in final effluent and 12 % to 28 % in settled sewage.

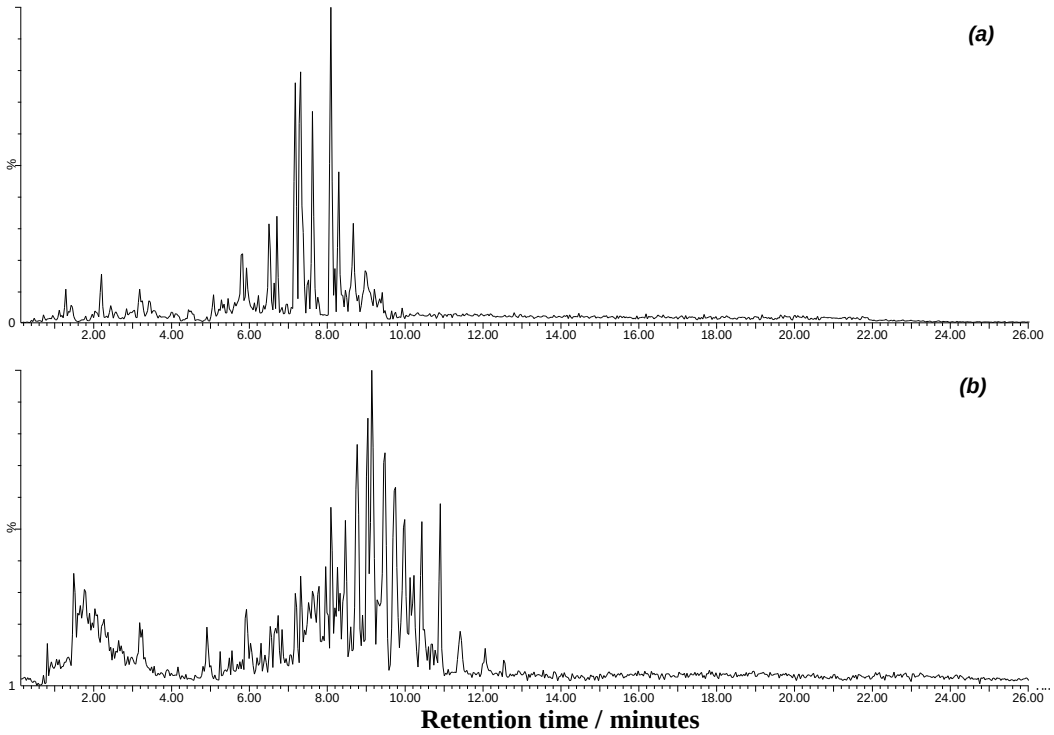


Figure 4.3. Full scan (50-1500 amu) base peak intensity plots of settled sewage in negative ion (a) and positive ion (b) monitoring modes

4.3.3. Nonylphenol and precursor removal in trickling filters

Corresponding grab samples of settled sewage and final effluent were collected to negate any intraday variation in receiving compound concentration. The presence of NPEOs in the sewage stream is mainly attributed to a local airfield. Nonylphenol ethoxylates have been previously observed in aircraft maintenance formulations such as de-icer and anti-icer (Corsi *et al*., 2003). The molar balance of all nonylphenolic moieties showed NP had the greatest molar concentration in settled sewage. In final effluent $NP_{1\text{-}2}EO$ predominated (Figure 4.4).

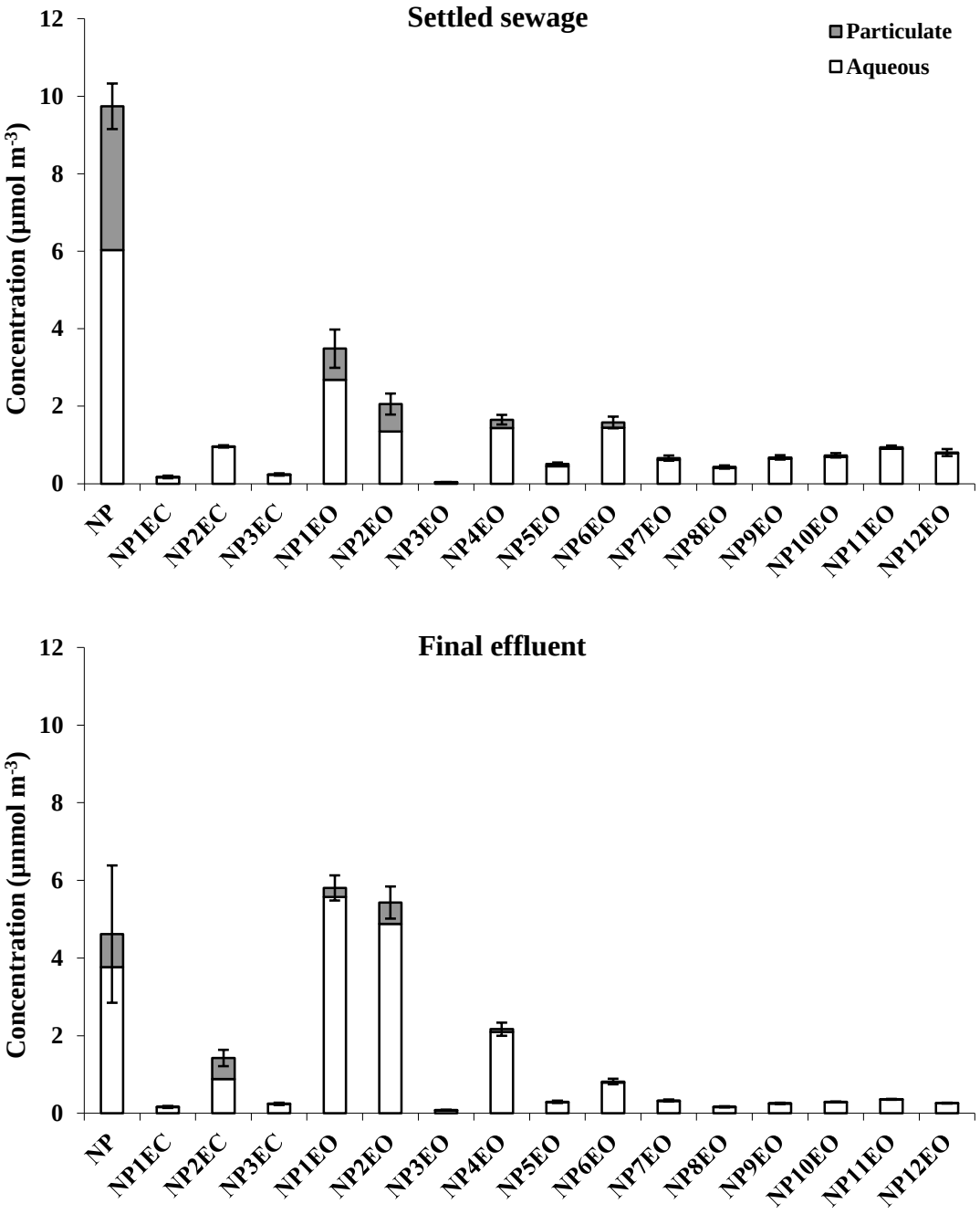


Figure 4.4. Molar concentration (corrected for extraction recoveries) of NP and its precursors in settled sewage and final effluent. Note: errors bars represent the standard deviation of the total sample concentration

In settled sewage a total of 602 ng l^{-1} of $NP_{12}EO$ was present, demonstrating the presence of longer chained compounds. A high concentration of NP (2,146 ng l^{-1}), relative to the other compounds was observed. The partitioning behaviour of the compounds was determined by calculating their partition coefficients (log K_d):

$$K_d = \frac{P}{A} \qquad (4.8)$$

where *P* is the concentration of the analyte in the particulate phase (ng kg^{-1}) and *A* is the concentration in the aqueous phase (ng l^{-1}). Of the total NP settled sewage concentration, 38 % was found bound to particulate matter with a log K_d of 4.1 l kg^{-1}. This reduced with increasing ethoxylate chain length to 0.5 for $NP_{12}EO$ which is the most hydrophilic of the ethoxylates monitored. The final effluent profile showed a reduction in concentration of NP by 53 % to a concentration of 1,017 ng l^{-1}. Nonylphenol removal across trickling filters in the literature varies greatly from -103 % to 86 % (Moreira *et al*., 2011; Barber *et al*., 2012) (Table 4.7). Similarly effluent concentrations vary from 6 ng l^{-1} to 1,820 ng l^{-1}. The removal of individual compounds across the trickling filter showed an interesting trend reducing from $NP_{12}EO$ to NP_2EO and a negative removal, which then increased to a positive removal for NP (Figure 4.5).

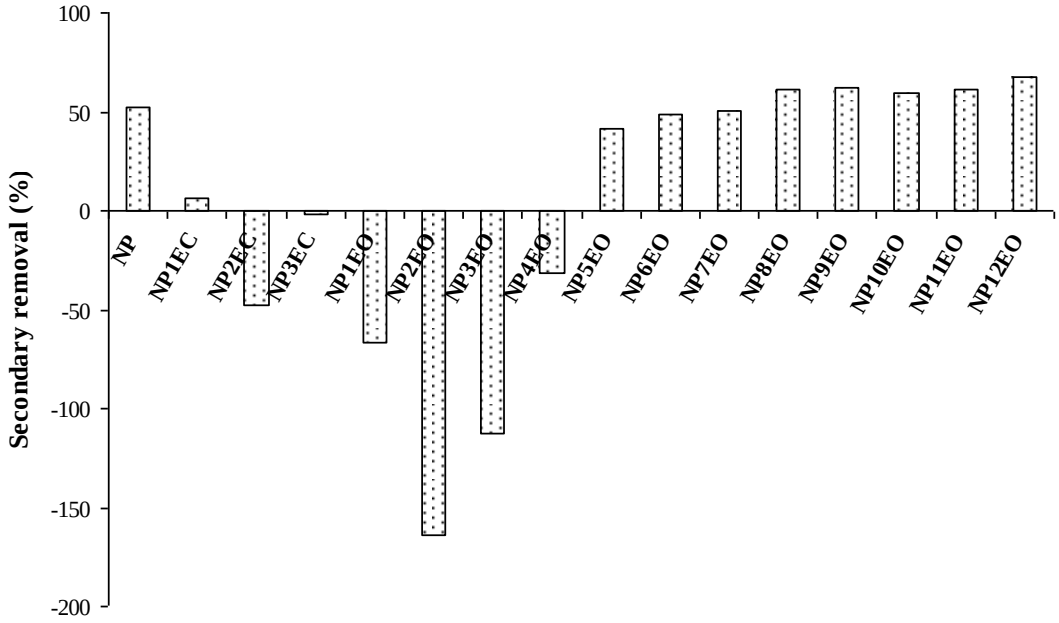


Figure 4.5. Removal of NP and its precursors by a trickling filter

Concentrations of the longer chained polyethoxylates ($NP_{5-12}EO$) showed reductions of between 42 % and 67 %. Conversely the concentrations of $NP_{1-4}EO$ increased during treatment. The mono and diethoxylate compounds displayed the greatest increase in concentration to 3,210 ng l^{-1} which accounted for 49 % of the total molar nonylphenolic load discharged in final effluent. The concentration of the NPECs in final effluent was 594 ng l^{-1}; an increase of 33 % during treatment. Both short chained NPECs and NPEOs have previously been observed in final effluent following trickling filter treatment (Table 4.7). Only an 8 % reduction in total nonylphenolic load was observed; caused by the accumulation of short chained NPECs and NPEOs.

Table 4.7. Removal of NP, NPECs and NPEOs by trickling filters

Compound	Configuration	Design	Media type	PE	Flow rate (m^3 d^{-1})	SS (ng l^{-1})	FE (ng l^{-1})	Removal (%)	Reference
NP	Nitrifying filter / 2 BOD filters	Diameter, 12 m; depth, 1.8 m	Plastic	2,800	650	2146	1017	52	This study
$NP_{1-3}EC$						447	594	-33	
$NP_{1-2}EO$						1556	3210	-106	
$NP_{3-12}EO$						4422	2490	44	
NP	-	2 x 379 m^3	-	-	-	2,400	340	86	Barber *et al.*, 2012
$NP_{1-4}EC$						39,600	155,600	-293	
$NP_{1-2}EO$						<1,850	6,270	-239	
NP	Tertiary process to an OD	-	-	41,043	9,447	3.1[a]	6.3	-103	Ifelebuegu, 2011
NP	Nitrifying filter / 2 BOD filters / tertiary filter	Diameter, 12 m; depth, 1.8 m; Tertiary 5 x 10 x 4 m	Plastic/stone (tertiary)	2,800	650	100	88	12	Koh *et al.*, 2008
$NP_{1-3}EC$						94	2308	-2355	
$NP_{1-2}EO$						305	253	17	
$NP_{3-12}EO$						5,513	1309	76	
NP	-	-	-	3,200	525	1,050	1,220	-16	Moreira *et al.*, 2011
NP	-	-	-	2,200	-	10,200[b]	1,820	82	Leusch *et al.*, 2006

[a]oxidation ditch effluent [b]raw sewage

Note: PE, population equivalent; SS, settled sewage; FE, final effluent; TF, trickling filter; OD, oxidation ditch; BOD, biochemical oxygen demand.

4.3.4. River predictions

Although the concentrations of NP observed in the effluents exceed the EQS for receiving waters, actual non-compliance would depend on the dilution factor and the potential in-stream formation of NP from various precursor compounds. Five compounds which were detected in the effluent streams were predicted to be readily biodegradable by BIOWIN ($NP_{1-3}EC$ and $NP_{1-2}EO$) and were denoted as substance type A. Nonylphenol was predicted not to be readily degradable by BIOWIN and is, therefore, assumed to degrade much more slowly in the river environment (substance type B). In each of the three scenarios, the effluent is assumed to mix completely and instantaneously with the flow of the receiving system with an initial dilution factor of 10 (European Commssion, 2003b). It should be noted that the dilution factor will vary significantly spatially (i.e., between wastewater treatment plants) and temporally (as flows change in the effluent stream and the receiving water body). Predicted changes in the concentrations of NP originating from the effluent, the total concentration of compounds assumed to behave as substance A and the total concentration of NP (i.e., the sum of the remaining NP concentration originating from the effluent and the concentration of new NP formed from the degradation of substance A) with distance downstream of the outfall are shown in Figure 4.6 for the three scenarios. Molar concentrations are used in order to facilitate the calculation of total concentrations of different compounds and the conversion of precursor compounds to NP. The concentration of the effluent-derived NP reduces exponentially with distance downstream as a consequence of degradation and dilution and is always well below the NP EQS. The concentration of the original NP present is predicted to reduce by between 31 % (for Scenario 3) and 75 % (for Scenario 1) over a 40 km reach. Concentrations of the readily degradable precursors were initially higher than the concentration of NP but decreased relatively rapidly with distance downstream owing to the high rate constants assumed. By 10 km, the predicted total concentration of readily degradable precursors varies between 0 % (Scenario 1) and 79 % (Scenario 3) of the initial concentration. This rapid degradation of precursors in Scenarios 1 and 2 is predicted to result in a total NP concentration which exceeds the EQS in the river over reach lengths of 6.6 and 10.2 km respectively. A peak NP concentration of 1.64 μmol m^{-3} (362 ng l^{-1}) was predicted for Scenario 1. The relatively slow degradation of readily degradable precursors in Scenario 3 resulted in predicted total NP concentrations which were below the EQS over the entire 40 km reach considered.

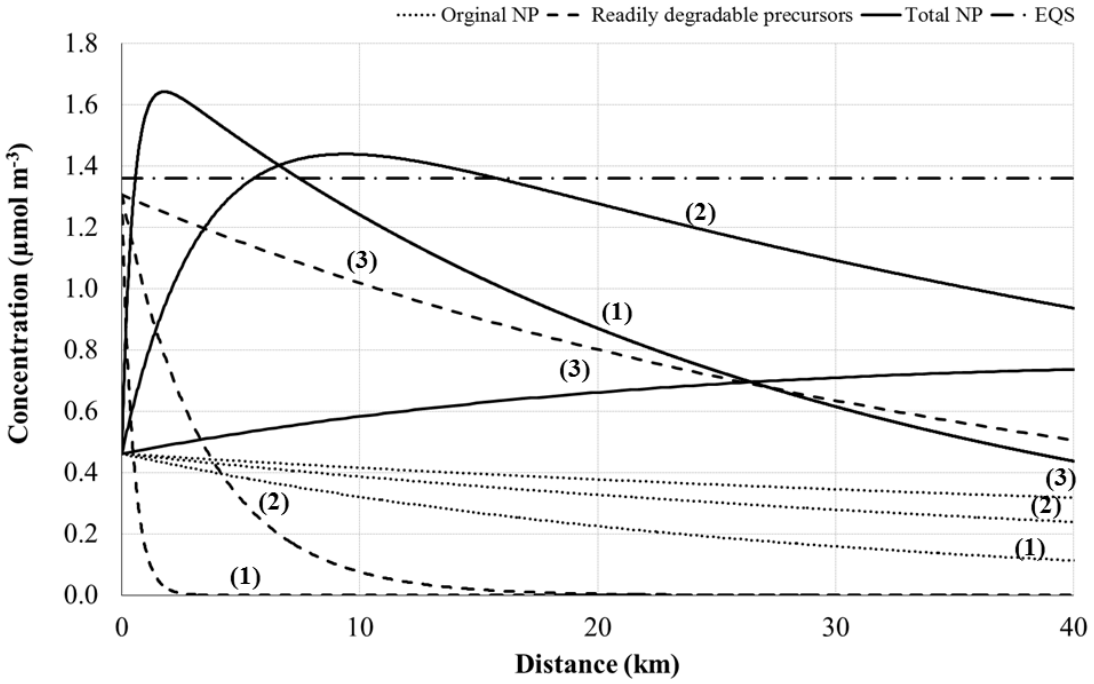


Figure 4.6. Predicted changes in riverine concentrations of NP and readily degradabale precursors with distance downstream of a wastewater outfall applying three hypothetical scenarios (1 – 0.03 m s^{-1} (Fox *et al.*, 2000), 2 – 0.1 m s^{-1} (Whelan *et al.*, 2007) and 3 – 0.9 m s^{-1} (Whelan *et al.*, 1999). In all cases the dilution factor was 10.

4.4. DISCUSSION

The extraction method exhibited recoveries greater than 50 % for the majority of analytes in both aqueous and particulate phases; typical of previously reported methods (Jahnke *et al*., 2004; Koh *et al*., 2008; Lara-Martin *et al*., 2012). The detection limits reported herein are comfortably below the EQS guideline for NP of 300 ng l^{-1} (European Commission, 2000) and are suitable for the analysis of typical NPEO concentrations encountered in wastewaters (Koh *et al*., 2008; McAdam *et al*., 2011). Nonylphenol monoethoxylate had the greatest detection limit of the ethoxylates due to its poor ionisation (Lara-Martin *et al*., 2012). A well-known problem of extracting trace amounts of a compound from wastewater is the influence of sample matrix on analyte signal strength. The full scan of settled sewage showed NP_1EC co-elutes with the majority of the matrix causing ~30 % signal suppression. Jahnke *et al* (2004) reported substantial signal suppressions of >39 % for all analytes using similar mobile phase compositions. The use of UPLC here may have aided the reduction of matrix effects by the improvement in the efficiency of chromatographic separations (Petrie *et al*., in press). However, differences in extraction methodology may contribute to this variation. An added advantage of using UPLC instead of HPLC is the reduced run time that it offers. Many studies which report similar method performances often require run times of between 40 and 60 minutes (Jahnke *et al*., 2004; Loos *et al*., 2007; Koh *et al*., 2008). Run time is often a rate

limiting step on sample number and turnover. A run time of 26 minutes using UPLC generally offers double the sample throughput in comparison to conventional HPLC systems.

The relative distribution of NP moieties in settled sewage and final effluents is similar to that observed by Ahel *et al* (1994), albeit at significantly lower concentrations. The concentration of NP determined in settled sewage was 2,146 ng l^{-1}, similar to that reported previously by other authors in the UK (Rule *et al*., 2006; McAdam *et al*., 2011), across the rest of Europe (Farré *et al*., 2002; Gunnarsson *et al*., 2009) and in the US (Kolpin *et al*., 2002; Loyo-Rosales *et al*., 2007). Care must be taken with the interpretation of findings from Kolpin *et al* (2002). Till (2003) identified possible shortcomings in the way in which data was processed. To demonstrate, reporting a median concentration for only those samples where chemicals were detected suggests higher median concentrations than actually present. Furthermore, the use of data with reported concentrations in field blanks overestimates the actual concentrations of these chemicals in real matrices (Till, 2003). The apparent partition coefficients of NP in settled sewage (4.09) and final effluent (5.27) are similar to the log octanol-water coefficient (log K_{ow}) of 4.48 (Corvini *et al*., 2006). Sorption to the biofilm and secondary sedimentation of particulate matter can contribute to the removal of NP by a trickling filter. However, biodegradation is considered to be the main removal mechanism of NP in secondary wastewater treatment processes (Tanghe *et al*., 1998; Langford *et al*., 2005). Removals of NP by trickling filters in the literature vary substantially from negative removals (Gomez *et al*., 2007; Ifelebuegu, 2011) to >80 % (Leusch *et al*., 2006; Barber *et al*., 2012). Without information of parent chain removal it is difficult to diagnose this variation in NP removal. Generally the trickling filter here showed poor removals of total nonylphenolic compounds with only an 8 % reduction in the total molar load causing a downward shift in the chain length distribution. The use of concentration (i.e., ng l^{-1}) overestimates total nonylphenolic removal. Ethoxylate chain shortening causes a loss of mass without effecting nonylphenolic molar load. The bulk of the load in final effluent consisted of $NP_{1-2}EO$ rather than the longer chained compounds found in settled sewage. This is of concern because shortening of ethoxylate chain length increases estrogenicity (White *et al*., 1994; McAdam *et al*., 2011; Lara-Martin *et al*., 2012). Similar removals were observed by Barber *et al* (2012) where a reduction of NP coincided with an increase in short chained NP precursors. The negative $NP_{1-4}EO$ removals across the trickling filter indicates sequential shortening of the long chained NPEOs through removal of ethoxy groups as previously proposed (Giger *et al*., 1984; Soares *et al*., 2008; McAdam *et al*., 2011). During the study only a small increase in NPECs was observed across the filter. Previous studies of biological processes have shown a large production of NPECs in comparison to the other compounds (Komori *et al*., 2006; Koh *et al*., 2008; Koh *et al*.,2009; McAdam *et al*., 2011; Barber *et al*., 2011). This increase in NPECs

coincided with a greater removal of the long chained NPEOs. Komori *et al* (2006) showed $NP_{1-5}EC$ contributed the greatest portion of the mass balance in final discharges from 20 WwTWs in Japan with only small concentrations of $NP_{1-10}EC$ found in settled sewage. Carboxylate production coincided with low observed ethoxylate chain shortening. This suggests that NPECs are produced following shortening of the ethoxylate chain and/or are more resistant to biological breakdown than their respective ethoxylates. The lack of NPEC production in this study indicates that their formation occurs following ethoxylate chain shortening. The proposed degradation pathway of long chained NPEOs during trickling treatment is outlined (Figure 4.7).

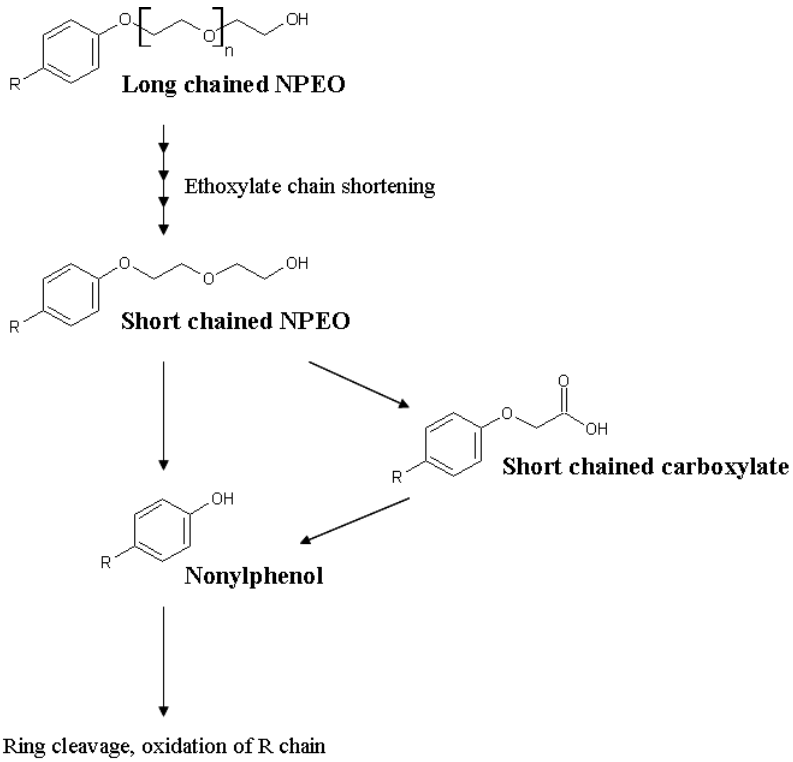


Figure 4.7. Proposed degradation pathway of long chained NPEOs during trickling filter treatment, adapted from (Warhurst, 1994; Soares *et al.*, 2008)

Here reasonable removals of NP corresponded with poor removals of total nonylphenolic load. It is postulated that the transformation of parent compounds had not reached the critical level at which NP is produced at a faster rate than it is removed. This supports studies conducted under controlled loading/feed conditions which have shown NP has a high susceptibility to biological removal during wastewater treatment (Tanghe *et al.*, 1999; Stasinakis *et al.*, 2008; Stasinakis *et al.*, 2010). However, removal is reduced substantially under environmental conditions by the production of NP from the biotransformation of its precursors. Negative removals of NP have been observed during activated sludge treatment (Farré *et al.*, 2002; Koh *et al.*, 2009; McAdam *et al.*, 2011) which, compared to trickling filter

treatment, is generally expected to achieve a greater removal of hazardous chemicals such as steroid estrogens (Koh *et al*., 2009; Petrie *et al*., in press). Longer residence time's characteristic of activated sludge may facilitate effective biotransformation of NPEOs and NPECs to NP, accounting for its poor removal and in some cases, its production. This will improve contact between NPEOs and bacterial cells; a process known to be critical for biodegradation to occur (Langford *et al*., 2005). During trickling filter treatment the contact time between an organic solute and a fixed bacterial cell is comparatively short. Whilst in this study the trickling filters were operated in single pass, effluent recycling which is common in some trickling filter assets, will improve biofilm contact potentially enabling greater biodegradation. It should also be noted that NP has a vapour pressure of 2.07×10^{-2} Pa and a Henry's law constant of 8.39×10^{-1} Pa m^3 mol^{-1} illustrating a semi-volatile nature (Soares *et al*., 2008). Consequently, NP can be removed by volatilisation during both trickling filter and activated sludge treatment.

The application of a simple model of chemical transport and degradation, in which readily biodegradable intermediate compounds are assumed to transform to NP (Soares *et al*., 2008; McAdam *et al*., 2011), suggests that under the environmental conditions considered, the NP EQS may be breached even though the initial NP concentration at the outfall is below the EQS. This is due to subsequent biotransformation of precursors to NP downstream of the outfall. Currently, many process performance evaluations have been undertaken using only NP data as the benchmark (Leusch *et al*., 2006; Gomez *et al*., 2007; Ifelebuegu, 2011). Whilst the intermediate compounds are not expected to be regulated in the short term (European Commission, 2000), this semi-quantitative assessment demonstrates that measurement of these intermediate compounds in parallel with NP could be advantageous in establishing compliant operation of existing wastewater treatment assets. There is a paucity of data currently available in the literature regarding the kinetic behaviour of NP and the short chain precursors under field conditions (e.g., rivers). Further work to underpin this preliminary fate evaluation to ascertain specific rates of degradation and transformation would therefore be advantageous. However, from this work it is clear that the analytical method is capable of detecting both NP and its precursors in environmentally relevant matrices and as such can support future field evaluations in addition to the optimisation of existing wastewater treatment assets through an enhanced understanding of intermediate compound fate and behaviour.

4.5. CONCLUSION

A significant reduction in total nonylphenolic concentrations has been observed since restrictions on the production of NPEOs was introduced. Nonetheless NP is still observed in

WwTW effluents and river waters at concentrations above the EQS of 300 ng l^{-1}. A paucity of information remains on NP fate and behaviour within WwTWs and in receiving environments which needs to be addressed by monitoring for a full range of nonylphenolic moieties. This is illustrated by the prediction of a transformation of NP precursors into NP which resulted in a breach of the NP EQS over several kilometres of a hypothetical river in some scenarios considered.

CHAPTER 5
A MULTI-RESIDUE METHOD TO DETERMINE THE FATE AND BEHAVIOUR OF PHARMACEUTICALS DURING SECONDARY WASTEWATER TREATMENT

IN PREPARATION: *Environmental Science and Pollution Research*

5. A MULTI-RESIDUE METHOD TO DETERMINE THE FATE AND BEHAVIOUR OF PHARMACEUTICALS DURING SECONDARY WASTEWATER TREATMENT

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Abstract

Recent trends of reducing analysis times and automating analytical methodologies has led to a lack of pharmaceutical analysis undertaken in the particulate phase of wastewaters. However, to better understand pharmaceutical fate and behaviour, determination here is essential. Reported is a multi-residue method for the determination of ten pharmaceuticals and the personal care product triclosan in the aqueous and particulate phases of wastewater. This was used to attain a complete process mass balance for a pilot-scale activated sludge plant operated under controlled, steady state conditions. Crude sewage was found to contain 63 % and 68 % of the antibiotics ofloxacin and ciprofloxacin within the particulate phase. Their high affinity to particulate matter and relatively low octanol-water coefficient (<1) indicates that electrostatic attractions are responsible for their solid partitioning. The mass balance revealed the chemicals studied were separable into three groups based on their dominant fate pathways (biological degradation, sorption onto activated sludge and no removal from the aqueous phase). Interestingly, diclofenac and fluoxetine are hydrophobic in nature (octanol-water coefficients >4) but exhibited no removal from the aqueous phase during treatment. Their charged nature in wastewater suggests they may be retained in the aqueous phase by dissolved organics (e.g., colloids). In final effluents, concentrations of triclosan, ofloxacin and ciprofloxacin were within the particulate phase at >10 % of their total concentration. Particulate concentrations were equivalent to 68.4, 104.5 and 25.7 ng l^{-1} respectively, providing a route for their release into the environment which often go unmonitored. Finally, the removal performance of activated sludge was directly compared to a trickling filter receiving the same influent sewage. This was used to assess the resilience of trickling filter treatment (characterised by very short contact times) for the removal of this diverse range of chemicals. The trickling filter demonstrated to be as effective as the ASP for the removal of most chemicals studied which rely on very different removal pathways.

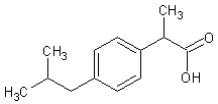
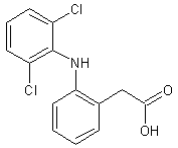
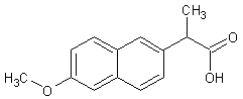
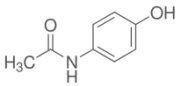
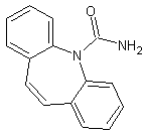
5.1. INTRODUCTION

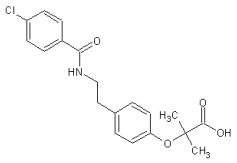
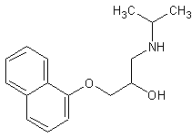
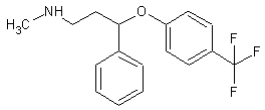
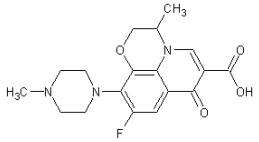
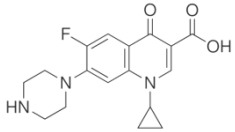
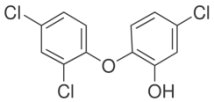
A large number of pharmaceuticals and personal care products, of varying concentrations are observed in river waters (Kasprzyk-Hordern *et al*., 2008; López-Serna *et al*., 2011), posing potential threats to aquatic biota (Kidd *et al*., 2007) and for water re-use (Martin *et al*., 2008). Their presence in surface waters is mainly attributed to their incomplete removal during wastewater treatment. Diagnosing wastewater treatment effectiveness for the removal of these organic contaminants relies on the application of analytical methodologies suitable for their determination at very low concentrations (ng l^{-1}) within complex organic matrices. Recent analytical trends generally focus on the development of methods for the rapid determination of a high number of chemicals (≥47) within the aqueous phase of wastewaters (Gracia-Lor *et al*., 2011; López-Serna *et al*., 2011; Gros *et al*., 2012). However, to better understand their fate, determination within the particulate fraction is essential (Petrie *et al*., 2013b). Although methods are available for particulate phase determinations (Radjenovic *et al*., 2009a; Baker and Kasprzyk-Hordern, 2011a), very little analysis is undertaken here due to the laborious sample collection and further extraction requirements. For example, one gram of dried solids is often required for each analysis (Radjenovic *et al*., 2009a). Although this is relatively straight forward to obtain for sludge samples where high solids concentrations are observed, the solids content typical of crude sewage (~200 mg l^{-1}), settled sewage (~100 mg l^{-1}) and final effluent (~20 mg l^{-1}) underlines the time and effort needed to collect suitable quantities of solids for analysis. As a result there is a paucity of information on the distribution of pharmaceuticals within the particulate phase of various wastewater matrices. Consequently, complete process mass balances have not been attainable which are essential to understand the pathways of their removal.

Trickling filters and activated sludge are widely used treatment methods which can remove pharmaceuticals and other hazardous chemicals to varying extents (Baker and Kasprzyk-Hordern, 2013; Petrie *et al*., 2013a; Petrie *et al*., 2013b; Petrie *et al*., in press). Removal by these biological processes is considered to be achieved mainly by biomass sorption and biodegradation (Andersen *et al*., 2005; Langford *et al*., 2005) which are thought to be influenced by their physico-chemical properties. It has been traditionally considered that hydrophobicity is a reasonable predictor of sorption. This assumption is applicable for some chemicals such as the steroid estrogens (Gomes *et al*., 2011; Petrie *et al*., submitted). However, it is inadequate to describe the behaviour of pharmaceuticals which exhibit a broad range of physico-chemical properties (Table 5.1). For example, recent research found hydrophobicity alone was insufficient to describe the sorption behaviour of pharmaceuticals and other interactions such as electrostatic attractions are important, particularly for charged chemicals (Hyland *et al*., 2012). Furthermore, it has been suggested that predicting sorption

cannot be fully ascertained using the properties of the sorbate. This is because sorption is also influenced by the specific properties of the sorbent (Sathyamoorthy and Ramsburg, 2013). These chemicals also vary significantly in their biodegradability. For example, ibuprofen is highly susceptible to biodegradation whereas carbamazepine and diclofenac are considered relatively resistant to biological attack (Petrie *et al.*, 2013b). This study was aimed at identifying the dominant removal pathways for a diverse range of organic hazardous chemicals during biological wastewater treatment. A multi-residue method for the determination of ten pharmaceuticals and the personal care product triclosan which represent a broad range of physico-chemical properties is reported. The method was used to assess their partitioning behaviour across a complete process mass balance for a pilot-scale activated sludge plant (ASP) operated under controlled steady state conditions. Furthermore, the removal performance of a trickling filter was directly compared to the ASP, whilst receiving identical influent sewage. This enabled the direct impact of process type on the removal of these chemicals to be assessed. Previous research has made the broad comparison between full-scale trickling filters and ASPs which receive varying compositions of influent sewage (Baker and Kasprzyk-Hordern, 2013). Therefore the direct impact of process type on the removal of trace organic contaminants has been difficult to ascertain.

Table 5.1. Physico-chemical properties of target chemicals (EPI suite, 2013)

Chemical of interest	Chemical class	Chemical structure	MW (g mol^{-1})	Water solubility (mg l^{-1})	Henry's law (atm m^3 mol^{-1})	VP (mm Hg)	pKa	Log K_{ow}	Log K_{oc}
Ibuprofen	NSAID		206.30	21.0	$1.52.10^{-7}$	$1.86.10^{-4}$	4.91[a]	3.79-3.97	2.35
Diclofenac	NSAID		296.15	2.4	$4.73.10^{-12}$	$2.18.10^{-6}$	4.15[a]	4.02-4.51	2.61
Naproxen	NSAID		230.26	15.9	$3.39.10^{-10}$	$1.27.10^{-6}$	4.15[a]	3.10-3.18	1.97
Acetaminophen	Aniline analgesic		151.17	14.10^3	$6.42.10^{-13}$	$1.94.10^{-6}$	9.38[a]	0.46	1.32
Carbamazepine	Anti-epileptic		236.27	112.0	$1.08.10^{-10}$	$8.80.10^{-8}$	13.90[a]	2.25-2.45	2.23

Bezafibrate	Lipid regulator		361.82	7.9	$2.12.10^{-15}$	$6.12.10^{-11}$	-	4.25	2.31
Propranolol	Beta-blocker		259.35	61.7	$7.98.10^{-13}$	$9.44.10^{-8}$	9.42	2.60-3.48	2.45
Fluoxetine	Anti-depressant		309.33	60.3	$8.90.10^{-8}$	$2.52.10^{-5}$	10.05[b]	4.05-4.65	4.97
Ofloxacin	Antibiotic		361.37	$2.8.10^{4}$	$4.98.10^{-20}$	$9.84.10^{-13}$	6.24[a]	<0	1.09
Ciprofloxacin	Antibiotic		331.35	$3.0.10^{4}$	$5.09.10^{-19}$	$2.85.10^{-13}$	6.09[a]	0.28	0.99
Triclosan	Anti-bacterial/ anti-fungal agent		289.55	10	$4.99.10^{-9}$	$4.65.10^{-6}$	7.90[a]	4.76	3.93

[a]Pubchem, 2013 [b]de Freitas *et al*., 2010

Key: MW, molecular weight; VP, vapour pressure; pKa, acid dissociation constant; Log K_{ow}, octanol-water coefficient; Log K_{oc}, organic carbon-water coefficient.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

The reference standards acetaminophen, carbamazepine and fluoxetine hydrochloride were purchased from Sigma-Aldrich (Dorset, UK) and were of ≥95 % purity. The standards bezafibrate, bezafibrate-d_6, carbamazepine-d_{10}, ciprofloxacin, ciprofloxacin-d_8, diclofenac, diclofenac-d_4, fluoxetine hydrochloride-d_5, ibuprofen, ibuprofen-d_3, naproxen, naproxen-d_3, ofloxacin, ofloxacin-d_3, propranolol, propranolol-d_7, triclosan and triclosan-d_3 were obtained from QMX laboratories (Thaxted, UK). The solvents methanol (MeOH) and acetonitrile (ACN) were purchased from Rathburn Chemicals (Walkerburn, UK) and were high-performance liquid chromatography (HPLC) grade. Formic acid and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) were obtained from Fisher Scientific (Loughborough, UK). Ultra-pure (UP) water of 18.2 MΩ quality (Elga, Marlow, UK) was used for the preparation of mobile phases. Ammonium acetate and Sigmacote® (silanising reagent for glass surfaces) was also purchased from Sigma-Aldrich (Dorset, UK). Sigmacote® was used to de-activate all glassware before use. Both individual stock standard and deuterated standard solutions of 1 mg ml^{-1} were prepared in MeOH and stored at -20 °C. Fresh stock solutions of the antibiotics were prepared monthly. Ten mixed working standard solutions ranging from 0 to 1,000 ng ml^{-1} (containing 200 ng ml^{-1} of each deuterated surrogate) were prepared daily in UP water: ACN: MeOH (90: 8: 2).

5.2.2. Wastewater treatment works

Samples for analysis were collected from a full-scale trickling filter works and a pilot-scale ASP situated in the East of England. These received municipal sewage of the same source containing indigenous concentrations of all chemicals. The trickling filter works serves a population equivalent of 3,000 and has an average flow of 650 m^3 d^{-1}. The works consisted of a roughing filter for bulk organics removal and two duplex filters for nitrification. Corresponding grab samples of settled sewage and final effluent was collected over three consecutive days in 2.5 l borosilicate glass bottles with Teflon lined caps. The pilot-scale ASP consisted of a primary sedimentation tank (0.18 m^3), an aerated basin (0.36 m^3) and a final clarifier (0.10 m^3). This was operated at a 30 day SRT whilst at a constant HRT of 24 hours. Samples of crude sewage, settled sewage, final effluent and return activated sludge (RAS) were collected on three consecutive days.

5.2.3. Extraction procedure

Samples (200 ml) for aqueous phase extraction were filtered using 0.7 µm GF/F filters (Fisher Scientific, Loughborough, UK) to remove particulates within 15 minutes of collection. Samples had Na_2EDTA added to achieve a concentration of 0.1 % (w/w) to improve extraction recoveries of the antibiotics (Hernandez *et al*., 2007). Each sample was then spiked with deuterated surrogates to achieve a concentration of 500 ng l^{-1}. To determine extraction efficiency, selected samples were spiked with an additional 500 ng l^{-1} of all reference standards. This was then subjected to solid phase extraction (SPE) using 200mg: 6cc Oasis HLB cartridges (Waters, Elstree, UK). The extraction protocol was similar to that described by Gros *et al* (2006). Cartridges were pre-conditioned with 5 ml MeOH followed by 5 ml UP water at a constant flow rate of 1 ml min^{-1}. Samples were then loaded at 5 ml min^{-1} and cartridges were rinsed with 5 ml UP water. These were then dried for 30 minutes under vacuum to remove excess water. Analytes were eluted on the same day using two 4 ml aliquots of MeOH at 1 ml min^{-1}. Extracts were then evaporated to dryness using a miVac Duo concentrator (Genevac, Ipswich, UK). These were then reconstituted in 0.5 ml UP water: ACN: MeOH (90: 8: 2) and transferred to auto-sampler vials. Extracts were stored at 4 ^{o}C and analysed within 24 hours.

For particulate extractions, large volumes of sample (crude sewage, settled sewage, final effluent and RAS) were centrifuged at 1,500 x *g* for 10 minutes and filtered to obtain the suspended solids. These were frozen immediately and then freeze-dried. Replicates of 0.3 to 0.5 grams were spiked with a mixed solution of deuterated standards in MeOH (absolute concentration of each individual chemical was 100 ng) and left for a minimum of 5 hours. Selected samples were also spiked at an additional 120 to 200 ng g^{-1} of the reference standards to assess extraction recovery. Samples were then mixed with ~20 g of Ottawa Sand obtained from Fisher Scientific (Loughborough, UK) and packed into 33 ml Dionex accelerated solvent extraction (ASE) cells. Two or three 2-4 µm Dionex glass fibre filters (Fisher Scientific, Loughborough, UK) were then packed into both ends of the cell. Extractions were performed using a Dionex ASE 200 (California, USA). The extraction pressure was set to 1,500 psi whilst at a temperature of 80 °C. The solvent mixture for extraction was UP water: MeOH (65: 35). For each cell, two extraction cycles were conducted with the following settings: pre-heat period (for the first cycle only) of 5 minutes, heating for 5 minutes, static extraction of 5 minutes, solvent flush volume of 60 % and a nitrogen purge time of 60 seconds. Extracts were combined and diluted with UP water to achieve a MeOH concentration of <5 % and then subjected to SPE as an aqueous sample.

5.2.4. UPLC-MS/MS analysis

Chromatography was performed using an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Manchester, UK). To maximise chromatographic resolution and sensitivity, chemicals were separated into two separate runs similar to that described by Gros *et al* (2012). Those chemicals exhibiting greater sensitivity in negative ion mode were separated on a reversed-phase BEH C18 column (2.1 mm 100 mm, particle size 1.7 µm) (Waters, Manchester, UK) using a gradient elution of 5 mM ammonium acetate in UP water (A) and an organic mixture of ACN: MeOH (80: 20) (B). Starting conditions were 10 % B which were maintained for 1.0 min. This was then increased linearly over 4.5 min to 80 % and maintained for 1.5 min before returning to starting conditions. The total run time was 9.0 minutes. Chemicals analysed in positive ion mode were separated using an Acquity HSS T_3 column (2.1 mm × 30 mm, particle size 1.8 µm) (Waters, Manchester, UK) and mobile phases consisting of 5 mM ammonium acetate in UP water containing 0.1 % formic acid (A) and an organic mixture of ACN: MeOH (80:20) (B). The elution started at 10 % B and was then linearly increased to 80 % B in 6.0 minutes, then maintained for 1.0 minute before returning to starting conditions. The total time of analysis was 10.0 minutes. In both runs, the flow rate was 0.35 ml min^{-1} with an injection volume of 10 µl. The columns were maintained at a constant 35 ºC and the sample manager at 4 ºC. The UPLC was coupled to a Waters Quattro Premier XE mass spectrometer with a Z-spray electrospray ionisation interface (Micromass, Watford, UK). The multiple reaction monitoring (MRM) transitions monitored for each chromatographic run are detailed (Table 5.2). The operational parameters for the mass spectrometer were: capillary voltage, 3.20 kV; multiplier voltage, 650 V; desolvation gas flow, 1000 l h^{-1}; cone, -25 V; RF lens, 0.2 V; cone gas flow, 50 l h^{-1}; desolvation temperature, 350 °C and source temperature, 120 °C.

Table 5.2. Optimised UPLC-MS/MS parameters for target chemicals analysed using the two different chromatographic runs

Chemical	Precursor ion (m/z)	SRM1	Cone voltage (V)	Collision Energy (V)	SRM2	Cone voltage (V)	Collision Energy (V)	Dwell time (seconds)	Retention time (minutes)	IDL (pg)
Column: C18 Mobile phase A: 5mM ammonium acetate in UP water. Mobile phase B: 80: 20 ACN: MeOH										
Acetaminophen	151.80 $[M+H]^+$	92.90	30	20	109.90	30	15	0.1	1.63	15
Naproxen	228.90 $[M-H]^-$	169.90	15	15	184.90	15	8	0.1	3.59	22
Naproxen-d_3	231.90 $[M-H]^-$	172.95	15	15	-	-	-	0.1	3.59	-
Bezafibrate	360.00 $[M-H]^-$	274.05	30	30	153.90	30	20	0.1	3.85	13
Bezafibrate-d_6	366.00 $[M-H]^-$	274.10	30	20	-	-	-	0.1	3.85	-
Diclofenac	293.80 $[M-H]^-$	250.00	20	10	-	-	-	0.1	4.35	17
Diclofenac-d_4	297.85 $[M-H]^-$	254.05	20	10	-	-	-	0.1	4.35	-
Ibuprofen	204.90 $[M-H]^-$	161.00	20	8	-	-	-	0.1	4.42	13
Ibuprofen-d_3	207.90 $[M-H]^-$	164.00	20	8	-	-	-	0.1	4.42	-
Triclosan	286.80 $[M-H]^-$	286.80	20	3	-	-	-	0.1	6.51	239
Triclosan-d_3	289.80 $[M-H]^-$	289.80	20	4	-	-	-	0.1	6.50	-
Column: T_3 Mobile phase A: 5mM ammonium acetate, 0.1 % formic acid in UP water. Mobile phase B: 80: 20 ACN: MeOH										
Ofloxacin	361.90 $[M+H]^+$	261.10	42	25	318.20	40	20	0.1	3.31	15
Ofloxacin-d_3	364.90 $[M+H]^+$	321.20	40	20	-	-	-	0.1	3.32	-
Ciprofloxacin	332.10 $[M+H]^+$	288.15	36	20	245.10	36	16	0.1	3.37	13
Ciprofloxacin-d_8	340.10 $[M+H]^+$	296.20	36	20	-	-	-	0.1	3.37	-
Propranolol	260.10 $[M+H]^+$	183.00	35	18	116.00	35	15	0.1	4.73	12
Propranolol-d_7	267.10 $[M+H]^+$	189.10	35	18	-	-	-	0.1	4.73	-
Carbamazepine	237.00 $[M+H]^+$	194.00	32	35	179.00	32	18	0.1	5.39	7
Carbamazepine-d_{10}	247.05 $[M+H]^+$	204.10	34	20	-	-	-	0.1	5.38	-
Fluoxetine	310.10 $[M+H]^+$	148.05	25	10	44.10	25	8	0.1	6.00	12
Fluoxetine-d_5	315.10 $[M+H]^+$	153.05	25	8	-	-	-	0.1	5.99	-

Key: SRM, selected reaction monitoring, IDL, instrument detection limit

5.3. RESULTS

5.3.1. Method performance

Mean recoveries of all chemicals ranged from 36 to 134 % and from 43 to 129 % in all matrices for aqueous and particulate phases, respectively (Figure 5.1). The influence of remaining matrix within sample extracts was quantified according to the algorithm reported by Vieno *et al* (2006). Matrix suppressions ranged from -51 (signal enhancement) to 67 % and from -50 to 78 % for aqueous and particulate phases (Figure 5.1). The chemicals analysed in negative ion mode (naproxen, bezafibrate, diclofenac, ibuprofen and diclofenac) all exhibited signal enhancement for some matrices. Method detection limits (MDLs) were calculated according to (Petrie *et al*., 2013b; Petrie *et al*., in press):

$$MDL = \frac{IDL.100}{(R.C)} . \frac{100}{(100-M)} \quad (5.1)$$

where *IDL* is the instrument detection limit (ng l^{-1}), *R* is recovery (%), *C* is the concentration factor and *M* is the matrix suppression (%). Pharmaceutical MDLs ranged from 2.3 to 22.4 ng l^{-1} in aqueous wastewater fractions whereas those for triclosan ranged from 46.4 to 86.5 ng l^{-1} (Figure 5.1). In particulate fractions, MDLs ranged from 2.4 to 29.0 ng g^{-1}. The MDLs of the personal care product triclosan were comparatively higher for both aqueous (46.4 to 86.5 ng l^{-1}) and particulate (52.5 to 86.8 ng g^{-1}) phases of all matrices studied. Chromatography for extracted environmental matrices exhibited Gaussian distribution for all target chemicals (Figure 5.2). However, poor peak shape was observed for acetaminophen when formic acid was present within the aqueous mobile phase. Consequently, this chemical was analysed using the mobile phase compositions for negative ion chemicals.

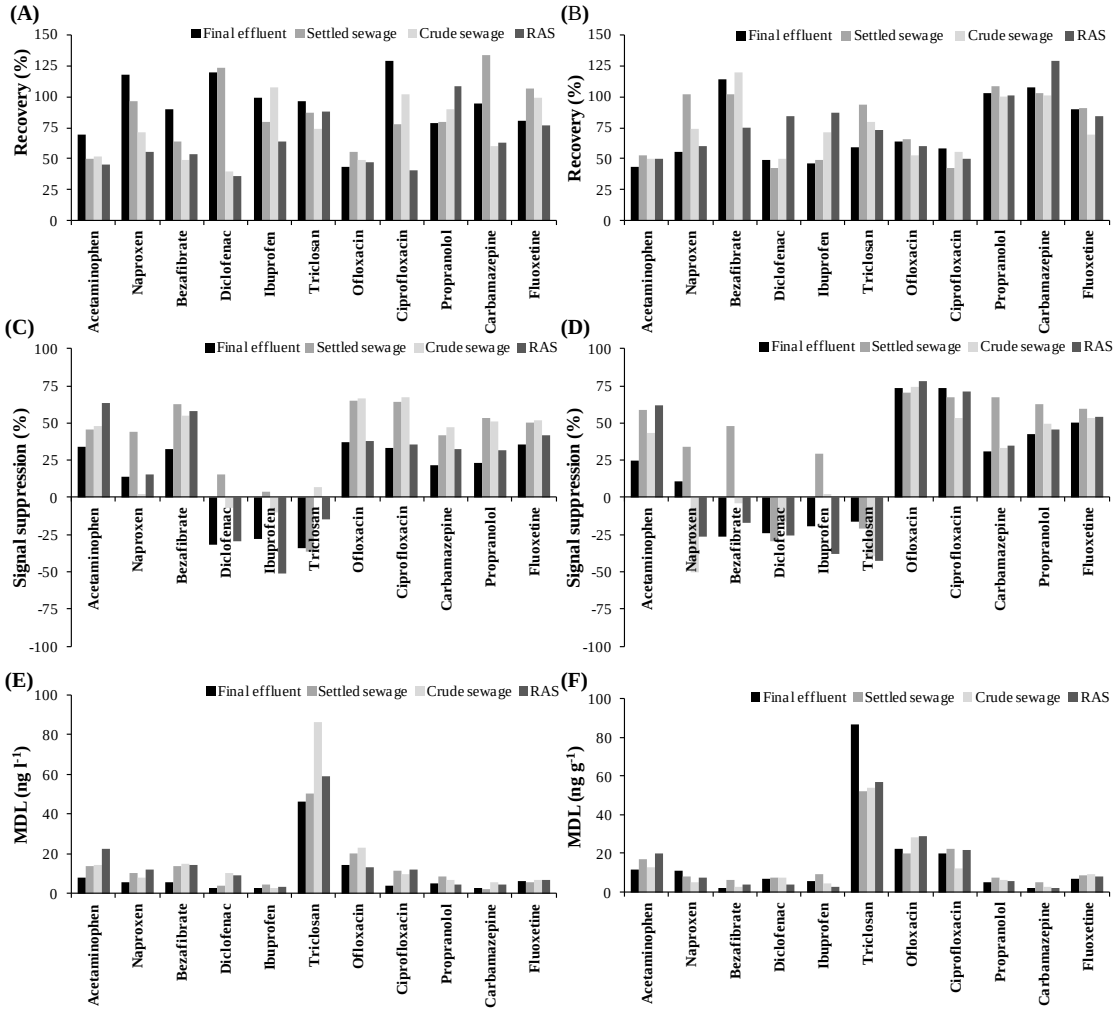


Figure 5.1. Method recoveries (aqueous – A, particulate – B), analyte signal suppressions (aqueous – C, particulate – D) and method detection limits (aqueous – E, particulate – F) for target chemicals in final effluent, settled sewage, crude sewage and RAS matrices.

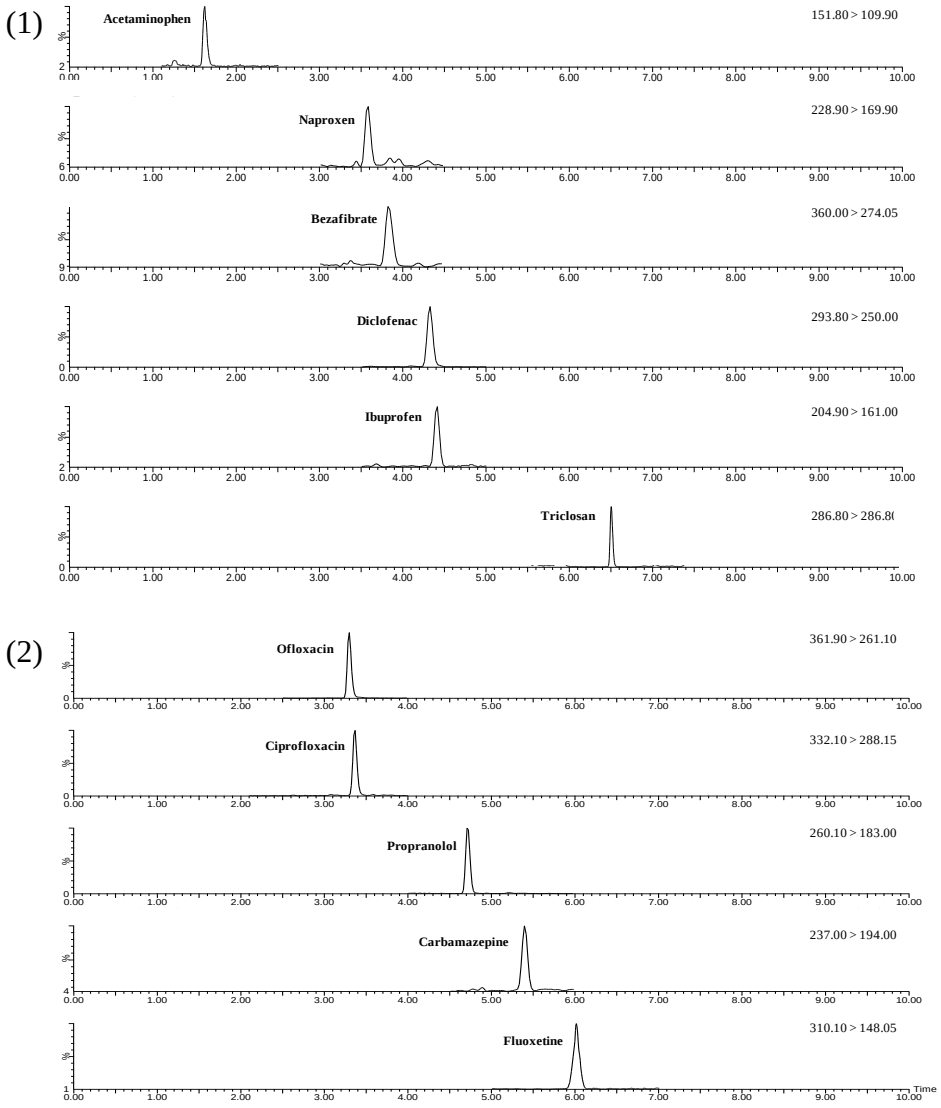


Figure 5.2. Multiple reaction monitoring chromatograms of target chemicals extracted from the particulate phase of crude sewage for chromatographic runs 1(BEH C18 column with mobile phases 5 mM ammonium acetate in UP water and 80: 20 MeOH: ACN) and 2 (HSS T_3 column with mobile phases 5 mM ammonium acetate, 0.1 % formic acid in UP water and 80: 20 MeOH: ACN)

5.3.2. Distribution within the particulate phase

In crude sewage, the antibiotics ofloxacin and ciprofloxacin exhibited greater distribution (63 and 68 %) within the particulate phase (Figure 5.3A). These correspond to partition coefficients (log K_d's) of 3.96 and 4.07, respectively. Propranolol, triclosan and fluoxetine all showed reasonable affinity to suspended solid material with relative distributions in the particulate phase of 15, 29 and 37 %, respectively. The remaining chemicals (acetaminophen, ibuprofen, bezafibrate, naproxen, carbamazepine and diclofenac) were all found to be distributed by <10 % in the particulate phase. Primary sedimentation resulted in a 71 % reduction in suspended solids concentration. Consequently, the contribution of particle bound chemicals to the total concentration was reduced (Figure 5.3B). Ofloxacin now exhibited the

greatest distribution within the particulate phase of settled sewage (41 %). Following a further 77 % reduction of suspended solids during secondary treatment, most chemicals were distributed mainly in the aqueous phase (>99 %) of final effluents (Figure 5.3C). Nevertheless, fluoxetine (5 %), ciprofloxacin (11 %), ofloxacin (24 %) and triclosan (28 %) still showed a reasonable proportion of their total concentration to be within the particulate phase. Despite the concentration of suspended solids being 12.5 mg l^{-1} in final effluents. The relatively high distribution of these chemicals in the particulate phase corresponded to log K_d's of 3.58, 3.98, 4.41 and 4.51, respectively.

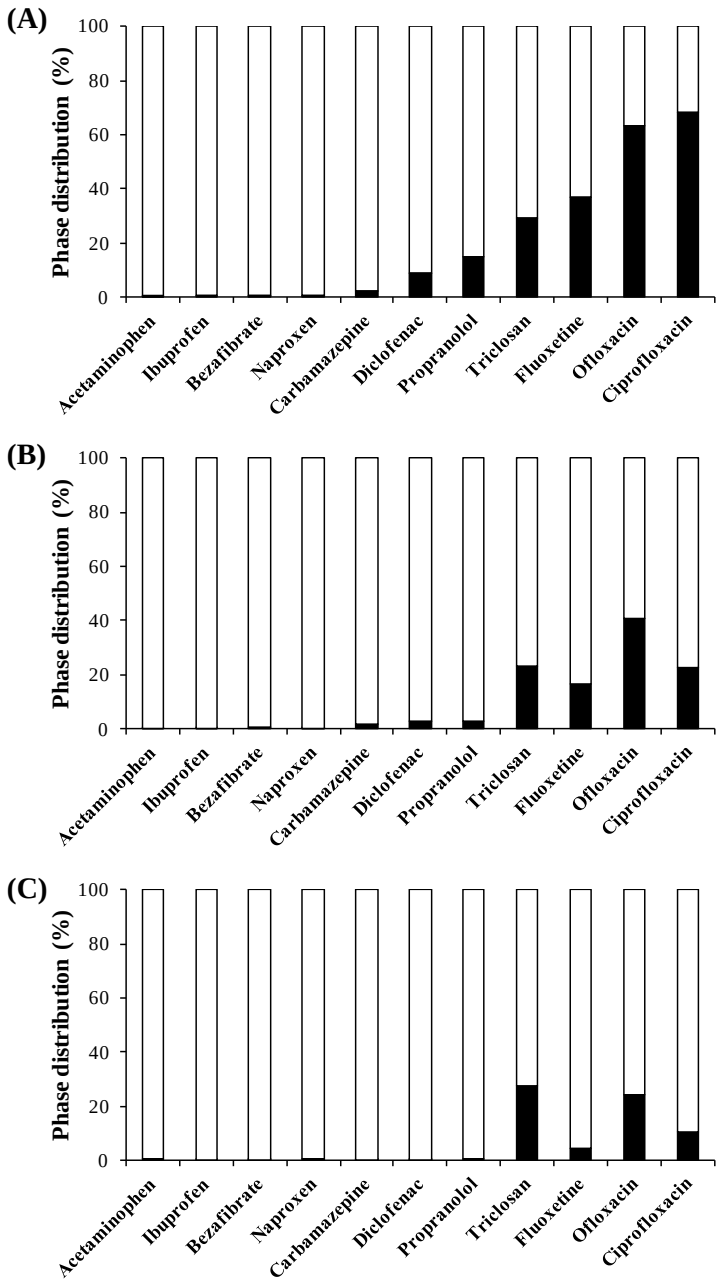


Figure 5.3. Relative distribution of target analytes within the dissolved (□) and particulate (■) phases of crude sewage (A), settled sewage (B) and final effluent (C)

5.3.3. Fate pathways

Attaining complete process mass balances enabled each chemical to be broadly separated into three groups, based on their respective fate pathway. These are chemicals predominantly removed by biodegradation (acetaminophen, ibuprofen, bezafibrate, and naproxen), by sorption onto activated sludge (triclosan, ofloxacin and ciprofloxacin) and those exhibiting no removal from the aqueous phase (carbamazepine, diclofenac, propranolol and fluoxetine), respectively (Figure 5.4). Each chemical was separated according to aqueous and particulate concentrations across crude sewage, settled sewage, RAS and final effluent. For example, secondary removal of ibuprofen was 97 % with no accumulation within the particulate phase of RAS suggesting its predominant removal pathway was biodegradation (Figure 5.5A). Interestingly, 55 % removal was observed from the aqueous phase of wastewater during primary sedimentation alone. In contrast, triclosan showed no removals from either the aqueous or particulate phases during primary sedimentation (Figure 5.5B). However, during secondary treatment 84 % removal was observed. An accumulation of triclosan within the particulate phase of RAS (2,469 ng g^{-1}) confirmed its removal by sorption. Considering the comparatively higher concentrations of ofloxacin (15,112 ng g^{-1}) and ciprofloxacin (3,964 ng g^{-1}) within RAS (and poorer secondary removal achieved) (Figure 5.4) suggests that triclosan is also removed by biodegradation. In contrast, dissolved concentrations of fluoxetine were similar in all sampling positions of the process mass balance (Figure 5.4C). Nevertheless, an accumulation of fluoxetine was observed in the particulate phase of RAS which corresponded to the removal of suspended solids (and particulate bound fluoxetine) during secondary treatment.

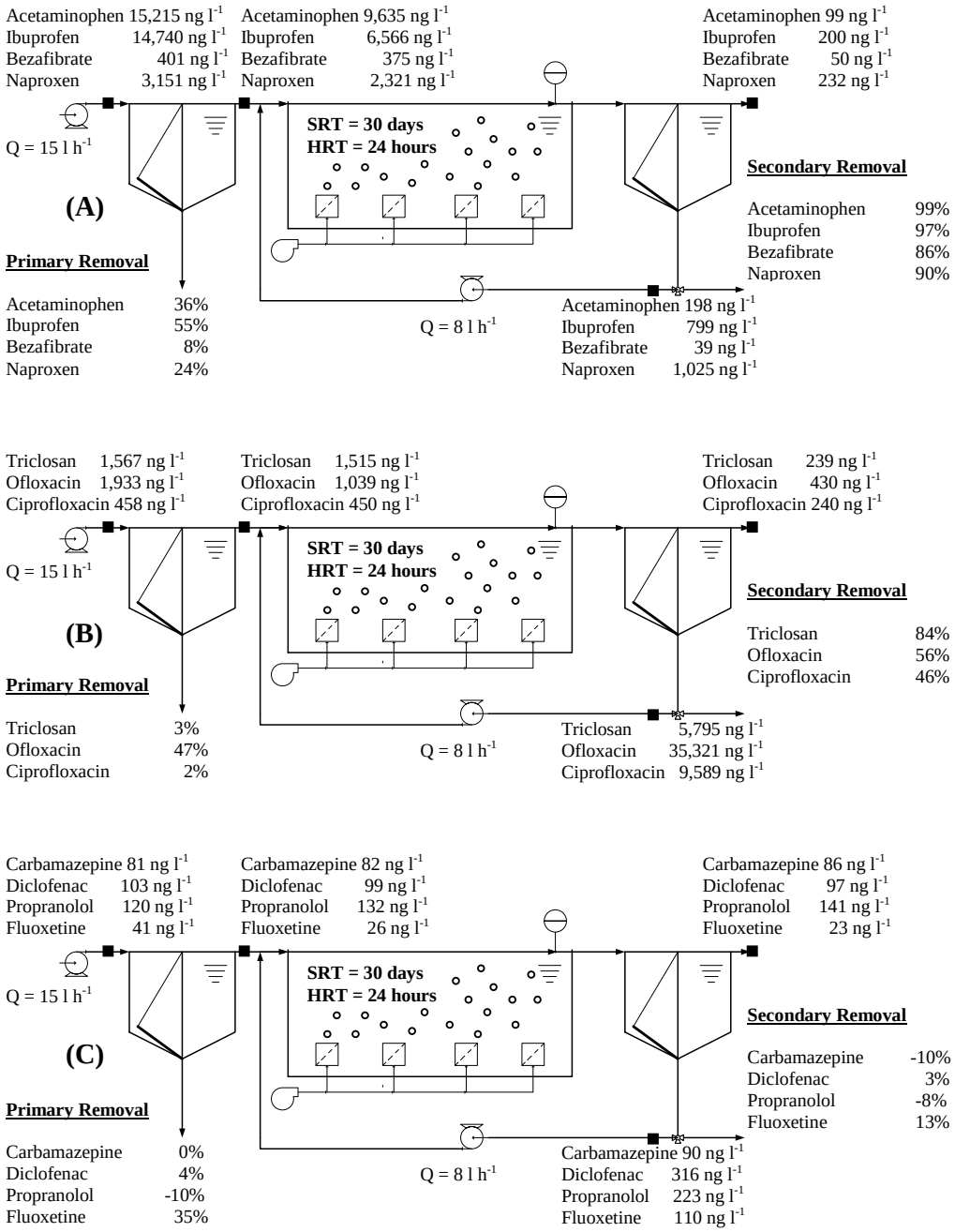


Figure 5.4. Mass balance information for target chemicals separated by their predominant removal pathways from the aqueous phase - biodegradation (A) and sorption (B) and those chemicals not removed by sorption or biodegradation (C), ■-sampling points

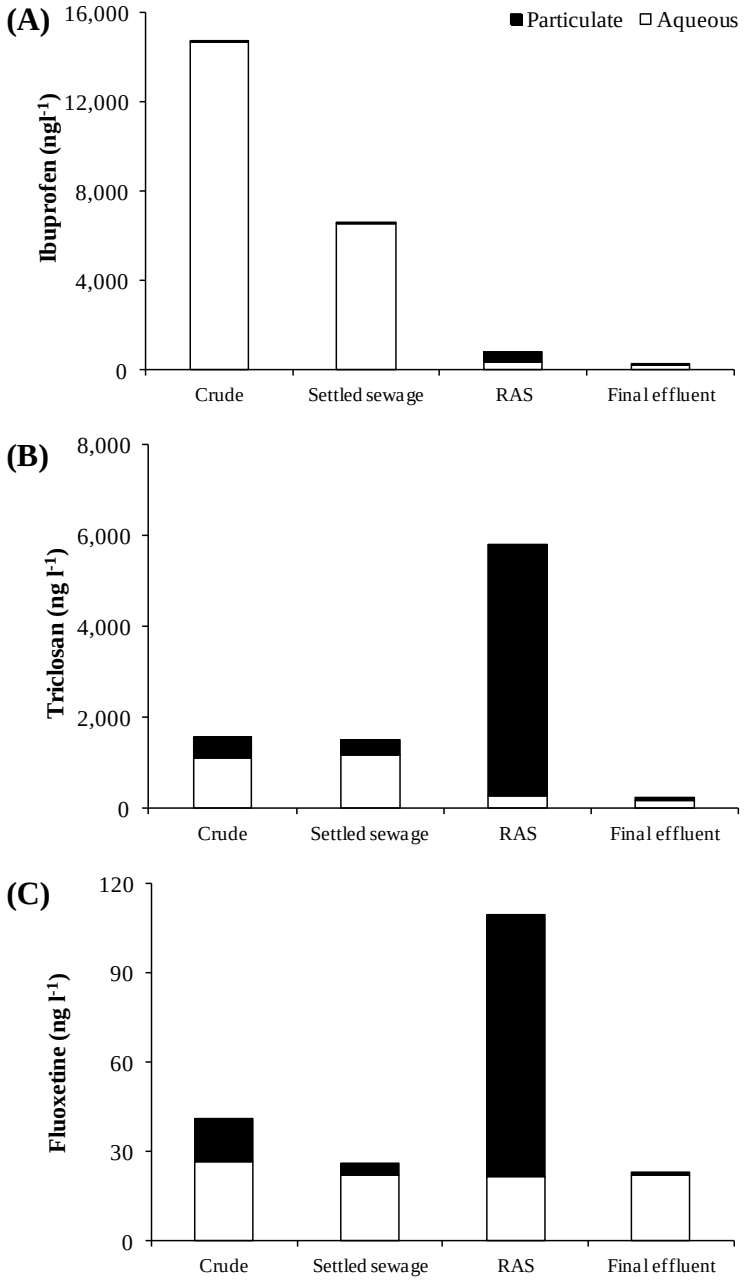


Figure 5.5. Aqueous and particulate concentrations of ibuprofen (A), triclosan (B) and fluoxetine (C) in crude sewage, settled sewage, return activated sludge and final effluents

5.3.4. Efficacy of trickling filter treatment

Corresponding settled sewage and final effluent samples were collected from a trickling filter to directly compare removal performance with activated sludge (Figure 5.6). Both treatment processes were monitored simultaneously whilst receiving the same influent sewage at 19 ºC. Pharmaceutical removal by the trickling filter ranged from -48 ±26 % for propranolol to >99 % for acetaminophen. Similar removal performance to activated sludge treatment was observed for the majority of target chemicals. This includes the antibiotics whose removal is attributable to sorption. Removals of ofloxacin were 56 ±12 % and 54 ±6 % and ciprofloxacin were 46 ±7 % and 42 ±15 % for activate sludge and trickling filter treatment respectively. However, secondary removals of bezafibrate and triclosan were comparatively lower by trickling filter treatment. Bezafibrate was removed by 86 ±2 % and 61 ±9 % for activated sludge and trickling filter treatment, respectively. Moreover, triclosan removals were 84 ±3 % (activated sludge) and 74 ±5 % (trickling filter). The remaining chemicals were all removed similarly by activated sludge and trickling filter treatment.

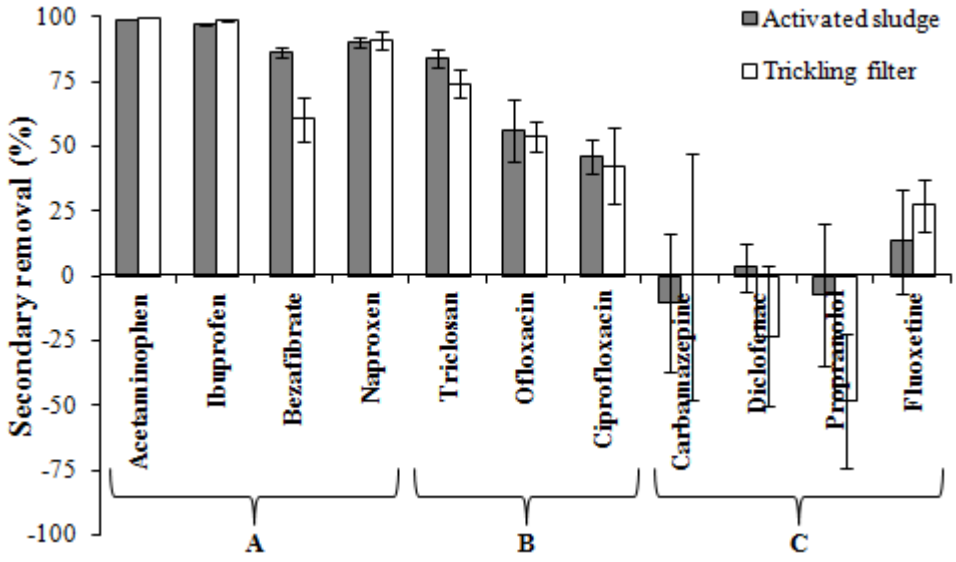


Figure 5.6. Comparison of target chemical secondary removal by activated sludge and trickling filter treatments receiving municipal sewage of the same source. Target chemicals separated by dominant removal mechanism - biodegradation (A), and sorption (B), and those chemicals not removed by sorption or biodegradation (C)

5.4. DISCUSSION

Chemicals were separated into two chromatographic runs (positive and negative ionisation modes) to achieve maximum sensitivity and lower MDLs (Figure 5.1). Those analysed in positive ion mode had 0.1 % formic acid added to the aqueous mobile phase. This was used to enhance their ionisation efficiency and sensitivity (Gros *et al*., 2012). Method detection limits were <10 ng l^{-1} for the majority of chemicals in final effluents (Figure 5.1). The MDLs of triclosan were comparatively higher (46 to 86 ng l^{-1}). This was due to its lack of product ions during MS/MS fragmentation. To illustrate, previous reported LC-MS/MS methods monitor for product ions with a mass to charge (m/z) ratio of 35 (Hyland *et al*., 2012; Bayen *et al*., 2013; Boleda *et al*., 2013) or the precursor ion again at a low collision energy (Behera *et al*., 2011; Zhao *et al*., 2010; Zhao *et al*., 2011) (replicating single MS mode). The latter approach was adopted here due to the mass range of the MS/MS used in this study. The comparatively lower signal to noise ratios for triclosan resulted in greater MDLs observed. Nevertheless, this enabled triclosan to be included in the method, without the need to run a separate LC-MS or gas chromatography method for its analysis. Furthermore, the MDLs achieved by this approach were adequate to monitor for triclosan at the concentrations encountered in all matrices studied. Analyte signal suppressions broadly ranged from -51 % (signal enhancement) to 78 % for all chemicals and matrices (Figure 5.1). This is caused by the influence of the complex heterogeneous composition of environmental matrices on analyte signal strength whilst using electro-spray ionisation, highlighting the necessity of including deuterated standards within the analysis.

The determination of the trace organic contaminants in both the aqueous and particulate phases of wastewaters revealed significant concentrations associated with particulates (Figure 5.3). Propranolol, triclosan, fluoxetine, ofloxacin and ciprofloxacin were all distributed in the particulate phase of crude wastewater at >10 % (15, 29, 37, 63 and 68 % of the total concentration, respectively). Consequently, determination in the particulate fraction of wastewater is essential for these chemicals to better understand fate and process performance for their removal (Petrie *et al*., 2013b). Interestingly, both ofloxacin and ciprofloxacin have log K_{ow}'s <1 (Table 5.1) illustrating their sorption is driven by other mechanisms such as electrostatic attractions. The remaining six chemicals were found in the particulate phase at <10 % of the total concentration. This broad range of distribution supports previous findings for a variety of illicit drugs and pharmaceuticals in crude sewage (Baker and Kasprzyk-Hordern, 2011a). However, distribution within final effluents has previously been unknown. This is due to the additional demands on sample collection and preparation caused by the very low suspended solids concentrations encountered. Nevertheless, this study revealed that significant concentrations of specific trace organic contaminants can be found within the

particulate phase of final effluents, even at low suspended solids concentrations (12.5 mg l^{-1}) (Figure 5.3). Triclosan, ofloxacin and ciprofloxacin were all within the particulate phase at >10 % of the total concentration, corresponding to particulate concentrations equivalent to 68.4, 104.5 and 25.7 ng l^{-1}, respectively. This is significant as relatively large quantities of these chemicals are released into the environment unmonitored and specific knowledge on the fate of particulate bound organic contaminants within the environment is unknown.

The complete process mass balance revealed that the chemicals studied were broadly separable into three groups based on their fate pathways (Figure 5.4); chemicals removed by biodegradation, those predominantly removed by sorption and chemicals which exhibited no removal from the aqueous fraction (Figure 5.5). The chemicals mainly removed by biodegradation exhibited no notable accumulation within the particulate phase of RAS despite significant secondary removal (≥86 %). Naproxen, acetaminophen and ibuprofen all exhibited removal from the aqueous phase (24 % to 55 %) during primary sedimentation (Figure 5.4A). This suggests that bacteria in receiving crude sewage biodegrade these contaminants during primary treatment. In contrast triclosan, ofloxacin and ciprofloxacin accumulated within the particulate phase of RAS confirming their removal by sorption (Zhou *et al*., 2013). Their concentrations were 2,469, 15,112 and 3,964 ng g^{-1}, respectively. The high partitioning of the antibiotics to RAS has been previously observed with concentrations of ofloxacin up to 24, 760 ng g^{-1} (Chen *et al*., in press). Interestingly, diclofenac and fluoxetine have log K_{ow}'s >4 yet no removal from the aqueous phase was observed during treatment. Shen *et al* (2012) successfully showed that the steroid estrogen estrone (log K_{ow} 3.13 to 3.43 – EPI Suite, 2013) could effectively be retained in solution by humic acid, a small molecular weight charged species. Both diclofenac (negative) and fluoxetine (positive) are charged at the pH typical of municipal wastewaters (Hyland *et al*., 2012). Therefore, knowledge of the behaviour of pharmaceuticals with charged colloidal species present within wastewater is required. This may help elucidate the poor removal of relatively hydrophobic chemicals during wastewater treatment.

Monitoring organic contaminant removal performance of activated sludge and trickling filter treatment simultaneously, whilst receiving the same influent sewage enabled the direct impact of process type to their removal to be evaluated. Previous research made the comparison between numerous full-scale processes (Baker and Kasprzyk-Hordern, 2013), which makes the impact of process type to removal difficult to ascertain. Findings here revealed that trickling filter treatment was as effective as activated sludge treatment for the removal of most chemicals which rely on very different removal pathways (Figure 5.6). For example, ibuprofen was considered to be removed by biodegradation and removals were 97 ±1 % and 98 ±1 % for activated sludge and trickling filter treatment, respectively. The antibiotics which are

removed by sorption exhibited removals of 46 to 56 % by activated sludge and from 42 to 54 % by trickling filter treatment, with no significant differences observed. Only removals of triclosan and bezafibrate were notably greater by ASP treatment. Furthermore, the ASP was operated at a long hydraulic retention time (HRT) which is considered favourable for enhanced organic contaminant removal (Petrie *et al*., in preparation). This demonstrates the effectiveness of trickling filter treatment to remove these organic contaminants which rely on very different removal pathways whilst operating at very short HRTs. It should be noted that the determination of metabolites must also be undertaken to better understand fate and removal (Huerta-Fontela *et al*., 2010; Ferrando-Climent *et al*., 2012). For example, there is a paucity of information available on ibuprofen metabolites, despite this being one of the most studied pharmaceuticals (Petrie *et al*., 2013b). Recent work by Ferrando-Climent *et al* (2012) revealed that at least three metabolites of ibuprofen exist at μg l^{-1} concentrations. These were found to be present in influent and effluent wastewater at higher levels than the parent chemical (Ferrando-Climent *et al*., 2012). Consequently, future work to assess chemical fate and process performance for their removal must include metabolites.

5.5. CONCLUSION

It is unlikely there will be a reduction of pharmaceuticals seen entering crude sewage due to their essential medical usage. Consequently, it is essential to better understand their fate and behaviour during wastewater treatment. It was found that these chemicals exhibit a range of dominant removal pathways (biological and physical) during activated sludge treatment. Interestingly, trickling filter treatment was as effective as activated sludge treatment for the removal of the majority of the organic contaminants studied. Nevertheless, significant concentrations of some chemicals were present within the particulate phase of final effluents which provide a route into the environment which commonly goes undetermined.

CHAPTER 6

DIAGNOSTIC INVESTIGATION OF STEROID ESTROGEN REMOVAL BY ACTIVATED SLUDGE AT VARYING SOLIDS RETENTION TIME

SUBMITTED: *Chemosphere*

6. DIAGNOSTIC INVESTIGATION OF STEROID ESTROGEN REMOVAL BY ACTIVATED SLUDGE AT VARYING SOLIDS RETENTION TIME

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Abstract

The impact of solids retention time (SRT) on estrogen removal in an activated sludge plant (ASP) was examined using a pilot plant to closely control SRT. *Ex-situ* analytical methods were simultaneously used to enable discrimination of the dominant mechanisms governing estrogen removal following transitions in SRT from short (3 day) to medium (10 day) and long (27 day) SRTs which broadly represent those encountered at full-scale. Total estrogen ($\sum_{EST}$) removals were 70 ±8, 95 ±1 and 93 ±2 % at 3, 10 and 27 day SRTs respectively. The improved removal observed following increasing SRT from 3 days to 10 days SRT was attributable to the augmented biodegradation of the natural estrogens estrone and 17β-estradiol. Interestingly, estrogen biodegradation per bacterial cell increased with SRT. These were 499, 1,361 and 1,750 ng$\sum_{EST}$ 1 x 10^{12} viable cells^{-1} d^{-1}. This indicated an improved efficiency of the same group or the development of a more responsive group of bacteria. In this study no improvement in absolute $\sum_{EST}$ removal was observed in the ASP when SRT increased from 10 days SRT to 27 days SRT. However, batch studies identified an augmented biomass sorption capacity for the more hydrophobic estrogens 17β-estradiol and 17α-ethinylestradiol at 27 days, equivalent to an order of magnitude. The limited influence on ASP operation can be ascribed to the receiving concentrations of the influent sewage being inadequate to challenge the system. However, this indicates that high SRT operation could be beneficial for wastewater characterised by higher receiving concentrations of the more hydrophobic estrogenic compounds 17β-estradiol and 17α-ethinylestradiol.

6.1. INTRODUCTION

The fate and behaviour of steroid estrogens in wastewater treatment works (WwTWs) is of great importance due to their detrimental environmental impact (Lai *et al*., 2002a; Lai *et al*., 2002b; Green *et al*., 2013) and possible adverse implications for water re-use (Martin *et al*., 2008). This is a consequence of their high estrogenic potency (Kidd *et al*., 2007) and ubiquity in municipal wastewaters (Joss *et al*., 2004; Clara *et al*., 2005; Koh *et al*., 2009; McAdam *et al*., 2010. Natural estrogens; estrone (E1), 17β-estradiol (E2) and estriol (E3) constitute the majority (typically ≥99 %) of free steroid estrogens observed in wastewaters (Gomes *et al*., 2009; Koh *et al*., 2009; McAdam *et al*., 2010). The synthetic estrogen 17α-ethinylestradiol (EE2) is present in much lower concentrations (Koh *et al*., 2009; McAdam *et al*., 2010; Baynes *et al*., 2012) but is arguably of greater concern due to its higher potency and the difficulty it poses to removal during conventional wastewater treatment. Such is the concern both E2 and EE2 were proposed to be included as priority hazardous chemicals under the Water Framework Directive (of the European Union) with suggested Environmental Quality Standards (EQS) of 0.4 ng l^{-1} and 0.035 ng l^{-1}, respectively (European Commission, 2012). However, as currently configured secondary WwTWs fail to consistently meet these proposed guidelines (Koh *et al*., 2009; McAdam *et al*., 2010). Activated sludge is a widely implemented secondary biological process with an established ability to remove numerous anthropogenic (Stoveland *et al*., 1979; McAdam *et al*., 2011; Helbling *et al*., 2012) and complex natural chemicals including estrogens to varying extents (Kreuzinger *et al*., 2004; Clara *et al*., 2005; Koh *et al*., 2009; McAdam *et al*., 2010). To ensure effluent quality exceeds proposed EQS's, optimisation of process operation is therefore required. Achieving this relies on understanding the mechanisms which drive removal at different operations. An extended solids retention time (SRT) is generally considered necessary to achieve enhanced removal of estrogens (Kreuzinger *et al*., 2004; Clara *et al*., 2005; McAdam *et al*., 2010). It has been considered that an increase in SRT enables the enrichment of a more diverse biocenosis which augments steroid estrogen biodegradation (Kreuzinger *et al*., 2004). It has also been suggested that the *k*-strategist concept maybe applicable (Graham and Curtis, 2003; Koh *et al*., 2009), hypothesising that micro-organisms characterised by low half saturation coefficients biodegrade estrogens as a primary carbon substrate at extended SRTs. The activity of nitrifying micro-organisms has long been associated with improved estrogen removal (Vader *et al*., 2000). However, the role of nitrifiers in environmentally representative conditions has been questioned (Gaulke *et al*., 2008) and it has been proposed that heterotrophic bacteria scavenging a broad spectrum of organics perform estrogen biodegradation in conditions conducive to nitrification (Bagnall *et al*., 2012).

Despite extensive research, the site of steroid estrogen biodegradation within the biomass matrix remains unknown. It is hypothesised that biological removal of organic chemicals can occur as an extracellular process within the bulk medium or, on the surface and/or within the activated sludge floc itself (Joss *et al*., 2004). Nitrifiers are known to produce extracellular ammonia mono-oxygenase (AMO) enzymes capable of co-oxidising a variety of organic chemicals (Vader *et al*., 2000). Thus, biodegradation may occur in the bulk medium outside activated sludge flocs. Khunjar and Love (2011) suggested a requirement for sorption to occur prior to biological breakdown being initiated. Pharmaceuticals which failed to sorb to extracellular polymeric substances (EPS) were not biodegraded. Thus sorption may be a prerequisite to the biodegradation process. Chemical hydrophobicity (log K_{ow}) is considered a reasonable predictor of sorption equilibrium (Gomes *et al*., 2011). McAdam *et al* (2010) highlighted the possible importance of changing floc physiology with SRT to estrogen sorption. Activated sludge biomasses ranging in age from 4 to 20 days have been observed to differ in their surface properties such as charge and, EPS and protein content (Liao *et al*., 2001). Both EPS and proteins concentration are thought to influence EE2 sorption. For example, when EPS was removed EE2 sorption coefficients were reduced by ~50 % (Khunjar and Love, 2011). Furthermore, EE2 was shown to sorb preferentially to EPS protein over carbohydrate. Activated sludge floc size is also likely to impact estrogen uptake as their size typically varies from 100 to 500 µm (Joss *et al*., 2004). On the basis of equivalent dry weight, activated sludge comprised of smaller floc size has an inherently greater surface area for sorption. To our knowledge no previous study has examined the impact of closely controlled SRT to estrogen removal. Research has traditionally compared full-scale activated sludge plants (ASPs) where receiving sewage differs between sites and process control is poor (Kreuzinger *et al*., 2004; Clara *et al*., 2005; Koh *et al*., 2009; McAdam *et al*., 2010). This study utilised municipal wastewater of the same source to circumvent variations in receiving sewage composition. Furthermore, a pilot-scale ASP was operated to enable close control of SRT. The implication of poor process control is a dynamic and highly variable SRT making the interpretation of its impact to estrogen removal difficult. This study specifically examined the impact of SRT on steroid estrogen removal. Supportive analysis was used to understand and assess the contribution of sorption and biodegradation to estrogen removal at each controlled condition.

6.2. MATERIALS AND METHODS

6.2.1. Pilot-scale activated sludge plant

A pilot-scale ASP was sited at a WwTWs in the east of England (3,000 population equivalent - PE) and consisted of a primary sedimentation tank, a 0.36 m^3 aerated basin and a final clarifier. The system was seeded using biomass from a full-scale nitrifying ASP (280,000 PE) and supplied with municipal crude wastewater containing indigenous concentrations of estrogens. The influent flow rate (1.08 m^3 d^{-1}) was controlled to achieve a constant hydraulic retention time (HRT) of 8 hours. Return activated sludge (RAS) was 0.55 of the influent flow (0.59 m^3 d^{-1}). Solids retention times of 3, 10 and 27 days were selected for monitoring and controlled by daily disposal of excess activated sludge (waste activated sludge (WAS)) following correction for loss of effluent solids. The system was operated for at least three SRTs prior to monitoring to ensure steady state conditions were established. Steroid estrogens and sanitary determinands were sampled once daily from influent settled sewage, final effluent and RAS (in duplicate) over seven consecutive days at each condition. Samples were collected in 2.5 l borosilicate glass vessels with Teflon lined caps and processed immediately.

6.2.2. Batch-scale sorption and biodegradation studies

To assess sorption, biomass of 3, 10 and 27 days SRT was collected and deactivated by pasteurisation at 65 °C for 15 minutes (Le Meur, 2011). Biomass was allowed to cool, washed 3 times using biochemical oxygen demand (BOD) water (1.25 x 10^{-4} mg l^{-1} ferric chloride; 0.028 mg l^{-1} calcium chloride; 0.025 mg l^{-1} magnesium sulphate in a buffered aqueous solution) and stored overnight at 4 °C. Biological inactivity was confirmed by monitoring soluble chemical oxygen demand (sCOD, 1.2 µm filtered) concentrations following spiking with a readily biodegradable carbon substrate (glucose) at 18 °C. Biomass was normalised to five different suspended solids concentration at 18 (+/- 0.5) °C using BOD water; 200, 400, 600, 800 and 1,000 mg l^{-1}. These were prepared in sacrificial 2 l borosilicate vessels and continually mixed using an orbital shaker (Stuart, Stone, UK) at 90 rpm. Each vessel was spiked with 100 ng l^{-1} of each steroid estrogen and 500 ml sample aliquots were taken after 8 hours. For the experimental design utilised here, the data was fitted to the Langmuir isotherm, enabling adsorption maxima to be determined (Del Bubbu *et al*., 2003; Schwarzenbach, 2003):

$$\frac{C_e}{q} = \left(\frac{1}{a.b} + \frac{C_e}{a} \right) \qquad (6.1)$$

Here a = maximum achievable surface concentration of a given estrogen (ng g^{-1}), b = Langmuir constant, C_e = the equilibrium concentration of estrogen in solution (ng l^{-1}) and q = concentration of estrogens sorbed per weight of biomass (ng g^{-1}). During the development

process, equilibrium time was established by monitoring estrogen concentrations at 0, 0.5, 1, 4, 8 and 24 hour time intervals for biomass concentrations of 200 and 1,000 mg l^{-1}. This also ensured no desorption occurred over the time period studied.

Biodegradation batch tests were also prepared with biomass of 3, 10 and 27 days SRT. This was achieved using borosilicate 10 l vessels (Fisher Scientific UK LTD, Loughborough, UK) containing 8 l of biomass. Initially biomass was washed with BOD water then normalised to a suspended solids concentration of 1,000 mg l^{-1} with filtered settled sewage to achieve an initial food: micro-organism (F: M) ratio of 0.12 ±0.01 g sCOD $gVSS^{-1}$. To assess the role of extracellular activity a test vessel containing 8 l of filtered (1.2 μm) nitrifying biomass was prepared (Langford *et al*., 2005). Furthermore, an additional vessel was prepared containing 5 mg l^{-1} allylthiourea (ATU) to inhibit nitrification (Zhou and Oleszkiewicz, 2010). Each vessel was spiked with an additional 100 ng l^{-1} of each steroid estrogen. The biomass was kept completely mixed using a magnetic stirrer and continuously aerated at 18 (+/- 0.5) °C. The test was initiated within 2 hours of collecting the biomass. Representative 500 ml samples were taken in triplicate at the following time intervals; 0 (immediately following estrogen spiking), 1, 4, 8 and 24 hours. All estrogens were determined in both aqueous and particulate phases. At each interval ammonium nitrogen (NH_4^+-N), nitrate (NO_3^-) and COD were also monitored.

6.2.3. Biomass characterisations

Biomass at the three SRTs was characterised by extracting EPS and soluble micro products (SMP) fractions. This was achieved using a method similar to that described by Le-Clech *et al* (2006). Initially 50 ml of biomass was centrifuged at 1,500 x *g* for 5 minutes. The supernatant was filtered through 0.2 μm filters to obtain the SMP fraction. The biomass was re-suspended in 50 ml of deionised water and placed in an oven at 105 °C for 1 hour (allowing the sample to reach 80 °C for 10 minutes). Once at room temperature the sample was centrifuged at 1,500 x *g* for 5 minutes and filtered (0.2 μm) to obtain the extracted EPS. Chemical oxygen demand measurements were taken as a means of quantifying organic composition. Protein and carbohydrate concentrations were quantified using the phenol–sulphuric acid method (Zhang *et al*., 1999) and modified Lowry method (Frølund *et al*., 1995), respectively. Charge (zeta potential) was measured using a Zetasizer 2000 (Malvern Instruments LTD, Worcestershire, UK) and biomass particle size by a Mastersizer 2000 (Malvern Instruments LTD, Worcestershire, UK).

Numbers of viable (intact) and dead (non-intact) cells in all biomasses was quantified using a modified method of that outlined by Ziglio *et al* (2002). Grab samples of biomass were taken from the aeration stage of the pilot-scale ASP and analysed immediately. The sample was

homogenised using a mechanical blender for 3 minutes. A 5 ml sample was then disaggregated using a homogeniser 125 (Fisher Scientific UK LTD, Loughborough, UK) at speed setting 4 (24,500 1/min). The samples were then diluted 1:20 (v/v) with a 0.22 µm filtered sodium chloride (NaCl) solution (0.085%) (Boulos *et al*., 1999). A further 5 minutes disaggregation step was undertaken to obtain maximum bacterial numbers without impacting viability. The bacterial cells were separated from the debris by differential centrifugation according to the method by Lunau *et al* (2005). Samples were centrifuged at 180 x *g* for 1 minute and the supernatant containing the cells was retained. The optical density (OD) of the cells was measured at 600 nm using a visible light spectrophotometer (Jenway 630) and the sample diluted to give an appropriate bacterial number per microscope field of view (FOV) (approximately 60 cells per FOV) with filtered NaCl solution (0.085%). Bacterial samples were stained with LIVE/DEAD® BacLight™ (Invitrogen, Glasgow, UK) according to manufacturer's guidelines. The BacLight test is an indirect measure of membrane viability based on the ability of cells with intact membranes to exclude propidium idodide. The total cell count is a measure of the non-specific SYTO -9 and the dead or membrane compromised cells are stained red by propidium iodide. The dyes dissolved in dimethyl sulfoxide were mixed together (1:1 (v/v)) and diluted in filtered NaCl solution (0.085%). During the staining procedure bacteria were incubated at 20º C for 15 minutes in a dark room prior to filtration (Boulos *et al*., 1999). A known volume of sample was then vacuum filtered onto a black polycarbonate membrane filter (0.22 µm pore size, 25 mm diameter; Nucleopore, Whatman, UK). The filter was washed with filtered NaCl solution (0.085%) and was placed (still wet) on a clean microscope slide, a drop of LIVE/DEAD® BacLight™ mounting oil was applied and a clean coverslip was placed over the filter. The corners were fixed with clear nail polish (Rimmel, London). Cells were viewed under oil immersion (63x) on an LSM 510 META confocal laser scanning microscope (CLSM) (Carl Zeiss, Inc.) and the Axiovision software (Carl Zeiss, Inc.). Images for cell counts were acquired using a Zeiss LSM camera (Carl Zeiss, Inc.). The imageJ program (Abràmoff *et al*., 2004) was used to prepare images for counting. Images were calibrated using a digital scale using the line selection tool and the command (Analyze > Set scale). Background corrections were made and converted into 8bit single colour images (Image > Colour > Split Channels) which split the image in the 3 channels, the blue was discarded. The bacteria were quantified using the image analysis software, CellC (Selinummi *et al*., 2005; Tempere University of Technology, 2012) and MatLab software (The Mathworks, Natick, MA, USA). This program counts particles on the basis of their relative fluorescent density compared with the background via thresholding, size discrimination and a cluster division algorithm. The relative proportion and absolute cell number of the viable (green) and dead (red) microbiota was then calculated taking into account dilutions.

6.2.4. Chemical analysis

Estrogen standards (>98 % purity) were purchased from Sigma Aldrich (Dorset, UK). Deuterated internal standards; estrone-2,4,16,16-d_4, 17β-estradiol-2,4,16,16,17-d_5, estriol-2,4,17-d_3 and 17α-ethynylestradiol-2,4,16,16-d_4 were obtained from QMX Laboratories (Thaxted, UK). The HPLC grade solvents; methanol, dichloromethane, ethylacetate and hexane were purchased from Rathburn Chemicals (Walkerburn, UK). Ammonium hydroxide was ACS grade and obtained from Sigma Aldrich (Dorset, UK). Chemical oxygen demand, NH_4^+-N and NO_3^- were determined using proprietary cell test kits purchased from VWR International (Leicestershire, UK). Steroid estrogen determination involved a three stage extraction and clean up procedure for determination in aqueous and particulate phases of wastewaters. Quantification was by ultra-performance liquid chromatography (Acquity, Waters, Manchester, UK) coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer with a Z-spray electro-spray ionisation interface. Full description of the methodology is available in Chapter 3.

6.3. RESULTS

6.3.1. Removal of estrogens at varying SRT

Total estrogen ($\sum_{EST}$) concentrations in settled sewage were 344 ±26, 344 ±20 and 331 ±9 ng l^{-1} during the 3, 10 and 27 day SRT sampling campaigns, respectively (Table 6.1). Variations in individual natural estrogen concentrations fluctuated by <10 % between SRT sampling campaigns. Total estrogen removals were 70 ±8 % at a 3 day SRT (Figure 6.1). During 10 and 27 day SRTs no significant differences in $\sum_{EST}$ removals were observed. Removals were 95 ±1 % and 93 ±2 %, respectively. Mixed liquor volatile suspended solids (MLVSS) concentrations were 972, 1,301 and 1,284 mg l^{-1} over the three studies (Table 6.2). Despite increasing sludge age, the concentration of estrogens in RAS reduced with SRT (Figure 6.1). Total estrogen concentrations in RAS were 372, 325 and 227 ng l^{-1} for 3, 10 and 27 day SRTs respectively. Individual estrogens exhibited greater removal with increased SRT except for E1. Removals of E1 were 93 ±4 % and 84 ±4 % for 10 and 27 day SRTs, respectively. The recalcitrant nature of EE2 was confirmed by its comparatively poor removal at all SRTs. Removals were 29-30 % for 3 and and 10 day SRTs. The highest removal (41 %) was observed at the longest SRT.

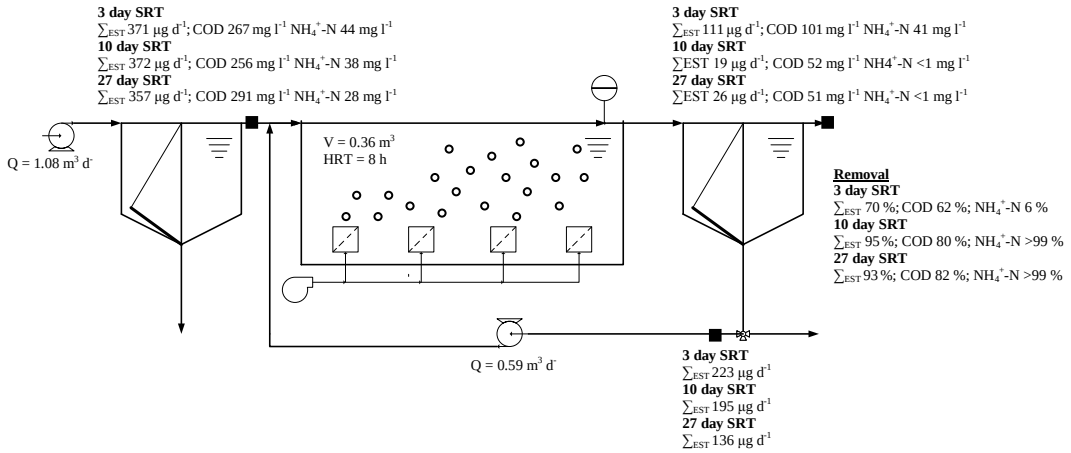

Figure 6.1. Process diagram inclusive of steroid estrogen mass balance data of varying SRT; 3, 10 and 27 days (n=7), sampling points (■)

Table 6.1. Steroid estrogen sorption and biodegradation information from pilot plant and batch studies

Steroid estrogen	SRT (d^{-1})	Pilot plant studies (continuous flow)				Sorption batch studies		Biodegradation batch studies	
		Settled sewage (ng l^{-1})	RAS log K_d	Final effluent (ng l^{-1})	Total removal[a] (%)	*a* (ng g^{-1})	r^2	*k*[b] (h^{-1})	r^2
E1	3	91.7 ±14.4	2.3	74.5 ±24.9	19	1.4×10^4	0.97	0.33	0.62
	10	100.5 ±12.2	2.3	7.1 ±2.7	93	3.3×10^4	0.91	0.51	0.95
	27	94.0 ±13.5	2.4	14.8 ±2.8	84	1.3×10^4	0.88	0.43	0.85
E2	3	30.1 ±4.8	2.7	9.7 ±2.9	68	3.3×10^4	0.97	0.26	0.76
	10	28.5 ±7.9	3.0	2.9 ±1.1	90	3.3×10^4	0.86	0.53	0.71
	27	30.4 ±6.2	3.7	2.4 ±0.8	92	2.0×10^5	0.97	0.45	0.99
E3	3	220.1 ±24.6	2.7	17.0 ±4.6	92	63	0.59	1.01	0.98
	10	214.4 ±32.9	3.6	6.8 ±1.7	97	280	0.92	0.99	0.99
	27	205.0 ±11.5	3.5	6.2 ±3.1	97	430	0.94	1.05	0.98
EE2	3	1.8 ±1.2	4.4	1.3 ±0.8	30	5.0×10^4	0.92	0.10	0.73
	10	0.9 ±0.8	3.9	0.7 ±0.5	29	3.3×10^4	0.86	0.09	0.96
	27	1.5 ±0.9	4.0	0.9 ±0.6	41	2.5×10^5	0.77	0.06	0.91

[a]Removal = (settled sewage-final effluent)/settled sewage x 100 % [b]Biodegradation rate constant calculated for initial 8 hour period

Key: SRT, solids retention time; RAS, return activated sludge; *a*, maximum achievable surface concentration of a given estrogen; *k*, biodegradation rate constant

Table 6.2. Physico-chemical biomass characteristics at varying SRT: 3, 10 and 27 days

Physico-chemical property	Biomass			Soluble micro-products			Extracellular polymeric substance		
	3 day SRT	10 day SRT	27 day SRT	3 day SRT	10 day SRT	27 day SRT	3 day SRT	10 day SRT	27 day SRT
Protein (mg $gVSS^{-1}$)	-	-	-	41.0	48.7	16.6	202.1	196.9	157.8
Carbohydrate (mg $gVSS^{-1}$)	-	-	-	21.4	23.7	12.7	28.9	46.3	43.2
P + C (mg $gVSS^{-1}$)	-	-	-	62.4	72.4	29.3	231.0	243.2	201.0
P: C	-	-	-	1.9	2.1	1.3	7.0	4.3	3.7
COD (mg $gVSS^{-1}$)	-	-	-	72.4	29.5	21.6	438.2	387.0	315.3
Zeta potential (mV)	-12.7	-12.2	-10.8	-8.7	-9.4	-6.1	-25.2	-18.2	-18.4
pH	8.0	7.0	7.4	-	-	-	-	-	-
Temperature (°C)	13.1	17.3	10.8	-	-	-	-	-	-
MLVSS (mg l^{-1})	972	1,301	1,284	-	-	-	-	-	-
d_{10} (µm)	79	74	52	-	-	-	-	-	-
d_{50} (µm)	358	274	164	-	-	-	-	-	-
d_{90} (µm)	1,163	692	408	-	-	-	-	-	-
SSVI (ml g^{-1})	217	84	105	-	-	-	-	-	-

Key: P, protein; C, carbohydrate; COD, chemical oxygen demand; MLVSS, mixed liquor volatile suspended solids; d_{10}, 10 % of particles lie below this diameter; d_{50}, median diameter; d_{90}, 90 % of particles lie below this diameter; SSVI, stirred sludge volume index

6.3.2. Function of SRT upon estrogen sorption and biodegradation

Activated sludge sorption capacity of varying SRT was determined in batch at normalised suspended solids concentrations. Equilibrium for all estrogens was achieved within 30 minutes of spiking. Estrogen sorption conformed well to log K_{ow} following EE2>E2>E1>E3 (Table 6.1). Sorption of those more hydrophilic estrogens was relatively unaffected by SRT. However, sorption of the more hydrophobic estrogens (E2 and EE2) was influenced by SRT. Sorption of E2 and EE2 to biomass of varying age was 27 day SRT>3 day SRT>10 day SRT. The maximum achievable surface concentration (*a*) for both E2 and EE2 was around an order of magnitude greater for the 27 day SRT biomass (Table 6.1). To demonstrate, EE2 *a*'s were 5.0 x 10^4, 3.3 x 10^4 and 2.5 x 10^5 l g^{-1} for 3, 10 and 27 day SRTs. However, this significantly greater sorption was not reflected in improved degradation in biodegradation batch studies. In biodegradation batch tests where the F: M ratio was normalised for each SRT, rate of biodegradation was E3>E1≥E2>EE2 (Table 6.1). Biodegradation rate constants (*k*) of E3 and EE2 were unaffected by SRT. Biodegradation of E3 was comparatively high in all biomasses with *k*'s ~1 h^{-1} (r^2 0.97-1.00). In contrast, biodegradation of EE2 was slow and similar at each SRT, exhibiting *k*'s <0.10 h^{-1} (r^2 0.78-0.99). Biodegradation rates of E1 and E2 were lowest for the 3 day SRT biomass. In the nitrifying biomasses (10 and 27 day SRTs) biodegradation was augmented.

6.3.3. Impact of SRT on sludge morphology and physico-chemical properties

At 3 days SRT, 6 % NH_4^+-N removals were observed with reasonable COD removals (70 %) indicative of a biomass dominated by heterotrophic metabolism (Figure 6.1). High removal of NH_4^+-N (>99 %) was achieved at 10 and 27 day SRTs. Nevertheless, numbers of viable bacterial cells in activated sludge reduced with SRT and was 1.45 x 10^{12}, 5.49 x 10^{11} and 4.09 x 10^{11} counts $gVSS^{-1}$ for 3, 10 and 27 day SRTs respectively. This was used to estimated $\sum_{EST}$ biodegradations in pilot plant studies of 499, 1,361 and 1,750 ng$\sum_{EST}$ 1 x 10^{12} viable $cells^{-1}$ d^{-1} for 3, 10 and 27 day SRTs respectively (Figure 6.2). In addition to viable cell number, SRT also impacted activated sludge physico-chemical composition. Concentrations of EPS ranged from 438 (3 day SRT) to 315 mg $gVSS^{-1}$ (27 day SRT) (Table 6.2). Also, total concentrations of proteins and carbohydrates extracted from the EPS were 231, 243 and 201 mg $gVSS^{-1}$ for 3, 10 and 27 day SRTs respectively. This corresponded with proteins to carbohydrates ratios of 7.0, 4.3 and 3.7. Median activated sludge floc size (d_{50}) was 358, 274 and 164 µm for 3, 10 and 27 day SRTs, respectively. No significant differences in charge (zeta potential) were observed. Zeta potential of all biomasses was broadly similar and ranged from -12.7 to -10.8 mV.

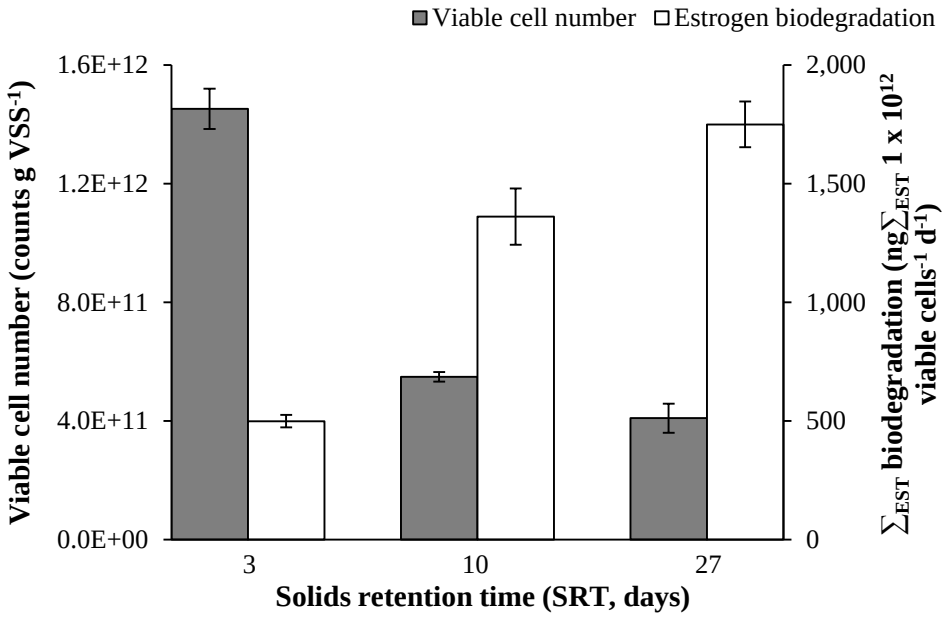

Figure 6.2. Impact of SRT to viable cell numbers in activated sludge and the quantity of total estrogens biodegraded per cell

6.3.4. Identification of the site and *modus* of estrogen biodegradation

The removal of cellular bound material and intact cells by filtration was used to assess the role of free extracellular enzymes in estrogen biodegradation. Filtration of the biomass resulted in the cessation of steroid estrogen biodegradation (Figure 6.3). Extracellular free enzymes present in the biomass medium did not facilitate any estrogen biodegradation. Initially, concentrations of total natural estrogens (E1, E2 and E3) and EE2 were 290 ±3 ng l^{-1} and 94 ±3 ng l^{-1}, respectively. Following 8 hours, concentrations of 296 ±1 ng l^{-1} and 100 ±4 ng l^{-1} were observed. In the presence of intact cells, initial concentrations of total natural estrogens and EE2 were 338 ±10 ng l^{-1} and 119 ±15 ng l^{-1}, respectively. Concentrations of 29 ±1 ng l^{-1} (natural estrogens) and 74 ±6 ng l^{-1} (EE2) were observed after 8 hours treatment. This corresponded with biodegradation rates of 0.28 h^{-1} (r^2 0.93) for natural estrogens and 0.06 h^{-1} (r^2 0.79) for EE2.

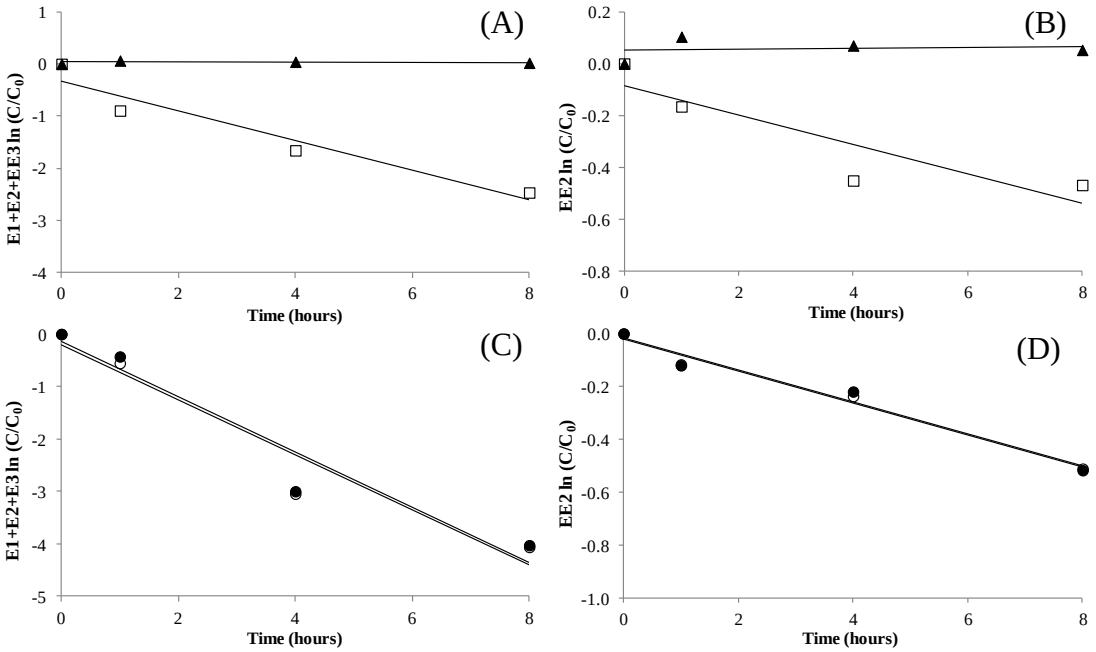

Figure 6.3. Function of free extracellular enzymes (A+B, □ 1.2 µm filtered biomass, ▲ unfiltered control) and oxygenase activity of ammonia oxidising bacteria (C+D, ○ ATU treated, ● non ATU treated control) in the biodegradation of E1+E2+E3, and EE2 in batch studies

6.3.5. Evaluation of the role of oxygenase activity during nitrification

The inhibition of nitrification by ATU was confirmed in the test vessel by the cessation of NH_4^+-N removal and NO_3^- production. In the control vessel the starting concentration of NH_4^+-N (17.6 mg l^{-1}) was completely transformed to NO_3^- within 8 hours. Heterotrophic activity was unaffected by the inhibition of nitrifying activity. Over an 8 hour period, sCOD removals were 61 ±4 % (control) and 64 ±2 % (test) of the starting concentration of 95 mg l^{-1}. Natural estrogen *k's* were 0.53 h^{-1} (r^2 0.94) and 0.53 h^{-1} (r^2 0.94) in nitrifying biomass (control) and biomass treated with ATU (test), respectively (Figure 6.3). Biodegradation of EE2 was considerably slower with *k's* of 0.07 h^{-1} (r^2 0.91) and 0.07 h^{-1} (r^2 0.95). No differences were observed for natural estrogens or EE2 biodegradation between control and test vessels.

6.4. DISCUSSION

Pilot-scale ASP operation at constant volumetric loading enabled the impact of SRT on estrogen removal to be evaluated. At a short SRT of 3 days, 70 % $\sum_{EST}$ biodegradation was achieved. This is consistent with previous observations of a full-scale ASP operated at a short SRT to enable oxidation of organics rather than to support nitrification (McAdam *et al*., 2010). However, in this study the removal of natural estrogens E1 and E2 was lower at the short SRT. Increasing SRT to 10 days facilitated NH_4^+-N removal and a concomitant increase in E1 and E2 removal specifically (Figure 6.1, Table 6.1). Despite increasing mixed liquor concentrations typical of lengthening SRT, sludge sorption was not responsible for greater removal here. To demonstrate, the quantity of $\sum_{EST}$ associated with RAS reduced with increased SRT. This was supported by sorption batch studies where no significant improvement in estrogen sorption was observed (Table 6.1). If sorption was driving increased removal at the 10 day SRT condition, an accumulation of sludge bound estrogens would be expected. These findings support those of Andersen *et al* (2005) which estimated <2 % of $\sum_{EST}$ to be removed in excess sludge if an equilibrium exists. Thus, it can be postulated that biodegradation is the key mechanism for improved removal of natural estrogens E1 and E2 removal here. This was corroborated by the enhanced biodegradation rates observed in batch studies at 10 days SRT (Table 6.1). The coincident initiation of NH_4^+-N removal at 10 days SRT infers nitrifier activity may be responsible for augmented natural estrogen biodegradation. However, investigation into the governance of free extracellular activity on estrogen removal indicated that free enzymes play no role in the biodegradation of estrogens (Figure 6.3). This suggests contact with activated sludge flocs was required for estrogen biodegradation. Biodegradation was either mediated by extracellular enzymes outside the bacterial cell but still contained within the floc (i.e., within EPS) and/or intracellular enzymes within viable bacterial cells. However, findings suggest that these are not nitrifiers as the inhibition of NH_4^+-N oxidation had no impact to estrogen biodegradation. Furthermore, it can be deduced that enzymatic reactions which follow NH_4^+-N oxidation (i.e., nitrite oxidation) do not contribute to estrogen biodegradation either. These findings are consistent with other investigations operated with synthetic sewage testing the role of nitrifiers in batch (Gaulke *et al*., 2008) and continuous flow systems (Bagnall *et al*., 2012). These studies postulated that heterotrophic bacteria were responsible for estrogen biodegradation.

In this study, viable cell quantitation revealed that cell specific estrogen biodegradation increased with SRT (Figure 6.2). This indicated an improvement in the efficiency of either the same group, or a more responsive group of bacteria. Larcher and Yargeau (2013) demonstrated that different strains of heterotrophs commonly found in activated sludge varied

in their efficacy of EE2 removal. Thus a shift in their relative abundance and their absolute numbers could explain increased cell specific biodegradation. Such a shift may be induced by process operation. For example, lengthening SRT provides conditions considered conducive to competitive exclusion (Saikaly and Oerther, 2004). It is postulated that increasing biomass age enabled bacteria more capable of non-favoured carbon source utilisation (e.g., estrogens) to prosper. This would explain increasing cell specific biodegradation with SRT.

Collated full-scale data by Clara *et al* (2005) and McAdam *et al* (2010) suggested that $\sum_{EST}$ removal may continue to increase with SRT beyond 10 days. To assess this, control at a SRT of 27 days was undertaken to encompass a broad range of typical full-scale operations. At 27 days SRT, removal of $\sum_{EST}$ showed no not further improvement and was 93 ±2 % (Figure 6.1). Nevertheless, the quantity of estrogen biodegraded per bacterial cell continued to increase as viable cell numbers further reduced. At an SRT of 27 days evidence suggests this may be due to improved transport to viable cells within activated sludge. This hypothesis is attributed to changes observed in the physico-chemical composition of biomass induced by increasing SRT (Figure 6.2). For example, the 27 day SRT biomass had the smallest median floc size. This results in shorter distances between bacteria and the floc surface suggesting shorter distances within the floc to be overcome by diffusion (Joss *et al*., 2004). Furthermore, EPS concentration reduced with SRT (Table 6.2). It has been suggested that EPS can behave as a selective barrier towards substrate diffusion, thereby limiting specific substrates to active bacteria (Lieleg and Ribbeck, 2011). Therefore it can be postulated that at lower EPS concentrations there is less restricted passage of estrogens to viable bacterial cells for biodegradation. Despite no increase in $\sum_{EST}$ removal in pilot studies, individually E2 and EE2 did show a moderate improvement in removal (Table 6.1). Biodegradation batch studies showed no increase in absolute biological removal between 10 and 27 day SRTs. However, batch sorption studies exhibited significantly greater sorption capacity of the 27 day SRT biomass for E2 and EE2 (Table 6.1). The smallest floc size observed here results in a greater total surface area for sorption to occur. Nevertheless, enhanced sorption was not reflected in the pilot study as estrogen concentrations in RAS did not increase. This is owed to the receiving concentrations of estrogens in this study. The enhanced sorption capacity of a high SRT biomass is likely to be appreciable for sites where the estrogen composition of receiving sewage is greater. For example, Clara *et al* (2005) reported mean receiving EE2 concentrations up to 70 ng l^{-1}. Such concentrations are significantly greater than those observed in this study (~70 times) and are more likely to challenge the system. Interestingly, >98 % removal was observed for a receiving EE2 concentration of 70 ng l^{-1} at an SRT of 48 days (Clara *et al*., 2005). Operation at a high SRT may therefore be beneficial for sites which

receive comparatively higher concentrations of the more hydrophobic estrogens E2 and EE2 due to the enhanced sorption potential of the biomass.

6.5. CONCLUSION

Solids retention time was critical for steroid estrogen removal as it influenced biodegradation and sorption mechanisms. Operation at a mid-ranged SRT was beneficial as it augmented biodegradation of the natural estrogens E1 and E2. The synthetic estrogen EE2 was relatively resistant to removal at all SRTs studied. However, the high sorption capacity of the high SRT (27 days) biomass may be beneficial to sites receiving comparatively higher concentrations of EE2.

CHAPTER 7
SIMULTANEOUS REMOVAL OF A DIVERSE RANGE OF HAZARDOUS CHEMICALS FROM WASTEWATER BY ACTIVATED SLUDGE

IN PREPARATION: *Water Research*

7. SIMULTANEOUS REMOVAL OF A DIVERSE RANGE OF HAZARDOUS CHEMICALS FROM WASTEWATER BY ACTIVATED SLUDGE

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Abstract

It is proposed that wastewater treatment facilities meet legislated discharge limits for a range of hazardous chemicals. However, the heterogeneity of these hazardous chemicals in receiving wastewaters make separation efficiency difficult to predict since their chemistry is so diverse. In this study, a range of organic and inorganic hazardous chemicals that are known to be preferentially removed via different separation mechanisms were therefore selected to challenge the activated sludge process (ASP) and determine its potential to achieve simultaneous hazardous chemical removal. At a fixed hydraulic retention time (HRT) of 8 hours, the influence of an increase in solids retention time (SRT) on removal was evaluated. Maximum achievable hazardous chemical removal was recorded for all chemicals (estrogen, nonlphenolics, metals) at the highest SRT studied (27 days). However, optimisation of HRT by extension to 24 hours further augmented organic biodegradation. Most notable was the enhancement in removal of the considerably recalcitrant synthetic estrogen 17α-ethinylestradiol which increased to 65 ±19 %. Regression analysis indicates that this enhanced hazardous chemical behaviour is ostensibly related to the concomitant reduction in food: microorganism ratio. Interestingly, extended HRT also initiated nonylphenol biodegradation which has not been consistently observed previously in real wastewaters. However, extending HRT increased the solubilisation of particulate bound metals. This is significant as only the dissolved metal fraction is to be considered for environmental compliance. Consequently, identification of an optimum process condition for generic hazardous chemical removal is expected to favour a more integrated approach where, for example, the improvement of primary sedimentation is demanded to reduce loading of the particle bound metal fraction onto the ASP, thereby enabling longer HRT in the ASP to be considered.

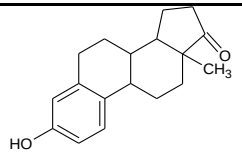
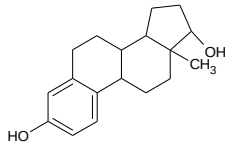
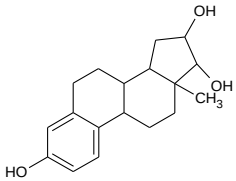
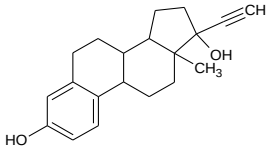
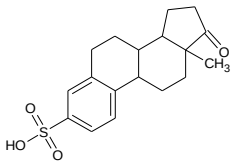
7.1. INTRODUCTION

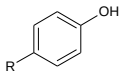
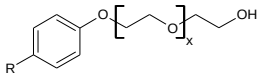
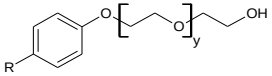
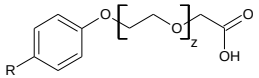
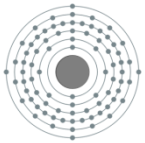
Current operations of secondary wastewater treatment works (WwTWs) do not enable compliance to Environmental Quality Standards (EQSs) and proposed legislative targets for a wide variety of hazardous chemicals (Gardner *et al*., 2012). However, the environmental and economic cost of implementing and operating advanced processes underpins the need to optimise these secondary processes (Jones *et al*., 2007a). Activated sludge is a widely used secondary process with a proven ability to remove a variety of hazardous chemicals (Clara *et al*., 2005; Kreuzinger *et al*., 2005; Koh *et al*., 2009; McAdam *et al*., 2010; McAdam *et al*., 2011). Hazardous chemical removal by activated sludge can be attributed mostly to sorption and biodegradation mechanisms (Langford *et al*., 2005; Radjenovic *et al*., 2009b; Petrie *et al*., submitted). Radjenovic *et al* (2009b) gave an excellent account of sorption and biodegradation behaviour for a variety of pharmaceuticals during full-scale activated sludge treatment. Measuring removal performance and process optimisation is needed on hazardous chemicals exhibiting extremities of propensity to these mechanisms. Natural and synthetic steroid estrogens, nonylphenolic surfactants (NPx) and metals encompass such diversity. These are describable as biodegradable and refractory organics (Petrie *et al*., submitted; Petrie *et al*., 2013a) and non-biodegradable inorganics (Santos *et al*., 2010), respectively. These exhibit a broad range of physico-chemical properties (Table 7.1) which contributes to their differing fate and behaviour during wastewater treatment. For example, hydrophobicity (log K_{ow}) can be used as a reasonable predictor for sorption behaviour of estrogens (Petrie *et al*., submitted). Nevertheless, biodegradation is accepted as their major removal pathway (Andersen *et al*., 2005). The removal of NPx is more complex as this family of chemicals possess a variety of physico-chemical properties and breakdown pathways. The relatively hydrophilic long chained NPx's are highly susceptible to biological attack (McAdam *et al*., 2011). However, their biotransformation can result in the formation of shorter chained ethoxylates and NP (Petrie *et al*., 2013a). These chemicals have log K_{ow}'s >5 (Table 7.1) and a relatively high propensity to sludge partitioning is expected (Byrns, 2001). In contrast, metals rely entirely on partitioning within the activated sludge matrix for removal. This is considered to be driven by three major processes which are; physical entrapment of insoluble metals, binding of soluble metals to bacterial walls and extracellular polymers, and active cellular uptake by bacterial cells (Ziolko *et al*., 2011).

Hazardous chemical removal is considered to be influenced by activated sludge process variables, solids retention time (SRT) and hydraulic retention time (HRT) (Svenson *et al*., 2003; Johnson *et al*., 2005; Hamid and Eskicioglu, 2012; Maeng *et al*., 2013). Despite a large volume of research undertaken on this subject area, it remains unclear whether operation can be optimised to achieve maximum removals of all hazardous chemicals simultaneously. To

illustrate, an extended SRT (≥10 days) is considered necessary to augment removal of those biodegradable hazardous chemicals (Kreuzinger *et al*., 2005; Clara *et al*., 2005; McAdam *et al*., 2010). This is thought to enable the enrichment of a more diverse bacterial community more capable of organic hazardous chemical biodegradation (Kreuzinger *et al*., 2005). However, such conditions are hypothesised to be detrimental to metals removal (Santos *et al*., 2010). It is anticipated that metals are solubilised by chelators produced by biomasses at SRTs >10 days and are therefore not available for removal within the activated sludge matrix (Santos *et al*., 2010). Furthermore, there is a paucity of information on the impact of HRT to hazardous chemical removal. It can be postulated that longer HRTs (and contact time) enable greater biodegradation of the organic hazardous chemicals. Previous research in this field has traditionally focussed on the broad comparison of different full-scale activated sludge plants (ASPs) operating at various SRT and HRTs (Svenson *et al*., 2003; Clara *et al*., 2005; Johnson *et al*., 2005; Kreuzinger *et al*., 2005; Koh *et al*., 2009; McAdam *et al*., 2010; McAdam *et al*., 2011). However, full-scale processes tend to suffer from poor process control resulting in a dynamic system and considerable variations in both SRT and HRT. To demonstrate, a full-scale ASP designed to be operated at a 10 day SRT 8 hour HRT saw considerable variations in flow (50 to 600 m^3 h^{-1}) and therefore HRT over an eight week period (Aboobakar *et al*., 2013) which based on a simple solids mass balance, implies and estimated SRT range of between 4 and 20 days. Consequently, understanding the impact of process variables at full-scale to hazardous chemical removal is challenging. In this study, a pilot-scale ASP was operated to enable good process control and circumvent variations in receiving sewage composition and flow. This enabled SRT and HRT to be de-coupled from one another and their individual impact to hazardous chemical removal assessed. The pilot-scale ASP was operated at: (i) 3, 10 and 27 day SRTs whilst at a constant HRT (8 hours) and (ii) 8, 16 and 24 hour HRTs at a constant SRT (27 days). To measure ASP resilience for the removal of a wide range of hazardous chemicals of varied chemistry and preferred removal mechanisms under a range of operation conditions - steroid estrogens, NPx's and metals were monitored. To our knowledge this is the first study which has closely controlled ASP operation and measured the impact to the removal of such a diverse range of chemicals simultaneously in municipal wastewater.

Table 7.1. Physico-chemical properties of hazardous chemicals found in wastewaters (EPI Suite, 2013)

Chemical of interest	Organic chemical structure/metal electron configuration	EQS/proposed legislative target (µg l^{-1})	Molecular weight (g mol^{-1})	Water solubility (mg l^{-1})	pKa	Vapour pressure (kPa)	Henry's law constant (atm m^3 mol^{-1})	Density (g cm^{-3})	log K_{ow}	log K_{oc}
Estrone (E1)		3.0 x 10^{-3a}	270.37	30.0	10.50[c]	3.00.10^{-8}	3.80.10^{-10}	-	3.13-3.43	3.02-4.38
17β-estradiol (E2)		4.0 x 10^{-4b}	272.39	3.6	10.71[c]	3.00.10^{-8}	3.64.10^{-11}	-	3.94-4.01	2.90-4.01
Estriol (E3)		-	288.38	441.0	-	9.00.10^{-13}	1.33.10^{-12}	-	2.45-2.81	1.62-3.08
17α-ethinylestradiol (EE2)		3.5 x 10^{-5b}	296.40	11.3	10.40[c]	6.00.10^{-9}	7.94.10^{-12}	-	3.67-4.15	2.71-4.65
Estrone 1-3sulfate (E1-3S)		-	350.43	960.0	-	1.97.10^{-11}	2.04.10^{-12}	-	0.95	1.81

Nonylphenol (NP)		0.3[d]	220.35	7.6	10.28[e]	$4.39.10^{-5}$	$4.30.10^{-6}$	0.95[e]	5.77	4.28
Nonylphenol mono, di and tri-ethoxylate ($NP_{1-3}EO$)		-	264.41-352.52	0.5-1.8	-	$2.38.10^{-8}$-$5.24.10^{-11}$	$1.25.10^{-6}$-$5.73.10^{-12}$	-	5.03-5.58	4.28
Nonylphenol polyethoxylates ($NP_{4-12}EO$)		-	396.57-749.00	3.3-248	-	$3.04.10^{-12}$-$5.43.10^{-20}$	$1.23.10^{-14}$-$5.35.10^{-36}$	-	2.56-4.75	1.35-3.17
Nonylphenol carboxylates ($NP_{1-3}EC$)		-	278.39-366.50	0.3-1.0	-	$1.31.10^{-6}$-$1.80.10^{-10}$	$1.79.10^{-7}$-$4.31.10^{-11}$	-	5.26-5.80	2.94-3.42
Zinc (Zn)		8-125[g]	65.39	3.4×10^5	-	-	-	7.15[f]	-	-
Copper (Cu)		1-28[g]	63.55	4.2×10^5	-	-	-	8.90[f]	-	-
Lead (Pb)		1.2[d]	207.20	9.6×10^3	-	-	-	11.34[f]	-	-

Cadmium (Cd)		0.08-0.25[d]	112.41	1.2×10^5	-	-	-	8.70[f]	-	-
Nickel (Ni)		4.0[d]	58.69	4.2×10^5	-	-	-	8.90[f]	-	-

[a]Environment Agency of England and Wales, 2000 [b]European Commission, 2012 [c]Liu *et al*., 2009 [d]European Commission, 2008 [e]Soares *et al*., 2008 [f]Lenntech, 2013 [g] Ziolko *et al*., 2011
Key: EQS, environmental quality standard; pKa, acid dissociation constant; log K_{ow}, octanol-water coefficient; log K_{oc}, organic carbon-water coefficient
0-50 mg l^{-1} calcium carbonate

7.2. MATERIALS AND METHODS

7.2.1. Chemicals

Estrogen standards (>98 % purity); estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2) and estrone sulphate (E1-3S) were purchased from Sigma Aldrich (Dorset, UK). Deuterated internal standards; estrone-2,4,16,16-d_4, 17β-estradiol-2,4,16,16,17-d_5, estriol-2,4,17-d_3, 17α-ethinylestradiol-2,4,16,16-d_4 and sodium estrone-2,4,16,16-d_4 sulfate were obtained from QMX Laboratories (Thaxted, UK). Technical 4-NP, 4-nonylphenol-monoethoxylate (NP_1EO), the diethoxylate compound (NP_2EO) and the long chain NPEOs were purchased from Sigma Aldrich (Dorset, UK). Long chain NPEOs were purchased as the technical mixtures CO210, CO520 and CO720. Nonyl-phenoxy acetic acid (NP_1EC) was obtained from QMX laboratories (Thaxted, UK). Single element metal solutions; zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), cadmium (Cd) and rhodium (internal standard), and OPTIMA trace metal grade nitric acid were obtained from Fisher Scientific (Leicestershire, UK). The high performance liquid chromatography grade solvents; acetone, methanol, dichloromethane, ethylacetate and hexane were purchased from Rathburn Chemicals (Walkerburn, UK). Ammonium hydroxide was ACS grade and obtained from Sigma Aldrich (Dorset, UK) and ultrapure water of 18.2 MΩ quality (Elga, Marlow, UK) was used in the preparation of mobile phases. Chemical oxygen demand (COD), ammoniacal nitrogen, nitrate, nitrite and total nitrogen proprietary cell test kits were purchased from VWR International (Leicestershire, UK).

7.2.2. Pilot plant operation

A pilot-scale ASP was sited at a WwTWs in the east of England (3,000 population equivalent) and consisted of a 0.18 m^3 primary sedimentation tank, a 0.36 m^3 aerated basin and a 0.10 m^3 final clarifier (Petrie *et al*., submitted). The plant was seeded with biomass from a full-scale ASP (280,000 population equivalent) and operated with municipal crude sewage containing indigenous concentrations of all hazardous chemicals. The influent flow rate was controlled to achieve a constant HRT. Return activated sludge (RAS) was 0.55 of the influent flow in all studies. Solids retention time was controlled by daily wastage of sludge following correction for loss of effluent suspended solids. The system was operated for at least three SRTs prior to monitoring at each different condition to ensure a steady state environmental was established. Once under steady state conditions sampling was undertaken over seven consecutive days for the determination of all hazardous chemicals and sanitary determinands at all ASP operations. The impact of SRT was investigated by maintaining a constant 8 hour HRT of the secondary treatment stage. Solid retention times of 3, 10 and 27

days were assessed. In HRT studies the SRT was maintained at 27 days and HRTs of 8, 16 and 24 hours examined.

7.2.3. Sanitary determinand analysis

Chemical oxygen demand, soluble COD, ammoniacal nitrogen, nitrate, nitrite and total nitrogen were determined using proprietary cell test kits (VWR International, Leicestershire, UK) and subsequent detection by spectrophotometry. The soluble fraction of wastewater was obtained by filtration through a 1.2 µm filter (Whatman, Maidstone, UK). Suspended solids, volatile suspended solids (VSS) and biochemical oxygen demand (BOD) were determined using standard methods (APHA, 1998). Biomass of each operational condition were characterised by separating extracellular polymeric substance (EPS) and soluble micro products (SMP) fractions (Le-Clech *et al*., 2006). Briefly, 50 ml of biomass was centrifuged at 1,500 x *g* for 5 minutes and the supernatant filtered (0.2 µm) to obtain the SMP fraction. The solid fraction was re-suspended in 50 ml of deionised water and placed in an oven at 105 °C for one hour. The extracted EPS was obtained by allowing the sample to cool to room temperature, centrifuging at 1,500 x *g* for 5 minutes and filtering (0.2 µm). Protein and carbohydrate concentrations were quantified using the phenol–sulphuric acid method (Zhang *et al*., 1999) and modified Lowry method (Frølund *et al*., 1995) respectively. Charge (as zeta potential) was determined using a Zetasizer 2000 (Malvern Instruments LTD, Worcestershire, UK) whereas activated sludge floc size was measured using a Mastersizer 2000 (Malvern Instruments LTD, Worcestershire, UK). Further details of biomass characterisations can be found in Chapter 6.

7.2.4. Hazardous chemical analysis

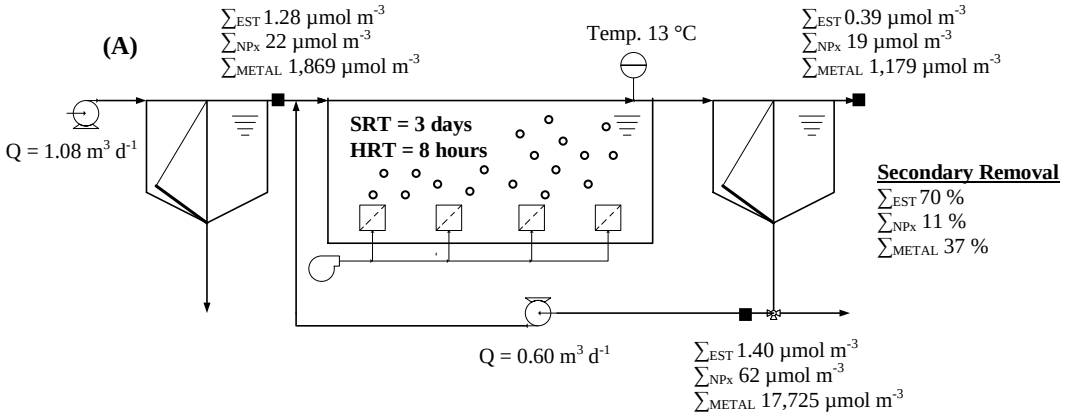
Steroid estrogen analysis involved a three stage extraction and clean up procedure with analysis by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as previously described in Chapter 3. Nonylphenolic analysis utilised a single stage solid phase extraction and quantitation by UPLC-MS/MS which has been detailed in Chapter 4. Sample preparation for metals analysis was similar to that reported by Santos *et al* (2010). Glass and plastic ware used for collection, storage, filtering and digestion of samples was soaked for a minimum of 24 hour in an aqueous 2.5 % v/v Decon 90 solution (Fisher Scientific, Loughborough, UK) and then rinsed with UP water three times. Subsequently they were soaked for a minimum of 24 hours in 2.5 % v/v OPTIMA trace metal grade nitric acid. Glass and plastic ware was then rinsed a minimum of five times with UP water before being

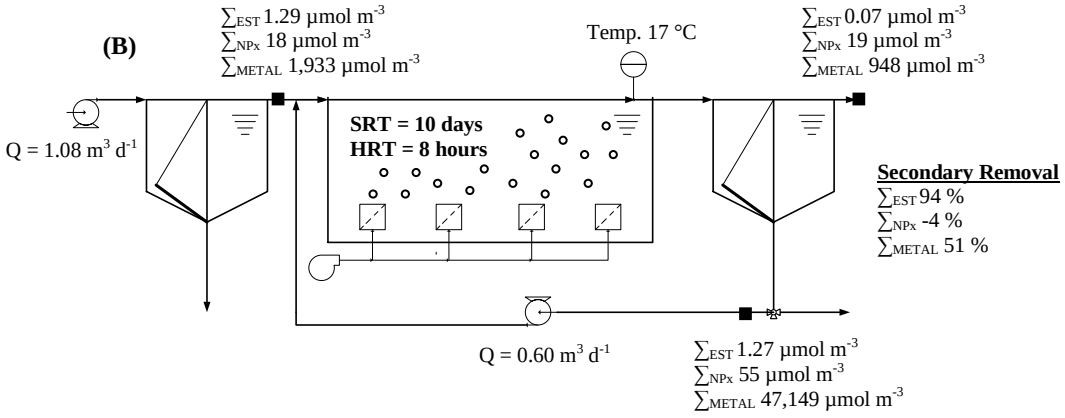
air dried. Total metal analysis was achieved by acid digestion. Samples for total metal analysis should contain a maximum of 100 mg of solids; if samples contained more than 100 mg of solids, dilution with acidified ultrapure water was used to reduce the solids content to ≤100 mg (Santos *et al*., 2010). Samples (30 mL) were placed in digestion tubes and acidified with 1.5 mL OPTIMA trace metal grade nitric acid. The samples were digested using a MARS Xpress microwave digester (CEM Microwave Technology, Buckingham, UK) using sample dissolution EPA method 3015 (US EPA, 2007). The samples were then transferred to 10 mL centrifuge tubes for analysis and spiked with the internal standard rhodium to achieve a concentration of 50 µg l^{-1}. All samples were analysed in duplicate and selected samples were spiked at low (20 µg l^{-1}) and high (100 µg l^{-1}) concentrations to determine recoveries. For dissolved metal analysis, each sample was vacuum-filtered through a Millipore all-glass three piece vacuum filtering set (Millipore, Cambridge, UK) using a 0.45 µm pore size cellulose nitrate membrane filter (Anachem Ltd, Luton, Bedfordshire, UK). A 10 mL aliquot of the sample filtrate was then placed in a 10 mL centrifuge tube and acidified with 0.75 ml OPTIMA trace metal grade nitric acid and internal standard added. All samples were then refrigerated at 4 °C prior to analysis. Samples were then analysed by an ELAN 9,000 inductively coupled plasma-mass spectrometer (Perkin Elmer, Beaconsfield, UK). The RF power was 1 kW with a nebuliser flow rate: 0.77 l min^{-1}. Plasma, auxiliary gas and sheath gas flow rates were all automatically controlled. The sample cone had a 1.1 mm diameter orifice and the skimmer cone with a 0.9 mm diameter orifice. The dwell time was 50ms/AMU and scan mode was peak hopping. The samples were determined in "dual detector mode" and "steady state mode" to introduce the sample continuously. Metal MDLs ranged from 0.10 to 0.54 µg l^{-1}.

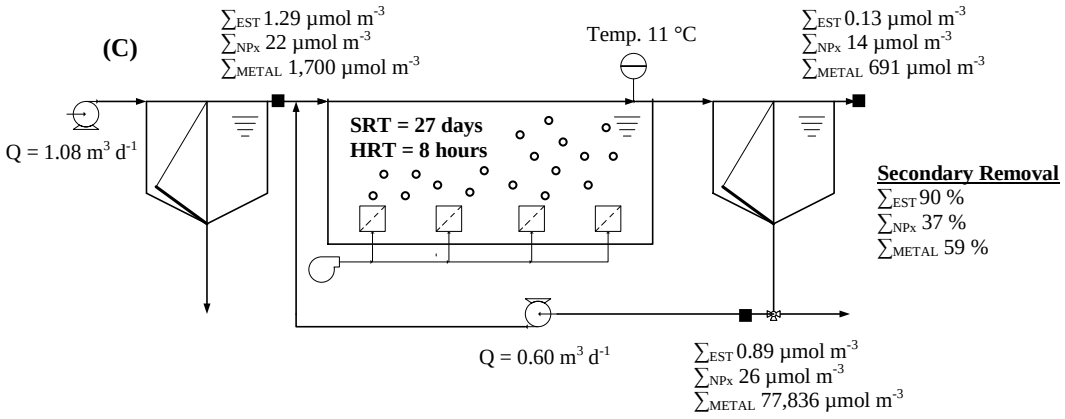
7.3. RESULTS

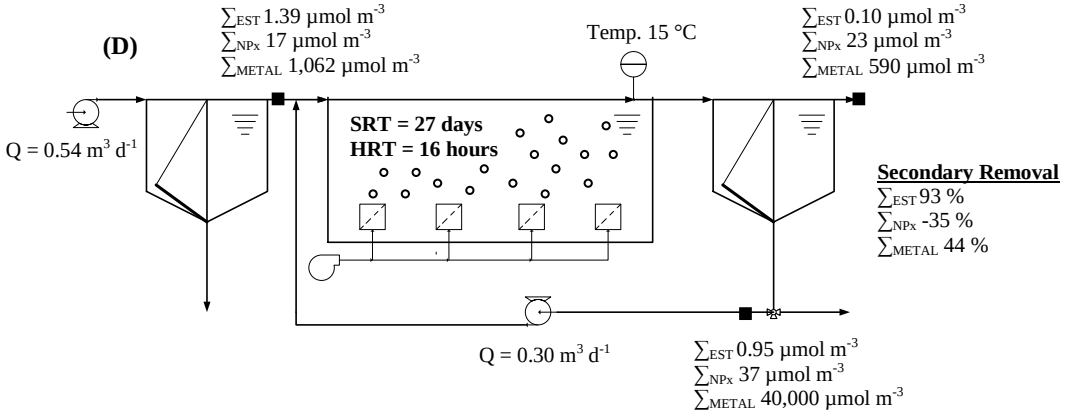
7.3.1. Impact of solids retention time on hazardous chemical removal

Removal of total estrogens ($\sum_{EST}$) at the 3 day SRT condition (8 hour HRT) was 70 ±7 % (Figure 7.1). Increased SRT (i.e., 10 or 27 days) was needed to achieve augmented removals. However, no improvement in $\sum_{EST}$ removal was observed when SRT was increased from 10 to 27 days. Removal of $\sum_{EST}$ was 94 ±1% and 90 ±2 % respectively. These higher $\sum_{EST}$ removals here were driven by improved natural estrogen (E1, E2 and E3) removals (Table 7.2). Removal of total nonylphenolic chemicals ($\sum_{NPx}$) was comparatively lower with the greatest being 37 ±5 % (Figure 7.1) which was achieved at the 27 day SRT condition. All ASP operations initiated NPEO chain shortening with removals of $NP_{4-12}EO$ ≥79 % for all SRTs studied (Table 7.2). This resulted in a substantial accumulation of $NP_{1-3}EC$. The lowest production of $NP_{1-3}EC$ was achieved at a SRT of 27 days and was -212 ±98 %. However, no net removal of the daughter chemical NP was observed with removals ranging from -6 to -39 %. The concentrations of both $\sum_{EST}$ and $\sum_{NPx}$ in RAS (aqueous and particulate fractions) reduced with increasing SRT (Figure 7.1). This included those individual chemicals which are relatively hydrophobic in nature (log K_{ow}'s > 3.5 - E2, EE2, NP, $NP_{1-3}EO$, $NP_{1-3}EC$ Table 7.1). In contrast to the organic hazardous chemicals, total metal ($\sum_{METAL}$) removals increased moderately with each SRT increase. Removals were 34 ±19, 51 ±10 and 59 ±11 % for 3, 10 and 27 day SRTs respectively (Figure 7.1). Improving removal was reflected in increasing concentrations observed in the particulate fraction of RAS for the three SRTs. Specifically, Cu removals were improved by increasing SRT and were 38 ±15, 53 ±9 and 82 ±4 % (Table 7.2). However, metal concentrations in the dissolved fraction of wastewater increased during treatment (Table 7.2). Nevertheless, the lowest dissolved $\sum_{METAL}$ concentration was observed for the 27 days SRT final effluent and was 34 ±8 µg l^{-1}. The concentrations of mixed liquor volatile suspended solids (MLVSS) concentrations were 972 ±100, 1,301 ±305 and 1,284 ±71 mg l^{-1} for 3, 10 and 27 day SRTs, respectively (Table 7.3). Also, in terms of biomass physiology both median activated sludge floc size and EPS concentration reduced with increased SRT (Table 7.4).


(A)
∑EST 1.28 µmol m-3
∑NPx 22 µmol m-3
∑METAL 1,869 µmol m-3
Temp. 13 °C
∑EST 0.39 µmol m-3
∑NPx 19 µmol m-3
∑METAL 1,179 µmol m-3
SRT = 3 days
HRT = 8 hours
Q = 1.08 m3 d-1
Secondary Removal
∑EST 70 %
∑NPx 11 %
∑METAL 37 %
Q = 0.60 m3 d-1
∑EST 1.40 µmol m-3
∑NPx 62 µmol m-3
∑METAL 17,725 µmol m-3


(B)
∑EST 1.29 µmol m-3
∑NPx 18 µmol m-3
∑METAL 1,933 µmol m-3
Temp. 17 °C
∑EST 0.07 µmol m-3
∑NPx 19 µmol m-3
∑METAL 948 µmol m-3
SRT = 10 days
HRT = 8 hours
Q = 1.08 m3 d-1
Secondary Removal
∑EST 94 %
∑NPx -4 %
∑METAL 51 %
Q = 0.60 m3 d-1
∑EST 1.27 µmol m-3
∑NPx 55 µmol m-3
∑METAL 47,149 µmol m-3


(C)
∑EST 1.29 µmol m-3
∑NPx 22 µmol m-3
∑METAL 1,700 µmol m-3
Temp. 11 °C
∑EST 0.13 µmol m-3
∑NPx 14 µmol m-3
∑METAL 691 µmol m-3
SRT = 27 days
HRT = 8 hours
Q = 1.08 m3 d-1
Secondary Removal
∑EST 90 %
∑NPx 37 %
∑METAL 59 %
Q = 0.60 m3 d-1
∑EST 0.89 µmol m-3
∑NPx 26 µmol m-3
∑METAL 77,836 µmol m-3


(D)
∑EST 1.39 µmol m-3
∑NPx 17 µmol m-3
∑METAL 1,062 µmol m-3
Temp. 15 °C
∑EST 0.10 µmol m-3
∑NPx 23 µmol m-3
∑METAL 590 µmol m-3
SRT = 27 days
HRT = 16 hours
Q = 0.54 m3 d-1
Secondary Removal
∑EST 93 %
∑NPx -35 %
∑METAL 44 %
Q = 0.30 m3 d-1
∑EST 0.95 µmol m-3
∑NPx 37 µmol m-3
∑METAL 40,000 µmol m-3

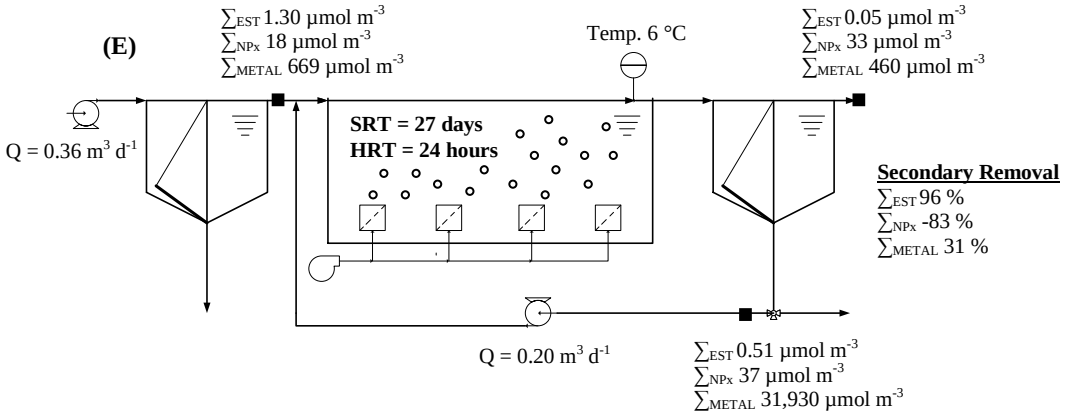


Figure 7.1. Mass balance data for estrogens, NPx and metals at varying operational conditions (A, 3 day SRT 8 hour HRT; B, 10 day SRT 8 hour HRT; C, 27 day SRT 8 hour HRT; D, 27 day SRT 16 hour HRT; E, 27 day SRT 24 hour HRT; ■, sampling points)

Table 7.2. Hazardous chemical quantitative information for SRT and HRT pilot plant studies

Chemical of interest	Crude sewage conc. (µg l^{-1})	Crude sewage log K_d	SRT (days)	HRT (hours)	Settled sewage conc. (µg l^{-1})	Settled sewage log K_d	Primary removal[a] (%)	RAS conc. (µg l^{-1})	RAS log K_d	Final effluent conc. (µg l^{-1})	Final effluent log K_d	Secondary removal[b] (%)
Zn	91.3	4.29	3	8	73.1	4.68		568	3.92	46.3	5.64	37
			10	8	88.5	4.33	14[d]	1,566	3.89	44.1	3.94	50
			27	8	75.1	4.41		2,856	3.99	38.0	4.07	49
			27	16	41.2	4.65	55	1,074	4.09	27.7	4.01	33
			27	24	25.8	4.46	72	941	4.07	22.1	3.95	14
Cu	45.8	5.11	3	8	45.6	5.13		548	4.72	28.3	4.96	38
			10	8	36.2	5.02	16[d]	1,389	4.95	16.9	4.99	53
			27	8	34.0	5.14		2,131	4.89	6.2	4.94	82
			27	16	27.0	5.39	41	1,418	4.99	10.3	4.82	62
			27	24	17.1	5.21	63	1,101	4.84	7.5	4.66	56
Pb	3.6	4.33	3	8	2.1	4.17		24.5	4.04	1.5	4.34	31
			10	8	1.0	4.29	70[d]	79.4	4.46	0.7	4.41	29
			27	8	0.2	-		60.6	4.75	<0.1	-	>49
			27	16	0.8	4.28	78	47.3	4.48	0.4	3.81	48
			27	24	0.8	3.72	78	10.8	4.23	0.6	4.07	28
Cd	-	-	3	8	0.2	-		1.9	3.67	0.1	-	43
			10	8	0.2	-	-	6.3	4.04	<0.1	-	>40
			27	8	0.2	-		6.4	4.05	<0.1	-	>48
			27	16	<0.1	-	-	-	-	<0.1	-	-
			27	24	<0.1	-	-	-	-	<0.1	-	-
Ni	-	-	3	8	1.3	4.03		16.1	3.69	1.0	4.16	25
			10	8	0.2	3.92	-	52.5	4.48	0.2	4.54	15
			27	8	0.8	3.86		46.6	3.51	0.7	4.35	22
			27	16	<0.1	-	-	-	-	<0.1	-	-
			27	24	<0.1	-	-	-	-	<0.1	-	-
E1	-	-	3[c]	8	0.0917	2.43		0.1903	2.28	0.0745	2.07	19
			10[c]	8	0.1005	2.64	-	0.1937	2.32	0.0071	3.39	93
			27[c]	8	0.0940	2.35		0.1307	2.38	0.0148	3.00	84
			27	16	0.1017	2.15	-	0.1678	2.46	0.0146	3.17	86
			27	24	0.0737	2.62	-	0.0615	3.63	0.0049	3.21	93

			3[c]	8	0.0301	3.20		0.1058	2.70	0.0097	3.43	68
			10[c]	8	0.0285	3.34	-	0.0892	3.02	0.0029	3.92	90
E2	-	-	27[c]	8	0.0304	2.99		0.0583	3.70	0.0024	3.54	92
			27	16	0.0407	3.32	-	0.0407	2.91	0.0033	3.43	92
			27	24	0.0417	3.78	-	0.0362	3.62	0.0022	4.08	95
			3[c]	8	0.2201	1.72		0.0430	2.65	0.0170	2.62	92
			10[c]	8	0.2144	2.02	-	0.0220	3.62	0.0068	3.65	97
E3	-	-	27[c]	8	0.2050	1.82		0.0229	3.48	0.0062	3.57	97
			27	16	0.2335	2.43	-	0.0175	3.73	0.0039	2.92	98
			27	24	0.2328	1.45	-	0.0220	3.27	0.0041	2.89	98
			3[c]	8	0.0018	4.25		0.0329	4.42	0.0013	4.57	30
			10[c]	8	0.0009	3.81	-	0.0203	3.92	0.0007	3.48	29
EE2	-	-	27[c]	8	0.0015	4.11		0.0148	4.01	0.0009	4.15	41
			27	16	0.0006	3.82	-	0.0113	4.76	0.0003	4.21	55
			27	24	0.0004	4.54	-	0.0133	4.63	0.0001	4.36	65
			3	8	0.0224	2.20		0.0183	2.51	0.0047	2.57	79
			10	8	0.0238	1.83	-	0.0286	2.68	0.0035	2.50	85
E1-3S	-	-	27	8	0.0410	1.57		0.0233	1.99	0.0147	1.88	64
			27	16	0.0197	1.80	-	0.0276	1.86	0.0056	2.34	71
			27	24	0.0242	1.96	-	0.0111	2.52	0.0044	2.65	82
			3	8	1.58	3.70		2.50	2.81	1.74	3.70	-10
			10	8	1.06	4.01	-	3.32	3.33	1.38	4.30	-30
NP	-	-	27	8	1.66	3.53		1.59	2.65	1.76	4.15	-6
			27	16	0.25	3.06	-	1.17	1.59	0.07	5.51	70
			27	24	0.63	3.45	-	1.04	2.92	0.24	2.94	63
			3	8	0.15	2.83-3.40		8.54	2.39-3.22	1.32	1.57-2.90	-763
			10	8	0.17	2.80-3.37	-	6.41	2.50-3.38	2.44	1.71-2.48	-1,416
$NP_{1-3}EC$	-	-	27	8	0.18	2.50-2.73		0.36	1.61-2.46	0.55	1.85-2.43	-212
			27	16	3.12	0.99-2.17	-	8.94	2.00-2.71	6.92	1.01-1.58	-128
			27	24	2.53	1.22-2.11	-	9.16	0.85-1.45	10.1	0.83-1.33	-297
			3	8	2.53	2.99-3.74		5.49	2.08-2.96	1.80	2.88-3.19	32
			10	8	2.16	3.77-4.19	-	5.02	3.55-4.03	1.14	3.88-3.99	49
$NP_{1-3}EO$	-	-	27	8	2.10	3.62-3.77		4.94	3.02-3.70	1.03	3.72-4.60	52
			27	16	0.30	1.65-2.96	-	0.02	4.11-4.20	0.02	2.87-3.78	93
			27	24	0.55	1.61-4.00	-	0.41	1.82-3.48	0.02	2.88-3.72	96

			3	8	2.74	3.10-3.69		2.33	2.58-4.79	0.49	2.94-4.58	79
			10	8	2.68	3.12-4.00	-	1.77	2.79-4.80	0.27	3.85-4.28	89
$NP_{4-12}EO$	-	-	27	8	3.55	2.68-3.27		0.46	2.45-3.38	0.27	3.76-5.16	92
			27	16	2.95	2.68-3.32	-	1.05	4.10-5.84	0.23	4.29-4.95	93
			27	24	3.07	3.28-3.81	-	1.09	2.17-4.31	0.25	3.63-3.95	92

[a]Primary removal (%) = (crude – settled)/crude x 100 [b]Secondary removal (%) = (settled – effluent)/settled x 100 [c]Petrie *et al*., submitted [d]Mean primary removal from 3 sampling campaigns

Key: SRT, solids retention time; HRT, hydraulic retention time; RAS, return activated sludge; -, not determined

Table 7.3. Physico-chemical properties of activated sludge, and extracted soluble micro-products and extracellular polymeric substances of varying SRT and HRT

Physicochemical property	SRT (days)	HRT (hours)	Activated sludge	Soluble micro-products	Extracellular polymeric substance
Protein (mg $gVSS^{-1}$)	3	8[a]	-	41	202
	10	8[a]	-	49	197
	27	8[a]	-	17	158
	27	16	-	13	133
	27	24	-	12	154
Carbohydrate (mg $gVSS^{-1}$)	3	8[a]	-	21	29
	10	8[a]	-	24	46
	27	8[a]	-	13	43
	27	16	-	10	22
	27	24	-	5	19
COD (mg $gVSS^{-1}$)	3	8[a]	-	72	438
	10	8[a]	-	30	387
	27	8[a]	-	22	315
	27	16	-	37	333
	27	24	-	38	347
Zeta potential (mV)	3	8[a]	-13	-9	-25
	10	8[a]	-12	-9	-18
	27	8[a]	-11	-6	-18
	27	16	-16	-9	-16
	27	24	-18	-8	-12
MLVSS (mg $gVSS^{-1}$)	3	8[a]	972	-	-
	10	8[a]	1,301	-	-
	27	8[a]	1,284	-	-
	27	16	1,067	-	-
	27	24	1,125	-	-
d_{50} (μm)	3	8[a]	358	-	-
	10	8[a]	274	-	-
	27	8[a]	164	-	-
	27	16	369	-	-
	27	24	236	-	-

[a]Petrie *et al*., submitted

Key: SRT, solids retention time; HRT, hydraulic retention time; COD, chemical oxygen demand; MLVSS, mixed liquor volatile suspended solids; d_{50}, median floc size

Table 7.4. Sanitary determinand quantitative information for SRT and HRT pilot plant studies

Sanitary determinand	SRT (days)	HRT (hours)	Settled sewage (mg l^{-1})	Final effluent (mg l^{-1})	Secondary removal (%)
COD	3	8	266	101	62
	10	8	258	52	80
	27	8	291	51	82
	27	16	252	43	83
	27	24	216	42	81
sCOD	3	8	109	42	62
	10	8	76	24	68
	27	8	103	30	71
	27	16	132	29	78
	27	24	103	34	67
BOD	3	8	104	27	74
	10	8	100	12	88
	27	8	105	5	96
	27	16	93	5	94
	27	24	59	2	96
Ammonium	3	8	44	41	6
	10	8	38	<0.5	>99
	27	8	28	<0.5	>99
	27	16	31	<0.5	>99
	27	24	30	<0.5	>99
Nitrate	3	8	<1	<1	-
	10	8	<1	35	<-3,400
	27	8	<1	29	<-2,780
	27	16	<1	34	<-8,425
	27	24	<1	30	<-5,857
Nitrite	3	8	<1	6	<-520
	10	8	<1	3	<-230
	27	8	<1	1	<-20
	27	16	5	<1	>79
	27	24	3	<1	>65
Total nitrogen	3	8	59	53	10
	10	8	52	47	10
	27	8	44	34	23
	27	16	42	38	9
	27	24	47	40	14
Suspended solids	3	8	110	69	37
	10	8	119	29	76
	27	8	139	18	87
	27	16	88	22	74
	27	24	64	17	67

Key: SRT, solids retention time; HRT, hydraulic retention time; COD, chemical oxygen demand; sCOD, soluble chemical oxygen demand; BOD, biochemical oxygen demand

7.3.2. Effect of hydraulic retention time to hazardous chemicals removal

Lengthening HRT of the secondary aerobic treatment stage (whilst at a constant SRT of 27 days saw a concomitant decrease in food to microorganism (F: M) ratios. Ratios were 0.25, 0.13 and 0.05 gBOD gVSS d^{-1} for 8, 16 and 24 hour HRTs, respectively. Reducing F: M ratio correlated well (r^2 0.91) with increased secondary removal of $\sum_{EST}$ (Figure 7.2).

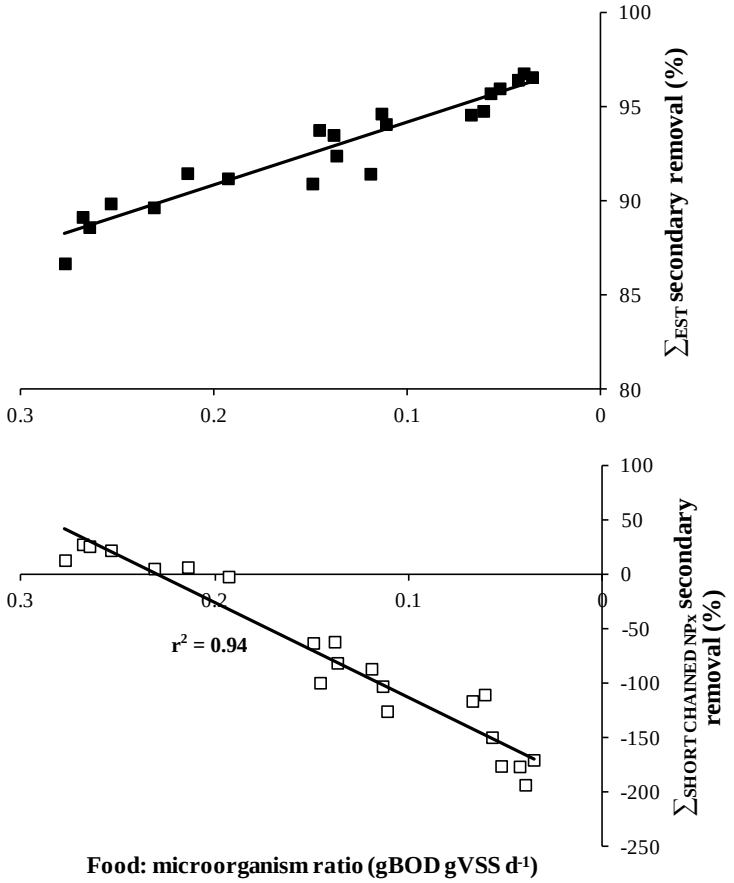


Figure 7.2. Significance of the food: microorganism ratio to secondary removal of $\sum_{EST}$'s (A, ■) and short chained NPx ($NP+NP_{1-3}EO+NP_{1-3}EC$) (B, □) in HRT studies

Removals of $\sum_{EST}$'s were 90 ±2 %, 93 ±1 % and 96 ±2 % for 8, 16 and 24 hour HRTs, respectively (Figure 7.1). Individually each estrogen showed improving removal with longer HRT. Most notably EE2 exhibited removals of 65 ±19 % at the 24 hour HRT condition (Table 7.2). However, lengthening HRT resulted in an apparent increase in $\sum_{NPEO}$ molar concentration with removals of 57 ±6%, -35 ±18% and -83 ±12% observed (Figure 7.1). A negative correlation (r^2 0.94) was observed between reduced F: M ratio and short chained NPx removal ($NP+NP_{1-3}EO+NP_{1-3}EC$) (Figure 7.2). This was mainly attributed to increased concentrations of $NP_{1-3}EC$ observed in final effluents (Table 7.2). Nevertheless, this counter-intuitively resulted in the greatest removals of NP. Positive removals of 70 ±16 % and 63 ±15 % were observed at 16 and 24 hour HRTs respectively (Figure 7.3).

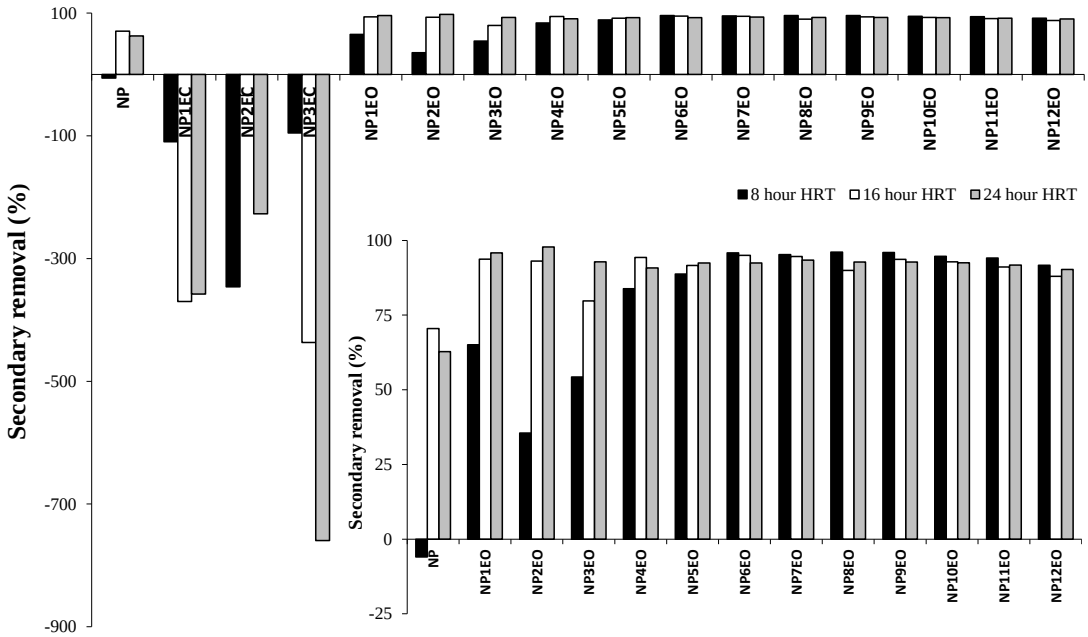


Figure 7.3. Secondary removal of individual nonylphenolic chemicals at varying HRT. Inset, individual removals of NP and $NP_{1-12}EO$

In contrast, lengthening HRT initially resulted in increased removal of $\sum_{METAL}$'s observed during primary sedimentation. This was because a high proportion of metals found in crude sewages were associated with particulates. To demonstrate, the percentage of Zn and Cu within the particulate fraction (>0.45 µm) of crude wastewater was 88 ±4 % and 97 ±2 %, respectively (Figure 7.4). Suspended solids concentration of crude sewage was 366 ±154 mg l^{-1} and reduced to 123 ±15 mg l^{-1}, 88 ±7 and 64 ±13 mg l^{-1} in settled sewage for primary sedimentation times of 4, 8 and 12 hours respectively (Table 7.4). This corresponded well to $\sum_{METAL}$'s behaviour. To demonstrate, $\sum_{METAL}$ concentrations in crude sewage were 141 ±29 µg l^{-1} with the majority of this attributed to Zn (91 ±27 µg l^{-1}) and Cu (46 ±11 µg l^{-1}) (Figure 7.4). In settled sewage Zn concentrations were 79 ±18, 41 ±6 and 26 ±5 µg l^{-1} corresponding to primary sedimentation residence times of 4, 8 and 12 hours (Table 7.2). Similarly, total Cu concentrations were reduced to 39 ±8, 27 ±4 and 17 ±2 µg l^{-1} in settled sewage. Primary removals of $\sum_{METAL}$'s were 69 ±8 % at the 12 hours sedimentation time. Interestingly though, dissolved metal concentrations (to which EQS's are applied) were unaffected by primary treatment and were similar to, or below their proposed legislative targets for consent in settled sewages (Figure 7.4).

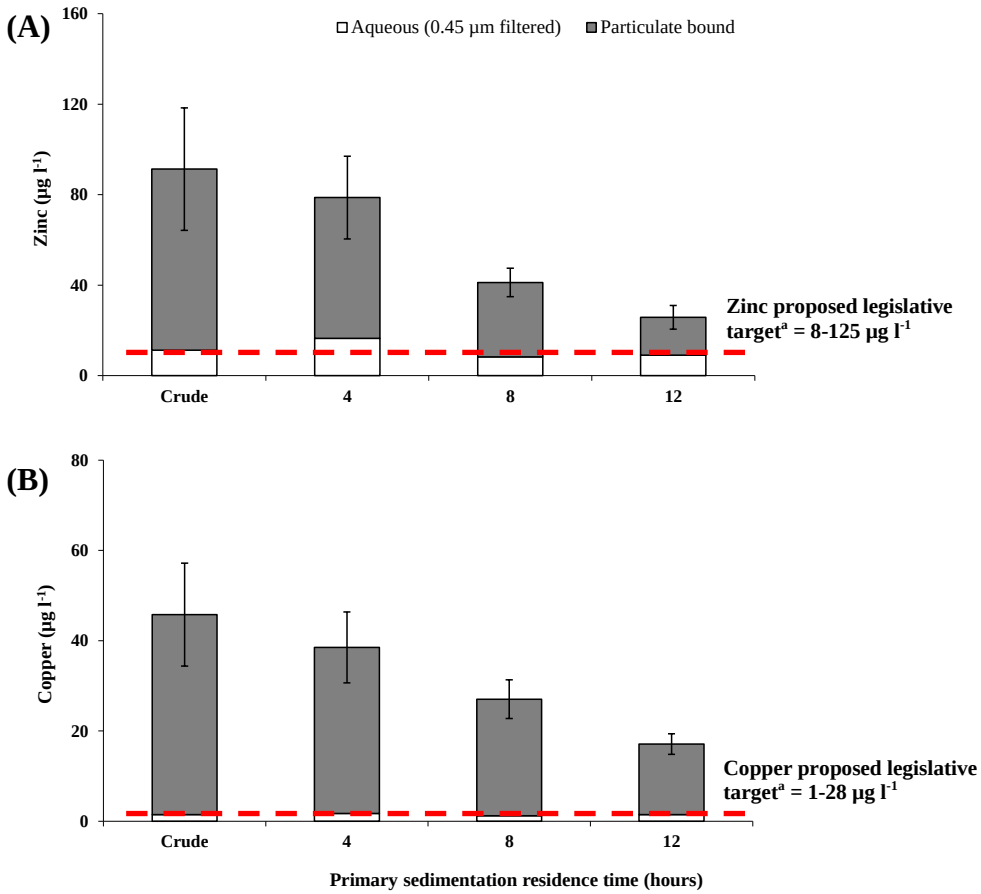


Figure 7.4. Effect of primary sedimentation tank residence time on Zn (A) and Cu (B) concentration in settled sewage. [a]EQS is applied to the dissolved concentration (i.e., pre-filtered) and is dependent on water hardness (European Commission, 2008). Note: error bars represent the standard deviation of the total concentration.

However, secondary treatment at longer HRT resulted in further increases in dissolved metal concentrations (Figure 7.5). To illustrate, dissolved metals removals were -88 ±9 % at an 8 hour HRT whereas removals at 16 and 24 hour HRTs were -176 ±9 % and -128 ±6 % respectively. Secondary removal of $\sum_{METAL}$'s decreased with longer HRT, explained by a linear correlation (r^2 0.92) between reduced $\sum_{METAL}$ concentrations in settled sewage with secondary removal (Figure 7.5).

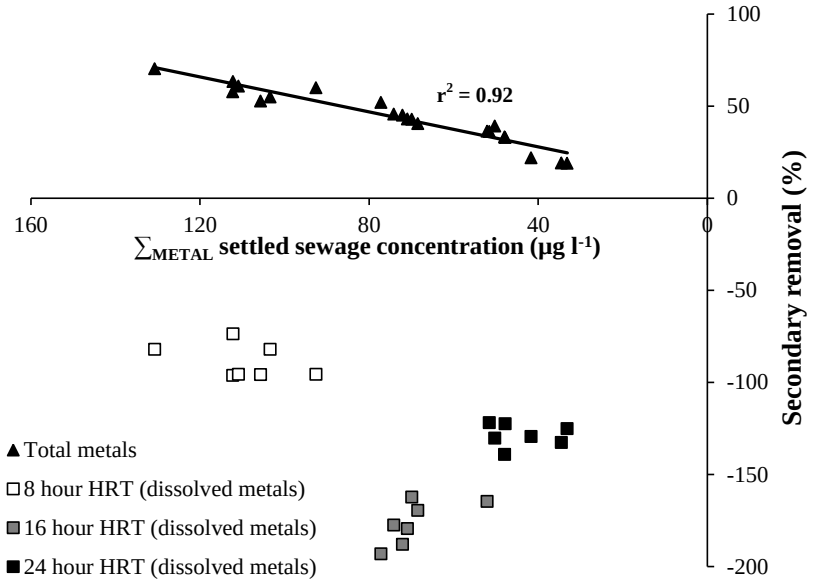


Figure 7.5. Impact of $\sum_{METAL}$ concentration (aqueous + particulate) in settled sewage to secondary removal of total metals and dissolved metals during HRT studies

7.3.3. Final effluent quality

The steroid estrogens E1, E2 and EE2 were at their lowest concentration in the final effluent of the 27 day SRT 24 hour HRT condition. Total concentrations of the natural estrogens E1 and E2 were 4.9 ±2.6 and 2.2 ±1.2, respectively (Table 7.2). In comparison, the synthetic estrogen was found here at a concentration of 0.14 ±0.07 ng l^{-1}. Noteworthy estrogen concentrations were found in the particulate fraction of final effluents. To demonstrate, the most hydrophobic estrogens E2 and EE2 had final effluent log K_d's of 4.1 and 4.4, respectively. Total concentrations of NP in final effluent were below its EQS of 300 ng l^{-1} (Table 7.1) at 16 and 24 hour HRT conditions. However, molar concentrations of $\sum_{NPx}$ in these final effluents were 23 ±8 and 37 ±6 µmol m^{-3} respectively (1.4 µmol m^{-3} of NP is equivalent to 300 ng l^{-1}) (Figure 7.1). Similar to E2 and EE2, substantial NP concentrations were observed in the particulate fraction exhibiting log K_d's up to 5.5. Overall, the concentration of $\sum_{METAL}$'s in final effluents reduced with increased SRT and longer HRT (Figure 7.1). At the 27 day SRT 24 hour HRT condition $\sum_{METAL}$ concentrations were 30 ±2 µg l^{-1}. Despite total concentrations reducing with longer HRT, concentrations in the aqueous fraction (0.45 µm filtered) increased. This is demonstrated by the general trend of reducing final effluent log K_d's for Cu and Zn with longer HRT (Table 7.2). Lowest dissolved concentrations were at the 27 day SRT 8 hour HRT ASP operation. To assess the likelihood of achieving compliance for each hazardous chemical within the receiving aqueous environment, the level of final effluent dilution required for consent was determined (Figure 7.6). This denotes the minimum river dilution requirement to achieve the EQS or proposed

legislative target for each ASP final effluent for EE2, NP and Cu. These chemicals were selected as they required the most dilution within the groups; steroid estrogens, NPx's and metals. Compliance was unequivocally assured for a dilution factor of 10 by the 27 day SRT 24 hour HRT operation. Modifying ASP operation had the greatest impact to the dilution requirement for EE2. This was due to EE2 having very low proposed legislative target of 0.035 ng l^{-1} (Table 7.1) and it being more responsive to changes in HRT. Dilution of ≥20 times was needed to ensure consent for ASPs where SRT was investigated (Figure 7.6). Conversely, at 16 and 24 hour HRTs the dilution required was ≤10 due to the increased EE2 removals. In comparison, the required dilution for consent for both NP and Cu was <10 for all ASP final effluents. However, the high SRT operation (27 day SRT 8 hour HRT) required significantly lower dilutions for Cu (2.4 ±0.4 times).

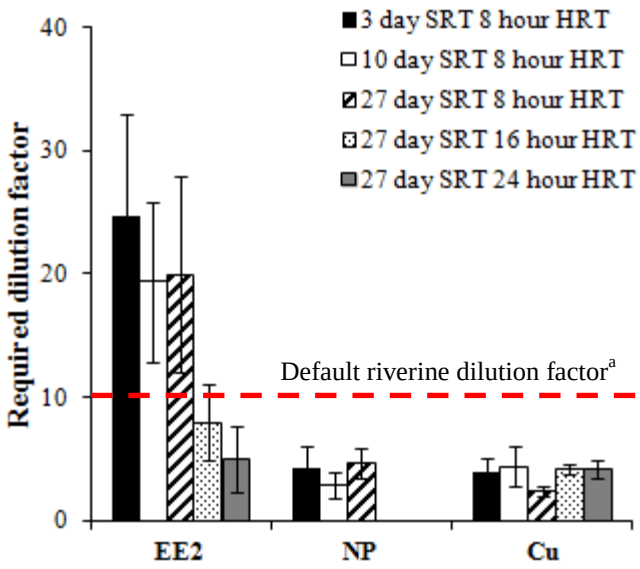


Figure 7.6. Riverine dilution required to ensure consent to EQS and proposed legislative targets for EE2 (proposed legislative target = 3.5 x 10^{-5} µg l^{-1}, respectively, NP (EQS = 0.3 µg l^{-1}) and Cu (proposed legislative target = 1-28 µg l^{-1}) at each ASP operation.

[a]Default riverine dilution factor of 10 as described by the European Commission (European Commission 2003b) Note: Dilution factor required for consent is calculated from the dissolved (i.e., pre-filtered) concentration only (European Commission 2008). Hazardous chemical concentration already present in dilution water is assumed to be zero as their presence will increase the water dilution requirement. For metals, a calcium carbonate concentration of 0-50 mg l^{-1} is assumed as EQSs are dependent on water hardness.

7.4. DISCUSSION

The highest SRT studied (27 days) achieved the greatest collective hazardous chemical removals. The SRT range studied broadly encompassed typical full-scale operations (Koh *et al*., 2009; McAdam *et al*., 2010; McAdam *et al*., 2011). Solids retention times of 10 and 27 days were required to achieve highest removal of $\sum_{EST}$ (≥90 %) (Figure 7.1). Despite increasing SRT, the quantity of estrogens within the dissolved and particulate fractions of RAS reduced. This relationship corresponded with that of NPx's. The majority of organic hazardous chemical removal was therefore attributed to biodegradation (Andersen *et al*., 2005; Petrie *et al*., submitted). This is supported by reducing concentrations of estrogens and NPx in RAS as SRT increased. Interestingly, secondary removal of $\sum_{NPx}$ was negligible at SRTs of 3 and 10 days (Figure 7.1). Nevertheless, substantial long chained ethoxylate removal was observed. This resulted in the accumulation of short chained intermediates (specifically $NP_{1\text{-}3}EC$) consistent with full-scale observations (Koh *et al*., 2009; McAdam *et al*., 2011). Operation at the highest SRT condition (27 days) was beneficial as $\sum_{NPx}$ removal was increased to 37 ±5 % (Figure 7.1). This demonstrates an improvement across the NPx removal pathway by assimilation and oxidation of breakdown intermediates. At 27 days SRT, higher $\sum_{NPx}$ removal was driven by an improved removal of $NP_{1\text{-}3}ECs$. The specific difficulty associated with this chemical type is the complexity of biotransformation reactions that this family of chemicals exhibit prior to NP biodegradation. To demonstrate, a number of structural changes (e.g., ethoxylate shortening, oxidations etc) are undertaken before cleavage of the NP ring takes place (Warhurst, 1994; Petrie *et al*., 2013a). In this study, removals of the daughter chemical NP ranged from -6 % to -39 % over the three SRTs studied (Table 7.2). As an independent chemical under controlled conditions, NP is known to be susceptible to biodegradation (Tanghe *et al*., 1998; Stasinakis *et al*., 2008), even at low SRT (Stasinakis *et al*., 2010). This suggests its production during activated sludge treatment by precursor biotransformation here was sufficient to compensate for its removal (i.e., net removal is zero). In contrast, $\sum_{METAL}$'s exhibited moderate improvements in removal with each increase in SRT (Figure 7.1). Partitioning within the biomass matrix was confirmed as the main removal pathway for metals as increased concentrations were observed in the particulate fraction of RAS as SRT increased (Figure 7.1). The moderate improvement (8 %) in $\sum_{METAL}$ removal between the 10 and 27 SRTs coincided with similar MLVSS concentrations (Figure 7.1, Table 7.3). This suggests an improvement in metal partitioning within the biomass matrix, likely to be induced by a physiological change to the biomass composition. For example, Laurent *et al* (2009) reported that smaller floc size was critical for increased Cd sorption. Activated sludge (of similar dry weight) comprised of smaller flocs has better availability of binding sites due to increased floc surface area. In this study median floc sizes reduced from

274 µm to 164 µm between SRTs of 10 and 27 days (Table 7.3). This may explain improved removal achieved by the 27 day SRT biomass. Although operation at a higher SRT improved simultaneous hazardous chemical removal, it has traditionally been associated with significantly greater aeration demands. However, it has been demonstrated that operation at a higher SRT achieves improved oxygen transfer due to smaller activated sludge floc size and better uniformity (Leu *et al*., 2012). Consequently, aeration requirements are not as great as previously considered.

Longer HRT substantially improved the biodegradation of $\sum_{EST}$ and NP. Steady state HRTs of 8, 16 and 24 hours were monitored to represent typical full-scale (dry weather flow) operational conditions (Koh *et al*., 2009; McAdam *et al*., 2010; McAdam *et al*., 2011), whilst at a fixed SRT of 27 days as this exhibited best overall removal performance previously. Longer HRT resulted in greater contact times and reduced F: M ratios. Reduced F: M correlated well with improved removal of $\sum_{EST}$ (r^2 0.91) (Figure 7.2). A lower F: M ratio is suggestive of a substrate limitation which may lead to the biodegradation of less-favoured carbon substrates (e.g., steroid estrogens). Coupled with increased contact time for biodegradation this may explain the observed improvement in biodegradation at longer HRTs (Figure 7.1). However, longer HRT resulted in an apparent increase of $\sum_{NPx}$ molar concentration (Figure 7.1). This illustrates that all chemicals within the NPx chemical family are not encompassed by current analytical methods. For example, longer chained NPEOs (Petrie *et al*., 2013a) and NPECs (Komori *et al*., 2006), and carboxylated carboxylates (CNPECs) (Jonkers *et al*., 2001) can be present. Without analytical reference standards available for their quantitation and inclusion in the nonylphenolic mass balance, their fate during treatment and resultant impact to NP removal remains unknown. Interestingly the high production of NPECs at the 16 and 24 hour HRT conditions resulted in good removals (63-70 %) of NP (Figure 7.3). The high removal of all NPEOs observed at 16 and 24 hour HRTs (Table 7.2) indicates that ethoxylate biotransformation is the dominant pathway for the production of NP during ASP treatment. Therefore longer HRT was advantageous as this initiated improved biotransformation across the NPx removal pathway resulting in significant NP removal. However, longer HRT during secondary treatment was detrimental to dissolved metal removal (Figure 7.5). To demonstrate, dissolved concentrations of Cu and Zn in crude and settled sewages were similar to their proposed legislative targets for consent (Figure 7.4). However, concentrations following secondary treatment were above these limits. Secondary HRTs of 16 and 24 hours resulted in the greatest increase of dissolved metals with removals of -176 ±9 % and -128 ±6 % respectively. These longer contact times may facilitate further oxidation of particulate organic matter (Ziolko *et al*., 2011). As a result metals were distributed more in the aqueous fraction of final effluents at longer HRT (Table 7.2). This is

significant as only the filtered (i.e., aqueous) fraction of water matrices are considered for EQS compliance (European Commission, 2008). Therefore, maximising particulate bound metals removal during primary sedimentation to circumvent their solubilisation during secondary treatment is essential. This is critical for sites which have low dilution ratios or where the receiving concentrations of metals are comparatively greater. For example, Cu and Zn are commonly found in crude wastewaters at concentrations >100 µg l^{-1} (Rule *et al*., 2006; Ziolko *et al*., 2009; Gardner *et al*., 2013). This study successfully demonstrated that primary treatment can effectively enhance metal removal prior to secondary treatment (Figure 7.4). However, enhancing suspended solids removal by existing assets may not be achievable without the continual dosing of coagulants. For example, there may not be sufficient space available onsite to increase primary sedimentation tank size to facilitate HRTs similar to those utilised in this study. As a trade off, the alternative or complimentary use of micro-screens can yield improved suspended solids removal at a comparatively smaller footprint (Salsnes Filter, 2011).

In this study, with the receiving concentrations observed, environment compliance was achieved for all hazardous chemicals by the 27 day SRT 24 hour HRT ASP when a default dilution factor of 10 was applied to final effluents (European Commission, 2003b) (Figure 7.6). Altering ASP operation had the greatest impact to the level of dilution needed for the steroid estrogen EE2. This is due to the very low proposed legislative target for consent of 0.035 ng l^{-1} (European Commission, 2012), and its comparative difficulty to remove during ASP treatment (Petrie *et al*., submitted). The more responsive removal of this chemical (65 %) at the 27 day SRT 24 hour HRT resulted in a <10 fold dilution requirement. In contrast, all ASP final effluents for NPx and metals were below a dilution threshold of 10. Nonylphenol is the only chemical within the NPx group which has an EQS but it has been successfully demonstrated that the biotransformation of readily degradable NP precursors in the environment (which are not legislated for) could theoretically lead to a breached EQS (Petrie *et al*., 2013a). It is therefore advantageous to operate the ASP at conditions which enhance biotransformation across the NPx family of chemicals (i.e., high SRT and long HRT conditions). However, the majority of existing full-scale ASPs are designed to operate at mid-ranged conditions (i.e., *circa* 10 day SRT 8 hour HRT) at yearly average flows (Aboobakar *et al*., 2013). Yet these tend to receive significant variations in sewage flow resulting in a dynamic system with significant variations in SRT and HRT. Therefore significant improvements in full-scale performance are achievable by better process control to improve process continuity and reduce variations in both SRT and HRT. For example, the use of *in-situ* suspended solids probes and real-time flow measurements could help avoid variability in SRT. Although achieving a significant improvement to HRT will require

infrastructure development. The extent of HRT alteration achievable for an existing process will depend on the available space onsite for expansion. Where sufficient space is not available to achieve a required HRT, remote holding tanks could be used as a buffer to counter-balance fluctuations in receiving sewage flow. This would enable steady state process conditions to be maintained whilst operating at a longer HRT. Furthermore, any operational change to lengthen secondary HRT requires suitable provisions for primary treatment to ensure greater removal of suspended solids are achieved. This will circumvent particle bound metals solubilisation which could lead reach to a breach of EQS.

7.5. CONCLUSION

Prospects of achieving EQS compliance through ASP operation improvements are highly site specific, governed by receiving hazardous chemical concentrations in crude sewage, available dilution water and quality, and available onsite space for infrastructure development. Solids retention times of 10 and 27 days were essential for augmented $\sum_{EST}$ removal and an increase of SRT from 10 to 27 days was beneficial to $\sum_{NPx}$ and $\sum_{METAL}$ removal. Longer HRT of the aerobic treatment stage was vital for achieving increased biodegradation of $\sum_{EST}$ (specifically EE2) and suitable precursor biotransformation such that positive NP removals were achieved. Enhanced removal of particulates from crude wastewater during primary sedimentation was critical for improved $\sum_{METAL}$ removals and avoiding increased concentrations of dissolved metals following secondary treatment. This study has identified that the broad range of hazardous chemical chemistries do result in trade-offs which should be considered for future studies.

CHAPTER 8
THESIS DISCUSSION

8. THESIS DISCUSSION

Previously reported method detection limits (MDLs) for some hazardous chemicals have been insufficient to consistently monitor their concentrations in wastewaters. Consequently, there has been a paucity of information on their fate and removal during wastewater treatment. A well-known example is the synthetic estrogen 17α-ethinylestradiol (EE2). This research involved improving current analytical methodologies mainly by the application of ultra-performance liquid chromatography (UPLC) to lower detection capabilities. Improved detection capabilities enabled hazardous chemicals to be monitored at environmentally typical concentrations over wastewater treatment works (WwTWs). Nevertheless, widely implemented secondary WwTWs such as activated sludge are not designed for the removal of hazardous chemicals. As a result these processes fail to meet environmental compliance for a wide range of hazardous chemicals. To achieve compliance more consistently with such processes, improvements in their operation is needed to specifically target the removal of hazardous chemicals. Therefore, understanding hazardous chemical pathways of removal and the impact of changing wastewater treatment operational conditions to these mechanisms was investigated. This was done under controlled conditions utilising real sewage containing typically encountered hazardous chemical concentrations. The information was then used to address the possibility of modifying the operation of activated sludge plants (ASPs) for the increased removal of hazardous chemicals. Finally, the impact of the operational parameters solids retention time (SRT) and hydraulic retention time (HRT) in a continuous flow pilot-scale system, to the removal of a diverse range of hazardous chemicals was assessed.

8.1. METHOD DEVELOPMENT FOR HAZARDOUS CHEMICALS

The determination of steroid estrogens in wastewater matrices was challenging due to the very low (ng l^{-1}) concentrations observed in wastewaters comprising comparatively high concentrations of bulk organics. It was particularly difficult for the synthetic estrogen 17α-ethinylestradiol (EE2) as it is typically encountered in wastewater <1 ng l^{-1} (Koh *et al*., 2009; McAdam *et al*., 2010). However, the MDLs achieved for steroid estrogens in settled sewages and final effluents ranged from 0.02 to 0.17 ng l^{-1} (Petrie *et al*., in press). Most notably, the MDL of EE2 was 0.06 ng l^{-1}, an improvement of >50 % from the previous lowest reported MDL (Koh *et al*., 2008). The application of UPLC enabled improved separation of the target analytes from remaining matrix within the sample extract. Consequently, reduced signal suppressions caused by the matrix aided the improved sensitivity observed. The newly reported MDL enabled complete process mass balances for EE2 to be attained. This helped in understanding their fate pathways and the influence of changing engineering conditions to

their removal at environmentally relevant receiving concentration. Previously reported methods have been restricted in monitoring EE2 concentrations during wastewater treatment by their detection capabilities (Braga *et al*., 2005; Chimchirian *et al*., 2007; Stanford and Weinberg, 2007; Kumar *et al*., 2009; Zorita *et al*., 2009; Gabet-Giraud *et al*., 2010; Zhou *et al*., 2012). However, the newly proposed legislative target for consent is 0.035 ng l^{-1} (European Commission, 2012). If this concentration became legislation in the future, additional improvements in analytical methodology would be needed, providing a challenge considering a significant improvement in sample extraction or chromatography is unlikely. More sensitive mass spectrometry detectors such as linear ion trap technology or new-generation triple quadrupole detectors offer the possibility of improved sensitivities (Barcelo and Petrovic, 2007). However, at present these are expensive restricting their widespread usage. Nevertheless, the method reported here is a substantial step towards achieving a MDL of 0.035 ng l^{-1}.

In contrast, the nonylphenolic (NPx) analysis did not require such a large concentration factor (400 to 1,000 times compared to 5,000 times for steroid estrogens) due to the inherently greater concentrations observed (Leusch *et al*., 2006; Koh *et al*., 2009; Moreira *et al*., 2011). Consequently, the extraction was more straight forward and the MDLs achieved for NPx's ranged from 1.4 to 45.3 ng l^{-1} (Petrie *et al*., 2013a). These were comparable to those reported in the literature (Koh *et al*., 2008; Loos *et al*., 2007) and sufficient to monitor nonylphenol (NP) and its precursors below the NP environmental quality standard (EQS) of 300 ng l^{-1} (European Commission, 2000). The relatively high concentration of precursors in trickling filter effluent led to an exploratory investigation of the possible environmental implication of their discharge. It was found that an initial dilution factor of 10 with river water would enable NP compliance. However, the biotransformation of precursors could lead to a breach of EQS downstream, over several kilometres. For example, NP concentrations could theoretically reach 362 ng l^{-1} in one river scenario studied (Petrie *et al*., 2013a). The main improvement achieved with this analytical method over those previously reported was the time saving achieved. For example, previously reported methods require run times of between 40 and 60 minutes (Jahnke *et al*., 2004; Loos *et al*., 2007; Koh *et al*., 2008). Run time is often a rate limiting step for such analysis, restricting sample number and turnover. However, the use of UPLC enabled adequate chromatographic separations within a 26 minute run time. This generally offers double the sample throughput in comparison to conventional high-performance liquid chromatography.

Method development for pharmaceuticals was more restrictive in sample extraction and clean-up. Extensive clean-up of sample extracts was not possible due to the wide ranging physico-chemical properties this group of hazardous chemicals exhibit. Otherwise, further

clean-up could be employed similar to the steroid estrogen method to achieve the required MDLs (Petrie *et al*., in press). Nevertheless, MDLs were lowered by improving sensitivity of detection. Chemicals were analysed in negative and positive ion modes separately, in different chromatographic runs. For example, 0.1 % formic acid was added to the aqueous mobile phase for chemicals analysed in positive ion mode. This improved their ionisation efficiency and therefore sensitivity of detection (Gros *et al*., 2012). Furthermore, separating the analytes in two chromatographic runs also limited the number of acquisitions registered for at the same time which can result in the loss of sensitivity (López-Serna *et al*., 2011). Despite, dividing into two separate chromatographic separations, run times were 9 and 10 minutes, respectively. The MDLs achieved for this diverse range of hazardous chemicals were generally <10 ng l^{-1}. These were adequate to monitor for typically encountered pharmaceutical concentrations in wastewater effluents (Metcalfe *et al*., 2003; Clara *et al*., 2005; Nakada *et al*., 2006; Zorita *et al*., 2009). Nevertheless, some pharmaceuticals such as the antibiotics are known to be present within the particulate fraction of sludge at relatively high concentrations (Chen *et al*., in press). Therefore to better understand their fate, determination in the particulate fraction of wastewaters was required (Petrie *et al*., 2013b). However, this provided a further challenge. To collect sufficient suspended solids for analysis and ensure detectable concentrations of all chemicals studied, a large quantity of wastewater (0.5 to 25 litres dependent on the matrix) was required for each sample analysis. The samples were then centrifuged and filtered to obtain the suspended solids. Nevertheless, the time spent on sample collection for particulate phase analysis was necessary as it enabled complete process mass balances to be attained which were used to reveal important information on removal pathways. This was specifically investigated for the pharmaceuticals as these hazardous chemicals exhibit a broad range of physico-chemical properties and therefore an expectant range of preferred removal pathways.

8.2. IDENTIFYING THE PATHWAYS OF HAZARDOUS CHEMICAL REMOVAL

Hazardous chemical removal by activated sludge is commonly described as being removed by sorption and biodegradation (Racz and Goel, 2010; Petrie *et al*., 2013b). However, there is limited evidence to support these assumptions due to the lack of particulate phase analysis undertaken. The complete process mass balances attained revealed that the pharmaceuticals studied were broadly separable into three groups based on their fate pathways (biodegraded, sorbed onto activated sludge and those exhibiting no removal from the aqueous phase) (Petrie *et al*., in preparation). Those chemicals mainly removed by biodegradation exhibited no

notable accumulation within the particulate phase of return activated sludge (RAS) despite significant secondary removal (≥86 %). For example, the total concentration of ibuprofen in settled sewage was 6,566 ng l^{-1}. This was reduced to 200 ng l^{-1} in final effluents with only 799 ng l^{-1} observed in RAS. In contrast, total concentrations of triclosan were 1,515, 239 and 5,795 ng l^{-1} for settled sewage, final effluent and RAS, respectively. The majority of triclosan in RAS was distributed within the particulate fraction (95 %) confirming that partitioning into sludge was its dominant pathway of removal. Sorption was also found to be the dominant removal pathway for the antibiotics ofloxacin and ciprofloxacin. This supports previous research which has postulated sorption to be their dominant removal pathway based only on the observation of high concentrations within the particulate phases of sludge (Chen *et al*., in press). The efficacy of trickling filter treatment to remove these chemicals which rely on very different mechanisms was directly compared to activated sludge. Findings revealed trickling filter treatment to be as effective as activated sludge for the removal of most of these chemicals. To demonstrate, ibuprofen was removed by 98 ±1 % and 97 ±1 % by trickling filter and activated sludge treatment, respectively. The antibiotics which rely on sorption for their removal exhibited removals of 42 to 54 % by trickling filter treatment and from 46 to 56 % by activated sludge, with no significant differences observed. This revealed the effectiveness of trickling filter treatment to remove these organic hazardous chemicals whilst operating at conditions thought to be challenging (i.e., very short HRTs). Considering the very different removal pathways observed for hazardous chemicals, specific knowledge of what drives these mechanisms and the impact of wastewater treatment operational conditions to these mechanisms is essential. Steroid estrogens were investigated because their removal was known to be more responsive to changing wastewater treatment operation.

8.3. DIAGNOSTIC INVESTIGATION OF STEROID ESTROGEN REMOVAL

To investigate the impact of process operation on estrogen removal a pilot-scale activated sludge plant (ASP) was operated on the same source of influent sewage at steady state conditions at varying SRTs of 3, 10 and 27 days. The activated sludge was then used in a series of controlled biodegradation and sorption batch studies. These were normalised for suspended solids concentrations to enable the direct comparison of SRT and spiked at environmentally relevant estrogen concentrations. Activated sludge of each SRT was also characterised (e.g., extracellular polymeric substances – EPS, floc size, cell viability etc) to establish whether a link could be made with estrogen biodegradation and sorption. In biodegradation tests, removal was augmented in nitrifying biomasses (10 and 27 day SRTs). To demonstrate, biodegradation rates were 0.27 ±0.01, 0.32 ±0.01 and 0.30 ±0.01 h^{-1} for 3, 10

and 27 day SRT biomasses. It has been previously reported that nitrifiers were responsible for increased biodegradation at higher SRTs (Vader *et al*., 2000). Allylthiourea was used to inhibit the action of nitrifiers in batch studies. However, no impact to estrogen removals were observed as degradation rates were 0.53 ±0.01 h^{-1} for activated sludge facilitating and inhibiting ammonia oxidation, respectively. These findings raised questions as to the site of biodegradation within the biomass matrix. It was unknown where biodegradation occurred within the activated sludge matrix (i.e., extracellularly or intracellularly) (Joss *et al*., 2004). This was investigated by monitoring the removal of estrogens in pre-filtered activated sludge in comparison to unfiltered sludge to establish the role of free extracellular enzymes to their biodegradation. Removal of cellular bound material by filtration resulted in the cessation of steroid estrogen removal. Starting concentrations of estrogens in filtered sludge were 384 ng l^{-1} and following 8 hours aeration, concentrations of 396 ng l^{-1} were observed. In contrast, starting concentrations in unfiltered activated sludge was 448 ng l^{-1} which reduced to 103 ng l^{-1} following treatment. Consequently, steroid estrogen biodegradation was by extracellular enzymes contained within the floc or by intact bacterial cells. Interestingly, the number of viable bacterial cells reduced with increased SRT. These were 1.45 x 10^{12}, 5.49 x 10^{11} and 4.09 x 10^{11} counts $gVSS^{-1}$ for 3, 10 and 27 day SRTs respectively. This corresponded to increasing estrogen biodegradations of 499, 1,361 and 1,750 ng 1 x 10^{12} viable $cells^{-1}$ d^{-1}. These findings indicate an improved efficiency of the same group, or a more responsive bacteria species type. Consequently, there is a need to elucidate these bacteria species for process performance improvement. Findings also suggest that the improved efficiency of bacterial cells at the 27 day SRT may be caused by changes to the physico-chemical composition of the activated sludge, facilitating less restricted passage of estrogens to viable cells for biodegradation. To demonstrate, 27 day SRT activated sludge had the smallest floc size (164 µm) resulting in shorter distances between the bacteria and the activated sludge floc surface suggesting shorter distances within the floc need overcome by diffusion (Joss *et al*., 2004). Furthermore, EPS concentration reduced with increased SRT from 438 to 315 mgCOD $gVSS^{-1}$. It has been suggested that EPS can act as a selective barrier towards substrate diffusion (Lieleg and Ribbeck, 2011). Therefore it can be hypothesised that lower EPS concentrations result in less restricted movement of estrogens to viable bacterial cells for biodegradation suggesting increased contact time could enable greater estrogen removal. Batch sorption studies exhibited significantly greater sorption capacity of the 27 day SRT biomass for the hydrophobic estrogens 17β-estradiol and EE2. This corresponded to the smallest floc size observed (164 µm) resulting in a greater total surface area for sorption to occur. It has been demonstrated that both SRT and HRT (contact time) can influence estrogen removal. Therefore, the individual impact of SRT and HRT to hazardous chemical removal was evaluated in a continuous flow system.

8.4. THE IMPACT OF CHANGING ACTIVATED SLUDGE OPERATION ON HAZARDOUS CHEMICAL REMOVAL

Previous research in this field has made the broad comparison of various full-scale ASPs operated at different SRT and HRTs (Svenson *et al*., 2003; Clara *et al*., 2005; Johnson *et al*., 2005; Kreuzinger *et al*., 2005; Koh *et al*., 2009; McAdam *et al*., 2010; McAdam *et al*., 2011). However, full-scale processes suffer from poor control of operational variables resulting in a dynamic system and considerable variations in both SRT and HRT. Operation of a pilot-scale ASP avoided these variations enabling the direct impact of SRT and HRT to hazardous chemical removal to be evaluated. To measure ASP resilience for the removal of hazardous chemicals of varying chemistry and preferred removal mechanisms - steroid estrogens, nonylphenolics and metals were monitored. Such a diverse range was chosen because it was unknown whether process conditions could be changed to effectively facilitate the removal of all hazardous chemicals collectively. For example an SRT of ≥10 days is considered necessary to augment removal of those biodegradable hazardous chemicals (Kreuzinger *et al*., 2005; Clara *et al*., 2005; McAdam *et al*., 2010). However, such conditions are considered detrimental to metals removal (Santos *et al*., 2010). In pilot plant trials investigating the impact of SRT whilst at fixed HRT, the highest SRT investigated (27 days) achieved the greatest removal of all hazardous chemicals collectively. The impact of altering HRT whilst at a fixed SRT of 27 days revealed that longer HRT was beneficial to the biodegradation of the organic hazardous chemicals. For example, total estrogen biodegradations were 90 ±2 %, 93 ±1 % and 96 ±2 % for 8, 16 and 24 hour HRTs, respectively. Furthermore, EE2 which is well-known for its recalcitrance (Petrie *et al*., in press) displayed its highest removal observed in this study of 65 ±19 % at the 24 hour HRT operation. Removals of NP were also greater (63-70 %) at longer HRTs. Interestingly, removals of steroid estrogens (r^2 0.91) and nonylphenolics (r^2 0.94) correlated well with the food: microorganism (F: M) ratio which reduced with increased HRT. This may be significant as full-scale F: M ratios are generally lower due to the higher mixed liquor suspended solids concentrations achieved at equivalent SRTs. To demonstrate, a full-scale works operated at a 13 day SRT 10-14 hour HRT had an F: M ratio of 0.09 to 0.10 gBOD gMLVSS d^{-1} (Koh *et al*., 2009). Whereas, the pilot-scale ASP operated under comparable conditions (10 day SRT 8 hour HRT) achieved an F: M ratio of 0.23 gBOD gMLVSS d^{-1}. Consequently, higher removals of both steroid estrogens and nonylphenol may be observed at full-scale whilst under the same operational conditions. However, longer HRT resulted in increased solubilisation of particulate metals during secondary treatment. Dissolved metals removals were -88 ±9 % at an 8 hour HRT. At 16 and 24 hour HRTs removals were -176 ±9 % and -128 ±6 % respectively. This is of concern as

only dissolved concentrations are measured for environmental compliance. Therefore careful consideration is required when implementing changes to ASP operation to ensure no detrimental impact is observed for a specific group of hazardous chemicals.

8.5. IMPLEMENTING CHANGES TO ASP OPERATION

Improvements in hazardous chemical removal required at full-scale ASPs to achieve environmental compliance are highly site specific. This is governed by receiving hazardous chemical concentrations in crude sewage, available dilution river water and the quality of this dilution water. Firstly, greater process control is needed. Full-scale ASPs are often designed to operate at mid-ranged conditions (i.e., 10 days SRT 8 hour HRT) at average yearly flows (Aboobakar *et al*., 2013). However, receiving flow can vary substantially resulting in a dynamic system with significant variations in both SRT and HRT. Therefore the use of *in-situ* suspended solids probes and real-time flow measurements would enable existing ASPs to be operated at a constant SRT. This would facilitate more consistent removal performance and final effluent qualities. The controlled pilot-plant studies revealed that the highest SRT studied (27 days) achieved greatest hazardous chemical removals collectively. Consequently, SRT can be increased with the knowledge that it will enhance removal of all hazardous chemicals. It has been traditionally considered that the aeration demands of high SRT operation are excessive for the benefits achieved. Leu *et al* (2012) demonstrated that improved oxygen transfer is achieved at higher SRTs due to smaller floc size and more uniformed floc distribution. Therefore, aeration costs are not as high as first thought for high SRT operation. Operation at a high SRT also has the benefit of producing less waste activated sludge which offers potential savings in sludge treatment. Longer HRTs may still be required to achieve compliance which is likely to require infrastructure development. Although this is unfavoured, the relatively high removal of EE2 achievable (65 %) could circumvent the need for controversial technologies (e.g., granular activated carbon) which have a proven ability to remove this chemical (Grover *et al*., 2011). However, there is a tension in HRT extension for improved organic hazardous chemicals as it increases solubilisation of metals. To overcome this, better solids removal at the primary sedimentation tank is needed. For example, using micro-screens in parallel to primary sedimentation tanks to enhance solids rejection is possible. An added advantage of producing more primary sludge is that it can be used to increase energy production through sludge treatment (Flores-Alsina *et al*., 2014). Overall, it has been demonstrated that ASP operation can be modified to specifically target the removal of hazardous chemicals exhibiting a range of chemistry and preferred removal pathways.

CHAPTER 9
CONCLUSIONS

9. CONCLUSIONS

- The application of ultra-performance liquid chromatography for steroid estrogen analysis lowered method detection limits by ~50 % over high performance liquid chromatography enabling complete process mass balances to be determined for 17α-ethinylestradiol (EE2) with a method detection limit of 0.06 ng l^{-1}.
- The relatively high concentration of nonylphenol (NP) precursors in final effluents could theoretically lead to a breach of the NP environmental quality standard of 300 ng l^{-1}within the receiving river, downstream from the point of discharge.
- The determination of pharmaceuticals in the particulate fraction of wastewaters was essential for diagnosing their different removal pathways during activated sludge treatment. Removal was dominated either by biological degradation or sorption to activated sludge.
- Biodegradation of steroid estrogens proceeds by the action of extracellular enzymes contained within the structure of activated sludge flocs and/or within viable bacterial cells as an intracellular process. Furthermore, these bacterial cells are not likely to be nitrifying micro-organisms.
- Steroid estrogen biodegradations were 499, 1,361 and 1,750 ng 1 x 10^{12} viable $cells^{-1}$ d^{-1} for 3, 10 and 27 day SRTs, respectively. This suggests the development of a specialised group of bacteria more capable of estrogen biodegradation or an improved response from bacteria already present within activated sludge at higher SRTs.
- The capacity for 17β-estradiol and EE2 sorption was an order of magnitude greater for activated sludge of higher SRT (27 days) in comparison to lower SRT (3 or 10 days). This was driven by changing biomass physiology (i.e., reducing floc sizes of 358, 274 and 164 μm at 3, 10 and 27 day SRTs, respectively).
- Operation of an activated sludge plant (ASP) at high SRT conditions (27 days) achieved maximum collective removals for all hazardous chemicals studied (steroid estrogens, nonylphenolics and metals).
- Longer hydraulic retention times (24 hours) augmented biodegradation of the organic hazardous chemicals, specifically NP and EE2. However, increased solubilisation of metals was observed. Consequently, improved solids removal during primary sedimentation is required to achieve maximum collective removals.
- Existing activated sludge works can be modified to specifically target the removal hazardous chemicals which have varied chemistry and preferred removal pathways.

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