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THE FATE OF ENDOCRINE DISRUPTING CHEMICALS DURING ACTIVATED SLUDGE TREATMENT

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ABSTRACT

A significant route for Endocrine disrupting chemicals (EDCs) such as natural and synthetic estrogens and nonylphenolics to enter the environment is via sewage treatment. Nanogram per litre levels of these compounds have been demonstrated to cause feminisation of aquatic organisms. The utilisation of treatments such as advanced oxidation and activated carbon could remove these compounds from effluent; however these technologies are expensive and energy intensive. Determining which operating parameters control the biodegradation of EDCs during activated sludge treatment, removal could be more sustainable. This study investigates which process parameters are responsible for controlling biodegradation of estrogens and nonylphenolics during activated sludge treatment. The role ammonia oxidising bacteria upon the breakdown of estrogens was also investigated. Field-based sampling campaigns were undertaken at three activated sludge plants and pilot-scale porous pot bioreactors were utilised to further examine the individual influences of loading, hydraulic retention time (HRT) and sludge retention time (SRT) upon EDC biodegradation.

It was evident that the volumetric loading of EDCs was an important factor in their biodegradation. Moreover, first-order reaction kinetics appeared to describe the biodegradation of estrogens and nonylphenolics at the ranges of 0.04 – 2.12 mg m^{-3} d^{-1} and 10 – 55 mg m^{-3} d^{-1} respectively, as observed at three sampled plants and under porous pot experimentation. The role of SRT appeared unimportant for EDC biodegradation as porous pot bioreactors with an SRT of three days were capable of on average >80% biodegradation of E1, E2 and E3. The effects of competitive inhibition on estrogen biodegradation by bulk organics could not fully be determined, although there was circumstantial evidence that the inherent biodegradability of the synthetic sewage led the porous pots to facilitate effective removal. However a negative linear relationship between $NP_{4\text{-}12}EO$ biodegradation and the decreasing proportion of $NP_{4\text{-}12}EO$ as COD in the settled sewage was observed. It was also demonstrated that reduction of ammonia oxidising bacteria from 5.15 x 10^9 to 4.27 x 10^7 mL^{-1} in activated sludge had no impact upon the biodegradation of estrogens including ethinylestradiol (EE2). Evidence is presented here that EDCs are biodegraded fortuitously by heterotrophs. To improve EDC removal during carbonaceous only activated sludge treatment, tertiary treatment may be necessary. The evidence presented by this research suggests that low energy low cost high rate processes, such as fixed film processes should be considered due to the biodegradation kinetics identified here.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AMO	Ammonium monooxygenase
AMP	Asset management plan
AOP	Advanced oxidation process
AP	Alkylphenol
APEOs	Alkylphenol polyethoxylates
APECs	Alkylphenoxy carboxylates
APx	Alkylphenolics
ASP	Activated sludge process
ASP_{carb}	Carbonaceous only removal activated sludge plant
ASP_{nit}	Nitrifying activated sludge plant
$ASP_{nit/denit}$	Nitrifying/denitrifying activated sludge plant
BOD	Biochemical oxygen demand
BNR	Biological nutrient removal
CAPEC	Dicarboxylated metabolites
COD	Chemical oxygen demand
DCM	Dichloromethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DI	Deionised water

DO	Dissolved oxygen
EA	Environment Agency
E1	Estrone
E1-d_4	Deuterated estrone
E1-3S	Estrone-3-sulfate
E1-3S-d_4	Deuterated estrone-3-sulfate
E2/17β-E2	17β-estradiol
17α-E2	17α-estradiol
E2-$d_{4/5}$	Deuterated 17β-estradiol
E3	Estriol
E3-d_4	Deuterated estriol
EE2	17α-ethinyl estradiol
EE2-d_4	Deuterated 17α-ethinyl estradiol
EDCs	Endocrine disrupting chemicals
EEq	Estradiol equivalent
ESI	Electrospray ionisation
ER	Estrogen receptor
ESTs	Estrogens
E-SCREEN	Estrogen-screen
EtOAc	Ethylacetate
EU	European Union
F/M	Food to microorganism ratio (mg BOD^{-1} mg MLVSS d^{-1})
GAC	Granular activated carbon

GC	Gas chromatography
GC/MS	Gas chromatography mass spectrometry
GC/MS/MS	Gas chromatography tandem mass spectrometry
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
IDL	Instrument detection limit
IFAS	Integrated Fixed Film Activated Sludge System
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MS-MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MBR	Membrane bioreactors
MCF-7	Human breast adenocarcinoma cell line
MDL	Method detection limit
MLSS/MLVSS	Mixed liquor (volatile) suspended solids
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MeOH	Methanol
ND	Not detected
NA	Not analyzed
NAS	Nitrifying activated sludge

NASP	No ammonia spiking period
NDP	National Demonstration Programme
NI	Negative ionisation
NP	4-nonylphenol
NPEO	Nonylphenol polyethoxylates
NP_1EO	Nonylphenol monoethoxylate
NP_2EO	Nonylphenol diethoxylate
NP_3EO	Nonylphenol triethoxylate
NP_4EO	Nonylphenol tetraethoxylate
NP_1EC	Nonylphenoxy acetic acid
NP_2EC	Nonylphenoxy monoethoxy acetic acid
NPx	Nonylphenolics
OP	4-tert-octylphenol
OPEO	Octylphenol polyethoxylates
OP_1EO	Octylphenol monoethoxylate
OP_2EO	Octylphenol diethoxylate
OP_3EO	Octylphenol triethoxylate
OP_4EO	Octylphenol tetraethoxylate
OP_1EC	Octylphenoxy acetic acid
OP_2EC	Octylphenoxy monoethoxy acetic acid
OPx	Octylphenolics
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls

PCDDs	Polychlorinated dibenzo-dioxins
PCDFs	Polychlorinated dibenzo-furans
PE	Population equivalent
PNECs	Predicted no effect concentrations
RAS	Return activated sludge
RSD	Relative standard deviation
SP	Spiking period
SPE	Solid phase extraction
SRT	Sludge retention time
STWs	Sewage treatment works
TF	Trickling filter
UK	United Kingdom
USA	United States of America
USEPA	United States environment protection agency
VFA	Volatile fatty acid
WAS	Waste activated sludge

LIST OF UNITS AND SYMBOLS

A_c	Peak area of the quantitation ion for the selected determinant identified
A_i	Peak area of the quantitation ion for the internal standard
dwt	Dry weight (g)
HRT	Hydraulic retention time (h)
K_p	Partitioning cooefficient (L kg^{-1})
K_{oc}	Octanol-carbon partition coefficient
K_{ow}	Octanol-water partition coefficient
L sec^{-1}	Litre per second
Log K_p	Log of portioning coefficient (L kg^{-1})
Log K_{ow}	Log of octanol-water partition coefficient
M_{diff}	Mass difference (g)
M_{in}	Mass influx (g)
M_{out}	Mass outflux (g)
m/z	Mass to charge ratio
NH_3	Ammonia
NH_4	Ammonium
NH_4OH	Ammonium hydroxide
N_2	Nitrogen
O_2	Oxygen
pK_a	Negative log to base 10 of the acid dissociating/acidity constant K_a

ppm	Parts per million
ppb	Parts per billion
ppt	Parts per trillion
Q	Volumetric flow rate ($m^3\ d^{-1}$)
rpm	Revolutions per minute
S/N	Signal to noise ratio
SRT	Sludge retention time (d)
V	Volt
Vol	Volume of tank (m^3)

LIST OF PUBLICATIONS

Koh, Y. K. K., Chiu, T. Y., Boobis, A. R., Scrimshaw, M. D., Bagnall, J. P., Soares, A., Pollard, S., Cartmell, E. and Lester, J. N. (2009) Influence of operating parameters on the biodegradation of steroid estrogens and nonylphenolic compounds during biological wastewater treatment processes. Environmental Science and Technology, 43,6646-6654.

McAdam, E. J., Bagnall, J.P., Koh, Y. K. K., Chiu, T. Y., Pollard S., Lester, J. N., and Cartmell, E. (2010) Removal of steroid estrogens in carbonaceous and nitrification activated sludge processes. Chemosphere, 81, 1-6

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Chapter 1

Introduction

1 Introduction

1.1 Endocrine Disrupting Chemicals in the Aquatic Environment

This research focuses upon the biodegradation of endocrine disrupting chemicals (EDCs) during activated sludge treatment. EDCs are potent aquatic micropollutants present at trace concentrations that continue to receive considerable public and scientific attention (Renner 1997, Gross *et al* 2004, Glassmeyer *et al* 2005 Richardson *et al* 2005, Sumpter and Johnson 2005). For the purposes of this research micropollutants are defined as a wide spectrum of substances with diverse physicochemical characteristics detected a trace concentrations and include steroid estrogens, nonylphenolic compounds and pharmaceuticals. Some of these micropollutants are endocrine disrupting chemicals (EDCs), including the steroid estrogens, nonylphenolic compounds and the synthetic estrogen pharmaceutical ethinylestradiol, which have been demonstrated to cause severe feminising effects in fish. Of emerging concern are other pharmaceuticals, which are designed to resist breakdown and are physicochemically active at low concentrations. Human and wildlife exposure to such micropollution (especially EDCs) during critical periods of development can result in permanent alteration of the adult organisms from a normal state (Colborn *et al* 1993). There is also evidence that these effects can occurs at trace concentrations.

Previous research over the past three decades has focused, although not entirely, upon environmental pollutants such as pesticides (DDT, DDE, lindane, atrazine), polychlorinated compounds (polychlorinated biphenols (PCBs), polycyclic aromatic hydrocarbons (PAHs), dioxins (PCDDs) and furans PCDFs)) (Langford and Lester 2003). In recent years research has refocused on EDCs and more recently pharmaceuticals (Ternes 1998, Hirsh *et al* 1999, Calamari *et al* 2003, Glassmeyer *et al* 2005a, Jones *et al* 2007b). This is due to the environmental risks these hazardous compounds pose and the realisation that emission is predominantly sewage mediated (Figure 1-1) and wastewater treatment only partially removes them (Hirsch *et al* 1998,

Hirsch *et al* 1999, Rodgers-Gray *et al* 2000; Kirk *et al* 2002, Calamari *et al* 2003 and Glassmeyer *et al* 2005, Langford *et al* 2005; Gibson *et al* 2005 Jones *et al* 2005b). Currently, further research into such priority substances as these has been generated by collaboration between the Environment Agency (EA) and UK Water Industry Research (UKWIR) (Comber *et al* 2009). Thus resulting in a substantial programme of investigations (The Chemical Investigation Programme or CIP), which aims to identify best strategies for improving water quality of aquatic environments.

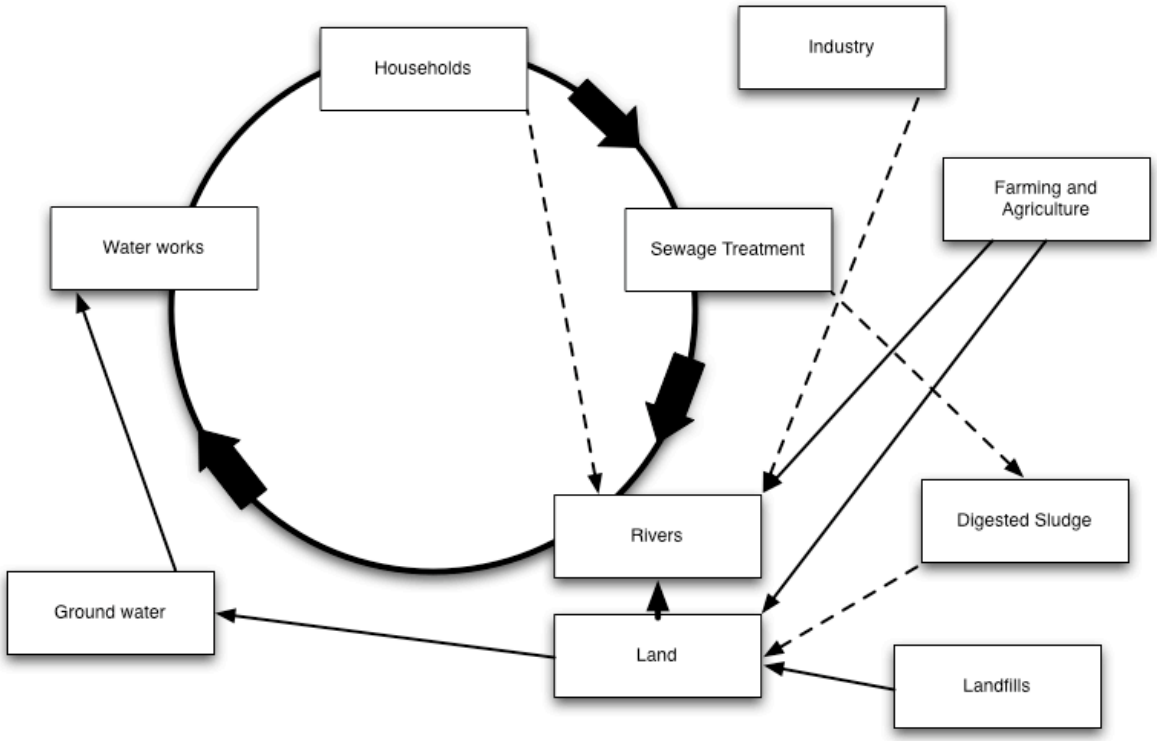


Figure 1-1 Components of a partially closed water cycle with indirect potable reuse indicated the possible routes micropollutants could take to end up within the aquatic system. Adapted from Petrovic et al (2003).

1.2 The Implications of Endocrine Disruption

The specific effects EDCs have upon fish has been well documented. Feminisation of fish caused by EDCs (Sumpter and Jobling 1995, Jobling *et al*., 1998) has been documented in several European countries (Jobling *et al* 1998, Larsson *et al* 1999, Flammarion *et al* 2000, Hecker *et al* 2002, Versonnen *et al* 2004), the USA (Folmar *et*

al 1996, Harshbarger *et al* 2000, Folmar *et al* 2001 and Sole *et al* 2002) and in the Asia Pacific region (Batty and Lim 1999, Hashimoto *et al* 2000, Chapman 2003 and Gong *et al* 2003). In the last decade, sewage treatment works have been implicated as sources of EDCs in the aquatic environment (Rodgers-Gray *et al* 2000 and Kirk *et al* 2002). In fact, the majority of EDCs in the aquatic environment are believed to have been released via sewage effluent. This has been observed in the UK (Jobling *et al* 1998), as well as other countries, including France (Cargouet *et al* 2004), Holland (Belfroid *et al* 1999), Italy (Baronti *et al* 2000), Germany (Spengler *et al* 2001), Sweden (Svenson *et al* 2003), Spain (Sole *et al* 2000), Norway (Knudsen *et al* 1997), Portugal (Diniz *et al* 2005) and Switzerland (Espejo *et al* 2002). In a comprehensive study of endocrine disruption on British Fish, steroid estrogens and alkylphenolics were observed to have a clear impact (Vos *et al* 2000). Furthermore, it has clearly been established that sewage effluent (Purdom *et al* 1994, Sumpter and Jobling 1995, Sheahan *et al* 2002) and many surface waters in the UK (Harries *et al* 1996) are estrogenic to fish. In these surveys, male roach (*Rutilus rutilus*) in rivers near sewage effluent outfalls had elevated vitellogenin (egg yolk protein precusor usually produced by females) in blood plasma. Up to 100% of these fish had ovotestis, a severe feminisation (Jobling *et al* 1998).

Evidence of endocrine disruption in wildlife has existed since the first half of the twentieth century (Dodds *et al* 1953). Despite this the environmental implications of endocrine disruption were not fully realized until relatively recently. Declining sperm quality and quantity has been observed since the 1990s (Carlsen *et al* 1992, Swan *et al* 2000, Handelsman 2001), as well as incidences of testicular cancer, hypospadias and cryptorchidism have also been on the rise (Sharpe and Skakkebaek 2003, Toppari *et al* 1996). Currently, there is no direct evidence that EDCs within the aquatic system are affecting human populations (Damstra 2002). Yet links exists between the frequency of male reproductive tract abnormalities such as hypospadias and crytorchidism and foetal exposure to estrogen (Martin *et al*., 2008). Therefore the implications of the environmental risk to wildlife, livestock and human populations cannot be underestimated.

The presence of EDCs in the aquatic environment is predominantly due to their incomplete removal during sewage treatment (Purdom *et al* 1994, Gomes *et al* 2003) and the generation of estrogenic products by deconjugation of estrogens or the degradation products of long chain alklphenolics (Ahel *et al* 1994, Langford *et al* 2005). It is therefore necessary to identify how optimisation of treatment parameters for the removal of EDCs can be done. Clearly working within the current infrastructure, without needing tertiary treatments, would be more sustainable. However, in order to develop optimal conditions for EDC removal during biological wastewater treatment, greater understanding of which process parameters are involved with their removal is necessary.

1.3 Rationale for Research

This research follows directly on from findings of Koh (2008), conducted as part of a £40 million National Demonstration Program (NDP), as undertaken by the water industry of the UK as part of the asset management plan (AMP) 4 settlement. This work was initiated by the Environment Agency (EA) to investigate the potential for removing EDCs from sewage effluent (Burke 2004). The main aim of Koh (2008) was to evaluate the factors contributing to the removal of estrogens and alkylphenolics during activated sludge treatment. Koh (2008) identified in line with other researchers that activated sludge with longer sludge ages had effective removal of EDCs. Koh (2008) also identified a disparity between the biomass activities (removal per tonne of biomass) between activated sludge plants and identified the potential for improving removal within the current treatment infrastructure. However, it could not be determined which specific process parameters were responsible for promoting effective removal.

This PhD was funded as part of the AMP 5 settlement by Anglian Water, Severn Trent Water, Thames Water, United Utilities and Yorkshire Water to examine which process parameters are principally responsible for the removal of steroid estrogens and nonylphenolics during activated sludge treatment; thereby determining whether

the aquatic environment can be protected from such micropollutants more sustainably by augmenting current infrastructure rather than using tertiary treatments. The need for this research has arisen from requirements of the Water Framework Directive, which requires by 2015, all water bodies achieve "good chemical water status" (European Commission 2000). In supplement to this legislation, the Priority Substances Daughter Directive establishes environmental quality standards that define the concentrations of chemical contaminants that are consistent with "good chemical water status" (European Commission 2008). Therefore, many operators will have to make informed decisions on the effectiveness, cost and benefit and sustainability of existing and enhanced wastewater treatment processes in dealing with the removal of EDCs from sewage effluent.

Chapter 2

Literature Review

2 Literature Review

2.1 Micropollutants of Interest

Steroid estrogens and non-ionic surfactants are two important groups of EDCs that demonstrate estrogenic effects. Their order of potency is as follows: ethinyl estradiol > estradiol > estrone > nonyl and octylphenol > estriol > polyethoxylated alkylphenols and their metabolites. Based on available data, the EA has derived the following predicted no effect concentrations (PNECs) for steroid estrogens: 0.1 ng L^{-1} for EE2, 1 ng L^{-1} for E2, and 3 ng *L-1* for estrone (Environment Agency, 2000). A combined PNEC value for total steroid estrogens of 1 ng L-1 EEq (E2 equivalence) was derived which takes into account the relative potency of each steroid and their additive effects since estrogens work synergistically (Environment Agency, 2002). The EA national survey of STW effluents showed that steroid estrogens are present in some effluents at concentrations at or higher than the PNEC derived for estrogens (Environment Agency, 2002). Furthermore, modelling studies undertaken as part of the EA risk assessment of effluents indicated predicted concentrations higher than these PNECs in the downstream receiving environment, thus leading to unacceptable exposure of aquatic life that inhabit certain river reaches below the STW.

The calculated PNEC values for NP_nEO, NP_2EC, NP_1EC, NP_2EO, NP_1EO, NP and OP are 0.9 µg L^{-1}, 0.99 µg L^{-1}, 2 µg L^{-1}, 0.11 µg L^{-1}, 0.11 µg L^{-1}, 0.021 µg L^{-1} (Fenner *et al* 2002) and 0.12 µg L^{-1} (Brooke *et al* 2003) respectively. The PNEC of NP for aquatic organisms of 0.33 µg L^{-1} was derived for three species representing three trophic levels (European Commission, 2002). Although the estrogenic potency of NP is less than the estrogens, the quantities of which this compound is released via effluent makes it a cause for concern, as estrogenic responses are dose-dependent. NP, which can cause intersexuality at "high" concentrations (Gray and Metcalfe, 1997), but not at lower concentrations (Nimrod and Benson, 1998). Nonetheless, the

longterm effects of low concentrations of NP on vitellogenin induction and gonadal disruption in fish are still uncertain (Gross-Sorokin *et al* 2006).

Pharmaceuticals pose a particular difficulty in that >3000 pharmaceuticals of various therapeutic classes are in use today (Ayscough *et al.* 2000). Therefore identifying which pharmaceuticals pose the greatest risk presents a challenge. Until recently, drug manufacturers have been able to develop and release products to the market without ecotoxicological consideration. Only recently have regulatory bodies produced details of how pharmaceuticals should be assessed for environmental effects (Fent *et al.* 2006). In the EU all new pharmaceuticals that do not occur naturally within the environment such as vitamins and electrolytes are required to undergo risk assessment according to European Union (EU) Directive 92/18 EEC (European Medicine Agency 2005). The initial assessment estimates predicted environmental concentration in surface waters PEC_w. If PECw is < 0.01µg L^{-1} no further assessment is required as it is unlikely that ecotoxicogical effects would occur below this level. Environmental fate and effects analysis is performed for compound with PECw >0.01µg L^{-1} by calculating the ratio of PEC with predicted no effects concentration (PNEC). If PEC/PNEC is <1, no risk is anticipated and no further tests are conducted. PEC/PNEC >1 then further risk assessment is required by testing the pharmaceutcial on algae, *Daphnia* and fish to specific OECD guidelines (Ayscough *et al.* 2000).

Removal of micropollution during wastewater is predominantly a result of by either adsorption to solids or by biological mechanisms (Gomes *et al* 2004 Langford *et al* 2005a). Estrogens are typically found in effluent at low nanograms per litre range: UK estrone concentrations of 4.3 to 5.5 ng L^{-1} have been reported by Koh *et al* (2009). Whereas is other country these estrone figures have been reported: Italy 9.3 ng L^{-1} (Baronti *et al* 2000), The Netherlands 4.5 ng L^{-1} (Belfroid *et al* 1999) and Canada 3 ng L^{-1} (Janex-Habibi *et al* 2009). All these concentrations exceed the estrone predicated no-effects concentration (PNEC) of 3 ng L^{-1} (EU 2002). It would therefore appear that as currently configured, sewage treatment as currently operated offers inadequate protection to the environment from EDCs. Once in the aquatic environment these compounds are amenable to biotransformation and

bioconcentration (Lai *et al* 2002) and may potentially bioaccumulate (Gomes *et al* 2004); as a consequence of this behaviour, complex issues for environmental health arise (Lai *et al* 2002).

To remove sufficient EDCs either the use of "bolt on" technologies such as advanced oxidation processes (AOP) or granular activated carbon (GAC) or the optimisation of current sewage treatment infrastructure are necessary. Advance treatments could completely remove these compounds from sewage effluent. However, using these technologies would adversely affect the capital operational and environmental costs of sewage treatment (Jones *et al* 2007a). Therefore, optimisation of treatment parameters for the removal of EDCs within the current infrastructure would clearly be more sustainable. However, in order to develop optimal conditions for pharmaceuticals and EDC removal during biological wastewater treatment, greater understanding of the mechanisms involved with their removal is necessary. This review aims to explore the current understanding of pharmaceutical and EDC removal during sewage treatment. This will be done, by identifying which process parameters are key for the removal of these compounds during conventional sewage treatment. By understanding how these process paraments influence the removal of these compounds, it may be possible yo manipulate seage treatment in order to achieve enhanced treatment of micropollution.

Estrogens

As established above, estrogens pose a significant threat to the aquatic environment. These compounds have been included in the Chemical Investigations Programme (CIP), the overall aim of which is to develop "cost effective and fairly apportioned measures to meet the requirements of the Water Framework Directive" (Gardener and Comber 2010). The estrogens included in the CIP are the natural and synthetic compounds: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are the most estrogenic. A large extent of the estrogenicity in the aquatic environment was a result of their presence in sewage effluent (Belfroid *et al* 1999, Jobling *et al* 1998, Larsson *et al* 1999, Rodgers-Gray *et al* 2000, Ternes *et al* 1999b). In addtion to these,

Estriol (E3) is a natural steroid albeit the least estrogenic, however, it is still worthy of consideration as it posses greater estrogenicity than many nonylphenolic compounds. Estrogens are predominantly excreted as conjugates, either glucuronides or sulfates in urine, with the sulfate conjugate of estrone (E1-3S) being the main urinary excretion product (Tang and Crone, 1989, Ternes *et al* 1999a). Due to the activity of β-glucuronidase, gluconuride conjugates are broken down before reaching the STW, however, concentrations of the conjugated steroid, E1-3S, may be important when considering total load reaching STW (Gomes *et al* 2005, Koh *et al* 2008a), since arylsulfatase enzyme is less common in the STW (Baronti *et al* 2000).

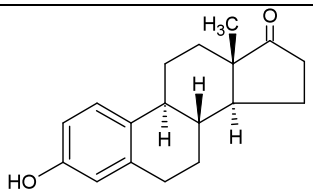
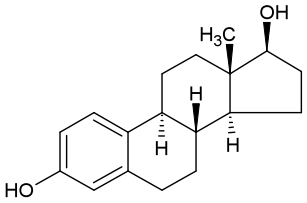
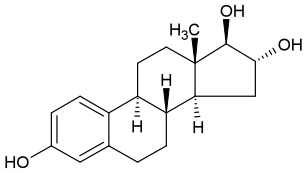
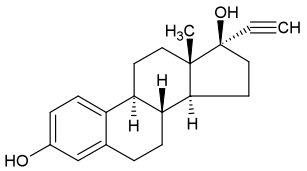
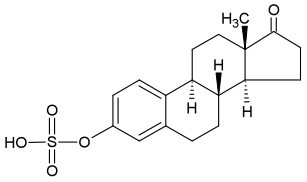
The chemical structure of the estrogens estrone (E1), estradiol (E2), estriol (E3), ethinylestradiol (EE2) and the sulphated conjugate of estrone (E1-3S) are detailed in Table 2-1. Information about their physicochemical properties is further elaborated in Table 2-2. Free unconjugated steroid estrogens have low water solubility ranging from 4.8-19 mg L^{-1}, in comparison to conjugated forms, which are more soluble. The synthetic estrogen EE2 is the least soluble estrogen and has the highest octanol-water partitioning coefficient (Log K_{ow}) 4.15, whereas the natural steroids range from 2.81-3.43. The sulphated conjugate of estrone (E1-3S) has the lowest Log K_{ow} of 0.95. Estrogens have low vapour pressures and pKa values above 10, making them weekly acidic. Therefore, unconjugated steroid estrogens are non–volatile, lipophillic and are likely to adsorb to environmental matrices (Lai *et al* 2000). Conjugated estrogens however, are 10 to 50 times more water-soluble than their parent compounds are resultantly less sorbative. Thus estrogenicity can increased during sewage transit.

Table 2-1 The physicochemical properties of selected estrogens

Estrogen	Vapour pressure (Pa)	Aqueous Solubility (mg l^{-1})	Henry's constant (atm m^3 mol^{-1})	Log K_{ow}
E1	3×10^{-8}	13	6.2×10^{-7}	3.43
E2	3×10^{-8}	13	6.3×10^{-7}	3.94
E3	9×10^{-13}	13	2×10^{-11}	2.81
EE2	6×10^{-9}	4.8	3.8×10^{-7}	4.15
E1-3S	1.08×10^{-14}[a]	9.6×10^{2}[b]	6.9×10^{-16}[c]	0.95[f]

Data taken from (Lai *et al* 2000). [a] Modified grain method (MPBPWIN v1.42). [b]Water solubility estimate from Log K_{ow} (WSKOW v1.41). [c] Henry's law constant (25°C) (HENRYWIN v3.10). [d] Estimated Log K_{ow} (KOWWIN v1.67 estimate). [g] (Huber *et al* 2003).

Table 2-2 Chemical structures, molecular weights and formulas of the steroid estrogens

Steroid estrogen	Type of steroid	Molecular formula	Molecular weight (g^{-1} mol)	CAS no.	Structure
E1	Natural	$C_{18}H_{22}O_2$	270.37	53-16-7	
E2	Natural	$C_{18}H_{24}O_2$	272.38	50-28-2	
E3	Natural	$C_{18}H_{24}O_3$	288.39	50-27-1	
EE2	Synthetic	$C_{20}H_{24}O_2$	296.40	57-63-6	
E1-3S	Natural	$C_{18}H_{22}O_5S$	350.50	481-97-0	

Alkylphenolics

The alkylphenolic (APx) base units nonylphenol (NP) and octylphenol (OP) are both priority substances listed in Annex I and II of the Priority Substances Daughter Directive (European Commission 2008). Furthermore these compounds have been included in the CIP. These compounds are the base units of alkylphenol polyethoxylates (APEOs) and are commercially important non-ionic surfactants used in both industrial and domestic detergent and emulsifier formulations (Ferguson *et al* 2001; Chiu *et al* 2010). These commercial APEOs are a mixture of nonylphenol ethoxylates (NPEOs) and octylphenol ethoxylates (OPEOs) with ethoxylate chain lengths ranging from 6 to 20 (Shao *et al* 2003). APEOs are ubiquitous in wastewater discharges and in the aquatic environment (Langford *et al* 2005; Gibson *et al* 2005). It has been established that APEOs degrade during wastewater treatment to generate the parent alkylphenols (AP), octylphenol (OP) and nonylphenol (NP); the shorter chain mono to triethoxylates (NP_1EO, NP_2EO and NP_3EO) and a range of carboxylated intermediate by-products, and carboxyalkylphenol polyethoxycarboxylates (CAPEC) can also occur (Giger *et al* 1984; Soares *et al* 2008). Numerous studies have shown that these APEO metabolites are more estrogenic than their parent substances and possess the ability to mimic natural hormones by interacting with the estrogen receptors (ER) (Jobling and Sumpter, 1993). Scientific and regulatory concerns have been raised over these APEO metabolites (NP, OP, $NP_{1-3}EO$) because of their ability to disrupt endocrine function in wildlife and humans (Johnson and Sumpter, 2001; European Commision 2005). It is therefore necessary to determine all possible alkylphenolics (APx) (parent and metabolites) in the sewage matrices and in particular final effluents in order to assess their environmental risk.

The physiochemical properties representing the spectrum of alkylphenolics (APx) are detailed in Table 2-3. Further details of their chemical structure and molecular weights are detailed in Table 2-4. Shorter chain APEO oligomers (EO<5) usually display lipophilic properties in contrast to the higher oligomers, which are hydrophilic (Ahel and Giger, 1993a). The solubility of OPEOs in water increased from 8 to 24.5

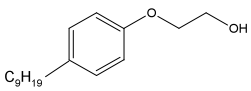
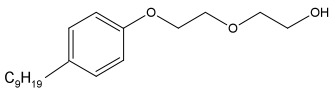
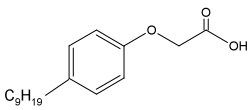
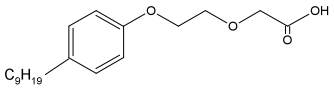
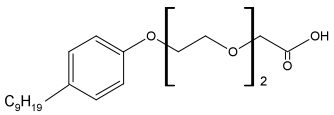
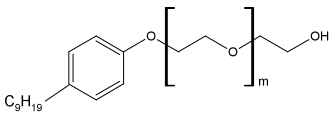
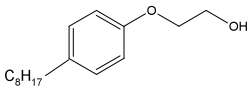
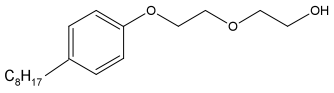
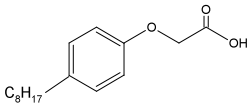
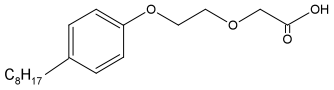
mg L^{-1} within increasing ethoxylation (OP_1EO – OP_4EO). The solubility of OP was 12.6 mg L^{-1}, which is higher than the solubility of OP_1EO but less than $OP_{2\text{-}4}EO$. NP had a water solubility of 5.43 mg L^{-1}, while the solubility of $NP_{1\text{-}4}EO$ increased with each additional ethoxylate from 3.02 and 9.48 mg L^{-1} at 20.5 °C (Ahel and Giger, 1993a). The solubility of $OP_{1\text{-}4}EO$ and OP were significantly greater than those of $NP_{1\text{-}4}EO$ and NP, thus indicating the influence of the hydrophobic chain length. Ahel and Giger (1993b) determined the partition coefficients of OP, NP and $NP_{1\text{-}3}EO$ in the octanol/water system and also calculated other log K_{ow} values from the solubility values (Ahel and Giger, 1993b). The logarithmic values (log K_{ow}) were between 3.90 and 5.53 for the APEO metabolites, suggesting that these substances may sorb to organic matter or sediments. The physicochemical data could help us predict the partitioning behaviour of these substances among different phases (air, water and sediment/soil) in the environment. Even so, from solubility and log K_{ow} data, it can seen that OP, NP and $O/NP_{1\text{-}4}EO$ could easily adsorb onto the sediment in an aquatic environment.

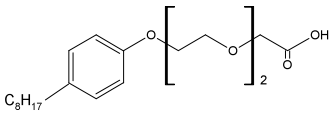
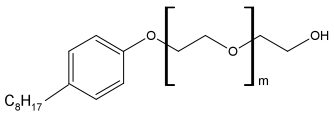
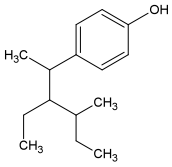
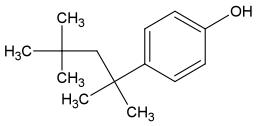
Table 2-3 Physicochemical properties of selected APx

Alkylphenolic compound	Vapour pressure (Pa)	Aqueous solubility (mg l^{-1} at 20°C)	Henry's constant (atm m^3 mol^{-1})	Log K_{ow}
NP	1.73 x 10^{-5}[i]	5.43[b]	1.23 x 10^{-5}[h]	4.48[c]
NP_1EO	1.34 x 10^{-9}[a]	3.02[b]	1.25 x 10^{-6}[h]	4.17[c]
NP_2EO	6.86 x 10^{-11}[a]	3.38[b]	2.68 x 10^{-9}[h]	4.21[c]
NP_3EO		5.88[b]		4.20[c]
NP_4EO		7.65[b]		4.30[d]
NP_1EC	7.37 x 10^{-8}[a]	0.45[g]	1.41 x 10^{-6}[h]	5.80[f]
NP_2EC	3.57 x 10^{-10}[a]	0.43[g]	3.01 x 10^{-9}[h]	5.53[f]
OP	3.89 x 10^{-6}[a]	12.6[b]	6.89 x 10^{-6}[h]	4.12[c]
OP_1EO	8.18 x 10^{-9}[a]	8.0[b]	5.87 x 10^{-7}[h]	4.10[e]
OP_2EO		13.2[b]		4.00[e]
OP_3EO		18.4[b]		3.90[e]
OP_4EO		24.5[b]		3.90[e]

[a] Modified grain method (MPBPWIN v1.42). [b] (Ahel and Giger, 1993a). c (Ahel and Giger, 1993b). [d] Half-life (days) in river water. [e] Calculated values using the solubility (Ying *et al* 2002b). [f] Estimated Log K_{ow} (KOWWIN v1.67). [g] Water solubiilty estimate from Log K_{ow} (WSKOW v1.41). [h] Henry's law constant (25°C) (HENRYWIN v3.10). [i] (Nielsen *et al* 2000).

Table 2-4 Chemical structures, molecular weights and formulas of selected APx

Alkylphenolic compound	Type of non-ionic surfactant	Molecular formula	Molecular weight (g/mol)	CAS no.	Structure
4-Nonylphenol monoethoxylate (NP_1EO)	Short-chain APEO	$C_{17}H_{28}O_2$	264.41	104-35-8	
4-Nonylphenol diethoxylate (NP_2EO)	Short-chain APEO	$C_{19}H_{32}O_3$	308.46	20427-84-3	
4-Nonylphenoxy acetic acid (NP_1EC)	Short-chain carboxylic acid	$C_{17}H_{26}O_3$	278.39	3115-49-9	
Nonylphenoxy monoethoxy acetic acid (NP_2EC)	Short-chain carboxylic acid	$C_{19}H_{30}O_4$	322.44	106807-78-7	
Nonylphenol diethoxy acetic acid (NP_3EC)	Short-chain carboxylic acid	$C_{21}H_{34}O_5$	366.50	NA	
Nonylphenol polyethoxylates (3–12)($NP_{3-12}EO$)	Long chain APEO	$(C_2H_4O)_n$ $C_{15}H_{24}O$ n=~1.5 – 12	-	68412-54-4	 NP_nEO, n = m+1
4-Octylphenol monoethoxylate (OP_1EO)	Short-chain APEO	$C_{16}H_{26}O_2$	250.38	2315-67-5	
4-Octylphenol diethoxylate (OP_2EO)	Short-chain APEO	$C_{18}H_{30}O_3$	294.44	2315-61-9	
4-Octylphenoxy acetic acid (OP_1EC)	Short-chain carboxylic acid	$C_{16}H_{24}O_3$	264.37	NA	
Octylphenol monoethoxy acetic acid (OP_2EC)	Short-chain carboxylic acid	$C_{18}H_{28}O_4$	308.42	NA	

Octylphenol diethoxy acetic acid (OP_3EC)	Short-chain carboxylic acid	$C_{20}H_{32}O_5$	352.47	NA	
Octylphenol polyethoxylates (3–12)($OP_{3–12}EO$)	Long chain APEO	$(C_2H_4O)_n$ $C_{14}H_{22}O$ n=~1.5 – 12.5	-	68987-90-6	 OP_nEO, n = m+1
4-nonylphenol (NP)	Alkylphenol	$C_{15}H_{24}O$	220.35	104-40-5	
4-tert-octylphenol (OP)	Alkyphenol	$C_{14}H_{22}O$	206.32	140-66-9	

2.1.1 Pharmaceuticals

Pharmaceutical is derived from the Greek *pharmakeutikos* and the Latin *pharmaceuticus* and can be defined as a chemical used for diagnosis, treatment or prevention of disease/health condition or structure/function of the body (Buser *et al* 1999). The term "medicine" can be used to distinguish therapeutic drugs from elicit substances. Therefore, 'pharmaceutical', 'pharmaceutically active compounds' (PhACs), 'pharmaceuticals and personal care products' (PPCPs) are general terms that encompass a board range of compounds with different physiochemical properties. They are a sophisticated group of compounds with specific targeted affects designed to work at low doses. In Europe, approximately 3000 pharmaceuticals of various therapeutic classes are in use today (Ayscough *et al* 2000). These compounds are excreted as metabolites or the unchanged parent compound in faeces and urine (Sanderson *et al* 2004). The propensity for pharmaceuticals to be removed during sewage treatment depends greatly upon their potential to biodegrade or sorb. Of the pharmaceuticals EE2 has been given the greatest focus, as this compound, as stated earlier, is the most estrogenic of all the estrogens. Selection of other pharmaceuticals for inclusion in this research needed to be based on a prioritisation procedure that takes into account toxicity and typical amount in the aquatic environment.

2.2 The typical concentrations of micropollutants entering sewage treatment works

Estrogens

Concentrations of the individual estrogens vary for different STWs. However on average, E3 and E1 typically have the highest concentrations in the settled sewage. In a UK study concentrations for E3 and E1 were detected at circa 70 ng L^{-1} and 50 ng L^{-1} respectively (Koh *et al* 2009). In a study of twenty Japanese STWs the influent concentrations of E1 (median 24 ng L^{-1}) were lower compared to E3 (median 110 ng L^{-1}) (Komori *et al* 2004). The concentration of E3 in European STWs also varied in the influent as E3 ranged between 2 to 120 ng L^{-1} compared to 3 – 100 ng L^{-1} for E1 (Johnson *et al* 2000) and an average 80 ng L^{-1} for E3 in 6 Italian STW (Baronti *et al* 2000). However further explanation as to the reason behind the higher concentration of E3 (>E1) in the influent is not given in these publications. However, deconjugation of E3-sulphate and/or glucuronide and excretion of E3 in pregnant women may offer an explanation (Xiao and McCalley, 2000). Other possible explanations for higher E3 influent concentrations compared to other estrogens could be the presence of enzymes in the microflora of the fecal material and biofilm in the sewerage system which allowed the biotransformation of E2 and E1 to E3 (D'Ascenzo *et al* 2003). Koh *et al* (2009) noticed higher a proportion of E3 in the influent at a site with longer transit time in the sewerage system.

The estrogen E2 has the greatest potency of the natural estogens and is 2.3-3.2 times more potent than E1 *in vivo* test in fish (Thorpe *et al* 2003). Many studies reported concentrations of E2 in the influent between <0.3 and 224 ng L^{-1} (Fawell *et al* 2001, Jiang *et al* 2005). Koh *et al* (2009) observed influents concentrations of circa 20 ng L^{-1} for E2 on average in settled sewage. This concentration is within the range of the predicted E2 influent concentration between 5 – 44 ng L^{-1} reported elsewhere (Johnson *et al* 2000). This is within the range of 5-10 times of the level of EE2 in other STWs (Johnson *et al* 2000, Ternes *et al* 1999b).

Synthestic estrogen EE2 is difficult to analyze as concentrations are close to the limit of detection. In a study by Koh *et al* (2008b) concentrations of EE2 were less than 3 ng L^{-1} in the settled sewage of three STWs. Elsewhere, concentrations of <0.3 to 5.9 ng L^{-1} were predicted for EE2 for influent of municipal STWs (De Mes *et al* 2005), however, predictions for this estrogen are particularly sensitive to assumptions on the number of people taking the oral contraceptive (Johnson *et al* 2000). These findings fall midway between STW influents of German (1.4 ng L^{-1}) and Brazilian (6 ng L^{-1}) (Ternes *et al* 1999b). These typically low EE2 concentrations where within the range found in other studies which showed a large variation in the actual sewage influent between the detection limit of <0.5 ng L^{-1} to 10 ng L^{-1} (average 5 ng L^{-1}) (Johnson *et al* 2000).

Estrone is excreted as sulphate rather than glucuronide conjugate in urine from pregnant women (Andreolini *et al* 1987). The concentration of steroid conjugate E1-3S has been found at concentrations circa 10 ng L^{-1} on average in the settled sewage (Koh *et al* 2008a). The conjugate was also detected at such level in the influent in other studies (D'Ascenzo *et al* 2003, Hu *et al* 2007). Although it is not estrogenic, this conjugated estrogen can be deconjugate during the STW treatment process and subsequently contribute to the overall estrogenicity of the effluent to the aquatic environment as estrone.

Alkylphenolics

Concentrations of the individual alkylphenolic compounds in the settled sewage vary widely between different STWs from <LOD to 3220 μg L^{-1} (Sole *et al* 2000, Ahel *et al* 1999a). APx influent concentrations have been reported to be declining in recent years as several countries have reduced the use of APx either through voluntary replacement or by legal regulations (Voutsa *et al* 2006). Supporting this, in a study where the same STW was sampled in 2004 and 2006, APx concentrations were one order of magnitude higher at the earlier occasion (Koh *et al* 2009).

In influent and settled sewage, long chain polyethoxylated APx ($AP_{3-12}EO$) tend to predominate. In a UK study, on average, $AP_{3-12}EO$ were higher in concentration ($NP_{3-12}EO$ ~25 μg L^{-1} average, $OP_{3-12}EO$ ~5 μg L^{-1}) compared to the short chain ethoxylates ($AP_{1-2}EO$ ~1 μg L^{-1} excluding STW2 2004) (Koh *et al* 2009). These observations indicate that $AP_{3-12}EO$ were readily degradable and were biotransformed to short chain $AP_{1-2}EO$, $AP_{1-3}EC$ or AP in the sewer prior to entry in to the STWs in agreement with other studies (Gonzalez *et al* 2007, Isobe and Takada, 2004, Melcer *et al* 2006). Long chain $NP_{3-12}EO$ are typically detected at higher concentrations to their octyl analogues (Petrovic *et al* 2002, Clara *et al* 2005). Koh *et al* (2009) found approximately 160 μg L^{-1} compared to the octyl- analogues at 25 μg L^{-1} in the settled sewage during the Summer 2004 compared to Winter 2006 ($NP_{3-12}EO$=18 μg L^{-1}, $OP_{3-12}EO$=2 μg L^{-1}). The concentrations of the nonylphenol derivatives ($NP_{3-12}EO$) are an order of a magnitude greater than those of the octylphenol analogues as a consequence because 80 – 90% of the APEO produced worldwide being NPEO whilst the remaining part consists of OPEO (Gunther *et al* 2001, Renner, 1997).

Low concentrations of $AP_{1-3}EC$ and AP (<3 μg L^{-1} for both) have been found in the settled sewage (Koh *et al* 2008a). Most of the carboxylated metabolites which were observed at low concentration in the settled sewage ($NP_{1-3}EC$<1 μg L^{-1}, OP1-3EC<0.2 μg L^{-1}) increased in concentration in the final effluent due to their formation in the secondary treatment, which corroborated with the findings in another study (Isobe and Takada, 2004). The percentage difference between NPEC and OPEC found in this study is in agreement with previous studies which have reported that NP_nEC (~60%) formed a bigger percentage than OP_nEC (~10%) in the STW (Ahel *et al* 1994b). Under anaerobic conditions AP are thought to be the final products (Montgomery-Brown and Reinhard, 2003). Some NP (<0.5 to 3.8 μg L^{-1}) and OP (<0.1 to 0.2 μg L^{-1}) were found in the settled sewage, which indicated the septicity of the sewerage system.

Pharmaceuticals

The concentration of a pharmaceutical entering a STW is dependant upon its usage. There are thousands of pharmaceutical compounds currently under license designed for chemical stability. Currently, only a relatively small fraction of the thousands of pharmaceuticals in use have been analysed in wastewater l*et al*one detected. Some commonly analysed and detected pharmaceuticals include paracetamol, salbutamol, mefanamic acid, ibuprofen, diclofenac and clofibric acid (Ternes 1998c, Stumpf *et al* 1999, Jones *et al* 2007b, Yu *et al* 2009). These pharmaceuticals are detected in microgram per litre ranges. In a study in Brazil, Stumpf (1999) recorded influent ibuprofen concentrations <0.4 μg L^{-1}. The highest concentrations of detected pharmaceutical were bezafibrate and clofibric acid at 1.2 and 1.0 μg L^{-1}. In a British study that mass balanced paracetamol, ibuprofen, salbutamol and mefenamic acid, settled sewage concentrations of these compounds ranged, 2.0-2.5, 3.8-4.8, 3.8-4.4 and 3.6-5.2 μg L^{-1} respectively (Jones *et al* 2002). In a recent study in Korea, influent ibuprofen concentration was on average 17.9 μg L^{-1} (Sim *et al* 2010).

To determine which pharmaceuticals need to be researched it is often necessary to estimate environmental concentrations. This must be based upon a quantification of the environmental hazard they pose, which takes into consideration concentration, toxicity and potential for bioaccumulation of the pharmaceutical. Therefore, records of pharmaceutical usage are useful references when estimating quantities released to the environment. In the UK, these records are kept by the department of health and the Propriety Association of Great Britain for prescribed and over the counter medicine respectively (Jones *et al* 2002). Other countries have similar systems for the record of pharmaceutical usage, which can be used to estimate amounts entering STWs.

In a Danish study, a risk assessment of 25 most used pharmaceutical in the primary health sector in 1997 was conducted (Stuer-Lauridsen *et al* 2000). Defined daily does (DDD) was used to calculate the amount of active substance used per year. Of these, ten of the most used pharmaceuticals are displayed in Table 2-5. As DDD is

effectively a quantification of prescription number, the quantity of pharmaceutical in weight is derived through multiplication by a conversion factor that defines the amount of active compound in a daily dose. In the case of estrogen, it was ranked as the fifth most used pharmaceutical (37.1 million DDD). When the quantities of active-compound are taken into account, 1.9 kg/year of estrogen is less significant compared to 33,792 kg/year for ibuprofen.

Further risk assessments in Germany and the UK have been performed using prescription data (Huschek *et al* 2003 and Jones *et al* 2002). These studied used prescription data to determine predicted environmental concentration (PEC) of surface waters. A summary of the data from both these risk assessments is presented in Table 2-6. Ibuprofen, paracetamol, amoxicillin and metformin were in both lists, however pharmaceutical usage is significantly different for Germany and the UK, which demonstrated the need for separate national risk assessments. Each of the top ten compounds for each country as (shown in Table 2-6) that exceeded the predicted environmental concentration (PEC) (equivalent to influent concentration) 0.01μg L^{-1} threshold proposed by European Medicines Agency (2003) and would warrant further risk assessment. In conjugation with ecotoxicological data, such predictions of environmental concentration are invaluable for determining the risk a pharmaceutical may pose. Both risk assessment have identified the risk posed by paracetamol, as its PEC exceeds its predicted no effects concentration PNEC. However, such studies tend to over estimate surface water concentrations (Carballa *et al* 2004) as the calculations are based on an assumption that effluent is diluted in rivers by a factor of ten and that there is no degradation during sewage treatment.

In a study by Ashton *et al* (2004) prioritising pharmaceuticals according the their PEC:PNEC ratio, paracetamol, ibuprofen, tamoxifen, clotrimazole and lofepramine were in the top ten pharmaceuticals with the highest environmental risk. Further assessment by application of the OSPAR dynamic selection prioritisation mechanism (DYNAMEC) (Wiandt and Heinz-Jochen 2002), to pharmaceuticals near the top of the prioritisation list highlighted, fluoxetine, trimethoprim, sulfamethoxazole, fenofibrate, erythromycin, propanolol and diclofenac as risk compounds due to their

toxicity and persistence. The OSPAR commission for the protection of the marine environment and the North-East Atlantic has listed substances of priority action and possible concern, these pharmaceuticals include diosdenin, clotrimazol miconazole, niflumic acid and chloropromazine are of possible concern (UNEP 2008).

For the CIP, the pharmaceuticals under examination are: ibuprofen; propranolol; erythromycin; ofloxacin; oxytetracycline; salicylic acid and fluoxetine (Gardener *et al* 2010). These pharmaceuticals were chosen for investigation as they include substances with a range of chemical structures and physico-chemical properties. This meant that they were likely to exhibit a range of different behaviours in sewage treatment. For example, some substances are susceptible to biodegradation; some might readily associate with suspended matter (and hence be removed from the aquatic phase); whilst others might never be expected to be removed by sorption processes. Understanding the fate and removal of compounds such as these would allow prediction as to whether environmental quality standards need to be applied.

Table 2-5 The ten most used pharmaceuticals in Denmark in 1997

No	Active Compound	Use	Amount used (million DDD)	Conversion factor (mg active compound/DDD)	Amount active compound used (kg-1 yr)
1	Furosemide	Diuretic	93.6	40	3744
2	Paracetamol	Analgesic	82.8	3000	248,250
3	Acetylsalicylic acid	Antithrombotic agent	70.9	3000	212,250
4	Bendroflumethiazide (and possasium)	Diuretic	66.8	2.5	167
5	Gestodene and estrogen combined	Sex hormone	37.1		
	Gestodene		37.1	1	3.7
	Estrogen		37.1	0.05	1.9
6	Acetylsalicylic acid, comb. Excl. phsocleptics	Analgesic	30.8	3000	92,460
7	Ibuprofen	Antirheumatic agent	28.2	1200	33,792
8	Lactic acid producing organisms	Antidiarrheal microoganisms	27.4	No data	No data
9	Possasium chloride	Mineral suppliment	26.7	3000	80,100
10	Amlodipine	Calicium channel blocker	26.4	5	132

Data taken from Stuer-Laurudsen *et al* (2000). DDD is defined daily dose

Table 2-6 A comparison between risk assessment data of the ten most used pharmaceuticals in Germany 2001 and the UK 2000.

	Germany						UK					
Pharmaceutical	**Annual usage (kg)**	**PEC µg L-1**	**Rank**	**PNEC**	**Test organism**	**PEC/ PNEC**	**Annual usage (kg)**	**PEC µg L-1**	**Rank**	**PNEC**	**Test organism**	**PEC/PNEC**
Acetylsalicylic acid	836256.7	13.95	1	167	*Daphnia*	0.08						
Paracetamol	621648.5	10.38	2	9.2	*Daphnia*	1.10	390954.3	11.96	1	1.29	*Daphnia*	1.29
Povidone-iodine	483293.1	8.07	3	4.6	Fish	1.75						
Metformin	516914.2	8.63	4	60	*Daphnia*	0.14	205795.0	6.30	2	511.57	Green alga	0.01
Ibuprofen	344884.6	5.76	5	30	*Daphnia*	0.19	162209.1	4.96	3	9.06	*Daphnia*	0.55
Metamizole sodium	213861.9	3.57	6	-	-	-						
Theophylline	137385.4	2.29	7	155	*Daphnia*	0.02						
Piracetam	121748.2	2.03	8	-	-	-						
Allopurinol	142.329.7	2.38	9	65000	Fish	<0.01						
Amoxicillin	115384.0	1.93	10	-	-	-	71466.8	2.19	4	<0.01	*Microcyctis aeruginosa*	588.02
Sodium valproate							47479.7	1.45	5	763.00	*Daphnia*	<0.01
Sulphasalazine							46430.4	1.42	6	38.99	*Daphnia*	0.04
Mesalazine							40421.7	1.24	7	6078.34	Green alga	<0.01
Carbamazepine							40348.8	1.23	8	6.36	*Daphnia*	0.19
Ferrous sulphate							37538.5	1.15	9	7.10	*Daphnia*	0.16
Ranitidine HCl							36319.2	1.11	10	-	-	-

PEC is predicted environmental concentration, PNEC is the predicted no effects concentration. Germany data is taken from Kuscjek *et al* (2004). UK data is taken from Jones *et al* (2002)

2.3 The Removal of Micropollutants During Sewage Treatment Processes

Conventional sewage treatment was developed to remove carbon, nitrogen to a lesser extent phosphorous. Thus any removal of micropollution is a fortuitous side effect rather than by design. Removal predominantly occurs either by adsorption to solids or by biological mechanisms (Gomes *et al* 2004, Langford *et al* 2005b). The most important removal mechanism for micropollution during wastewater treatment is thought to be biodegradation (Andersen *et al* 2003), typically ranging from 60-95% for total estrogens (Baronti *et al* 2000, Johnson *et al* 2000). Whereas non-toxic long-chain ethoxylated alkylphenols are typically biodegraded >95%, however, more recalcitrant and estrogenic metabolites such as alkylphenols are formed (Giger *et al*. 1984). Such removals are achieved via conventional sewage treatment, typically as a three-part process, consisting of preliminary, primary sedimentation and secondary treatments. In this section, removal of EDCs will be described by conventional sewage treatment.

Secondary biological treatment is thought to be the principle process for the removal of micropollution (Anderson *et al* 2003, Pickering and Sumpter 2003, Carballa *et al* 2004). Biodegradation and biotransformation are believed to have significant roles in the removal of micropollutants, as some microorganisms can utilise such compounds as soul carbon sources for metabolism. However, the population dynamics of these xenotrophic microorganisms are immensely complex; as their ecology is dependant upon process type i.e. trickling filters, activated sludge and membrane bioreactors and the variables used to control the operation of the STW.

2.3.1 Removal of Micropollution by trickling filters

Estrogens

Trickling filters are generally less effective at removing estrogens than activated sludge STW, possibly due to the lower contact times. In Sweden, less than one third of estrogenicity was removed in a trickling filter (Svenson *et al* 2003). A Canadian study of 18 STWs, poor estrogen removal was noted at sites that incorporated trickling filters (Servos *et al* 2005). In fact, E1 and E2 concentrations increased by 62.4 and 18.5% respectively, possibly as a result of deconjugation. Steroid estrogens are particularly stable and can withstand sewage treatment (Turran 1995). However, with the addition of further treatment processes, the removal of these compounds becomes more appreciable. Jiang *et al* (2005) demonstrated this when removals of E1 and E2 of 58 and 82% were observed in a STW employing a two stage trickling filter and two-stage sedimentation. Thus achieving comparability with an activated sludge process

Higher removal rate may be achieved at STW with treatment regimes that combine the removal of organics with nitrogen and phosphorous, but not using trickling filters. In an American study, BNR activated sludge with a solid retention time (SRT) of 10-13 days was shown to have superior removal in comparison with a trickling filter (Johnson and Sumpter 2001). The difference in removal efficiency was attributed to hydraulic retention time (HRT). European activated sludge STWs have retention times between 4 and 14 hours (Johnson and Sumpter 2001), which is significantly greater compared to trickling filters, which have an HRT usually less than one hour. It would therefore appear that lower contact time coupled with smaller biomass surface area in comparison with activated sludge contribute to the inadequate estrogens removals that have been documented.

Alkylphenolics

Few studies document the fate of APx during trickling filter treatment. However, two studies document relatively high removals of APEOs, 68-77% (Gerike 1987) and about 77% (Brown *et al* 1987). High removal of these compounds was attributed to high removal of COD compared to an activated sludge plant. Where removal of $AP_{4+}EO$ is witnessed, formation of $AP_{1-3}EO$, $AP_{1-3}EC$ and AP can be expected. However, due to stratification and the occurrence of anaerobic zones within the fixed film, less oxidative metabolites i.e. $AP_{1-3}EO$ and AP, would be formed.

Pharmaceuticals

Few studies have documented the removal of pharmaceuticals by trickling filters. However, as with the estrogens and APx, evidence suggests that removal by trickling filter is less effective than activated sludge. Kanda *et al* (2003) noted that the removal of ibuprofen was higher in activated sludge compared with a trickling filter. This is in agreement with Bartlet-Hunt *et al* (2009) who observed that streams impacted with effluent from trickling filters or trickling filters run in tandem with activated sludge had the highest observed in-stream pharmaceutical concentration. In a British study monitoring the fate of 55 pharmaceuticals, a trickling filter works demonstrated consistently poor removal (Kasprzyk-Horden *et al* 2009). In this study, antibiotics sulfamethoxazole and erythromycin-H_2O were not removed during trickling filtration, although removal efficiencies of over 50 and 70% were achieved respectively by activated sludge. The authors noted that paracetamol, a readily biodegradable compound, although removed 100% by activated sludge treatment persisted in effluent from the trickling filter.

2.3.2 The Removal of Micropollution by Activated sludge

Estrogens

Conventional activated sludge is commonly used to treat domestic sewage by means of a suspended biomass rather than a fixed film as with trickling filters. However, as run and currently configured, estrogens are still detected in activated sludge effluents (Langford and Lester 2004, Koh *et al* 2008a). In batch experiments, activated sludge did not fully remove E1 or EE2 (Johnson and Sumpter 2001). In laboratory test using activated sludge, EE2, was shown to be persistent under aerobic conditions compared with E1 and E2 which degraded rapidly (Ternes *et al* 1999). In the field, the situation is more varied. Whilst E1 removal can vary, removals of E2, E3 and EE2 were greater than 85% (Johnson and Sumpter 2001). Elsewhere, average removals of E1, E2, E3, and EE2 were 61%, 85%, 95% and 85% respectively in six STWs in Rome (Baronti *et al* 2000). Conversely, Ternes *et al* (1999) found lower removal of E1 and EE2 (>10%), but significantly higher removal of E3 (circa 66%). In agreement with this, 45% reduction in E1 was observed by Komori *et al* (2004), which was considerably lower than the removals of E2 and E3. By conventional activated sludge treatment, the removal of steroid estrogens is highly variable. However, each of the above studies will have observed activated sludge treatment utilising different process parameters. Therefore, determining which factors are key for the removal of estrogens and fundamental to developing strategies for the removal of EDCs.

The key parameters that require control during activated sludge treatment are hydraulic retention time (HRT) and sludge retention time (SRT). The process parameter HRT indicates the length of time a reactant stays within a reaction vessel and is defined by its volume in relation to its flow rate. The process parameter SRT indicates the age of the bacteria within a reactor. These two parameters are closely associated. Usually an increase in HRT occurs concurrently with an increase in SRT. Increasing HRT results in longer reaction times, as less food in terms of lower volumetric loading is available. Therefore, bacterial growth rates are affected. Maintaining a short SRT whilst increasing HRT would result in decreasing mixed liquor suspended solids (MLSS) concentration, as the bacterial population would not be able

to sustain itself. Despite the integral importance of these parameters, researchers do not consistently detail the operational characterises of the ASP sampled.

From the early of the twenty-first century, researchers have noted improved removal with increased SRT (Holbrook *et al* 2002, Kreuzinger *et al* 2004,). Retention time of 10 – 12.5 has been suggested as the period required for the growth of organisms to remove E2 and E1 (Clara *et al* 2004). Kreuzinger *et al* (2004) proposed that higher SRTs allowed the enrichment of slow growing bacteria and consequently the establishment of a more diverse biocoenosis. Research that focuses on the removal of estrogens during activated sludge treatment but includes details of HRT and or SRT utilised by the ASP are shown in Table 2-7. Indeed, improved estrogen removal efficiency does appear to correlate with ASP with longer SRT.

Table 2-7 The influent, effluent and percent removals of estrogen observed independently by researchers under differing HRT and SRT

Reference	HRT	SRT	E1			E2			E3			EE2			E1-3S		
			Inf	Eff	Removal	Inf	Eff	Removal	Inf	Eff	Removal	Inf	Eff	Removal	Inf	Eff	Removal
[a]Cargouet *et al* (2004)	**10**		15.2	6.5	**57**	17.4	7.2	**59**	15.2	5.0	**67**	7.1	4.4	**38**			
[b]Baronti *et al* (2000)	**2-3**		11.2	6.2	**44**	17.1	8.6	**49**	11.4	6.8	**40**	6.8	4.5	**34**			
	12		71.0	9.6	**86**	16.1	1.5	**89**	83.8	3.7	**94**	3.9	0.6	**79**			
	14		50.4	7.7	**84**	9.3	0.8	**91.5**	65.6	0.8	**99**	2.3	0.4	**82**			
	12		67.0	4.1	**93.9**	9.2	0.9	**88**	70.8	1.2	**98**	3.4	0.7	**80**			
	14		36.8	13.9	**64.3**	11.5	1.0	**91.5**	79.4	2.6	**97**	3.0	0.5	**84**			
	12		35.2	30.3	**9**	8.6	1.9	**76.2**	54.4	8.7	**84**	2.9	0.7	**78.7**			
	14		50.6	44.6	**17.6**	14.7	2.4	**84**	128.6	1.1	**99**	2.5	0.8	**75**			
[c]Carballa *et al* (2004)	**24**		3.4	4.4	**-42%**	2.4	<LOQ	**47**									
[d]Servos *et al* (2005)	**8.5**	**5.5**	40	12	**66.7**	7	1.5	**82.9**									
	6.6	**9.6**	46	12	**72.7**	22	1	**96.8**									
	6.7	**2.7**	60	96	**-54.8**	24	14.5	**39.5**									
	2.8	**0.9**	68	16	**76.7**	22	2	**92.7**									
	5.0	**4.1**	52	8	**85.4**	26	<1	**98.3**									
	6.5	**4.7**	56	70	**-45.8**	10	2.5	**75.9**									
	43	**13.6**	18	<1	**96.5**	2	<1	**93.3**									
	16.8	**53**	78	2	**97.8**	16	<1	**98.8**									
	12.3	**35.5**	60	3	**95.1**	15	<1	**98.2**									
[e]Onda *et al* (2003)	**4**	**7-10**	43.1	12.3	**69.2**	28.1	1.2	**94.7**	381.5	5.6	**96.9**						

Reference	HRT	SRT	E1			E2			E3			EE2			E1-3S		
			Inf	Eff	Removal	Inf	Eff	Removal	Inf	Eff	Removal	Inf	Eff	Removal	Inf	Eff	Removal
[f]Clara *et al*		**2**	35	38	**-8.6**	57	18	**68.4**				28	106	**-278.6**			
(2005)a		**19**	35	1	**97.1**	57						28	8	**71.4**			
		48	35	2	**94.3**	57						28	11	**60.7**			
		>100	35	2	**94.2**	57						28	10	**64.3**			
[g]Joss *et al*		**21**	7.3	>0.1	**>98.5**	4.9	<0.1	**94**				0.7	<0.1	**71**			
(2006)		**12**	24	0.3	**98**	7.6	<0.1	**>94.5**				4.3	0.9	**94**			
		12	75	>0.1	**>99.8**	11	<0.1	**>99**				5.2	0.3	**>93**			
[h]Gunnarsson	**4.5**	**14**	32	6.3	**98**	17	0.3	**98**				1.1	0.2	**82**			
et al (2009)																	
[i]Braga *et al*	**4**	**16**	54.8	14.3	**85**	22.0	1.0	**96**									
(2005)	**6**	**6**	66	28	**58**	62	nd	**100**	17	6	**65**	54	13	**75**			
[j]Esperanza *et*	**6**	**6**	37	18	**52**	30	2	**94**	5	6	**-20**	29	15	**50**			
al (2007)																	
[k]Koh *et al*	**13**	**13.6**			**89**			**94**			**99** [k]			**68**			**79**
(2009)	**13**	**10.2**			**89**			**96**			**99** [k]			**65**			**59**
	9-13	**12.1**			**91**			**94**			**98** [k]			**60**			**88**
[l]Andersen *et*		**11-13**	74.9	<1	**>98**	10.9	<1	**>98**				5.2	<1	**>80**			
al (2002)																	

Unless stated Removal = Influent-Effluent. Nd = not detected. a Presumably only aqueous phases were analysed. N=5. b Only aqueous phases were analysed. N=5. c Presumably, aqueous and solids phases extracted and analysed together. Influent is settled sewage, removal rate are from secondary treatment only. Twenty-four hour composite taken. d Presumably, aqueous and solids phases extracted and analysed together. Influent and effluent data is estimated from graphical data. Twenty-four hours composites take over an unknown period. e Only aqueous phases analysed for influent and effluent. Data are mean values from one year sampling. Removals calculated from daily masses in influent and effluent. Twenty-four hours composites take over an unknown period. f Presumably aqueous and solid phases were analysed together. Removals calculated as from influent and effluent data from paper. N=unknown. g Aqueous and solid phases extracted and analysed together. Removals calculated by mass balance. Twenty-four hours composites take over an unknown period. h Presumably, aqueous and solids phases extracted and analysed together. Twenty-four hours composites take over a two-week period. i Only aqueous phases analysed for influent and effluent. Samples taken periodically over a four-month period. j Aqueous and solid phases extracted and analysed separately. Synthetic wastewater, influent is settled sewage, only liquid phases analysed. N=3. k Aqueous and solid phases extracted and analysed separately. Removals calculated by mass balance. Removal = ((settled sewage – (effluent + waste activated sludge)). l Aqueous and solid phases extracted and analysed separately. Twenty-four hours composites take over an unknown period.

The link between SRT and increasing estrogen removal efficiency is demonstrated in Figure 2-1. Above an SRT of 20 days, it appeared that using the combined data displayed in Table 2-7, the removal efficiency of E1, E2 and E3 was consistently high (>90%). At three ASP each with SRT with SRT >10 days, E1, E2 and E3 removal efficiency was >90%, whilst EE2 was greater than 60%. In agreement with this, Joss *et al* (2006) also observed >94% removal of E1 and E2 at three ASP with SRT in excess of 10 days. Below an SRT of ten days, estrogen removal was less consistent. At an ASP with an SRT of 2 days, E1 and E2 removal efficiencies were reported to be -8.6 and 68.4% respectively (Clara *et al* 2005a). These findings are in agreement with those of Servos *et al* (2005), who reported low E1 and E2 removal efficiencies of -54.8 and 39.5 respectively at a site with an SRT of 2.8 days. However, this was not a perfect correlation and cannot fully explain differences between removals at sites with the same SRT. Braga *et al* (2005) and Esperanza *et al* (2007) both observed estrogen removal at site with an SRT of 6 days. Braga *et al* (2005) reported E1, E2, E3 and EE2 removals of 58, 100, 65 and 75% respectively. Esperanza *et al* (2007) reported E1, E2, E3 and EE2 removals of 52, 94, -20 and 50% respectively. The apparent improved removal witnessed by Braga *et al* (2005) could not be explained by SRT alone. Agreement with this, Servos *et al* (2003) noted that greater removal of estrogens was observed in works with nitrifying capabilities (longer SRT). Yet, no statistical correlation between SRT and estrogen removal could be shown. Despite this SRT is an important parameter to consider when studying the removal of estrogens in ASP, as this is the primary design operating parameter and engineers and operators use to influence the sorption potential of the biological solids by adjusting the SRT.

The design parameter HRT is often cited by researchers as influencing estrogen removal efficiencies (Cargouet *et al*., 2004, Johnson and Sumpter, 2001, Kirk *et al*., 2002, Svenson *et al*., 2003). Long HRT allows for more adsorption and biodegradation to occur. Higher removals of E1, E2 and EE2 were achieved at UK ASP with longer HRT (circa 13 hours) compared to sites with HRTs of 2–5 hours (Kirk *et al* 2002). Concentrations of estrogens were removed to below the LOD were obtained at an ASP with an HRT of 20 hours plus percolation through wetland with an HRT of 7 days (Svenson *et al* 2003). Cargouet *et al* (2004) found superior removals for E1 (58%) and E2 (60%) in ASP with HRT of 10–14 hours compared to a plant with an HRT of 2–3 hour where only 44% for E1 and 49% of E2 was achieved.

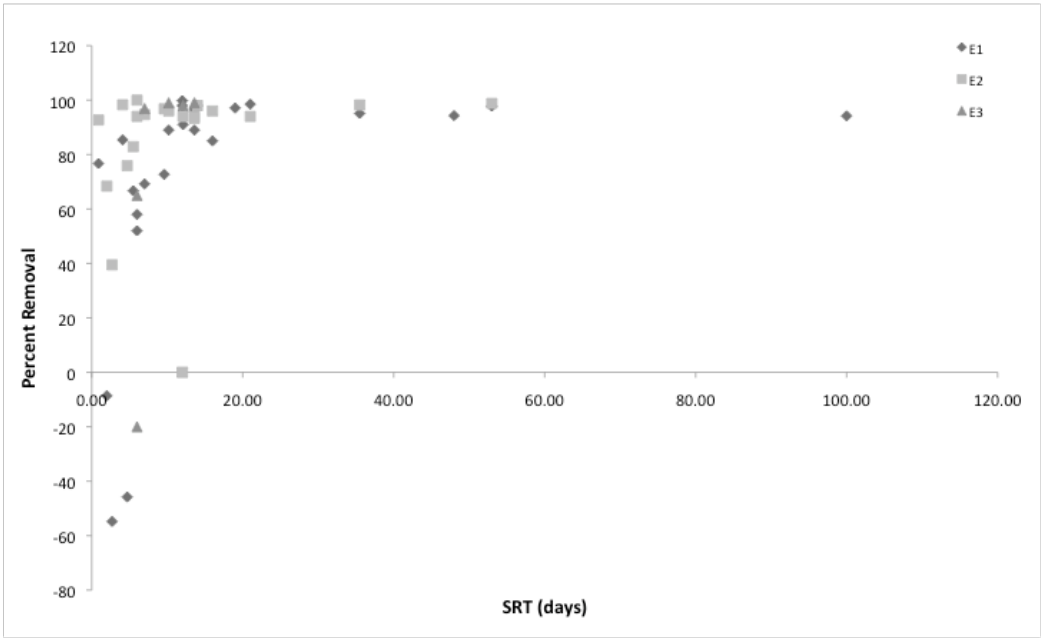


Figure 2-1 The link between improved percent removal of E1, E2 and E3 and increased SRT. Data derived from percent removals of estrogen observed independently by researchers under differing SRT as displayed in Table 2-7

As SRT correlates closely with HRT, these parameters cannot easily be separated. Whether removal of EDCs is a function of rate kinetics, or greater bacteriological diversity is unclear as these variables are invariably linked. Using the data available in Table 7-2 no link between improved removal and increasing HRT could be established as had been done with SRT. Johnson *et al* (2005) however observed a weak significant (α=5%) correlation between E1 removal and HRT or SRT. In a Canadian study (Servos *et al*., 2005), there was little or no statistical correlation (r^2 <0.53) between HRT and estrogen removal at 9 ASP. However, observations were reported that plants with high HRT (>27h) had relative high removal of E1 and E2 while low HRT plants had more variability and lower removal. Ternes *et al* (1999) proposed that estrogen degradation follows a pseudo first-order reaction. Likewise, Johnson and Sumpter (2001), postulated that pseudo first-order kinetics could explain incomplete biodegradation (circa 62%) of E1 at a full scale ASP. This was confirmed during batch testing, as an E1 rate constant (k 0.0693 h^{-1}) was derived. As lower organic loading is an

artefact of extended HRT, which in turn is inextricably linked with SRT, enhanced removal from longer SRTs may simply result from longer reaction times rather than bacteriological differences between carbonaceous and nitrifying ASP.

It has been suggested that estrogen removal could be inhibited by presence of more easily degradable substrate in settled sewage (Joss *et al* 2004). These authors hypothesised that when low BOD occurs, microorganisms are forced to mineralise more recalcitrant compounds. However, there is no clear evidence of this: no correlation between estrogen removal and organic loading has been demonstrated to date. Onda *et al* (2003) could not demonstrate a relationship between E1 removal and loading. However, it was generally noted that lower removal of E1 occurred when organic loading was high. Similarly, Johnson *et al* (2000) attempted to correlate E2 removal with flow per head. These authors observed decreased estrogen removal with increased percentage of flow (an indicator of loading). In a review of MBRs, enhanced removal of EDCs have been attributed to lower loading in comparison with AS (Merlin *et al* 2006).

The presence of aerobic, anoxic and anaerobic zones during sewage treatment appears to permit effective removal of estrogens. Whether this is due to the variety of degradation mechanisms or the prolonged treatment, the evidence is less clear. In a recent study, a nitrifying ASP demonstrated higher removal efficiencies for total estrogens compared with an ASP with nitrification and dinitrification (Koh *et al* 2009). These authors alluded to the importance of loading and its correlation with biomass activity (estrogen removed per tonne of biomass) implying a pseudo first-order rate kinetic.

Alkylphenolics

Monitoring of surface waters for APEOs has been extensively undertaken (Ahel *et al* 2000a, Ahel *et al* 1996, Blackburn *et al* 1999, Blackburn and Waldock, 1995, Sole *et al* 2000). However, due to the lipophillic nature of APEO degradation products, sludge (primary and secondary), and sediment (marine, estuarine and river bed), may accumulate large quantities of these compounds and therefore act as reservoirs leaching their estrogenicity in a prolonged

manner (Ying *et al* 2002). The ubiquity of NPEOs and their metabolites within the aquatic environment, result from sustained anthropogenic usage. Physiochemically, they exist as non-volatile compounds with either high or low water solubility and can partition to solids readily. Therefore biodegradation, biotransformation and sorption are all important mechanisms for the removal of these compounds: Adsorption to solids is a particular issue as biosolids are often applied to land (Ying *et al* 2006). Generally, long chain NPEO are removed >90% in ASP (Di Corcia *et al* 2000). However, their breakdown produces short chain NPEO and NPECs, which are more estrogenic and recalcitrant and are of greater concern. These residuals, such as NPECs, after leaving ASP are expected to further degrade in the aquatic environment (Melcer *et al* 2006). The NPEOs and their degradation products have been detected in ASP effluents. The typical concentrations of these compounds in sewage effluent varies from <LOD to 343 μg L^{-1}. Only a few authors report the occurrence of APECs, which are also estrogenic, thus providing a more complete picture of the degradation NPEOs (Field and Reed 1996, Fujita *et al* 2000a, Lee *et al* 1998, Loos *et al* 2007, Koh *et al* 2009). Nor has it been common practice until recently to include process parameters of the ASP under investigation in published research.

For opportunities for commenalism, synergism and co-metabolism to occur and facilitate the degradation of NPEO, it has been suggested that longer SRTs are necessary. This hypothesis, is supported by Clara *et al* (2005) and Kreuzinger *et al* (2004) who investigated the influence of extended SRT upon the removal of EDCs and pharmaceuticals. These authors investigated the influence of SRT upon removal efficiency using four-bench scale reactor (SRT 1 to 26 days) and four ASP (SRT 2 to >100 days). There was a correlation between SRT and removal of NP compounds (NP, $NP_{1-3}EO$ and $NP_{1-3}EC$). However, these authors did not include long chain NPEOs in their work making it difficult to fully assess removal of these compounds.

Table 2-8 The influent, effluent and percent removals of NPx observed independently by researchers under differing HRT and SRT conditions during activated sludge treatment

Reference	HRT	SRT	Long Chain NPEO			Short Chain NPEO			NPEC			NP			∑NPEO		
			Inf	Eff	%	Inf	Eff	%	Inf	Eff	%	Inf	Eff	%	Inf	Eff	%
[a]Clara *et al* (2005)b	**319.2**	**114**				$NP_{1-2}EO$ 7982	153	**98.1**	∑1+2 1086	1101	**-1.4**	4031	487	**87.9**			
	300	**237**				8066			844	1407	**-66.7**	2673	280	**89.5**			
	326.4	**52**				5285	142	**97.3**	900	3192	**-254.6**	3129	326	**89.6**			
	1.9	**2**				4660	3940	**15.5**	1330	13260	**-897.9**	1950	370	**81.0**			
	28.8	**46**				115500	219	**98.1**	5960	1295	**-118.0**	1280	285	**77.7**			
[b]Esperanza *et al* (2004)	**6**	**6**	$NP_{>4}EO$ 910070	14650	**98**	$NP_{1-3}EO$ 17860	20020	**-12.1**				14650	950	**94**			
	6	**6**	1026630	8210	**99**	31038	28180	**9.2**				20420	8210	**99**			
[c]Gonzalez *et al* (2007)		**9**													$NP_{1-15}EO$ 244000-465000	12000-72000	**87**
[d]Gunnarson *et al* (2009)	**4.5**	**14**										3200	870	**73**			
[e]Koh *et al* (2009)	**13**	**13.6**			**$NP_{4-12}EO$ 96**									**30**			

Reference	HRT	SRT	Long Chain NPEO			Short Chain NPEO			NPEC			NP			∑NPEO		
			Inf	Eff	%	Inf	Eff	%	Inf	Eff	%	Inf	Eff	%	Inf	Eff	%
	13	**10.2**			**93**						**-157**			**-12**			
	9-13	**12.1**			**93**						**-110**			**11**			
[f]Shao *et al* (2003)		**18**	$NP_{4-12}EO$ 15291	3160	**79**	$NP_{1-3}EO$ 31235	11672	**62**				9275	1461	**84**	$NP_{1-12}EO$ 46527	14831	**68**
[g]Loyo-Rosales *et al* (2007)	**7-13**	**5-10**	$NP_{4-12}EO$ 47400-74500	272-5390	**>99**										NP+$NP_{1-3}EO$ 47600-262000	39200-32.3	**63-99**
	14-20	**15-21.5**	$NP_{4-12}EO$ 265000-722000	42-8420	**>99**										NP+$NP_{1-3}EO$ 68500-160000	44200-20700	**77-97**
	7-12	**7-14**	$NP_{4-12}EO$ 610000	5080	**99**										NP+$NP_{1-3}EO$ 50200	21500	**57**

Unless stated Removal = Influent-Effluent. Nd = not detected. a Presumably aqueous and solids phases were extracted and analysed together. N=unknown. b Presumably aqueous and solids phases were extracted and analysed together. Synthetic sewage spiked with NPx used. N=3 c Only aqueous phases were analysed. Influent was settled sewage. Daily twenty-four hour composite samples taken for two weeks. d Presumably, aqueous and solids phases extracted and analysed together. Twenty-four hours composites take over a two-week period. e Aqueous and solid phases extracted and analysed separately. Removals calculated by mass balance. Removal = ((settled sewage – (effluent + waste activated sludge)). f Aqueous and solid phases extracted and analysed separately. Samples taken from one day sampling. N=unknown. g Aqueous and solid phases extracted and analysed separately for NPEO. Aqueous phases analysed only for NPEC. N=>10. Averages of summer and winter data used.

Global removal rates for NPEOs are variable. In the USA, Naylor *et al* (1995) reported removal of NPEO to be 93-99%. Influent concentrations were 987 and 33'700 μg L^{-1} for NP and NPEO respectively: High compared with other studies. The effluent concentration for NP were 15 to >1 μg L^{-1}, whilst NPEO varied from 260 to >5 μg L^{-1}. In Japan, removal rates of NPEO varied form 86-99% in autumn to 66-99% in winter (Nasu *et al* 2001). In an Italian STW, removal rates ranges form 74-98% (Crescenzi *et al* 1995, Di Corcia *et al* 1994). A Swiss STW demonstrated less affective removal, ranging from 47-89% (Ahel *et al* 1994). Details of from other researchers that have included details of the HRT and/or SRT associated with the ASP sampled are detailed in Table 2-8. The most easily removed compounds were long chain NPEO. These were consistently removed >98% with SRTs as low as 6 (Esperanza *et al* 2004) and as high as 21 days (Loyo-Rosales *et al* 2007). These finding would suggest the extended SRT is not necessary for the removal of long chain NPEOs.

Possibly due to extended SRTs, membrane bioreactors (MBRs), in comparison with AS, exhibit higher removal efficiencies for NPx (Gonzalez *et al* 2007, Terzic *et al* 2005). NPEO removals of 74 and 87% coupled with formations of 1471 and 429% of NPEC occurred with conventional ASP and MBR respectively (Terzic *et al* 2005). The NPEC accounted for 67 and 42% of the total NPx composition of the final effluents of the ASP and MBR respectively. Such differences were attributed to longer SRTs achieved during MBR treatment and the development of bacterial biomass capable of biotransforming the more persistent oligmers (Terzic *et al* 2005). Although, these authors did not determine whether, the increased removal was due to degradation or sorbtion. In another study, 54 and 94% removal of NPx was achieved by AS STW and MBR respectively (Gonzalez *et al* 2007). These authors found that $NP_{1\text{-}2}EC$, contributed to 60 and 35% of the final effluents of the ASP and the MBR respectively. Additionally, it was determined, after further tests that enhanced removal for the MBR was due to better degradation rather from sorbtion. Better removal was attributed to the higher SRT and lower sludge load, which would allow for a more diverse bacterial assemblage and lower resource competition from more easily degraded compounds. However, neither of these studies published the operational SRTs, making these claims difficult to substantiate. The impact of SRT on the removal efficiencies at an ASP and MBRs operated at different SRTs, was evaluated by Clara *et al* (2005b). The ASP with SRTs an SRT > 10 day exhibited increased removal potential for NPEOs and

NPECs. These findings were in agreement with Ahel *et al* (1994a) who observed the highest removal rates for NPEOs in low-loaded ASP operated at high SRTs. Productions of NPEC of 1.4% and 49%, corresponded to SRTs of 114 and 237 days respectively, observed ASPs had comparable HRTs, food to microorganism ratios, and temperature.

From the literature available there it is suggested that SRT has an important role in the removal of APx. However, in isolation SRT cannot be separated from organic loading and HRT. Since MBR have low organic loading in comparison to ASP, bacteria could be forced to metabolise recalcitrant compounds such as APECs and $AP_{1-3}EO$. Ahel *et al* 1994 and Clara *et al* 2005 demonstrated this indirectly. Formation of $NP_{1-2}EC$ was 240 and 289% in low and high loaded ASP respectively (Ahel *et al* 1994). Low loaded systems also have extended HRT. This is an important parameter, as it extends the potential reaction time of the compounds, providing more opportunity to degrades or adsorb (Langford *et al* 2005). APECs are weakly hydrophobic; therefore longer HRT could result in greater removal. However, there is limited work to demonstrate this.

Pharmaceuticals

There is a paucity of information surrounding pharmaceutical removal during sewage treatment. Many authors have only analysed pharmceuticals in sewage effluents (Aherne *et al* 1985, Hirsch *et al* 1999). Adding to the knowledge, other authors have in addition to effluent analysed influent, therefore making it possible to determine removal efficiencies (Ternes 1998, Stumpf *et al* 1999, Soulet *et al* 2002, Metcalfe *et al* 2003, Kanda *et al* 2003). However, limited information is given regarding the type of STW sampled and even less can be determined of the process parameters such as SRT and HRT.

An extensive study by Ternes (1998) investigated the occurrence and removal of 32 pharmaceuticals including analgesics, lipid regulators, psychiatric drugs, β-blockers, β2-sympathomimetics as well as five drug metabolites in a German STW. Removals ranged from 7% for carbamazepine and >99% for aspirin. Greater than 60% of the influent load was removed by the AS process, with the exception of carbamazepine, clofibric acid, phenazone

and dimethylaminophenazone. Fenofibrate, paracetamol and the metabolites of aspirin where not detected in the final effluent. Due to extremely high loading of aspirin (3 kg d^{-1}), up to 54μg L^{-1} was detected in the final effluent.

During an UK study of 6 STP, micropollutants, including pharmaceuticals where measured in influent and effluent samples. It was noted that ibuprofen removal was higher at ASP compared to trickling filters (Kanda *et al* 2003). These authors concluded that processes with increased retention time demonstrated improved micropollutant removal. It was also noted that a nitrifying activated sludge (NAS) STW had the best removal of ibuprofen. However, the authors did not indicate the process parameters associated with each STW. It is therefore difficult to ascertain which process parameters are responsible for the differences in removal efficiency.

2.3.3 The Anoxic and Anaerobic Treatment Processes

For the removal of nitrogen and phosphorous during sewage treatment, biological nutrient removal (BNR) is utilised. For this, an anoxic/anaerobic zone as part of the treatment process is necessary. It has been observed that significant removal of estrogens can be achieved by BNR (Koh *et al* 2008a). From observations such as these it has been postulated that a variety of redox conditions are required for significant removal of micropollutants such as estrogens (Joss et al 2004).

Estrogens

Removal of estrogens occurs under aerobic conditions. However, ASP with nitrification and denitrification, have demonstrated effective elimination of natural and synthetic estrogens (Andersen *et al* 2003, Leusch *et al* 2005, Koh *et al* 2008a). At a German ASP with nitrification and denitrification natural estrogens largely degraded in the anoxic zones whereas the synthetic estrogen (EE2) only degraded aerobically in the nitrifying zone. This is in agreement with Vader *et al* (2000) who concluded that EE2 degradation was associated

with the nitrifying activity of the activated sludge. Joss *et al* (2004) investigated the effect of redox condition upon removal kinetics of estrogens using a model. These authors noted that maximum removal rate of E1 occurred during aerobic treatment. Yet an increase in rate was observed by factors for 3 to 5 for transitions from anaerobic to anoxic and anoxic to aerobic. E2 oxidation was observed at a high rate under all redox conditions, although the difference between anaerobic compared to aerobic sludge was three times less. Under anaerobic conditions E1 was shown to reduce to E2. EE2 demonstrated removal only during aerobic treatment. Similar observation was accounted by Leusch *et al* (2005). These authors measure estrogenicity using sheep ER binding assay and the MCF-7 breast cell proliferation assay. After secondary treatment of nitrification/denitrification (SRT 25.8 days) >95% removal of estrogenicity was observed. At two ASP utilising nitrogen removal AS in Sweden, >97% removal of estrogens (based on EEq ng L^{-1}) was shown (Svenson *et al* 2003).

Under purely anaerobic conditions, evidence of removal is sparse and contradictory. Andersen *et al* (2003) measured E1 and E2 in primary sludge and subsequent anaerobic digestate and concluded that under methanogenic conditions estrogens are not degraded considerably. However, de Mes *et al* (2008) reported that the type of inoculum is fundamental for the reductive anaerobic capabilities. Contrary to this, conversion of E2 to E1 under anaerobic conditions was shown with river sediments (Czajka and Londry 2006).

In a lab scale batch study, it was reported that alternating microbial populations from anaerobic/aerobic/anoxic conditions do not necessarily affect E2 removal (Pholchan *et al* 2008). These authors noted that batch reactor SRTs <5.7 days adversely affected E2 removal. Despite this, removals of E1, E2 and EE2 accounted for 60%, 90% and 35% respectively, although the EE2 removal was attributed to sorption. Similar EE2 removals were reported by Esperanza *et al* (2007), but the exact anaerobic conditions were not specified in their report. The contradictory nature of estrogen removal is further illuminated, as Czajka and Londry (2006) reported that EE2 demonstrated no reduction during three years of anaerobic digestion, using river sediment as the inoculum.

Under continuous mixed sludge digestion, removals of circa 85% for E1, E2 and EE2 under mesophilic and thermophilic anaerobic conditions have been shown (Carballa *et al* 2006, 2007). Strictly anaerobic desulphating strains isolated from human excreta have demonstrated ability to cleave sulphate conjugates of E1 and E2. (Johnson and Williams 2004).

Alkylphenolics

Few reports detail the fate of APEOs and their metabolites during BNR. In a comprehensive study of the fate EDCs, including NP, by measuring effluent concentrations across Europe (Johnson *et al* 2005), many of the effluents monitored were from ASP that incorporated BNR. Although only effluent was measured, these authors demonstrated trends that plants with longer HRT and SRT (incorporating BNR) had greater NP removal efficiencies. In another study, declining NP residuals displayed weak correlation with increasing SRT (Drewes *et al* 2005). Lack of statistical correlation from these studies, demonstrates that the relationship between removal efficiency and BNR is not clear. Koh *et al* (2009), reported APEO removal of >90% for two nitrifying/denitrifying ASPs, one of which incorporated P removal. Although removal efficiencies were similar, the plant that incorporated P removal was 50-60% less efficient that the straight nitrifying/denitrifying ASP.

In anaerobic as well as aerobic conditions, the ethoxylate chain of higher ethoxylate APEOs is shortened until persistent short-chained $AP_{1\text{-}2}EOs$ are formed. This breakdown proceeds by the stepwise removal mechanism of one ethylene glycol unit (Chiu *et al* 2010*,* Montgomery-Brown and Reinhard, 2003), in the similar way as the anaerobic biodegradation of alcohol polyethoxylates, proposed by (Wagener and Schink, 1988).

Pharmaceutical

Few reports detail the fate and removal of pharmaceuticals during BNR and fewer during strictly anaerobic treatment. Much work has been done regarding pharmaceutical removal

during sewage treatment. However, few authors provide sufficient detail of the STW in order to assess how process type influences removal efficiency.

2.4 The Biodegradation of Micropollutants During Activated Sludge Treatment

Estrogens

Distinguishing how EDCs are removed is a complex issue. It has been suggested by many authors that NAS possesses the necessary bacteriology to degrade estrogens particularly EE2 (Vader *et al* 2000, Layton *et al* 2000, Clara *et al* 2005). During biotransformation batch testing, AS (non-nitrifying) showed little or no EE2 transformation over 20h. However, using NAS, good removal of EE2 (with a half life 24h) was witnessed (Vader *et al* 2000). These authors concluded that greater elimination of EE2 would occur with STW with nitrifying activated sludge. A conclusion supported by Layton *et al*, 2000, who found that sludge that failed to nitrify also failed to remove EE2 significantly. This, and evidence that the best estrogen removal efficiencies are achieved with ASP with longer SRT (Clara *et al* 2005) that consequently have nitrifying consortia has led to hypotheses that ammonia oxidising bacteria are fundamental to biodegradation of such compounds.

The co-metabolic properties of ammonium monoxygenase (AMO) have been suggested to fortuitously oxidise estrogens. Ren *et al*. (2007), demonstrated removal of estrogens during batch tests with NAS. To test whether such removal was due to ammonia oxidising bacteria (AOB) or heterotrophs, tests were performed at 4˚C and after AS had been heat treated for 30 minutes at 45˚C to suppress the activities of autotrophic and heterotrophic bacteria respectively. On the basis of different of activity of AOB and heterotrophs at 4˚C, they postulated that degradation was due to co-metabolism of AOB. However, a recent study by Gaulke *et al* (2008) refutes these findings. These authors demonstrated that EE2 can abiotically transform to nitro-EE2 with conditions of low pH and high nitrite that are typical during small scale tests. Additionally, these researchers demonstrated that EE2 does not

degrade in pure culture of the NAS species *Nitrosomonas europaea* with low ammonia conditions.

Other research found that during batch tests EE2 was biotransformed to catechol-EE2 when incubated with enriched NAS enzyme extract (Yi and Harper 2007). Since, AMO is unstable outside the cell (Moir *et al* 1996), extracellular degradation is unlikely. The presence of heterotrophic enzymes would also have been present; and biotransformation could have been attributed to these bacteria. Therefore, if estrogen removal occurs extracellularly, the enzymes involved may be from heterotrophic sources. Therefore the role of heterotrophic organisms to degrade estrogens should not be over looked, as enzymes from heterotrophic organisms have been show to oxidise estrogens including EE2.

It is known that ligninolytic fungi produce extracellular enzymes with low substrate specificity (Cajthaml *et al* 2009). These enzymes, such as manganese peroxidase and laccase from the fungi, *Phanerochaete chrysosporium and Trametes versicolor were able to transform E2 and EE2, completely removing estrogenicity within one hour (Suzuki et al 2003)*. However, it should be noted that the concentrations of E2 and EE2 used were one order of magnitude greater than found in environmental samples. In agreement with this work, Auriol *et al* (2006a, 2007, 2008), found that estrogens at environmentally typical concentrations were effectively removed by horseradish peroxidase and lactase.

Heterotrophic bacteria that are capable of degrading estrogens have been identified including *Rhodococcus zopfii, R. equi, R. erythropolis* and *Mycobacterium fortuitum* (Yoshimoto *et al* 2004, Ferreira *et al* 1984, Watanabe *et al* 1987). Using strains of *Rhodococcus* isolated from STWs, Yoshimoto *et al* (2004) found that high concentrations (100mg L^{-1}) of E1, E2, E3 and EE2 were rapidly degraded. Also isolated from activated from AS, *Sphingobacterium sp.* JCR 5 was reported to degrade EE2. This bacterium grew on EE2 as the sole carbon source, metabolising 87% of the substrate added (30mg L^{-1}) at 30˚C within ten days (Haiyan *et al* 2007); and could also utilise other estrogens as carbon sources.

At these concentrations (mg L^{-1}), estrogens are many orders of magnitude greater than found typically in sewage influent. Therefore the relevance of these studies is in question. At the typical concentrations found within sewage treatment, these bacteria are unlikely to contribute to biodegradation, as they would be out competed by species with greater resource affinity at lower concentrations. Heterotrophic organisms capable of biodegrading EDCs are likely to be k strategists. These are organisms that occupy specific niches and utilise low concentrations of a resource (Graham and Curtis 2003).

Although, there is disagreement regarding which organisms are responsible for the removal of estrogens, high SRT low loading conditions are generally agreed to be the most favourable. By determining whether estrogen removal is intra or extracellular, it would be possible to elucidate whether degradation is facilitated by AOB or heterotrophs, as AMO is unstable out of cell control.

Alkylphenolics

Even though the biodegradation pathways of APEOs have been studied for 45 years, the mechanisms behind which, are still not fully understood. The fact that carboxyalkyphenol polyethoxycarboxylates (CAPECs), were not discovered until 1996 (Ding *et al* 1996), is testament to this. It has been acknowledged that for the complete degradation of APEO, a consortium of bacteria is necessary, due to the limited metabolic capabilities of individual species (van Ginkel 1996). Langford *et al* (2005) reported that nonylphenol polyethoxylated were biodegraded intracellularly. However, this study was conducted with artificial sludge. Using real sludge is necessary in order to demonstrate biodegradation is intracellular within sewage treatment works (STWs).

The oxidative shorting of the polyethoxylate chain of long chain APEO occurs rapidly under aerobic conditions is undisputed; yet the formation of oxidative metabolites is less consistent. The formation of $AP_{1\text{-}2}EC$ from long chain APEOs is reported to be an aerobic process during activate sludge treatment (Giger *et al* 1981, 1984, 1987; Stephanou and Giger 1982;

DiCorcia *et al* 1994). Few reports detail APEO degradation during anaerobic conditions. Some researchers observed the formation of APECs from the oxidation of APEOs under strict anaerobic condtions (Field and Reed 1999, Schroder 2001, Ferguson and Brownawell, 2003).

Further biotransformation of APEO metabolites is reported to occur with greater ease aerobically (Ying *et al* 2002) rather an anaerobically. Therefore it could be concluded that microbial communities with higher sludge ages (nitrifying populations) would be most effective at biotransformation. However, to date no link between AMO and alkylphenol removal has been established.

The complete mineralisation of APEOs is rare; due to the highly branched alkyl group on the phenolic ring. However, the hydrophillic polyethoxylated chain contains carbon that is more available to oxidation. Resultantly, the successive removal of ethoxy groups is a potential source of energy for bacteria (Auriol *et al* 2006). This chain shortening results in the formation of recalcitrant compounds such as NP, OP and $NP_{1\text{-}3}EO$ (Ying *et al* 2002). Biodegradation of these compounds occurs much slower, due to the presence of the benzene ring. Additionally, their relative lipophillic nature further confounds their biodegradations due to their tendency to sorb to solids and therefore limiting their availability for degradation.

It is generally believed that NP_1EC is transformed to NP only under anaerobic conditions (Ahel *et al* 1994a) and anoxic conditions (Ike *et al* 2002). However, Liu *et al* (2006) reported that NP_2EO was transformed to NP via NP_1EC and NP_2EC as intermediates under aerobic conditions by *Ensifer sp.* strain S08 and *Pseudomonas sp.* strain AS90. This is in agreement with Maki *et al* (1996) who postulated that NP_2EC could be further degraded to NP_1EO and/or NP_1EC. Although, under aerobic conditions, NPECs are more resistant to biodegradation than long chained NPEO, they are eventually biodegraded (Staples *et al* 2001). These authors, determined biodegradability using OECD screening tests (OECD., 1980) from $NP_{1\text{-}2}ECs$ and $OP_{1\text{-}2}EC$ in a laboratory study using AS inoculum.

It was reported that under anaerobic (as well as aerobic) conditions, the stepwise shortening of ethylene glycols of nonylphenol ethoxylates (NPEOs) was carried out by *Pseudomonas putida* (John and White, 1998). Recently, Zhang *et al* (2008) studied the biodegradation of NPEOs in a lab-scale upflow anaerobic sludge blanket (UASB) and reported that the predominant species during the 90 days of anaerobic digestion were $NP_{1-3}EO>NP$ which were formed immediately after the commencement of the experiment. Nucleotide sequence analysis identified that *Clostridium sp*., gram-positive bacteria, (oxygen-independent prokaryotes) were the dominant species in the UASB system but NPEO degrading bacteria were the dominant indigenous species in sewage.

Pharmaceuticals

Pharmaceuticals are subject to biological and chemical processes, therefore, degradation rates are determined by environmental conditions, with some drug groups having greater persistence than others (Velagaleti, 1997) In STW, biodegradation is likely to be an important mechanism for the elimination of pharmaceuticals.

Long chain and unbranched compounds are generally considered to be more degradable than short chained or branched compounds. The presence of halogen groups, sulphates or methoxy groups also adds to a compound's recalcitrance (Rogers 1996). Groups such a halogens cause electron deficiency making a compound less susceptible to catabolism. Pharmaceuticals that are hydrophilic tend not to sorb to solids: by remaining within the aquatic phase a compound is more susceptible to biodegradation. Simple molecules such as aspirin and paracetamol are readily degraded within STWs (Jones *et al* 2005a). Whilst larger more complex molecules such as anti cancer drugs, x-ray contract media and certain antibiotics have been shown to be non-degradable in the environment (Kalsch 1999, Kummerer and Al-Ahmad 1997, Kummerer *et al* 2000a, Kummerer *et al* 2000b) are more stable. Even readily biodegradable compounds such as ibuprofen and paracetamol exhibit pseudo-persistent characteristics due to the amounts in which they are used and the inability of STWs to remove 100% of the compound (Daughton and Ternes 1999).

Molecules with long, highly branched side chains are less amenable to biodegradation compared with compounds with short side chains (Schwarzenbach *et al* 2003). In addition, unsaturated aliphatic compounds are generally more accessible to biodegradation than saturated analogues or aromatic compounds with complicated aromatic ring structures (Rogers 1996), such as X-ray contrast media. These compounds are used exclusively in human medicine; therefore aquatic contamination is solely from this source. Ternes and Hirsch (2000) examined the occurrence of 4 X-ray contrast media (diatrizoate, iopamidol, iopromide, iomeprol) in eight German STWs. These compounds were found ubiquitously in raw sewage and were not significantly degraded. However, degradation of iopamidol by AS, was observed by Kalsch (1999), as 85% was transformed into two metabolites. Further degradation within the river was even more significant, with a halflife of 3.1 days.

The biodegradation of three cynically important antibiotics, (ciprofloxacin, ofloxacin and metronidazole) was assessed by Kummerer *et al* (2002b). None of these antibiotics were found to be biodegradable. In a comprehensive review of antibiotics in the environment, Hirsch *et al* (1999) described the analysis of various water samples for 18 antibiotics, including macrolide antibiotics, sulphonamides, penicillins and tetracylines. Frequently, both STW effluents and surface waters were contaminated with sulphamethoxazole and roxithromycin (a degradation product of erythromycin) at concentrations up to 6μg L^{-1}. Tetracylines and penicillins were 50 and 20 ng L^{-1} respectively.

2.5 Summary

It unclear whether micropollutants such as estrogens, nonlylphenols and pharmaceutical, will be regulated as other hazardous chemicals. The threat to aquatic and terrestrial environments is still unproven. However, the risk posed by these compounds should not be underestimated. Whether conventional sewage treatment can be optimised using the current infrastructure to reduce emission of these compounds should be explored. Yet substantial research is needed to determine this.

Estrogens and Nonylphenolics

At ASP with extended HRT and SRT, estrogens are biodegraded readily. However, the mechanisms behind this are not fully understood. As organic loading, SRT and HRT are interconnected, research needs to be undertaken to disentangle these process parameters from each other to determine which variables are fundamental for the removal of estrogens and alkylphenolics. Carbonaceous ASP has demonstrated some estrogen removing capacity indicating the importance of heterotrophic organisms. Therefore the role of heterotrophic and autotrophic organisms with the removal of estrogens needs clarification.

Pharmaceuticals

The research of pharmaceuticals removal during activated sludge treatment appears to be many years behind that of estrogens and nonylphenolics. Limited links between process parameters such as HRT and SRT have been alluded to. However, the sheer number of pharmaceuticals makes it difficult to predict which one will have the greatest potential to elicit an environmental effect. Each country has a different prescription policy and the pharmaceuticals used are different. Prior to the development of an analytical method for the detection of pharmaceuticals in wastewater samples a selection exercise is necessary to determine which compounds pose the greatest UK risk.

Chapter 3

Aims and Objectives

3 Aim and objectives

The main aim of this study was to determine which process parameters were responsible for the effective removal of EDCs during activated sludge treatment. This study aimed to investigate whether the current UK treatment infrastructure is equipped to deal with present and future contamination by EDCs. If additional technologies are necessary can it be done more sustainably without the use of expensive and energy intensive processes? The desired outcome of this study was to inform operators how best to optimize current sewage treatment plants in order to minimize discharge of hazardous micropollution. With the information gathered here, modification to existing processes and management practices could be made that would better protect the aquatic environment

The overall aims were met by the following objectives:

- Sampling of estrogens and nonylphenolics within the settled sewage, effluent and WAS/RAS at three ASP (representing a broad spectrum of process conditions) in order to mass balance the removal of these compounds across the processes
- Using observational data from the sampled works, (a carbonaceous ASP, a nitrifying/denitrifying ASP and a nitrifying ASP), gain evidence as to which process parameters are responsible for the effective removal of estrogen and nonylphenolics during activated sludge treatment using field-based sampling campaigns
- Conduct experiments using pilot-scale porous pot bioreactors to elucidate separate function of process parameters such as hydraulic and sludge retention times, loading and the influence of bulk organics on the biodegradation of estrogens
- Elucidate the role of ammonia oxidising bacteria upon the biodegradation of estrogens during activated sludge treatment at environmentally typical loadings
- Advise how activated sludge treatment can be optimised for effective removal of these compounds

Chapter 4

Materials and Methods

4 Materials and Methods

The quantification of estrogens and nonylphenolic compounds in complex environmental matrices requires the application and understanding of analytical chemistry techniques. The EDCs were present at ng/μg L^{-1} concentrations, below the limit of detection (LOD) for many analytical detectors. Their quantification therefore incorporated the use of solid phase extraction (SPE) to concentrate environmental samples within the detection range of high performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS). Due to the complexity of sewage matrices, gel permeation chromatography (GPC) was used for estrogen analysis to ameliorate the influence of signal suppression. Both aqueous and solid samples were analysed for EDCs. The methodology for the analysis of EDCs was established by Koh *et al* (2007, 2008) and are detailed below (see sections 4-1 – 4-4). The estogens E1, E2, E3, EE2 and the conjugate E1-3S were focused on during this work. Since NPx are typically found at significantly higher concentrations than the OPx, as discussed earlier in Section 2.2 and the OPx also exhibit similar biodegradation to the NPx (Koh 2008) NPx were selected as representatives of the APx for the remainder of this work.

Field based sampling was carried out at two full-scale STWs (a carbonaceous ASP and a nitrifying/denitrifying ASP) and a medium-scale pilot plant (nitrifying ASP) to determine the impact of process type and operational parameters upon EDC removal. Pilot scale porous pot bioreactors were utilised to further determine the separate effects of organic loading and hydraulic and sludge retention times on the removal of estrogens and nonylphenolics. Furthermore porous pots were used to elucidate the role of nitrifying bacteria for the biodegradation of estrogens.

4.1 The Carbonaceous, Nitrifying and Nitrifying/Denitrifying Activated Sludge Sewage Treatment Works

4.1.1 Description of Sewage Treatment Works

Two full-scale STWs (a carbonaceous ASP and a nitrifying/denitrifying ASP) and a medium-scale pilot plant (nitrifying ASP) were used for this study: Full process configurations including SRT are detailed in Figure 4-1. The pilot $ASP_{nit.}$ was operated on real sewage at a full scale STW and had an HRT of 5.6 hours. The $ASP_{nit/denit.}$ was an 'Orbal' oxidation ditch design and was thus subject to extended HRT (16.9 to 26.4 hours). The ASP_{carb} had an HRT of 8 hours.

4.1.2 Sampling regime

The nitrifying ASP (ASP_{nit}) was sampled in late summer 2007; both the nitrifying/denitrifying ASP ($ASP_{nit/denit}$) and the carbonaceous ASP (ASP_{carb}) were sampled in spring 2008. Five day sampling campaigns were undertaken at each site with a sampling interval of four to six hours to account for residence time and flow variation. Samples were collected in glass borosilicate jars with Teflon lined caps and extracted onto solid phase extraction (SPE) cartridges within fifteen minutes of collection. Settled sewage and final effluent were collected from each interval at SP1 and SP2 respectively. Primary sludge and WAS/RAS were collected twice in 24-hour periods from points A and B respectively (see Figure 4-1). Duplicates and spike recovery samples were taken to ensure analytical robustness and to check that recovery rate and MDLs were comparable with those of Koh *et al* (2007 and 2008).

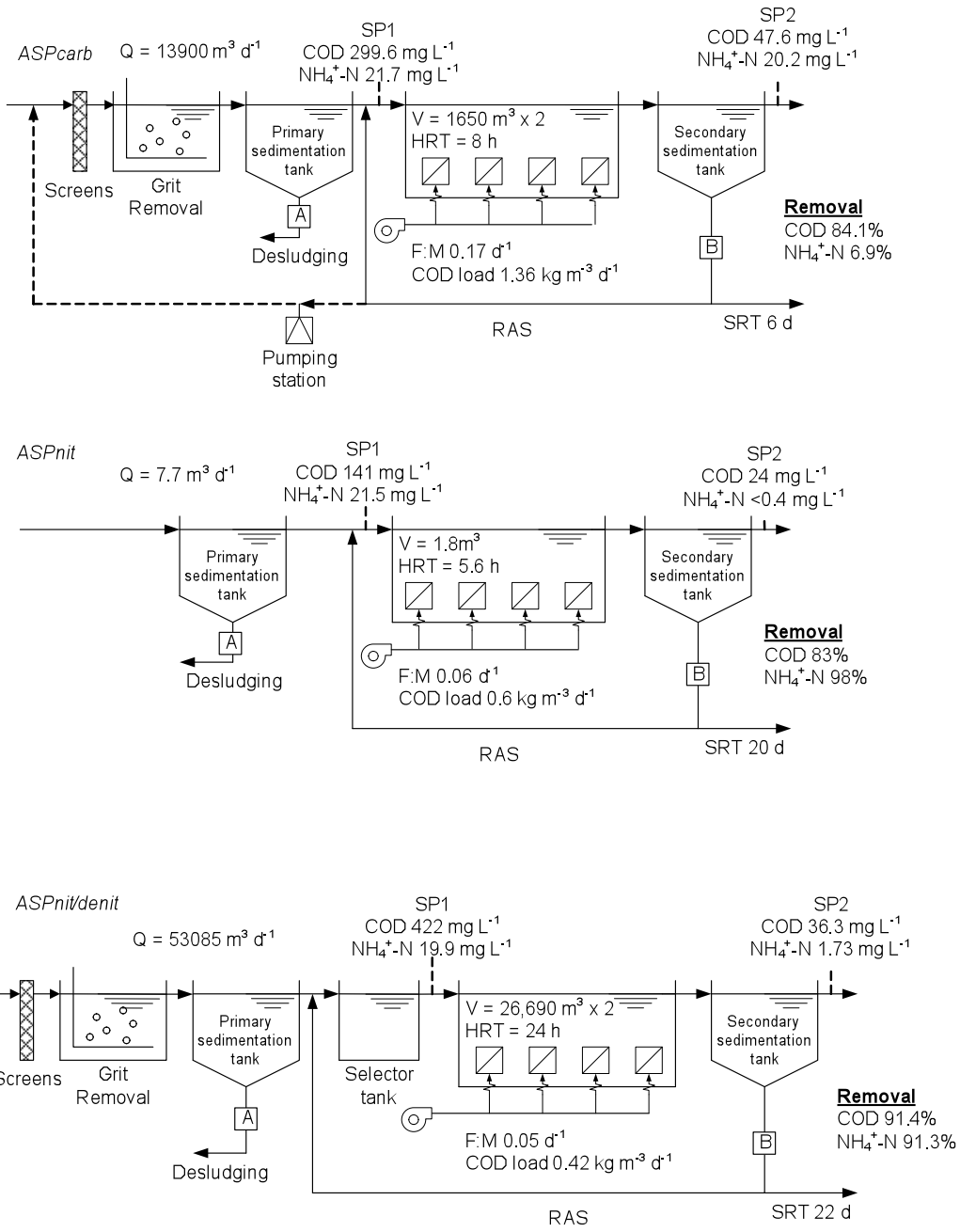


Figure 4-1 The Process Configuration and Operation Parameters of ASP_{carb}, ASP_{nit} and $ASP_{nit/dinit}$ and the Location of the Sampling Point. The samples taken were: settled sewage (SP1); effluent (SP2); primary sludge (A); and WAS/RAS (B)

4.2 Determining the influence of loading, HRT and SRT upon the removal of estrogen

4.2.1 Preparation of Synthetic Sewage

To determine the role of nitrification for the removal of EDCs it was necessary to remove ammonia during the final spiking period and replace with nitrate. Therefore the carbon sources used were solely carbohydrate as more complex COD contains nitrogen and could have contributed to the available ammonia. Synthetic wastewater (COD circa 500 mg L^{-1}) was made daily by preparing the carbon source, basic media and 190mL of a trace mineral solution separately and adding to 75L of tap water. The carbon sources were heated and dissolved prior to mixing with other components. The precise formula required to make the synthetic sewage is shown in table 4-1. The formula and proportion of the trace mineral solution is detailed in Table 4-1.

Table 4-1 The proportions and formula of each component used to prepare 75L of synthetic sewage

Component	Amount per 75L of synthetic sewage	Element	Ratio
Carbon Source			
Soluble starch	25.32	C	75
Golden syrup	11.41	C	25
Basic Media			
K_2HPO_4	0.86	K	1.14
KH_2PO_4	0.32	K	0.49
$MgSO_4\ 7H_2O$	6.00	Mg	
$CaCl_2\ 2H_2O$	7.00	Ca	
$NaHCO_3$	2.67		
EDTA	1.00		
Nitrogen Source			
NH_4Cl[a]	6.96	N	12.16
KNO_3[b]	13.16	N	12.16

[a] NH_4Cl was used as the nitrogen source for spiking periods 1-5 [b]KNO_3 was used as the nitrogen source during spiking period 6 (no ammonia spiking period)

Table 4-2 The chemical constituents used to prepare 1L of trace minerals

Trace mineral	Concentration g L^{-1}
$FeCl_3.6H_2O$	1.5
H_3BO_3	0.15
$CuSO_4.5H_2O$	0.03
KI	0.03
$MnCl_2.4H_2O$	0.12
$Na_2MoO_4.2H_2O$	0.06
$ZnSO_4.7H_2O$	0.12
$CoCl_2.6H_2O$	0.15

4.2.2 Configuration of porous pot bioreactors

A pilot scale arrangement of six porous pot bioreactors was used to replicate wastewater treatment conditions (Water Research Centre, UK). Each pot consisted of an impermeable outer pot and a porous highdensity polyethylene inner membrane with a pore size 65-90μm that contained the activated sludge. The capacity of each pot was 3.8L and was fed with a constant flow of synthetic sewage (Figure 4-2). The experiments conditions were defined as **Low HRT/SRT** and **High HRT/SRT** and are described in Table 4-3

Table 4-3 The Process Parameters for the Low HRT/SRT and High HRT/SRT experimental conditions

Process Parameter	Process Condition	
	Low HRT/SRT	High HRT/SRT
HRT (hours)	5	20
SRT (days)	3	20
Flow rate (L hr^{-1})	0.76	0.19
Sludge wastage rate (L d^{-1})	1.26	0.19

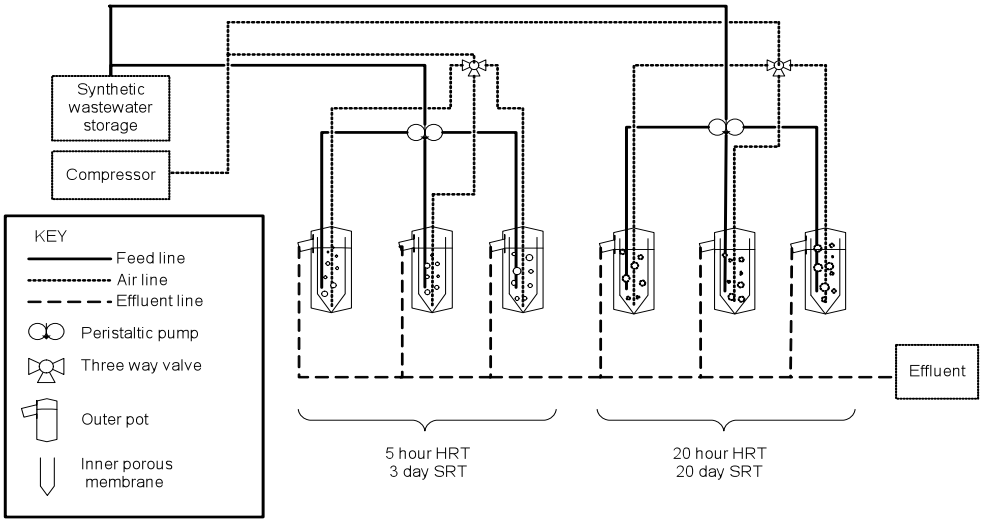


Figure 4-2 The Process configuration and operational parameters of the porous pot experiments

4.2.3 Estrogen and Loading Experiments

Nitrifying mixed liquors were collected from a nitrifying ASP. Each pot was seeded with 10% of its volume with the mixed liquors. The pots were fed for five days without wastage in order to grow up the biomass. Measurements of temperature, dissolved oxygen, COD, NH_4^+ and MLSS were taken five days per week. Spiking with estrogens began when COD removal was on average >80% and >90% for the Low HRT/SRT and High HRT/SRT conditions respectively and MLSS concentration had stabilized and was >1.5 and >1.8g L^{-1} for the Low HRT/SRT and High HRT/SRT respective conditions. The mean concentration and their proportions, of estrogens from five UK STWs were used as the basic formula for the EDC loading experiments (Table 4-4). This ratio of estrogens was kept constant during the experimentation.

Table 4-4 The average concentration of estrogens from five UK ASPs

Compound	Concentration ng L^{-1}
E1	27
E2	10
E3	41
EE2	1
E1-3s	11
Σ EST	89

Averages taken from Koh *et al* (2009), McAdam *et al* (2010)

To assess the impact of estrogen loading upon biomass activity and removal efficiency, the concentration of EDCs was increased from 42 to 667 ng L^{-1} over five spiking periods. Thus achieving a range of total estrogen (Σ EST) loadings of 0.05 – 0.8 mg m^3 d^{-1} and 0.2 – 3.2 mg m^3 d^{-1} for Low HRT/SRT and High HRT/SRT conditions respectively (see Table 4-5). Each spiking period lasted for one week, with the no ammonia spiking period (NASP), which ran for 21 days to ensure nitrifying bacteria had significantly reduced in number. Samples from each of the six pots were taken on days 1, 2, 3, 5, 7, (14 and 21 for the NASP). Samples were collected in glass borosilicate jars and extracted onto solid phase extraction (SPE) cartridges within one hour of collection. Duplicates and spike recovery were done daily to ensure analytical robustness and to check that recovery rate and MDLs were comparable with those of Koh *et al* (2007).

Table 4-5 The concentration and loading of Σ EST (E1, E2, E3, EE2, E1-3) during the each spike period

Spiking period	Σ EST		
	Concentration ng L^{-1}	HRT 5 Loading mg m^3 d^{-1}	HRT 20 Loading mg m^3 d^{-1}
1	42	0.05	0.2
2	83	0.1	0.4
3	167	0.2	0.8
4	333	0.4	1.6
5	667	0.8	3.2
NASP	667	0.8	3.2

NASP is No ammonia spiking period

4.2.4 Quantification of the ammonia and nitrite oxidising bacterial component of nitrifying sludge

Immuno latex Kenshutsukun kits - two given as gifts from the Department of Civil Engineering Iwate University, and two purchased from Yakult Central Institute for Microbiological Research, Yakult Honsha Co. Ltd (Tokyo Japan) - were used for the quantification of nitrifying bacteria in activated sludge. Bacterial quantification involved the use of polyclonal antibodies for ammonia oxidising and nitrite-oxidizing bacteria sorbed to latex particles, which were provided in the kit. Enumeration of nitrifying bacteria was done according to the manufacturer's protocol, which advised homogenisation of the activated sludge prior to the immuno assay. Homogenisation was performed using the methodology of Ziglio *et al* (2002). 150mL of activated sludge was homogenised using an IKA T25 digital Ultra Turrax (Wolf Laboratories, York, UK) for 4 minutes. Subsequently, 5mL aliquots were further disaggregated for 10 minutes.

Processed sludge was diluted serially by a factor of two for 10-15 diluted depending upon the expected autotrophic bacterial concentration. The kits were used to determine abundance of both ammonia and nitrite oxidising bacteria. The test for ammonia and nitrite oxidising bacteria only varied in the immuno latex reagent used. A volume of 0.5 mL of each serial

dilution were subsequently pipetted onto a 96 well microtiter plate, and one drop (127 μL) immuno latex reagent was added and incubated for 4–30 hours at room temperature. The presence of coagulations indicated a positive result. Enumeration, was done by determining the highest dilution where coagulation could still be detected. Concentration was calculated using following equation.

$C=dk$

Where:
c = concentration of nitrifying bacteria (mL^{-1})
d = the maximum dilution factor where coagulated could be detected
$k = 2x10^5$ (The titer value of the antibody provided by the kit)

These tests were done weekly; from before estrogen spiking began to the end of the experiment. Each week waste activated sludge (WAS) from each porous pot was used to calculate ammonia and nitrite oxidising bacteria abundance. The limit of detection for ammonia oxidising bacteria (AOB) was $2x10^5$ mL^{-1}. The limit of detection for nitrite oxidising bacteria (NOB) was $2x10^4$ mL^{-1}. Details of accuracy and errors associated with this test were not available. However, Konuma *et al* (2001) compared this test with other techniques of the enumeration of ammonia oxidising bacteria including, most probable number (MNP), fluorescence *in situ* hybridization (FISH) and dot plot hybridization. These authors commented that the antibody technique gave consistent results with dot plot hybridization.

4.3 Reagents and Chemicals

Solvents and eluents

HPLC-grade organic solvents, dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), hexane and ethyl acetate (EA) were purchased from Rathburn Chemicals (Walkerburn, UK). Ammonium hydroxide (30-33% NH_3 in H_2O), ammonium acetate (≥99%), triethylamine (>99%) and acetic acid (≥99.99% purity) were purchased from Sigma-Aldrich (Dorset, UK).

Estrogens

All estrogen standards (>98% chemical purity) were purchased from Sigma Aldrich (Dorset UK). Deuterated ($d_{3/4/5}$) labeled internal standards of 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-ethinylestradiol-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 C/D/N Isotopes (QMX Laboratories, Thaxted, UK) with >98% chemical purity. Stock solutions were prepared in MeOH. The solid phase extraction cartridges tC18 (500mg/6cc) and silica (500mg/3cc) were obtained from Waters (Watford, UK) and the aminopropyl (NH_2) anion-exchange (500mg/6cc) was obtained from Varian (Oxford, UK).

Nonylphenolic compounds

The technical 4-nonylphenol mixture of various chain isomers and were obtained from Sigma-Aldrich. The long-chain NPEO (Igepal CO210, CO520, CO720) and were available in a commercial surfactant mixture containing different oligomers also purchased from Sigma-Aldrich. Nonyl-phenoxy acetic acid (NP_1EC), 4-nonylphenol-diethoxylate ($NP_{1-2}EO$) were obtained from QMX Laboratories. Standards for NP_2EC, and NP_3EC, were not available commercially and these determinants were therefore estimated by their respective NP_1EOs. Stock solutions were prepared in ACN. Single standard stock solutions of the analytes in the high mg L^{-1} range were prepared by weighing out milligram amounts of the compounds and dissolving in ACN. Reagent grade MilliQ water (18.2 MΩ) (Millipore, Watford, UK) was used for spikes and preparation of solutions. The working standard solutions were prepared by further diluting the stock standard solutions with acetonitrile/MilliQ-water (50:50 v/v). The solid phase extraction cartridges tC18 (500mg/3cc) and silica (500mg/3cc) were obtained from Waters (Watford, UK).

Synthetic Sewage

The following reagents used to make synthetic sewage: K_2HPO_4; KH_2PO_4; $MgSO_4.7H_2O$; $CaCl_2.2H_2O$; NH_4Cl; KNO_3; EDTA; $FeCl_3.6H_2O$; H_3BO_3; $CuSO_4.5H_2O$; KI; $MnCl_2.4H_2O$; $Na_2MoO_4.2H_2O$; $ZnSO_4.7H_2O$ and $CoCl_2.6H_2O$, were purchased from Fisher Scientific (Loughborough, UK).

4.4 Reference Standard Preparation

Estrogens

Standard solutions prepared from stock solutions were used to calibrate the response of the HPLC-MS-MS. Stock solutions were made by dissolving 1 mg of each compound (deuterated or non-deuterated) with 10 mL MeOH in a volumetric flask. Intermediate (1 μg mL^{-1}) solutions were prepared (10 μL of each stock solution to a 10 mL volumetric flask with MeOH/H_2O (10:90 v/v). Calibration standards containing all 5 analytes in MeOH/H_2O (10:90) were used to produce an eight-point calibration at 1 – 100 ng mL^{-1} and the concentration of the deuterated internal standards was 75 ng mL^{-1}.

Nonylphenolic compounds

Pure polyethoxylate compounds were not commercially available; therefore compounds with an average number of ethoxylate groups were used. The values derived for the average number of ethoxylate groups are numerical averages based on the surfactant molar distribution. Conversion from molar distributions to mass distributions, was achieved by scaling the molar concentration of each oligomer by its molecular mass and then normalizing the results so their sum was equal to one. Working standard solutions were prepared from stock solutions and used to calibrate the response of the HPLC-MS-MS. Standard stock solutions of 1 mg of each compound were dissolved into a 10 mL volumetric flask with

ACN. Intermediate solutions of (1 μg mL^{-1}) were prepared (10 μL of each stock solution to a 10 mL volumetric flask containing acetonitrile/H_2O (50:50 v/v). As individual standards were available for NP, $NP_{1-2}EO$ and NP_1EC, these were combined to give 5 mg L^{-1} mixed solutions. Calibration was performed using an eight point calibration curve at 0.01 – 25 mg L^{-1} $NP_{3-12}EO$. Calibration was performed using an eight point calibration curve at 0.01 – 5 mg L^{-1} for NP, NP_1EC and $NP_{1-2}EO$.

4.5 The Analytical Procedure for Aqueous and Solid Sewage Matrices

4.5.1 Estrogens

The natural and synthetic estrogens: estrone (E1); 17β-estradiol (E2); estriol (E3); sulphate conjugate of estrone (E1-3S); and 17α-ethinylestradiol (EE2) were extracted from aqueous and adsorbed matrices and then quantified using the methodology of Koh *et al* (2007). The extraction methodology is elaborated upon below. The analytical methodology used for the quantification of estrogens is elaborated upon in section 4.6.

Aqueous samples

Aqueous samples (1L) including settled sewage, effluent and decant sludge liquors were filtered through GF/C filters (VWR International, Leicestershire, UK) prior to solid phase extraction (SPE). Settled sewage and effluents solids retained on filters, were frozen and freeze dried for extraction later (see section ***Solid samples***). The filtrate were spiked with 0.15mL of a 0.1 μg mL^{-1} methanolic solution of mixed deuterated estrogens and loaded onto tC18 cartridges (Waters Ltd, Hertfordshire UK) preconditioned with 5mL MeOH followed by 5mL MQ water. The flow rate was kept between 5-10mL min^{-1} under vacuum using a SPE manifold. When the sample had been loaded the cartridges were washed with 5mL of MQ water and then dried thoroughly under vacuum for 30-60 minutes prior to elution. Estrogens, were eluted using 10mL MeOH followed by 10mL DCM. The eluate were dried under rotary

evaporation (Heidolph Instruments, Kelheim, Germany) and then reconstituted with 0.2mL DCM/MeOH (9:1 v/v) prior to gel permeation chromatography (GPC), which was performed using a PLgel column, 5µm 50Å, 300 x 7.5 mm (Varian, Oxford, UK). Conjugated and unconjugated steroids were collected in a 6mL fraction collected between 5.5 and 11.5 minutes of a 15 minutes isocratic elution ran with DCM/MeOH (9:1 v/v) at 1 mL min^{-1}. This fraction was dried using nitrogen to approximately 0.2mL. This was reconstituted to 2 mL with hexane and loaded onto a NH_2 SPE (Varian, Oxford, UK) cartridge preconditioned with 2 mL hexane at a flow rate between 5-10 mL min^{-1}. This was then washed with 4mL 10% ethylacetate (EtOAc)/hexane (1:9 v/v). Non-polar steroids E1, E2 and EE2 were then eluted using 6mL EtOAc. The polar estrogens E1-3S and E3 were subsequently eluted in a second fraction using 6mL 3% NH_4OH in methanol. The separate eluates were dried under rotary evaporation reconstituted with 0.2 mL MeOH/H_2O (10:90 v/v) and transferred to auto-sampler vials prior to analysis using HPLC-MS-MS.

Sludge liquors

Sludge liquors were distributed between 4 x 250mL Teflon bottles and centrifuged at 4700 RCF for 10 minutes. The 1 litre aqueous phase from the sample bottle was decanted prior to filtration. The solid phase from the bottle was transferred by spatula into 50 mL centrifuge tubes, labelled and frozen. The estrogens in the dissolved phase were extracted and analyzed as above (see ***Aqueous samples***).

Solid samples

For sludge or solids retained on filters, a multi-reax system (Heidolph UK, Germany) was used to extract estrogens. Freeze-dried sludge (0.1g) and filter papers associated with 1L of settled sewage or effluent were extracted using 10-20 mL EA and mechanically shaken for an hour in a 25mL Teflon tube. This procedure was repeated twice with centrifugation at 4700 RCF for 10 minutes each time. The supernatant was decanted and dried to approximately 0.2mL under rotary evaporation. This was reconstituted to 2 mL with hexane and loaded onto a Silica 500mg/3cc SPE cartridge (Waters, Hertfordshire, UK) conditioned with 2 mL hexane

at a flow rate between 5-10 mL min^{-1}. The cartridges were not allowed to dry and were eluted using 3mL EA followed by 2mL MeOH whilst keeping the solid phase media saturated. The purified sample was then subjected to further clean up by GPC, anion-exchange and then quantified by HPLC-MS-MS as described above (see section ***Aqueous samples***).

4.5.2 Nonylphenolic compounds

The nonylphenolic compounds (NPx): nonylphenol; nonylphenol polyethoxylates ($NP_{3-12}EO$); nonylphenol mono and di ethoxylate ($NP_{1-2}EO$) and nonylphenol mono and di carboxylate ($NP_{1-2}EC$) were determined by the methodology of Koh *et al* (2008) based on that of Langford *et al* (2005b). These compounds were determined in solid and aqueous phases for all samples. The extraction methodology is elaborated upon below. The analytical methodology used for the quantification of estrogens is elaborated upon in section 4.6.

Aqueous samples

Aqueous samples (1L) including settled sewage, effluent and decant sludge liquors were filtered through GF/C filters (VWR International, Leicestershire, UK) prior to solid phase extraction (SPE). Settled sewage and effluents solids retained on filters, were frozen and freeze dried for extraction later (see ***Solid samples***). Filtrate aliquots of 100 mL settled sewage and sludge liquors and 250mL of effluent we loaded onto tC18 cartridges preconditioned with 5mL MeOH followed by 5mL MQ water. The flow rate was kept between 5-10 mL min^{-1} under vacuum using an SPE manifold. When the sample had been loaded the cartridges were washed with 5mL of MQ water and then dried thoroughly under vacuum for 30-60 minutes prior to elution. The NPx of the dissolved phase were eluted using 10 mL EA, 10 mL DCM followed by 5 mL 0.1% acetic acid in methanol and were dried using a rotary evaporation and reconstituted with 0.25mL ACN/MQ water (1:1 v/v) before being put into auto-sampler vials and analysed by HPLC-MS-MS.

Sludge liquors

Sludge liquors were distributed between 4 x 250mL Teflon bottles and centrifuged at 4700 RCF for 10 minutes. The aqueous phase from the sample bottle was decanted prior to filtration. The solid phase from the bottle was transferred by spatula into 50 mL centrifuge tubes, labelled and frozen. The NPx in the dissolved phase were extracted and analyzed as above (see section ***Aqueous samples***).

Solid samples

For sludge or solids retained on filters, the multi-reax system was used to extract NPx. Freeze-dried sludge (0.2 g) and filter papers associated with 1L of settled sewage or effluent were extracted using 10-20 mL MeOH/Acetone (1:1 v/v) and mechanically shaken for 30 minutes in a 25mL Teflon tube. This procedure was repeated twice with centrifugation at 4700 RCF for 10 minutes each time. The supernatant was decanted and dried to approximately 0.2mL under rotary evaporation. This was reconstituted to 2 mL with hexane and loaded onto a Silica 500mg/3cc SPE cartridge (Waters, Hertfordshire, UK) conditioned with 2 mL hexane at a flow rate between 5-10 mL min^{-1}. The cartridges were not allowed to dry and were eluted using 10mL MeOH with 10% acetic acid whilst keeping the solid phase media saturated. This purified sample was then dried using a rotary evaporation and reconstituted with 0.25mL ACN/MQ water (1:1 v/v) before being put into auto-sampler vials and analysed by HPLC-MS-MS.

4.6 Instrumental Analysis

Estrogens

Concentration of steroid estrogens were determined using HPLC-MS-MS consisting of an HPLC (Waters Alliance HPLC system 2695) coupled to a Waters Quattro Premier XE mass spectrometer with a Z-Spray ESI source (Micromass, Watford, UK). The steroids were separated on a Gemini C18 column (3µm particle size, 100mm x 2mm i.d., Phenomenex, Cheshire, UK). The mass spectrometer was operated in the negative electrospray ionisation mode using multiple reaction monitoring (MRM). The desolvation gas was nitrogen and the collision gas was argon. The conditions for detection by the mass spectrometer were as follows: capillary voltage, 3.20kV; multiplier voltage, 650V; desolvation gas flow, 1000 l h^{-1}; cone at -55V; RF lens at 0.2V; cone gas flow at 49 l h^{-1}; desolvation temperature at 350°C and source temperature at 120°C.

Chromatographic separation of the estrogens was achieved using two solvents, solvent (A) water containing 0.1% ammonium hydroxide (NH_4OH) and solvent (B) MeOH containing 0.1% NH_4OH. Gradient conditions were initiated with 20% B followed by an increase to 50% B (over 3.5 min). The proportion of solvent B was then increased to 60% maintained for 9 min before the column was returned to starting conditions 20% B (over 3 min) and held for 2.5 min to equilibration. The total run time was 18 min and a sample volume of 20µl of was injected into the HPLC. Instrument control, data acquisition and evaluation were performed with MassLynx software 4.1 (Waters Ltd, Hertfordshire, U.K.).

The optimal conditions necessary (such as dwell time, cone and collision voltage) required to achieve the specific mass transitions for quantfication and confirmation for the estrogens are detailed in Table 4-5. The method detection limits (MDLs) recoveries and relative standard deviations (RSDs) of the methodology are listed in Table 4-7. The estrogen method was robust and demonstrated to achieved recovery rates >80% and RSDs <10% for all estrogens in both aqueous and solid phases (Koh *et al* 2007).

Table 4-5 The HPLC-MS-MS conditions for MRM chromatographic separation and detection of the estrogens

Compounds	*m/z* precursor	Dwell time (msec)	Cone (V)	Collision energy (eV)	Retention time (min)
E1	[a]269.10>144.85	85	70	40	13.97
	[b]269.10>158.80	85	70	45	
E2	[a]271.10>144.85	85	60	45	14.37
	[b]271.10>158.80	85	60	40	
E3	[a]287.10>170.85	95	55	50	8.90
	[b]287.10>144.85	95	55	50	
EE2	[a]295.15>144.85	85	60	40	14.67
	[b]295.15>158.80	85	60	40	
E1-3S	[a]349.05>144.85	60	50	65	6.77
	[b]349.05>269.00	60	50	40	
E1-d_4	[a]273.10>146.85	85	60	45	13.91
E2-d_5	[a]276.10>146.85	85	55	50	14.23
E3-d_3	[a]290.15>146.85	90	50	65	8.86
EE2-d_4	[a]299.15>146.85	85	60	50	14.60
E1-3S-d_4	[a]353.10>146.85	60	50	65	6.75

[a]MRM used for quantification (primary ion); [b]MRM used for confirmation (daughter ion)

Nonylphenolic compounds

Analytes were determined using HPLC-MS-MS as for the estrogens above. The NP, NPEC and NPEO were separated on a Gemini C18 column (3µm particle size, 100mm x 2mm i.d., Phenomenex, Macclesfield, U.K.). The mass spectrometer was operated in the negative electrospray ionisation (ESI^-) (i.e. NP and NPEC) or positive electrospray mode (ESI^+) (i.e. NPEO) using multiple reaction monitoring (MRM). The conditions for detection by the mass spectrometer were as follows, capillary voltage, 3.20kV in the positive mode and -2.3kV in the negative mode, extractor lens at 3.0V, RF lens at 0.5 V in the positive mode and 1.0V in the negative mode, multiplier voltage, 650V, desolvation gas flow, 1000 l h^{-1}, cone at 50V, cone gas flow at 50 l h^{-1}, desolvation temperature at 350°C and source temperature at 120°C. The applied analyser parameters for MRM analysis were LM 1 and HM 1 resolution 11.0, ion energy 1 1.0, entrance 1 (negative mode), 2 (positive mode), exit 0, LM2 and HM 2 resolution 10.0, ion energy 2 1.0. The MRM inter-channel delay was 0.05 and the inter-scan delay 0.02.

Chromatographic separation of the NPx was achieved using a Gemini C18 column (Phenomenex, Macclesfield, UK). A gradient separation was achieved using two solvents: solvent A, MQ water containing 20mM NH_4OH; solvent B, ACN containing 20mM NH_4OH.

For the separation of $NP_{3-12}EO$, gradient conditions were initiated with 45% solvent B (maintained for 5 min) followed by a gradual increased gradient to 80% solvent B which was maintained for 40 min. Following this the gradient was again increased from 80 to 90% solvent B, the conditions were maintained at 90% solvent B for 5 min before the column was re-equilibrated to starting conditions at 20% solvent B. The total run time was 50 min and 10µl of each sample was injected into the HPLC. For the separation of NP, $NP_{1-3}EC$ and $NP_{1-2}EO$, gradient condition was initiated with 20% solvent B and linearly increased to 45% over 10 min. This was followed by a gradual linear increased to 80% solvent B which was maintained for 20 minutes. Following the increase gradient from 80 to 90% solvent B, the conditions were maintained at 90% solvent B for 5 min before the column was re-equilibrated to starting conditions at 20% solvent B for 10 min prior to next run. Similarly, the total run time for this analysis was 50 min and sample volume injected was 10 µl. The flow rate was kept at 0.2 ml min^{-1}.

The optimal conditions necessary (such as dwell time, cone and collision voltage) required to achieve the specific mass transitions for quantification and confirmation for the NPx are detailed in Table 4-6. The method detection limits (MDLs) recoveries and relative standard deviations (RSDs) are listed in Table 4-7. The NPx methodology was robust and was demonstrated to deliver >65%, >69% and >55% recovery rates for settled sewage, final effluent and sewage sludge solids respectively (Koh *et al* 2008). The RSDs for the NPx were ≤10%.

Table 4-6 The HPLC-MS-MS conditions for MRM chromatographic separation and detection of the NPx

Compounds	*m/z* precursor	Dwell time (msec)	Cone (V)	Collision energy (eV)	Retention time (min)
NP_3EO	[a]370.0>353.0 [b]370.0>226.5	100	40	10 20	27.87
NP_4EO	[a]414.0>397.0 [b]414.0>270.0	90	30	14 20	27.71
NP_5EO	[a]458.0>440.0 [b]458.0>315.0	90	30	20 25	27.63
NP_6EO	[a]502.0>485.0 [b]502.0>359.0	90	30	20 30	27.47
NP_7EO	[a]546.0>529.0 [b]546.0>403.0	90	30	23 25	27.23
NP_8EO	[a]634.0>617.0 [b]634.0>335.0	90	30	25 27	27.07
NP_9EO	[a]634.0>617.0 [b]634.0>335.0	90	30	25 30	26.82
$NP_{10}EO$	[a]378.0>661.0 [b]378.0>132.5	90	30	25 40	26.66
$NP_{11}EO$	[a]772.0>705.0 [b]772.0>291.0	90	30	25 40	26.42
$NP_{12}EO$	[a]766.0>749.0 [b]766.0>291.0	90	30	30 35	26.26
NP_1EO	[a]282.0>127.0 [b]282.0>265.0	120	25	25 25	29.11
NP_2EO	[a]326.0>183.0 [b]326.0>121.0	100	40	40 40	28.88
NP_1EC	[a]263.5>205.0 [b]263.5>106.0	100	20	20 30	11.32
NP_2EC	[a]307.0>205.0	100	20	20	11.32
NP_3EC	[a]351.1>205.0	100	20	20	11.32
NP	[a]219.0>132.5 [b]219.0>147.0	100	30	30 35	10.87

[a]MRM used for quantification (primary ion); [b]MRM used for confirmation (daughter ion)

Table 4-7 The percent recovery efficiency, relative standard deviation (RSD) and method detection limits for estrogen and nonylphenolic compounds analysed by HPLC-MS-MS

Compounds	Percent recovery efficiency (RSD)			Method detection limits	
	Aqueous phase Settled Sewage	Aqueous phase Final Effluent	Solid phase Sewage sludge	Aqueous Phase	Solid Phases
E1	[a]95 (4)	[b]88 (3)	[c]100 (2)	[g]0.1	[h]2.1
E2	[a]88 (1.6)	[b]88 (4)	[c]99 (1)	[g]0.2	[h]4.6
E3	[a]98 (0.3)	[b]86 (6)	[c]84 (2)	[g]0.2	[h]4.2
EE2	[a]88 (5)	[b]83 (5)	[c]100 (1)	[g]0.2	[h]4.6
E1-3S	[a]95 (4)	[b]99 (1)	[c]100 (3)	[g]0.1	[h]2.5
NP_3EO	[d]90(7)	[e]99(8)	[f]83(2)	[i]0.1	[j]0.5
NP_4EO	[d]67(7)	[e]74(2)	[f]84(8)	[i]1.5	[j]7.5
NP_5EO	[d]61(2)	[e]66(4)	[f]76(10)	[i]2.1	[j]10
NP_6EO	[d]65(5)	[e]64(8)	[f]62(10)	[i]2.7	[j]13
NP_7EO	[d]63(2)	[e]66(7)	[f]77(7)	[i]0.8	[j]4
NP_8EO	[d]63(2)	[e]62(2)	[f]67(9)	[i]0.7	[j]4
NP_9EO	[d]63(2)	[e]62(2)	[f]84(5)	[i]2.2	[j]11
$NP_{10}EO$	[d]61(2)	[e]68(7)	[f]77(7)	[i]2.1	[j]10
$NP_{11}E$	[d]63(2)	[e]65(2)	[f]71(3)	[i]0.9	[j]4.5
$NP_{12}EO$	[d]72(2)	[e]68(2)	[f]81(9)	[i]1.1	[j]5.5
NP_1EO	[d]70(5)	[e]72(2)	[f]79(8)	[i]1.2	[j]6
NP_2EO	[d]62(7)	[e]60(3)	[f]52(5)	[i]2.5	[j]12
NP_1EC	[d]77(2)	[e]81(2)	[f]66(5)	[i]2.5	[j]12
NP_2EC	[d]66(4)	[e]89(2)	[f]53(8)	[i]NA	[j]NA
NP	[d]72(2)	[e]81(2)	[f]55(8)	[i]2.3	[j]11

a1L of settled sewage spiked with 15ng of each estrogen (n=3); b1L of final effluent spiked with 15ng of each estrogen (n=3); c0.1g of freeze dried seage sludge spiked with 15ng of each estrogen (n=3); d100mL of settled sewage spiked with 250ng of each NPx (n=3); e250mL of final effluent spiked with 250ng of each NPx; f0.2g of sewage sludge spiked with 250ng of each NPx; [g]determined from spiked settled sewage; [h]detemined from 0.2g of sewage sludge; [i]determined from 250mL MQ water; [j]determined from 0.2 g of sewage sludge. Data taken from Koh *et al* (2007, 2008)

4.7 Physicochemical Determinants

BOD

Solids analysis

The analysis of liquids for total suspended solids (TSS) and sludge for MLSS and MLVSS were undertaken according to Standard Method 2540D (APHA, 1998) using Whatman GF/C (1.2µm pore size) filters (VWR International, Leicestershire, UK).

COD

The analysis of COD was carried out using Merck Spectroquant COD cell test having the range of 25 – 3500 mg L^{-1} (VWR, Leicestershire, UK) using the method analogous to Standard Method 5220D (APHA, 1998) and values are reported as mg L^{-1} O_2.

Ammonium (NH_4^+)

The analysis of ammonium was carried out using Merck Spectroquant Ammonium cell test having a range of 0.01 – 80 mg L^{-1} NH_4-N (VWR, Leicestershire, UK) using the method analogous to Standard Method 4500-NH_3D (APHA, 1998) and values are reported as mg L^{-1} NH_4-N.

Nitrate (NO_3^-)

Nitrate concentration was carried out using Merck Spectroquant nitrate cell test 0.5 – 50 mg L^{-1} NO_3-N (VWR, Leicestershire, UK) using the method analogous to (ISO7890/1, 1985) and values are reported as mg L^{-1} NO_3-N.

pH

The pH measurements were carried out a Hanna HI 8424 handheld pH meter (Hanna Instruments Ltd, Leighton Buzzard, UK). The meter was calibrated prior to use each day prior to use.

Temperature

The temperature of the wastewater and the ambient temperature were measured using a mercury thermometer –10 to +110°C (VWR International Ltd, UK).

Dissolved oxygen

The dissolved oxygen (DO) was determined using the Hach HQ20 meter equipped with a Luminescent Dissolved Oxygen (LDO®) probe (Hach Lange Ltd, Manchester, UK).

Food to microorganisms ratio

(F/M) ratio is a process parameter to characterize process designs and operating conditions. It is also the reciprocal of the sludge age. The calculation to determine F/M ratio for the STWs can be found in Equation 4-1

Log K_p

Log K*p* is a process parameter that characterizes the partitioning of a compound between aqueous and solids phases. Log K*p* =((ng chemical sorbed/kg biomass))/(ng chemical dissolved)

Equation 4-1 The calculation of food to microorganism ratio

$$F:M = \frac{BOD_{\text{inf}} \times Q}{MLVSS \times Vol}$$

Chapter 5

Estrogen Biodegradation at Carbonaceous, Nitrifying and Nitrifying/Denitrifying Activated Sludge Treatment Plants

5 Estrogen Biodegradation at Carbonaceous, Nitrifying and Nitrifying/Denitrifying Activated Sludge Treatment Plants

In order to assess which process parameters are key for the effective removal of estrogens during activated sludge treatment, field based sampling were performed at three separate ASP. Sampling for nonylphenolic compounds was done concurrently with the estrogens. Five day sampling was carried out at four to six hour intervals. Samples were taken from settled sewage, final effluent and WAS/RAS. Both aqueous and solid phases were analysed using HPLC-MS-MS in order to accurately mass balance each ASP.

5.1 Operating parameters and general performance of the activated sludge plants

Field based sampling was carried out at two full-scale ASP (a carbonaceous ASP (ASP_{carb}) and a nitrifying/denitrifying $ASP_{nit/denit}$) and a medium-scale nitrifying ASP pilot plant (ASP_{nit}) to determine the impact of process type and operational parameters upon EDC biodegradation. The ASP_{carb} and $ASP_{nit/denit}$ were both sampled in spring 2008. The ASP_{nit} was sampled in late summer 2007. All three ASPs were designed for the removal of organic pollution BOD/COD and suspended solids. The ASP_{nit} and $ASP_{nit/denit}$, were designed additionally for the removal of ammonia and nitrate respectively and had typically long SRT, 20 and 22 days in that order. Since the ASP_{carb} was designed for carbonaceous removal only, its SRT was 6 days. The ASP_{carb} had an HRT of 8 hours. The ASP_{nit} has an HRT of 5.6 hours, considering its process type, typically less compared to 20.4 hours of the $ASP_{nit/denit}$. A summary of these process parameters and the general performance of the three ASP are detailed in Table (5-1).

The $ASP_{nit/denit}$ was the largest ASP (53085 m^3) and subsequently had the greatest average flow rate (53085 m^3 d^{-1}). The ASP_{nit} was a medium sized pilot plant (1.8 m^3) and had a lowest average flow rate of (7.7 m^3 d^{-1}). The ASP_{nit} had the highest ambient and sewage temperature of 17°C in comparison to the ASP_{carb} and $ASP_{nit/denit}$ as it was sampled during the summer. The highest average MLSS concentration was found at the ASP_{nit} (4520±363.4 mg L^{-1}), which was comparable to the ASP_{carb} (4328±1488 mg L^{-1}) but higher than the $ASP_{nit/denit}$ (2428±157.7 mg L^{-1}). F:M ratios were 0.17, 0.06 and 0.05 mg BOD^{-1} mg MLVSS d^{-1}, for the ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ respectively. The removal of COD and BOD was >79.5% and >76.6% respectively at all sites. Both the ASP_{nit} and $ASP_{nit/denit}$ fully nitrified, with ammonia removal >96%. The ASP_{carb}, had limited nitrification 8.84%, due to an SRT of 6 days. Removal of TSS was >77% at all sites. The average TSS (±1SD) were, 6.4±0.89, 16.2±8.8 and 12.80±8.90 g L^{-1} at the ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ respectively.

No precipitation occurred during the sampling of the ASP_{carb} and ASP_{nit} and estrogen occurrence represented a worst-case scenario. Heavy rain however, occurred during the first two days of sampling at the $ASP_{nit/denit}$ and would therefore have resulted in dilution of EDCs. Flow profiles of each ASP were relatively stable including the $ASP_{nit/denit}$, which received significant precipitation (Figure 5-1). This was due to the necessity to use storm tanks, which allowed the $ASP_{nit/denit}$ to maintain a normal flow profile. However, average flow rates at the $ASP_{nit/denit}$ demonstrated appreciable decrease from day three onwards 57395 to 46313 m^3 d^{-1}, presumably as the storm tanks had emptied after the heavy rain and flow to works was solely sewage once again.

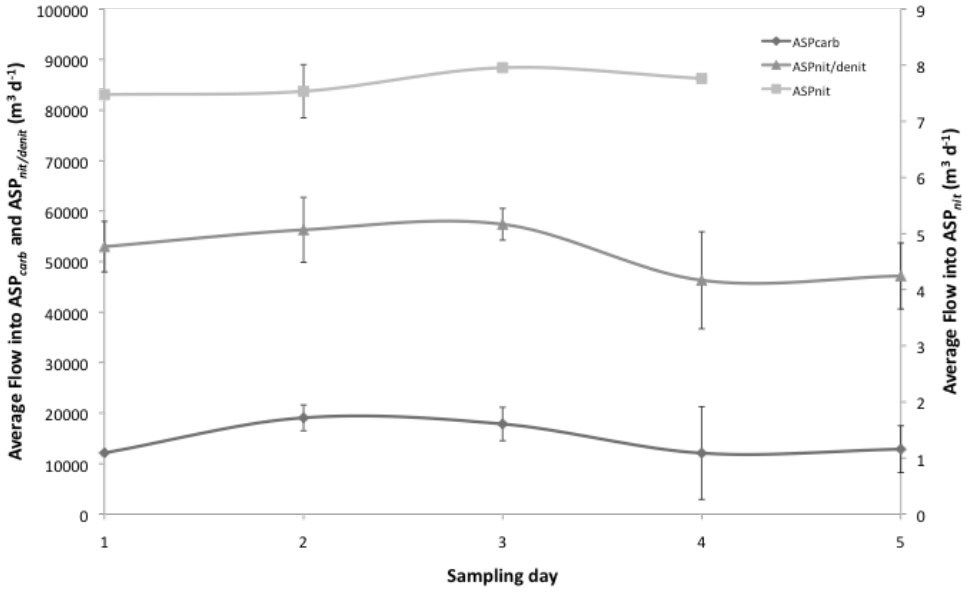


Figure 5-1 The daily average flow profiles at the ASP$_{carb}$, ASP$_{nit/denit}$ and ASP$_{nit}$ during sampling

Table 5-1 Process parameters and general performance of the three ASPs during sampling. Values displayed as averages during period of sampling

Process Variable		**ASP_{carb}**	**ASP_{nit}**	**$ASP_{nit/denit}$**
Biological Process		Carbonaceous	Nitrifying	Nitrifying/denitrifying
Total flow rate (m^3 d^{-1})		13900	7.7	53085
Volume (m^3)		3300	1.8	53380
HRT (h)		8	5.6	24
SRT (d)		6	20	22
DO (g m^{-3})	Eff**	6.0	6.5	8.0
MLSS (g m^{-3})		4328	4520	2428
F:M ratio (mg BOD^{-1} mg MLVSS d^{-1})		0.17	0.06	0.05
pH	SS*	7.5	7.5	7.4
	Eff**	7.7	7.8	7.4
Trade input		1-5%	<1%	<10%
Temperature (°C)	Ambient	10.4	17.0	5.7
	SS*	11.1	17.0	10.4
	Eff**	11.5	15.0	11.4
COD (g m^{-3})	SS*	326.9	279.6	423.0
	Eff**	51.7	57.3	35.8
	Removal %	83.8	79.5	89.6
BOD (g m^{-3})	SS*	137.1	92.1	153.4
	Eff**	9.3	16.9	35.8
	Removal %	93.4	79.6	76.6
NH_4^--N (g m^{-3})	SS*	21.7	21.5	20.5
	Eff**	20.0	0.4	0.7
	Removal %	8.8	97.9	96.1
TSS (g m^{-3})	SS*	80.6	84.8	107.6
	Eff**	6.4	16.2	12.8
	Removal %	91.4	80.3	83.9
NO_3^--N (g m^{-3})	SS*	3.3	0.39	0.4
	Eff**	2.8	30.9	7.0
	Removal %	15.1	-7742	-1650

*SS is settled sewage **Eff is effluent

5.2 The estrogen concentrations and partitioning of the settled sewage

Deconjugation of estrogens was occurring in the sewerage system. This was apparent due to the detection of unconjugated estrogens E1, E2, E3 and EE2 in the settled sewage. Estrogens were found principally in the aqueous phase (Figure 5-2). The proportion of which total estrogens were associated with the solids was 7.7, 4.7 and 12.7% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. ΣEST, log K_p values were, 3.7, 2.7 and 3.2 at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The most sorbent estrogen was EE2 at the $ASP_{nit/denit}$ and ASP_{nit}, 51.1 and 42.8% of EE2 respectively was found in associated with solids. Log k_p for EE2 at the $ASP_{nit/denit}$ and ASP_{nit} were respectively 4.0 and 4.3. However, at the ASP_{carb}, EE2 was associated 9.27% with the solids: less than the other sites and resultantly had a lower log k_p of 3.8 for this compound. At ASP_{carb}, the most adsorbent estrogen was E1, of which 29.7% was associated with the solids and had a log k_p for of 4.4 for this compound. Proportionally, E3 was the most abundant estrogen at all sites. At ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} E3 represented 34.7, 40.2 and 57.4% of total (aqueous and solids phase) estrogen. After E3, E1 was the second most abundant estrogen in settled sewage representing 25.4, 39.2 and 31.8% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively from the total of aqueous and solids phases.

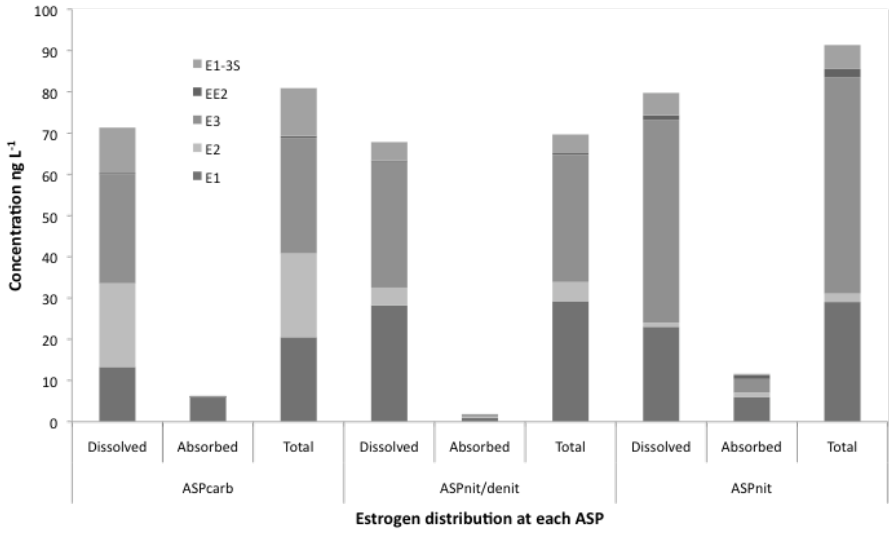


Figure 5-2 The average distribution in settled sewage of individual estrogens and their concentrations within dissolved, absorbed and total (dissolved plus adsorbed) at each ASP over the entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8

The daily average total (solid and aqueous) concentrations of individual estrogens in the settled sewage are detailed in Figure 5-3. There was no evidence of any correlation between average daily flow to ASP and settled sewage concentration. The $ASP_{nit/denit}$ had the most variable flow rate (Figure 5-1), however this did not translate to significant variation in estrogen concentration from day to day. On the contrary, day one at the $ASP_{nit/denit}$ had the highest E1 and E3 concentrations of 74.2 and 51.1 ng L^{-1} respectively although flow into works was relatively high, on average 52970 m^3 d^{-1}. In contrast, day three at the $ASP_{nit/denit}$ had the highest E1 and E3 concentrations of 16.7 and 18.0 ng L^{-1} respectively although flow into works was lowest, on average 46312.5 m^3 d^{-1}. Between days variation of individual estrogens could not be attributed to a specific pattern, highest solid plus aqueous average daily concentrations peaked on different days for individual estrogens at all three ASP. For example, at ASP_{carb}: the highest concentration of E1 (28.8 ng L^{-1}) was on day three; the highest concentration of E2 (24.5 ng L^{-1}) was on day four; the highest

concentration of E3 and E1-3S (31.3 and 13.7 ng L^{-1} respectively) was on day five and the highest concentration of EE2 (0.62 ng L^{-1}) was on day two. At $ASP_{nit/denit}$: the highest concentrations of E1, E3 and E1-3S (74.2, 51.1 and 13.1 ng L^{-1} respectively) were on day one; the highest concentration of E2 (8.8 ng L^{-1}) was on day two and the highest concentration of EE2 (0.9 ng L^{-1}) was on day four. At ASP_{nit}: the highest concentrations of E1, EE2 and E1-3S (52.2, 2.4 and 6.5 ng L^{-1} respectively) were on day one; the highest concentration of E2 (2.2 ng L^{-1}) was on day two and the highest concentration of E3 (55.0 ng L^{-1}) was on day three. As between days variation of individual estrogens could not be attributed to flow variation and appeared to be random, further qualification of settled sewage characteristics was done on data averaged over the entire sampling period.

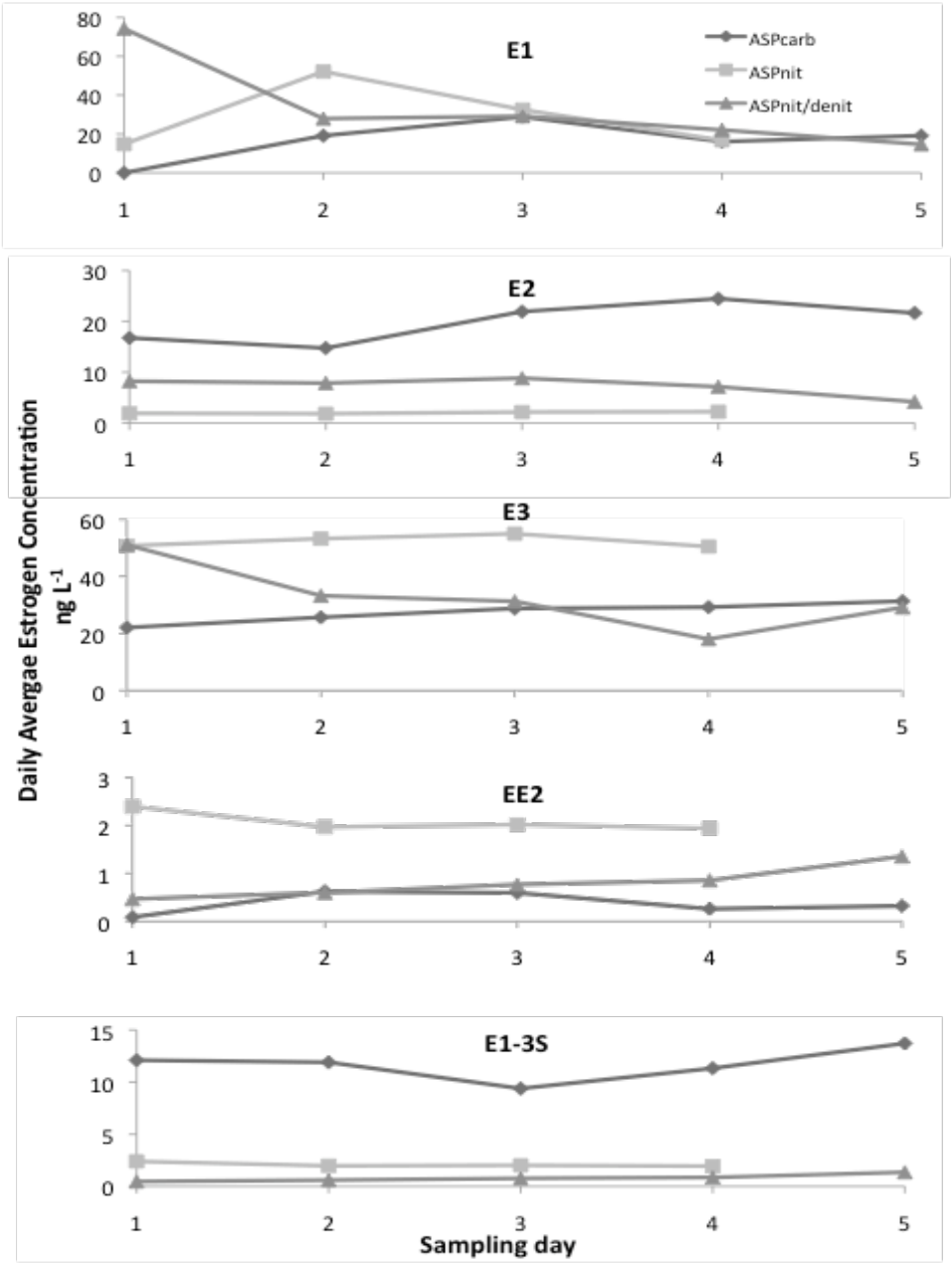


Figure 5-3 The average daily total (solid and aqueous) concentrations of individual estrogens in settled sewage. At ASP_{carb}, n=1 on day 1, n=5 on day 2, n=4 on day 3, n=5 on day 4, n=3 on day 5. At $ASP_{nit/denit}$, n=2 on day 1, n=5 on day 2, n=4 on day 3, n=5 on day 4 and n=2 on day 5. At ASP_{nit}, n=2 on day 1-4.

Similar total average ∑EST (sum of E1, E2, E3, E1-3S and EE2 in both dissolved and adsorbed phases) settled sewage concentrations of 80.1, 70.0 and 91.4 ng L^{-1}, were found at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively (Figure 5-4). The average total (adsorbed and aqueous phase) concentration of E1 was, 20.5, 29.2 and 29.1 ng L^{-1}, at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The average total (adsorbed and aqueous phase) concentration of E2 was, 20.3, 4.6 and 2.0 ng L^{-1}, at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The average total concentration of E3 was, 28.0, 30.9 and 52.1 ng L^{-1}, at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The average total concentration of EE2 was, 0.4, 0.4 and 0.9 ng L^{-1}, at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The average total concentration of E1-3S was, 11.6, 4.6 and 5.9 ng L^{-1}, at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Of the sites, $ASP_{nit/denit}$ had the greatest variability in estrogen concentrations. This was demonstrated by the greatest standard deviation of ∑EST (Figure 5-2). At $ASP_{nit/denit}$, the relative standard deviation (RSD) of average ∑EST was 46.2%, higher than ASP_{carb} and ASP_{nit}, whose respective equivalent RSDs were 15.7 and 18.9%.

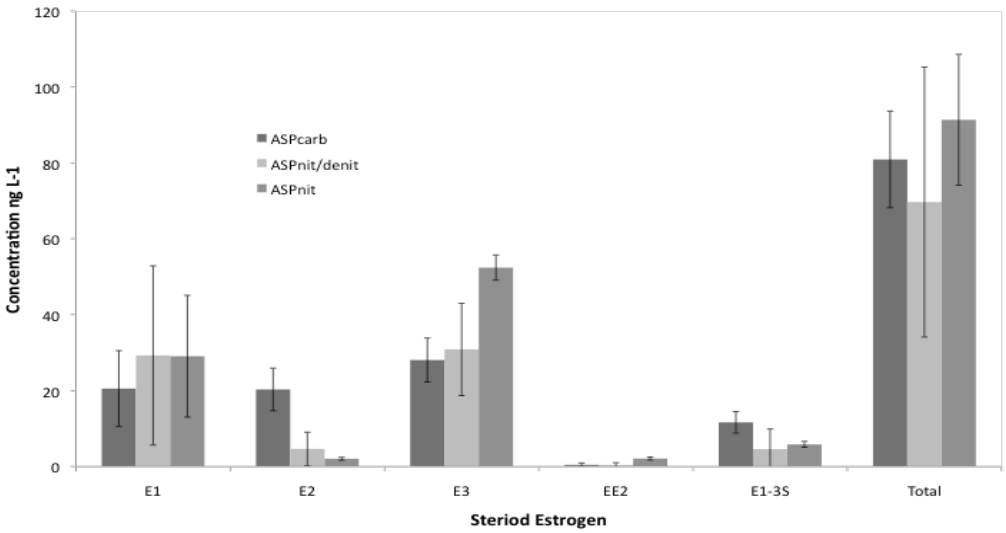


Figure 5-4 Average total (aqueous plus solids phase) ∑EST (E1, E2, E3, E1-3S and EE2 concentrations in settled sewage at the three ASP over entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8. Standard deviations displayed as bars.

5.3 The estrogen concentration and partitioning of the effluent

Estrogens were also found principally in the aqueous phase in the effluent (Figure 5-5). The proportion of total estrogens associated with the solids in the effluent was 10.2, 19.5 and 24.5% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. ΣEST, log K_p values were, 3.7, 3.2 and 3.5 at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Each site had different absorbency profiles. For the ASP_{carb}, the three most absorbent estrogens in the effluent were E1, E3 and EE2, which were associated with the solid, 29.0, 17.8 and 3.1% respectively. At the ASP_{carb} the log K_p values for E1, E3 and EE2 were 4.3, 4.1 and 3.0 respectively. For the $ASP_{nit/denit}$, the three most absorbent estrogens in the effluent were E2, E3 and EE2, which were associated with the solid, 96.3, 57.7 and 53.4% respectively. At the $ASP_{nit/denit}$ the log K_p values for E2, E3 and EE2 were 4.7, 4.0 and 4.9 respectively. For the ASP_{nit}, the three most absorbent estrogens in the effluent were E2, EE2 and E3, which were associated with the solid, 43.2, 31.5 and 21.7% respectively. At the ASP_{nit} the log K_p values for E2, EE2 and E3 were 4.8, 4.6 and 4.4 respectively. Proportionally, E1 was the most abundant estrogen at ASP_{carb} and represented 28.8% of total (aqueous and adsorbed) concentrations in the effluent. At ASP_{nit}, E2 and E3 represented the most abundant estrogen in aqueous and adsorbed phases, respectively 26.9 and 25.6%.

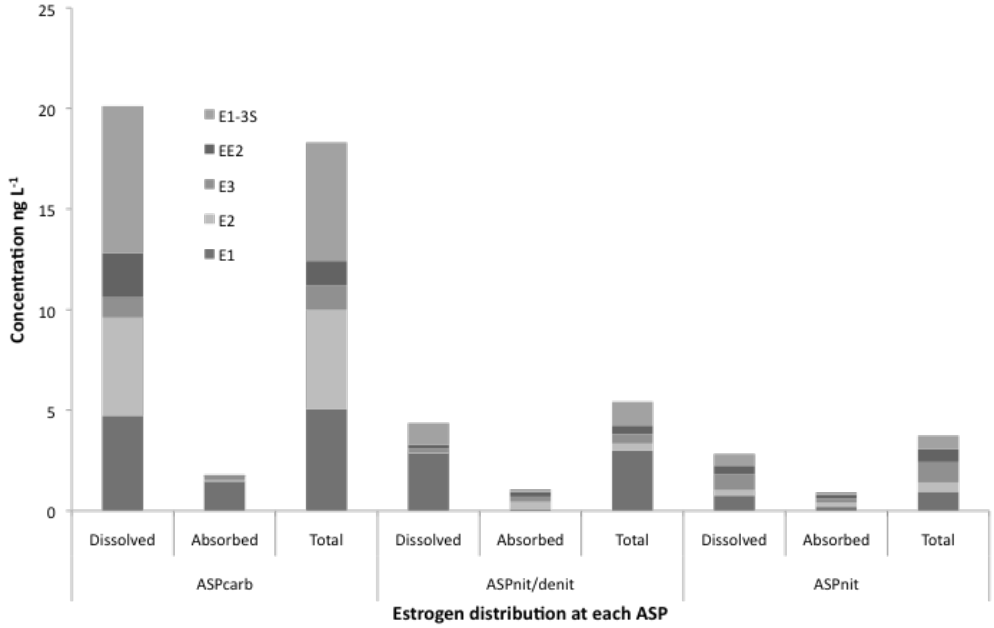


Figure 5-5 The average distribution in effluent of individual estrogens and their concentrations within dissolved, absorbed and total (dissolved plus adsorbed) at each ASP over the entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8

The daily average total (solid plus aqueous phases) concentration of individual estrogens within the final effluent are detailed in Figure 5-6. There was no evidence of any correlation between average daily flow to ASP and final effluent concentration. The $ASP_{nit/denit}$ had the most variable flow rate (Figure 5-1), however this did not translate to significant between days estrogen concentrations. Between days variation of individual estrogens was not attributed to a specific pattern, highest average daily concentrations peaked on different days for individual estrogens at ASP_{carb} and ASP_{nit}. Except at $ASP_{nit/denit}$ where highest concentrations of E1, E2, E3 and EE2 (6.8, 2.2, 1.3 and 0.9 ng L^{-1} respectively) were found on day one and the highest concentration of E1-3S (2.75 ng L^{-1}) was on day five. However, these concentrations were similar day on day. At ASP_{carb}: the highest concentration of E1 (5.8 ng L^{-1}) was on day one; the highest concentration of E2 (6.0 ng L^{-1}) was on day two; the highest concentrations of E3 and E1-3S (1.8 and 9.3 ng L^{-1} respectively) was on day five and

the highest concentration of EE2 (1.5 ng L^{-1}) was on day two. At ASP_{nit}: the highest concentrations of E1 (1.3 ng L^{-1}) were on day four; the highest concentration of E2 (0.5 ng L^{-1}) was on day two and four; the highest concentration of E3 (1.2 ng L^{-1}) was on day three and the highest concentrations of EE2 and E1-3S (0.8 and 1.0 ng L^{-1} respectively) were on day one. There was little evidence for correlation with highest settled sewage concentrations and settled sewage concentration. Only at $ASP_{nit/denit}$ whose E1 day one concentration spiked at an appreciable 74.2 ng L^{-1} did this translate to a significant final level of 6.8 ng L^{-1}. Therefore, as between days variation of individual estrogens could not be attribute to flow variation, further qualification of effluent characteristics was done on data average over the entire sampling period.

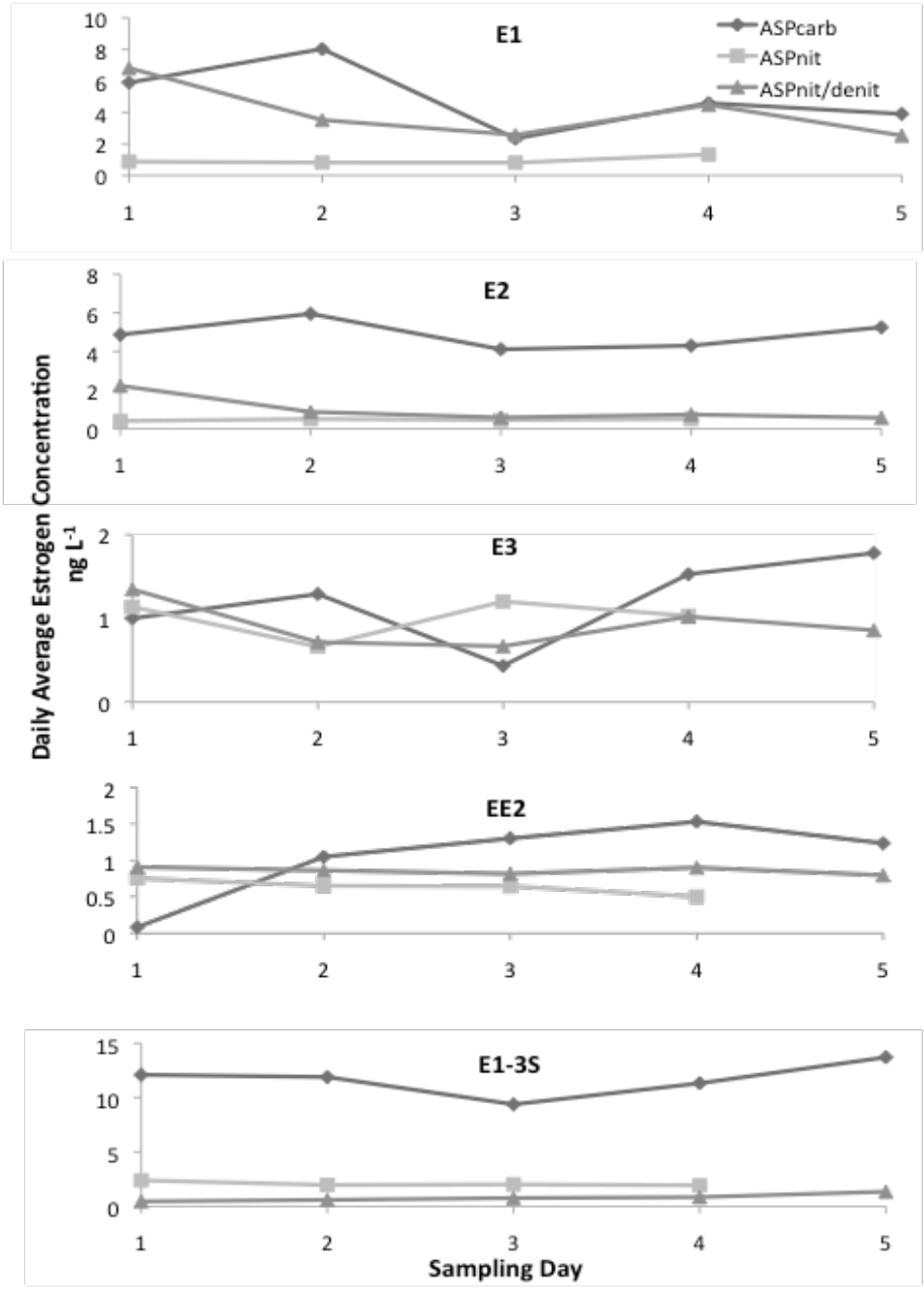


Figure 5-6 The average average daily total (solid and aqueous) concentrations of individual estrogens in the effluent. At ASP_{carb}, n=1 on day 1, n=5 on day 2, n=4 on day 3, n=5 on day 4, n=3 on day 5. At $ASP_{nit/denit}$, n=2 on day 1, n=5 on day 2, n=4 on day 3, n=5 on day 4 and n=2 on day 5. At ASP_{nit}, n=2 on day 1-4.

Average total (dissolved and adsorbed phases) ∑EST concentrations in effluent at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were, 17.6, 5.4 and 3.7 ng L^{-1} respectively (Figure 5-7). Based on effluent concentration, it would appear that the ASP_{nit} was the best performing site. At the ASP_{carb} and $ASP_{nit/denit}$ E1 effluent quality exceeded the predicted no-effects concentration (PNEC) of 3 ng L^{-1}. Total (adsorbed and aqueous) E1 concentrations at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were, 5.1, 3.0 and 1.0 ng L^{-1} respectively. At the ASP_{carb}, E2 effluent concentration exceeded the E2 predicted no-effects concentration (PNEC) of 1 ng L^{-1}. Total (adsorbed and aqueous) E2 concentrations at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were, 4.9, 0.3 and 0.5 ng L^{-1} respectively. Every site exceeded the predicted no-effects concentration (PNEC) for EE2 of 0.1 ng L^{-1}. Total (adsorbed and aqueous) EE2 concentration at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} was, 1.2, 0.4 and 0.6 ng L^{-1} respectively. Non-conformity was compounded when evaluating the cumulative affect of E1+E2+EE2. Based upon effluent quality normalised on the basis of toxic equivalence (Equation 5-1), EEQ values of were 18.6, 5.7 and 7.2 determined for the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively.

Equation 5-1 Combined PNEC

$$\frac{17\alpha - ethinylestradiol}{PNEC = 0.1} + \frac{17\beta - estradiol}{PNEC = 1} + \frac{Estrone}{PNEC = 3} =< 1$$

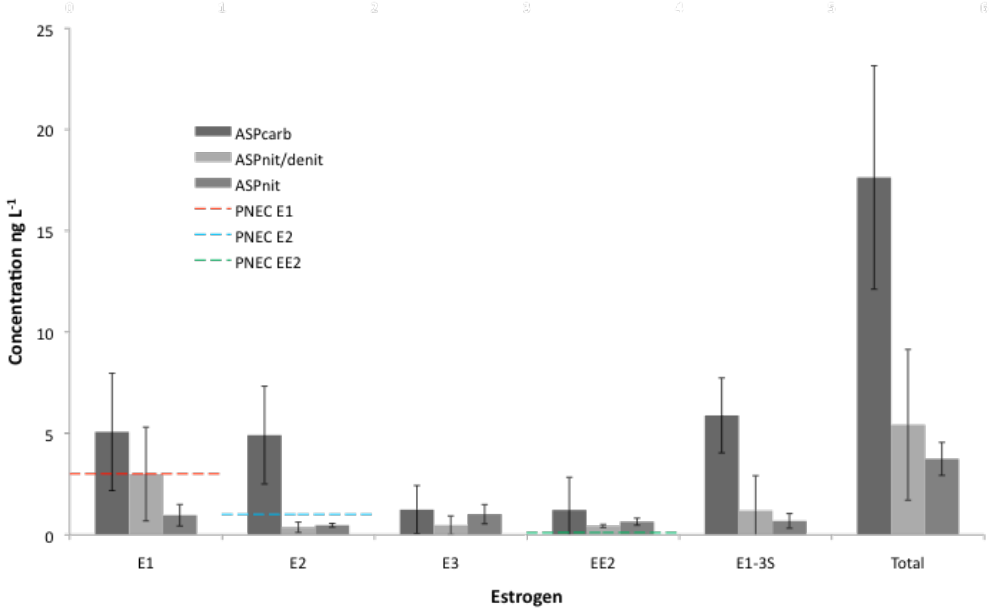


Figure 5-7 Average total (aqueous plus solids phase) ∑EST (E1, E2, E3, E1-3S and EE2 concentrations in effluent at the three ASP over entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8. Standard deviations displayed as bars.

5.4 The estrogen concentration and partitioning of the WAS

Estrogens were also found principally in the aqueous phase of the WAS (Figure 5-8). The proportion to which estrogens were associated with the solids in the WAS was on average 1.1, 21.8 and 49.0% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. ΣEST, log K_p values were, 0.3, 1.7 and 2.3 at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. At the ASP_{carb}, estrogens were associated with the solids <3%, with the exception of E3. This compound was found to be 41.6% in association with the solids, its log k_p value was 2.1. At the $ASP_{nit/denit}$, the three most absorbent estrogens in the effluent were E3, EE2 and E2, which were associated with the solid, 82.2, 66.7 and 63.3% respectively. At the $ASP_{nit/denit}$ the log K_p values for E3, EE2 and E2 were 2.9, 2.5 and 2.5

respectively. At the ASP_{nit}, the three most absorbent estrogens in the effluent were E1, E3 and EE2, which were associated with the solid, 70.9, 61.2 and 38.1% respectively. At the ASP_{nit} the log K_p values for E1, E3 and EE2 were 2.7, 2.5 and 2.5 respectively.

These log k_p values were much lower in comparison to the settled sewage and final effluent particularly at the ASP_{carb}. Despite elevated solids of the WAS, the concentration to which estrogens adsorbed did not exceed those of the settled sewage and the final effluent. At the ASP_{carb}, average ΣEST concentrations (±1SD) of adsorbed fractions were 6.2±6.8, 1.8±5.2 and 0.6±0.5 ng L^{-1} in the settled sewage, final effluent and the WAS respectively. At the $ASP_{nit/denit}$, average ΣEST concentrations (±1SD) of adsorbed fractions were 3.6±7.4, 2.1±0.7 and 2.2±1.3 ng L^{-1} in the settled sewage, final effluent and the WAS respectively. At the ASP_{nit}, average ΣEST concentrations (±1SD) of adsorbed fractions were 11.6±1.1, 0.9±0.3 and 2.3±0.7 ng L^{-1} in the settled sewage, final effluent and the WAS respectively. During the course of sewage treatment, average ΣEST concentrations of adsorbed fractions decreased between settled sewage and WAS from 0.36 – 0.0001, 0.03 – 0.0004 and 0.12 – 0.0005 ng ΣEST^{-1} mg SS at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. However, in the final effluent, average ΣEST concentrations of adsorbed fractions were comparable with settled sewage concentration, 0.22, 0.13 and 0.77 at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. This could indicate that estrogens were associated with solid fractions that were not removed by the secondary sedimentation tank. These fractions would have been lighter in weight and smaller in size compared with the floccules undergoing sedimentation in order to leave the sites as final effluent.

In terms of individual estrogens, proportionally, E1 was the most abundant estrogen at $ASP_{nit/denit}$ and ASP_{nit}, and represented 74.0 and 32.0% of total (aqueous and adsorbed) concentrations in the WAS (Figure 5-8). At ASP_{nit}, E2 represented the most abundant estrogen in aqueous and adsorbed phases, on average 64.1%. At the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit}, total (aqueous and adsorbed) E1 concentrations were 12.7, 7.4 and 1.6 ng L^{-1} respectively (Figure 5-9). At the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit}, total

(aqueous and adsorbed) average E2 concentrations were 35.9, 0.6 and 1.1 ng L^{-1} respectively. Total (aqueous and adsorbed) average ΣEST concentrations were 55.9, 2.2 and 5.1 ng L^{-1} respectively at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit}. Whilst total average WAS concentration were comparable with total average final effluent concentrations at the $ASP_{nit/denit}$ and ASP_{nit}, this was not so at the ASP_{carb}. Here, WAS concentrations exceeded those of the final effluent by 317.3%. Since activated sludge was not analysed directly, it has not been possible to determine the reason for this. Further work is needed to understand partitioning and its affect upon the fate to EDCs during activated sludge treatment. It may be that during secondary sedimentation, a great deal of estrogen enter WAS adsorbed and therefore accumulate due to higher solid concentrations. Subsequent desorption may explain elevated aqueous concentrations.

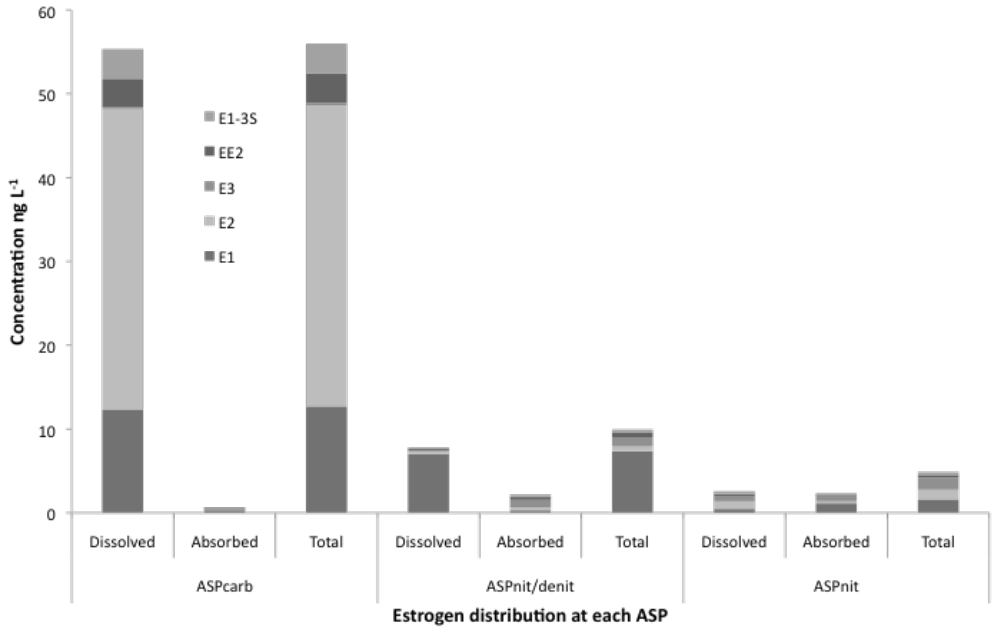


Figure 5-8 The average distribution in WAS of individual estrogens and their concentrations within dissolved, absorbed and total (dissolved plus adsorbed) at each ASP over the entire sampling period. At ASP_{carb} n=6. $ASP_{nit/denit}$ n=4. ASP_{nit} n=4

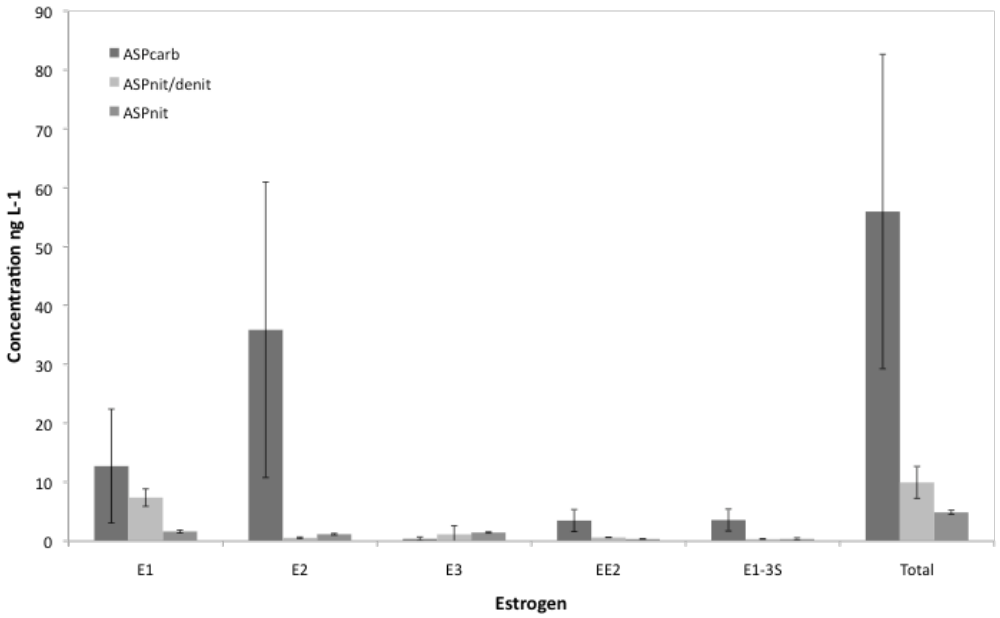


Figure 5-9 Average total (aqueous plus solids phase) ∑EST (E1, E2, E3, E1-3S and EE2 concentrations in WAS at the three ASP over entire sampling period. At ASP_{carb} n=6. $ASP_{nit/denit}$ n=4. ASP_{nit} n=4. Standard deviations displayed as bars.

5.5 Mass Flux of Estrogen at each ASP

The greatest average daily mass of total ∑EST entering the three sites was 4035 mg d^{-1} at the $ASP_{nit/denit}$, unsurprisingly as it was the largest site (Figure 5-10). Since ASP_{nit} was a pilot plant, it had the lowest average daily mass of total ∑EST in settled sewage, 702 μg d^{-1}. The average daily mass of total ∑EST at the ASP_{carb} was 1219 mg d^{-1}. The total ∑EST average daily mass in effluent was 259 mg d^{-1}, 29 μg d^{-1} and 620 mg d^{-1} at ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ respectively. Based on this mass balance, site removal efficiencies (based on settled sewage minus effluent masses) were 79, 95.9 and 84.6% at ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ respectively. The effective site removal observed of ASP_{carb} was interesting as this site had an SRT of just 6 days and

would have had a limited nitrifying population. This therefore implied that heterotrophic bacteria were responsible for the biodegradation of estrogen as this site. Determining removal in terms of biodegradation, after accounting for the dissolved and sorbed estrogens recycled in the RAS, further supported this hypothesis. Based upon this removal calculation, 51, 91.3 and 72.9% could be demonstrated (Figure 5-10).

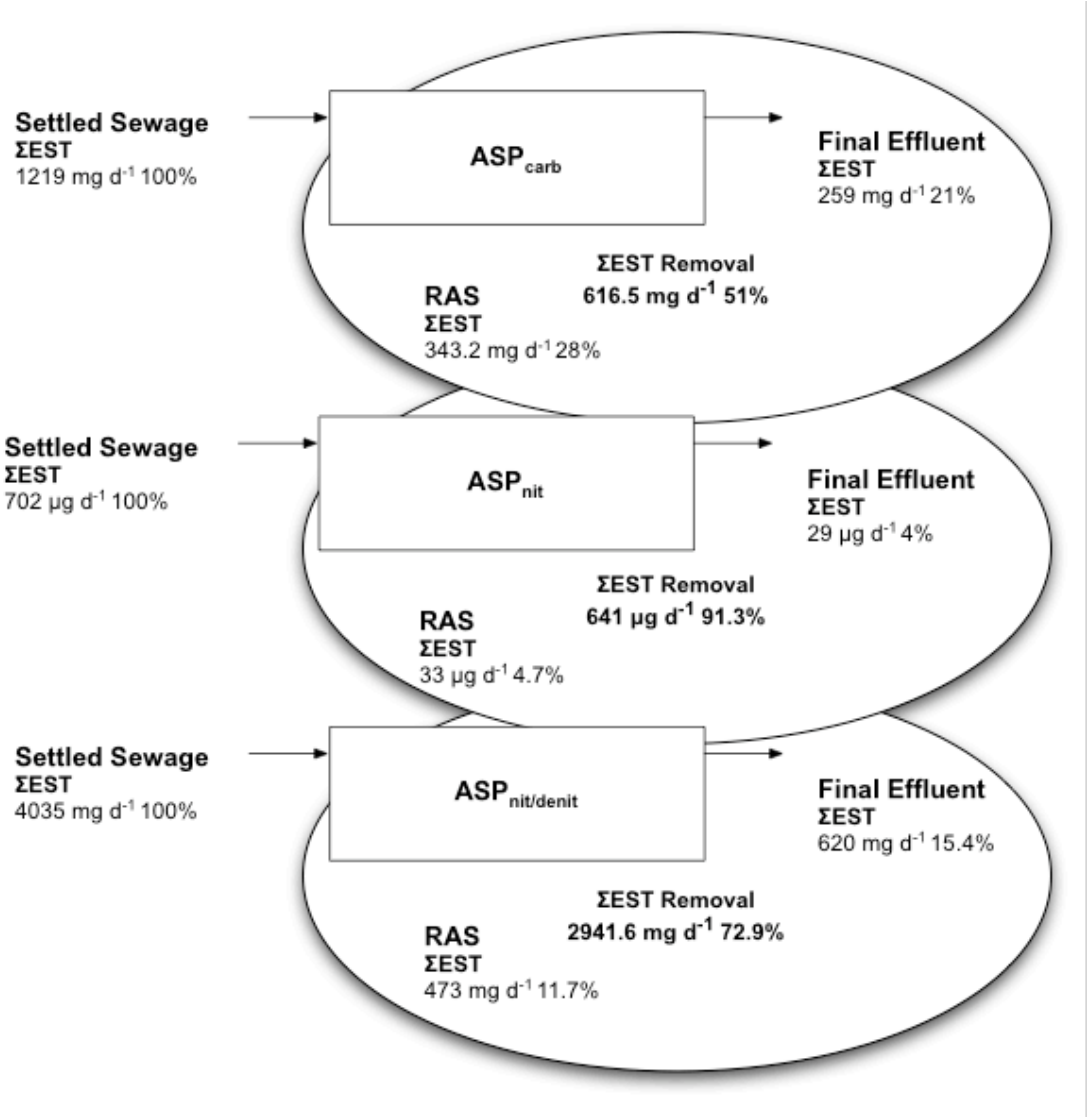


Figure 5-10 Mass balance of the three ASP indicating average masses and the percent proportions entering settled sewage and accounted for in effluent and returned activated sludge (RAS). Removal calculated as (settled sewage – (effluent + RAS).

5.6 Biodegradation of estrogens at each ASP

In order to assess the potential role of heterotrophic bacteria in removal of estrogens, biodegradation calculations were performed based upon waste activated sludge (WAS) (Figure 5-11). By doing this, distinctions between removal due to sorption and biodegradation could be made. Therefore, biodegradation (settled sewage – (effluent + WAS), at the ASP_{nit}, was 95.5% (671 μg d^{-1}) whilst only 0.2 μg d^{-1} (0.2% of ∑EST entering site as settled sewage) was lost in waste activated sludge (WAS). At $ASP_{nit/denit}$, 3390 mg d^{-1} was biodegraded (84.0% biodegradation), whilst only 24.9 mg d^{-1} (0.6%) was lost in waste activated sludge (WAS). At ASP_{carb}, the sludge wastage rate was more significant due to the lower SRT. Here 657 mg d^{-1} was biodegraded (54% biodegradation) and 298 mg d^{-1} (24% wastage) was lost to the WAS. This therefore indicated that wastage rate was an important factor in the effective removal of estrogens at the ASP_{carb}. The ASP_{carb} had higher ΣEST WAS concentration in comparison to other two sites (section 5.4), which resulted from the lowest ΣEST biodegradation rate of 54%. However, the ASP_{carb} was clearly capable of moderate estrogen biodegradation despite having a heterotrophic biomass.

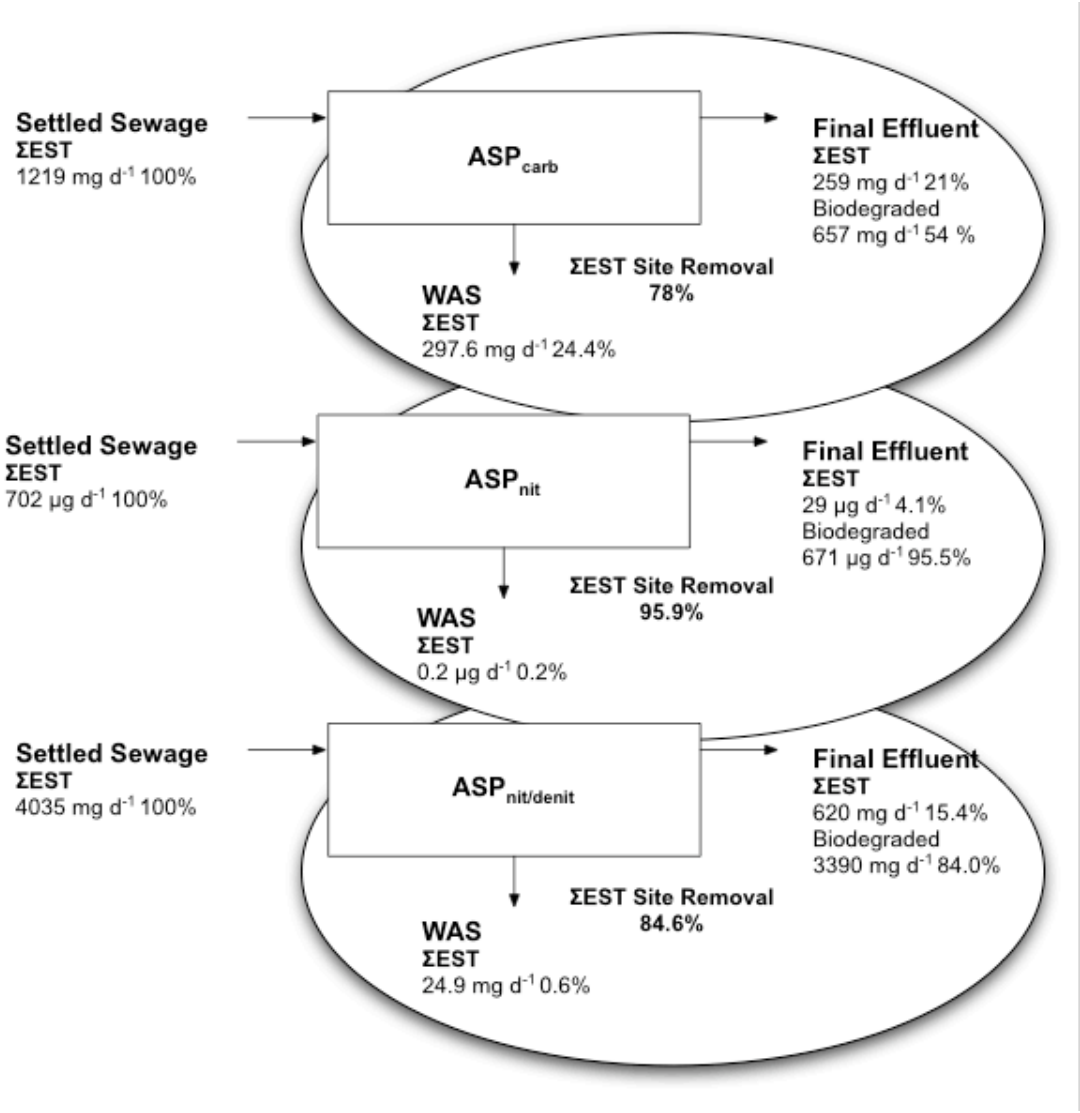


Figure 5-11 Mass Balance of the three ASP indicating average masses and the percent proportions entering settled sewage and accounted for in effluent, waste activated sludge (WAS). Site removal = (settled sewage - effluent). Biodegradation = (settled sewage – (effluent + WAS).

The average individual percent estrogen removal and biodegradation profiles for ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} are shown below (Figure 5-12). At all sites, the most easily removed estrogen was E3, which was removed from the final effluent on average >86.2%. Biodegradation was the predominant form of removal for this compound and was on average 95.2, 89.5 and 96.4% at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. At the $ASP_{nit/denit}$, after E3, E1 was the next most easily biodegraded followed by E2>E1-3S>EE2. Biodegradation efficiency was the predominant form of removal for all estrogen (with the exception of EE2) and was on average 87.6, 84.1 and 59.4% respectively for E1, E2 and E1-3S. Only minor losses to WAS, on average >0.5% of the estrogen entering the $ASP_{nit/denit}$, indicating that sorbtion was an insignificant mode of removal for these compounds. At the ASP_{nit}, after E3, E1 was the next most easily biodegraded followed by E1-3S>E2>EE2. Biodegradation efficiency was on average 96.4, 88.5, 74.6 and 69.3% respectively for E1, E1-3S, E2 and EE2. This was the only site where evidence of removal or biodegradation of EE2 could be demonstrated. Biodegradation was the predominant form of removal of every estrogen. The lower biodegradation and removal efficiency of E2 could be explained due to its low average mass of 15.6 μg d^{-1}entering the ASP_{nit}, which was the lowest of all estrogens at this site. At the ASP_{carb}, after E3, E1 was the next most easily biodegraded followed by E1-3S and E2. Biodegradation efficiency was on average 63.7, 43.8 and 8.9% respectively for E1, E1-3S and E2. Average site removals were 75.6, 77.9, and 48.9% for E1, E2 and E1-3S respectively. For E1 and E2 on average, respectively 3.2 and 17.0% of their masses entering the ASP_{carb} were associated with WAS, which was comparably greater than the other sites. This therefore indicates that unlike the other sites, sorption played a greater role in the removal of estrogens here.

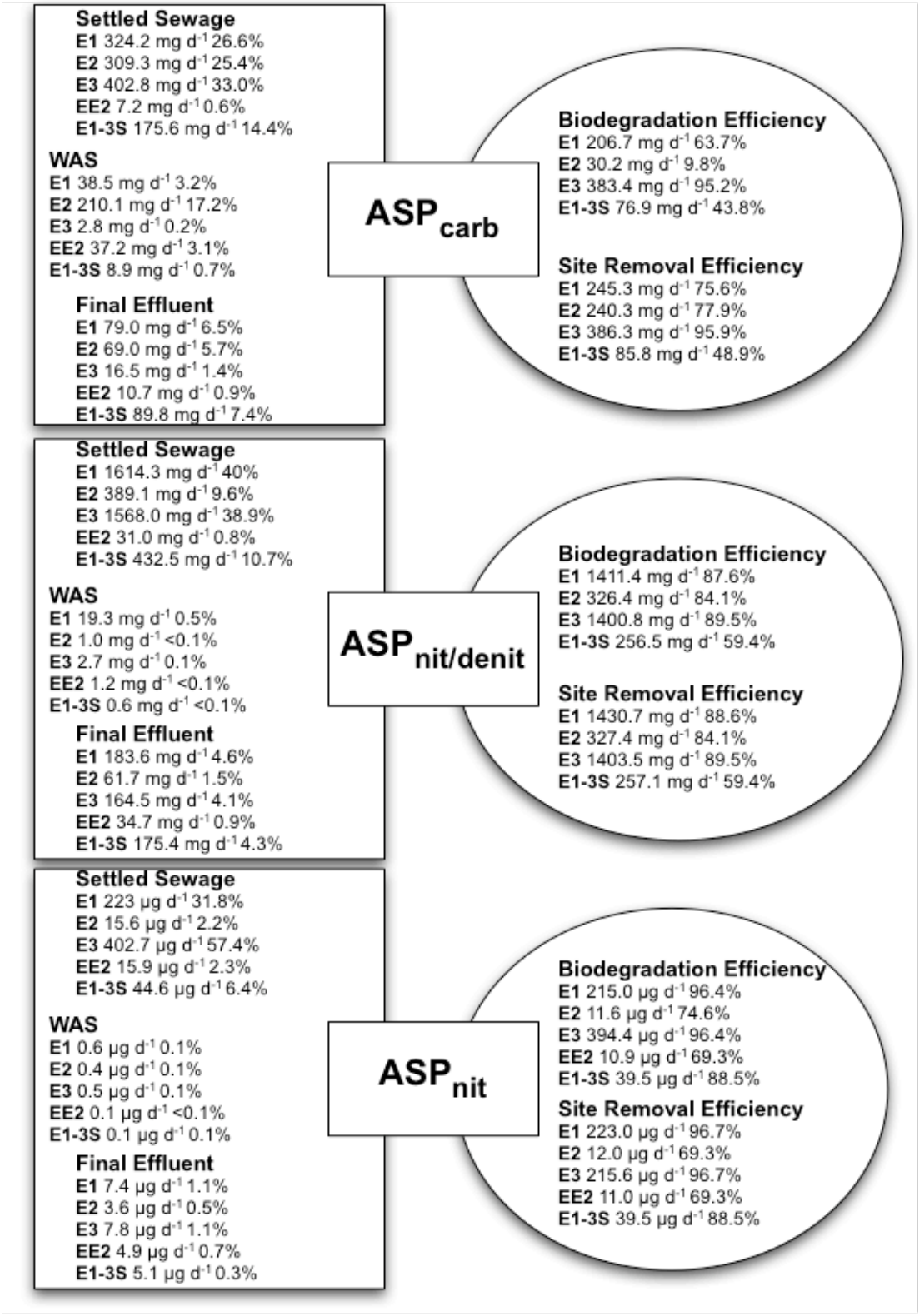


Figure 5-12 The average percent removal and biodegradation profiles of each estrogen at each ASP. Biodegradation = Settled sewage – (final effluent + WAS). Site removal = Settled sewage – final effluent.

5.7 The role of sorption upon the biodegradation of estrogens

There appeared to be a link between estrogen sorption and biodegradation at the ASP_{carb}. Here, where biodegradation was the lowest and losses via WAS was a significant route for estrogen removal, a link between Log K_p of individual estrogens and biodegradation appeared to exist (Figure 5-13). It appeared that there was a positive trend between increasing sorption and biodegradation efficiency. At the ASP_{carb}, E3 was on average the most sorbent estrogen within the WAS having a Log K_p of 1.9 coupled with an average biodegradation efficiency of 95.2. Conversely E2, which had the lowest biodegradation rate (8.9%) and demonstrated periods where it was being formed, the average Log K_p was -0.7. The low partitioning for E2 could indicate that it was released from cells after formation. Whereas the other compounds that demonstrated higher biodegradation had higher Log K_p, this could indicate that sorption was necessary before biodegradation could proceed. At the $ASP_{nit/denit}$ and ASP_{nit} the same relationship was not established. Sorption in relation to biodegradation appeared not to have had any connection. However, at these sites, biodegradation was comparably higher, which could indicate that preceding sorption had already occurred.

At the ASP_{carb}, losses to WAS was more important, as the concentration of estrogens within the WAS at the ASP_{carb} was >3 times higher than the effluent. However, most of the estrogens were in the aqueous phase, which indicated that these compounds had subsequently desorbed following settlement in the secondary sedimentation tank. At the other sites proportions in WAS were less significant. This was further evidence that biodegradation proceeded sorption in these cases. However at the ASP_{carb}, despite it being likely that estrogen were in association with bacterial cell capsules whilst in the activated sludge treatment tank, based upon higher aqueous WAS concentration compared with effluent, biodegradation to the degree found at the other sites did not occur. This could indicate that higher biodegradation could have been possible at the ASP_{carb}, as the sorption necessary had occurred within the activated sludge tank. However, determining the factor that had inhibited further biodegradation is necessary to understand how to improve removal of the ASP_{carb}.

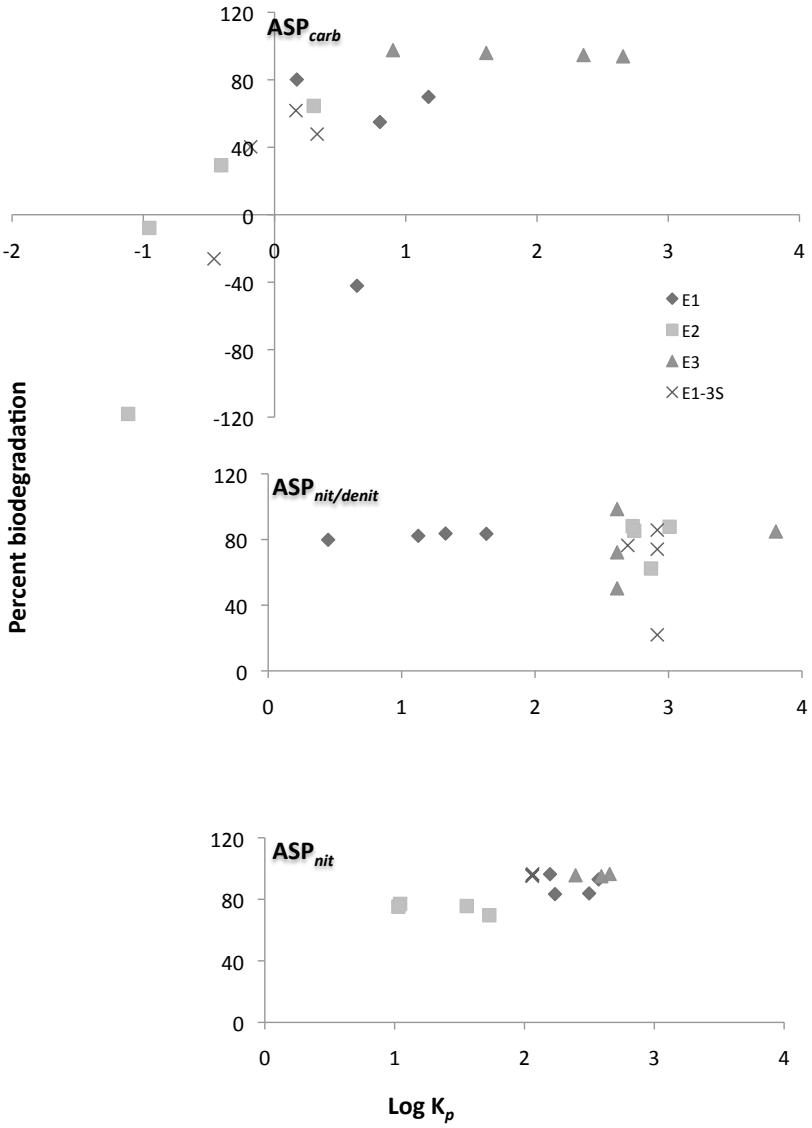


Figure 5-13 The average daily percent biodegradations of E1, E2, E3 and E1-3S plotted against average daily Log K*p* values within the WAS at individual sites indicating the requirement for bacteria to sorb estrogens prior to biodegradation

5.8 The resource dependent removal of estrogens

To compare the different sites effectively, estrogen biodegradation was normalised to MLSS concentration and reactor volume. This resulted in the descriptor "biomass activity" which describes the amount ∑EST removed per tonne of biomass per day. Average biomass activities were, 40.7, 22.8 and 60.4 (mg ∑EST removed $tonne^{-1}$ day^{-1}) for ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Indicating that despite ∑EST biodegradation efficiency of 54%, per tonne of biomass, the ASP_{carb} was removing comparably more estrogens than the $ASP_{nit/denit}$. Loading appeared to be a key driver of biomass activity. Due to the reactor volume at the $ASP_{nit/denit}$, despite being the largest site, its ∑EST volumetric loading was on average circa 0.05 mg m^{-3} d^{-1}, significantly lower than the other sites. Comparably lower volume in comparison to their flow rates resulted in average ∑EST volumetric loadings of 0.36 and 0.38 mg m^{-3} d^{-1} at the ASP_{carb} and ASP_{nit} respectively. Clearly the ASP_{carb} and ASP_{nit} had greater estrogen resources available to the biomass than the $ASP_{nit/denit}$. On average, maximum potential biomass activities were 69.1, 27.4 and 63.1 mg ∑EST removed $tonne^{-1}$ day^{-1} at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively.

Since the variation in biomass activity witnessed between the three ASPs appeared to be driven by loading. This research demonstrated that resource availability (as defined by volumetric loading of estrogens) appeared to be proportional to biodegradation (normalised to MLSS concentration). Using this normalised data from all three sites, there appeared to be a moderate linear relationship ($R^2 = 0.60$) between, volumetric loading (mg m^{-3} d^{-1}) and biomass activity (∑EST removed $tonne^{-1}$ day^{-1}) (Figure 5-14). This is evidence that first order reaction kinetics descibe the relationship between removal and loading. In which case resource dependent removal may have been occurring at full-scale; which has only previously been reported under controlled bench scale experiments (Vader *et al* 2000). This therefore implies that effective biodegradation may be more dependent upon concentration and flow conditions than whether a site has nitrifying or nitrifying/denitrifying activity.

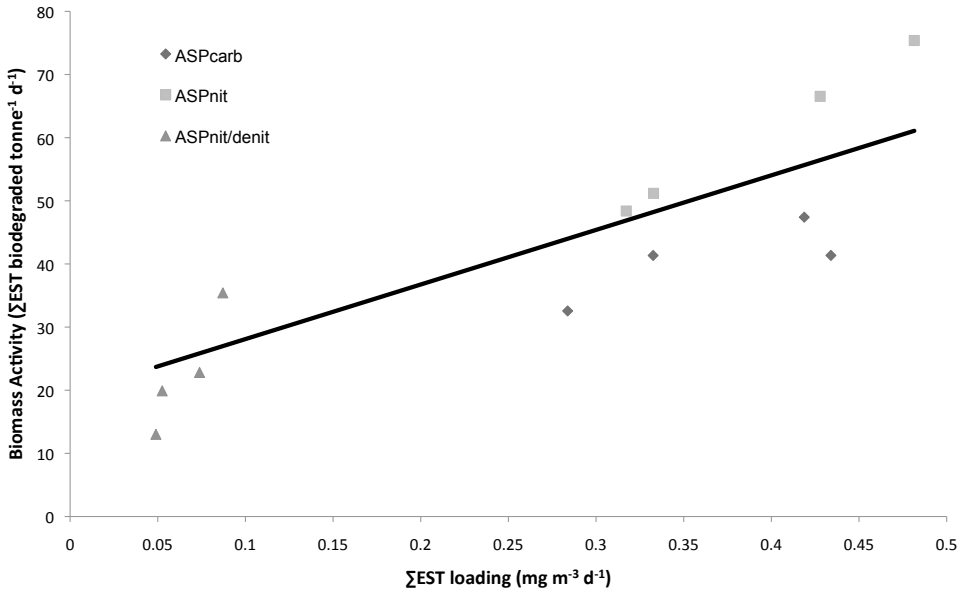


Figure 5-14 The relationship between average daily ∑EST volumetric loading and biomass activity at the three ASP suggesting biodegradation follows first-order reaction kinetics

5.9 The influence of competing carbon sources upon the biodegradation of estrogens

Data from all three sites were combined in order to assess whether the presence of bulk organics influenced the biodegradation of estrogens. It has been hypothesised high proportions of bulk organics may inhibit the biodegradation of estrogen, as bacteria may favour more readily biodegradable carbon sources that are present at higher concentrations to the estrogens. In this research, it was noticed that the highest ∑EST biomass activities (>38.6 mg ∑EST biodegraded tonne^{-1} day^{-1}) were recorded when the settled sewage COD/∑EST ratio was <6 x10^{6} mg COD^{-1} mg ∑EST L^{-1}. Furthermore, a power relationship between COD/∑EST ratio and biomass activity could be demonstrated (Figure 5-15). Through this relationship, it appeared that biomass activities were lowest when the ratio of COD/∑EST increased (when the

proportion of ∑EST as organic carbon decreased). This could indicate that suppression of ∑EST biodegradation was influenced by the greater availability of more readily available bulk organics.

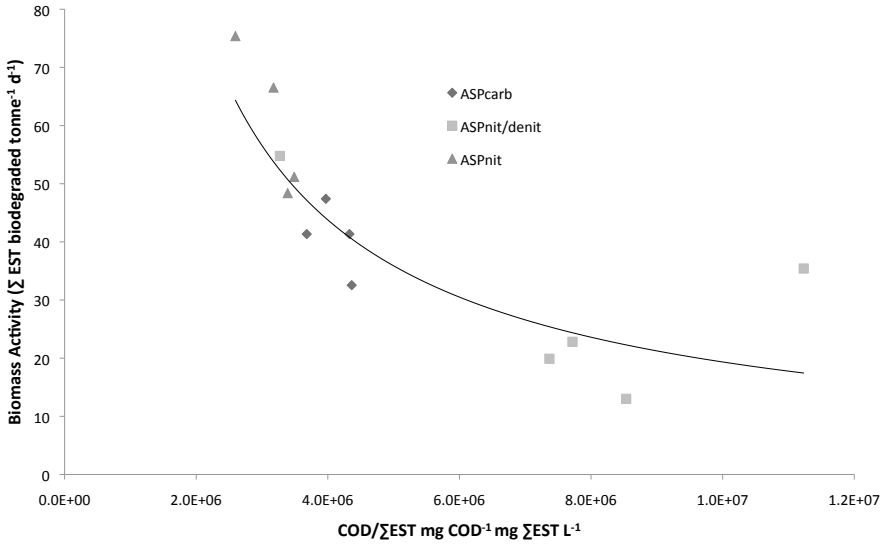


Figure 5-15 The power relationship between the daily average ratio of COD/∑EST (mg COD-1 mg ∑EST L^{-1}) and ∑EST biomass activity (mg ∑EST removed $tonne^{-1}$ d^{-1}) suggesting the potential for competitive inhibition of estrogens by bulk organic material

However, biomass activity was linked with loading, as established in section 4.5. Furthermore, plotting COD/∑EST ratio against loading questioned whether such competitive inhibition was occurring at all. In fact, a power relationship between COD/∑EST ratio and estrogen loading also described how ∑EST loading >0.28 mg m^{-3} d^{-1} tended to occur at COD/∑EST ratio circa 4 x 10^6 mg COD^{-1} mg ∑EST L^{-1} (Figure 4-16). This meant that the highest estrogen loading coincided with the highest proportion of estrogen as organic carbon within settled sewage. Therefore, instances of COD/∑EST ratio > 6 x 10^6 mg COD^{-1} mg^{-1} ∑EST could not have produced biomass activities in excess of 50 mg ∑EST removed $tonne^{-1}$ day^{-1} as loading was not sufficient to achieve this.

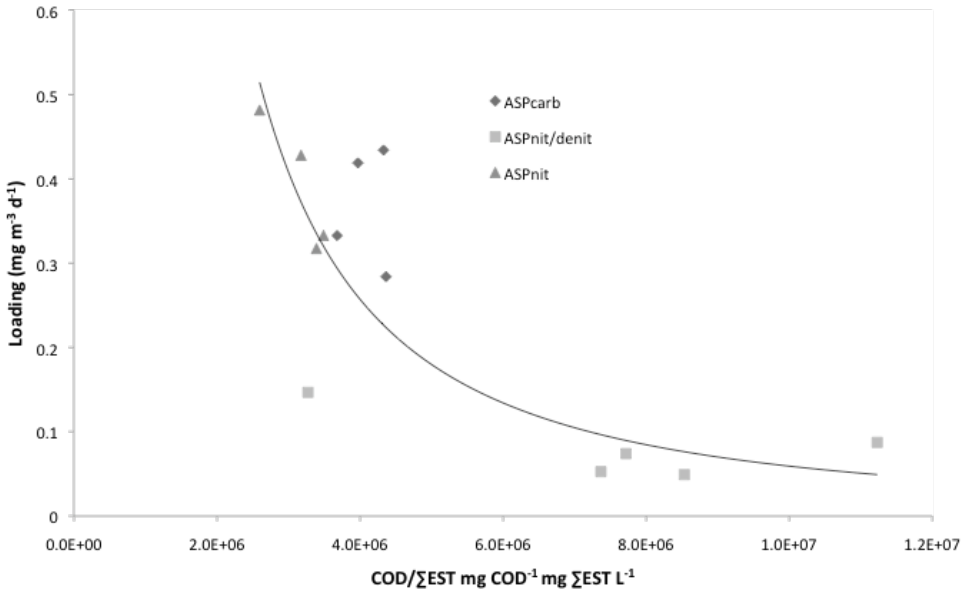


Figure 5-16 The power relationship between the ratio of COD/∑EST (mg COD-1 mg ∑EST L^{-1}) and ∑EST biomass activity (mg ∑EST biodegraded $tonne^{-1}$ d^{-1}) indicating that loading is driving COD/∑EST ratio

To explore now biodegradation could be influenced by the availability of other carbon sources, the maximum potential biomass activity was calculated. This demonstrated that maximum potential biomass activities did not drastically alter the shape of previous power curves (Figure 5-17). Only the ASP_{carb} demonstrated a major difference between its maximum potential biomass activity and biomass activity, on average 69.1 and 44.1 mg ∑EST removed $tonne^{-1}$ day^{-1} respectively. This difference from the maximum potential was predominantly due to the high sorption and amount of ∑EST removed through this process.

Since loading and biomass activity were proportional, it seems likely these power curves (Figures 5-14 to 5-16) describe how the highest ∑EST loading were associated

with the lowest COD/∑EST ratios. It is therefore unclear what impact bulk organic loading has had upon ∑EST biodegradation. These relationships make it difficult to determine whether competitive inhibition of estrogens by other more readily available carbon has occurred but rather suggests loading is the key driver of biodegradation.

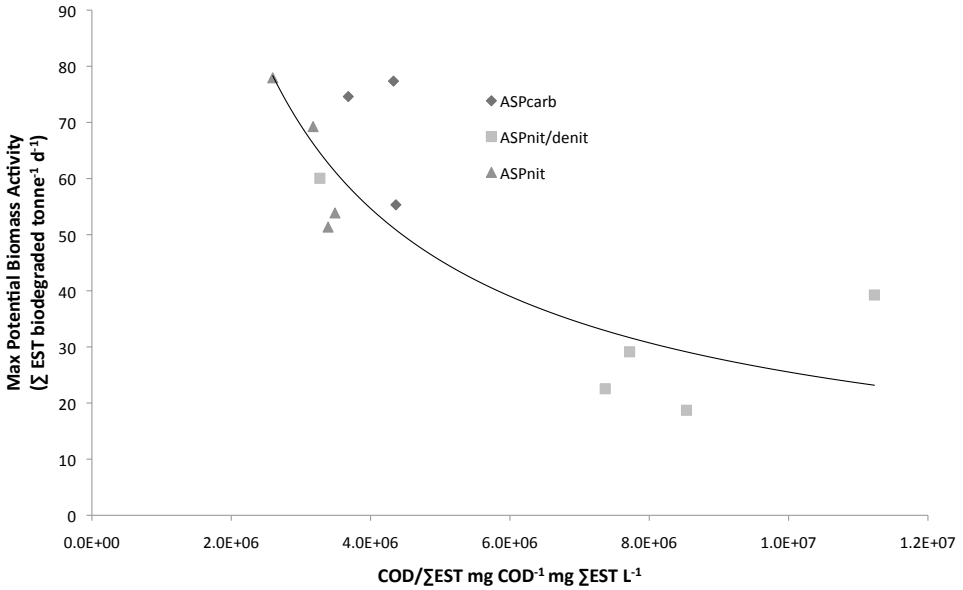


Figure 5-17 The power relationship between the ratio of COD/∑EST (mg COD-1 mg ∑EST L^{-1}) and maximum potential ∑EST biomass activity (mg ∑EST biodegraded $tonne^{-1}$ d^{-1}) further suggesting that loading is driving COD/∑ EST ratio

Chapter 6

Nonylphenolic Biodegradation at Carbonaceous, Nitrifying and Nitrifying/Denitrifying Activated Sludge Treatment Plants

6 Nonylphenolic Biodegradation at Carbonaceous, Nitrifying and Nitrifying Denitrifying Activated Sludge Treatment Plants

Sampling for nonylphenolic compounds was done concurrently with the estrogens. The ASP_{carb} and $ASP_{nit/denit}$ were both sampled in spring 2008 and the ASP_{nit} was sampled in late summer 2007. As with the estrogen (Chapter 5) the objective of sampling was to assess which process parameters were key for the effective removal of nonylphenolic compounds during activated sludge treatment. Five day sampling was carried out at four to six hour intervals. Samples were taken from settled sewage, final effluent and WAS/RAS. Both aqueous and solid phases were analysed in order to accurately mass balance each ASP. Details of the operating parameters and general performance of the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} can be found in Section 5-1.

6.1 The Nonylphenolic characteristics of the settled sewage

To simplify explanation of NPx distribution, similar compounds were grouped. There groups comprised: long-chain polyethoxylates $NP_{4-12}EO$; short-chain polyethoxylates $NP_{1-3}EO$; short-chain carboxylates $NP_{1-3}EC$ and NP was kept on its own. Proportionally, $NP_{4-12}EO$ were the most abundant NPx. At ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} $NP_{4-12}EO$ represented 81.6, 89.9 and 78.5% of total average (both liquid and solid phases) NPx respectively. NP was the second most abundant NPx in settled sewage representing 16.9, 7.8 and 17.0% total (solid and aqueous phase) concentration at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Overall, ASP_{nit} had the highest total (solid and aqueous phase) average ∑NPx (sum of $NP_{1-12}EO$, $NP_{1-3}EC$ and NP) settled sewage concentration (± standard deviation), 22340±6006 ng L^{-1}; explained possibly due to its placement within a hospital catchment. Similar average total ∑NPx concentrations (± standard deviation) of 11306±4572 and 10779±2794 were found at ASP_{carb} and $ASP_{nit/denit}$ respectively (Figure 6-1). The variability of total ∑NPx concentration was greatest at ASP_{carb}, the RSD of the mean was 40.4%, higher than ASP_{nit} and $ASP_{nit/denit}$ whose RSDs were 26.9 and 25.9% respectively.

Nonylphenolics were found principally in the adsorbed phase at ASP_{carb} and $ASP_{nit/denit}$, with the exception of ASP_{nit}, where they were found chiefly in the aqueous phase (Figure 6-1). The proportion of which NPx were associated with the solids was 8521 ng L^{-1} (75.4 %), 7254 ng L^{-1} (67.3 %) and 2495 ng L^{-1} (11.2%) at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The NPx log Kp values for the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were 5.24, 4.29 and 3.69 respectively. Each site had different absorbency profiles. At ASP_{carb}, the most adsorbent NPx were NP, NP_2EO and NP_3EC, with 99.9, 94.6 and 83.7% associated with solids respectively. At $ASP_{nit/denit}$ the most adsorbent NPx were NP_2EO, NP_3EO and NP_4EO, with 96.9, 83.5 and 80.5% associated with the solids respectively. At ASP_{nit} the most adsorbent NPx were, NP_3EO, NP_4EO and NP, with 98.4, 35.8 and 28.0% associated with solids respectively. At all sites, NP and $NP_{1-3}EO$ represented the most absorbent compounds (Figure 6-1). However, concentrations of $NP_{1-3}EO$ in settled sewage were much less significant in comparison to NP.

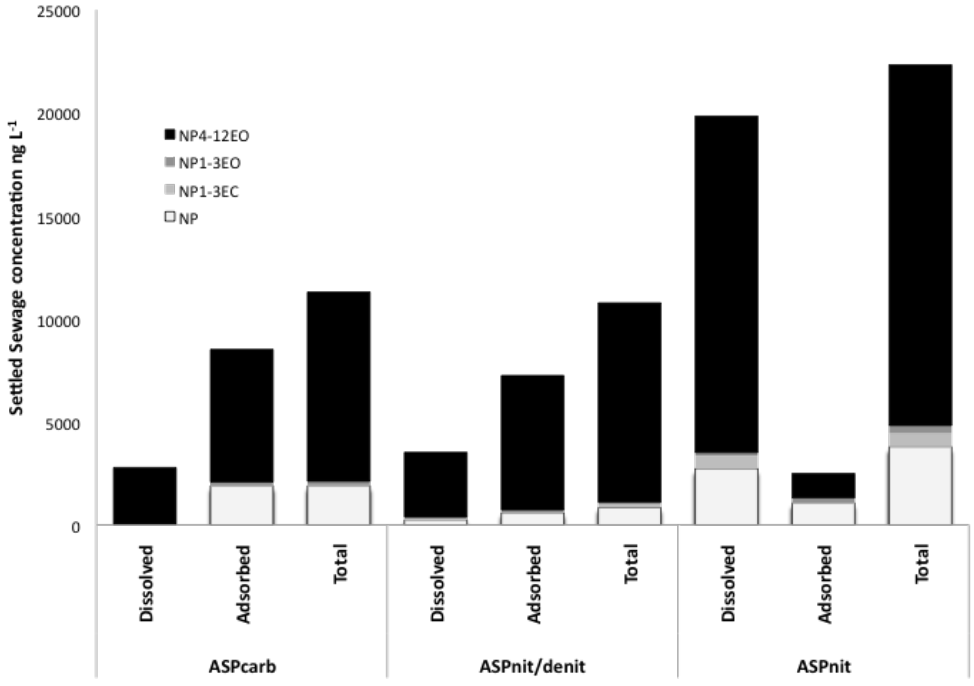


Figure 6-1 The average distribution and concentrations in settled sewage of $NP_{4-12}EO$, $NP_{1-3}EO$, $NP_{1-3}EC$ and NP within dissolved, absorbed and total (dissolved plus adsorbed) at each ASP over the entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8

The distribution of total NPx (in both aqueous and solid phases) in settled sewage is further elaborated in Figure 6-2. The absence of short chain NPx ($NP_{1-3}EO$ and $NP_{1-3}EC$) in settled sewage was apparent. At all sites, the most abundant ethoxylated compounds were mid range ethoxylates. At the ASP_{carb}, this was NP_6EO whose average total (both solid and aqueous) concentration was 1949 ng L^{-1}. At the $ASP_{nit/denit}$, this was also NP_6EO whose average total (both solid and aqueous) concentration was 2200 ng L^{-1}. At the ASP_{nit}, this was NP_7EO whose average total (both solid and aqueous) concentration was 3121 ng L^{-1}.

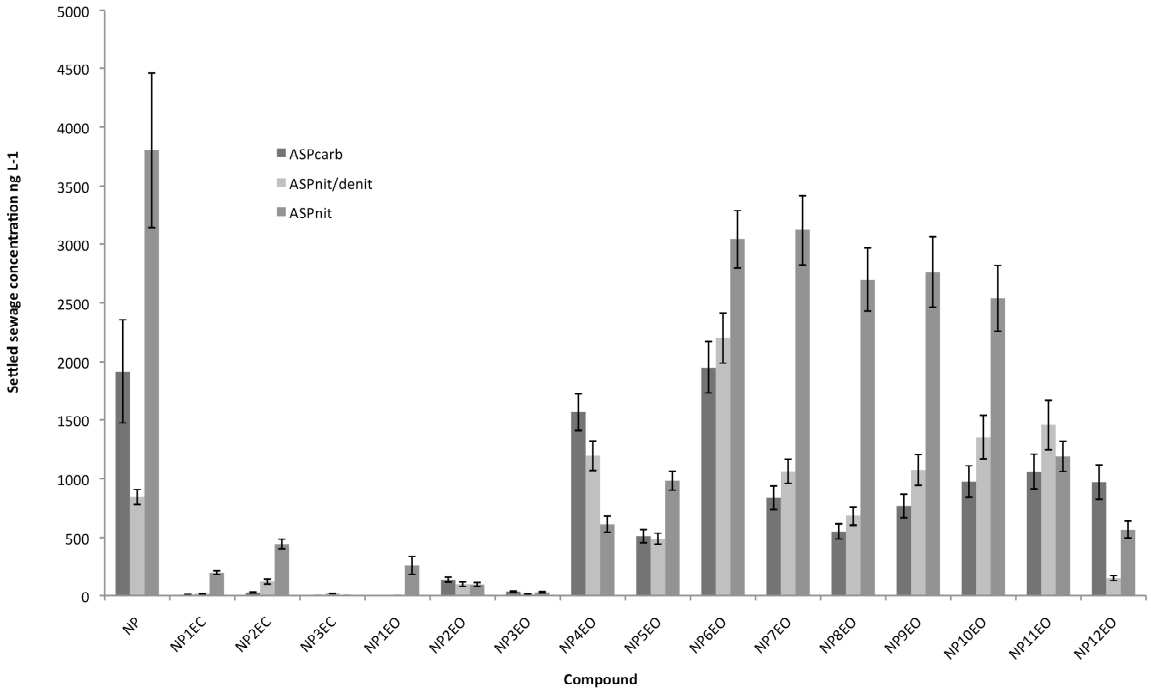


Figure 6-2 Average total (aqueous plus solids phase) individual NPx concentrations in settled sewage at the three ASP over entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8. Standard deviations displayed as bars.

6.2 The Nonylphenolic characteristics of the effluent

The most abundant NPx at ASP_{carb} and $ASP_{nit/denit}$ representing on average 53.1 and 58.9% of total (both liquid and solid phases) NPx respectively were $NP_{4-12}EO$. At ASP_{nit}, $NP_{4-12}EO$ represented much less (14.2% of total NPx), here NP was the most

abundant compound and represent 35.1% of the total. Based on settled sewage and effluent concentrations, $NP_{4-12}EO$ was removed on average 66.5, 47.5 and 95.6% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} to 3087.3, 5087.8 and 773.2 ng L^{-1} respectively, demonstrating net removal of long chain compounds. For ASP_{carb} and $ASP_{nit/denit}$, NP was not removed, in fact this compound increased by 12.6 and 27.9% from settled sewage to effluent. Only ASP_{nit} demonstrated removal of NP (49.8%). Similarly for $NP_{1-2}EO$ ASP_{nit} was the only site to demonstrate net removal. Average removal for this site was 53.5% (a reduction to 45.2ng L^{-1} for $NP_{1-3}EO$). At ASP_{carb} and $ASP_{nit/denit}$ it appeared that $NP_{1-3}EO$ was produced (81.5 and 92.8% produced respectively). At ASP_{carb} and $ASP_{nit/denit}$ concentration of $NP_{1-3}EO$ increased to 247.8 and 682.78 ng L^{-1} respectively. It appeared that $NP_{1-3}EC$ was formed in the activated sludge process at all sites. Here occurred, 823.3, 1191.9 and 223.8% production, which translated to concentrations of 322.5, 1987.5 and 2081.3 ng L^{-1} of $NP_{1-2}EC$ at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively.

In comparison to the estrogens, nonylphenolics were more sorbent. The proportion of which NPx were associated with the solids in the effluent was 66.1, 50.0 and 36.1% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively (Figure 6-3). The NPx log Kp values for the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were 5.37, 4.82 and 4.69 respectively. Each site had different absorbency profiles. For the ASP_{carb}, the three most absorbent groups of NPx in the effluent were NP, $NP_{1-3}EO$ and $NP_{4-12}EO$, which were associated with the solid, 92.9, 66.1 and 52.8% respectively. For the $ASP_{nit/denit}$, the three most absorbent groups of NPx in the effluent were $NP_{1-3}EO$, NP and $NP_{4-12}EO$, which were associated with the solid, 98.7, 66.3 and 65.6% respectively. For the ASP_{nit}, the three most absorbent groups of NPx in the effluent were NP, $NP_{1-3}EO$ and $NP_{4-12}EO$, which were associated with the solid, 77.7, 50.5 and 28.6% respectively. At ASP_{carb} and $ASP_{nit/denit}$, average final effluent $\sum$NPx (sum of NP, $NP_{1-12}EO$ and $NP_{1-3}EC$) absorbency respectively decreased overall by 9.3 and 17.3% in comparison to the settled sewage. At ASP_{nit}, final effluent $\sum$NPx absorbency increased overall by 24.9% in comparison to the settled sewage.

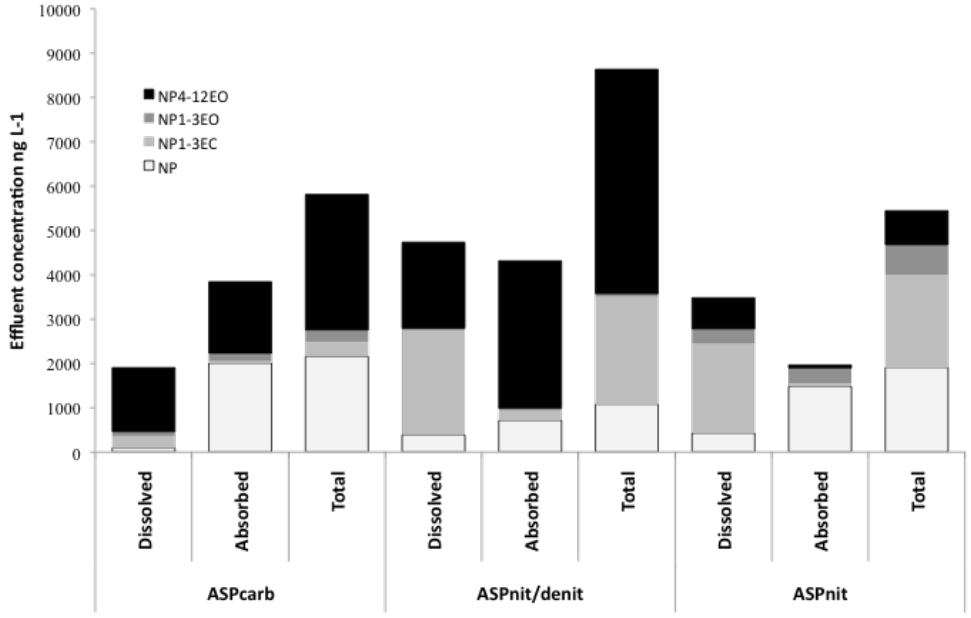


Figure 6-3 The average distribution and concentrations in effluent of $NP_{4\text{-}12}EO$, $NP_{1\text{-}3}EO$, $NP_{1\text{-}3}EC$ and NP within dissolved, absorbed and total (dissolved plus adsorbed) at each ASP over the entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8

Average total ∑NPx (sum of NP, $NP_{1\text{-}12}EO$ and $NP_{1\text{-}3}EC$ in both dissolved and adsorbed phases) effluent concentrations at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were, 5813.9, 8638.2 and 5446.1 ng L^{-1} respectively (Figure 6-3). Based on total average removal, it would appear that the ASP_{nit} was the best performing site as it removed 75.6% of NPx in comparison to ASP_{carb} and $ASP_{nit/denit}$ that removed 48.6 and 19.8% respectively. The individual site average effluent NPx profiles are shown in Figure 6-4. A reduction in net average ethoxylate chain length as well an accumulation in carboxylates could clearly be observed. The most abundant ethoxylate was NP_4EO at ASP_{carb} and $ASP_{nit/denit}$ (1096.7 and 1084.2 ng L^{-1} respectively). At ASP_{nit}, the most abundant ethoxylate was NP_1EO (533.3 ng L^{-1}). The most abundant carboxylate was NP_2EC, which represented 226.7, 1810.0 and 1508.4 ng L^{-1} at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The NP concentrations were 2156.2, 1078.8 and 1909.8 ng L^{-}

[1] at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Far in excess of NP predicted no-effects concentration (PNEC) of 330 ng L^{-1}.

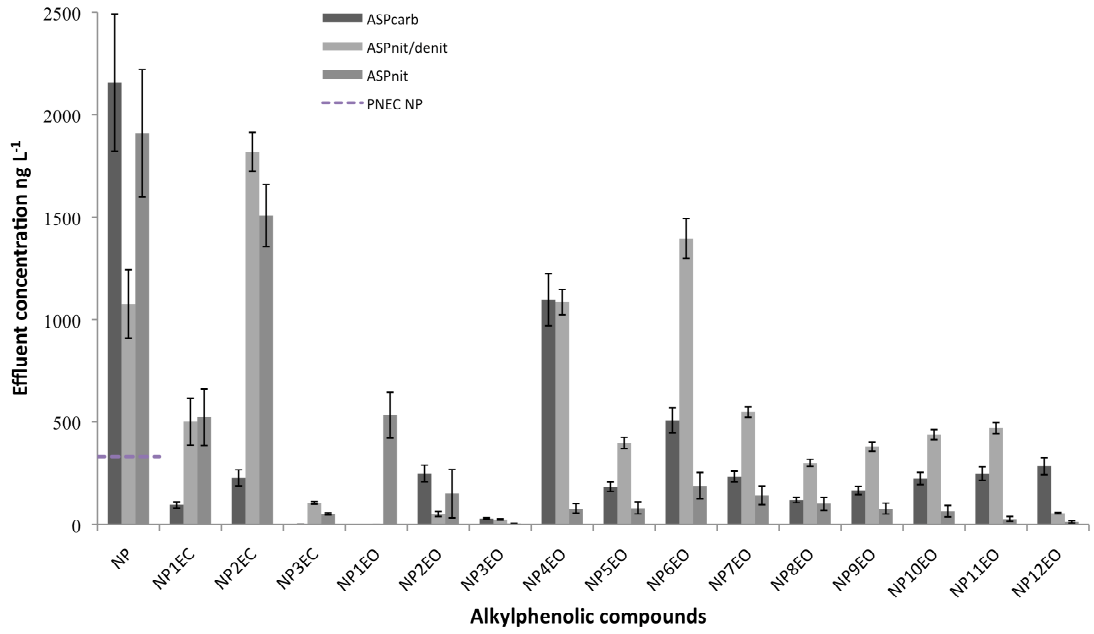


Figure 6-4 Average total (aqueous plus solids phase) individual NPx concentrations in effluent at the three ASP over entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8. Standard deviations displayed as bars. All three sites exceeded the NP NPEC of 330 ng L^{-1}.

6.3 The Nonylphenolic characteristics of the WAS

In the WAS, proportionally, $NP_{4\text{-}12}EO$ were the most abundant NPx at ASP_{carb} and $ASP_{nit/denit}$ representing 66.3 and 50.6 of total average (both liquid and solid phases) NPx respectively. At ASP_{nit} $NP_{1\text{-}3}EC$ were the most abundant NPx in WAS representing 64.0%. The second most abundant NPx at the ASP_{carb} was NP representing 37.0% of total average concentration (both liquid and solid phases). The second most abundant NPx at the $ASP_{nit/denit}$ was $NP_{1\text{-}3}EC$ representing 37.0% of total average concentration (both liquid and solid phases). The second most abundant NPx at the ASP_{nit} was NP representing 35.0% of total average concentration (both liquid and solid phases). Overall, ASP_{nit} had the highest total (solid and aqueous phase)

average ∑NPx (sum of $NP_{1-12}EO$, $NP_{1-3}EC$ and NP) WAS concentration (± standard deviation), 4108±722 ng L^{-1}. Total ∑NPx concentrations (± standard deviation) of 1264±340 and 3769±1008 were found at ASP_{carb} and $ASP_{nit/denit}$ respectively (Figure 6-5). There was no correlation between absorbency and WAS NPx concentration. The ASP_{nit} had the lowest log kp value but the highest NPx WAS concentration. NPx in settled sewage appeared to have greater influence upon WAS concentration.

Nonylphenolics were found principally in the adsorbed phase at ASP_{carb}. At the $ASP_{nit/denit}$ and ASP_{nit}, where they were found chiefly in the aqueous phase (Figure 6-5). The proportion of which ∑NPx were associated with the solids was 1140 ng L^{-1} (90.2 %), 2077 ng L^{-1} (44.9 %) and 3769 ng L^{-1} (10.8%) at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. The ∑NPx log Kp values for the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} were 3.21, 2.14 and 1.92 respectively. Log kp values were appreciably lower in comparison to the settled sewage and final effluent values due to the considerably higher solids content. Each site had different absorbency profiles. At ASP_{carb}, every NPx was found in association with the solids >85% with the exception of NP_1EC which was only 18.1%. At $ASP_{nit/denit}$, as with the settled sewage, the most adsorbent NPx were NP_2EO, NP_3EO and NP_4EO, with 98.0, 99.7 and 73.0% associated with the solids respectively. All other compounds at this site were less absorbent, being in association with the solids <56%, including NP whose absorbency was 15.0%. At ASP_{nit} the most adsorbent NPx were, NP_1EO, NP_3EO and NP_1EC, with 72.5, ~100 and 50.5% associated with solids respectively. All other compounds at this site were less absorbent, being in association with the solids <32%, including NP whose absorbency was 16.2%. At all sites $NP_{1-3}EO$ were the most absorbent compounds (Figure 6-5), however, these NPx were only significant constituents of the WAS at ASP_{carb}.

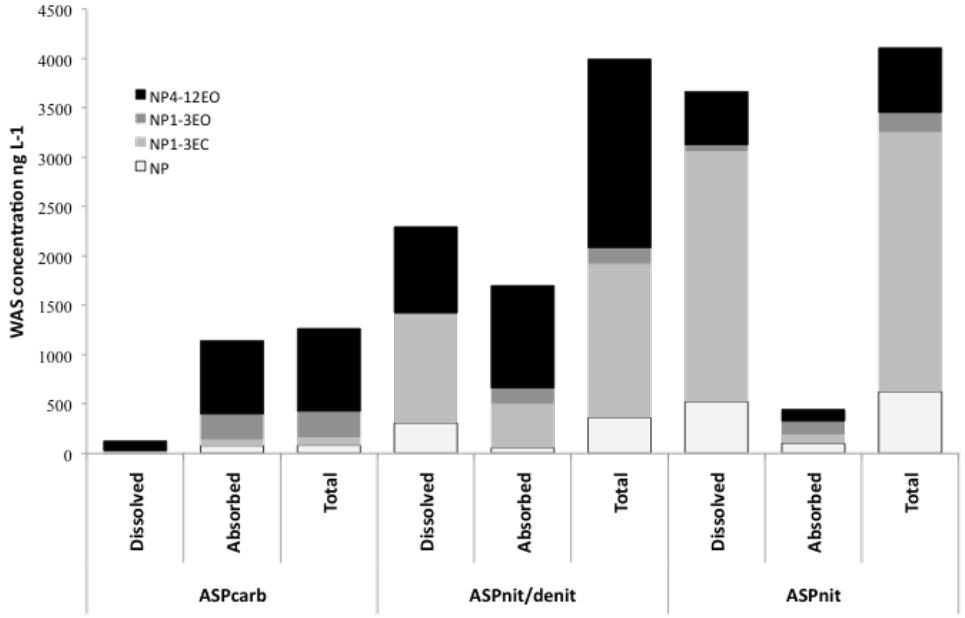


Figure 6-5 The average distribution and concentrations in WAS of $NP_{4-12}EO$, $NP_{1-3}EO$, $NP_{1-3}EC$ and NP within dissolved, absorbed and total (dissolved plus adsorbed) at each ASP over the entire sampling period. At ASP_{carb} n=6. $ASP_{nit/denit}$ n=4. ASP_{nit} n=4

The distribution of total NPx (in both aqueous and solid phases) in WAS is further elaborated in Figure 6-6. In comparison to the settled sewage, the presence of short chain NPx ($NP_{1-3}EO$ and $NP_{1-3}EC$) in WAS was apparent. Except for the ASP_{carb} where total ∑NPx concentration was significantly lower than the other two sites and representatives of $NP_{4-14}EO$ were the most abundant group. Here, NP_2EO, NP_4EO and NP_6EO were the most abundant NPx, with concentrations of 204, 309 and 230 ng L^{-1} respectively. At the $ASP_{nit/denit}$, the most abundant long chain NPEO was NP_4EO with a concentration of 506 ng L^{-1}. At the ASP_{nit}, the most abundant long chain NPEO was NP_8EO with a concentration of 114 ng L^{-1}. At the $ASP_{nit/denit}$, the most abundant NPx was NP_2EC with a concentration of 1023 ng L^{-1}. At the ASP_{nit}, the most abundant NPx was also NP_2EC with a concentration of 1967 ng L^{-1}.

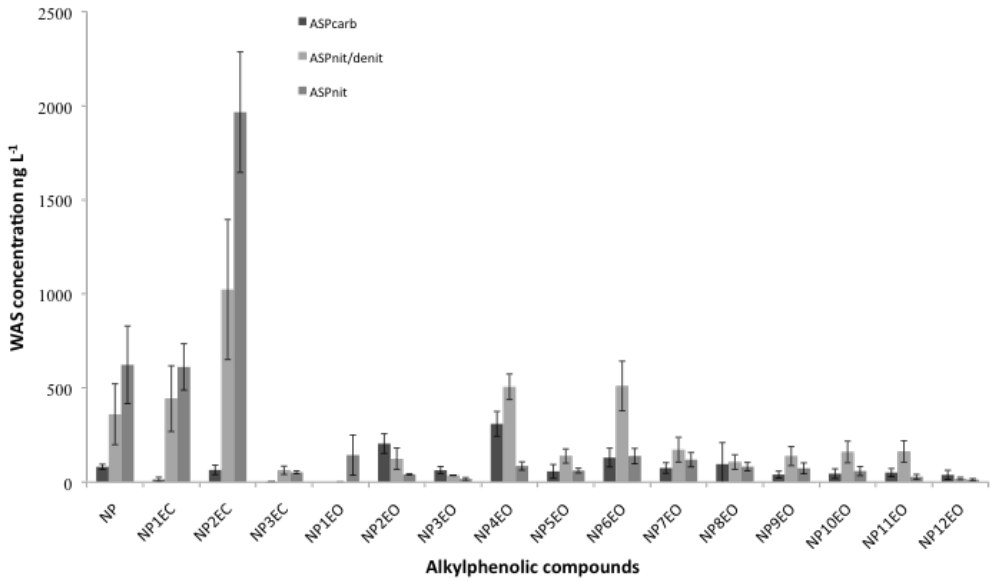


Figure 6-6 Average total (aqueous plus solids phase) individual NPx concentrations in WAS at the three ASP over entire sampling period. At ASP_{carb} n=6. $ASP_{nit/denit}$ n=4. ASP_{nit} n=4. Standard deviations displayed as bars.

6.4 Mass Flux and Biodegradation and Bioformation of Nonylphenolics at each ASP

The greatest average daily mass of ∑NPx entering the three sites was 508530 mg d^{-1} at the $ASP_{nit/denit}$, unsurprisingly as it was the largest site (Figure 6-7). Since ASP_{nit} was a pilot plant, it had the lowest average daily mass of NPx in settled sewage, 200.9 mg d^{-1}. The average daily mass of NPx at the ASP_{carb} was 174954 mg d^{-1}. The ∑NPx average daily mass in effluent was 86015 mg d^{-1}, 56.5 mg d^{-1} and 360777 mg d^{-1} at ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ respectively. Based on this mass balance, removal efficiencies were 49.2, 71.9 and 35.1% at ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ respectively. At the and ASP_{nit}, biodegradation was the predominant removal mechanism. At ASP_{nit}, 142.8 mg d^{-1} was biodegraded (71.1 % biodegradation), whilst only 1.6 mg d^{-1} (0.8%) was lost in waste activated sludge (WAS). At $ASP_{nit/denit}$, 182167 mg d^{-1} was

biodegraded (32.8% biodegradation), whilst only 12943 mg d^{-1} (2.3%) was lost in waste activated sludge (WAS). At ASP_{carb}, the sludge wastage rate was more significant due to the lower SRT. Here 86015 mg d^{-1} was biodegraded (39.7% biodegradation) and 16583 mg d^{-1} (9.5%) was lost in WAS. This therefore implies that effective removal may be more dependent upon wastage rate than specifically the age of the sludge bacteria, as decreasing the volume of WAS would increase the overall removal efficiency of this site.

The mass flux of specific NPx groups is further elaborated in Figure 6-8. At all sites, the long chain ethoxylates ($NP_{4-12}EO$) demonstrated biodegradation. ASP_{nit} had the highest biodegradation efficiency (95.2%). The ASP_{carb} and $ASP_{nit/denit}$ had lower $NP_{4-12}EO$ biodegradation efficiencies of 59.3 and 56.0% respectively. The relative standard deviation (RSD) of the average percent biodegradation of $NP_{4-12}EO$ was 25.8, 34.4 and 3.1% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Thus $NP_{4-12}EO$ were only biodegraded (and not formed) presumably by ethoxylate chain shortening. The variability of biodegradation was the least for $NP_{4-12}EO$. At all sites, the short chain carboxylates ($NP_{1-3}EC$) demonstrated bioformation. ASP_{nit} had the lowest bioformation efficiency (242.0%). The ASP_{carb} and $ASP_{nit/denit}$ had higher $NP_{1-3}EC$ bioformation efficiencies of 976.9 and 1160.3% respectively. The relative standard deviation (RSD) of the average percent bioformation of $NP_{1-3}EC$ was 63.4, 50.1 and 38.7% at ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Of the short-chained ethoxyate compounds $NP_{1-3}EO$ demonstrated 174.6 and 79.9% bioformation at ASP_{carb} and ASP_{nit}, respectively. At $ASP_{nit/denit}$ average $NP_{1-3}EO$ biodegradation of 47.7% was witnessed. However here, the RSD of this average was 135.9% indicating that biodegradation was highly variable and during the sampling, periods of biotransformation probably occurred. The only site that demonstrated net a NP biodegradation of 49.8% was ASP_{nit}. However, despite NP biotransformation efficiencies of 12.4 and 6.6%, the RSDs for this compound at all sites was >100%. Evidently this indicated that all sites had periods of NP bioformation as well as biodegradation.

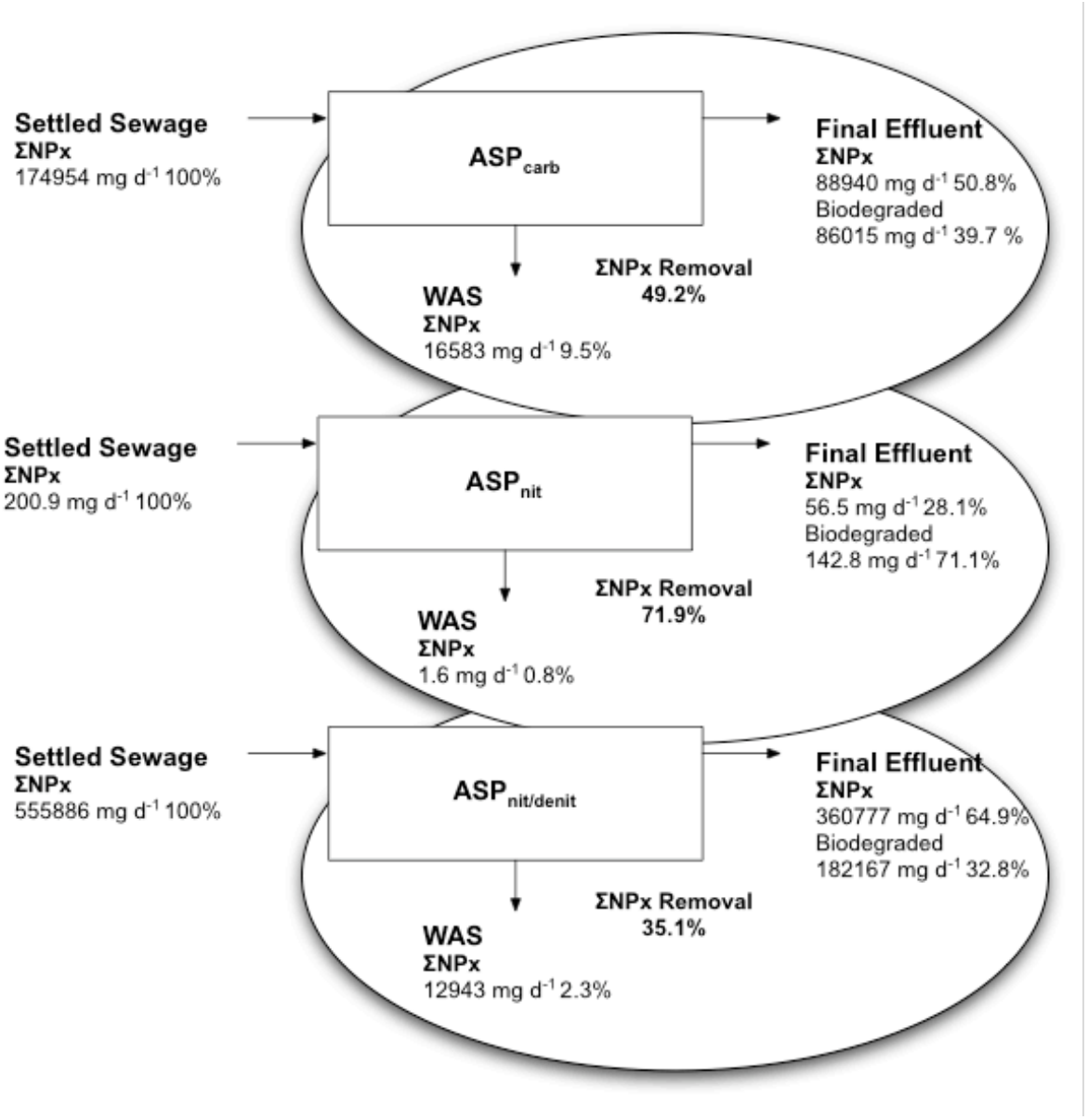


Figure 6-7 Mass Balance of the sites indicating ∑NPx daily average masses and percent proportions entering settled sewage and accounted for in effluent, waste activated sludge (WAS), by biodegradation and total site removal.

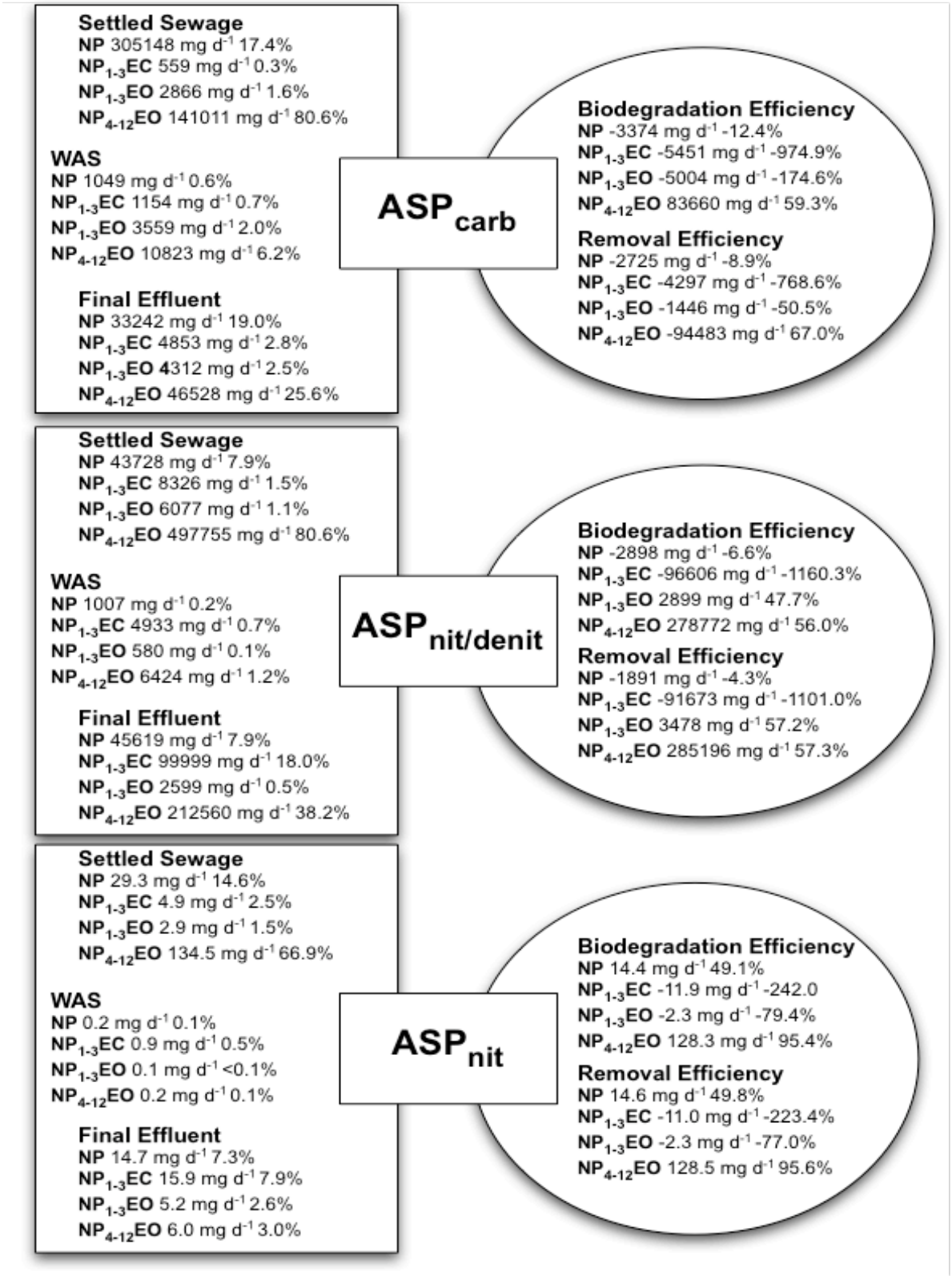


Figure 6-8 Mass Balance of the sites indicating NP, $NP_{1-3}EC$, $NP_{1-3}EO$ and $NP_{4-12}EO$ daily average masses and percent proportions entering settled sewage and accounted for in effluent, waste activated sludge (WAS), by biodegradation and total site removal.

To fully compare the three ASP, biodegradation was normalised to MLSS concentration (Figure 6-9). Confirming previous finding, the ASP_{nit} was the most effective site for the biodegradation of NPx. Here average biomass activities were -1086, -227, 11543 and 1286 mg NPx biodegraded $tonne^{-1}$ day^{-1} for $NP_{1-3}EC$, $NP_{1-3}EO$, $NP_{4-12}EO$ and NP respectively. At the ASP_{carb} biomass activities were -365, -298, 6709 and -2147 mg NPx biodegraded $tonne^{-1}$ day^{-1} for $NP_{1-3}EC$, $NP_{1-3}EO$, $NP_{4-12}EO$ and NP respectively. At the $ASP_{nit/denit}$ biomass activities were -927, 19, 2172 and -71 mg NPx biodegraded tonne day for $NP_{1-3}EC$, $NP_{1-3}EO$, $NP_{4-12}EO$ and NP respectively. Interestingly, despite being the largest site and receiving the largest daily mass of NPx, the $ASP_{nit/denit}$ preformed the worst of all sites. Here ∑NPx biomass activity was 1193 mg ∑NPx biodegraded $tonne^{-1}$ day^{-1}. At the ASP_{carb}, 3900 mg ∑NPx was biodegraded $tonne^{-1}$ day^{-1}. At the ASP_{nit} 11516 mg ∑NPx was biodegraded $tonne^{-1}$ day^{-1}.

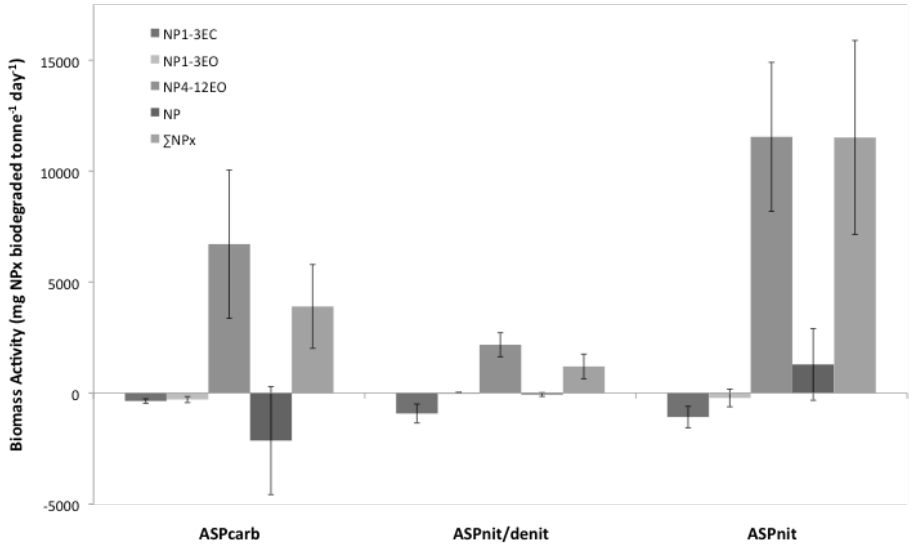


Figure 6-9 Average $NP_{1-3}EC$, $NP_{1-3}EO$, $NP_{4-12}EO$, NP and ∑NPx biomass activities (mg NPx bniodegraded^{-1} tonne d^{-1}) at the three ASP over entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8. Standard deviations displayed as bars.

6.5 The Molar biodegradation of the Nonylphenol Base Unit and the interconnection between Nonylphenolic Species

Nonylphenolic biodegradation witnessed as (mg NPx biodegraded per day) was mainly due to ethoxylate chain shortening and not the elimination of the nonylphenol base unit. Nonylphenolic biodegradation in terms of micromoles biodegraded per day is detailed in Figure 6-10. The average ∑NPx biodegradation was 105.0, 0.25 and 87.0 mMol d^{-1}. This was equivalent to 23134.1, 19174.4 and 54.3 mg NP biodegraded day^{-1} at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively. Of the NPx mass biodegradation detailed in Figures 6-7 and 6-8, it would appear that much of this can be attributed to ethoxylate chain shortening. However, the standard deviation of the averages at ASP_{carb} and $ASP_{nit/denit}$ were vast, resulting in RSDs of 540.0 and 194.6% respectively, indicating that bioformation could have been occurring as well. This evidence of formation of NPx could be due to accumulated uncertainty as a result of the analytical method as well as, the presence of CAPEX (which were not analysed for) in settled sewage could have contributed to ∑NPx in the effluent. Due to the variability of molar biodegradation of NPx at ASP_{carb} and $ASP_{nit/denit}$, it is unclear whether the nonylphenol base unit was net eliminated or not.

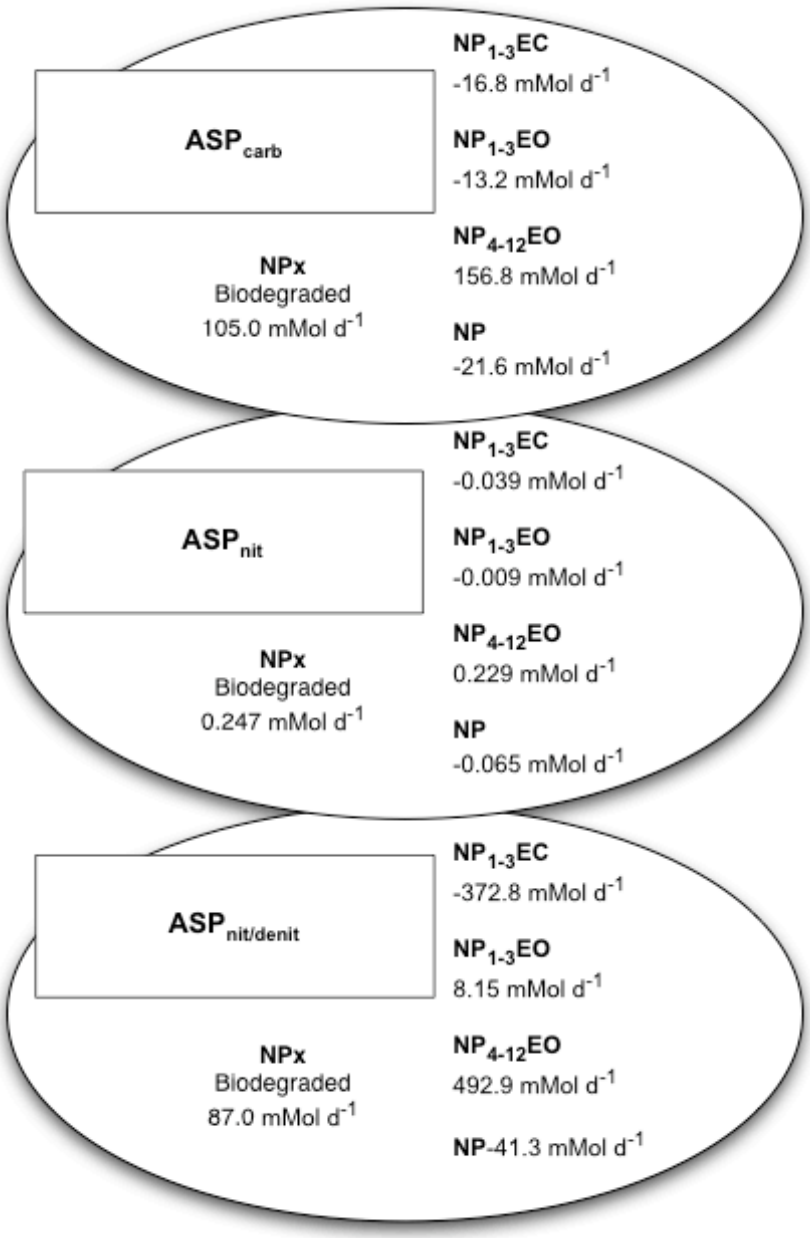


Figure 6-10 The average molar biodegradation of ∑NPx, $NP_{1-3}EC$, $NP_{1-3}EO$ and $NP_{4-12}EO$ at the three ASP over entire sampling period. At ASP_{carb} n=18. $ASP_{nit/denit}$ n=18. ASP_{nit} n=8.

Using molar biodegradation, it was possible to demonstrate that long chain ethoxylates were converted to more recalcitrant nonylphenolics at ASP_{carb} and $ASP_{nit/denit}$. Due to net biodegradation at the ASP_{nit}, a relationship between the breakdown of $NP_{4-12}EO$ and the formation of NP, $NP_{1-3}EO$ and $NP_{1-3}EC$ could not be demonstrated. There appeared to be a linear relationship between molar biomass activity (mMol NP4-12EO biodegraded $tonne^{-1}$ day^{-1}) and the biotransformation

(mMol NP, $NP_{1-3}EO$ and $NP_{1-3}EC$ biotransformed $tonne^{-1}$ day^{-1}) at the ASP_{carb} and $ASP_{nit/denit}$ (Figure 6-11). At the ASP_{nit}, the net molar biodegradation observed at this site precluded the linear formation of NP, $NP_{1-3}EO$ and $NP_{1-3}EC$ as witnessed at the other sites.

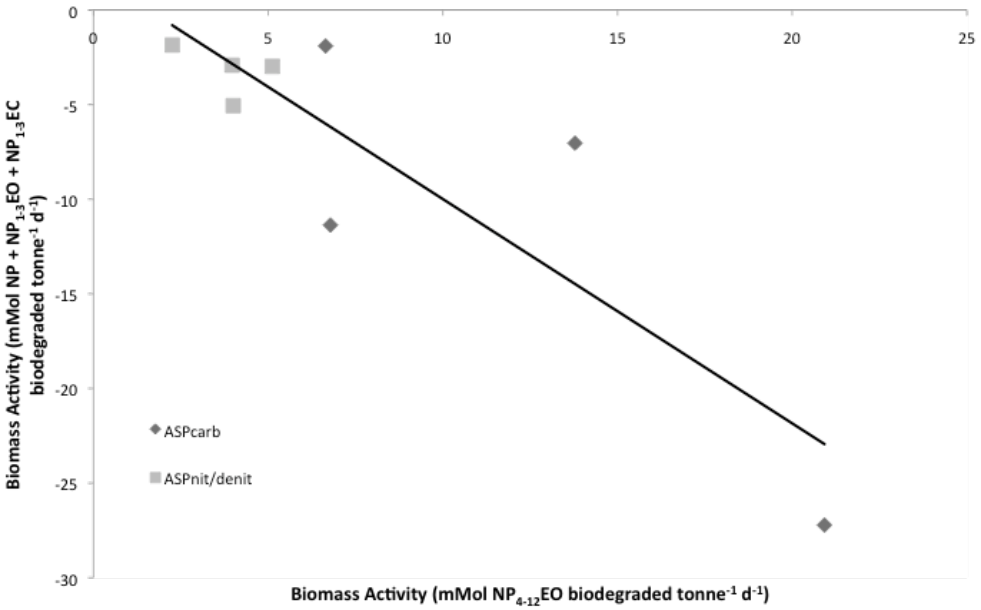


Figure 6-11 The relationship between daily average molar biomass activities of $NP_{4-12}EO$ and $NP+NP_{1-3}EO+NP_{1-3}EC$ at ASP_{carb} and $ASP_{nit/denit}$. At the ASP_{carb} and $ASP_{nit/denit}$ it appears that NP, $NP_{1-3}EO$ and $NP_{1-3}EC$ are formed linearly with the biodegradation of $NP_{4-12}EO$. At the ASP_{nit}, this relationship did not exist, as NPx were net biodegraded.

6.6 The resource dependent biodegradation of long chain nonylphenolics

The long chain nonylphenolic compounds ($NP_{4-12}EO$) were the most readily biodegradable compounds. Therefore this compound group was used to assess how resource availability influenced biodegradation. Average biomass activities were, 6678, 1888 and 11543 (mg $NP_{4-12}EO$ biodegraded $tonne^{-1}$ day^{-1}) for ASP_{carb},

$ASP_{nit/denit}$ and ASP_{nit} respectively. Indicating that despite an average $NP_{4\text{-}12}EO$ biodegradation efficiency of 67.0%, per tonne of biomass, the ASP_{carb} was removing comparably more NPx than the $ASP_{nit/denit}$. As with the estrogens, the variation in biomass activity witnessed between the three ASPs appeared to be driven by loading, and biodegradation appeared to increase proportionally with resource availability (volumetric loading). Due to the reactor volume at the $ASP_{nit/denit}$, despite being the largest site, its $NP_{4\text{-}12}EO$ volumetric loading was on average circa 10 mg m^{-3} d^{-1}, significantly lower than the other sites. Comparably lower volume in comparison to their flow rates resulted in average $NP_{4\text{-}12}EO$ volumetric loadings of 43 and 55 mg m^{-3} d^{-1} at the ASP_{carb} and ASP_{nit} respectively. Clearly the ASP_{carb} and ASP_{nit} had greater NPx resources available to the biomass than the $ASP_{nit/denit}$. On average, maximum potential biomass activities were 9902, 4018 and 11721.7 mg ∑EST removed $tonne^{-1}$ day^{-1} at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively.

Using normalised data from all three sites, a linear relationship (R^2=0.76) between, volumetric loading (mg m^{-3} d^{-1}) and biomass activity ($NP_{4\text{-}12}EO$ biodegraded $tonne^{-1}$ day^{-1}) could be observed (Figure 6-12). This is evidence of first order relationship between biodegradation and loading and nonylphenolics behave similarly to estrogens. This relationship could demonstrate that resource dependent biodegradation was occurring at full-scale. This therefore implies that effective biodegradation may be more dependent upon concentration and flow conditions than whether a site has nitrifying or nitrifying/denitrifying capability. Coupled with the biodegradation of $NP_{4\text{-}12}EO$, there was bioformation $NP_{1\text{-}3}EC$ at all sites and $NP_{1\text{-}2}EO$ and NP in some cases. For this reason, clear relationships between loading and biodegradation could only be established for $NP_{4\text{-}12}EO$.

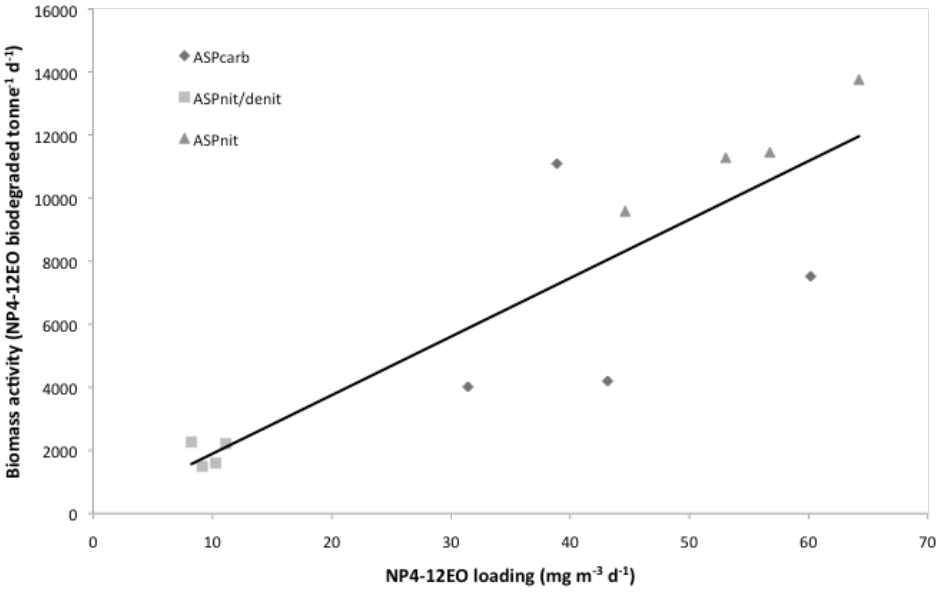


Figure 6-12 The relationship between daily average $NP_{4\text{-}12}EO$ loading and biomass activity at the three ASP suggesting biodegradation may follow pseudo-first order reaction kinetics

Assessing molar biodegradation, it can be demonstrated that ∑NPx also followed a first-order relationship (Figure 6-13) as could be seen with mass biodegradation of $NP_{4\text{-}12}EO$ (Figure 6-14). However, this did not occur at the origin as with mass biodegradation of $NP_{4\text{-}12}EO$ (Figure 6-12). With molar biodegradation of ∑NPx there appeared to be lag between volumetric loadings of 0.02 and 0.14 mg ∑NPx m^{-3} d^{-1}, mMol ∑NPx biomass activity remained <3.7 mMol ∑NPx biodegraded $tonne^{-1}$ d^{-1}. Only when loadings exceeded 0.14 mMol ∑NPx m^{-3} d^{-1}, did molar biomass activity proceed in a linear fashion. Between loading of 0.02 and 0.14 mMol ∑NPx m^{-3} d^{-1}, molar biodegradation of ∑NPx was minimal. Mass losses during these loading could be attributed predominantly to de-ethoxylation.

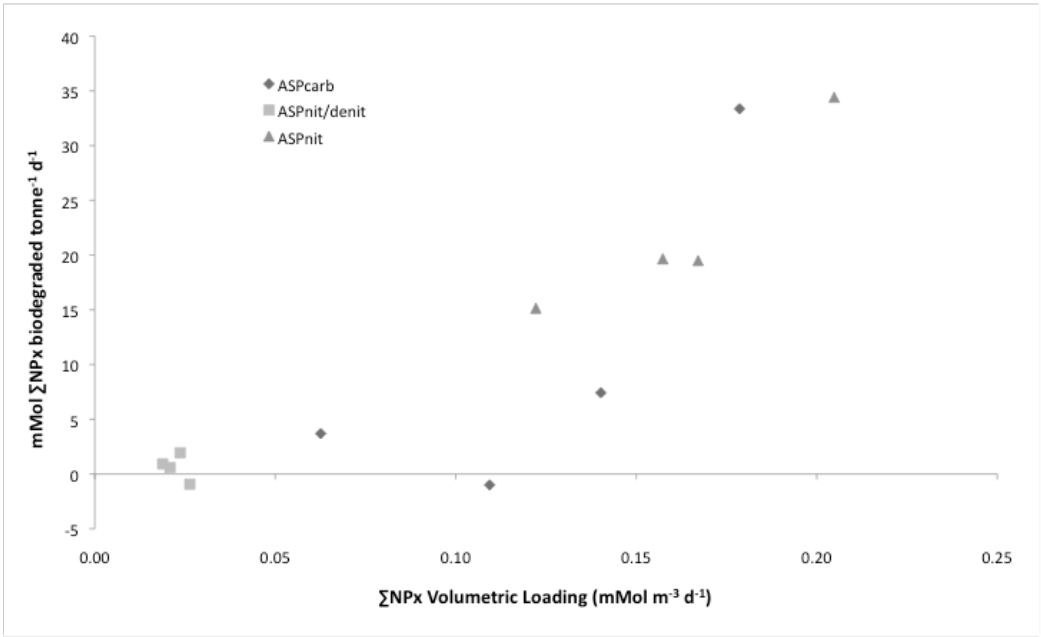


Figure 6-13 The relationship between daily average mMol ∑NP loading and biomass activity at the three ASP suggesting biodegradation may follow first-order reaction kinetics after ∑NP loading of 0.14 mMol ∑NP m^{-3} d^{-1}.

6.7 The influence of competing carbon sources upon the biodegradation of Nonylphenolics

The inability of ASP biomass to net biodegrade NP, $NP_{1-3}EO$ and $NP_{1-3}EC$ at loadings of 0.02 to 0.14 mg ∑NPx m^{-3} d^{-1}, could have been due to the presence of competing carbon sources. The presence of more readily biodegradable carbon could have had an inhibiting effective upon the biodegradation of NPx. Unlike the estrogens, it appeared that there was a clearer impact from bulk organics upon the overall biodegradation of nonylphenolic compounds, particularly with regards to the nonylphenol base unit. The only site that demonstrated appreciable net molar biodegradation of NPx was ASP_{nit}. This site had the highest proportion of NPx as carbon available to the activated sludge biota. To determine the influence of bulk organics upon biodegradation of NPx, $NP_{4-12}EO$ where assessed. These compounds demonstrated biodegradation at all sites and

are the first link in a complete removal pathway resulting in the formation of all other NPx. By assessing the biodegradation of these compounds under variable proportions as available carbon, $NP_{4\text{-}12}EO$ gave insight into how competing carbon influences the removal of NPx.

The highest daily average $NP_{4\text{-}12}EO$ biomass activities (>9590 mg $NP_{4\text{-}12}EO$ biodegraded $tonne^{-1}$ day^{-1}) were recorded when the settled sewage $sCOD/NP_{4\text{-}12}EO$ ratio was each day on average <20828 mg $sCOD^{-1}$ mg $NP_{4\text{-}12}EO$ L^{-1} (Figure 6-14). Furthermore, a power relationship between $sCOD/NP_{4\text{-}12}EO$ ratio and biomass activity could be clearly demonstrated (Figure 6-14). The significance of this relationship could be demonstrated by an r^2 of 0.76, much higher than equivalent relationship with the estrogens (see Section 5.6). It therefore appeared that biomass activities were lowest when the ratio of $sCOD/NP_{4\text{-}12}EO$ increased (when the proportion of $NP_{4\text{-}12}EO$ as organic carbon decreased). This could indicate that suppression of $NP_{4\text{-}12}EO$ biodegradation was influenced by the greater availability of more readily available bulk organics. However, since biomass activity was also linked with loading, as established in Section 5.6, a relationship between $sCOD/NP_{4\text{-}12}EO$ ratio and volumetric loading could also be demonstrated (r^2 0.62). Therefore, disentangling the effect $NP_{4\text{-}12}EO$ loading has from the influence of other organic compounds had been difficult, as the highest loading of $NP_{4\text{-}12}EO$ occurred when these compound comprised their largest proportion as COD, which also coincided with the greatest biomass activities.

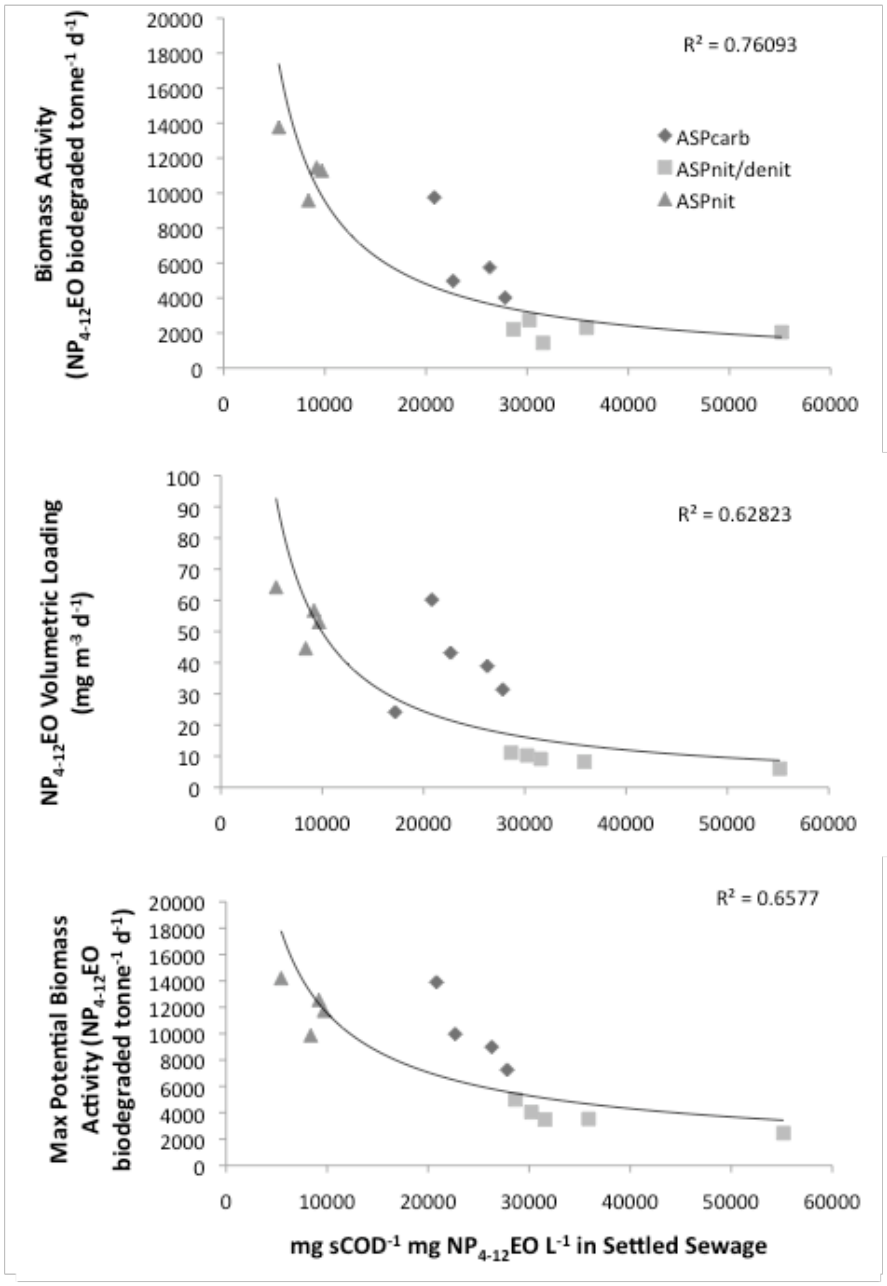


Figure 6-14 The Relationship between daily average COD/$NP_{4\text{-}12}EO$ settled sewage ratio and $NP_{4\text{-}12}EO$ biomass activity, volumetric loading and maximum potential biomass activity suggesting inhibition from COD upon the biodegradation of NPx

Due to the correlation between volumetric loading biomass activity, plotting COD/$NP_{4\text{-}12}EO$ ratio against volumetric loading and maximum potential biomass activity resulted in similar power relationships: Interestingly, these curves had a lower r^2 values, 0.62 and 0.66 respectively in comparison to the relationship between COD/$NP_{4\text{-}12}EO$ ratio and biomass activity. Lower r^2 values resulted from high $NP_{4\text{-}12}EO$ loadings (> 30 mg $NP_{4\text{-}12}EO$ m^{-3} d^{-1}) at the ASP_{carb} that occurred when COD/$NP_{4\text{-}12}EO$ ratio was between 20000 and 30000 mg $sCOD^{-1}$ mg $NP_{4\text{-}12}EO$ L^{-1}. Furthermore the highest loadings did not necessarily correspond with instances where $NP_{4\text{-}12}EO$ proportion of the COD was highest. Although the $ASP_{nit/denit}$ had at volumetric loading <11.1 mg $NP_{4\text{-}12}EO$ m^{-3} d^{-1} and subsequently could not have had a biomass activity exceeding 5005 mg $NP_{4\text{-}12}EO$ $tonne^{-1}$ d^{-1}.

Yet despite the limitations of these relationships, the growing disparity between actual and maximum potential biomass activity, from the lowest to the highest COD/$NP_{4\text{-}12}EO$ ratios was evident. It was observed that the relative difference between potential and actual biomass activities increased with sCOD/$NP_{4\text{-}12}EO$ ratio. This relative difference, as described by biodegradation efficiency, decreased as the availability of other organic material increased. A linear relationship between biodegradation efficiency and sCOD/$NP_{4\text{-}12}EO$ ratio (Figure 6-15) could be demonstrated (R^2=0.78). $NP_{4\text{-}12}EO$ percent biodegradation was 96.5% at the ASP_{nit} when the sCOD/$NP_{4\text{-}12}EO$ ratio was 5451 mg $sCOD^{-1}$ mg $NP_{4\text{-}12}EO$ L^{-1}. At the $ASP_{nit/denit}$, $NP_{4\text{-}12}EO$ percent biodegradation was 42.6% when the sCOD/$NP_{4\text{-}12}EO$ ratio was 31570 mg $sCOD^{-1}$ mg $NP_{4\text{-}12}EO$ L^{-1}. This decrease in biodegradation efficiency could be due to the increasing availability of other carbon sources to the biota. The resulting linear regression of daily $NP_{4\text{-}12}EO$ percent biodegradation and sCOD/$NP_{4\text{-}12}EO$ ratio would indicate that at 54330 mg $sCOD^{-1}$ mg $NP_{4\text{-}12}EO$ L^{-1}, biodegradation of $NP_{4\text{-}12}EO$ could not occur. This is strongest evidence to date that bulk organics can have in an inhibitory effect upon the biodegradation of NPx.

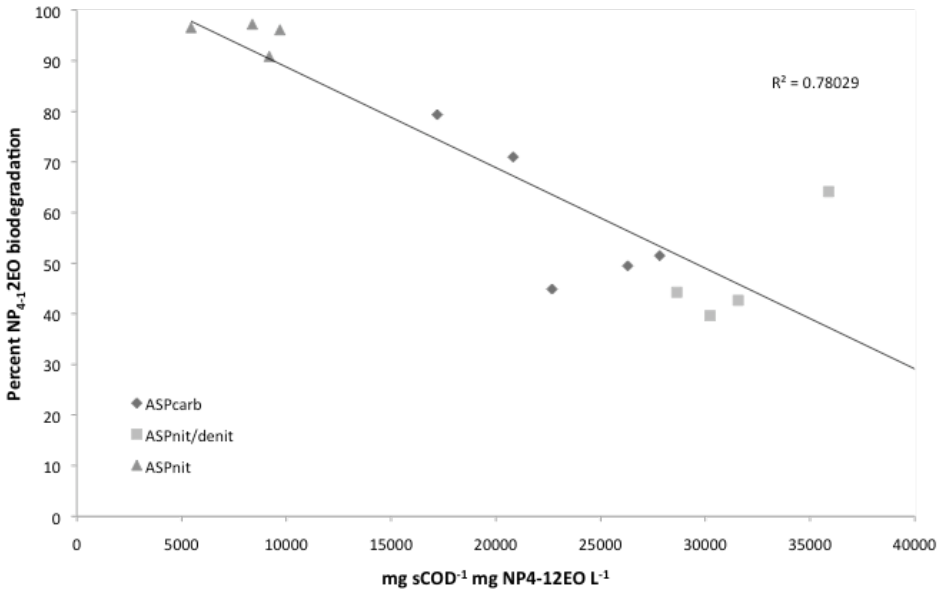


Figure 6-15 The linear relationship between daily average COD/$NP_{4\text{-}12}EO$ settled sewage ratio and percent $NP_{4\text{-}12}EO$ biodegradation indicating that NPx may be inhibited by increasing proportion of other more readily available organic material.

Chapter 7

The Biodegradation of Estrogen Exponentially Loaded under Pilot Conditions

7 The biodegradation of estrogen exponentially loaded with estrogen under pilot conditions

Findings from previous chapters have indicated that EDC loading was key to driving removal of these compounds during activated sludge treatment. Additionally, 54% estrogen removal was observed at the ASP_{carb} indicating the potential role of heterotrophic bacteria in the removal of these compounds. However, due to the interrelationship between SRT, HRT and loading, the separate function of these parameters needed to be determined. Furthermore, the influence of competitive inhibition from more readily available carbon needed clarification, as EDC loading correlated with increasing proportion of these compounds as COD. To understand how removal of EDCs can be optimised during activated sludge treatment is was necessary to design an experiment in order to elucidate the individual roles of these parameters.

Using porous pot bioreactors estrogen loading was exponentially increased independently of HRT and SRT in two separate experimental conditions. These were Low HRT/SRT and High HRT/SRT experimental conditions. Altogether the E1+E2+E3 loading ranged from 0.04 to 2.12 mg^{-1} m^{-3} d^{-1}. At three of the spiking periods, estrogen loadings were identical between the Low HRT/SRT and High HRT/SRT experimental conditions. Thus allowing the possibility to establish the role of HRT and SRT upon biodegradation of estrogens. Additionally, estrogen concentrations approximately doubled each subsequent spiking period whilst influent COD concentration remained constant. Doing this, the objective was to determine how increasing the proportion of estrogens as overall COD influenced the biodegradation of these compounds. Thus determined whether competitive inhibition of estrogens by more readily available carbon does occur. To determine the role of ammonia oxidising bacteria (AOB), synthetic sewage was used. This made it possible to control the nitrogen source the activated sludge was receiving. At the highest spiking concentration, ammonia was removed in order to reduce AOB populations. This was done in order to assess the role of these bacteria upon the biodegradation of estrogens.

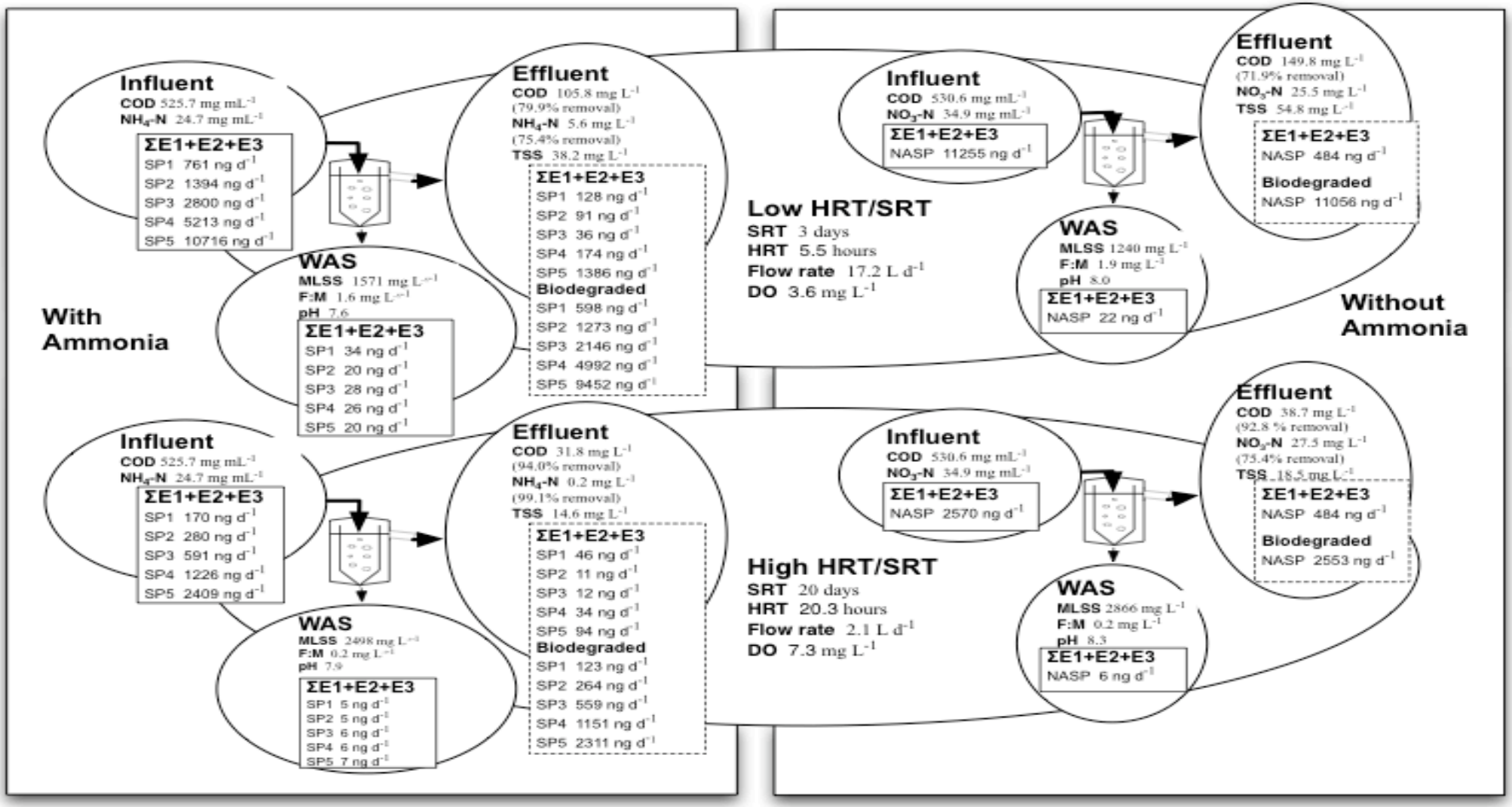


Figure 7-1 The E1+E2+E2 mass balance and general performance of the Low HRT/SRT and High HRT/SRT experimental conditions. The schematic details average ng d^{-1} masses of E1+E2+E3 in influent, effluent and waste activated sludge (WAS) during spiking periods were ammonia was the nitrogen source (SP1-5) and when ammonia was removed (NASP)

7.1 The general performance of the porous pot bioreactors

The average general wastewater characteristics and process parameters associated with the synthetic sewage and porous pot bioreactor effluents are detailed in Figure 7-1. During the 57 day sampling period of the porous pot bioreactors, flow rates were kept as constant as possible. Total average flow rates (±1SD) were 0.71±0.09 and 0.19±0.02 L hr^{-1} respectively for the Low HRT/SRT and high HRT/SRT experimental conditions respectively. Therefore, average HRTs (±1SD) were 5.39±0.82 and 20.30±2.14 hours respectively for the Low HRT/SRT and high HRT/SRT experimental conditions. Fixed volumes of MLSS were wasted daily ensuring constant respective SRTs of 3 and 20 days. These SRTs influenced average MLSS concentration (±1SD) of 1671±409 and 1240±386 mg L^{-1} respectively for SP1-5 and the NASP of the Low HRT/SRT experimental condition. In comparison, the High HRT/SRT experimental condition had the highest average MLSS concentration of 2498±410 and 2866±457 mg L^{-1} respectively for SP1-5 and the NASP. Due to higher bulk organic and estrogen loading of the Low HRT/SRT experimental condition, the F:M ratio was 1.55±0.72 mg BOD^{-1} mg MLVSS d^{-1}; comparably higher than the F:M ratio of the high HRT/SRT experimental condition (0.24±0.08 mg BOD^{-1} mg MLVSS d^{-1}).

During the total sampling period, both experimental conditions complied with upper 95 percentile COD effluent quality limits of 125mg L^{-1}, as specified by the urban wastewater treatment directive. However, the Low HRT/SRT experimental condition had the highest total average COD. This concentration (±1SD) was, 105.84±67.85 mg L^{-1}, higher in comparison the average COD effluent quality of the High HRT/SRT condition whose concentration (±1SD) was, 31.79±14.79 mg L^{-1}. In comparison, the High HRT/SRT experimental condition removed circa 94% compared with the Low HRT/SRT bioreactors, which removed circa, 80%. This superior COD removal in the High HRT/SRT condition was typical of a system with longer hydraulic and solids retention times. The comparably lower SRT of three days clearly influenced its biomass, forcing the activated sludge into a state of net anabolism. Not only was the MLSS concentration lower, but also the amount of biomass varied significantly in comparison to the high HRT/SRT experimental condition. The relative

standard deviation (RSD) of MLSS concentration was 27.43% for the Low HRT/SRT condition, higher in comparison to the High HRT/SRT condition whose RSD was 18.05%. This indicated that the Low HRT/SRT stability, in terms of performance consistency of removal was the lowest of the experimental conditions.

7.2 Estrogen sorption into solids phases

At the ASP_{carb}, the high wastage rate coupled with high estrogen concentrations in the WAS resulted in significant losses to the WAS. In the porous pot experiment, estrogens were principally found in association with aqueous phases. In fact, estrogens were observed to be associated with the solids at or just above the analytical LOD. The LODs for solids phase estrogens were, 2.1, 4.9, 4.5 and 5.3 ng g^{-1} for E1, E2, E3 and EE2 respectively. This typically equated to <0.08, <0.19, <0.17 and <0.20 ng L^{-1} using the average effluent TSS concentration of the Low HRT/SRT experimental condition for E1, E2, E3 and EE2 respectively. For the High HRT/SRT experimental condition, this typically equated to <0.04, <0.07, <0.07 and <0.08 ng L^{-1} average effluent TSS concentration for E1, E2, E3 and EE2 respectively. For MLSS, due to higher solids concentrations, the LOD biased higher concentrations. For the Low HRT/SRT experimental conditions, MLSS concentration were, <3.3, <7.7, <7.1 and <5.3 ng L^{-1} for E1, E2, E3 and EE2 respectively. For the Low HRT/SRT experimental conditions, MLSS concentration were, <5.2, <12.2, <11.2 and <13.2 ng L^{-1} for E1, E2, E3 and EE2 respectively.

Estrogens therefore were only detected reliably in the aqueous phases. For this reason, it has not been possible to calculate log K*p* values. All concentrations reported in this chapter are a sum of concentrations detected in the aqueous plus the LOD for solids analysis normalised to solids concentration. For this reason, it has not been possible to determine the impact of sorbtion upon estrogen biodegradation. Any attempts to do so would have resulted in bias based upon the analytical LOD for solids analysis. However, despite this limitation, it was clear that sorption was less important in the porous pots than the actual ASP sampled.

7.3 The impact of HRT and SRT upon estrogen biodegradation in the porous pots

From spiking period one-four (SP1-4), average E1, E2, E3 and EE2 total (solid and aqueous phase) concentrations of the effluent were all less than 7.2 ng L^{-1} for both the Low HRT/SRT and High HRT/SRT experimental conditions (Figure 7-2). This occurred as ∑EST influent concentration increasing from 42 – 333 ng L^{-1} from SP1-4. Only during SP5 and the NASP did individual estrogen total (solid and aqueous phase) average concentrations elevate and the experimental conditions be differentiated from one another in terms of effluent concentration. During SP5 when total (solid and aqueous phase) ∑EST influent concentrations increase by the greatest amount from 333 to 667 ng L^{-1}, E1, E2, E3 and EE2 total (solid and aqueous phase) average effluent concentrations were 44.5, 11.5, 2.9 and 0.2 respectively for the Low HRT/SRT experimental condition. This was higher than the average E1, E2, E3 and EE2 effluent SP5 concentrations of 18.5, 1.4, 1.0 and 0.2 ng L^{-1} respectively for the High HRT/SRT experimental condition. During NASP total (solid and aqueous phase) average effluent concentration decreased, presumably due to acclimation to influent ∑EST concentration of 667 ng L^{-1}. During the NASP, E1, E2, E3 and EE2 total average effluent concentrations were 17.0, 7.1, 1.7 and 1.0 ng L^{-1} respectively for the Low HRT/SRT experimental condition. These were higher than the total average E1, E2, E3 and EE2 effluent NASP concentrations of 3.3, 0.3, 0.4 and 0.4 ng L^{-1} respectively for the High HRT/SRT experimental condition. These results indicated that the Low HRT/SRT and High HRT/SRT were behaving comparably (in terms of biodegradation efficiency and effluent concentrations). Despite an HRT of five and an SRT of three days, average biodegradation of the entire sampling period was 90.7% for the Low HRT/SRT pots. This was comparable with 92.0% average biodegradation of the entire sampling period for the High HRT/SRT pots.

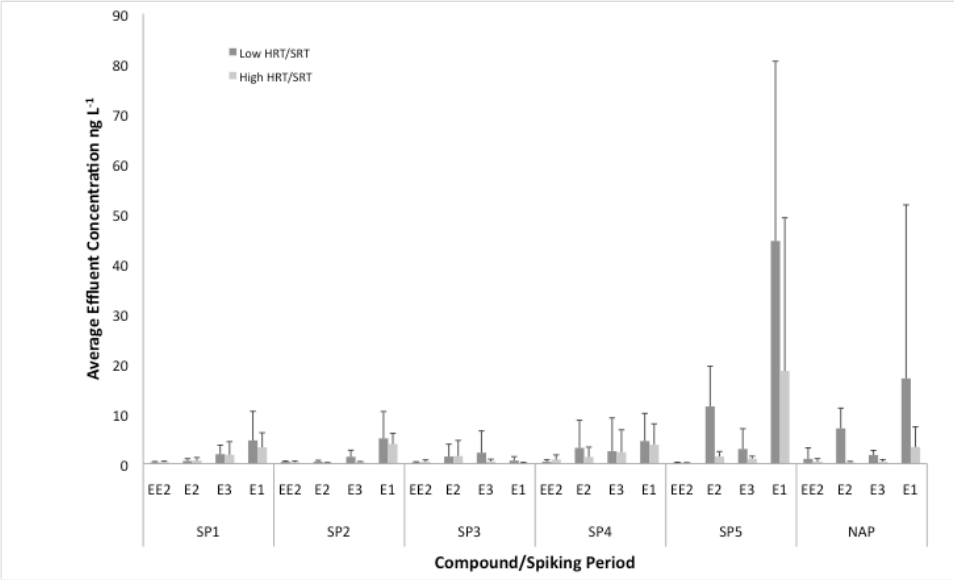


Figure 7-2 The average E1, E2, E3 and EE2 effluent concentration during each spiking period. SP1, influent ∑EST concentration was 42 ng L^{-1}. SP2, influent ∑EST concentration was 83 ng L^{-1}. SP3, influent ∑EST concentration was 167 ng L^{-1}. SP4, influent ∑EST concentration was 333 ng L^{-1}. SP5 and NASP, influent ∑EST concentration was 667 ng L^{-1}. During the NASP, ammonia was removed from the synthetic sewage. SP1-5 n=12, NASP n=18

7.4 Biodegradation of estrogens by biomass acclimatised without addition of estrogen

Biomass, grown without addition of estrogens was capable of significant biodegradation of estrogen. Despite a sludge age of just three days, estrogens were effectively biodegraded by the Low HRT/SRT bacteria. Virgin biomass, grown and established without estrogens was capable of biodegrading estrogen without an excessive equilibration period. Twenty-four hours after initial estrogen spiking, 88.54% and 78.36% biodegradation was witnessed at the Low HRT/SRT and High HRT/SRT conditions respectively. Thus indicating that bacteria grown without previous estrogen addition were capable of biodegradation and bacteria responsible for this were already present. Since the Low HRT/SRT experimental condition had an SRT of just three days, the biomass consisted of fast growing bacteria capable of

proliferating in such as short duration. Therefore the bacteria responsible for the biodegradation could have been no older than three days. Beyond spiking period one (SP1), evidence for the necessity of biomass to acclimate to the exponentially increasing influent estrogen concentration could not be established. Only on day one of spiking period 3 (SP3) was average percentage biodegradation <70%. Nevertheless, the first day biodegradation data was not used in any subsequent calculation within this results section to ensure that conclusions were draw from fully acclimated biomass.

It was unanticipated that the estrogens would be so biodegradable. Individually, E1, E2 and E3 all exhibited high biodegradability on average from SP1-5, 79.1, 93.9 and 94.9% respectively. Clearly, E1 was the most recalcitrant natural estrogen, noticeably less than E2 and E3 for both experimental conditions during SP1, SP2 and SP5 (Figure 7-2). During SP1, average E2 and E3 biodegradation was >80% for both experimental conditions. However for the Low HRT/SRT and High HRT/SRT pots respectively, average E1 biodegradation was 57.4 and 71.9%. During SP2, average E2 and E3 biodegradation was >93.9% for both experimental conditions. However for the Low HRT/SRT and High HRT/SRT pots respectively, average E1 biodegradation was marginally lower 81.4 and 89.2%. During SP5, when total (solid and aqueous phase) ∑EST influent concentration had it largest increase from 333 to 667 ng L^{-1}, average E2 and E3 biodegradation was >92.5% for both experimental conditions. However for the Low HRT/SRT and High HRT/SRT pots respectively, E1 biodegradation was 64.3 and 89.9%. This was a notable reduction in efficiency for the previous spiking period; during SP4, E1 biodegradation was 94.7 and 95.7% from the Low HRT/SRT and High HRT/SRT experimental condition respectively. After the change in nitrogen source from ammonia to nitrate, biodegradation efficiency was appeared to be unaffected. In fact E1 biodegradation improved to 88.7 and 98.3% for the Low HRT/SRT and High HRT/SRT experimental condition respectively.

Biodegradation efficiency for EE2 could not be determined during SP1-4. This was because this compound was detected at or just above the analytical LOD. This resulted in calculation errors as the LODs for solids phases were not sufficiently low enough and resulted in negative biodegradation. However, for SP5 and the NASP, EE2 biodegradation could be reasonably calculated. During SP5, EE2 biodegradation was 72.1±6.9 and 74.8±3.9% for the

Low HRT/SRT and High HRT/SRT experimental condition respectively. During the NASP, EE2 biodegradation was 68.8±11.0 and 72.6±316.8% for the Low HRT/SRT and High HRT/SRT experimental condition respectively. This data would indicate that EE2 was the most recalcitrant estrogen during these periods.

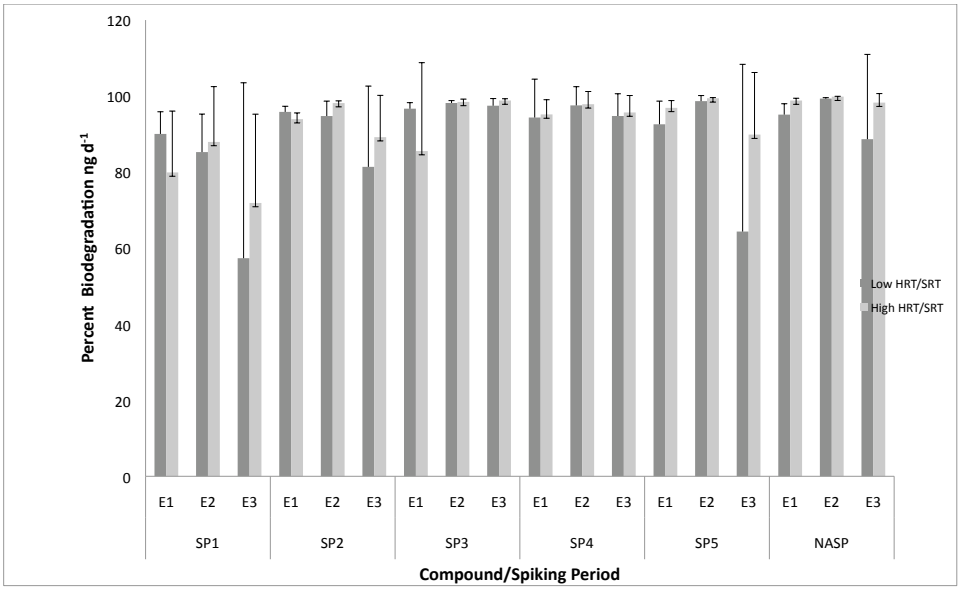


Figure 7-3 The percent biodegradation individually of E1, E2 and E3 of the Low HRT/SRT and High HRT/SRT experimental conditions during spiking periods were ammonia was the nitrogen source (SP1-5) and when ammonia was removed (NASP). SP1-5 n=12, NASP n=18. Standard deviations displayed as bars.

7.5 Mass biodegradation of estrogens

For the mass balance, ∑E1+E2+E3 was used. EE2 was not included in the calculation as it was detected at or just above the method detection limit and the accuracy of the balance would have been compromised. For the Low HRT/SRT experimental condition, E1+E2+E3 loading increased on average (±1SD) from 761±99 ng d^{-1} to 10716±1771 ng d^{-1} from spiking periods 1 to 5 (SP1-5) approximately double after each successive period (Figure 7-3). For the High HRT/SRT experimental condition, E1+E2+E3 loading increased on average (±1SD) from 170±18 ng d^{-1} to 2409±112 ng d^{-1} from spiking periods 1 to 5 (SP1-5) approximately

double after each successive period (Figure 6-3). The amount of average E1+E2+E3 biodegraded from SP1-5 also approximately doubled. E1+E2+E3 biodegradation for the Low HRT/SRT condition increased on average (±1SD) from 598±825 ng d^{-1} to 9452±1876 ng d^{-1} from SP1-5. E1+E2+E3 biodegradation for the High HRT/SRT condition increased on average (±1SD) from 123±71ng d^{-1} to 2311±211 ng d^{-1} from SP1-5. During the no ammonia spiking period (NASP), E1+E2+E3 loading was kept equivalent that of spiking period 5 (SP5), on average (±1SD) 11255±2218 ng d^{-1} and 2570±180 ng d^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. Despite the removal of ammonia, biodegradation remained at a rate equivalent to SP5, on average (±1SD) 11056±2530 ng d^{-1} and 2553±187 ng d^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively.

The proportions to which estrogens were proportioned during activated sludge treatment in the porous pot bioreactors is elaborated in Figure 7-6. As with the $ASP_{nit/denit}$ and ASP_{nit} it was confirmed that biodegradation was the predominant means of removal. Percent biodegradation of both the Low HRT/SRT and High HRT/SRT experimental conditions was high, >78.5%. For the Low/HRT/SRT experimental condition, average (±1SD) percent biodegradation was 78.7±15.3 92.1±8.7 97.7±17.4, 96.2±5.5, 86.9±12.7 and 95.5±5.6% respectively during spiking period 1-5 (SP1-5) and the no ammonia spiking period (NASP). For the High/HRT/SRT experimental condition, average (±1SD) percent biodegradation was consistently higher than the Low HRT/SRT experimental condition, 81.7±9.3, 94.4±4.1, 97.7±2.5, 96.2±3.1, 95.8±6.0 and 99.1±0.9% respectively during spiking period 1-5 (SP1-5) and the no ammonia spiking period (NASP).

The losses of estrogen to WAS for the Low HRT/SRT pots appeared to be much lower in comparison to what was observed ASP_{carb}. Clearly, the effective estrogen biodegradation that was occurring within the Low HRT/SRT pots, despite the short sludge age, ensured that concentrations within WAS remained low. However, as stated earlier, estrogen solid concentrations were at the method detection limit. This has added in unavoidable bias in that for the Low HRT/SRT and High HRT/SRT pots respectively, E1+E2+E3 lost in WAS was >34 and >5 ng d^{-1} (Figure 7-4). This equated to >4.4 and >2.8% of E1+E2+E3 lost via sludge wastage (Figure 7-4). Actual losses could have been lower, but analytical restraints have

made it impossible to determine where the true value may lie. Despite this, it is clear that estrogen losses to WAS were insignificant and in line with observations made at the $ASP_{nit/denit}$ and ASP_{nit}.

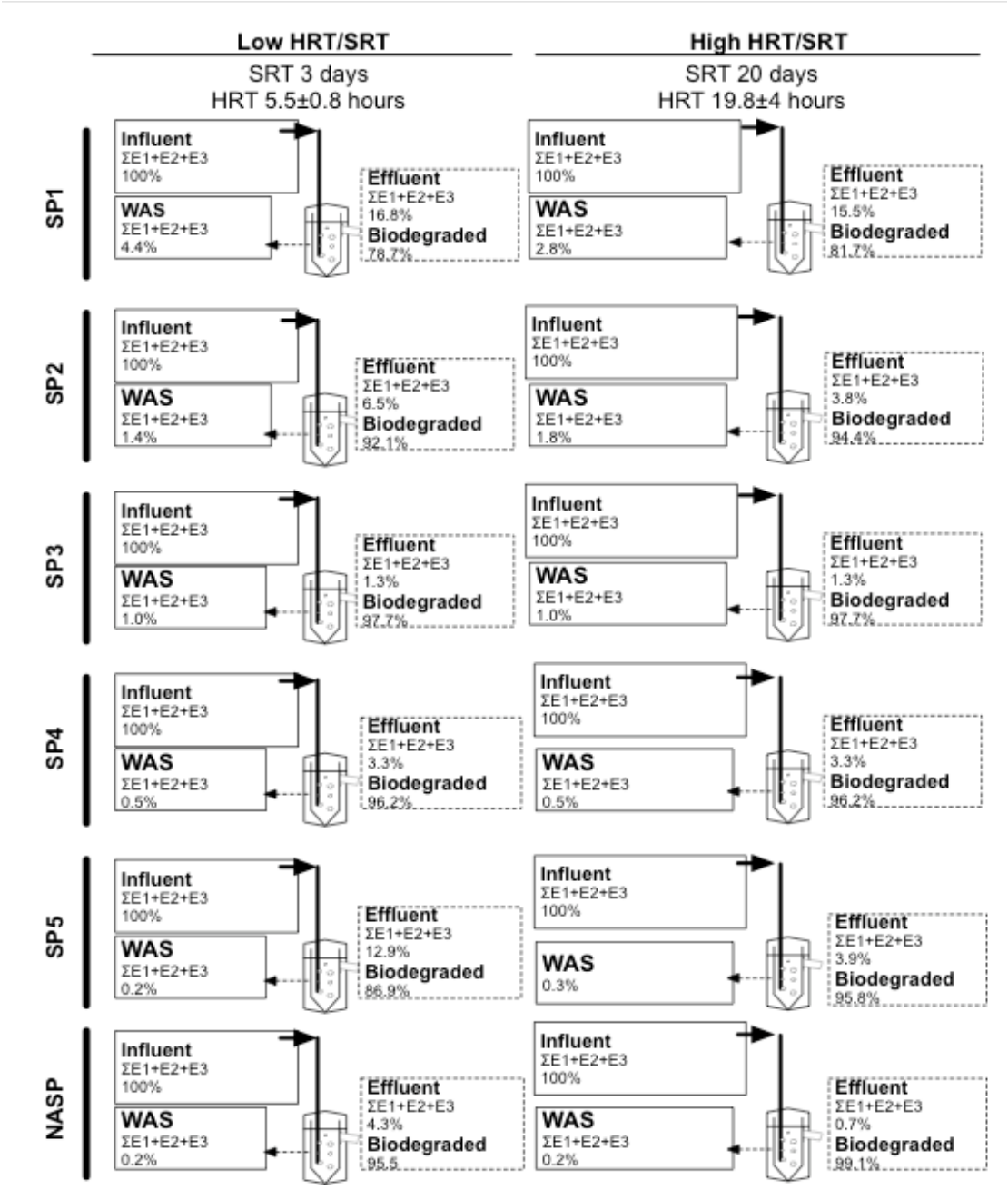


Figure 7-4 The E1+E2+E2 proportional mass balance of the Low HRT/SRT and High HRT/SRT experimental conditions. The schematic details proportions biodegraded, in the effluent and WAS during spiking periods were ammonia was the nitrogen source (SP1-5) and when ammonia was removed (NASP).

7.6 Nitrification, growth of ammonia oxidising bacteria and its influence upon estrogen biodegradation

To determine the role of ammonia oxidising bacteria (AOB) and their influence upon the biodegradation of estrogens, ammonia was removed during the NASP (Figure 7-5). From SP1-5 influent ammonia was circa 25 mg L^{-1}. After the removal of ammonia from the synthetic sewage, the equivalent mass of nitrogen was replaced with nitrate. Prior to this, the source of nitrate found in the influent was from tap water. During the NASP, ammonia could not be detected above the LOD in either effluent of the Low HRT/SRT of High HRT/SRT experimental condition. This demonstrated that nitrification had effectively stopped and the electron donor used by AOB was no longer available.

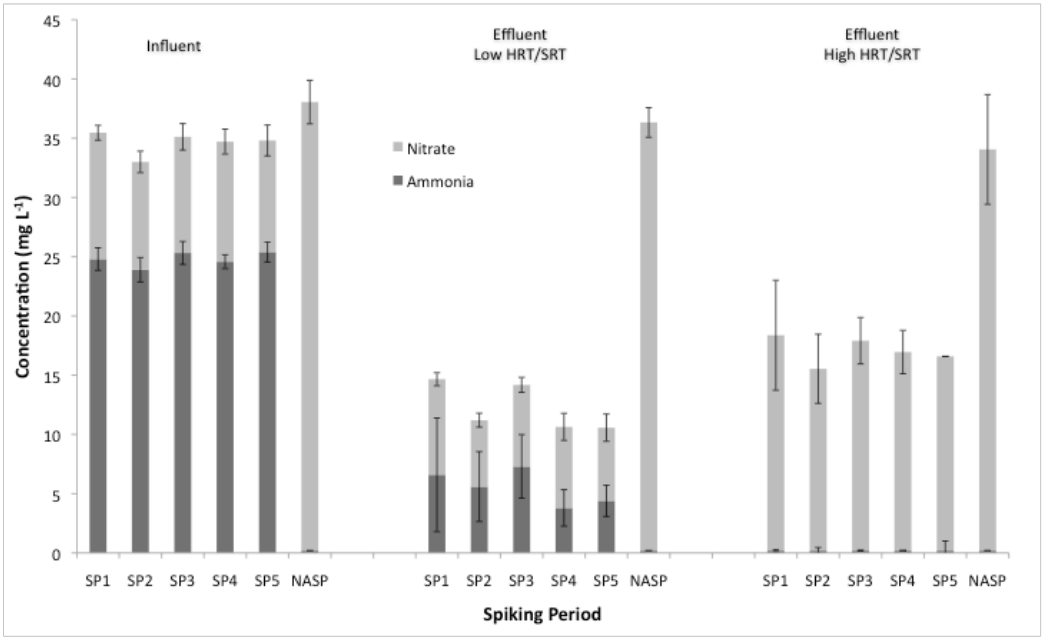


Figure 7-5 The ammonia and nitrate concentrations within the influent and effluents of the Low HRT/SRT and High HRT/SRT experimental conditions. SP1-5 n=12, NASP n=18. Standard deviations displayed as bars

From spiking period 1-5 (SP1-5) with ammonia as the synthetic sewage nitrogen source, both the experimental conditions experienced exponential growth of ammonia oxidising bacteria (AOB) (Figure 7-6). The High HRT/SRT condition had >1.44x10^8 AOB mL^{-1} than the Low HRT/SRT condition. Except at the end of the no ammonia spiking period (NASP) where the difference was 3.1x10^7 mL^{-1}. AOB grew on average, 1.49 x 10^7 – 2.47 x 10^8 and 3.75 x 10^8 – 5.15 x 10^9 mL^{-1} from SP1-5 for the Low HRT/SRT and High HRT/SRT conditions respectively. When ammonia was removed from the synthetic sewage on day 36, abundance of AOB began to exponentially decrease (Figure 7-5). During the no ammonia spiking period (NASP), AOB decreased exponentially to 1.17 x 10^7 and 4.27 x 10^7 mL^{-1}, below SP5 abundance for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. Growth of AOB during SP1-5 for both experimental conditions probably resulted from unequilibrated nitrifying biomass. An explanation for the continued growth of AOB may have been the formation of a nitrifying biofilm upon and within the porous pot membrane, which had a pore diameter of circa 70 μm. This was unanticipated as the membranes were cleaned and replaced weekly by spares that had been stored in 5% sodium hypochlorite solution.

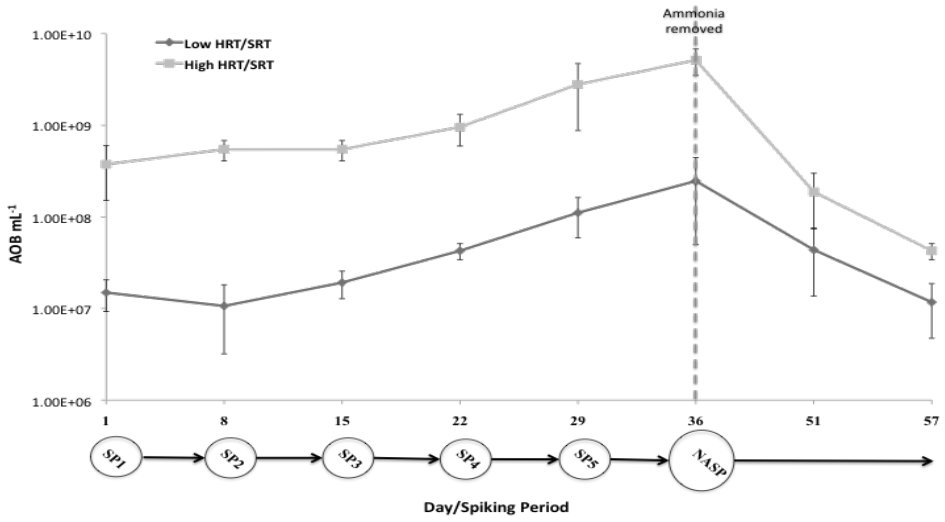


Figure 7-6 Abundance of ammonia oxidising bacteria in the Low HRT/SRT and High HRT/SRT experimental conditions over the 57-day sampling period. Standard deviations are shown as bars. SP1, influent ∑EST concentration was 42 ng L^{-1}. SP2, influent ∑EST concentration was 83 ng L^{-1}. SP3, influent ∑EST concentration was 167 ng L^{-1}. SP4, influent ∑EST concentration was 333 ng L^{-1}. SP5 and NASP, influent ∑EST concentration was 667 ng L^{-1}. N=3

From SP1-5 as AOB increased, nitrite oxidising bacteria (NOB) abundance remained constant (Figure 7-6). NOB abundance for the Low HRT/SRT and High HRT/SRT experimental conditions was on average $6.22x10^4$ and $2.89x10^5$ mL^{-1} respectively during SP1-5. NOB abundance was higher for the High HRT/SRT experimental condition presumably due to the longer sludge age and a higher rate of nitrification in comparison to the Low HRT/SRT experimental condition. After ammonia was removed from the synthetic sewage, both experimental conditions experienced decreases in NOB abundance. For the Low HRT/SRT condition, NOB was not detected. For both conditions, NOB abundance decreased to below the limit of detection ($1x10^4$) by day 57.

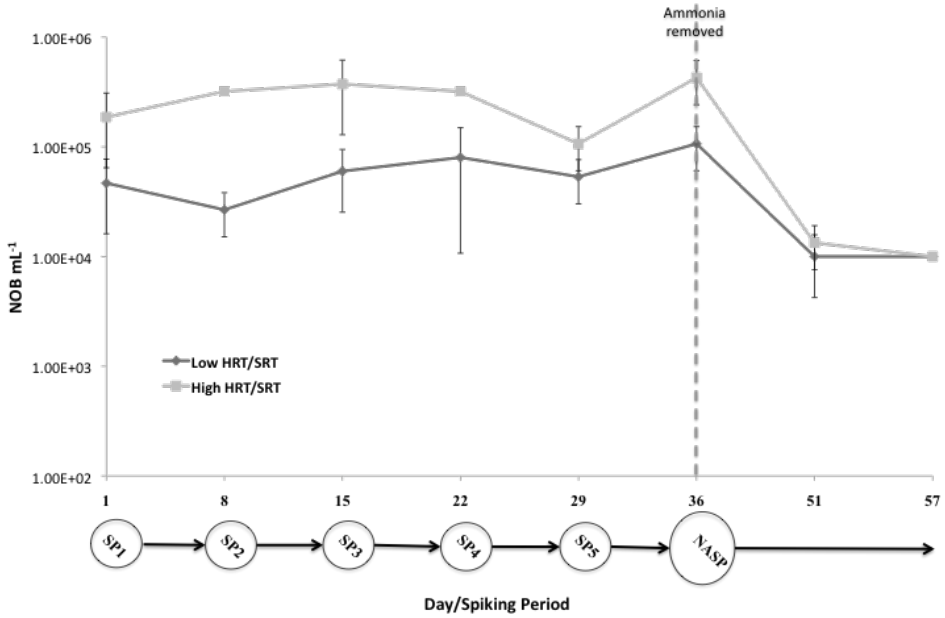


Figure 7-7 Abundance of nitrite oxidising bacteria in the Low HRT/SRT and High HRT/SRT experimental conditions over the 57-day sampling period. Standard deviations are shown as bars. SP1, influent ∑EST concentration was 42 ng L^{-1}. SP2, influent ∑EST concentration was 83 ng L^{-1}. SP3, influent ∑EST concentration was 167 ng L^{-1}. SP4, influent ∑EST concentration was 333 ng L^{-1}. SP5 and NASP, influent ∑EST concentration was 667 ng L^{-1}. N=3

These reductions in AOB did not appear to influence the biodegradation of any estrogen. As estrogen loading was the same during SP5 and the NASP, biomass activities (biodegradation normalised to MLSS concentration) between these periods were used to determine whether

significantly reducing AOB abundance influenced biodegradation. A Kruskal-Wallis non-parametric analysis was used due to unequal sample sizes that compared the similarity between median estrogen biomass activities for the two experimental conditions (Table 7-1). Despite the removal of ammonia and the reduction in Nitrosomonas, biomass activity (biodegradation tonne^{-1} day^{-1}) for E1, E2, E3 and EE2 did not change significantly (Kruskal-Wallis P>0.05). This would indicate that reduction in AOB did not influence the estrogen biodegradation ability of activated sludge.

Table 7-1 Median biomass activity during spiking period 5 (SP5) and the no ammonia spiking period (NASP)

	Median biomass activity (mg biodegraded tonne^{-1} day^{-1})			
Estrogen	Low High/SRT		High HRT/SRT	
	SP5	NASP	SP5	NASP
E1	509.02	558.78	76.18	79.17
E2	676.82	527.86	25.53	31.16
E3	786.27	956.92	112.56	120.03
EE2	7.25	8.04	0.68	0.65

For both the Low HRT/SRT and High HRT/SRT pots n=12 for SP5 and n=16 for NASP

To further test this hypothesis AOB abundance was extrapolated for each sampling day using log-linear equations describing the respective exponential increase and decrease over SP1-5 and the NASP. This was done in order to marry AOB abundance with daily biomass activity data. For statistical robustness, 95% confidence limits were calculated. The resulting plot of extrapolated AOB abundance against biomass activity (biodegradation normalised to MLSS concentration) demonstrates that bacterial abundance increased linearly with biodegradation of E1+E2+E3 (Figure 7-7). Data from the High HRT/SRT experimental condition was used for this purpose as the reduction in AOB was more pronounced here than the other condition. During the High HRT/SRT experimental period when ammonia was the nitrogen source, AOB numbers increased from $3.5x10^8$ to $3.1x10^9$ mL^{-1} in correlation with biomass activity, which increased from 19.6 to 216.5 mg E1+E2+E3 biodegraded tonne^{-1} day^{-1}. Since biomass activity also increased with time over the experimental period, it is likely that growth of AOB

was not due to increase in estrogen loading, but resulted from an unequilibrated nitrifying assemblage.

For the High HRT/SRT experimental condition AOB decreased from $3.08x10^{9}$ mL^{-1} to its lowest abundance of $4.27x10^{7}$ mL^{-1} after the removal of ammonia (Figure 7-3). However, during this period biomass activity remained within the 95% confidence zone of 62.3 – 332.8 mg E1+E2+E3 biodegraded $tonne^{-1}$ day^{-1}. This was statically identical to the biomass activity of SP5 (31.7 – 331.2 mg E1+E2+E3 biodegraded $tonne^{-1}$ day^{-1}), which had the same influent estrogen concentration ∑EST 667 ng L^{-1}, but with ammonia. If AOB were linked with biodegradation of estrogens, it would be expected that biomass activity would decrease. As stated previously, biomass activities for all estrogen (including EE2) were statically the same regardless of nitrogen source. Confirming this, biomass activity and AOB abundance did not demonstrate correlation as witnessed during SP1-5 (Figure 7-7), after the removal of ammonia. Different 95% confidence zones during estrogen spiking with and without ammonia as the nitrogen source can be clearly observed (Figure 7-7). This is evidence that AOB was not linked to the biodegradation of estrogen and that increased biomass activity did not result in proliferation of AOB.

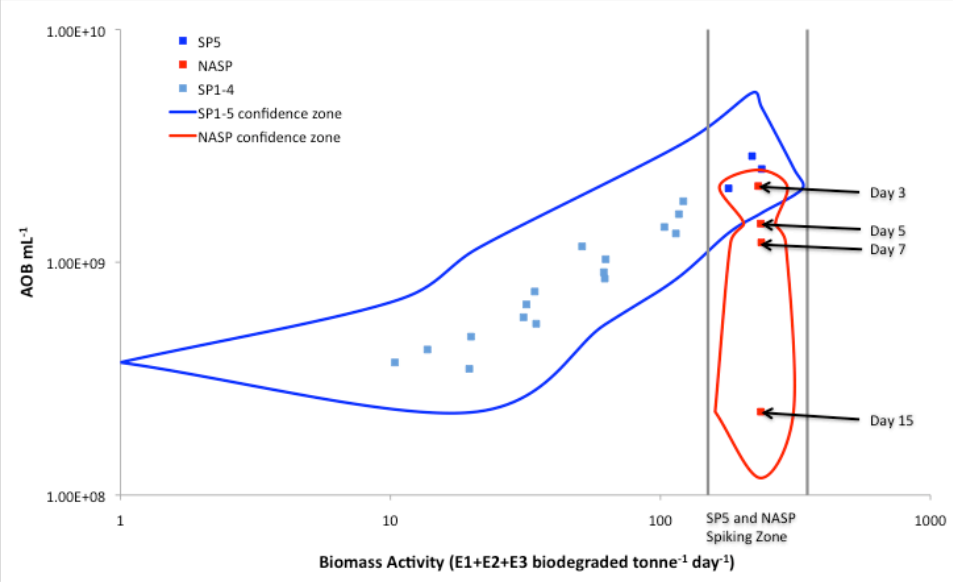


Figure 7-8 A semi conceptional illustration indicating the linear relationship between ammonia oxidising bacteria (AOB) abundance (extrapolated with Figure 7-6 data) and average daily biomass activity (biodegradation of E1+E2+E3 normalised to MLSS concentration) for High HRT/SRT experimental conditions. Solid lines encircling average daily biomass activity and daily AOB extrapolations denote confidence zones. These zones illustrate zone where within, one can be 95% sure the true values for biomass activity and AOB abundance exists.

7.7 Resource dependent biodegradation of estrogens

Biodegradation was normalised to MLSS concentration (biomass activity) and E1+E2+E3 loading was normalised volumetrically in order to compare with full-scale observations. Biomass activity of E1+E2+E3 increased proportionally with loading (Figure 7-8). During SP1-5, as average E1+E2+E3 (±1SD) volumetric loading increased from 0.20±0.03 to 2.12±1.46 mg m^{-3} d^{-1}, average (±1SD) biomass activities increased from, 103±132, to 1883±716 mg E1+E2+E3 biodegraded $tonne^{-1}$ d^{-1} for the Low HRT/SRT experimental condition. For the High HRT/SRT experimental condition, average E1+E2+E3 (±1SD) volumetric loading increased from 0.04±0.00 to 0.48±0.32 mg m^{-3} d^{-1}, average (±1SD) biomass activities increased from, 17±9, to 237±41 mg E1+E2+E3 biodegraded $tonne^{-1}$ d^{-1}.

Thus indicating that biodegradation of estrogen followed a first-order relationship and that the availability of estrogen to activated sludge, was fundamental for biodegradation capacity. Biomass activities during the NASP also fell within this first-order relationship. This was further evidence that removing ammonia in the synthetic sewage had little affect upon the biodegradation potential of either experimental condition.

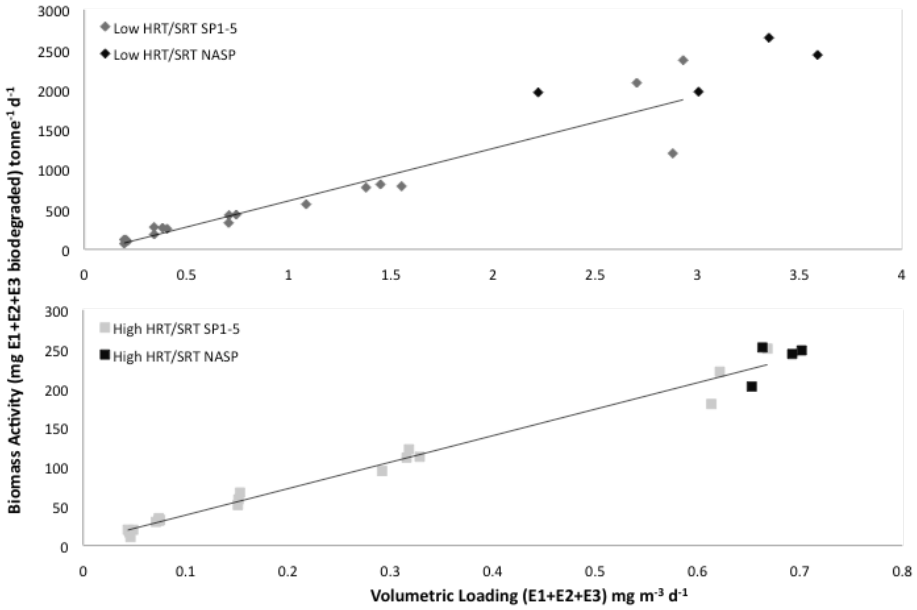


Figure 7-9 The first order relationships between biomass activity and loading of daily average E1, E2 and E3 in both low HRT/SRT and high HRT/SRT experimental conditions during spiking periods were ammonia was the nitrogen source (SP1-5) and when ammonia was removed (NASP). N=3

These relationships between loading and biomass appeared to be strongly correlated (R^2 values of 0.88 and 0.97 respectively for the Low HRT/SRT and High HRT/SRT experimental conditions). Furthermore, these linear relationships could be established individually for E1, E2 and E3. However, between the Low HRT/SRT and High RT/SRT experimental conditions, these regressions were different from each other, with their own distinct slopes (Equation 7-1). Using these equations, the biomass activities achieved at a volumetric loading of 1 mg m^{-3} d^{-1} would be 606 and 342 mg E1+E2+E3 biodegraded $tonne^{-1}$ d^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. Grouped linear

regression with covariance analysis was used to compare the slopes of the Low HRT/SRT and High HRT/SRT regressions, statistical difference was demonstrated between each experimental condition ($P<0.05$). Therefore, the Low HRT/SRT experimental condition biomass was biodegrading per tonne comparably more estrogens than the High HRT/SRT pots.

Equation 7-1 Linear equations describing the proportional relationship between volumetric loading and biomass activity of the Low HRT/SRT and High HRT/SRT experimental conditions

$$y_{LowHRT/SRT} = 655.91x - 49.872$$
$$y_{HighHRT/SRT} = 337.42x + 4.5787$$

7.8 The Kinetics of Estrogen Biodegradation

The speed at which the Low HRT/SRT experimental condition was biodegrading estrogens was significantly greater than the High HRT/SRT pots. Plotting the calculated mass of E1+E2+E3 biodegraded during one HRT and regressing this through the origin could demonstrate this (Figure 7-10). This was done on the assumption that estrogen biodegradation followed first-order reaction kinetics and that estrogens are biodegraded linearly over time after spiking. In reality, the porous pots were a continuous system and received estrogen spiking continually. However, Figure 7-10 demonstrates that the Low HRT/SRT condition was the faster system, would have biodegraded 22.3 mg E1+E2+E3 $tonne^{-1}$ biomass within one HRT (5.2 hours) during SP1. The High HRT/SRT condition in comparison would have biodegraded 13.5 mg E1+E2+E3 $tonne^{-1}$ biomass within one HRT (18.7 hours). Within 5.2 hours, the same HRT/reaction time of the Low HRT/SRT condition, the High HRT/SRT are predicted to have biodegraded 3.7 mg E1+E2+E3 $tonne^{-1}$. As loading was increased from SP1-5, both experimental conditions demonstrated that rate of biodegradation increased. Within one HRT, the Low HRT/SRT experimental condition increased from 22.3 to 429.3 mg E1+E2+E3 $tonne^{-1}$ from SP1-5. Within one HRT, the Low HRT/SRT experimental condition increased from 13.5 to 183.1 mg E1+E2+E3 $tonne^{-1}$ from SP1-5.

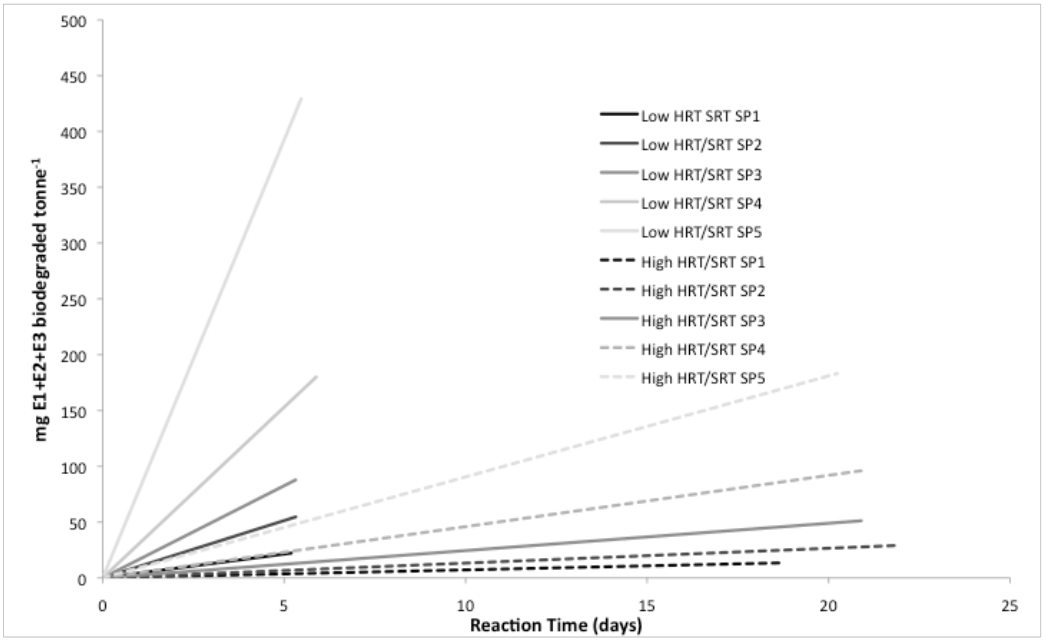


Figure 7-10 The rate kinetics of E1+E2+E3. Assuming first-order reaction kinetics for the biodegradation of E1+E2+E3, calculated E1+E2+E3 biodegraded during one HRT for each sampling period and regressed through the origin to indicate reaction biodegradation rates.

The rate kinetics for separate estrogens, were also calculated using the method described above (Table 7-2). The rate at which biodegradation of each estrogen occurred appeared to increase significantly each spiking period. This was except for the NASP, which was similar to the SP5 as the loadings of estrogen for these periods were identical. For both experimental conditions E3 was the fastest biodegraded estrogen. This occurred consistently during every spiking period. From SP1-5 the rate at which E3 was biodegraded increased from 2.17 to 33.75 mg biodegraded tonne^{-1} hour^{-1}, for the Low HRT/SRT experimental condition. For the High HRT/SRT experimental condition the rate at which E3 was biodegraded increased from 0.61 to 3.05 mg biodegraded tonne^{-1} hour^{-1}. These findings suggest that estrogen biodegradation was not limited at the loadings used during the porous pot experimentation. Furthermore, increasing loading appeared to increase the rate of biodegradation. If this were

the case, then sizing activated sludge reactor volume is critical to ensure that estrogen loading and HRT are balanced in order to achieve full biodegradation.

Table 7-2 The rate kinetics (mg biodegraded tonne^{-1} hour^{-1}) of E1, E2, E3 and EE2 during sampling periods 1-5 (SP1-5) and the no ammonia spiking period (NASP) for the Low HRT/SRT and High HRT/SRT experimental conditions. Rates derived from E1+E2+E3 biodegraded during one HRT/HRT

Estrogen	Low HRT/SRT						High HRT/SRT					
	SP1	SP2	SP3	SP4	SP5	NASP	SP1	SP2	SP3	SP4	SP5	NASP
E1	1.07	2.81	5.08	9.05	16.00	24.36	0.38	0.76	1.32	2.52	5.78	7.21
E2	1.64	3.36	5.08	9.13	22.74	25.17	0.17	0.33	0.49	1.04	2.63	3.05
E3	2.17	4.73	7.34	13.37	33.74	38.38	0.61	1.19	1.88	3.71	9.27	10.52
EE2					0.34	0.32					0.10	0.10

7.9 The influence of bulk organics upon estrogen biodegradation

During the sampling period, influent COD concentration was kept at circa 526 mg L^{-1}, whilst estrogen loading approximately doubled each subsequent spiking period. Resultantly the influent ratio of COD/E1+E2+E3 mg^{-1} mg L^{-1} for both experimental periods decreased on average (±1SD) from, $9.51x10^5 \pm 1.55x10^4$ to $1.29x10^7 \pm 4.11x10^6$ from SP1-5 respectively (Figure 7-11). Meaning that the proportion of estrogen, as organic carbon available to the biomass, approximately doubled each period from SP1-5. When the ratio of COD/E1+E2+E3 was at its highest, biomass activity was at its lowest level, but decreased exponentially as the proportion of estrogen increased. It could appear that the relationship between COD/E1+E2+E3 ratio and biomass activity indicated the occurrence of competitive inhibition of estrogen biodegradation via the availability of bulk organic material. That at biomass activity >734 and >110 mg E1+E2+E3 biodegraded tonne^{-1} day^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively, only occurred at when estrogen constituted its highest proportion as COD (COD/E1+E2+E3 $<2x10^6$ mg^{-1} mg L^{-1}).

However, biomass activity was connected with loading. Subsequently, maximum potential biomass activities could only be on average (±1SD) 118.6±37.8, 94.1±94.1, 499.9±80.6, 755.5±101.1 and 1475.8±326.2 for the Low HRT/SRT experimental condition from SP1-5

respectively. For the High High/SRT experimental condition, maximum potential biomass activities were on average (±1SD) 20.9±4.1, 36.0±5.4, 68.0±9.6, 123.8±16.4 and 210.1±39.9 mg E1+E2+E3 biodegraded tonne^{-1} day^{-1} from SP1-5 respectively. Therefore, at COD/E1+E2+E3 of 1.29x10^{7} mg^{-1} mg L^{-1}, biomass activity could potentially only have been on average 118.6 and 20.8 mg E1+E2+E3 biodegraded tonne^{-1} day^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. Thus indicating that loading was the key driver of biodegradation in this case.

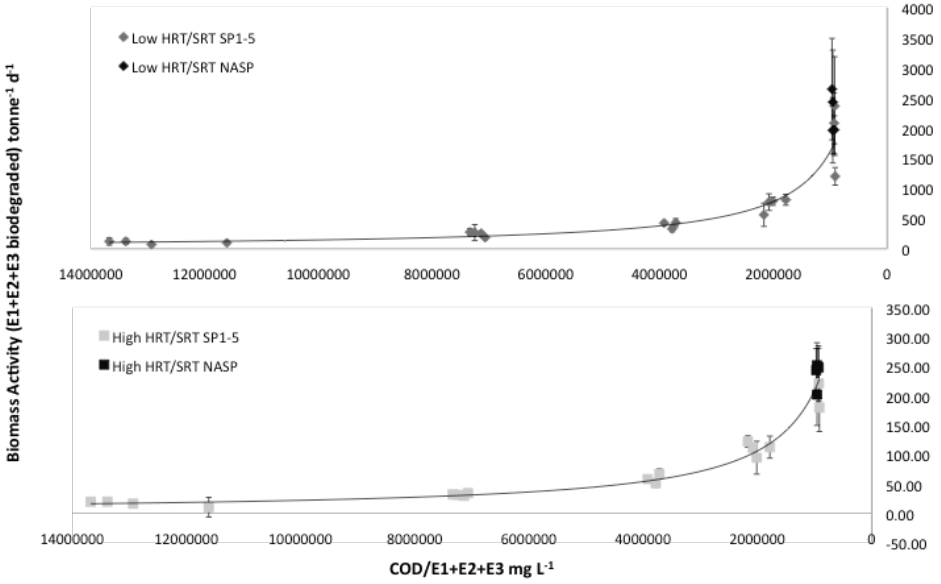


Figure 7-11 The exponential relationship between decreasing COD/E1+E2+E2 mg-1 mg L-1 and biomass activity during spiking periods where ammonia was the nitrogen source (SP1-5) and when ammonia was removed (NASP). N=3

Despite this, as the proportion of COD/E1+E2+E3 increased it appears that percent biodegradation potential was suppressed. Although biodegradation remained high on average >87.49% ± 12.63 SD (standard deviation) and > 94.51% ± 5.64 SD for the low HRT/SRT and high HRT/SRT experimental conditions respectively during SP2-5 when COD/E1+E2+E3 mg^{-1} mg L^{-1} ratios were 9.21 x 10^{5} to 7.30 x 10^{6}. Above 8.0 x 10^{6} COD/E1+E2+E3 mg^{-1} mg L^{-1} ratios, percent biodegradation was at its lowest. During SP1, when the proportion of

estrogen as COD was highest, the lowest percent biodegradation for both experimental conditions could be witnessed here. Biodegradation potential was on average 78.46% ± 15.29 SD and 81.35 ± 8.61 SD respectively for the low HRT/SRT and high HRT/SRT experimental conditions when COD/E1+E2+E3 mg^{-1} mg L^{-1} was circa 1.31 x 10^7. This could indicate that when the proportion of estrogen in COD was lowest during SP1, suppression of full biodegradation potential could have occurred due to the presence of more readily available carbon compared with estrogen. Alternatively, this could have been due to the biomass encountering estrogens for the first time and the necessity for a period of acclimation.

Establishing whether biodegradation of estrogen is competitive inhibited by other more readily available carbon could not fully be established by this experiment. The biodegradability of the estrogen was high (typically >90%) as low concentrations of estrogen were observed in the effluents (typically >7 ng L^{-1}). Extending the ratio between COD and estrogen influent concentrations beyond those encountered at the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} did not solve this issue. During the pots experiment, loading was the dominant factor controlling biodegradation. Biodegradation appeared to be uninhibited by bulk organics. This could have been due to the biodegradability of the carbohydrate based influent. The rapid biodegradation of the COD could have facilitated the biodegradation of estrogen observed here. In which case, bulk organics still potentially play a significant role influencing the rate estrogens are biodegraded.

Chapter 8

Discussion

8 Discussion

8.1 The characteristics of bacteria responsible for estrogen biodegradation

This study demonstrated that activated sludge heterotrophic communities could achieve effective estrogen removal. This was demonstrated by the 54% biodegradation observed at the ASP_{carb}. Furthermore the Low HRT/SRT porous pots with SRT as low as three days could achieve typically >90% estrogen removal. Whereas previously it was understood that ASP that commonly achieved the highest removal efficiencies of estrogens by activated sludge that had longer SRTs (Clara *et al* 2005, Johnson *et al* 2005, Joss *et al* 2004). These observations were based upon an assumption that diverse bacterial communities are promoted by increasing SRT. Kreuzinger *et al* (2004) stated that "higher SRTs allow the enrichment of slowly growing bacteria and consequently the establishment of a more diverse biocoenosis with broader physiological capabilities compared to STPs with low SRT". It is assumed that short SRT equals low bacterial diversity. However, a study assessing the impact of changing SRT upon bacterial diversity, evidence was presented contrary to this axiom (Saikaly *et al* 2005). These authors demonstrated bacterial diversity counter intuitively increased as SRT decreased. More recently in a similar study, it was demonstrated that bacterial community similarity between reactors, run at different sludge ages, were indistinguishable from the similarities of reactors run at the same sludge age (Akarsubasi *et al* 2009). However, these authors did note that diversity increased slowly with sludge age, an observation counter to that of Saikaly *et al* (2005).

The work of Saikaly *et al* (2005) and Akarsubasi *et al* (2009) implies that the High HRT/SRT experimental condition may not have greater bacterial diversity than the Low HRT/SRT pots. Whilst both sets of porous pots would undoubtedly have had distinctly different bacterial communities, it cannot be assumed that either experimental condition had an advantage due to species richness. Clearly the porous pot bioreactors were run under ideal conditions and receiving a highly biodegradable bulk organic in the form of carbohydrate, however, this research demonstrated that activated sludge run at low HRT and SRT conditions is

theoretically capable of effective estrogen biodegradation. The implication is that the ASP_{carb} therefore has the potential to achieve higher biodegradation given modifications to its process control. Furthermore, estrogen biodegrading bacteria can be fast growing and capable of proliferating within three days. Identifying the inhibitory factor that prevents actual AST achieving effective estrogen biodegradation is of key importance. It was unanticipated that the Low HRT/SRT porous pots would prove so effective. Identifying the reasons for this success could be transposed to actual ASPs and be used to deliver enhanced environmental protection from endocrine disruption.

8.1.1 The role of Autotrophic bacteria upon the biodegradation of estrogens

Based upon the observation that extended SRT ASP have the most effective estrogen removals, it has been suggested that ammonia oxidising bacteria (AOB) are linked with this (Andersen *et al* 2003, Clara *et al* 2005, Matsui *et al* 2000, Servos *et al* 2005, Svenson *et al* 2003). This hypothesis has had much support with research seemingly supporting this (Vader *et al* 2000, Shi *et al* 2004, Yi and Harper *et al* 2007). Until a study by Gaulke *et al* (2009), demonstrated that EE2 was abiotically transformed to nitro-EE2 under nitrite concentrations equivalent to those of batch tests used by Vader *et al* 2000 and Shi *et al* 2004. Furthermore, Gaulke *et al* (2009) questioned the validity of work by Yi and Harper (2007). The latter authors demonstrated transformation of EE2 to catecol-EE2 using enzyme extract from nitrifying activated sludge. Retrospective consideration of these results calls into question whether ammonia monooxygenase was the enzyme responsible for the conversion of EE2. The presence of heterotrophic enzymes within the extract, which could have been responsible of the conversion of EE2, cannot be ruled out. Within the literature the role of ammonia oxidising bacteria within the biodegradation of estrogens is unclear at best.

This research has presented evidence demonstrating there is no link between nitrification and estrogen biodegradation at the estogen loadings and AOB concentrations typically found during activated sludge conditions and supports hypotheses of Gaulke *et al* (2009), who indicated that heterotrophic bacteria play an important role. Exponential increases in AOB abundance encountered during the spiking experimentation could not have been linked with increased biodegradation activity. Reduction of AOB abundance from 2.47×10^8 to $1.17 \times$

10^7 and 5.15 x 10^9 to 4.27 x 10^7 mL^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively did not affect estrogen biodegradation. Biodegradation of estrogen for both experimental conditions was statistically the same despite SP5 and the NASP both receiving 667 ∑EST ng L^{-1} influent concentration. Removing nitrogen for the influent and reducing the abundance of AOB appeared to have no impact upon estrogen biodegradation potential of the porous pot bioreactors.

It was not possible to completely eliminate AOB during the NASP. This meant that AOB, albeit with reductions of circa 1.35 x 10^8 and 5.11 x 10^9 cells mL^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively, influence upon estrogen biodegradation could not be ruled out. However, no research to date has demonstrated estrogen biodegradation/biotransformation at environmentally relevant concentrations. It has been demonstrated that pure cultures of the AOB *Nitrosomonas europea* biotransformed EE2 concentrations of 0.01 mg L^{-1} and 1 mg L^{-1} (Skotnicka-Pitak *et al* 2009). Using NMR these authors demonstrated that the resulting metabolites were not only nitrated-EE2 compounds as reported by Gaulke *et al* (2008), but were the products of biotic transformation. Although these nitrated compounds were present due to high concentrations of nitrite, other metabolites of EE2 were deemed to have been biotransformed, as they were not produced abiotically unlike the nitrated compounds. Therefore at concentrations >0.01 mg L^{-1} EE2, AOB (particularly *N. europea*) appears to exhibit co-metabolic biodegradation of estrogens. Concentrations >0.01 mg L^{-1} EE2, are not environmentally relevant however and significantly higher than those observed at the three sampled ASP and spiked in porous pot biomass. At environmentally relevant concentrations, Gaulke *et al* (2008) reported no biotranformation of EE2 by pure cultures of *N. europea.* This evidence supports the findings of this research. At significant concentrations of EE2, AOB may well be important for the removal of this compound. However, at these concentrations, numerous heterotrophic bacteria have also demonstrated capability to biodegrade EE2 (Yoshimoto *et al* 2004 and Pauwels *et al* 2008).

It seems unlikely that the affinity of ammonia-monoxygenase for estrogens (particularly EE2) is high enough to support biotransformation at ng L^{-1} concentrations. Significant differences in concentrations of *N. europea* and EE2 used during these studies may explain these

apparent contradictions. Gaulke *et al* (2008) spiked batch tests with 500 ng L^{-1} EE2 with an *N. europea* abundance of 15-41 mg L^{-1} (equivalent to 21.3-61.5 mg L^{-1} theoretical COD (Grady *et al* 1999)). Skotnicka-Pitak *et al* (2009) spiked batch tests with 1 and 0.01 mg L^{-1} EE2 with an *N. europea* abundance of 1100 mg L^{-1} theoretical COD (tCOD) (pers comm. Love 2010). Therefore, ratio of EE2 and *N. europea* concentration was 0.023-0.008 and 0.009-0.909 mg $EE2^{-1}$ tonne tCOD *N. europea* for Gaulke *et al*., (2008) and Skotnicka-Pitak *et al* (2009) respectively. Both studies covered similar ranges. Although the ratio of EE2 and *N. europea* were similar, increasing micropollutant and bacterial concentration leads to increased degradation rates (McAdam *et al*., 2010, Skotnicka-Pitak *et al* 2009, Ternes *et al*., 2004). During the batch test performed by Skotnicka-Pitak *et al* (2009), high concentrations of EE2 and *N. europea* would have resulted in increased availability of active sites and substrate and therefore appreciable biotransformation occurring at a faster rate to the abiotic nitro-transformation could have occurred.

In addition to batch testing, Skotnicka-Pitak *et al*., (2009) also conducted continuous-flow experiments that can be directly compared with the finding of this research. These authors noted biotransformation of EE2 during continuous-flow experiments. Their EE2 loadings were circa 129.5 mg $EE2^{-1}$ kg *N. europea* tCOD d^{-1}, significantly higher than those of this study, 0.008-0.74 mg $EE2^{-1}$ kg AOB tCOD d^{-1}, based on the Kuai and Verstraete (1998) relationship of 2.5 x 10^{10} $cell^{-1}$ g VSS and further conversion to tCOD (Grady *et al* 1999). This supports the hypothesis of this research that environmental concentrations of AOB and EE2 are insufficiently high enough to support co-metabolism during activated sludge treatment. Whilst it is postulated that the fortuitous activity of monoxygenase is important for the removal of organic contaminants such as trihalomethanes and chloroform (Chang *et al*., 2002, Skotnicka-Pitak *et al*., 2009), to date, there is no compelling research indicating that AOB can biotransform these chemicals at ng L^{-1} concentrations. Ammonium monoxygenase is reported to have fortuitous co-metabolism of numerous organic contaminants such as trihalomethanes and chloroform (Chang *et al* 2002, Skotnicka-Pitak *et al* 2009). However, these compounds demonstrated degradability at significantly higher concentrations than the environmentally relevant estrogen concentrations used in this research. In the study by Chang *et al* (2002), naphthalene concentrations of 80 and 240 µM were used. This is equivalent to spiking 10.2 and 30.2 mg L^{-1} of naphthalene, approximately one million times greater than the concentration of estrogens used in this study.

8.1.2 Resource dependent biodegradation of estrogens and nonylphenolics

Both estrogens and nonylphenolics (NPx) appeared to follow first-order relationship as proposed by Ternes *et al* (2004) and McAdam *et al* (2010). This was demonstrated with observations from the three ASP sampled and the porous pot bioreactor experimentation. At the ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ it was possible to observe concentration dependant biodegradation of estrogen and $NP_{4\text{-}12}EO$ biodegradation. Something that had only been reported under controlled bench conditions previously (Vader *et al* 2000). Up to loadings of circa 0.5 mg ∑EST m^{-3} d^{-1} and 64 mg $NP_{4\text{-}12}EO$ m^{-3} d^{-1} biodegradation following first-order kinetics (Equation 8-1) was observed at the ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$.

Equation 8-1 The first order relationship describing the microbial decomposition of estrogens

$$r_{decompostion}=k_{decompostion}xC_{decomposition}$$

Modified from Ternes *et al* (2004). Where $r_{decomposition}$ is the rate of decomposition, $k_{decomposition}$ is the rate constant and $C_{dissolved}$ is the dissolved steroid estrogen concentration.

At the ASP_{carb}, ASP_{nit} and $ASP_{nit/denit}$ loadings of circa 0.05 – 0.5 mg ∑EST m^{-3} d^{-1} and 10.02 – 75.79 mg $NP_{4\text{-}12}EO$ m^{-3} d^{-1} were observed, however, biomass activities showed no signs of abating. Furthermore, neither did increasing the loadings range from circa 0.04 to 2.8 E1+E2+E3 m^{-3} d^{-1} during the porous pot experiments, demonstrate the occurrence of a threshold of biomass activity. This therefore implies that given higher volumetric loadings of estrogen, ASP would biodegrade more estrogens and NPx. The enzymatic activity required to biodegrade EDCs appeared not to be limited either at actual ASP or at porous pot bioreactor level. These ranges of estrogens and $NP_{4\text{-}12}EO$ loadings at the three ASPs and the porous pot experiments represent gradients of resource availability (Graham and Curtis 2003). As resources increase, a greater amount of bacteria would be able to utilise them. Over these ranges, $NP_{4\text{-}12}EO$ biomass activity increased 529% (2629.68 – 13925 mg $NP_{4\text{-}12}EO$ tonne $biomass^{-1}$ d^{-1}). ∑EST biomass activity increased 379% (19.9 – 75.4 mg ∑EST tonne $biomass^{-1}$ d^{-1}). During the porous pots experimentation E1+E2+E3 biomass activity increase 1833 and 1313% (102.8 – 1883.2 and 16.5 – 217.2 mg ∑EST tonne $biomass^{-1}$ d^{-1}) for the Low HRT/SRT and High HRT/SRT experimental conditions respectively.

This was evidence that the variety of niches available for different bacteria could occupy changed as EDC loading increased. In a study of the influence of resource availability upon diversity of soil bacteria, an increase in benzoate (a common intermediate of pollutants) resulted in changes in the composition of biodegraders at every concentration (Langenheder and Prosser 2008). It is therefore feasible that biodegradation of estrogens and NPx are also carried out by different members of activated sludge biota as resource availability changed. Based upon an observation that benzoate-degrading communities were dominated by taxa with high substrate affinity at lower benzoate concentration, Langenheder and Prosser (2008) concluded, specialist bacteria primarily drove biodegradation. Applicable to this research of this PhD is an understanding that multiple and variable bacteria are responsible for micropollution biodegradations. However, evidence from estrogen biodegradation suggests that the bacteria responsible may not be specialists. This research demonstrated that activated sludge communities with SRT as low as three days could achieve effective estrogen elimination.

Within 24 hours of initial spiking, E1+E2+E3 biodegradation efficiency of 88.54% and 78.36% was witnessed at the Low HRT/SRT and High HRT/SRT conditions respectively. Furthermore, as estrogen volumetric loading subsequently doubled each progressive spiking period, biodegradation was capable of dealing with additional estrogen without needing a period of equilibration. Within 6 hours of doubling of spike, estrogen biodegradation was never less than 63 or 93% for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. Whereas with Langenheder and Prosser (2008), community structure was observed to have altered after soil bacteria had been incubated with 90, 240 and 960 mg benzoate^{-1} kg soil, such swift response to significant increases in estrogen as witnessed during the porous pot experiments, and indicated that biodegradation was carried out by bacteria that were already present and did not need to proliferate. The fact that no appreciable equilibration period could be detected as estrogen spike loading was increased during the porous pots experimentation is evidence that the number of bacteria responsible for estrogen biodegradation were increasing with resource availability. The apparent seamless transition from one spiking period to the next indicated that the enzymatic ability to deal with increasing estrogen loading was already present.

Since estrogen biodegradation within the porous pots were carried by bacteria that were already present and performing activated sludge function, this could indicate that bacteria responsible for estrogen biodegradation may vary and change according to resource availability and are likely to scavenge a number of carbon sources. In agreement with this, Koh *et al* (2009) suggested that the presence of the environmentally low concentrations of EDCs are unlikely to specifically selected for bacteria that biodegrade them as these compounds would only sustain small cell numbers. Gaulke *et al*., (2008), suggests that it is more likely that EDCs are biodegraded fortuitously by bacteria scavenging a broad spectrum of organic material. In agreement with this hypothesis, a recent study had demonstrated that the bacterial communities responsible for biodegradation of E1 changed in response to increasing concentration (Thayanukul *et al* 2010). These authors found that as E1 concentrations increased from 540 ng L^{-1} to 2000 ng L^{-1}, the communities responsible for biodegradation changed from those dominated with *Alphaproteobacteria* to those associated with *Beteproteobacteria* and *Gammaproteobacteria*. This study confirms the finding of this PhD, that substrate concentration is key of determining the bacteria responsible for the biodegradation of estrogens.

8.1.3 Bacteria responsible for the heterotrophic biodegradation of estrogens

It has become increasing accepted that heterotrophic bacteria are important for the biodegradation of estrogens (Gaulke *et al* 2008, Koh *et al* 2009, McAdam *et al* 2010) and has been further confirmed by the results of this research. Indeed, at higher than environmental concentrations (mg L^{-1}), numerous heterotrophic bacteria capable of degrading estrogens have been isolated and identified (Fujii *et al* 2002, Yoshimoto *et al* 2004, Haiyan *et al* 2007, Yu *et al* 2007, Muller *et al* 2010). The bacterium *Novosphingobium* species was isolated from activated sludge with growth media containing 30 mg L^{-1} E2 as the sole carbon source. This bacterium degraded 5, 10 and 30mg L^{-1} E2 as the sole carbon source (Fujii *et al* 2002). In a similar study *Sphingobacterium* JCR5 isolated using growth media containing 10mg L^{-1} EE2 as the sole carbon source. This bacterium grew on 30 mg L^{-1} EE2 as the sole carbon source (Haiyan *et al* 2007). Additionally, this bacterium was also capable of degrading equivalent concentrations of E1, E2 and E3. In another study, fourteen bacteria were isolated from activated sludge capable of degrading 3 mg L^{-1} E2 (Yu *et al* 2007). Of these, only three stains

KC6-8 were capable of degrading 3 mg L^{-1} E1. These E1 and E2 degraders were representatives from the genera *Aminobacter* and *Sphingomonas*. More recently, Muller *et al* (2010) demonstrated that a consortium of bacteria isolated from activated sludge with a growth media containing 2 mg L^{-1} E1. These mixed cultures were made up of species including, *Alcaligenes faecalis*, *Pusillimonas sp., Denitrobacter sp.,* and *Brevundimonas diminuta*. However, in isolation these species were unable to metabolise estrogens, with the exception of *B. diminuta*, which could convert E1 to E2, perhaps indicating the importance of cometabolism. Jiang *et al* (2010) demonstrated that five isolates from the genus *Bacillus* were capable of E2 to E1. Of these, two strains were capable of biotranforming E2.

Current research has demonstrated the ability of heterotrophic bacteria to biodegrade estrogens. These bacteria are phylogenetically diverse and represent numerous genera of which new discoveries are continually being added to. Muller *et al* (2010) demonstrated that estrogen biodegradation occurred with syntrophic relationships between estrogen and ACN degrading organisms. This supports the theory that co-metabolism is necessary for the biodegradations of estrogens. Although the key criticism for all these estrogen degrader isolation studies, is that none of the bacteria were isolated with environmentally relevant concentrations of estrogen. Nor were they further grown with environmentally relevant concentrations of estrogen either. There is lack of evidence that *Aminobacter sp., Sphingomonas sp., Novosphingobium sp., Sphingobacterium sp., Alcaligenes faecalis*, *Pusillimonas sp., Denitrobacter sp.,* and *Brevundimonas diminuta* or *Bacillus sp.* can biodegrade estrogens at environmentally relevant levels. However, the authors above have all demonstrated that heterotrophic bacteria are capable of degrading estrogens. Furthermore, their findings would suggest that a diverse variety of bacteria within the ASP sampling during this PhD were capable of biodegrading estrogens. The variety of bacteria listed here that have been demonstrated to biodegrade estrogens at a variety of concentrations supports the hypothesis that biodegraders change with changing supply of resource availability.

8.1.4 Bacteria responsible for estrogen biodegradation of NPx

Bacteria identified with NPx biodegrading capabilities are predominantly heterotrophic (Maki *et al* 1994, Frassinetti *et al* 1996, John and White 1998, Tanghe *et al* 1999, Fujii *et al* 2000, Gioia *et al* 2008, Gu *et al* 2010). As with estrogen degrading heterotrophs, bacteria isolated with NPx enrichment resulted in cultures capable of biodegrading mg L^{-1} concentrations of these compounds. The same issue also exists in that the bacteria identified have not been evidenced to degrade NPx at environmentally relevant concentrations. However these studies indicate that the bacteria involved have diverse phylogeny. Biodegradation of long chain NPEO has been demonstrated by strains of the genera: *Pseudomonas* (Frassinetti *et al* 1996, Maki *et al* 1998, John and White 1998, Gu *et al* 2010); *Stenotrophomonas* (Salvadori *et al* 2006); *Xanthomonas* (Frassinetti *et al* 1996); *Sphingomonas*; *Sphingobium*; *Cupriavidus*; *Ralstonia*; *Achromobacter* and *Staphylococcus* (Gu *et al* 2010). Biodegradation of NP has been demonstrated by stains of the genera: *Sphingomonas* (Tanghe *et al* 1999, Fujii *et al* 2000, Corvini *et al* 2004); *Alicaligenes* (Tanghe *et al* 1999) and *Pseudomonas* (Tanghe *et al* 1999, Fujii *et al* 2000). Although these studies demonstrated biodegradation at significantly higher concentrations than those found in sewage, it is undeniable, the diversity of bacteria capable of such a task.

8.2 Competitive Inhibition of EDCs

A food to micro-organisms ratio (F:M) of 0.17 mg BOD^{-1} mg MLVSS d^{-1} was observed at the ASP_{carb} compared to 0.06 and 0.05 mg BOD^{-1} mg MLVSS d^{-1} at the ASP_{nit} and $ASP_{nit/denit}$ respectively. These Food to Micro-organism (F:M) ratios for the three ASPs were within the range suggested for effective EDC biodegradation (Chiu *et al*., 2010, Joss *et al*., 2004). Bulk-organic loading has been suggested to be an important parameter in the biodegradation of EDCs, due to the potential for competitive inhibition from bulk organics (Onda *et al* 2003, Svenson *et al* 2003, Joss *et al*., 2004). Despite this, previous full scale surveys (Onda *et al* 2003) have failed to provide clear correlation between EDC removal efficiency and F:M. However F:M is not an adequate parameter to explain the influence bulk organic loading have upon estrogen biodegradation (McAdam *et al* 2010), as this parameter is used to evaluate systems designed based upon SRT. At the three ASP it was observed that COD and estrogen loadings increased commensurately in correlation (r^2 = 0.59). High F:M ratios are typically due to the adoption of shorter SRT rather than variations in settled sewage substrate

concentration, thus an increases in EDC loading occurred as F:M increases, as was observed in this study.

8.2.1 Estrogen

Relative settled sewage organic composition of ΣEST and bulk organic follow a trend inverse to this. Average ΣEST and COD concentrations ratios were lowest at the ASP_{nit} (3.16×10^6 mg COD^{-1} mg ΣEST L^{-1}) and highest at the $ASP_{nit/denit}$ (7.62×10^6 mg COD^{-1} mg ΣEST L^{-1}). Above 4×10^6 mg COD^{-1} mg ΣEST L^{-1}, there appeared to be thresholds at which estrogen biomass activity respectively reduced. However, it was impossible to separate the influence of loading in order to assess the effect bulk-organics has upon biodegradation. As the sites with the highest estrogen loading (ASP_{carb} and ASP_{nit}) also had higher proportions of ΣEST as organic carbon and subsequently had the highest biomass activities. Therefore, as with other research (Onda *et al* 2003), using full-scale data, it has not been possible to elucidate the role of bulk organics upon estrogen biodegradation.

The porous pot experiment was designed to clarify the role of bulk-organics upon biodegradation of estrogens. However, this study failed to separate the role competing carbon sources had upon the biodegradation of estrogens. A gradient of COD/ ΣEST concentration ratios was created, from 9.51×10^5 to 1.29×10^7 mg COD^{-1} mg ΣEST L^{-1}. Throughout the porous pots experiment estrogen biodegradation was consistently high for both experimental conditions. Furthermore, COD removal was on average 80 and 94% for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. It is unlikely that much of the carbohydrate constituent of the influent remained within the effluent. Within effluents, COD concentrations were 105.8 and 31.8 mg L^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. Additionally, TSS concentrations were 38.2 and 14.6 mg L^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively. This indicated that much of the effluent was organic waste generated by activated sludge biota and may not have been readily biodegradable. It is possible that the carbohydrates within the synthetic sewage were too rapidly biodegradable and due to this the activated sludge biota of both sets of pots were capable of biodegrading the estrogens as they were on only available carbon source after the bulk organics had been utilised.

Broadening the ratio of COD and estrogen concentration ratios was not sufficient to elucidate the role of competing organics due to the underling biodegradability of the synthetic sewage. COD concentration needed to have been increased independently from estrogen loadings to elucidate this issue further. Rogers and Reardon (2000) demonstrated in a kinetics and substrate interaction study of a *Burkholderia species JS150,* inhibition of alternative food sources by the availability of its preferred source. These researchers found that phenol biodegradation was inhibited by the presence of toluene (the favoured carbon source). This is clear evidence of competitive inhibition as *Burkholderia species JS150,* preferentially utilised toluene over phenol. Inhibition induced by the availability of alternative carbon sources could suggest that a wider range of bacteria could degrade EDCs, but it is only competitive for them to do so when alternative food sources had become limiting. The porous pots experiment may represent an opposite to the scenario present by Rogers and Reardon (2000). Perhaps the synthetic sewage was completely biodegradable and estrogen biodegradation was therefore uninhibited.

8.2.2 NPx

In contrast to the estrogen, it has been observed that NPx biodegradation appeared to be influenced by the presence of bulk organics more clearly. An inverse linear relationship between daily average data at all three ASP between COD/$NP_{4\text{-}12}EO$ settled sewage ratio and percent biodegradation demonstrated this. As the proportion of bulk organics in proportion to $NP_{4\text{-}12}EO$ increased, biodegradation efficiency decreased. This is the first time evidence demonstrating a link between bulk organics and NPx biodegradation has been presented. Moreover, the complexity of NPx biodegradation and the cascade in biodegradation and bioformation appeared to be closely linked to the presence of alternative carbon sources.

A prominent feature of the results, were high removals of $NP_{4\text{-}12}EO$ compared to $NP_{1\text{-}3}EO$. This finding illustrates successive shortening of long chain oligomers through deethoxylation (Ahel *et al* 1994, Langford *et al* 2005). Shortening of $NP_{4\text{-}12}EO$ long chain ethoxylates coincided with the formation of $NP_{1\text{-}3}EO$ ethoxylates and $NP_{1\text{-}3}EC$ carboxylate intermediates.

Alternative aerobic mechanisms, where initial ethoxylate degradation occurs through ω-carboxylation of the individual ethoxylate chains, terminating at the formation of dicarboxylated metabolites (without the formation of short chain ethoxylates or nonylphenol) has been demonstrated elsewhere (Jonkers *et al* 2001, Zhang *et al* 2007). In this study, based on the rate of NP_1EO metabolite formation and the correlation of NP_1EC metabolite formation to biodegradation rates of long chain ethoxylated compounds, ω-carboxylation is unlikely to have occurred. Short chain ethoxylate oligomers and nonylphenol were formed, thus it can be assumed that biodegradation at all surveyed ASPs corresponded to the more conventional aerobic mechanism proposed (Thiele *et al* 1997). At the ASP_{nit} net NP biodegradation was observed. Furthermore, this site demonstrated net molar removal of the NP subunit. Several full-scale studies have proposed that high nonylphenol effluent concentrations are evidence of compound recalcitrance (Ahel et 1994). In contrast, under laboratory controlled batch (Staples *et al* 1999) and dynamic conditions (Tanghe *et al* 1998), high nonylphenol compound reduction has been demonstrated under aerobic conditions.

In this study NPx biodegradation occurred with >90% $NP_{4\text{-}12}EO$ at the ASP_{nit}. From this result, it may be hypothesised that in the absence of competing substrate such as the $NP_{4\text{-}12}EO$ compounds, activated sludge bacteria preferentially attack NP. Tanghe *et al*. (1998) made a similar assertion following observations in high concentration nonylphenol experiments with a semi-continuous ASP. Koh *et al* (2009) also identified nonylphenol degradation at a full scale $ASP_{nit/denit}$ which was heavily loaded with alkylphenolic compounds (242 mg m^{-3} d^{-1}), operated under low bulk COD primary effluent concentrations (252 mg L^{-1}) and achieved 95% ΣNPEO degradation (sum of $NP_{1\text{-}12}EO$). The data presented here suggest that at low bulk COD/ΣNPEO ratios, the kinetic rate at which ethoxylated compounds are biotransformed increases, and in turn, increases the likelihood of metabolite and nonylphenol removal. Further work is required to ascertain the impact of multi-component micropollutant matrices on the biodegradation kinetics of individual micropollutant compounds.

8.3 Future control of EDCs emission using activated sludge treatment

8.3.1 Designing ASP for optimal EDC biodegradation by controlling reactor volume, loading and sludge wastage

Reactor Volume in relation to flow rate

The high biomass activity and modest ∑EST removal determined at the ASP_{carb} contradicts previous hypotheses that imply that effective EDC biodegradation may be dependent upon process SRT and nitrification. Clearly, process parameters that coincidentally favour nutrient removal are important design parameters for the consideration of EDC elimination. It has been postulated that increasing total MLSS concentration for such sites (i.e. gMLSS L^{-1} normalised for reactor volume) would increase first-order rate kinetics (McAdam *et al* 2010). This is analogous to nutrient based ASP design, in which extensive basin volumes assure much higher total mixed liquor concentrations, for example $ASP_{nit/denit}$ had significantly higher biomass in relation to its flow (128112 kg, Q 53380 m^3 d^{-1}) in comparison to solely carbonaceous ASP (ASP_{carb} 14190 kg, Q 15000 m^3 d^{-1}). Cao *et al* (2008) observed that using increasingly concentrated activated sludge liquors in batch conditions rate kinetics increased. These authors demonstrated rate differences between estrogen biodegradation in river water and activated sludge, which was probably due to the spark difference in bacterial concentration between these two mediums. However there was no correlation between estrogen biodegradation rate and increasing solids concentration of activated sludge.

Increasing reactor volume to ensure greater total mixed liquors concurrently increases HRT and the reaction time available for biodegradation. Assuming that EDC biodegradation is first-order, as proposed in this research and by others (Ternes *et al* 2004, McAdam *et al* 2010), enzyme concentrations would greatly exceed those of the substrate (Schnell and Mendoza 2004). According to researchers such as Schnell and Mendoza (2004), increasing overall biomass would only add to an over abundance of EDC degrading enzymes which in itself would not affect first-order reaction kinetics. Therefore, the benefits achieved from extensive reactor volume would not be due to increased total MLSS, but the extra reaction

time achieved. However, by providing extensive reactor volume, EDC loading would be reduced. However, this reduced loading would also subsequently decrease reaction rate due to increased resource scarcity.

Where biodegradation follows first-order kinetics sizing of reactor volume in relation to it flow rate is of utmost importance for the optimisation of EDC biodegradation. ASP volume governs its volumetric loading and HRT, two parameters that are intimately involved with first-order kinetics. Increased resource loading increases the rate of degradation. However, loading and HRT have an inverse negative relationship. Higher EDC loading, achieved with conservative reactor sizing would yield shorter HRT. This phenomenon may explain the incomplete biodegradation witnessed at the ASP_{carb}. Where conversely, low EDC loading achieved by over-sizing reactor volume would result in longer HRT. There is currently a paucity of information regarding the kinetics of estrogen biodegradation. In order to design AST capable of effective EDC biodegradation, the correct balance between loading and HRT need to be established. Only with thorough kinetic investigation of EDC biodegradation will it be possible to determine reaction time necessary for specific loadings.

Optimising Sludge Wastage

At the ASP_{carb}, overall removal of estrogen was 79%. However 35% of this removal was attributed to the fraction leaving the works at WAS. Estrogen losses via WAS would have equated to 162 g y^{-1}. These significant quantities of estrogens were due to the higher sludge wastage rate in comparison to the other sites and an elevated WAS concentration. At the ASP_{carb} higher WAS concentrations compared to effluent were observed, this could be due to higher colloidal proportions in the activated sludge. It was not possible to directly sample activated sludge and determine estrogen partitioning at this stage of treatment. Recent research has indicated that pharmaceuticals such as propranolol and carbamazepine can associate with colloidal phases 7-70% (Maskaoui and Zhou 2009). Further research investigating the role of colloids as sinks for EDCs is necessary in order to determine how best to optimise removal. Due to their lipophillic characteristics, it is widely understood that EDCs pose significant environmental threats due to these potential to sorb to waste primary

and secondary sewage sludge (Rogers 1996, Ternes *et al* 1999, Dizer *et al* 2002). However, data about EDC losses via WAS are sparse, inconsistent and lacking in information regarding AST process parameters that would permit direct comparison with the finding of this research. Carballa *et al* (2007) preformed mass balance upon an ASP with no appreciable E1 or E2 biodegrading ability. These authors noted that only circa 6% of E1+E2 ended up as WAS. In another study in China, up to 45% of EE2 was removed by WAS (Shao and Ma 2009 abstract only). The disparity between these results and those of this PhD indicate that sorbtion as a mechanism of removal is poorly understood and further research is needed to illuminate this area. Despite this, the results of this PhD indicate that moderate biodegradation observed at the ASP_{carb} was not necessarily due to bacteriological limitations that resulted from an SRT of six days. The significant wastage rate required to maintain this SRT effectively removed 35% of estrogen that the activated sludge had not biodegraded. This significant proportion of estrogen in the WAS appeared to be a result of incomplete biodegradation in the activated sludge tank. Although biodegradation could potentially have been greater due to evidence that estrogens had sorbed and subsequently desorbed in the WAS. This inhibition from the maximum potential could posabily have been from competing carbon sources. This could inticate that a longer reaction time was necessary. Therefore increasing SRT may only be important insofar that this parameter increases dependandtly with HRT.

8.3.2 Enhancing the performance of established AST and achieving sub-PNEC effluent concentrations

If it is assumed that first-order kinetics describe the mechanism of EDC biodegradation in activated sludge, then it is then understood that the enzymatic potential to carryout this breakdown is already present. This was evident due to the estrogen degrading ability of the porous pots that had been fed on easily biodegradable carbon sources. However at actual ASP, the correlation between EDC resource availability (volumetric loading) and biomass activity is not perfect. To achieve effective EDC removal, this inhibitory element that prevents complete biodegradation needs to be determined. It has been established in the previous section that balancing HRT and loading could be necessary for optimal biodegradation of EDCs. However, this research has also demonstrated that in order to

activate the potential within activated sludge, it may be necessary to remove competitive carbon first, particularly with regards to NPx. The $ASP_{nit/denit}$ had the worst ∑NPx biodegradation and the highest proportion of bulk organics in its settled sewage. Conversely, the ASP_{nit} had the best ∑NPx biodegradation and the lowest proportion of bulk organics in its settled sewage.

Effective EDC elimination may therefore be possible by manipulating the bulk organic constituent within activated sludge either by using pre or post treatment. In fact, for established ASPs this may be the only option available. Established ASP cannot be retro-fitted in order to manipulate loading and HRT, as it would be unfeasible to alter reactor volume for most current ASP due to the capital investment retained within the facility. Out of necessity, UK sewage treatment has a long tradition of adding bolt-on technologies to deal with stricter consent requirements. In terms of achieving sub-PNEC effluent concentrations, it may be necessary to use a pre or tertiary treatment, especially at carbonaceous ASP with poor removal efficiencies. It has been established that treatment technological utilising activated carbon (Fukuhara *et al* 2006, Snyder *et al* 2007, Filby *et al* 2010), ozonation (Huber *et al* 2004, Hashimoto *et al* 2006, Snyder *et al* 2007, Filby *et al* 2010) and chlorination (Lee and von Gunten 2009, Schiliro *et al* 2009 and Filby *et al* 2010). However, using these technologies would adversely affect the capital operational and environmental costs of sewage treatment (Jones *et al* 2007b).

Effective removal of excess EDCs not removed during ASP, could be done by passive low-tech means. Sand-filters receiving effluent from a presumably carbonaceous ASP reduced E1 concentrations from 6.3 to 0.67 ng L^{-1} (Gunnarsson *et al* (2009). This was an additional removal of >90% on top of what had been achieved by the ASP. The findings of these authors support the hypothesis of this research that tertiary treatment of EDC need not be high energy or high cost. Such processes receive effluent, low in competitive bulk organics and are highly loaded. All evidence points to effectiveness of EDC biodegradation by highly loaded high rate processes provided it is competitive for bacterial communities to degrade them. Therefore, removing competitive bulk organics from influent may also be an effective future strategy. In an evaluation of septic systems, treating on-site wastewater, it had been demonstrated that estrogenic activity could be effectively reduced using pre-treatments such

as aerobic sand filters (Stanford and Weinberg 2010). YES responses were reduced from 80 to 0.54 ng L^{-1} indicating the effectiveness of such technologies. Such pre-treatments removed bulk organics and therefore increase the ability of activated sludge to remove EDCs. This area of research is promising as it has the potential to deliver better environmental protection whilst reducing the tertiary treatment investment necessary.

8.4 Further Work

This research has highlighted several key areas in which further study is necessary in order to progress this field of study. Evidence strongly suggests that first-order reaction kinetics describe the mechanism by which EDC biodegrade. Therefore greater understanding into how volumetric loading of both EDCs and bulk organics affect these kinetics is necessary. The findings of this research suggest that the sizing of an activated sludge reactor is essential for enabling effective EDC biodegradation. A reactor must be sized to allow sufficient volumetric loading of EDCs but without a deleteriously short HRT so that full biodegradation can occur. A comprehensive kinetics study would provide invaluable information for wastewater treatment designers. However, greater understanding into the influence of bulk organics is also necessary. This PhD demonstrated that NPx may have been inhibited by the presence of more readily available carbon. Further study is necessary to establish how estrogens biodegradation is affected by bulk organics. A similar experiment to the one utilising porous pots in this PhD could accomplish this. During this PhD, estrogen loading was increased and COD remained constant. By keeping estrogen loading constant and increasing COD independently, it could be possible to test the hypothesis that bulk organics inhibit estrogen biodegradation. Were this information available, it may be possible to predict and model EDC biodegradation for any activated sludge process. With greater understanding it may be possible to design an ASP that would achieve the full biodegradation of EDCs. Whilst understanding biodegradation kinetics would be a distinct advantage to wastewater treatment designers, it is unlikely that current ASPs can be retrofitted to make use of this knowledge. Once in operation, reactor volume cannot be altered significantly. A hypothesis developed as a result of this research is that high rate passive tertiary technologies could enhance EDC biodegradation if necessary. Clearly, the cost and benefits of any added stage treatment would need to be assessed. Prior to this, testing of the hypothesis is necessary.

Chapter 9

Conclusions

9 Conclusions

The overall aim of this research was to determine which process parameters were responsible for the effective removal of EDCs during activated sludge treatment. This was assessed through: observations made from sampling three ASP and mass balancing estrogen and NPx removal throughout the treatment process; and then by experimentation using porous pot bioreactors.

Of all three sites, the ASP_{nit}, with an SRT of 20 days had the most effective biodegradation of ∑EST (95.5%) and NPx (71.9%). This was higher in comparison to the ASP_{carb}, with an SRT of 6 days that biodegraded, ∑EST 54% and NPx 39.7%. The $ASP_{nit/denit}$, which had the highest SRT (22 days), was less active at biodegradation of estrogen (84%), which was lower than the ASP_{nit} and NPx (32.8%), which was lower than the ASP_{carb}. It appeared that SRT in isolation could not explain these differences in biodegradation. Neither could HRT (in isolation) effectively explain the differences in biodegradation at the three ASP, as the ASP_{nit} and ASP_{carb} both had comparable HRTs (5.6 and 8 hours respectively). However, it was evident that volumetric loading of EDCs was an important factor in biodegradation as it appeared to correlate with biomass activity. At the ASP_{carb}, $ASP_{nit/denit}$ and ASP_{nit} respectively, ∑EST loadings of 0.05, 0.36 and 0.38 mg m^{-3} were recorded. Subsequently, at the $ASP_{nit/denit,}$ ASP_{carb} and ASP_{nit} respectively, biomass activities of 22.8, 40.7 and 60.4 mg ∑EST $tonne^{-1}$ day^{-1} were recorded. At the $ASP_{nit/denit,}$ ASP_{carb} and ASP_{nit} respectively, $NP_{1-4}EO$ loadings of 10, 43 and 55 mg m^{-3} were recorded. At the $ASP_{nit/denit,}$ ASP_{carb}, and ASP_{nit} respectively, biomass activities of 1888, 6678 and 11543 mg $NP_{4-12}EO$ biodegraded $tonne^{-1}$ day^{-1} were recorded. The higher volumetric loading of EDC at the ASP_{carb} in comparison with $ASP_{nit/denit}$ explained the disparity between biomass activities at these two sites.

The role of more readily available carbon on the biodegradation of EDCs was also assessed at the three ASP. It was evident that under instances when estrogen

contributed its highest proportion to overall COD, biomass activities were highest. The highest ∑EST biomass activities (>38.6 mg ∑EST biodegraded tonne^{-1} day^{-1}) were recorded when the settled sewage COD/∑EST ratio was <6 x10^{6} mg COD^{-1} mg ∑EST L^{-1}. However, since volumetric loading also correlated with biomass activity, the separate role of competing carbon could not be established. However, evidence that competing carbon influenced biodegradation of NPx was more compelling. The most effective site for the biodegradation of NPx was the ASP_{nit}. This was the only site capable of net molar biodegradation of the NP subunit. This site had the highest proportion of NPx as carbon available to the activated sludge biota. The highest daily average $NP_{4\text{-}12}EO$ biomass activities (>9590 mg $NP_{4\text{-}12}EO$ biodegraded tonne^{-1} day^{-1}) were recorded when the settled sewage sCOD/$NP_{4\text{-}12}EO$ ratio was each day on average <20828 mg sCOD^{-1} mg $NP_{4\text{-}12}EO$ L^{-1}. Yet the same situation was true for the NPx as of estrogens. Volumetric loading correlated with biomass activity and the separate role of competing carbon could not be established. However, a linear relationship between percent $NP_{4\text{-}12}EO$ biodegradation and the proportion of $NP_{4\text{-}12}EO$ as COD could be observed. This relationship implied that at 54330 mg sCOD^{-1} mg $NP_{4\text{-}12}EO$ L^{-1}, biodegradation of $NP_{4\text{-}12}EO$ could not occur. This is strongest evidence to date that bulk organics can have in an inhibitory effect upon the biodegradation of NPx.

Following the field based sampling, the porous pot bioreactors were used to gain better understanding of the influences of SRT, HRT, loading and competing carbon sources and to test the hypothesis that ammonia oxidising bacteria and not involved in the biodegradation of estrogen at the concentrations encountered within sewage treatment. Effective estrogen biodegradation (typically > 79.1% for E1, E2 or E3) was demonstrated by the Low HRT/SRT experimental condition, despite an SRT of three days. This suggested that the ASP_{carb}, could achieve better estrogen biodegradation following optimisation. The porous pot experiments confirmed that volumetric loading was an important driver of EDC biodegradation. The porous pots responded to increases in loading with linear increases of biomass activity from 17 to 1883 mg E1+E2+E3 tonne^{-1} day^{-1}. This was strong evidence that EDC biodegradation followed first-order biodegradation. However, the role of bulk organics and the effects of competitive inhibition of EDC biodegradation by more readily available carbon were

not determined by the porous pot experimentation. At the porous pot bioreactor level, it is probable that the biodegradability of the synthetic sewage contributed to the effective estrogen biodegradation during the porous pot experimentation. This could imply that availability of bulk organics plays an important role in estrogen biodegradation. However, this evidence was circumstantial and therefore this research has not been able to adequately determine whether competitive inhibition of estrogens biodegradation by more readily available carbon had occurred.

This research has given valuable insight into the bacteria responsible for the biodegradation of estrogens and NPx. The key observation from sampling the three ASP was that the ASP_{carb} was capable of 54% biodegradation despite being a carbonaceous only removal process. Previously is had been hypothesised that AOB were responsible for the biodegradation of estrogens. The porous pots confirmed that, ammonia oxidising bacteria appeared not to have a role in biodegradation of estrogens (including EE2) at typical loadings encountered within sewage treatment. Reduction of AOB abundance from 2.47×10^8 to 1.17×10^7 and 5.15×10^9 to 4.27×10^7 mL^{-1} for the Low HRT/SRT and High HRT/SRT experimental conditions respectively did not affect estrogen biodegradation. Within activated sludge treatment, it is unlikely that neither the abundance of ammonia oxidising bacteria nor the concentration of estrogens is sufficient to permit fortuitous co-metabolism via monooxygenase activity. Therefore, it is more likely that heterotrophic bacteria are responsible of the biodegradation of estrogens. Furthermore, these heterotrophic bacteria demonstrate first-order biodegradation of estrogen and NPx. Given higher loadings, it is likely that activated sludge would biodegrade more EDCs as the enzymatic activity required to biodegrade EDCs appeared not to be limiting either at actual ASP or at porous pot bioreactor level. The porous pots also demonstrated that virgin biomass were capable of biodegrading estrogens. This was evidence that estrogens are biodegraded fortuitously by bacteria utilising a variety of carbon sources and that a broader range of (heterotrophic) bacteria than previously thought can utilise EDCs. As no threshold in biodegradation was achieved, and given the gradient of EDC resource loading used in this research, is it likely that communities already in situ carry out that biodegradation once resources are at sufficient level for them to so.

Since this research has presented evidence that first-order reaction kinetics describe the mechanism of EDC biodegradation. Therefore, reaction time is closely linked with effective biodegradation and decreases as EDC volumetric loading increases. At sites like the ASP_{carb}, it may not be possible to achieve sub-PNEC effluent emission. The HRT may not have been long enough to achieve full EDC biodegradation. Increasing the reactor volume (in relation to its flow) could provide the additional contact time necessary, however, volumetric loading would be been reduced as well. Clearly further work is required to illuminate the controlling factors surrounding first-order reaction kinetics, which would be required to future design of activated sludge treatment. To improve inadequate EDC removal typical of carbonaceous only AST, tertiary treatment may be necessary. The evidence presented by this research suggests that highly loaded processes with short HRTs, such as those indicative of fixed film treatment options such as trickling/sand filters should be considered as tertiary treatment options.

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