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AN EVALUATION OF THE FATE AND BEHAVIOUR OF ENDOCRINE DISRUPTING CHEMICALS DURING ANAEROBIC DIGESTION OF SEWAGE SLUDGE

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Supervisor: Dr Elise Cartmell

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ABSTRACT

Endocrine disrupting chemicals such as steroid estrogens and alkylphenol polyethoxylates entering the environment via regular domestic or industrial discharges have been demonstrated to cause feminization of aquatic organisms at trace levels. Despite these discharges, the solid-end product of wastewater treatment i.e. digested sludge, poses a potential source of these compounds in the environment when sewage sludge is recycled onto land. Greater concentrations of alkylphenolic metabolites such as alkylphenols and short-chained one to three ethoxy units, ethoxylates have been reported to occur in digested sludge than the parent compounds.

This study investigates the fate and behaviour of these chemicals in mesophilic and thermophilic anaerobic digestion by using primary sludge and a mixture of primary and secondary sewage sludges. The analytical methodologies used for the determination of these endocrine disrupting compounds allowed accurate quantification at microgram per kilo of dry-sludge weight concentrations in the complex sludge matrices. Four mesophilic and two thermophilic semi-continuous lab-scale anaerobic digesters were examined. In addition, acclimated sludges were dosed with high nonylphenolic concentrations to observe the capacity of biomass to remove these compounds.

Sewage sludge type has significant impact on the removal of estrogens which is favoured at mesophilic temperatures. Removal efficiencies for these compounds were >53% in primary sludge and >39% in mixed sludge during mesophilic digestion. Removal of nonylphenolics was favoured during thermophilic digestion. The biochemical activity of the primary sludge biomass was found to be more efficient, irrespective of digestion temperature for both steroid estrogens and nonylphenolics.

It was found that the maximum nonylphenolic sludge content of 50 mg kg^{-1} dw proposed in the European Commission for the use of sludge as land conditioner was not exceeded by neither mesophilic nor thermophilic anaerobic digestion of primary or mixed sludge.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AP	Alkylphenol
APEOs	Alkylphenol polyethoxylates
APECs	Alkylphenoxy carboxylates
BOD	Biochemical oxygen demand
BNR	Biological nutrient removal
CAPEC	Dicarboxylated metabolites
DCM	Dichloromethane
EA	Environment Agency
E1	Estrone
E1-3S	Estrone-3-sulfate
E2/17β-E2	17β-estradiol
E3	Estriol
EE2	17α-ethinyl estradiol
EDCs	Endocrine disrupting chemicals
EEq	Estradiol equivalent
ESTs	Estrogens
EU	European Union
HRT	Hydraulic retention time
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
MeOH	Methanol
NP	4-nonylphenol
NPEO	Nonylphenol polyethoxylates
NP_1EO	Nonylphenol monoethoxylate
NP_2EO	Nonylphenol diethoxylate
NP_3EO	Nonylphenol triethoxylate
NP_1EC	Nonylphenoxy acetic acid

NP_2EC	Nonylphenoxy monoethoxy acetic acid
OP	4-tert-octylphenol
OPEO	Octylphenol polyethoxylates
OP_1EO	Octylphenol monoethoxylate
OP_2EO	Octylphenol diethoxylate
OP_3EO	Octylphenol triethoxylate
OP_1EC	Octylphenoxy acetic acid
OP_2EC	Octylphenoxy monoethoxy acetic acid
PNECs	Predicted no effect concentrations
SRT	Sludge retention time
STWs	Sewage treatment works
UK	United Kingdom
USA	United States of America
VFA	Volatile fatty acid
WAS	Waste activated sludge
Σ = sum	
PSM	primary sludge digester
MSM	Mixed sludge digester
ΣPSM	refers to both primary digesters
ΣMSM	refers to both mixed digesters
$\Sigma NP_{3\text{-}12}EOs$	the sum of NP_3EO to $NP_{12}EO$
$\Sigma NP_{1\text{-}2}EOs$	the sum of NP_1EO and NP_2EO
ΣNPECs	the sum of NP_1EC to NP_2EC
ΣPSM	the sum of primary mesophilic digesters
ΣPST	the sum of primary thermophilic digesters
ΣMSM	the sum of mixed mesophilic digesters
ΣMST	the sum of mixed thermophilic digesters
APEOC	Alkylphenolics ($AP_{1\text{-}12}EO + AP_{1\text{-}3}EC$ + AP)

LIST OF UNITS AND SYMBOLS

±	Standard error
=	Equal
>	More than
<	Less than
%	Percentage
°C	Degrees Celsius
CO_2	Carbon dioxide
CH_4	Methane
dw	Dry weight
d	Day
h	Hour
H_2O	Water
K_d	Absorption constant
K_{oc}	Octanol-carbon partition coefficient
K_{ow}	Octanol-water partition coefficient
l	Litre
Log K_{ow}	Log of octanol-water partition coefficient
M_{diff}	Mass difference
M_{in}	Mass influx
M_{out}	Mass outflux
mg	Milligram
mg kg^{-1}	Milligram per kilogram
mg d^{-1}	Milligram per day
mg l^{-1}	Milligram per litre
ml	Millilitre
ml min^{-1}	Millilitre per minute
mV	Millivolts
µg	Microgram

$\mu g\ l^{-1}$	Microgram per litre
$\mu g\ g^{-1}$	Microgram per gram
$\mu g\ kg^{-1}$	Microgram per kilogram
$\mu g\ d^{-1}$	Microgram per day
n.d.	Not detected
NH_4OH	Ammonium hydroxide
N_2	Nitrogen
ORP	Oxidation/reduction (redox) potential

LIST OF PUBLICATIONS

Chiu, T.Y., Paterakis, N., Cartmell, E., Scrimshaw, M.D., J.N., L. (2010). A critical review of the formation of mono and dicarboxylated metabolic intermediates of alkylphenol polyethoxylates during wastewater treatment, Critical Reviews in Environmental Science and Technology, 40, 3, 199-238.

Chiu, T.Y., Koh, Y.K.K., Paterakis, N., Boobis, A.R., Cartmell, E., Richards, K.H., Lester, J.N., Scrimshaw, M.D. (2009). The significance of sample mass in the analysis of steroid estrogens in sewage sludges and the derivation of partition coefficients in wastewaters, Journal of Chromatography A, 1216, 4923-4926.

IN PREPARATION

Behavior of nonylphenol ethoxylates and steroid estrogens during anaerobic digestion of sewage sludge.

1. INTRODUCTION

1.1. Endocrine Disrupting Chemicals (EDCs) - Problem

Environmental pollution has been receiving increased attention in both scientific and public sectors (Renner, 1997). Endocrine disrupting chemicals (EDCs) comprise a wide spectrum of substances with diverse physicochemical characteristics which have recently caused concerns to fish (Cargouët et al., 2004; Routledge et al., 1998; Desbrow et al., 1998; Johnson & Sumpter, 2001a; Purdom et al., 1994a; Cargouet et al., 2004; Kramer et al., 1998).

Although environmental pollutants such as pesticides (DDT, DDE, lindane, atrazine), polycyclic aromatic hydrocarbons (PAHs), dioxins (PCDDs, PCDFs) and polychlorinated compounds i.e. polychlorinated biphenyls (PCBs) have received great research emphasis in the last three decades (Birkett, 2003), in recent years, research has focused on the hazard posed by EDCs. The rationale behind this shift in research lies to the estrogenic potency of EDCs and in particular the steroid estrogens (ESTs) and alkylphenol polyethoxylates (APEOs). These classes of EDCs enter the aquatic and terrestrial environment via regular domestic or industrial discharges and by sludge recycling applications (Gibson et al., 2005; Koh et al., 2005) (Figure 1-1). Critical exposure to these chemicals can alter the development of many tissues of the organism (humans and wildlife) which ultimately could result in permanent character changes in the mature organism (Colborn et al., 1993). It has been shown that these effects can occur at trace concentrations and it has been suggested that EDCs may work synergistically (Environment Agency, 2000).

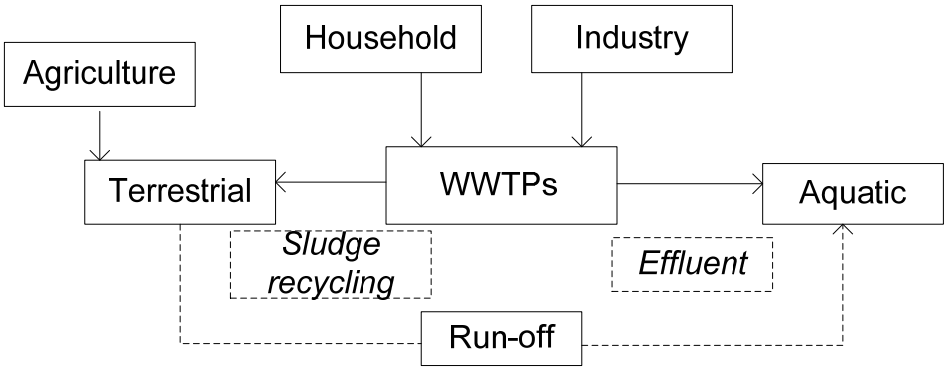


Figure 1-1 Components of anthropogenic EDCs inputs into the environment.

1.2. Phenomena of EDCs

The feminization of male fish has been detected in several European countries (Jobling et al., 1998; Larsson et al., 1999; Flammarion et al., 2000; Versonnen et al., 2004), USA (Folmar et al., 2001; Folmar et al., 1996; Solé et al., 2000) and in the Asia Pacific region (Batty & Lim, 1999; Chapman, 2003; Gong et al., 2003). Research has clearly implicated that certain sewage treatment works (STWs) (Petrović & Barceló, 2004; Kirk et al., 2002; Rodgers-Gray et al., 2000) and pulp mill effluents (Durhan et al., 2002) are sources of EDCs in sewage sludge and in the aquatic environment. It is believed that the majority of EDCs reach the aquatic environment by sewage effluent as it has been observed in the UK (Jobling et al., 1998), Germany (Spengler et al., 2001), France (Cargouët et al., 2004), Italy (Baronti et al., 2000), Sweden (Svenson et al., 2003), Spain (Solé et al., 2000), Portugal (Diniz et al., 2005), Norway (Knudsen et al., 1997), Switzerland (Espejo et al., 2002) and The Netherlands (Belfroid et al., 1999). By contrast to the aqueous sewage effluents**,** sewage sludge is a potential source of EDCs (Birkett, 2003) since it contains major anthropogenic organic contaminants (Lee et al., 2004; Petrović & Barceló, 2000; Brunner et al., 1988; Birkett, 2003). Potential risks may therefore result for ecosystems (Harrison et al., 2006; Maguire, 1999) through the recycling applications of sewage sludge to agricultural land. Besides the uptake of

EDCs by indigenous organisms including plants (Günther & Pestemer, 1990), leachate and run-off can be potential sources to aquatic environments and groundwater (Kouloumbos et al., 2008b; Birkett, 2003).

One of the most comprehensive studies of endocrine disruption in wildlife is that on the impact of steroid estrogens and non-ionic surfactants on British fish (Vos et al., 2000). It has clearly been established that the STWs effluent (Purdom et al., 1994b; Sumpter & Jobling, 1995) and several receiving surface waters in U.K. are estrogenic to fish (Harries et al., 1996). These surveys demonstrated an increased plasma level of vitellogenin (egg yolk protein precursor that is normally produced by mature females) in male roach (*Rutilus rutilus*) in rivers near sewage effluent discharges. A high prevalence (locally up to 100%) of intersex (ovotestis) observed in these fish (Jobling et al., 1998). At present, most studies on endocrine disrupting phenomena have been focused on male fish as opposed to the female fish. Even though the precise mechanisms of action are poorly understood and the contributing chemicals are not always known, extensive evidence is available that sewage effluents can disturb endocrine function in fish (Harries et al., 1999).

Regardless of the early evidence of endocrine disruption in wildlife (Dodds et al., 1938), emphasis on the EDCs phenomena was only attributed in 1990s where reduction of human semen quality had occurred in countries such as U.K., France, Belgium and Denmark (Carlsen et al., 1992; Handelsman, 2001; Swan et al., 2000). During the same period of research, testicular cancer increased (Sharpe & Skakkebaek, 1993; Toppari et al., 1996). Other anthropogenic pollutants such as organochlorines (PCBs and DDE) are constantly receiving attention since certain species of wildlife exposed to the particular EDCs experiencing endocrine disruption to their reproductive functions (Pickford et al., 2000). At present, human exposure to these chemicals are only postulated to induce endocrine disrupting effects since no strong evidence exists to link them to a risk to human (Damstra et al., 2002).

1.3. Rationale for Research

As a result of endocrine disrupting phenomena in the aquatic environment and the EU Legislation in force, a £40 million National Demonstration Program (NDP) is being undertaken by the water industry of the U.K., as part of the asset management plan 4, which was initiated by the Environment Agency (EA) of England and Wales, to investigate the potential removal of these EDCs from the final effluents (Burke, 2004).

One of the objectives of the EA NDP involves collecting baseline data across 17 STWs in the U.K. that will be used to evaluate the ability of treatment options (conventional and enhanced sewage treatment processes) to remove EDC (namely ESTs and APEOs) and reduce estrogenic activity of final effluents.

Since the presence of EDCs in surface waters and in sludge has been primarily attributed to their incomplete removal in the STWs (Gong et al., 2003; Purdom et al., 1994b) due to deconjugation of estrogens or persistent metabolic products from APEOs such as alkylphenols (AP) and short-chain APEOs (Ahel et al., 1994a; Langford et al., 2005), it is valuable to investigate the EDCs in both solid (sludge) and aqueous phases in order to obtain a holistic view of the EDC levels arising from STWs. By investigating the fate and removal of EDCs under anaerobic conditions it will potentially provide extensive information to the industry with emphasis the configuration and operation of anaerobic digesters which in turn will aid the national decision makers and EA to tackle effectively and consistently the EDCs in anaerobically treated sludge.

It is therefore necessary to determine whether the EDCs are brought into the aquatic environment via wastewater at levels that could be harmful to aquatic life, and to assess STW efficiency at removing EDCs from wastewater effluent.

Urban Waste Water Treatment Directive 91/271/EC requires that all EU wastewater will have to be collected and subjected to secondary treatment (biological, with secondary settlement) before being discharged into the environment, to eliminate the input of potentially active estrogenic compounds (European Commission, 1999). With the impending deadline within the European Community Water Framework Directive

(WFD) to phase out or reduce all discharges of priority hazardous substances by 2015 to achieve "good chemical surface water status", many wastewater operators will have to make informed decisions on the effectiveness, cost benefits, and sustainability of existing or enhanced wastewater treatment processes in dealing with the present estrogenic activity of sewage effluent discharged from their works (European Commission, 2000c).

In addition the OSPAR Convention (OSPAR Commission, 2006) for the protection of the marine environment of the North-East Atlantic has agreed between all fifteen Governments the monitoring of a list of chemicals for priority action, including alkylphenol ethoxylates.

In the past sewage sludge disposal routes such as sea or landfill have been banned; the Sewage Sludge Directive 86/278/EEC (CEC, 1986a) encourages the use of sewage sludge in agriculture in such a way as to prevent harmful effects on soil, vegetation, animals and man. Parallel to the Urban Waste Water Treatment Directive 91/271/EC the Working Document on Sludge, 3rd Draft of Directive (86/278/EEC) presented to the European Commission proposes cut-off limits for sludge contaminants including alkylphenol ethoxylates but currently these compounds are not regulated in the sludge.

The primary scope of this study is to evaluate the removal efficiencies of EDCs during anaerobic digestion of domestic sewage sludge. Two different sludge types were sampled on two occasions (April 2007 and April 2008) from a large scale STWs to implement the objective. They were subjected to lab scale mesophilic (35^{o}C) and thermophilic (53^{o}C) anaerobic digestion. The sampled sludges comprised of a primary sludge collected from the primary clarifier and a mixed sludge collected from the balancing tank which was comprised of primary and secondary sludge (60:40 w/w respectively). The mesophilic inoculum was sampled from the outlet of the large-scale anaerobic digester of the STWs. The thermophilic inoculum was cultured in the laboratory from the mesophilic digester inoculum by a gradual increase in temperature. Anaerobic digestion studies were carried at semi-continuous manual feed mode. Nominal sludge retention time for the mesophilic digesters was 30 days and 15 days for

the thermophilic digesters and the digestion period lasted for six retention times, respectively. Under mesophilic conditions, different APEO receiving concentrations were considered in order to evaluate the behaviour and fate of the relevant metabolites.

The anaerobic digestion research was funded by Yorkshire Water (Kelda Group Plc) and Thames Water. Anglian Water Plc, Severn Trent Plc and United Utilities Plc were also the co-sponsors for the EDC project. The research was carried in Cranfield University, U.K.

1. The first (*H1*E) and second research hypotheses (*H2*E) regarding the steroid estrogens are outlined in chapter 5, section 5.2

2. The first (*H1*N) and second research hypotheses (*H2*N) regarding the nonylphenol ethoxylates are outlined in chapter 6, section 6.2.

3. The research hypothesis (*H1*ND_0) of the shock loading experiment with nonylphenol ethoxylates are outlined in chapter 7, section 7.

2. LITERATURE REVIEW

2.1. Endocrine disrupting chemicals of environmental interest

Steroid estrogens and metabolites of the non-ionic alkylphenolic surfactants are two important classes of compounds, which are endocrine disrupting chemicals (EDCs) (Table 2-1). Male fish exposed to trace concentrations of steroid estrogens and non-ionic surfactant metabolites downstream of STWs have shown increased levels of oocytes in their testes (Jobling et al., 1998; Jobling et al., 1996; Purdom et al., 1994b; Villeneuve et al., 2002; White et al., 1994).

The order of relative potency relative to E2 equivalent is: EE2 > E2 > E1 > NP&OP > E3 > APEOs metabolites. The Environment Agency (EA) derived the following predicted no effect concentrations (PNECs) for individual steroid estrogens: 0.1 ng l^{-1} for EE2, 1 ng l^{-1} for E2, and 3 ng l^{-1} for E1 (Environment Agency, 2002) and a combined PNEC value for total steroid estrogens of 1 ng l^{-1} EEq (E2 equivalents) was also derived for aquatic organisms. The combined PNEC value takes into account the relative potency of each steroid and their additive effects since estrogens work synergistically (Environment Agency, 2002; Thorpe et al., 2003). Modelling studies that were carried out as part of the EA risk assessment of STWs effluents indicated predicted concentrations higher than these PNECs values in the downstream receiving environment, thus leading to unacceptable exposure of aquatic life that inhabit certain river reaches below the STWs (Environment Agency, 2002). The calculated PNEC values (for river) for NPnEO, 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 $PNEC_{water}$ of NP for aquatic organisms (algae) of 0.33 µg l^{-1} was derived for three species representing three trophic levels whereas the PNEC for soil representative of the most sensitive species of three trophic levels is 0.3 mg Kg^{-1} wet weight (calculated from the no observed effect concentration (NOEC) for earthworms (*Apporec-todea calignosa*) (European Commission, 2002). The

estrogenic potency of NP for aquatic organisms suggests that, in isolation, it is unlikely to educe an estrogenic response in fish downstream of STWs effluents once dilution is taken into consideration. This is because estrogenic responses are dose-dependent for NP, which can cause intersexuality at "high" concentrations (Gray & Metcalfe, 1997), but not at lower concentrations (Nimrod & Benson, 1998). Nonetheless, the long-term effects of low concentrations of NP on vitellogenin induction and gonadal disruption in fish are still uncertain (Gross-Sorokin et al., 2006).

Table 2-1 Endocrine disrupter concentrations in sewage effluent and potential impacts on wildlife.

Estrogen	*In vitro* E2 equivalent	Typical effluent concentration (ng l^{-1})	Typical predicted E2 equiv (*in vitro*)	*In vivo* VTG response in trout E2 equiv	Typical predicted E2 equiv (*in vivo*)	Judgement
E1	0.5	5	2.5	0.5	2.5	Concern
E2	1	1.5	1.5	1	1.5	Concern
E3	0.005, 0.04	20	0.1	0.001	0.02	Little concern
EE2	1 – 2	0.5	0.5 – 1	25	12	Greatest concern
4t-NP or 4t-OP	0.0001	2 000	0.2	0.001, 0.0006	2 – 20?	concern
NPEOs, NPECs, & CNPEC	0.00001	20 000	0.2	no significant NPEOs mixture effect at 100 µg l^{-1}	?	less concern
Total			5 – 5.5		18.1 – 36	

Adapted from (Johnson & Sumpter, 2001b)

2.1.1. Steroid estrogens

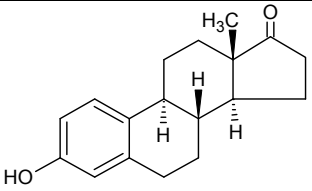
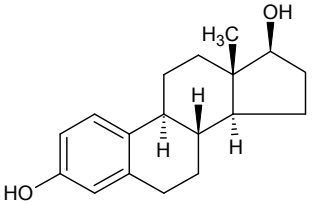
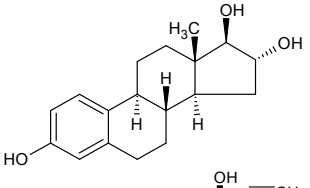
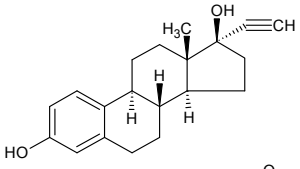
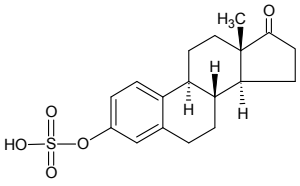
A large extent of the estrogenicity in STWs effluents is a result of the presence of natural and synthetic estrogens: estrone (E1); 17β-estradiol (E2); and 17α-ethinylestradiol (EE2) (Belfroid et al., 1999; Jobling et al., 1998; Larsson et al., 1999; Rodgers-Gray et al., 2000; Ternes et al., 1999b) that exhibit the strongest estrogenic effects (Environment Agency, 2000). Although the natural steroid Estriol (E3) is less estrogenic than E1, E2 and EE2 is still worthy of consideration since it has higher estrogenic activity compared to EDCs of industrial origin i.e. NPEOs. Estrogens are excreted by humans and animals through their urine as inactive polar conjugates such as glucuronides(E1-3G) and sulphates (Ternes et al., 1999a) with the sulphate conjugate of estrone (E1-3S) being the main urinary excretion product (Tang & Crone, 1989; Ternes et al., 1999a; Shi et al., 2004). Although many of the glucuronide conjugates are cleaved due to the activity of β-glucuronidase before they reach STWs, concentrations of the conjugated steroid E1-3S may be important when considering total estrogen loads reaching sewage treatment works (STWs) (Gomes et al., 2005), since arylsulfatase is less common in STWs (Baronti et al., 2000). The physiochemical properties of the steroid estrogens researched in this study (Table 2-2) and further information of these EDCs are shown in (Table 2-3). A survey of various estrogen concentrations in the influent of STWs can be found in Table 2-4. The synthetic steroid, EE2 has the highest octanol-water partitioning coefficient (Log K_{ow}) whilst the natural free estrogens range from 2.81 to 3.94, whereas the sulphate conjugate estrogen exhibits the lowest value 0.95 (Koh, 2008). The sulphate conjugated estrogen has also the highest water solubility compared to the unconjugated steroid estrogens which their solubilities range from 4.8 to circa 13 mg l^{-1}. All of the steroids have very low vapour pressures and relatively high pK_{α} values (above 10), therefore they are weakly acidic (Koh, 2008). Hence, the steroid estrogens under investigation are non-volatile, highly lipophilic chemicals that can be expected to adsorb to solids in environmental matrices (Lai et al., 2000). Studies on river water have demonstrated that due to high Log K_{ow}, steroid estrogens will readily sorb onto particulates, which consist largely of decaying natural organic matters (NOM) (Xiao & McCalley, 2000).

Table 2-2 Physicochemical properties of the investigated steroid estrogens.

Steroid compound	Vapour pressure (Pa)	Aqueous Solubility (mg l^{-1})	Henry's constant (atm m^3 mol^{-1})	K_{oc} (l kg^{-1})	Log K_{ow}	pK_a
E1	3×10^{-8}	13	6.2×10^{-7}	4882[d]	3.43	10.4[g]
E2	3×10^{-8}	13	6.3×10^{-7}	3300[d]	3.94	10.4[g]
E3	9×10^{-13}	13	2×10^{-11}	1944[d]	2.81	10.4[g]
EE2	6×10^{-9}	4.8	3.8×10^{-7}	4770[d]	4.15	10.4[g]
E1-3S	1.08×10^{-14}[a]	9.6×10^{2}[b]	6.9×10^{-16}[c]	29660[e]	0.95[f]	

After (Lai et al., 2000; Koh et al., 2008b), [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] (Ying et al., 2002a), [e] Estimated K_{oc} (PCKOCWIN v1.66), [f] Estimated Log K_{ow} (KOWWIN v1.67 estimate), [g] (Huber et al., 2003).

Table 2-3 Molecular properties of the investigated steroid estrogens.

Steroid estrogen	Type of steroid	Molecular formula	Molecular weight (g/mol)	CAS no.	Structure
Estrone (E1)	Natural	$C_{18}H_{22}O_2$	270.37	53-16-7	
17β-Estradiol (E2)	Natural	$C_{18}H_{24}O_2$	272.38	50-28-2	
Estriol (E3)	Natural	$C_{18}H_{24}O_3$	288.39	50-27-1	
17α-Ethinylestradiol (EE2)	Synthetic	$C_{20}H_{24}O_2$	296.40	57-63-6	
Estrone 3-sulfate (E1-3S)	Natural	$C_{18}H_{22}O_5S$	350.50	481-97-0	

Data taken from (QMX Laboratories, 2008)

Table 2-4 Steroid estrogen concentrations (ng l^{-1}) in STWs influents.

	E1	E2	E3	EE2	E1-3S	Reference
United kingdom	2-4	<0.3	-	<LOD[a]	-	(Fawell et al., 2001)
United kingdom	57-59	132-224	-	-	-	(Jiang et al., 2005)
United kingdom	78-81	185-189	-	-	-	(Jiang et al., 2005)
United kingdom	21	40	70	n.d	10	(Gomes et al., 2005)
France	9.6-17.6	11-17	11-15	5-7	-	(Cargouet et al., 2004)
Netherlands	11-140	9-48	<LOD[b]	0.5-9	-	(Johnson et al., 2000)
Netherlands	20-130	17-150	-	<0.3-6	-	(Vethaak et al., 2005)
Italy	0.5-75	0.5-20	2-120	0.5-10	-	(Johnson et al., 2000)
Italy	25-132	4-25	24-188	0.4-13	-	(Baronti et al., 2000)
Italy	44	11	72	-	-	(D'Ascenzo et al., 2003b)
Italy	15-60	10-31	23-48	<LOD[c]	-	(Lagana et al., 2004)
Italy	100	2	100	20	8	(Gentili et al., 2002)
Spain	<2.5-12	<5-30	<0.25-71	<5	-	(Petrovic et al., 2002)
Spain	2	3	-	<LOQ[d]	-	(Carballa et al., 2004b)
Brazil	40	21	-	6	-	(Ternes et al., 1999b)
Canada	19-78	2-26	-	-	-	(Servos et al., 2005a)
Japan	-	5	-	-	-	(Behnisch et al., 2001)
Japan	32-197	13-29	83-255	-	-	(Nakada et al., 2006a)
Japan	15-18	4-23	-	<LOD[e]	-	(Nakada et al., 2006b)
Japan	31-70	19-31	174-731	-	-	(Onda et al., 2003)
Japan	10-57	n.d-21	27-410	-	12-170	(Komori et al., 2004)
Austria	29-670	14-125	23-660	3-70	-	(Clara et al., 2005a)
Switzerland	7.3-75	5-11	-	1-5	-	(Joss et al., 2004a)
Germany	27	15	-	-	-	(Ternes et al., 1999b)

	E1	E2	E3	EE2	E1-3S	Reference
Germany	27-100	10-24	41-580	-	<4-26	(Schlusener & Bester, 2005)
Singapore	27.81	n.d	n.d	n.d	23.06	(Hu et al., 2007)
USA	24-24	7.5-8	-	-	29-39	(Reddy et al., 2005)

[a]0.3 ng l^{-1}, [b]0.1 to 1.8 ng l^{-1}; [c]1.6 ng l^{-1}, [d]1ng l^{-1}, [e]2 ng l^{-1}, LOD: Limit of detection; n.d.: Not detected.

2.1.2. Alkylphenol polyethoxylates

Alkylphenol polyethoxylates (APEOs) are both commercially and industrially important non-ionic surfactants which are incorporated as additives in detergents, pesticide formulations, dispersing agents for wool scouring, hydrogen peroxide bleaching and dyeing processes (Ferguson et al., 2001). The worldwide production of APEOs was estimated at 500,000 tons in 1997 (Renner, 1997). Production of APEOs in Western Europe has declined between 2000 to 2002 from 116,000 to 83,000 tons respectively (Cefic, 2002). The usage of NPEO in the U.S. was approximately 130,600 tons in 2006 (ICIS & Chemical Business Americas, 2007) whilst the consumption of these compounds in the growing Asian economies is expected to rise including an increased demand for nonylphenol (NP) (Dutta, 2008).

Eighty percent of commercial APEOs are comprised of nonylphenol ethoxylates (NPEOs) whereas 20% are octylphenol ethoxylates (OPEOs) isomers (Staples et al., 1999). As a result, complex mixtures of ethoxy homologues and alkyl isomers (European Commission, 2002, 2003; Petrović et al., 2002; Knepper et al., 2003) are discharged to sewage treatment works (STWs) or released directly into the aquatic environment (Gibson et al., 2005; Koh et al., 2005; Langford et al., 2004; Bennie et al., 1997; Blackburn et al., 1999; Ferguson et al., 2001; Naylor et al., 1992b; Tabata et al., 2001).

Some of the reported data on the levels of APs, APECs and APEOs in STWs influents around the world are shown in . The mean concentrations of the nonyl-group metabolites in sewage influents varied widely among various STWs from <LOD to 3285 ng l^{-1} whereas for the octyl-group the varied mean concentration ranged from <LOD to 243 ng l^{-1}. However, after secondary wastewater treatment usually more than 95% of the complex mixtures are degraded to various stable and more persistent metabolites such as short-chained APEOs: mono- to tri-ethoxylates (NP_1EO, NP_2EO and NP_3EO), alkylphenols (APs), carboxylated alkylphenols i.e. carboxyalkylphenol polyethoxycarboxylates (CAPECs) and alkylphenol ethoxycarboxylates (APECs) (Giger et al., 1984). Numerous studies have shown that APEOs metabolites (APs, short-

chained APEOs and APECs) are more toxic than their parent substances and possess the ability to mimic natural hormones by interacting with estrogen receptor (ER) (Field & Reed, 1999; Jobling et al., 1996; Jobling & Sumpter, 1993; Renner, 1997; Routledge & Sumpter, 1996; Soto et al., 1991).

Furthermore, during wastewater chlorination, carboxylated alkylphenoxy ethoxylates (CAPEC) can act potentially as sources of AP, short-chained APEOs and halogenated APECs (such as Br-APECs, or Cl-APECs) which are estrogenic to fish and other aquatic organisms (Kinae et al., 1981b, a; Reinhard, 1982; Ball et al., 1989b). The formation of halogenated derivatives of the alkylphenols and acidic alkylphenols, mostly brominated compounds, were reported at μg l^{-1} levels in wastewater effluent and receiving river water after disinfection with chlorine in the presence of bromide ions in the wastewater treatment plant (Kinae et al., 1981b; Reinhard, 1982; Fujita et al., 2000b; Ventura et al., 1989). The toxicity and estrogenicity of halogenated APECs were found to retain a significant affinity for the estrogen receptors in in-vitro tests and their acute toxicity to Daphnia magna was higher than their non-brominated precursors APEOs and APECs (Maki et al., 1998; García-Reyero et al., 2004). However, the scientific and regulatory concerns have been raised over APEO metabolites (NP, OP, $NP_{1-2}EO$) present in the environment above the threshold necessary to disrupt endocrine function in wildlife and humans. The physiochemical properties of APEOs researched in this study are shown in (Table 2-5) whereas (Table 2-6) includes the molecular properties of APEOs.

The solubility of APEOs depends on the number of polar groups forming the hydrophilic part of the molecule. Lower APEO oligomers (EO<5) usually display lipophilic properties in contrast to the higher oligomers which are hydrophilic (Ahel & Giger, 1993a). The solubilities of $OP_{1-4}EO$ ranged from 8 to 24.5 mg l^{-1} in water, while the solubility of OP is 12.6 mg l^{-1}. NP has a water solubility of 5.43 mg l^{-1}, while $NP_{1-4}EO$ have solubilities ranging between 3.02 and 9.48 mg l^{-1} at 20.5 °C (Ahel & Giger, 1993a). The solubilities of $OP_{1-4}EO$ and OP were significantly greater than those of $NP_{1-4}EO$ and NP, indicating the predominant influence of the hydrophobic chain length on APEOs solubility.

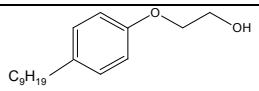
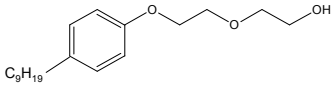
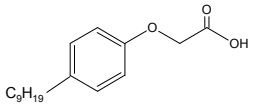
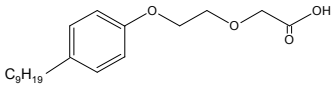
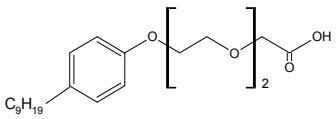
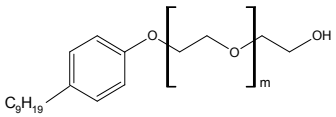
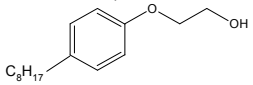
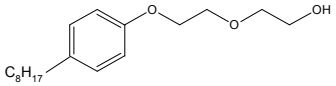
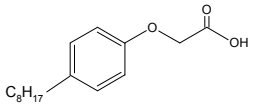
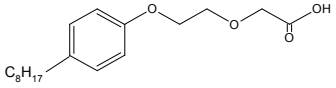
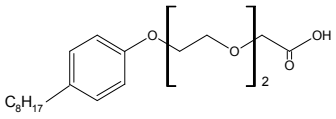
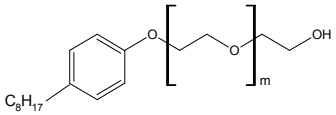
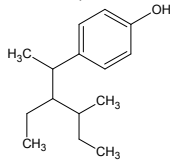
Table 2-5 Physicochemical properties of the investigated alkylphenolics.

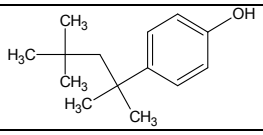
Alkylphenolic compound	Vapour pressure (Pa)	Aqueous solubility (mg l^{-1} at 20°C)	Henry's constant (atm m^3 mol^{-1})	K_{oc} (l kg^{-1})[j]	Log K_{ow}[k]	*pKa*
NP	1.73 x 10^{-5}[i]	5.43[b]	1.23 x 10^{-5}[a]	245,470[c]	4.48[e]	10.7[g]
NP_1EO	1.34 x 10^{-9}[a]	3.02[b]	1.25 x 10^{-6}[a]	288,403[c]	4.17[e]	
NP_2EO	6.86 x 10^{-11}[a]	3.38[b]	2.68 x 10^{-9}[a]	151,356[c]	4.21[e]	
NP_3EO		5.88[b]		74,131[c]	4.20[e]	
NP_4EO		7.65[b]			4.30[a]	
NP_1EC	7.37 x 10^{-8}[a]	0.45[a]	1.41 x 10^{-6}[a]	2496[a]	5.80[i]	
NP_2EC	3.57 x 10^{-10}[a]	0.43[a]	3.01 x 10^{-9}[a]	852[a]	5.53[i]	
OP	3.89 x 10^{-6}[a]	12.6[b]	6.89 x 10^{-6}[a]	151,356[c]; 3500 – 18,000[d]	4.12[e]	~9.9 – 10.9[h]
OP_1EO	8.18 x 10^{-9}[a]	8.0[b]	5.87 x 10^{-7}[a]	1078[a]	4.10[f]	
OP_2EO		13.2[b]			4.00[f]	
OP_3EO		18.4[b]			3.90[f]	
OP_4EO		24.5[b]			3.90[f]	

[a](Koh, 2008), [b](Ahel & Giger, 1993a), [c](Ferguson et al., 2001), [d](Johnson et al., 1998), [e](Ahel & Giger, 1993b), [f]Calculated values using the solubility (Ying et al., 2002b), [g](Deborde et al., 2004), [h](Shiu et al., 1994), [i](Nielsen et al., 2000), [j]Organic carbon sorption constant $K_{oc,}$ [k]Octanol-water partition coefficient K_{ow}

The logarithmic values (Log K_{ow}) for OP, NP and $NP_{1-3}EO$ are lying between 3.90 and 4.48 suggesting that these substances may become associated with organic matter in sediments. However, NPECs are more likely to sorb onto organic matter as presented in (Table 2-5). Calculated vapour pressure for NP and OP (0.0023 mm Hg) are higher compared to the rest of APEOs metabolites (Nielsen et al., 2000). From the physicochemical data, solubility and Log K_{ow} values indicate that OP, NP and $AP_{1-4}EOs$ could easily adsorb onto sediments in aquatic environments.

Table 2-6 Molecular properties of the investigated alkylphenolics.

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	

Alkylphenolic compound	Type of non-ionic surfactant	Molecular formula	Molecular weight (g/mol)	CAS no.	Structure
4-tert-octylphenol (OP)	Alkylphenol	$C_{14}H_{22}O$	206.32	140-66-9	

Data taken from (QMX Laboratories, 2008), NA: Not applicable

Table 2-7 Alkylphenol ethoxylate concentrations (ng l^{-1}) in STWs influents.

Sampling location	AP		Short-chained AP_nEOs		Long-chained AP_nEOs		AP_nECs		Reference
	OP	NP	OPEOs	NPEOs	OPEOs	NPEOs	OPECs	NPECs	
Austria[a]	0.12-0.7	1.3-4.0	(n=1-2) n.d-0.7	(n=1-2) 0.6-7.3	-	-	-	(n=1-2) 0.1-4	(Clara et al., 2005b)
China	-	9.3	-	(n=1-2) 3-28	-	(n=3-23) 0.002-3.1	-	-	(Shao et al., 2003)
Canada	n.d-2	2-23	-	(n=1-2) 3-37	-	(n=3-17) 99-403	(n=1-2) n.d-8	(n=1-2) 2-17	(Lee & Peart, 1998)
Japan[b]	-	-	-	(n=1-3) n.d-938	-	(n=4-18) 5.1-1035	-	(n=1-3) n.d-26	(Fujita et al., 2000a)
Japan	n.d-5	1.3-75	-	(n=1-4) 6-92	-	(n=5) 9.5-810	-	-	(Nasu et al., 2001)
Japan[c]	0.04-0.09	1	-	(n=1) 11	-	-	-	(n=1-2) 0.1-0.1	(Isobe & Takada, 2004)
Italy	-	2-40	-	-	-	(n=1-18) 50-360	-	-	(Di Corcia et al., 1994)
Italy[d]	-	-	-	-	-	(n=3-20) 29-145	-	-	(Di Corcia et al., 2000)
Italy[e]	-	-	(n=2) <0.002-0.08	(n=1-2) <0.002-4	-	(n=9) 1.5-6	-	(n=1-3) 0.12-15	(Loos et al., 2003)
Spain[f]	-	40-343	-	-	-	(n=4-6) n.d-938	-	(total) n.d-80	(Sole et al., 2000)
Spain	-	17-58	-	(n=1-2) 10-150	-	(n=3-15) 244-465	-	(n=1-2) ~3-90	(Gonzalez et al., 2007)
Spain	4	82	-	-	(n=~9) 78	(n=~10) 1850	(n=1-2) 2-9	(n=1-2) 7-13	(Petrovic et al., 2001)
Spain[g]	<0.1-5	1-80	-	-	(n=2-15) 8-84	(n=2-15) <0.05-2	(n=1) <0.05	(n=1) 1-65	(Petrovic et al., 2002)
Greece[j]	-	<0.03-1.04	-	(n=1-2) 0.2-21	-	-	-	-	(Stasinakis et al., 2008)
Belgium	-	-	-	-	-	25	-	-	(Cohen et al., 2001)
Denmark	-	4.5-9.6	-	(n=2) 55-181	-	-	-	-	(Fauser et al., 2003)
Switzerland[h]	-	-	-	-		(n=1-18) 840-2250	-	-	(Ahel & Giger, 1985)
Switzerland[i]	-	110-430	-	(n=1-2) 310-840	-	(n=3-20) 1310-3220	-	(n=1-2) 80-270 -	(Ahel et al., 1994c)
USA	-	-	-	-	-	(n=1-18) 2400	-	-	(Naylor et al., 1992a)
USA	0.4	4	(n=1-2) 0.3-0.4	(n=1-2) 8-9	(n=3-5) 1	(n=3-16) 4-46	(n=1) <1	(n=1-2) <1-1	(Loyo-Rosales et al., 2007)

[a]3 STWs; [b]40 STWs; [c]2 STW; [d]2 STWs (textile and municipal sewage); [e]3 STWs; [f]4 STWs; [g]4 STWs; [h]5 STWs; [i]11 STWs; [j]6 STWs; n.d: Not detected.

2.2. Legislation

Extensive scientific research has evidently indicated that the majority of EU sewage treatment works (STWs) pose significant sources of alkylphenol ethoxylate (APEOs) metabolites ($AP_{1\text{-}2}EO$, APECs, APs) and steroid estrogens (ESTs) for the aquatic and terrestrial environments (European Commission, 1999; Kirk et al., 2002; Rodgers-Gray et al., 2000; Svenson et al., 2003; Espejo et al., 2002; Snyder et al., 1999; Knudsen et al., 1997; Solé et al., 2000). These sources arise from effluent discharges and agricultural sewage sludge recycling applications Figure 1.1 (Laturnus et al., 2007; Cefic, 2002; Diniz et al., 2005; Jobling et al., 1998; Petrović & Barceló, 2004; Spengler et al., 2001). As a result of the widespread phenomena of EDCs in particular, E1, E2, EE2 and NP (Cargouët et al., 2004; Johnson & Darton, 2003; Routledge & Sumpter, 1996; White et al., 1994), a number of voluntary, PARCOM Recommendation (92/8/2000) (OSPAR Convention, 2006) and legal European actions were established as prevention actions against EDCs phenomena. These actions include: the Water Framework Directive of the European Community (European Commission, 2000b), the Urban Waste Water Treatment Directive 91/271/EC (European Commission, 2000c), the OSPAR Convention for the protection of North-East Atlantic (OSPAR Commission, 2006), the Council Directive (2003/53/EC) that regulates the uses of APEOs (European Commission, 2000a) and the (3rd Draft) of the Council Directive (86/278/EEC) which has proposed the concentration limits of 50mg NPEOs kg dw^{-1} (NP, $NP_{1+2}EO$) in sludge (European Commission, 1986). The legislation in force i.e. Council Directive (2003/53/EC), will most likely cause alteration of APEO loadings in STWs and possible changes concerning the removal efficiencies of these EDCs.

2.3. Overview of STWs

Sewage treatment works incorporate physical, chemical and biological processes to treat and remove a wide spectrum of contaminants from wastewater following domestic and industrial use. The primary objective of STWs is to produce treated effluent suitable for discharge and reuse into the environment, whilst sludge is rendered suitable for appropriate disposal or reuse (Birkett, 2003). During the STWs treatment train different processes provide various levels of treatments known as preliminary (gross solids such as large objects and grit are removed), primary (solids sedimentation), secondary (biological processes to remove organic matter) and tertiary treatment (removal of residual suspended solids and nutrient removal) (Lester, 1996). Despite the fact that conventional sewage treatment was developed to remove carbon, nitrogen and to a lesser extent phosphorous (Birkett, 2003), EDCs removal does occur, by either adsorption to solids or by microbial degradation (Langford, 2005; Ahel et al., 1994a). Biodegradation is considered the most important removal mechanism of EDCs within STWs (Andersen et al., 2003), typically ranging from 60-95% for total steroid estrogens (Johnson et al., 2000; Baronti et al., 2000) and >95% for the parent nonylphenol ethoxylates (Giger et al., 1984). Although biodegradation of nonylphenol ethoxylates occurs, more recalcitrant and estrogenic metabolic products such as nonylphenol (Giger et al., 1984) and NP_1EC who is toxic to both marine and freshwater species (Jobling & Sumpter, 1993; Purdom et al., 1994b; Comber et al., 1993; McLeese et al., 1981) are formed. Research was focused on the secondary treatment (activated sludge) due to the complex biodegradation patterns observed and because it was formerly believed that aerobic treatment comprised the dominant removal mechanism of non-ionic nonylphenol ethoxylate surfactants (Stephanou & Giger, 1982; Reinhard, 1982; Giger et al., 1981). However, anaerobic treatment for the removal of nonylphenol ethoxylates received particular attention after the finding that anaerobically digested sewage sludges contained extraordinary high concentrations of nonylphenol (Giger et al., 1984) and carboxylated nonylphenolic metabolites (Ball et al., 1989a) which once was believed to be exclusively formed in the aerobic environments. Scientific research was since received interest to see whether anaerobic and aerobic degradation pathways were

different since sludge recycling practices onto land could prove to be potential sources of EDCs contamination. Ecotoxicological and regulatory issues were aided the research towards the fate of EDCs in anaerobic conditions.

2.3.1. Primary sedimentation

2.3.1.1. Steroid estrogens

The mean concentrations of steroid estrogens in sewage influents vary and mean concentrations range from <LOD to 528 ng l^{-1} Table 2-4. Nevertheless, despite the small amount of organic solid removal during preliminary treatment, no removal of EDCs (steroid estrogens and nonylphenol ethoxylates) has been observed during this process (Ternes et al., 1999b). Deconjugation of the steroid estrogens occurred in the sewers and this was apparent from the detection of the unconjugated steroidal estrogens E1, E2, EE2 and E3 in the settled sewage. During primary sedimentation, estrogen removal is achieved primarily by adsorption onto fats, oils and greases. The degree of EDCs removal is influenced by the lipophilicity of the compound, the suspended solids concentration and their settling rate, the retention time and loading rate (Langford & Lester, 2003). Insignificant removal of E1 and E2 was observed at STWs in Canada where only primary sedimentation was employed (Servos et al., 2005b). Increase of E1 was reported at a Norwegian STWs where primary sedimentation and phosphate removal was employed (Johnson et al., 2005). These results were in line with a UK where during primary sedimentation no significant removal occurred for E1 and E2, however removal did occur during biological secondary treatment i.e. biological treatment process (Jiang et al., 2005). Although E1-3S is not an endocrine disrupter it has been suggested that during primary sedimentation the release of sulphate which is used as terminal electron acceptor (D'Ascenzo et al., 2003a; Van Eldere et al., 1988) yields E1. Due to the relatively polar and hydrophilic nature of steroid estrogens, suggestive of low adsorption potential, concentrations in the solid phase of primary sludge i.e. (µg kg^{-1} dw) are not available.

2.3.1.2. Alkylphenolic compounds

Some of the reported concentrations of APs, APECs and APEOs in primary sludge around the world are shown in Table 2-8. Concentrations vary widely among various STWs. Nonylphenol (NP) in primary sludge ranges from 0.04-470 mg kg^{-1} dw whilst $NP_{1-2}EOs$ are less varied (0.42-100 mg kg^{-1} dw) in the same sludge matrix. Less variation in primary sludge is observed for NPECs (0.04-69 mg kg^{-1} dw). The range of the nonyl-group metabolites in primary sludge is 0.42-470 mg kg^{-1} dw. The reported range for the octyl-group in primary sludge is less than that of the nonylphenolics group as is shown in Table 2-8.

Partial biodegradation (aerobic/anaerobic transformation) has been observed for alkylphenol ethoxylates in crude (Ahel et al., 1994a; Lee et al., 1998) and settled sewage (Koh, 2008). Three to 9.6% of dissolved carbon was attributed to nonylphenol ethoxylates (Ahel et al., 1994a). These observations were attributed to the septicity of the sewerage system (Ahel et al., 1994a; Koh, 2008). Short-chained $AP_{1-3}EOs$, $AP_{1-3}ECs$ and alkylphenols (NP and OP) were present in crude sewage which confirms that biodegradation/biotransformation may occur in sewerage as these metabolites are not present in commercial formulations (Ahel et al., 1994a; Lee et al., 1998). While long-chained nonylphenol ethoxylates (NP_nEOs, n>4) remained constant in the influent, reduction of the lipophilic nonylphenolic metabolites (NP and $NP_{1-2}EO$) and increase (3%) of NPECs was observed during primary sedimentation (Giger et al., 1987) which suggested that lipophilic nonylphenol ethoxylates were removed by adsorption during this treatment stage. The presence of long-chained $NP_{4-8}EOs$, short-chained $NP_{1-3}EOs$ and small amounts of short-chained NPECs (<5% of total NPEOs) in primary effluents was observed by (Fujita et al., 2000b) in 40 full-scale STWs; however, no NP was detected possibly due to adsorption. According to (Bennie et al., 1998), short hydraulic retention time (HRT) of primary sedimentation inadequately removes $NP_{3-20}EOs$ (82%), $NP_{1-2}EOs$ (12%), $NP_{1-2}ECs$ (3%) and NP (3%).

Table 2-8 Concentrations of alkylphenol ethoxylate metabolites in raw (primary), activated sludge (secondary) and anaerobically digested sludges

Sampling location	Compound	Sludge matrix	Concentration (mg kg^{-1} dw)	Reference
Germany	NP	Primary	3.7	(Bolz et al., 2001)
Canada	NP	Primary	137–470	(Lee & Peart, 1995)
Germany	NP	Primary	4.6	(Jobst, 1998)
Greece	NP	Primary	0.04-0.45	(Stasinakis et al., 2008)
Greece	NP_1EO	Primary	1-41	(Stasinakis et al., 2008)
Greece	NP_2EO	Primary	1-25	(Stasinakis et al., 2008)
-	NP	Primary	0.2-0.3	Current study[a]
-	$NP_{1-2}EOs$	Primary	2-15	Current study[a]
-	$NP_{3-12}EOs$	Primary	1.3-1.5	Current study[a]
-	$NP_{1-3}ECs$	Primary	0.04-26.5	Current study[a]
Italy	OP	Primary	14	(Bruno et al., 2002)
Italy	NP	Primary	242	(Bruno et al., 2002)
Italy	NP_1EC	Primary	6.8	(Bruno et al., 2002)
Italy	NP_2EC	Primary	69	(Bruno et al., 2002)
Italy	NP_3EC	Primary	11	(Bruno et al., 2002)
Italy	NP_4EC	Primary	3	(Bruno et al., 2002)
Italy	NP_2EO	Primary	100	(Bruno et al., 2002)
Italy	$NP_{3-6}EOs$	Primary	7.5	(Bruno et al., 2002)
Denmark	NP	Primary	12	(Fauser et al., 2003)
Denmark	NP_2EO	Primary	39	(Fauser et al., 2003)
Belgium	NPEOs	Primary	44	(Cohen et al., 2001)
Spain	NP	Primary	4.3-49	(Aparicio et al., 2009)
Spain	NP_1EO	Primary	$<LOD^{+}$-72	(Aparicio et al., 2009)
Spain	NP_2EO	Primary	$<LOD^{++}$-47	(Aparicio et al., 2009)
Spain	NP	Secondary	4.5-1.8	(Aparicio et al., 2009)
Spain	NP_1EO	Secondary	$<LOD^{+}$-26	(Aparicio et al., 2009)
Spain	NP_2EO	Secondary	$<LOD^{++}$	(Aparicio et al., 2009)
-	NP	Mixed[b]	0.1-0.2	Current study[a]
-	$NP_{1-2}EOs$	Mixed[b]	1.7-90	Current study[a]
-	$NP_{3-12}EOs$	Mixed[b]	0.7	Current study[a]
-	$NP_{1-3}ECs$	Mixed[b]	0.08-241.5	Current study[a]
Belgium	NPEOs	Digested	$<LOD^{*}$	(Cohen et al., 2001)
-	OP	Digested	17	(Bruno et al., 2002)[a]
-	NP	Digested	308	(Bruno et al., 2002)[a]
-	NP_1EC	Digested	1.9	(Bruno et al., 2002)[a]
-	NP_2EC	Digested	18	(Bruno et al., 2002)[a]
-	NP_3EC	Digested	4.2	(Bruno et al., 2002)[a]
-	NP_4EC	Digested	1.1	(Bruno et al., 2002)[a]
-	NP_2EO	Digested	40	(Bruno et al., 2002)[a]
-	$NP_{3-6}EOs$	Digested	3.9	(Bruno et al., 2002)[a]
Taiwan	NP	Digested	250	(Lin et al., 1999)

Sampling location	Compound	Sludge matrix	Concentration (mg kg^{-1} dw)	Reference
USA	NP	Digested	13 and 24	(Keller et al., 2003)
Spain	OP	Digested	7.5	(Petrović et al., 2001)
Spain	OPEOs	Digested	12	(Petrović et al., 2001)
Switzerland	NP	Digested	78	(Brunner et al., 1988)
Switzerland	NP	Digested	450–2530	(Giger et al., 1984)
UK	NP	Digested	326 and 638	(Sweetman, 1994)
Spain	NP	Digested	172	(Petrović et al., 2001)
Germany	NP	Digested	80–120	(Schnaak et al., 1997)
Canada	NP_1EO	Digested	51–304	(Lee et al., 1997)
Canada	NP_2EO	Digested	4–118	(Lee et al., 1997)
Spain	NP_nEOs	Digested	133	(Petrović et al., 2001)
Canada	NP_nEOs	Digested	9–169	(Lee et al., 1997)
Canada	NP_1EC	Digested	<0.5–25	(Lee et al., 1997)
Canada	NP_2EC	Digested	<0.5–38	(Lee et al., 1997)
Canada	NP_1ECs	Digested	19	(Field & Reed, 1999)
Canada	NP_2ECs	Digested	83	(Field & Reed, 1999)
Canada	NP_3ECs	Digested	11	(Field & Reed, 1999)
-	$NP_{>5}EC$	Digested	n.r.	(Schröder, 2001)[a]
Spain	NP	Digested	6.5-323	(Aparicio et al., 2009)
Spain	NP_1EO	Digested	1.3-78	(Aparicio et al., 2009)
Spain	NP_2EO	Digested	$<LOD^{++}$-12	(Aparicio et al., 2009)

[a]lab-scale study; [b]mixed (60:40 v/v primary: SAS); *LOD (0.1 mg Kg^{-1}); $^{+}$LOD (0.75 mg kg^{-1} dw); $^{++}$LOD (0.42 mg kg^{-1} dw); n.r. detected but concentrations were not reported

2.3.2. Secondary biological treatment

It thought that secondary biological treatment (activated sludge) is the principal process to most if not all estrogenic activity (Pickering & Sumpter, 2003; Carballa et al., 2004a; Andersen et al., 2003). Biodegradation and biotransformation are believed to have significant roles in the removal of micropollutants, as some microorganisms can utilise such compounds as sole carbon sources for metabolism (Corvini et al., 2006). It has been reported that STWs employed Biological nutrient removal (BNR) exhibit the most significant overall estrogen removals (Tan et al., 2007; Dorabawila & Gupta, 2005).

2.3.2.1. Steroid estrogens

Conventional activated sludge is commonly used to treat domestic sewage however; estrogens are still detected in activated sludge effluents. Lab-scale batch experiments indicated partial E1 and EE2 removal (Johnson & Sumpter, 2001b). Degradation of steroid estrogens requires consortia of microorganisms (Vader et al., 2000) and therefore the reported removals vary from one STWs to another since the process parameters within STWs vary considerably. Reported removals for E2, E3 and EE2 from real-scale STWs were greater than 85%, whereas E1 removal was more variable (Johnson & Sumpter, 2001b). Removals from 6 STWs in Rome for E1, E2, E3 and EE2 were 61%, 85%, 95% and 85%, respectively (Komori et al., 2004). The significant removal of EE2 has only been observed under aerobic conditions (Joss et al., 2004b; Andersen et al., 2003; Koh et al., 2008a) and to a lesser extent in trickling filters (Koh, 2008). Recently, a pathway for the degradation of EE2 by using ozonation in aqueous solution was proposed by (Zhang et al., 2006). However, further studies would be required to identify the complete mineralization of these recalcitrant estrogenic compounds in the environment. A study by (Andersen et al., 2003) indicated that BNR with sludge recirculation and alternating redox conditions could appreciably eliminate natural and synthetic estrogens. It was observed that natural estrogens were degraded to a large extent biologically in the denitrifying and aerated nitrifying tanks of the BNR system, whereas EE2 was only degraded in the nitrifying tank (Andersen et al., 2003). This is in agreement with (Vader et al., 2000) who indicated a correlation between the

nitrifying activity and EE2 degradation capability. The significance of alternating redox conditions within BNR systems was investigated by (Joss et al., 2004b) in which it was shown that the maximum removal rate occurred under anaerobic conditions without nitrate when E1 was reduced to E2. E1 degraded under all redox conditions and increased by factors of five and three in the transition between anoxic to aerobic and anaerobic to anoxic respectively, whereas the oxidation of E2 was higher at lower redox conditions (Joss et al., 2004b). However, the removal of EE2 occurred at a significant rate only under aerobic conditions (Joss et al., 2004b).

The conversion of E2 to E1 has been reported in aerobic lab-scale studies where approximately two-thirds of spiked E2 were quantiitatively oxidised to E1 (Lee & Liu, 2002). Co-metabolic reactions using enzyme(s) already present in the organisms obtained from activated sludge have been shown to have the ability to convert E2 to E1 in a rapid and stoichiometric manner (Yu et al., 2007). The authors proposed 3 possible degradation pathways, with varying degradation conversion rates.

In a study which involved a BNR system estrogenic activity was removed >95% at the effluent whereas the estrogenicity was below the detection limits at the STWs effluent (Leusch et al., 2005). In two Swedish STWs that incorporate nitrogen removal with activated sludge, the removal of steroid estrogens (based on EEq ng l^{-1}) from the effluent was >97% (Svenson et al., 2003). Removal of up to 19.5 ng l^{-1} was achieved in an activated sludge with subsequent anoxic stages for nitrification due to the prolonged duration of the biological treatment. However, in a survey carried out on the distribution of E2 and E1 in 18 selected municipal STWs effluents across Canada, no statistical correlation observed between the HRT or (solid retention time) SRT and the apparent steroid removals, even though plants or lagoons with high SRT exhibited high steroid hormone removal efficiencies. Furthermore, it was observed that nitrifying plants exhibited greater removals than those without nitrifying systems (Servos et al., 2005b). The presence of alternating redox conditions within a BNR system allows for most of the removal processes i.e. anaerobic/anoxic/aerobic biodegradation and adsorption to occur. A high sludge age is usually required to achieve nitrification and nutrient removal because the autotrophic bacteria involved grow very slowly (Metcalf and Eddy,

2003). Increased removal of steroid estrogens with increased SRT has been observed by (Andersen et al., 2003; Ternes et al., 1999b; Holbrook et al., 2002).

Little research on E3 degradation is currently available in the literature but it was postulated that E3 and E1 metabolism could have similar degradation pathways (Koh, 2008).

A retention time of at least 10 to 12.5 days has been suggested as the minimum period required for the growth of organisms that decompose E2 and E1 (Saino et al., 2004). The influence of increased SRT is illustrated by a German STWs which has been upgraded from a BOD removal plant to a BNR plant, with substantial higher SRT, increasing from <4 days to 11 – 13 days. Batch experiments with sludge from the old plant did not show any reduction of EE2 (Ternes et al., 1999a) while at the increased SRT a reduction of around 90% was established in the full scale plant, which indicates the growth of microorganisms capable of degrading EE2 (Andersen et al., 2003). Further evidence of the effects of SRT on natural estrogens elimination from the wastewater is reported by (Ternes et al., 1999b; Holbrook et al., 2002). At $SRT_{10°C}$ >10 days, almost complete estrogen (E1, E2 and E3) removals were achieved with critical $SRT_{10°C}$ between 5 and 10 days, however, no critical $SRT_{10°C}$ could be established for EE2. Solids retention time affects the microbial population and its growth rates influencing in that way the structural and physical nature of the flocs, which in turn affects their hydrophilic or hydrophobic properties (Metcalf and Eddy, 2003). Since flocs are largely comprised of polysaccharide and protein coatings (Metcalf and Eddy, 2003) their sorptive affinities could be significant for certain steroid estrogens which in turn will accumulate in the resulting sludges. The nutrient status of the flocs, could also influence their hydrophilic-hydrophobic balance (Jorand et al., 1998). From the literature studies appears that SRT who dictates the contact time between pollutants and microorganisms, when is adequate allows the establishment of appropriate bacteria, presumably the slow growing bacteria who result in a diversified and acclimated microbial consortium that is capable for steroid metabolism.

A general pathway of steroid degradation has been demonstrated by (Sih & Wang, 1963) in which various environmental microorganisms of the genera *Nocardia,*

Pseudomonas, Mycobacterium and *Arthrobacter* were capable to cleave and utilize the steroid nucleus as a sole carbon source (Talalay, 1957). A novel mode of degradation of steroids was proposed which involved the cleavage of the A-ring prior to the B-ring (Coombre et al., 1966) (Figure 2-1). Recently a study by (Yi & Harper Jr, 2007) has also demonstrated the cleavage of A-ring prior to B/C-ring modification required for the biotransformation of EE2. There are also reports of other genera of wastewater or environmental microorganisms able to degrade these compounds (Yoshimoto et al., 2004; Shi et al., 2002).

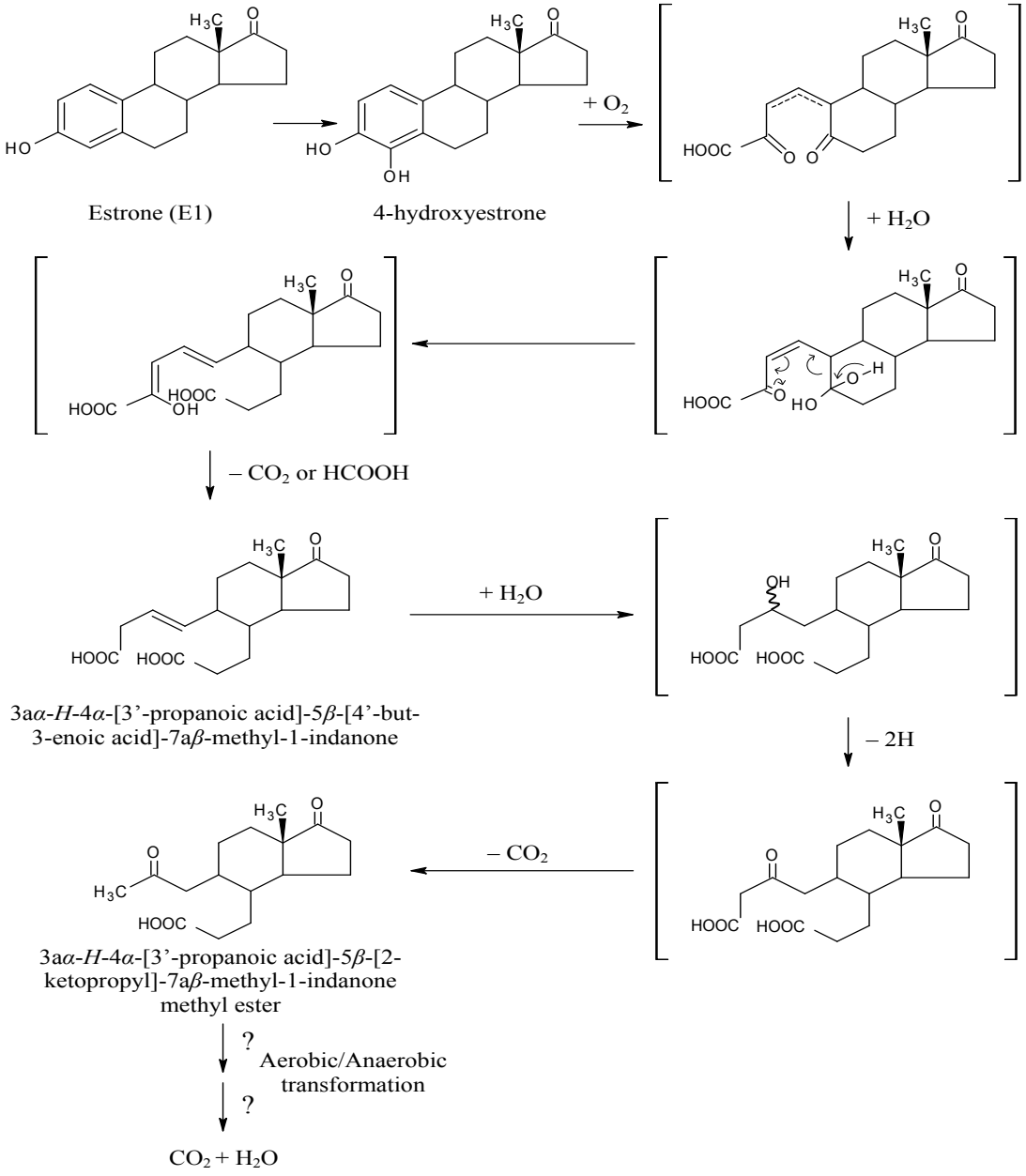


Figure 2-1 Scheme for the aerobic metabolism of estrone. Adapted from (Koh, 2008).

2.3.2.2. Alkylphenolic compounds

Despite the fact that APEO degradation has been studied for 45 years and their mineralization pathway has been extensively reviewed (Montgomery-Brown & Reinhard, 2003), mechanisms for certain APEO metabolites remain unknown and inconsistent (Chiu et al., 2010). Under aerobic conditions such as the activated sludge process the ethoxylate chain of higher ethoxylate APEOs is shortened until persistent short-chained $AP_{1-2}EOs$ are formed (Di Corcia et al., 1998). This breakdown proceeds by the stepwise removal mechanism of one ethylene glycol unit (Figure 2-2) (Montgomery-Brown & Reinhard, 2003; White et al., 1994). It was reported that the stepwise shortening of ethylene glycols of nonylphenol ethoxylates (NPEOs) was carried out by *Pseudomonas* and *Sphingomonas* spp (Takeo et al., 2006; Tanghe et al., 2000; Fujii et al., 2000; Fujii et al., 2001; Maki et al., 1994; John & White, 1998). Further degradation of these metabolites during activated sludge treatment occurs via the oxidative shortening of polyethoxylate chain (i.e. APECs) (Di Corcia et al., 1998; Di Corcia et al., 1994) thus suggesting oxidative mechanisms (Chiu et al., 2010; Ahel et al., 1994b). Complete de-ethoxylation with formation of APs has only been observed under anaerobic conditions (Giger et al., 1984). The abundance of lipophilic alkylphenolic isomers and the presence of the highly branched alkyl group on the phenolic ring restrict the ultimate biodegradation. Nonetheless the existence of rich consortium of microorganisms under variable redox conditions within activated sludge could enhance their degradability. However, the formation of dicarboxylated metabolites has only been reported during biological activated sludge treatment and in the final effluents (Di Corcia et al., 1998). Reports on fate of APEOs and their metabolites during BNR systems are scarce. A study by (Johnson et al., 2005) that investigated 14 municipal STWs indicated the existence of a correlation between the BNR configuration of some of these plants and NP removals, however NP was only considered at the effluents. These findings were consistent with those obtained by (Drewes et al., 2005) which also showed declining trends of NP with increasing SRT with BNR systems. High SRT, circa 10 days, increases the removal of APEO and their metabolites (Clara et al., 2005a; Kreuzinger et al., 2004). These authors investigated the effect of SRT on APEO

removal efficiencies in various bench scale and real scale STWs utilising different treatment processes and variable SRTs (Kreuzinger et al., 2004; Clara et al., 2005a). Their studies showed the correlation of SRT on the removal of total nonylphenolic compounds (molar sum of NP, $NP_{1-3}EO$, and $NP_{1-3}EC$). However, due to the lack or raw influent data it was not possible to assess the effect of SRT with respect to specific NP or NPEO loadings.

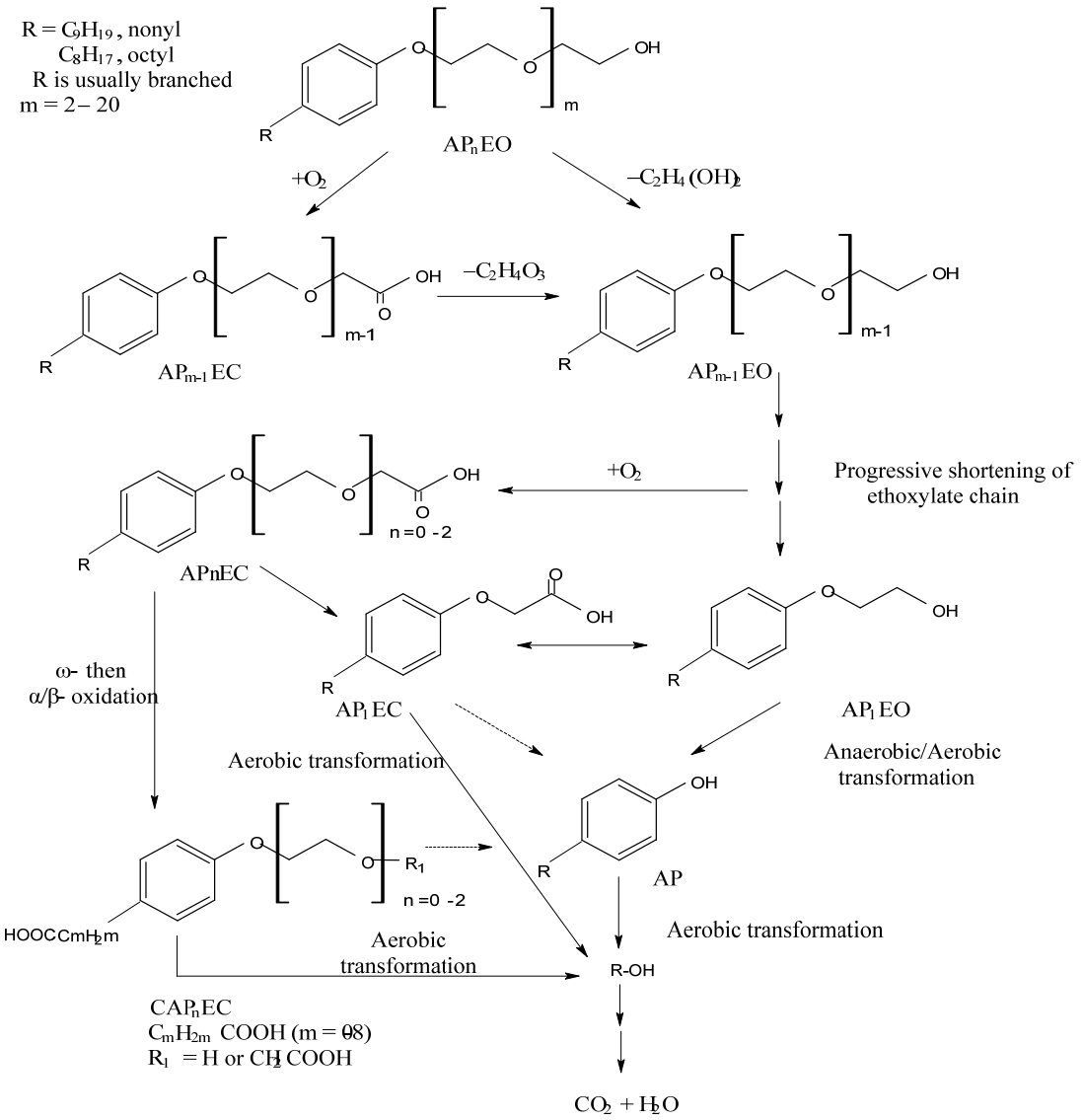


Figure 2-2 Aerobic degradation pathways of APEOs. Adapted with modifications from (Renner, 1997).

2.4. Anaerobic digestion processes

The major anthropogenic organic contaminants including EDCs are contained within the anaerobically digested sludge (Kouloumbos et al., 2008a, b; Lee et al., 2004; Birkett, 2003; Petrović & Barceló, 2000; Brunner et al., 1988). Post anaerobic treatment, the most beneficial usage of sewage sludge is its application as agricultural fertilizer (Smith, 1996) in comparison to landfill or incineration disposal practices, since plant nutrients and organic matter are recycled in that way into soil (Laturnus et al., 2007). Sewage sludge use in agriculture is encouraged through the Directive 91/271/EEC, (European Commission, 1999) as the most beneficial and sustainable rout, therefore potential risks may result for ecosystems through the use of sludge contaminated with EDCs (Harrison et al., 2006; Maguire, 1999). Besides, the uptake of EDCs by indigenous organisms and plants (Günther & Pestemer, 1990), leachate on the other hand and run-off can also be potential sources to aquatic environments, including groundwater (Kouloumbos et al., 2008b; Birkett, 2003). Stricter regulations of wastewater industry (CEC, 1991), for example increased level of treatment and the use of newer technologies will most likely increase the quantity of sludge production (European Commission, 1999) and possibly the sludge-associated EDCs i.e. lipophilic species.

Although, anaerobic digestion (AD) is the most sustainable and preferred process of sewage sludge stabilisation (compared to lime stabilisation or composting), studies on ESTs and APEOs in sludge under anaerobic conditions are limited and somehow inconsistent. These inconsistencies are attributed to number of factors: The mode of anaerobic digestion (AD) (i.e. batch, continuous) and the scale of the process (i.e. full or lab scale), since lab scale studies do not necessarily reflect the realistic environmental conditions hence, result in overestimation of the biodegradability potencies of organisms. Full scale AD periodically exposed to diverge influent flows and various engineering failures, resulting in variations of operating parameters, which ultimately affect their performance. In addition, steady state conditions are therefore difficult to be achieved (de La Rubia et al., 2006) as opposed to controlled lab scale systems. The matrix subjected to digestion is also important since sludges from primary

sedimentation tanks, secondary treatments (activated sludge, BNR), or combinations of those two, all have different physicochemical characteristics (Metcalf and Eddy, 2003) compared to i.e. synthetic sewage and therefore may differ significantly in biodegradability. The type of sludge treatment prior to digestion, typically referred as sludge disintegration treatments including physical, chemical, thermal, enzyme treatments or freezing and thawing (Chu et al., 1999) that aim to increase sludge biodegradability (Kampas et al., 2007b, a; Muller, 2000) would also have significant impacts to EDC digestibility and their biotransformation. EDC loadings, digestion's operating conditions (i.e. temperature, pH, SRT, alkalinity), type of anaerobic inoculants and extent of acclimated inoculums used would also significantly account towards the fate of EDCs under anaerobic conditions.

2.4.1. Steroid estrogens

Reports on the fate of estrogens under strict anaerobic conditions are limited. The reduction of E1 to E2 has been reported to occur in anaerobic conditions, without nitrate (Joss et al., 2004b). The authors hypothesised that electron acceptors other than nitrates i.e. Fe^{3+} and organic compounds present in sludge may have accounted as possible electron acceptors for E2 oxidation under anaerobic conditions (Joss et al., 2004b). In their study, transitional redox conditions, anaerobic/anoxic as well as anoxic/aerobic, increased E1 degradation by a factor between three and five respectively intensifying the strong influence of redox in E1 elimination (Joss et al., 2004b). It was reported that the extent of the biological reduction of E1 to E2 under anaerobic conditions depends on the type of inoculum (de Mes et al., 2008). This finding has serious implications since E2 is an estrogen with great concern. However, (Andersen et al., 2003) measured similar inlet (primary tanks) and outlet loads (anaerobic digestate) from a real scale STWs of E1 and E2 and concluded that under methanogenic conditions (33^{o}C , 20 days retention time, 30-40% TS reduction) estrogens are not degraded considerably. In contrary to the findings of (Joss et al., 2004b; de Mes et al., 2007, 2008), in a study involved river sediment it was reported that inter-conversion of E2 to E1 occurs under anaerobic conditions (Czajka & Londry, 2006). However, (Matsui et al., 2000) observed that the aqueous phase of anaerobically digested sludge contained higher levels of E2 than those entering the plant, suggesting the reduction of E1 to E2.

During a lab scale batch study, (Pholchan et al., 2008) reported that alternating microbial populations under anaerobic/aerobic/anoxic conditions do not necessarily affect E2 removal. The authors observed that sorter than 5.7 days SRT in batch reactors E2 removal was adversely affected. Nevertheless, removals of E1, E2 and EE2 accounted circa 60%, 90% and 36% respectively and the removal of EE2 was attributed to sorption considering its high K_{oc} value (Table 2-2). Similar EE2 removals 38.5% were reported by (Esperanza et al., 2007) but the exact anaerobic conditions were not specified in their report. In contrast, no EE2 reduction was observed under anaerobic batch experiments (28^{o}C) by using sludge or river sediment inoculated with seed from a real scale STWs during 3 years of anaerobic digestion (Czajka & Londry, 2006). During

a continuous mixed sludge (70:30v/v primary and secondary sludge) digestion study and after sludge adaptation, similar removals circa 85%, for both, E1+E2 combined and EE2 under mesophilic and thermophilic anaerobic conditions were reported by (Carballa et al., 2006; Carballa et al., 2007). The authors reported that chemically (lime) or thermally (60 min. at 130^{o}C) pre-treated sludges, SRT and digestion temperature had no significant influence on estrogen removals.

Strictly anaerobic desulphating strains isolated from human excreta have shown their ability of cleaving sulphate conjugates of E1 (E1-3S) (Johnson & Williams, 2004) as well as the sulphate conjugate of E2. This ability is believed to be associated with the use of sulphate as a terminal electron acceptor (Van Eldere et al., 1988). However, little research has been performed for the more persistent sulphate conjugates (D'Ascenzo et al., 2003a) in contrast to the glucuronide conjugates under anaerobic conditions. Desulphating activity of the sulphate conjugates is due to the fact that the principal organisms responsible are obligate anaerobes (Van Eldere et al., 1988). Despite the relative hydrophobicity that is associated with some steroid estrogens indicating the potential for accumulation in sludge matrices (primary and secondary) researchers do not report the quantities in the solid phase (μg kg^{-1} dw).

2.4.2. Alkylphenolic compounds

Concentration ranges for NP in digested sludges varies from 6.5-2530 mg kg^{-1} dw whilst the range for $NP_{1-2}EOs$ is 0.75-304 mg kg^{-1} dw which is similar to that of NPECs i.e. 0.08-242 mg kg^{-1} dw. In anaerobic as well as aerobic conditions, the ethoxylate chain of higher ethoxylate APEOs is shortened until persistent short-chained $AP_{1-2}EOs$ are formed Figure 2-4. This breakdown proceeds by the stepwise removal mechanism of one ethylene glycol unit (Figure 2-4) (Chiu et al., 2010; Montgomery-Brown & Reinhard, 2003), in the similar way as the anaerobic biodegradation of alcohol polyethoxylates, proposed by (Wagener & Schink, 1988). 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 & 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.

Although carboxylated APEOs (APECs) believed to be exclusive aerobic intermediates (Stephanou & Giger, 1982; Di Corcia et al., 1994), the formation of short chained OPECs and their persistence over 190 days during the mesophilic anaerobic digestion of mixed sludge, (a mixture of primary and waste activated sludge from a municipal STWs), was firstly reported by (Ball et al., 1989b). Substantial oxidation of APEOs resulted in the formation of APECs in mesophilic anaerobically digested sludges obtained from real scale STWs was also observed by (Field & Reed, 1999). Reported concentrations of $NP_{1-4}EC$ in anaerobically digested sludges from real scale STWs were in the range 27–113 mg kg^{-1}. Nonylphenoxy ethoxy carboxylate (NP_2ECs) was the most abundant oligomer, and with *ortho*-to-*para* isomer ratios ≥ 1, which indicated the depletion of *para* NPEC isomers relative to *ortho* isomers during anaerobic sludge treatment. By contrast, sludge that had not undergone anaerobic treatment contained only *para* isomers, which implied specific isomeric degradation (Chiu et al., 2010). The presence of NPECs in lab-scale anaerobic reactors was observed on the 9th day of the commencement of the experiment (Schröder, 2001) indicating that even under anaerobic conditions NPEC formation could be prompt. During anoxic batch lab-scale studies of sediment slurry (Ferguson & Brownawell, 2003) reported the presence of carboxylated APEOs. In another study, (Minamiyama et al., 2006) investigated NPEO degradation in lab-scale mesophilic anaerobic digesters (28days SRT) in which NP_2EC showed the highest resistance (20 days) in degradation. It has been suggested that concentrations of $NP_{1-3}EO$>NPEC indicate that the predominant degradation route has been under anaerobic conditions, while if $NP_{1-3}EO$<NPEC then this would indicate that aerobic degradation is predominant (Tarrant et al., 2005; Petrović et al., 2001; Petrović & Barceló, 2001; Barber et al., 2000). According to the current literature, long-chained carboxylated nonylphenol ethoxylates i.e. $NP_{>4}EC$ (i.e. in (Bruno et al., 2002), alkyl

chain carboxylated APEOs i.e. CAPEs or dicarboxylic APEO metabolites i.e. CAPECs have not been reported under anaerobic conditions. This is presumably attributed to the lack of available analytical methods for anaerobically digested sludges and to a lesser extent of the absence of these species under anaerobic conditions.

According to the current reports, it appears that formation of APECs is not exclusively oxygen dependant but it also depends on the predominating microbial consortia and substrate and it seems of being irrespective of aerobic or anaerobic conditions. Since under anaerobic conditions a food chain (hydrolysis, acidogenesis, acetogenesis, methanogenesis) is required to degrade aromatic macromolecules (i.e. APEOs) to oligomers, formation of NPECs could occur co-metabolically as it has been suggested by (Hayashi et al., 2005) for aerobic environments.

Under relatively high redox potential (-100 – -50 mV), sulphate reduction is favoured over methanogenic bacteria (Gerardi, 2003) because sulphate reducers have higher affinity for hydrogen or other reducing equivalents (Omil et al., 1998)**.** Although the reduced equivalents of APEOs would require electron acceptors such as SO_4^{-2}, Fe^{3+}, Mn^{4+}, CO_2 for microbial metabolism, mixed anaerobic cultures would be required for ultimate APEOs reduction since methanogens can only utilise simple low molecular organic molecules such as acetate or methylamines (Whitman et al, 1992 and Winter, 1984) the products of hydrolysis and fermentation.

It is well documented that under anaerobic conditions, alkylphenols APs (NP and OP) the degradation products of APEOs, are major metabolites (Soares et al., 2005; Montgomery-Brown & Reinhard, 2003; Schröder, 2001; Ejlertsson et al., 1999; Brunner et al., 1988; Stephanou & Giger, 1982). This is attributed to the presence of highly branched alkyl group on the phenolic ring, the high hydrophobicity of APs and due to the large number of isomers present in commercial products that defer from one manufacturer to another, including the reference materials used for quantitative analytical studies. Theoretically, 211 constitutional NP isomers exist in which many of these isomers posse three chiral C-atoms, so that in total 550 NP compounds are possible of which 50-80 are estimated to be present at the same time in biological

matrices (Guenther et al., 2006; Montgomery-Brown et al., 2008). A spectra analysis of a reference material revealed the presence of 22 *p*-NP isomers (five distinct *p*-NP isomeric groups were identified which were differed by the substitution of the alpha-carbon on the alkyl chain (Wheeler et al., 1997). As a result, bioavailability of APs is reduced (Ejlertsson et al., 1999; Schröder, 2001) and this is primarily attributed to the luck of a large number of hydrolysing exoenzymes which are required for the conversion (hydrolysis) of NP isomers to CH_4, CO_2, (in the absence of sulphate) or H_2S and CO_2, (in the presence of sulphate). Since sources of APEOs in STWs and ultimately within ADs are highly diversified, short SRT does not allow stabilization, adaptation and proliferation of mixed anaerobic cultures for adequate production of selected exoenzymes required for APs degradation (oxidation) as it is the case for the reductive dechlorination requirements (Kafkewitz et al., 1996). Besides, irregular APEOs loadings, in terms of both mass and speciation, abate degradation potential that otherwise i.e. during permanent APEOs loadings it would be risen. Because of the aforementioned issues, an approximate mass balance has never been reported as yet.

Nonetheless, (Ferguson & Brownawell, 2003) in their study on the fate of NP under anoxic mesophilic batch lab-scale of sediment slurry they did not observe NP production. Recently, the degradation of spiked NP within 84 days of incubation, during batch mesophilic anaerobic digestion by using different electron acceptors on river sediment and sewage sludge was reported by (Chang et al., 2005; Chang et al., 2004). Nonylphenol degradation rates in their lab scale studies were sulphate-reducing conditions > methanogenic conditions > nitrate reducing conditions at pH 7.0. Nonylphenol monoethoxylate was also degraded, however the presence of NPECs was not reported. Bacteria that showed the highest NP degradation rates were identified as being *Bacillus cereus* (strain TN1), rod-shaped gram positive and *Acinetobacter baumannii* (strain TN6), coccus-shaped gram-negative (Chang et al., 2005). In another study, during continuous mesophilic (20 d HRT) anaerobic digestion of mixed sludge (50:50v/v primary and secondary sludge), (Patureau et al., 2008) and co-workers observed that the complete degradation of NP_2EO and the partial degradation of NP_1EO (80%) did not contribute to NP accumulation. This observation and the absence of NPECs in their samples led them to conclude that NP degraded by circa 25%. Authors

attributed the low NP degradation to the high content of organic matter in sludge (75% of TS) compared to that in river sediment or sludge reported by (Chang et al., 2005; Chang et al., 2004) in which NP degraded within 84 days. Patureau et al (2008) observed increased NPEOs removals during continuous thermophilic anaerobic (20 d HRT) digestion of mixed sludge (50:50v/v primary and secondary sludge) compared to their mesophilic anaerobic digestion study. A 60% degradation of spiked NPEOs within 3 days under Fe^{3+} reducing conditions was reported by (Lu et al., 2007). Although degradation of NP or presence of NPECs was not observed during the batch anaerobic (30^0C) experiment by using river sediment and synthetic sewage, NPEOs degradation was coupled to Fe^{3+} reduction.

2.5. Literature review summary

The limited studies indicate that under anaerobic conditions steroid estrogens are difficult to digest. It has been shown that reduction of E1 to E2 may occur under anaerobic conditions (de Mes et al., 2008; Carballa et al., 2007; Carballa et al., 2006; Joss et al., 2004b) whereas the extent of this reaction depends on the inoculum. However, the reverse reaction i.e. E2 to E1 under anaerobic conditions is unlikely to provide energy to the cells involved and probably this reaction represents the use of E2 as an alternative electron acceptor to regenerate co-factors (NAD) (Czajka & Londry, 2006). It was hypothesised that electron accepting conditions i.e. redox potential or the amount of thermodynamically available free energy would determine the rate and the extent of this reaction which is independent of the total amount of steroid estrogens added (Czajka & Londry, 2006). In addition, inter-conversion of E2 to E1 and back to E2 was observed under methanogenic, sulphate-reducing and iron-reducing conditions (Czajka & Londry, 2006) (Figure 2-3). Similarly, limited studies indicate that EE2 is recalcitrant in anaerobic conditions whereas there are no published data on the fate of E3 and E1-3S in relation to anaerobic digestion.

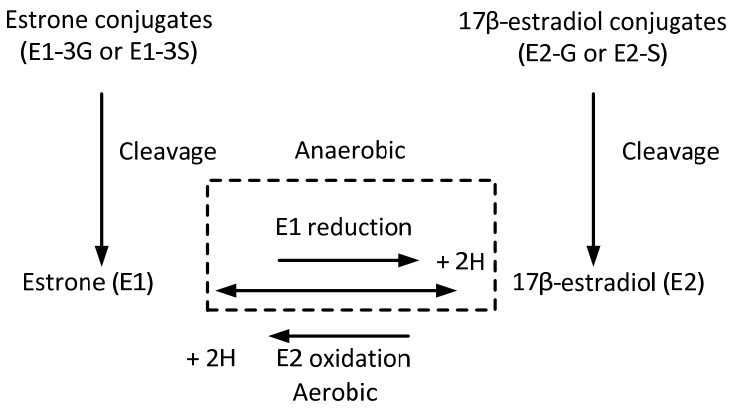


Figure 2-3 Relationship between E1 and E2 in anaerobic and aerobic conditions.

At present, the reported breakdown mechanisms of APEOs and their associated metabolites under anaerobic conditions are inconsistent. The shortening of long-chained ethoxylates proceeds rapidly both aerobically and anaerobically (Figure 2-4), (Chiu et al., 2010). However, the presence of carboxylated nonylphenol ethoxylates during anaerobic digestion has been reported but due to the limited studies these metabolites have been overlooked in anaerobic conditions. Despite the fact that the regulated NP is a typical recalcitrant by-product of the anaerobic degradation of nonylphenol ethoxylates, recent studies were carried in anaerobic environments have shown its disappearance.

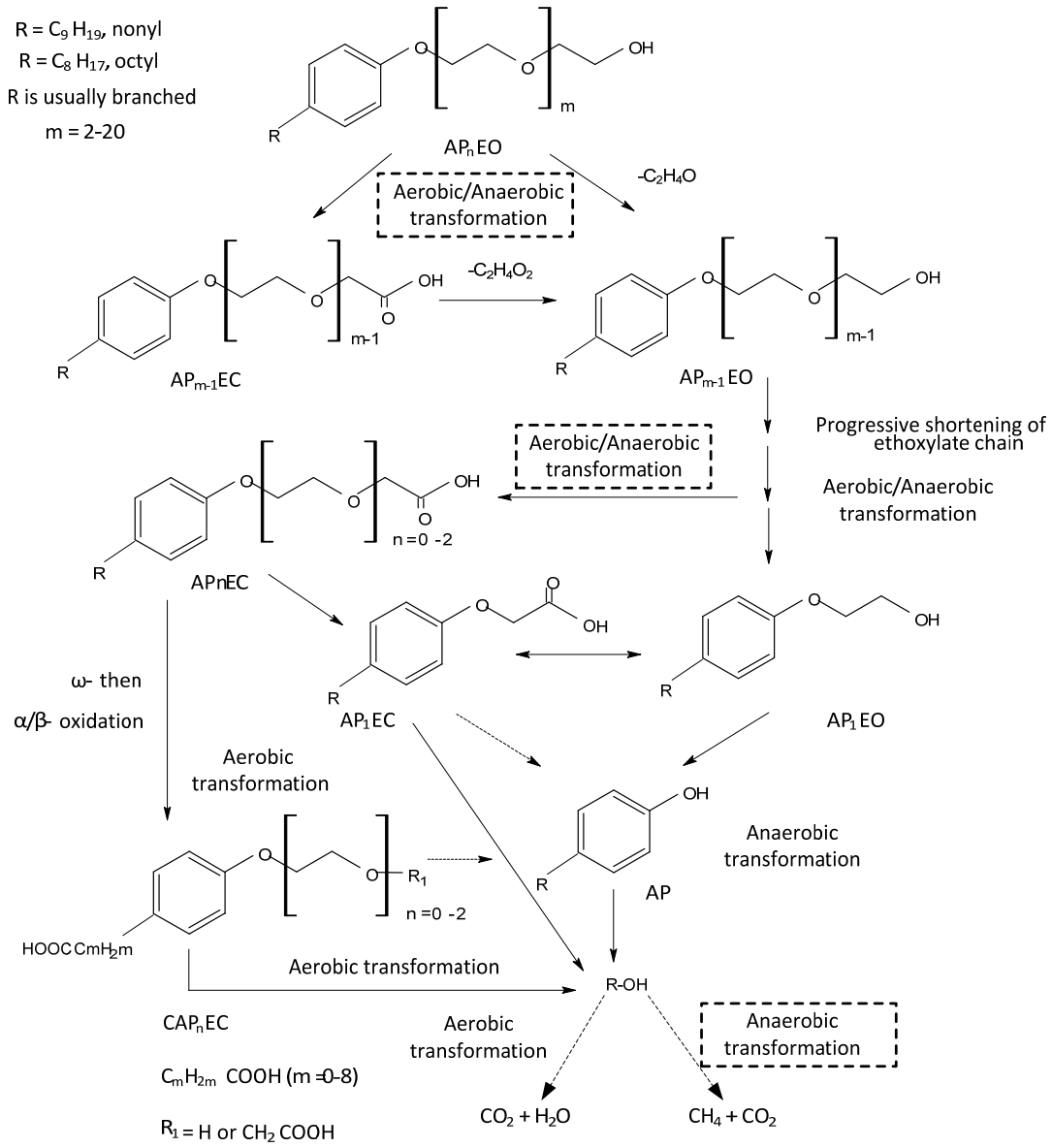


Figure 2-4 Anaerobic degradation pathways of APEOs. Adapted with modifications from (Renner, 1997).

3. MATERIALS AND METHODS

3.1. Feed sludge sampling

Sludge samples for both mesophilic and thermophilic experiments were collected from Sheffield STWs, (Woodhouse Mill) U.K. during dry weather conditions on two occasions, April 2007 and April 2008, respectively. One sampling event per trial ensured constant feed and background EDCs levels during mesophilic or thermophilic digestion. The specific STW was an activated sludge plant with a population equivalent (PE) of 155,000, trade ~10%. Sludge stabilisation on site was carried out by two continuous mesophilic (32ºC) anaerobic digesters (450 m^3 d^{-1}) with 28 days nominal retention time (Figure 3-1). Digesters were fed every hour for 20 minutes continuously with mixed sludge (primary / surplus activated sludge (SAS) 60/40 v/v) from the balancing tank.

Figure 3-1 Woodhouse Mill, STW sampling site.

Primary sludge was collected directly from the pump room from the first primary sedimentation tank; after withdrawing several 25L containers ensuring sampling of fresh sludge whereas mixed sludge (primary/SAS) was sampled directly from the balancing tank. For the mesophilic trial, approximately 50L of primary and 40L of mixed sludge were collected respectively to ensure the continue operation for 360 days. For the thermophilic trial, approximately 30L of primary and 20L of mixed sludge were collected respectively to ensure the continue operation for 180 days. Mesophilic digestate (seed) (see section 3.2.1) was sampled directly from the outlet of digester 1. Feed sludges were collected in 25L plastic containers whereas the seed sludge was stored in 10L plastic containers that had been completely filled up to minimise air entrance into the container. All sludges were then transported immediately to the Cranfield laboratories where sludge processing commenced within 6hrs according to sections 3.1.1 and 3.2.4.

3.1.1. Sludge handling and processing

After transportation to the laboratories, all the sludge types were manually homogenised in 75L PTFE containers (Fisher Scientific, UK) and packed to pre-labelled polypropylene containers (Fisher Scientific, UK) for storage in the freezers at -25ºC. All containers used, had been previously washed as it is described in section 3.2.1.

Primary sludge was sieved through a 2mm fish net (Alana Ecology Ltd, UK) prior to homogenization in order to remove debris and the fibrous materials so as to prevent blockage of the feeding and wasting lines in the laboratory reactors.

3.1.2. Experiments and investigated variables

For the fulfilment of the research hypotheses a series of experiments were carried out as shown on Table 3-1. The investigated variables for all experiments were: sludge type i.e. primary and mixed sludge, digestion temperature, sludge retention time, pH, total solids, volatile fatty acids, alkalinity and redox potential.

Table 3-1 Conducted experiments

Digestion temperature	Sludge type	Number of digesters	Retention time (days)
Six retention time experiments			
Mesophilic 35^{o}C	Primary (PSM)	Two	30
Mesophilic 35^{o}C	Mixed (MSM)	Two	30
Thermophilic 53^{o}C	Primary (PST)	One	15
Thermophilic 53^{o}C	Mixed (MST)	One	15
Shock loading experiment			
Mesophilic 35^{o}C	Primary (PSM)	One	30
Mesophilic 35^{o}C	Mixed (MSM)	One	30

Key: PSM (primary sludge mesophilic), PST (primary sludge thermophilic), MSM (mixed sludge mesophilic), MST (mixed sludge thermophilic)

3.2. Anaerobic digesters (set up)

The set up used for both mesophilic and thermophilic anaerobic digesters was similar. Four, 2L borosilicate lab scale digesters with an effective capacity of 1.5L each and 100 mm flange bore flask lids (QUICKFIT-19/26, Fisher Scientific, UK) were used for the mesophilic anaerobic digestion whereas two identical digesters were used for the thermophilic anaerobic digestion. Although the utilisation of more thermophilic digesters was initially considered for this study, it was later proven impractical due to space constraints of the available set up (rig).

Anaerobic conditions within the digesters were ensured by using vaseline and PTFE o-rings between the vessels and the five socket flask lids which were secured with appropriate size flask clips, QUICKFIT stirring glands (with 5ml glycerol in the gland, the air trap), QUICKFIT cone/screwthreads and QUICKFIT joint clips (Fisher Scientific, UK), as shown in (Figure 3-2).

Figure 3-2 Setup of anaerobic digesters/bioreactors.

QUICKFIT feed and waste lines were custom prepared by (Soham Scientific, Cambridge, UK) equipped with straight bore PTFE BiBi valves (I.D. 8mm) having an upper I.D. 10mm and a total length of 200mm. Feed lines were secured on the 5º socket on the flask lid, whereas waste lines on the 10º socket ensuring the waste line was located above the feed line (Figure 3-6).

To ensure complete mixing conditions for effective digestion conditions i.e. to overcome problems such as poor mixing and solids' precipitation, overhead stirrers (Heidolph Instruments, Schwabach, Germany) with stainless steel stirring paddles of 450mm total length (O.D.8mm) equipped with 4-blade propellers (Fisher Scientific, UK) were safely secured on the central QUICKFIT gland socket (0º) of each reactor. Digestion was carried under continuous stirring conditions at 90-100rpm for the whole digestion period.

Digesters were immersed in a thermostatically controlled water bath (170L capacity) by using a VFP Thermostatic Circulator (Grant Instruments Ltd, Shepreth, UK). Floating polypropylene spheres were added to the water's surface to minimize water evaporation and a black die was added in the water bath to prevent light entering the digesters through the transparent water tank.

Biogas was conveyed from each bioreactor to its dedicated gas collection cylinder (Figure 3-3) via Nalgene® 380 tubing (11.1mm Fisher Scientific, UK) through the fourth flange socket (0º) equipped with the appropriate hollow QUICKFIT borosilicate glass.

Figure 3-3 Gas measuring apparatus of the lab scale anaerobic digesters.

The fifth socket on the flange lid was constantly blocked with a hollow blown Pyrex stopper secured with QUICKFIT joint clips (Fisher Scientific, UK) appropriately. The setup of the lab scale anaerobic rig is shown in (Figure 3-4) and a schematic diagram in Figure 3-5.

Figure 3-4 Lab scale anaerobic set up used for the evaluation of EDCs.

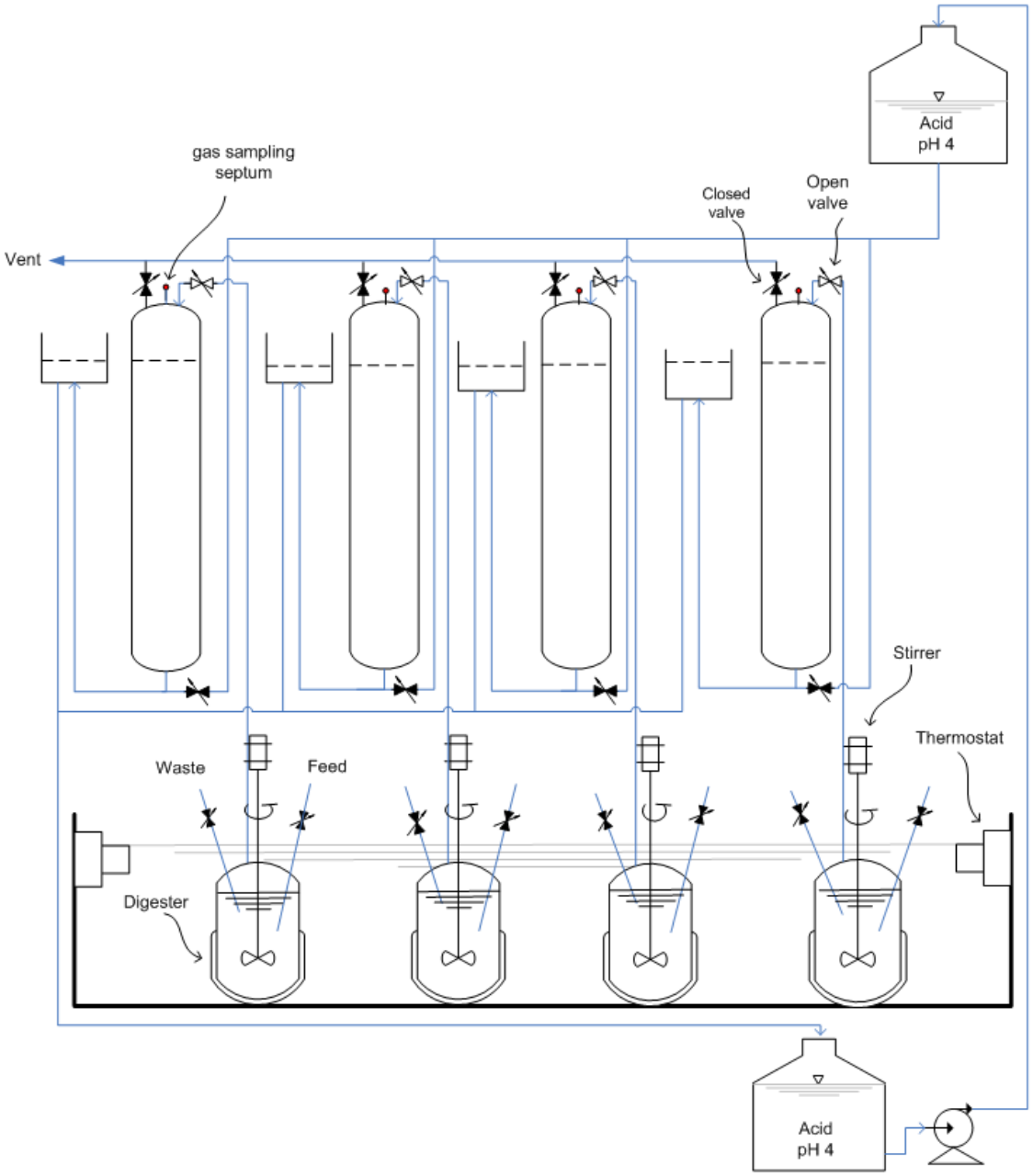


Figure 3-5 Schematic diagram of anaerobic digesters used in the EDC project.

3.2.1. Mesophilic digesters start-up

Mesophilic digestion (35º ± 0.2ºC) of primary and mixed sludges commenced on the sampling day by utilising four digesters. Two digesters (duplicate) were running with primary sludge (PSM) and two digesters (duplicate) with mixed sludge (MSM). All four digesters were run at semi-continuous feed mode i.e. once daily manual feeding.

To commence digestion, each digester was seeded with 1.5L mesophilic seed (see section 3.1). After seeding, N_2 gas (99.99%) (BOC Gases, Manchester, UK) was flashed into each digester for 10 minutes at a flow rate of ~80ml min^{-1} ensuring the absence of atmospheric air in each reactor. On the same day, 50ml of each digester were wasted (removed) and the same volume was replaced with either primary or mixed sludge according to the type of digester. Feed was at (35º ± 0.2ºC) prior to addition to each digester. The nominal retention time for the mesophilic digesters was 30 days. No supernatant was removed from the digesters and there was no grit accumulation due to the effective constant stirring conditions. In order to avoid gas-outlet blockages from possible scum and foaming effects, each digester's total sludge volume did not exceed the digesters effective volume e.g. 1.5L.

All digesters' parts e.g. all QUICKFIT components (flasks, lids, feed and waste lines), syringes, pipettes and stirring rods were appropriately washed before use. The washing procedure involved: initial acid wash in the dishwasher (without detergent), then immersion in 5% v/v sodium hypochlorite for 24hrs followed by deionised water and then further immersion in 5% v/v hydrochloric acid solution for further 4hrs and then final wash with deionised water.

For the whole period of digestion, each digester was allocated its own dedicated syringes (for feeding and wasting), pipettes and any other materials required for maintenance and /or cleaning avoiding cross contamination between the digesters.

3.2.1.1. Temperature conversion (mesophilic to thermophilic)

Due to unavailable thermophilic seed, mesophilic seed was used after it was converted to thermophilic seed (53ºC ± 0.2ºC). After the successful temperature conversion, the thermophilic trial commenced by removing (waste) 100ml of sludge of each digester and by replacing the same volume with either primary or mixed sludge according to the type of digester. Thermophilic nominal retention time was 15 days.

Two different strategies were utilised for the change of the mesophilic to thermophilic culture which involved slow and gradual temperature increase with intermittent feeding, in order to ovoid digesters' overloading. The first trial involved a temperature increase rate of 0.71ºC d^{-1} but this rate proved unsuccessful. A second and successful attempt involved a much lower temperature increase rate of 0.29ºC d^{-1} (Appendix I).

3.2.2. Thermophilic digesters (start-up)

Thermophilic digestion (53ºC ± 0.2ºC) of primary and mixed sludges commenced after the successful temperature conversion by utilising two 5L digesters with 1.5L sludge volume content. One digester was running with primary sludge (PST) and the other digester with mixed sludge (MST). Both digesters were run at semi-continuous feed mode i.e. once daily manual feeding.

Thermophilic digesters' start up was similar to the start up of the mesophilic digesters. After seeding (see section 3.2.1.1), N_2 gas (99.99%) (BOC Gases, Manchester, UK) was flashed into each digester for 20 minutes at a flow rate of ~80ml min^{-1} ensuring the absence of atmospheric air in each reactor. Nominal retention time for the sludge in each thermophilic digester was 15 days. No supernatant was removed from the digesters and there was no grit accumulation due to the effective constant stirring conditions. In order to avoid gas-outlet blockages from possible scum and foaming effects, each digester's total sludge volume did not exceed the digesters effective volume e.g. 4L. Digesters' size and their sludge volume was primarily dependant on the available rig space and the freezers' storage capacity for storing two different sludge types for approximately 180 days (for two digesters).

3.2.3. Anaerobic digesters' operating schedule

Sampling and feeding for the whole digestion period for all types of digesters was carried out by following the sequential steps as shown in Table 3-2. All digesters were sampled and fed manually once on a daily basis between 11:00 – 12:00 hours for the whole period of digestion. Feeding the digesters at more frequent intervals by using peristaltic pumps was considered impractical since all feed types were kept frozen for the whole digestion period.

Table 3-2 Anaerobic digester's operation mode

Steps	Activity	Section
1	Biogas measurement	3.2.3.1
2	Biogas composition analysis	3.2.3.2
3	Sludge sampling (wasting)	3.2.3.3
4	Sludge feeding (feeding)	3.2.3.4
5	Acid levelling	3.2.3.1
6	Rig maintenance	3.2.3.1

3.2.3.1. Biogas measurement

Separate biogas collection units were dedicated for each digester. The collection and measurement of biogas was achieved by using 'the manual liquid displacement principle' ISO/DIS 14853 (1999) by utilising an Eudiometer. Eudiometers are used as anaerobic biodegradability techniques in aqueous or sludge systems (Guwy 2004, Valke and Verstraete 1983, Kirk et al, 1982) by measuring gas at atmospheric pressure. Although this method is labour intensive and requires careful adjustment of the displacement liquid for accurate measurement of biogas the particular apparatus was chosen as the most appropriate method for the specific set up.

Other types of gas measurement during the initial anaerobic trials were also utilised e.g. gas displacement method by using inverted cylinder immersed in an acid bath (HCl, 1.0

mol, pH 4), and the use of biogas bags (8L). Both methods resulted to identical and comparable results in terms of biogas volume and composition to that of the Eudiometer.

The biogas measurement apparatus utilised, consisted of a sealed graduated glass cylinder I.D. 100mm (5L effective volume) filled with acidified water (HCl, 1.0 mol, pH 4) connected to a balancing reservoir (open to the atmosphere) (Figure 3-6). Biogas from the headspace of the digester was transferred to the headspace of the graduated glass cylinder by displacing the acidified barrier solution in the balancing reservoir tank. The excess acidified water in the balancing reservoir was over flown into the acid collection tank. Biogas was measured by graduation from the acid height difference at atmospheric pressure by converting the height loss of the acidified solution in the graduated cylinder into volume of gas produced according to Equation 1.

$$Gas\ volume\ (ml) = 77.8 \times h \times \frac{(1019.7 - 62 + h)}{1019.7}$$

Equation 1

Where, 77.8 ml was the glass cylinder calibration for 1 cm, *h* was the height loss of acid (cm), 62cm was the working length of the gas-measuring cylinder and 1019.7 stands for the standard atmospheric pressure in cm water gauge. The vent valve (V3) on each graduated cylinder was permanently connected to the fume cupboard for the whole period of digestion to avoid suffocation and/or explosion. For the detailed description of the Eudiometer operation see Appendix II.

3.2.3.2. Biogas composition

After biogas measurement, an aliquot of biogas was sampled from the Suba seal on the top of the graduated cylinder by using a concentric Luer Lock gas tight amber syringe (VWR International, Dorset, UK) with a bevel tipped needle (Fisher Scientific Ltd, UK). Ten millilitres of biogas were injected into the gas analyser Servomex 1440 D

infra–red (Servomex Group Ltd, Crowborough, UK) modified for CH_4 analysis. One biogas sample of each reactor was analysed for methane content. Between injections a stream of N_2 gas (99.99%) (BOC Gases, Manchester, UK) was passed through the infra-red cell of the gas analyser to remove residual methane molecules and to prevent carry over effects. The Servomex gas analyser was calibrated weekly by using N_2 gas (99.99%) (BOC Gases, Manchester, UK) to set the zero point for calibration and CH_4 gas (99.999%) canister (Sigma-Aldrich Co Ltd) to set the 100% span. The septum (Servomex Group Ltd, Crowborough, UK) on the sample delivery port on the gas analyser was replaced regularly according to Servomex Group Ltd® specifications.

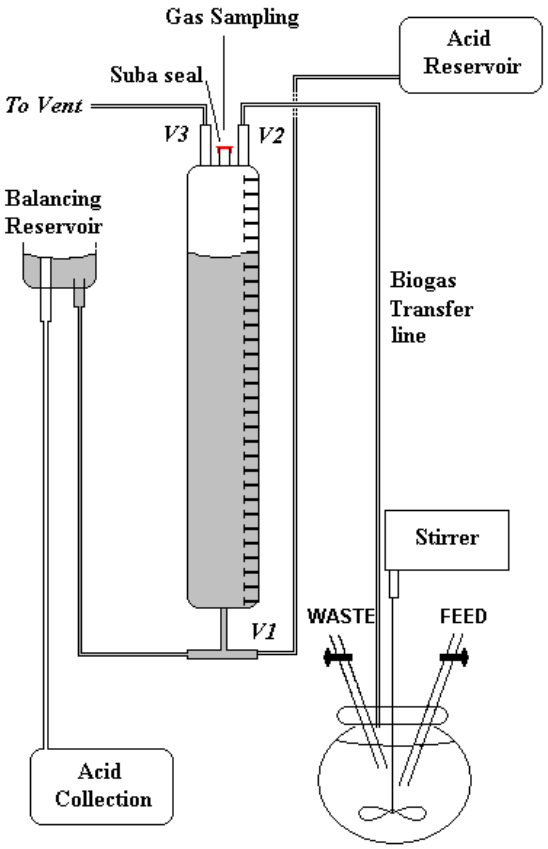


Figure 3-6 Schematic diagram of the Eudiometer apparatus used in the EDC project.

3.2.3.3. Sampling regime (digestate)

Following biogas measurement and composition analysis, each digester was sampled (wasted) before feeding to avoid removing the freshly added feed. Sampling/feeding regime was semi-continuous. Sampling occurred daily on one occasion between 11:00 – 12:00 hours. Table 3-3 summarises the sampling protocol by using the difference in hydrostatic pressure between the acid level in the acid reservoir to that level in the graduated cylinder. The sampling regime for the determination of EDCs is described in section 3.5.

Table 3-3 Sampling protocol by using the hydrostatic pressure difference

Step	Valve	Balancing reservoir port	Activity
1	-	-	Connect syringe to waste line
2	-	x	Close BR's lower port
3	V1	-	Open
4	-	-	Wait for 2cm acid raise in graduated cylinder
5	Waste line	-	Open to obtain sample and then Close
6	V1	-	Close
7	-	x	Open BR's lower port
8	-	-	Observe and record acid level drop

BR: Balancing reservoir

3.2.3.4. Feeding regime

After the completion of sampling, immediately the same sludge volume (50 ml for the mesophilic and 100 ml for the thermophilic digesters) was replaced with primary or mixed sludges respectively. The frozen sludges were left to thaw adequately in the laboratory. To minimize temperature variability within each digester, each feed bottle container was semi-immersed in the rig's water bath (35º ± 0.2ºC or 53º ± 0.2ºC) for adequate time before feeding. Each sludge type was vigorously shaken up by hand before feeding. The complete sampling / feeding protocol is shown in Table 3-4. For the detailed description of sampling/feeding see Appendix III.

Table 3-4 Protocol for sampling and feeding.

Step	Valve V1	Valve V2	Valve V3	BR lower port	Waste line	Feed line	Activity	Observation
1	Closed	Open	Closed	Open	Closed	Closed	Biogas measurement & biogas analysis	Record acid height
2	Closed	Open	Closed	Open	Closed	Closed	Connect syringe to WASTE line	-
3	Closed	Open	Closed	Closed	Closed	Closed	BALANCING RESERVOIR - lower port	-
4	Open	Open	Closed	Closed	Closed	Closed	V1	2cm acid raise in graduated cylinder
5	Open	Open	Closed	Closed	Open	Closed	WASTE LINE – obtain sample	-
6	Open	Open	Closed	Closed	Closed	Closed	WASTE LINE	-
7	Closed	Open	Closed	Closed	Closed	Closed	V1	-
8	Open	Open	Closed	Open	Closed	Closed	BALANCING RESERVOIR - lower port	Acid level drop
9	Open	Open	Closed	Open	Closed	Closed	Connect syringe to FEED line and introduce feed sludge	-
10	Open	Open	Closed	Open	Closed	Open	FEED LINE – feed sample	-
11	Open	Open	Closed	Open	Closed	Closed	FEED LINE	-
12	Open	Close	Open	Open	Closed	Closed	V2 & V3	Acid raise in graduated cylinder above BR level

13	Closed	Close	Open	Open	Closed	Closed	V1	Wait for acid to level between BR and graduated cylinder
14	Closed	Open	Close	Open	Closed	Closed	V3 & V2	Record acid height

BR: Balancing Reservoir

3.2.4. Monitoring digesters' performance

The digesters were monitored on a weekly basis from the commencement of the experiment by measuring total and volatile solids destruction, gas production and methane content, pH, alkalinity, redox (ORP), volatile fatty acids (VFAs) and temperature (Table 3-5). Chemical Oxygen Demand (COD) and soluble COD (SCOD) was also measured intermittently.

Table 3-5 Routine analysis for feed and digestates (sampled sludges) in all digesters.

Parameters measured	Frequency	Sludge type
Biogas	Daily	All digesters
Methane	Daily	All digesters
pH	Daily	All digesters
Temperature	Daily	Feed and Waste
Total solids	Weekly	Feed and Waste
Volatile solids	Weekly	Feed and Waste
VFAs	Weekly	Waste
Alkalinity	Weekly	Waste
Redox	Weekly	Waste

3.2.4.1. pH and temperature

Sampled sludges were measured daily for pH and temperature as part of the digesters monitoring regime by using a VWR pH meter-100 (VWR International Limited, UK) calibrated weekly to ensure its reliability and accuracy, by using pH buffers pH=4 - 10 (VWR International Ltd).

3.2.4.2. Redox

Sampled sludges were measured weekly for their redox potential as part of the digesters monitoring regime by using an Ion Meter 3340 (Jenway Ltd, Essex, UK) coupled to a platinum (924003) probe, calibrated weekly to ensure its reliability and accuracy, by using redox standard (465mV) (Jenway Ltd, Essex, UK).

3.2.4.3. Total solids

Total Solids (TS) content was determined by the Standard Method 2540B (APHA, 1998). A clean pre ignited (550°C for 4 hours required for the consecutive volatile solids analysis) weighted porcelain crucible (Fisher Scientific UK Ltd) that had been previously cooled in a desiccator was used to accommodate a well mixed homogenised sludge sample (10ml). The loaded crucible with sample was reweighed and evaporated overnight at 105°C in a drying oven. After drying, crucible contained the sample were put in a desiccator for adequate time (~20 minutes) and then reweighed.

Total solids analysis of both feed and digestates in all digesters was carried out in duplicate. However, the available sludge volume for the solids analysis, particularly in sampled sludges was only 10 ml (x2) as the remaining portion of the sample was subjected for further analysis. However, when it was possible larger sludge volumes were analysed for solids.

Total solids (TS) content was determined according to Equation 2, where A value is the weight after drying at 105^0C (g), B is the weight of the pre ignited crucible (g) and C is the samples volume (ml).

$$\mathrm{TS\,(g\,L^{-1})} = \frac{(A-B)}{C} \mathrm{x}\,1000$$

Equation 2

3.2.4.4. Volatile solids

Volatile Solids (VS) content was determined by Standard Method 2540E (APHA, 1998). The residue obtained from the total solids analysis was ignited in a furnace (550° C for 4 hours), then cooled in a desiccator and re weighed.

Volatile solids (VS) content was determined according to Equation 3, where A is the weight of the dry crucible (105^0C) from total solids determination (g), B is the weight of

the crucible after ignition at (550^0C) (g) and C is the sample volume (ml) (see 3.2.4.3 section).

$$VS\,(g\,L^{-1}) \;=\; \frac{(A-B)}{C} \text{x}\, 1000$$

Equation 3

3.2.4.5. Preparation of solid free sludge fraction

Preparation of the solid fraction was required for the determination of soluble VFAs and alkalinity. The solid free fractions were prepared by centrifugation (~30 ml) of sampled sludges for 10 minutes at 4193 *g* in a Rotanta 96 R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) and then filtering the supernatant through a 70 mm Schleicher & Schuell Grade GF 52 glass fibre filter paper (Patterson Scientific, Bedfordshire, UK) to remove any residual suspended matter.

3.2.4.6. Volatile fatty acids (VFAs)

Total VFAs were analysed according to the HACH method 8196 (HACH, 1992) for digested sludges (0 – 2800 mg L^{-1} acetic acid). All reagents for the total VFAs test were supplied by Camlab Ltd, Cambridge, UK.

Sludge samples for total VFAs analyses were prepared according to section 3.2.4.5. An aliquot of 500µl (suspended solid-free prepared sample) was added to a 25 ml HACH sample cell followed by the addition of 1.5 ml ethylene glycol and 200µl sulphuric acid (19.2 N). After esterification, the sample was digested at 100°C in a water bath for 3 minutes. The sample was cooled under running tap water and 500µl of hydroxylamine hydrochloride was added and the cell was swirled. Two hundred microliters (200µl) of sodium hydroxide (4.5 N) were added to the sample cell followed by swirling. Ten millilitres (10 ml) of ferric chloride were added followed by swirling and finally, 10 ml of deionised water were added. The sample was swirled and simultaneously a 3 minute reaction time was started. The HACH DR/2010 portable data logging

spectrophotometer was then calibrated with a zero VFAs sample (blank) and respective samples were subjected for total VFAs analysis. Results obtained in mg L^{-1} as acetic acid. All samples were analysed in duplicates.

3.2.4.7. Total alkalinity

Sludge samples for total alkalinity determination were prepared according to section 3.2.4.5. Alkalinity was determined by titrating 10ml of prepared sample (suspended solid-free) against freshly prepared 0.02 M hydrochloric acid to pH (4.5) end point according to Standard Method 2320B.4c (APHA, 1998). The pH in solution was measured by immersing the pH probe (Hanna HI 8424, pH meter) and titration with HCl carried out until the end point was reached. Total alkalinity was measured as mg $CaCO_3$ L^{-1}. All samples were analysed in duplicates.

Where A refers to the acid volume used (ml) and N is the Normality of the acid used. HCl is a monoptotic acid so its normality is 1.0.

$$\text{Total Alkalinity } (\text{mg } CaCO_3\ L^{-1}) = \frac{A \times N \times 50{,}000}{\text{sample volume (ml)}}$$

Equation 4

3.2.4.8. Calculation for first order kinetics

First order kinetic calculations (*k*) assumed the mass balanced cumulative input versus cumulative output for each compound of interest and the changing biomass in order to account for the continuous addition of EDCs. First order kinetics were considered for the 6th retention time digestion period and follows equation 5. Where, S_0 = volumetric cumulative influent substrate concentration (µg m^{-3} d^{-1}), Se = volumetric cumulative effluent substrate concentration (µg m^{-3} d^{-1}), V = reactor volume (m^3), R = solid retention time (days).

$$\text{mass flux} = (S_0 - S_e) \times \left(\frac{V}{R}\right)$$

Equation 5

In practice the first order kinetic constant, k (d^{-1}), is derived according to equation 6.

$$k = \left(\frac{\text{mass flux}}{V \times S_e} \right)$$

Equation 6

3.3. Shock loading experiment

After the completion of the six-retention time experiment, mesophilic digesters received higher NPEOs loadings to investigate the effects of higher NPEOs receiving concentrations on the biomass as well to see whether higher concentrations had an effect on the anaerobic digestion and/or the biodegradation process (Chapter 7). Feed sludges were dosed on a daily basis for one retention time (30 days) in order to observe the ability of biomass to constant shock NPEOs loadings. During one retention time, the initial biomass is wasted and replaced by new bacteria therefore the constant exposure to high NPEOs levels would enable the evaluation of the performance of the 30 days old biomass. Shorter than one retention time exposure periods would rather reflect the intermittent effects of shock loading onto biomass.

Dosing occurred after each digester had been wasted. After thawing, homogenization (hand shaken) and temperature adaptation of the different feed sludges, 50 ml of each sludge type were transferred to clean pre-labelled glass beakers. The exact transfer of sludge volume was ensured by using volumetric cylinders (100 ml). The dosing solution (1 ml) was transferred directly onto the sludge surface by using pipettes. After hand mixing (swirling) for 10 seconds, the dosed feed was immediately introduced into each respective digester. However, at the commencement of the experiment (day 1), each digester was received an additional concentration of NPEOs in order to replace the lost mass of nonylphenolics that was present in the wasted sludge of day 1 and to reach the target concentration immediately. The replenishment concentrations for each digester were calculated according to the total solids content for each respective waste at a level equal to the concentrations obtained during the sixth retention time trials for each respective digester.

All dosing solutions were prepared in distilled water. The use of solvents for the preparation of the dosing solutions was considered inappropriate since their presence could exhibit adverse effects on the digesters' performance and/or unforeseen matrix effects. Dosing solutions were freshly prepared every day for the first five days and then every two days for the remaining period of the dosing experiment due to time

constraints. Dosing solutions were always kept in the fridge during the non-dosing periods.

The feed sludges were dosed with the commercial surfactant mixture (Igepal CA210, CA520, and CA720) from Sigma-Aldrich (Gillingham, Dorset, U.K) containing only non-ionic nonylphenolics $NP_{1-12}EOs$. It should be pointed out that dosing with the mixture of (Igepal CA210, CA520, and CA720) contributed only to non-ionic species ($NP_{1-12}EOs$) and not to NP or NPECs. Dosing concentrations were according to the respective total solids content of the respective primary or mixed sludge mesophilic digesters. The dosing concentrations of $NP_{1-12}EOs$ for each different feed type are shown in Table 3-6. The dosing solution aimed to increase the concentration of the 'parent' $NP_{1-12}EOs$ in each feed sludge to a significant higher level of 10 times higher the feed concentration that digester was receiving before spiking. However, due to inconsistencies of the stock solutions the dosed concentrations varied between the primary and mixed feed sludges. Dosing onto primary feed sludge resulted to 7.8x higher (28.7 mg kg^{-1} dw) the background concentration of $NP_{1-12}EOs$ (3.7 mg kg^{-1} dw) whereas in mixed sludge the increase was 9.0x higher (21.8 mg kg^{-1} dw) the background $NP_{1-12}EOs$ concentration (2.7 mg kg^{-1} dw).

Table 3-6 Dosing concentrations for nonylphenol ethoxylates in primary and mixed feed sludges with standard errors.

Digester	Background $NP_{1-2}EOs$ mg kg^{-1} dw	Background $NP_{3-12}EOs$ mg kg^{-1} dw	10x background $NP_{1-12}EOs$ mg kg^{-1} dw	Total solids in digester kg	Received dose in feed sludge mg
Primary sludge	2.15±0.34	1.54±0.20	36.9±0.0	0.07665±5.55	2.826
Mixed sludge	1.68 ± 0.27	0.75±0.16	24.2±0.0	0.08565±6.45	2.076

3.4. Steroid estrogens and alkylphenolic ethoxylate analysis

3.4.1. Surfactants (Alkylphenolic compounds)

The technical 4-nonylphenol mixture of various chain isomers and 4-*tert*-octylphenol were obtained from Sigma-Aldrich (Gillingham, Dorset, U.K). The long-chain APEO, OPEO (Igepal CO210, CO520, CO720) and NPEO (Igepal CA210, CA520, CA720) were available in a commercial surfactant mixture containing different oligomers also purchased from Sigma-Aldrich (Gillingham, Dorset, U.K). Nonyl- and octyl - phenoxy acetic acids (NP_1EC, OP_1EC), 4-nonyl- and octyl-phenol-mono- and diethoxylates (NP_1EO, NP_2EO-OP_1EO, OP_2EO) were available as individual reference materials obtained from QMX Laboratories (Thaxted, Essex, U.K).

Single standard stock solutions of the analytes were prepared by weighing out milligram amounts of the reference materials and then dissolving them in acetonitrile (ACN). Reagent grade MilliQ water (18.2 mΩ) (Millipore, Watford, UK) was used in the preparation of the working standard solutions. The working standard solutions and spiking solutions were prepared from the standard stock solutions by further dilution with acetonitrile/MilliQ water (50:50 v/v). HPLC-grade organic solvents that were used for extracting alkylphenolic compounds from sewage and sludge matrices were methanol (MeOH), acetone, hexane, acetonitrile (ACN), purchased from Rathburn Chemicals (Walkerburn, UK) whereas acetic acid was purchased from Sigma-Aldrich (Gillingham, Dorset, U.K). Silica, solid phase extraction cartridges (Sep-Pak 500 mg 3cc) purchased from (Waters Ltd, Hertfordshire, UK).

There are no pure polyethoxylates compounds 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. Conversions from molar distributions to mass distributions were achieved by scaling the molar concentration of each oligomer by its molecular mass and then normalising the results so their sum was equal to one. Working

standard solutions prepared from standard stock solutions used to calibrate the response of the instrument.

A standard stock solution containing 1 mg of each compound was transferred into a 10 ml volumetric flask containing HPLC grade (ACN) and then made up to the mark by using (ACN) HPLC grade. Alkylphenolic working standard solutions of concentration 1 μg ml^{-1} were prepared (10 μl of each stock solution to 10 ml volumetric flask containing acetonitrile/MilliQ water (50:50 v/v). Available individual standards of NP, $NP_{1-2}EO$, NP_1EC, OP, $OP_{1-2}EO$ and OP_1EC, were combined to give 5 mg l^{-1}. Calibration was performed using an eight point calibration curve at 0.01 – 25 mg l^{-1} for long chain alkylphenol polyethoxylates oligomers (NPEO and OPEO). Calibration was performed using a eight point calibration curve at 0.01 – 5 mg l^{-1} for APs (NP and OP), APECs (NP_1EC and OP_1EC) and lower chain APEOs ($NP_{1-2}EO$ and $OP_{1-2}EO$).

3.4.2. Steroid estrogens

All estrogen standards (>98% chemical purity) were purchased from Sigma Aldrich (Dorset, UK). Deuterated ($d_{3/4/5}$) labelled 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-ethynylestradiol-2,4,16,16-d_4 (EE2-d_4) and sodium estrone-2,4,16,16-d_4 sulphate (E1-3S-d_4) were obtained from C/D/N Isotopes (QMX Laboratories, Essex, UK) with >98% chemical purity. HPLC-grade organic solvents, ethyl acetate (EtOAc), hexane, methanol (MeOH), dichloromethane (DCM) were purchased from Rathburn Chemicals (Walkerburn, UK) and ammonium hydroxide from Sigma-Aldrich (Gillingham, Dorset, U.K). Silica, solid phase extraction cartridges (Sep-Pak 500 mg/3cm^3) purchased from (Waters Ltd, Hertfordshire, UK) and Varian aminopropyl (NH_2) anion-exchange (500 mg/3cm^3) from (Varian Inc, Oxford, UK).

Standard stock solutions were prepared in MeOH. Working standard solutions prepared from standard stock solutions were used to calibrate the response of the instrument. Standard stock solutions of 1 mg of each compound (deuterated or non-deuterated) were dissolved into a 10 ml volumetric flask containing methanol HPLC grade respectively and then made up to the mark. Secondary standard steroid solutions of concentration 1

µg ml^{-1} were prepared (10 µl of each stock solution to a 10 ml volumetric flask containing MeOH/MilliQ water (10:90 v/v). Calibration standards containing all five analytes in MeOH/MilliQ water (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}.

3.5. Extractions of analytes from sewage sludges

The methods employed to extract surfactants and estrogens from sludge and in the dissolved phase were based on (Koh et al, 2008; Koh et al, 2007) (see sections 3.5.1 and 3.5.2), respectively.

Homogenous, weekly composite digested sludge samples (digestates) collected from each digester at the end of each respective retention time were stored in the freezer at -26º ± 1.5ºC prior to EDC analysis. Frozen digestates were lyophilised, extracted and then analysed for their steroid estrogens and alkylphenolic concentrations. Two individual samples from each respective digestate obtained out of each digester were separately extracted (n=2) and were analysed for steroid estrogens or alkylphenolics according to method developed by (Koh, 2008) (see section 3.6).

3.5.1. Surfactants (Alkylphenolic compounds)

Alkylphenolic compounds were eluted from solid phase extraction (SPE) cartridges by using a sequence of polar, non-polar and acidified solvents to elute all analytes of interest without the need for an additional clean-up procedure. The steps followed resulted to rapid processing time and facilitated quantitative analysis.

Combined aqueous and solid phases of either untreated or anaerobically digested sewage sludges (digestates) were lyophilized in a ModulyoD-115® freeze-drier (Thermo Fisher Scientific Inc. UK) to minimize biodegradation of polyethoxylates, then stored in acetone-cleaned Amber glass vials (25ml) (Fisher Scientific, UK) and kept in a desiccator until analysis. Weighted 0.2 g (±0.002 g) of lyophilized sludge was extracted using 10ml MeOH/Acetone (1:1) (Figure 3-7). The suspensions were then mechanically shaken at 447g (Heidolph UK, Germany) for 30 minutes in acetone-cleaned centrifuge Teflon tubes (25 ml) followed by centrifugation at 4192.5g for 10 min. This procedure (mechanical shaking and centrifugation) was repeated twice. The supernatants obtained (20ml) were decanted in acetone-cleaned pear–shape glassware (25ml) and subjected to a rotary evaporator (Heidolph Instruments, Kelheim, Germany) to concentrate the extracts to approximately 0.2 ml. Hexane, 1.8 ml was then added to the extract and the

total 2 ml aliquot was passed through a pre-conditioned (Sep-Pak) silica cartridge (500mg, 3cc) (Waters Ltd, Hertfordshire, UK) to remove the matrix's impurities (Koh et al, 2008).

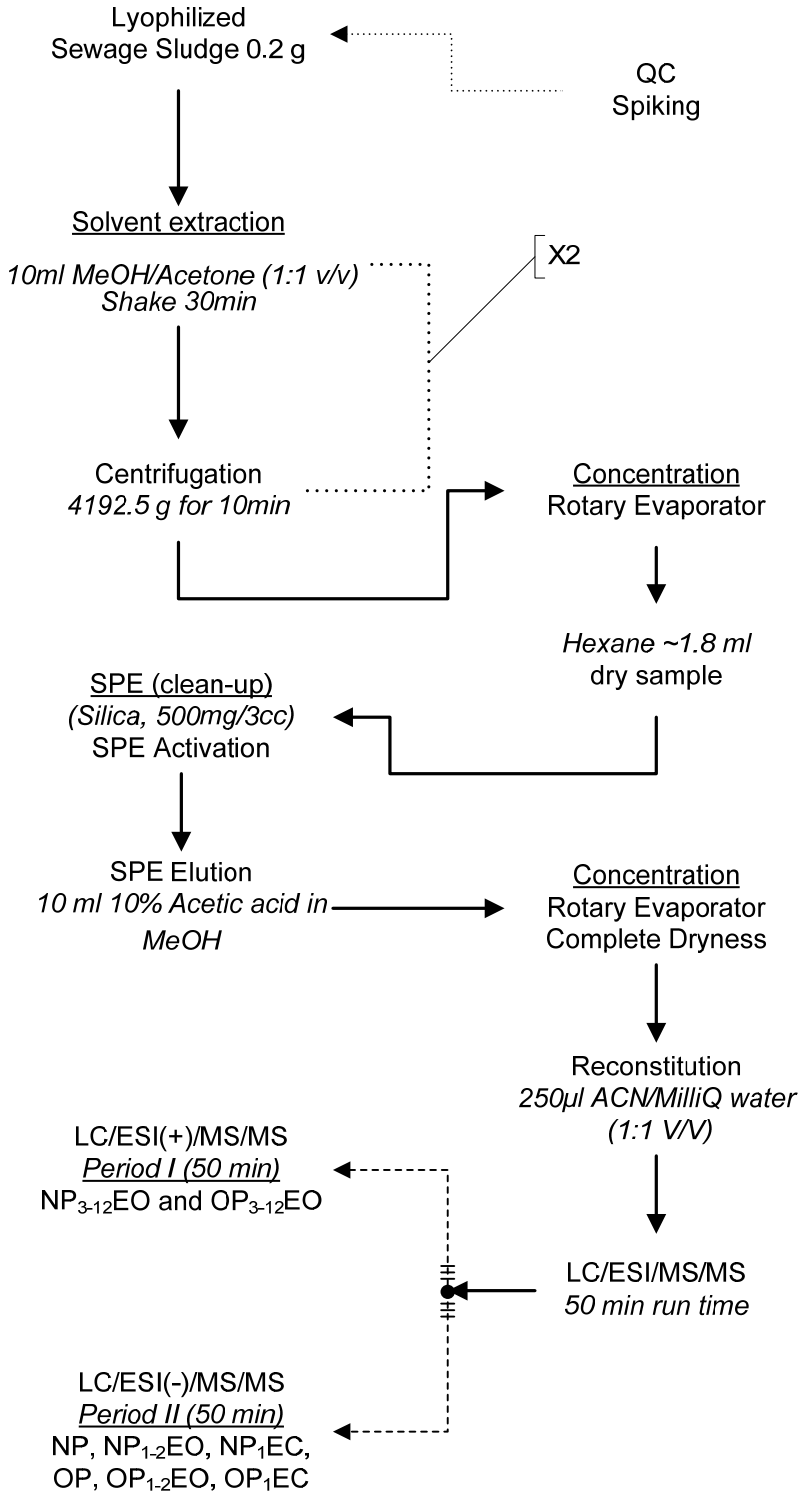


Figure 3-7 Analytical procure used for the determination of the alkylphenolic compounds in sludge. (modified from Koh 2008).

Solid phase extraction (SPE) was carried out using a Waters Sep-Pak Vacuum Manifold and a vacuum pump (Waters, Watford, UK) at constant pressure and flow rate (5mb, 5ml min^{-1}). SPE elution with 10ml of 10% acetic acid in MeOH was followed and the eluate was subjected to rotary evaporator followed by N_2 gas until complete dryness. Reconstitution with 250 µl of ACN:Water (1:1 v/v) was followed and subsequently the sample was subjected to LC/MS/MS for quantification by employing (Koh et al, 2008) method. Quality control of individual samples was performed by spiking two different samples with high spike (HS) (250 µg l^{-1}) and low spike (LS) (25 µg l^{-1}) concentrations, equivalent to 1.25 and 0.125 µg g^{-1} respectively.

3.5.2. Steroidal oestrogens

Steroid oestrogens were extracted from sewage sludges by using SPE, two clean up stages and deuterated internal estrogen standards. All lyophilised sludge samples were spiked with 15 ng l^{-1} deuterated oestrogens as internal standard.

Combined aqueous and solid phases of either untreated or anaerobically digested sewage sludges (digestates) were lyophilized in a ModulyoD-115® freeze-drier (Thermo Fisher Scientific Inc. UK), then stored in acetone-cleaned Amber glass vials (Fisher Scientific, UK) and kept in a desiccator until analysis. Weighted 0.1 g (±0.002 g) of lyophilized sludge was spiked with (15 ng l^{-1}) deuterated estrogens as internal standard and were extracted with ethyl acetate (10 ml) (Figure 3-8). The suspensions were then mechanically shaken at 447*g* (Heidolph UK, Germany) for 60 minutes in acetone-cleaned centrifuge Teflon tubes (25 ml) followed by centrifugation at 4192.5*g* for 10 min. This procedure (mechanical shaking and centrifugation) was repeated twice. The supernatants (20ml) were decanted in acetone-cleaned pear–shape glassware (25ml) and subjected to a rotary evaporator (Heidolph Instruments, Kelheim, Germany) in order to concentrate the extracts to approximately 0.2 ml. Hexane, 1.8 ml was then added to the extract and the total 2 ml aliquot was passed through a pre-conditioned with 2 ml hexane (Sep-Pak) silica cartridge (500mg, 3cc) (Waters Ltd, Hertfordshire, UK) to remove the matrix's impurities without letting the cartridge to dry.

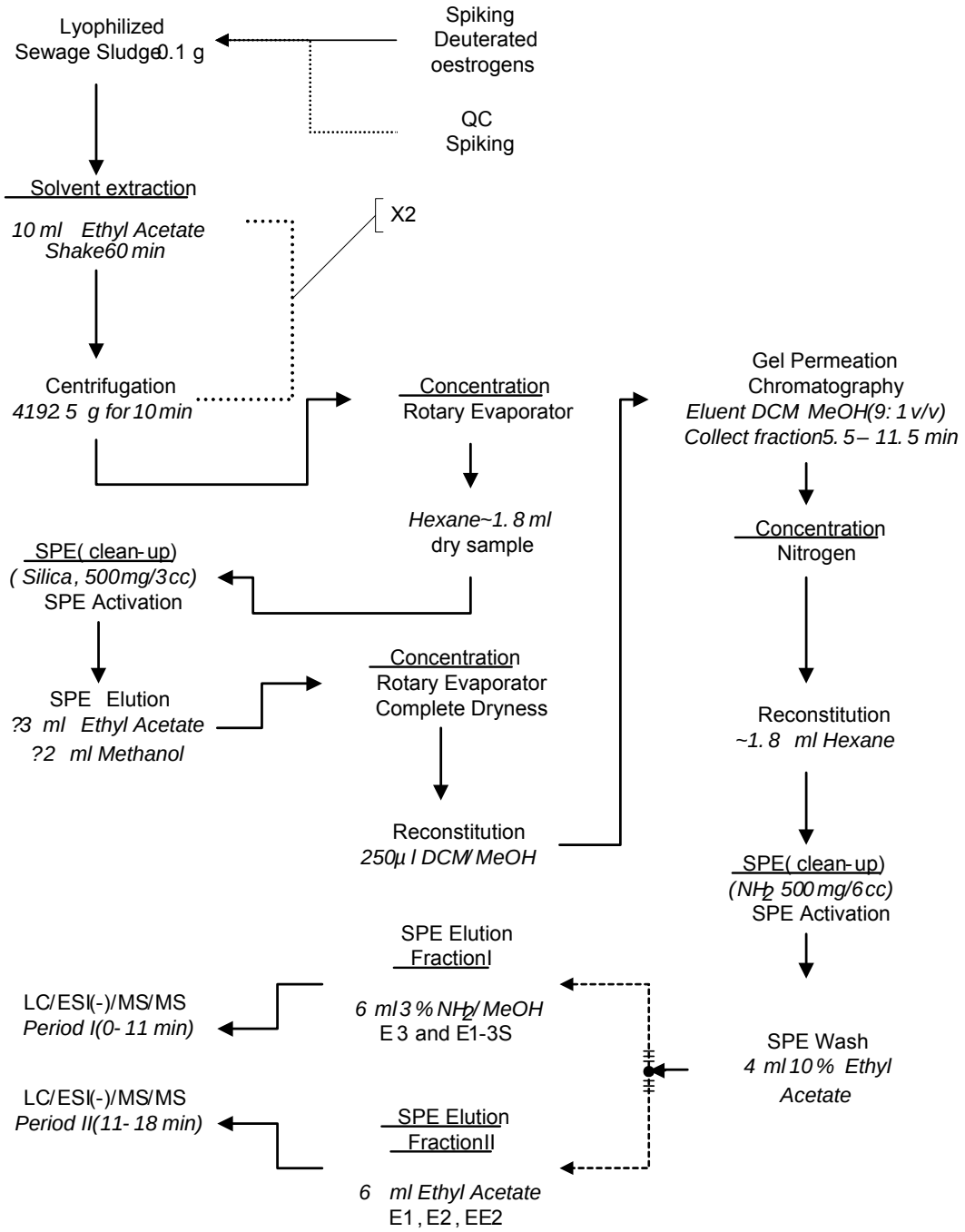


Figure 3-8 Analytical procure used for the determination of the steroidal estrogens in sludge. (modified from, Koh 2008).

Solid phase extraction (SPE) was carried out using a Waters Sep-Pak Vacuum Manifold and a vacuum pump (Waters, Watford, UK) at constant pressure and flow rate (5mb,

5ml min^{-1}). The semi dry Sep-Pak cartridges were then eluted sequentially with 3ml EtOAc followed by 2ml MeOH without letting the cartridge dry between the different elutions. The collected eluates were subjected to the rotary evaporator until complete dryness followed by reconstitution with 200 µl of DCM:MeOH (9:1) v/v). Samples were then subjected to a further clean up step by using gel permeation size exclusion chromatography (GPC) clean up step utilising an HPLC LC-9A-System (Shimadzu UK Ltd) coupled to a SPD-10ADvp UV-detector (Shimadzu UK Ltd). Instrument control, data acquisition and evaluation were performed with a CLASS-VP^{TM} software 7.1. A PLgel column, 5µm 50Å, 300 x 7.5 mm (Polymer Laboratories, Shropshire, UK) was used to elute the conjugated and unconjugated steroids which were detected at 280nm. A 6ml fraction was collected from the column using an isocratic elution of DCM/MeOH (90:10 v/v) at a flow rate of 1 ml min^{-1}. All steroids were eluted between 5.5 to 11.5 min, and a single fraction corresponding to this time window was collected. This fraction was dried by rotary evaporation to a final volume of approximately 0.2 ml. This was then reconstituted to 2 ml with hexane and loaded onto a conditioned with 2 ml hexane NH_2 SPE cartridge (Varian Inc, Oxford, UK) at a flow rate (5 ml min^{-1}). The NH_2 SPE cartridge was then washed with 4 ml (10% v/v EtOAc/Hexane) at a flow rate (5 ml min^{-1}). The non-polar steroids E1, E2 and EE2 were then eluted using 6ml EtOAc. The more polar conjugate (E1-3S) and E3 were subsequently eluted in a second fraction using 3% NH_4OH in MeOH. The separate eluates were blown to dryness under a gentle stream of nitrogen, reconstituted with 0.2 ml MeOH/H_2O (10:90 v/v) and transferred to vials prior to their quantification by using LC-MS/MS. Quality control of individual samples was performed by spiking all samples with 15 ng l^{-1} of deuterated oestrogens (internal standard). In addition to the internal standard samples, individual samples were spiked either with high spike solution (HS) (15 ng l^{-1}) or with low spike solution (LS) (2 ng l^{-1}) of free estrogens.

3.6. Instrumentation

All analytes (alkylphenolic compounds and estrogens) were determined using LC/ESI/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, Manchester, U.K.).

3.6.1. Surfactants (Alkylphenolic compounds)

Alkylphenolic compounds, (APs, APEOs and APEC) 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 positive electrospray mode (ESI+) for APEOs or negative electrospray ionisation (ESI–) mode for APs and APECs. The mass spectrometer was operated using multiple reaction monitoring (MRM). Instrument control, data acquisition and evaluation were performed with MassLynx software 4.1 (Waters Ltd, Hertfordshire, U.K.). Nitrogen was used as the nebuliser gas and argon as the collision gas. The conditions for detection by the mass spectrometer were as follows, capillary voltage, 3.2kV in the positive mode and -2.3kV in the negative mode, RF lens at 0.5 V in the positive mode and 1.0V in the negative mode, extractor lens at 3.0V, 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), entrance 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 (Koh, 2008).

3.6.2. Steroid oestrogens

The steroid estrogens 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 (ESI^-) mode using multiple reaction monitoring (MRM). Instrument control, data acquisition and evaluation were performed with MassLynx software 4.1 (Waters Ltd, Hertfordshire, U.K.). Nitrogen was used as the

nebuliser gas and argon as the collision gas. The conditions for detection by the mass spectrometer were as follows: capillary voltage, 3.2kV, RF lens at 0.2V, multiplier voltage 650V, desolvation gas flow 1000 l h^{-1}, cone at -55V, cone gas flow at 49 l h^{-1}, desolvation temperature at 350°C and source temperature at 120°C (Koh 2008).

4. RESULTS: ANAEROBIC DIGESTION TRIALS (SIX RETENTION TIMES)

This chapter reports the results from the mesophilic and thermophilic anaerobic digestions which were carried out over 180 and 90 days, respectively. The performance and stability of each type of digester under mesophilic and thermophilic conditions is presented in this chapter.

4.1. Anaerobic digesters performance

Four (1.5L) mesophilic (35^{o}C ± 0.2^{o}C) and two (1.5L) thermophilic (53^{o}C ± 0.2^{o}C) anaerobic digesters were utilised in this study to observe the fate of steroid estrogens and alkylphenol ethoxylates compounds during anaerobic digestion of sewage sludge. Two different sewage sludge types were used in this study to determine their possible differences in relation to alkylphenolic surfactants and estrogen degradation. The sludge types used were primary sludge collected from the primary sedimentation tank and mixed sludge collected from mixed tank of Sheffield sewage treatment works (STWs). Mixed sludge comprised of 60% (v/v) primary sludge and 40% (v/v) surplus activated sludge (SAS). Nominal digesters retention times in this study were 30 days for the mesophilic and 15 days for the thermophilic digesters. Both mesophilic and thermophilic trials lasted for six retention times respectively (i.e. 180 days under mesophilic temperatures and 90 days under thermophilic temperatures). A stabilization period of 90 days (3 retention times) preceded prior the commencement of the mesophilic anaerobic trials whereas the stabilization period for the thermophilic trials was 60 days (4 retention times). The results presented below are referred to the respective six retention time periods i.e. post the stabilisation periods for each type of digestion, mesophilic or thermophilic.

4.1.1. Anaerobic digesters stability

The data from the anaerobic digestion trials are shown in Table 4-1. Mean pH values of each type of digester were within the accepted ranges (Gerardi, 2003) and their values

reflected their respective alkalinities. During mesophilic digestion, mean pH value in the primary sludge digesters (7.1 ± 0.05) was lower than the mean value in mixed digesters (7.5 ± 0.03). Under thermophilic conditions, mean pH values were (7.2 ± 0.01) and (7.6 ± 0.02) for primary and mixed digesters respectively. Primary sludges for both mesophilic and thermophilic trials were always within the optimum pH range (6.8 – 7.2) whereas mixed sludges were lying between the marginal pH ranges (7.2 – 7.7) according to (Gerardi, 2003; Metcalf and Eddy, 2003).

Volatile fatty acid (VFAs) content remained at low levels during both mesophilic and thermophilic digestion indicative of the establishment of methanogenic bacteria (Gerardi, 2003).

Volatile fatty acid contents during mesophilic digestion were within the optimum levels (50-500 mg acetic acid l^{-1}) whereas during thermophilic digestion VFAs were well within the marginal levels (500-2000 mg acetic acid l^{-1}) (Table 4-1) according to (Gerardi, 2003; Metcalf and Eddy, 2003).

Measured total alkalinities ($CaCO_3$) in all digesters under both mesophilic and thermophilic conditions were above 2000 mg l^{-1} without any buffer additions. Alkalinities were stable in primary and mixed thermophilic digesters during the entire digestion period, however during mesophilic digestion alkalinities reached a plateau after the 3rd retention time (RT). The relatively high levels of alkalinities in all digesters were indicative of their respective good buffering capacities and the establishment of anaerobic fermentation (Gerardi, 2003). Mixed digesters during both mesophilic and thermophilic digestion exhibited higher alkalinities (5130 mg l^{-1} and 4763 mg l^{-1} respectively) than primary digesters (2720 mg l^{-1} and 3700 mg l^{-1} respectively) throughout the entire period of digestion. Total alkalinity values for both mesophilic and thermophilic trials were within the optimum (1500-3000 mg l^{-1}) or accepted values for methane-forming bacteria (Gerardi, 2003; Metcalf and Eddy, 2003).

Redox potential (ORP) at the start of the experiment (after the stabilization period) was circa -300 mV. Redox decreased in all types of digesters with increasing digestion period. Redox was within the optimum range (-200 to -400 mV) for both survival and

substrate degradation and the proper activity of methane-forming bacteria (Gerardi, 2003) during the entire digestion periods. Mesophilic digestion exhibited slightly higher (less negative) redox potential than thermophilic digestion. Good correlations obtained between the 2 primary mesophilic digesters (0.914, p=0.011) and the 2 mixed mesophilic (0.998, p=0.000) digesters.

4.1.2. Anaerobic digesters efficiency

Overall, organic loading rates (OLR) were lower during mesophilic digestion trials than during the thermophilic trials. Primary mesophilic digesters (1.3 ± 0.1 kg VS m^{-3} d^{-1}) were receiving circa 1.5 times lower volatile solids than the respective primary sludge for the thermophilic trial (1.9 kg VS m^{-3} d^{-1}) (Table 4-1). Mixed mesophilic digester OLR (1.5 ± 0.1 kg VS m^{-3} d^{-1}) was circa 1.6 times lower than it were during thermophilic digestion (2.5 kg VS m^{-3} d^{-1}). The organic loading rates for both sludge type mesophilic digesters (0.8-1.6 kg VS m^{-3} d^{-1}) were within the typical values (Metcalf and Eddy, 2003; CIWEM, 1996) as it was the case for the respective thermophilic (1.9 -3.0 kg VS m^{-3} d^{-1}) digesters.

With regards the volatile solids (VS) content between the primary (36.5 g l^{-1}) and the mixed (44.0 g l^{-1}) feed sludges for the mesophilic digesters were statistically different ($p<0.05$) according to the two-sample t-test (Appendix III). Statistically significant differences of the volatile solids content between primary (29.2 g l^{-1}) and the mixed (38.1 g l^{-1}) feed sludges for the thermophilic digesters were also observed at the ($p<0.05$) level of significance (Appendix III), according to the two-sample t-test.

The highest VS removals (%) were achieved during the mesophilic digestion. Primary digesters yielded the highest VS removals compared to the mixed digesters (Table 4-1). Primary mesophilic digesters achieved the highest VS removals (50.2% ± 0.6%), after the 3rd RT, followed by the respective mixed digesters (40.8% ± 0.6%) which were in accordance with typical or better VS removals under mesophilic conditions (40-50%) (Metcalf and Eddy, 2003; CIWEM, 1996).

A slight increase in VS removals after the 3rd RT, was achieved within the primary mesophilic digesters. A paired t-test (Appendix III) confirmed that VS removal differences between primary mesophilic digesters were insignificant at ($p>0.05$). Thermophilic digestion of primary sludge showed a slight increase of VS removal post the 5th RT. Volatile solids removals in the mixed sludge mesophilic digesters demonstrated similar VS removals after the 2nd RT for the entire digestion period with an exemption of an increase during the 5th RT. A paired t-test (Appendix III) confirmed that VS removal differences between mixed mesophilic digesters were insignificant at ($p>0.05$). Correlations obtained within the primary mesophilic digesters regarding the VS removals were strong (0.968, $p=0.002$) (Appendix III). Digestion of primary sludge under thermophilic conditions achieved higher VS removals (39.2% ± 1.3%) than the respective digestion of mixed sludge (32.7% ± 1.2%). Volatile solids removals were inversely proportional to the VS loadings in each digester (Table 4-1).

Total solids contents between the two respective feed primary sludges of the mesophilic and thermophilic digesters were observed to differ significantly ($p<0.05$) (Appendix III). Likewise, their respective volatile solids contents (Appendix III) were also statistically different ($p<0.05$). Differences between the mixed feed sludges of the mesophilic and thermophilic digesters in terms of their total solids and volatile solids, were similarly significant ($p<0.05$) according to the respective two-sample t-tests (Appendix III).

Total solids (TS) content of the feed primary (PS) sludge for the mesophilic digesters (51.1 g l^{-1}) were statistically different ($p<0.05$) to the TS content of the feed mixed (MS) sludge (57.1 g l^{-1}) according to the two-sample t-test (Appendix III). Similarly, the respective TS contents in primary (39.5 g l^{-1}) and mixed (49.7 g l^{-1}) feed sludges for the thermophilic digesters (Appendix III) were found to be significantly ($p<0.05$) different. The highest TS removals were achieved through the digestion of primary sludges. Mesophilic digesters yielded the highest TS removals (47.3% ± 8.5), after the 3rd RT, followed by the thermophilic digesters (37.04% ± 4.4), (Table 4-1). Mixed mesophilic digesters, after the 3rd RT, also yielded higher TS removals (33.7% ± 4.6) followed by the respective thermophilic digesters (29.85% ± 2.6). In general, TS removals were

according to typical values or better (30-35%) (CIWEM, 1996) with the exception of mixed sludge digestion under thermophilic conditions. Good correlations obtained between TS removals within the primary mesophilic digesters (0.946, p=0.004) (Appendix III).

Total solids removals in all digesters increased with increasing digestion period. Primary mesophilic digesters yielded to steep TS removals up to the 3rd RT, followed by a smoother TS removal increase. After the 3rd RT, a slight (4%) decrease in TS removals observed until the 5th RT, followed by an increase at the end of the digestion period. A paired t-test (Appendix III) confirmed that TS removal differences between primary mesophilic digesters were insignificant at ($p>0.05$). Thermophilic digestion of primary sludge showed a constant increase of TS removal, after the 2nd RT, for the entire digestion period. However, TS removals in the mixed sludge mesophilic digesters demonstrated similar TS removals after the 2nd RT for the entire digestion period. A paired t-test (Appendix III) confirmed that TS removal differences between mixed mesophilic digesters were insignificant at ($p>0.05$). Total solids removals of the mixed sludge thermophilic digestion remained similar after the 3rd RT, throughout the end of the digestion period. Good correlations were obtained between TS removals of the mixed mesophilic digesters (0.945, p=0.004) (Appendix III). According to the volatile solids (VS) content of each type of digester, biogas production was higher during thermophilic digestion due to the faster rate of digestion. Mixed sludge thermophilic digesters produced more biogas (1.62 l d^{-1}) than primary digesters (1.01 l d^{-1}). However, under mesophilic conditions the differences in biogas production between mixed and primary sludges were negligible (0.78 l d^{-1} against 0.76 l d^{-1}), respectively.

Primary mesophilic digesters produced higher methane values than the respective mixed digesters; however, that was not the case for the respective thermophilic digesters. The methane yield from the VS destruction was higher in mixed thermophilic digesters (0.74 m^3 ± 0.06 CH_4 $kgVS_{destroyed}^{-1}$) which was in accordance with VS loadings (2.5 kg ± 0.1kg m^{-3} d^{-1}) (Table 4-1), whereas the lowest value was observed for the primary thermophilic digesters (0.44 m^3 ± 0.07m^3 CH_4 $kgVS_{destroyed}^{-1}$). Primary mesophilic

digesters yielded higher methane (0.70 m^3 ± 0.09m^3 CH_4 $kgVS_{destroyed}^{-1}$) than the respective mixed digesters (0.60 m^3 ± 0.06m^3 CH_4 $kgVS_{destroyed}^{-1}$).

Primary mesophilic and mixed thermophilic digesters produced closer methane values to the typical values (~1m^3 $kgVS_{destroyed}^{-1}$) (Metcalf and Eddy, 2003) than the rest of the digesters. Since temperature is important in determining the rate of digestion, particularly the rates of hydrolysis and methane formation, the latter depends on the ratio of proteins, carbohydrates and lipids within the substrate (Stronach et al., 1986; Gerardi, 2003). Variations therefore in this ratio between primary and mixed sludges as well as the temperature of digestion could explain the observed differences and similarities regarding the methane production. However, the mean methane during the entire mesophilic digestion trials was greater than 75.0% ± 1.1% whereas for the thermophilic trials mean value for both sludge types was greater than 73.0% ± 0.6%. Methane content levels during mesophilic digestion were higher than typical methane values (Gerardi, 2003; CIWEM, 1996; Metcalf and Eddy, 2003) indicative of good methanogenic digestion.

Table 4-1 Feed sludge characteristics, anaerobic digestion performance at the sixth retention time and digestate quality.

	Mesophilic		Thermophilic	
Feed sludges	Primary sludge	Mixed sludge	Primary sludge	Mixed sludge
TS (g l^{-1})	51.1±3.7	57.1±4.3	39.5±0.1	49.7±0.1
VS (g l^{-1})	36.5±2.6	44.0±3.0	29.2±0.1	38.1±0.1
VFA (mg acetic acid l^{-1})	1314±68	1592±44	1168±98	1470±52
Operational conditions				
T (^{o}C)	35±0.2	35±0.2	53±0.2	53±0.2
SRT (d)	30	30	15	15
OLR (kg VS m^{-3} d^{-1})	1.3±0.1	1.5±0.1	1.9±0.0	2.5±0.0
TS (g l^{-1})	26.7±2.3	38.5±1.3	22.7±1.8	33.9±1.3
VS (g l^{-1})	19.5±1.6	23.9±2.0	11.5±4.5	22.0±2.2
pH	7.1±0.1	7.5±0.1	7.2±0.0	7.6±0.1
ORP (mV)	-320.8±12.8	-380.6±29.8	-411.6±36.9	-419.0±34.9
VFA (mg acetic acid l^{-1})	76.4±7.3	132.9±17.3	1098.5±189.6	829.3±145.9
Total alkalinity (mg l^{-1})	2399±37	5362±63	4000±453	4770±85
Biogas				
Daily production (l d^{-1})	0.8±0.0	0.8±0.1	1.0±0.1	1.6±0.1
GRP (m^3 m^{-3} d^{-1})	0.51±0.0	0.52±0.0	0.67±0.0	1.08±0.0
SGP (m^3 CH_4 kg $VS^{-1}_{removed}$)	0.7±0.1	0.6±0.1	0.4±0.1	0.7±0.1
Biogas yield (m^3 kg $VS^{-1}_{removed}$)	0.95±0.2	0.80±0.1	0.60±0.1	1.02±0.1
Removal efficiencies (%)				
VS	53.5±6.9	40.1±2.1	43.2±3.0	32.4±1.0
TS	47.3±8.5	33.7±4.6	37.0±4.4	29.8±2.6
g $VS_{removal}$ d^{-1}	1.07±0.1	0.98±0.1	2.24±0.2	1.84±0.1

Mean values (*n*=4) and standard error (±); TS: total solids; VS: volatile solids; VFA: volatile fatty acids; T: temperature; SRT: solids retention time; OLR: organic loading rate; ORP: oxidation-reduction potential; GPR: gas production rate; SGP: specific gas production.

5. RESULTS: FATE OF STEROID ESTROGENS DURING ANAEROBIC DIGESTION (SIX RETENTION TIMES)

5.1. Feed characterization for the primary and mixed sludges for mesophilic and thermophilic anaerobic digesters

Steroid estrogens were obtained from the combined (aqueous and solid phases) freeze-dried feed sludge samples; hence, the concentrations refer to the total amount of each analyte present in each feed sample. Three individual samples (n=3) from each sludge type (primary or mixed) were extracted and analysed for steroid estrogens. The respective concentrations obtained were then combined to produce an average value (arithmetic mean) representative for each type of feed i.e. primary or mixed feed. The feed concentrations represent the start concentrations or the influent. The individual concentrations from each digester are presented in Appendix IV.

5.1.1. Primary sludge

The observed background mean concentration of E1 in primary sludge over the two sampling periods was 111 ± 62-184 μg kg^{-1} dw. The mean background E3 concentration in primary sludge was 7 ± 5-9 μg kg^{-1} dw whereas the mean background concentration of E2 was 8 ± 5-10 μg kg^{-1} dw. Mean background EE2 concentration in primary sludge was 14 ± 8-23 μg kg^{-1} dw whereas, mean background concentration of E1-3S in primary sludge was 6 ± 3.5-8 μg kg^{-1} dw.

The sum of all steroid estrogen concentrations (ΣEST) in primary sludge for the first sampling period (mesophilic digestion) was 202 ± 8-158 μg kg^{-1} dw whereas the sum for the second sampling period (thermophilic digestion) was 89 ± 4-64 μg kg^{-1} dw.

Overall, mean E1 background concentration for the mesophilic trials (first sampling period) was 2.5 times higher than the feed for the thermophilic trials (second sampling period). The respective feed contributions of E1 to the sum of steroid estrogens (ΣEST) were 78% and 77% in primary sludge for the mesophilic and thermophilic trials, respectively (Figure 5-1). E1 was the dominant steroid estrogen in both primary sludges.

Mean background concentrations of E3 and E2 were higher in the mesophilic feed by a factor of 1.5 compared to the thermophilic feed. E3 contribution was 4% for the mesophilic feed and 3% for the thermophilic feed whereas the contribution of E2 was 5% and 7%, respectively. Mean concentrations of EE2 and E1-3S in the feed for the mesophilic trials were higher by a factor of 2 compared to the thermophilic feed. The contribution of EE2 was 9% and 11% whereas the contribution of E1-3S was 4% and 2% for the mesophilic and thermophilic feeds, respectively.

5.1.2. Mixed sludge

Mean concentration of E1 in mixed sludge over the two sampling periods was 60.8 ± 30-97 μg kg^{-1} dw. The mean background E3 concentration in mixed sludge was 6.5 ± 2-6 μg kg^{-1} dw, whereas mean E2 background concentration in mixed sludge was 4.7 ± 3-7 μg kg^{-1} dw. Mean background EE2 concentration in mixed sludge was 10 ± 10-11 μg kg^{-1} dw whereas mean background concentration of E1-3S was 5.4 ± 4-8 μg kg^{-1} dw. The sum of all steroid estrogen concentrations (ΣEST) in mixed sludge for the first sampling period (mesophilic digestion) was 120 ± 6-89 μg kg^{-1} dw whereas the sum for the second sampling period (thermophilic digestion) was 55 ± 3-32 μg kg^{-1} dw.

Mean E1 background concentration for the mesophilic trials was 3 times higher than the feed for the thermophilic trials. The feed contributions of E1 to the sum of steroid estrogens (ΣEST) were 74% and 69% (Figure 5-1). E1 was the dominant steroid estrogen in mixed sludges as it was the case in the primary sludges.

Regarding the mean background E3 concentration in the feed for the mesophilic trials, it was higher by a factor of 1.5 compared to the mixed sludge feed for the thermophilic trials. The contribution of E3 to the respective feeds was 6% and 4%. With respect to E2, the mean concentration was higher by a factor of 2 in the feed for the mesophilic trials than it was in the feed for the thermophilic trials. The contribution of E2 in the respective feed sludges was 5% and 6%, respectively. With regards the mean EE2 concentration, it was higher by a factor of 1 in the mesophilic feed than it was in the thermophilic feed. Contribution of EE2 in the mesophilic feed was 8% whereas its contribution to the thermophilic feed was higher (19%). The mean E1-3S concentration was higher by a factor of 1.6 in the mesophilic feed than it was in the thermophilic feed and its contribution was 6% and 2%, respectively.

Higher concentrations of natural and synthetic steroid estrogens were observed in the first sampling period (April 2007) than in the second sampling period (April 2008) presumably due to the different sampling periods.

Overall, steroid estrogen levels in the primary feed sludge were found in greater amounts than in the mixed feed sludge by a folder of 1.7. This observation is typical since mixed sludge had undergone some degree of biotransformation/biodegradation compared to primary sludge. Mixed sludge contained the aerobically treated sludge i.e. surplus activated sludge (40% by volume) and hence explains the greatest E1 concentration. Concentrations of steroid estrogens in the mixed feed sludge were in the order of (E1>EE2>E3>E1-3S≈E2) whilst primary feed sludge demonstrated similar trend (E1>EE2>E2≈E3>E1-3S) which is indicative of biodegradation/ biotransformation. There are two possible explanations for this phenomenon, namely the consequence of the biologically active returned liquors or sludge to the head of the works and/or biodegradation/biotransformation in the sewerage system.

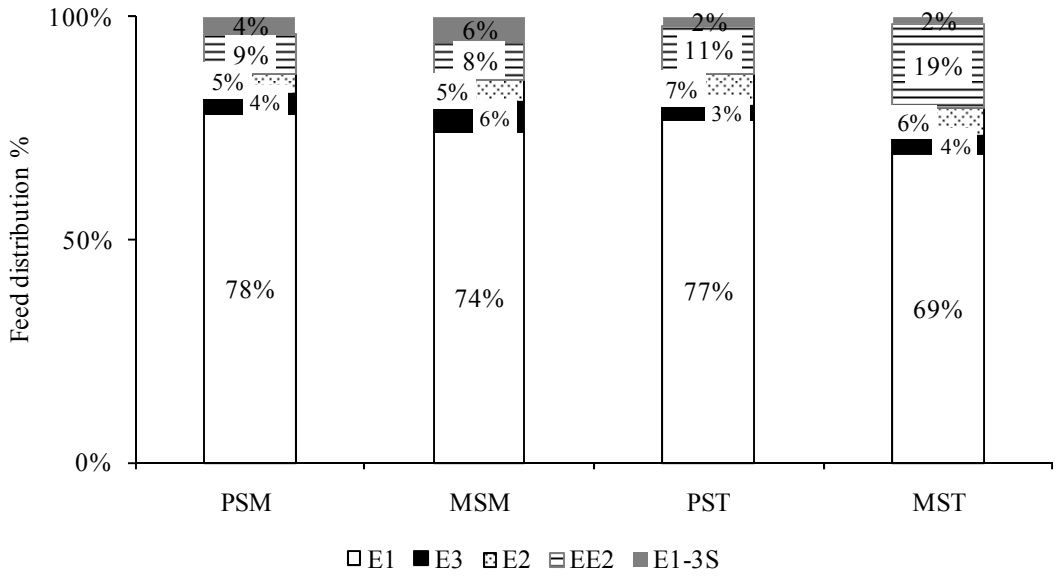


Figure 5-1 Mean (*n*=3) composition of steroid estrogens for the mesophilic trials in the primary (PSM) and mixed feed sludges (MSM) and the thermophilic trials (*n*=3) in primary (PST) and mixed feed sludges (MST), respectively.

5.2. Trends for steroid estrogens during anaerobic digestion of primary and mixed sludges under mesophilic and thermophilic conditions

This chapter reports the results from the mesophilic and thermophilic trials for steroid estrogens over 180 and 90 days, respectively. The aim was to understand if there were any differences in terms of removals in relation to different sludge types i.e. primary or mixed (60:40 v/v, primary sludge: SAS) sludge during mesophilic and thermophilic digestion temperatures. The first (*H1*E) and second research hypotheses (*H2*E) were:

*H1*E_0: Anaerobic digestion of steroid estrogens by using mixed or primary sludge will not result to significantly different mass removal.

*H1*E_α: Anaerobic digestion of steroid estrogens by using mixed or primary sludge will result to significantly different mass removal.

*H2*E_0: Digestion temperature will not result to significant effect on steroid estrogen removals.

*H2*E_α: Digestion temperature will result to significant effect on steroid estrogen removals.

The objectives were to:

1. Examine the effect of primary and mixed sludge (60:40 v/v, primary sludge: SAS) on steroid estrogen removals during anaerobic digestion.

2. Investigate the effects of mesophilic ($35^oC \pm 0.2^oC$) and thermophilic ($53^oC \pm 0.2^oC$) digestion on steroid estrogen removals.

At the end of each retention time i.e. 30 days for the mesophilic and 15 days for thermophilic digestion, a composite sample from each individual digester was used for the quantification of steroid estrogens. The composite sample was comprised of all digestates collected from each individual digester over a period of the last week of each

retention time, respectively and were kept frozen (-26º ± 1.5ºC) until analysis. Each digestate (50 ml) was extracted and analysed twice for steroid estrogen concentrations to produce an average value (n=4), for mesophilic digesters and (n=2) for the thermophilic digesters representative for each type of digester i.e. primary or mixed sludge digester. All values reported represent the mean values of steroid estrogens from the primary or the mixed digesters, respectively. The individual concentrations from each separate digester are presented in Appendix IV. Effluent refers to digestate i.e. total solids and aqueous phase combined and similarly, influent refers to the feed i.e. total solids and aqueous phase combined.

5.2.1. Primary sludge (mesophilic digestion)

Primary sludge mesophilic (30 days) digestates, for each retention time, are shown in (**Error! Reference source not found.**). The sum of the concentration of all steroid estrogens (ΣEST) at the end of the 1st retention time (RT) was 167 µg kg^{-1} dw. A reduction of 35 µg kg^{-1} dw from the feed concentration. Concentrations of ΣEST at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT were 132 µg kg^{-1} dw, 112 µg kg^{-1} dw, 104 µg kg^{-1} dw, 105 µg kg^{-1} dw and 101 µg kg^{-1} dw, respectively.

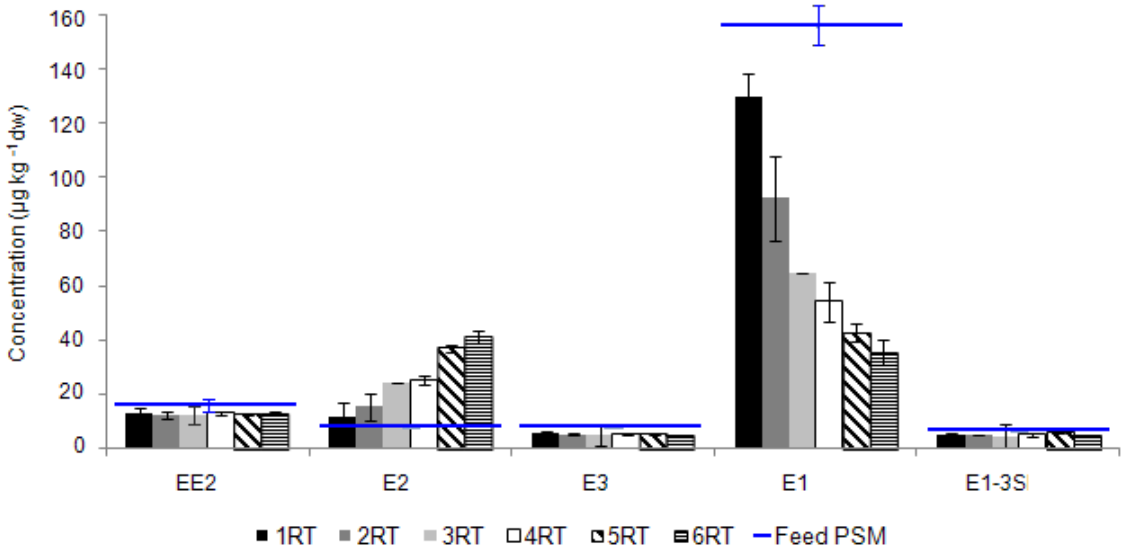


Figure 5-2 Mean (*n*=4) trends of steroid estrogen concentrations (µg kg^{-1} dw ± SE) per retention time (30 days) during mesophilic digestion (35°C ± 0.2°C) of primary sludge (PSM). The start concentration is displayed as follows: ⊣⊢.

At the 1st RT, E1 was reduced by 28 µg kg^{-1} dw from the feed concentration (158 µg kg^{-1} dw) and reached 130 µg kg^{-1} dw. Concentration of E1 at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 93 µg kg^{-1} dw, 65 µg kg^{-1} dw, 54 µg kg^{-1} dw, 43 µg kg^{-1} dw and 36 µg kg^{-1} dw, respectively. Overall, E1 indicated a progressive and steady reduction in concentration during the entire period of mesophilic digestion. The change in concentration of -123 µg E1 kg^{-1} dw, which resulted from the difference between the

effluent obtained from the 6th retention time and the influent levels, is indicative of the significant reduction potential of E1 in primary sludge.

The mass reduction of E1 at the 6th retention time during mesophilic digestion of primary sludge was 3.6 μg d^{-1}. Interestingly, during the entire mesophilic digestion period, a significant mass of E1 was biodegraded (14.4 μg d^{-1}) whilst less (2.4 μg d^{-1}) was biotransformed to E2. It should be noted that biodegradation refers to E1 removed – E2 formed. This finding implies that more E1 was biodegraded than it was converted to E2. The conversion of E1 to E2 occurred progressively during the entire mesophilic digestion and the respective masses were equilibrated during the 6th retention time. The reduction of E1 to E2 has also been manifested under various anaerobic lab scale set ups at pH 6.8-7.8 i.e. batch, continuous, psychrophilic, mesophilic and thermophilic experiments, according to (de Mes et al., 2008; Carballa et al., 2007; Carballa et al., 2006; Joss et al., 2004b). However, in terms of flux removal, E1 indicated 79% removal.

In contrast to E1, at the 1st RT, E2 concentration increased by 3 μg kg^{-1} dw from the feed concentration (9 μg kg^{-1} dw) and reached 12 μg kg^{-1} dw. Concentration of E2 at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 16 μg kg^{-1} dw, 25 μg kg^{-1} dw, 25 μg kg^{-1} dw, 37 μg kg^{-1} dw and 42 μg kg^{-1} dw, respectively.

Overall, E2 indicated a progressive increase in concentration during the entire period of mesophilic digestion (**Error! Reference source not found.**) and it was the dominant estrogen at the 6th retention time. The accumulation potential of E2 during the mesophilic digestion of primary sludge was significant as it was indicated from the difference of 32 μg E2 kg^{-1} dw i.e. between the effluent obtained from the 6th retention time and the influent levels. It should be noted that the increase of 32 μg E2 kg^{-1} dw, was attributed to the biotransformation of E1 to E2 since the feed E2 concentration remained constant i.e.9 μg kg^{-1} dw.

However, the mass of the accumulated E2 at the end of the mesophilic digestion period (6th retention time) of primary sludge was 0.9 μg d^{-1} and E2 indicated a negative flux removal (accumulation) of -324%. The contribution to the mass of E2 (2.4 μg d^{-1})

during the entire period of mesophilic digestion was congregated from the biotransformation of E1. Interestingly, the sum of E1 and E2 (ΣE1+E2) decreased with increasing digestion period. This finding implies that both E1 and E2 were biodegraded in primary sludge. Furthermore, it demonstrates a) the presence of suitable substrates and nutrients to promote the growth of certain bacteria to degrade E1 and E2 b) a substantial diversified anaerobic consortia in primary sludge and c) delayed acclimation to E1 and E2. Sludge adaptation for the increasing removal of ΣE1+E2 has also been reported by (Carballa, 2006) during continuous mesophilic (37.5^{o}C ± 0.5^{o}C, 20 days retention time, pH=7.8) anaerobic digestion of mixed sludge (70:30 v/v primary:SAS). In contrary, (de Mes et al., 2008; Czajka & Londry, 2006) did not observe a reduction of ΣE1+E2 under anaerobic conditions.

Regarding E3, its concentration at the 1st RT (6 μg kg^{-1} dw) reduced by 3 μg kg^{-1} dw from the feed concentration (9 μg kg^{-1} dw). For the remaining digestion period, the concentration of E3 indicated recalcitrance and remained unchanged (6 μg kg^{-1} dw) as opposed to E1 and E2. Overall, the mass reduction of E3 at the end of the mesophilic digestion period of primary sludge was 0.1 μg d^{-1} or 45% flux removal.

The concentration of E1-3S at the 1st RT (5.5 μg kg^{-1} dw) was reduced by 2 μg kg^{-1} dw from the feed concentration (7.6 μg kg^{-1} dw). For the remaining digestion period, E1-3S remained unchanged and recalcitrant as it was the case for E3. At the end of the mesophilic digestion period of primary sludge the mass reduction of E1-3S was 0.1 μg d^{-1}. In terms of flux removal, E1-3S removed by 36%.

At the 1st RT, EE2 concentration (13 μg kg^{-1} dw) was reduced by 5 μg kg^{-1} dw from the feed concentration (18 μg kg^{-1} dw) whilst for the remaining digestion period, i.e. 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT its concentration remained unchanged as it was the case for E3 and E1-3S. The change of -5 μg EE2 kg^{-1} dw which resulted from the difference between the 6th retention time effluent and the influent levels indicated the recalcitrant character of EE2 in these conditions. These results are in agreement with (de Mes et al., 2008) that EE2 is persistent under anaerobic conditions but in contrary with (Carballa et al., 2006) which observed EE2 removal with sludge adaptation. However,

at the end of the mesophilic digestion, the mass of EE2 was reduced by 0.2 μg d^{-1} and indicated 33% flux removal.

5.2.2. Primary sludge (thermophilic digestion)

Primary sludge thermophilic (15 days) digestates, for each retention time, are shown in (Figure 5-3). The sum of the concentration of all steroid estrogens (ΣEST) at the end of the 1st retention time (RT) was 60 µg kg^{-1} dw. A reduction of 29 µg kg^{-1} dw from the feed concentration. Concentrations of ΣEST at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT were 58 µg kg^{-1} dw, 39 µg kg^{-1} dw, 38 µg kg^{-1} dw, 41 µg kg^{-1} dw and 46 µg kg^{-1} dw, respectively.

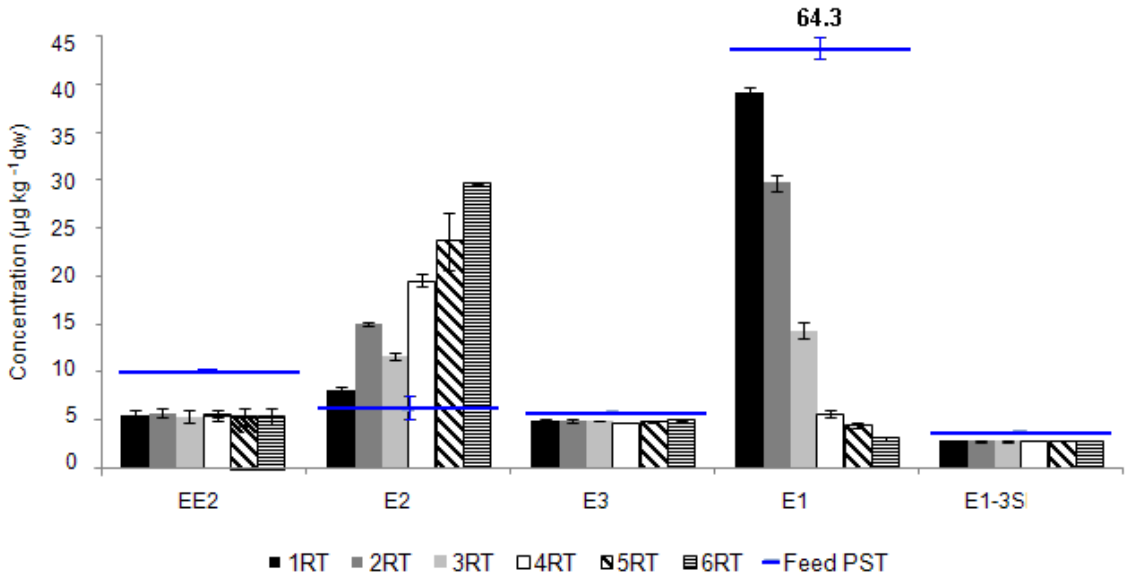


Figure 5-3 Mean (*n*=2) trends of steroid estrogen concentrations (µg kg^{-1} dw ± SE) per retention time (15 days) during thermophilic digestion (53°C ± 0.2°C) of primary sludge (PST). The start concentration is displayed ⊣⊢.

During thermophilic digestion of primary sludge, concentration of E1 (39 µg kg^{-1} dw) at the 1st RT reduced from the feed concentration (64 µg kg^{-1} dw). Concentration of E1 for the remaining digestion period was 30 µg kg^{-1} dw, 14 µg kg^{-1} dw, 6 µg kg^{-1} dw, 4 µg kg^{-1} dw and 3 µg kg^{-1} dw at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT, respectively. Although E1 indicated a progressive reduction during the entire thermophilic digestion period, its reduction was more pronounced during the first three retention times presumably due to higher bacterial activity at the initial digestion period. In contrast,

during mesophilic digestion, E1 reduction was steady throughout the entire digestion period implying a more balanced bacterial community in these digesters.

The concentration of E1was significantly reduced at the end of the thermophilic digestion period of primary sludge.

The reduction of E1 in primary sludge under thermophilic conditions is in agreement with its reduction under mesophilic conditions. Overall, during the entire 90 days of thermophilic digestion, E1 was partially biodegraded by 12.5 μg d^{-1} whereas a smaller amount of mass (3.5 μg d^{-1}) was converted to E2. This finding is in agreement with the mesophilic trial that more E1 was biodegraded than it was biotransformed to E2 under thermophilic conditions. In addition, the reduction of E1 to E2 is consistent with (de Mes et al., 2008; Carballa et al., 2007; Carballa et al., 2006; Joss et al., 2004b) but contradictory to (Czajka & Londry, 2006) where they observed production of E1 from the spiked E2, under anaerobic conditions. Interestingly, at the last three retention times, the concentration of E1 hardly changed whereas the concentration of E2 was increasing with time. One possible explanation is the intrinsic high endogenous death rates that are usually associated with the thermophilic anaerobes that lead to the lack of bacterial diversity. In terms of flux removal, E1 was removed by 96%.

Concentration of E2 at the 1st RT, was increased by 2 μg kg^{-1} dw from the feed concentration (6 μg kg^{-1} dw) and reached 8 μg kg^{-1} dw. Concentration of E2 at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 15 μg kg^{-1} dw, 12 μg kg^{-1} dw, 19 μg kg^{-1} dw, 24 μg kg^{-1} dw and 30 μg kg^{-1} dw, respectively. As was seen in the mesophilic digesters, E2 concentration was increased during the entire thermophilic digestion period (Figure 5-3) and E2 was the dominant estrogen at the 6th retention time. The biotransformation potential of E1 to E2 at thermophilic digestion temperature was apparent during digestion of primary sludge. Interestingly, this conversion was manifested faster than the mesophilic digestion possibly because of the considerable faster rate of thermophilic digestion, the higher bacterial activity and the faster acclimation period.

Overall, the accumulated mass of E2 at the end of the thermophilic digestion period of primary sludge was 1.2 μg d^{-1} and indicated a negative flux removal (accumulation) of -

367% . The contribution of E1 to E2 accounted for 0.8 μg d^{-1} for the entire thermophilic digestion of primary sludge.

Under thermophilic conditions, ΣE1+E2 indicated similar trend as it was during mesophilic conditions. The decreasing mass of ΣE1+E2 with increasing digestion period implied sludge adaptation and abundance of E1 and E2 eliminating bacteria at thermophilic temperatures and conditions. This observation is in agreement with the mesophilic digestion of primary sludge and with (Carballa et al., 2006) but in dispute with (de Mes et al., 2008; Czajka & Londry, 2006) that did not observe a reduction of ΣE1+E2 under anaerobic conditions.

However, E3 concentration at the 1st RT was reduced by 2 μg kg^{-1} dw from the feed concentration (6 μg kg^{-1} dw) and reached 5 μg kg^{-1} dw and remained unchanged until the end of the digestion period.s it was seen in the mesophilic digesters, E3 indicated recalcitrance under thermophilic conditions. The mass reduction of E3 at the end of the thermophilic digestion period of primary sludge was 0.05 μg d^{-1} and its flux removal was 17%.

The concentration of E1-3S at the 1st RT (3 μg kg^{-1} dw) was reduced by 1 μg kg^{-1} dw from the feed concentration (4 μg kg^{-1} dw). For the remaining of the digestion period, i.e. 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT, E1-3S concentration remained unchanged and at low levels (3 μg kg^{-1} dw). Low flux removal (30%) was demonstrated by E1-3S.

The concentration of EE2 at the 1st RT (5 μg kg^{-1} dw) was reduced from that of the feed concentration (9 μg kg^{-1} dw) and remained unchanged for the entire digestion period. In general EE2 indicated recalcitrance under thermophilic conditions as it was the case under mesophilic conditions. Overall, the mass reduction of EE2 at the end of the thermophilic digestion period of primary sludge was 0.2 μg d^{-1} and indicated 44% flux removal.

5.2.3. Mixed sludge (mesophilic digestion)

Mixed sludge mesophilic (30 days) digestates, for each retention time, are shown in (Figure 5-4). The sum of the concentration of all steroid estrogens (ΣEST) at the 1st retention time (RT) was 85 µg kg^{-1} dw. A reduction of 35 µg kg^{-1} dw from the feed concentration. Concentrations of ΣEST at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT were 64 µg kg^{-1} dw, 67 µg kg^{-1} dw, 66 µg kg^{-1} dw, 73 µg kg^{-1} dw and 72 µg kg^{-1} dw, respectively.

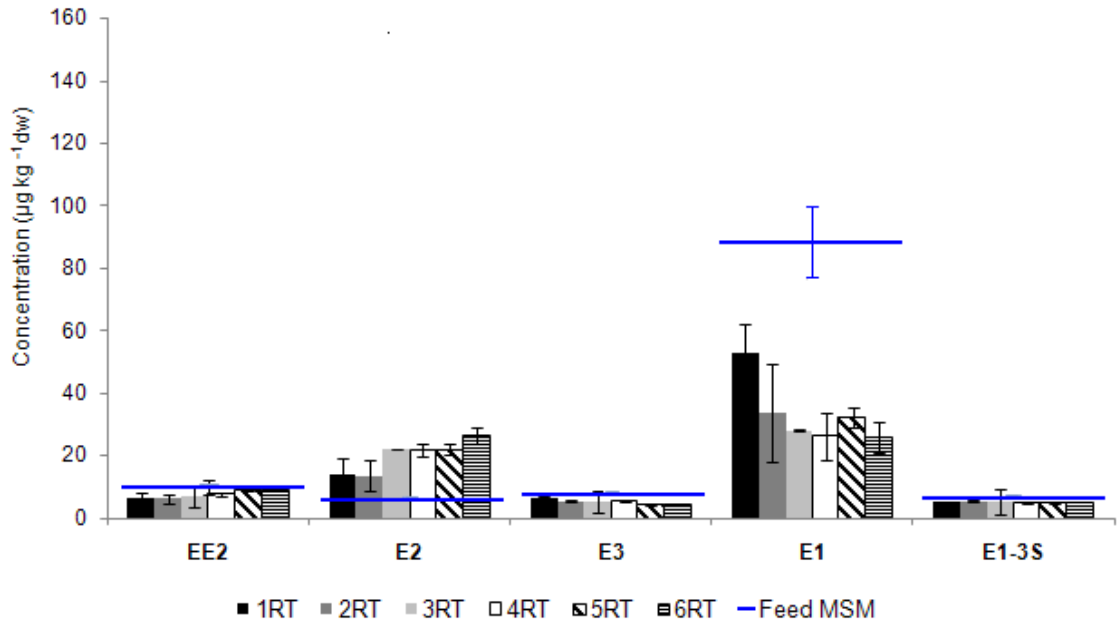


Figure 5-4 Mean (*n*=4) trends of steroid estrogen concentrations (µg kg^{-1} dw ± SE) per retention time (30 days) during mesophilic digestion (35°C ± 0.2°C) of mixed sludge (MSM). The start concentration is displayed ⊣⊢.

During mesophilic digestion of mixed sludge, at the 1st RT, E1 was reduced (53 µg kg^{-1} dw) from the feed concentration (90 µg kg^{-1} dw). Concentration of E1 at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 34 µg kg^{-1} dw, 28 µg kg^{-1} dw, 26 µg kg^{-1} dw, 32 µg kg^{-1} dw and µg kg^{-1} dw, respectively. Overall, E1 indicated a significant reduction in concentration only at the 1st RT and it was not mirrored in the subsequent retention times during the mesophilic digestion of mixed as it was observed in primary sludge. It

should be noted that the concentration of the 2nd retention time digestate was associated with a large standard error. However, at the 6th retention time E1 was the dominant steroid estrogen as it was the case for E2.

The change in concentration of -63 μg E1 kg^{-1} dw between the 6th retention time and the feed indicated the overall reduction of E1 during the mesophilic digestion of mixed sludge.

The reduced mass of E1 at the 6th retention time was 1.6 μg d^{-1}. However, the overall biodegradation of E1 in mixed sludge was 7 μg d^{-1} whereas 2 μg d^{-1} were biotransformed to E2. More E1 was biodegraded in mixed sludge than it was biotransformed to E2. The biotransformation of E1 to E2 occurred faster in mixed sludge than it was in primary sludge possibly due to the shorter acclimation period of the mixed sludge bacteria to E1 and E2. However, the reduction of E1 to E2 is in agreement with (de Mes et al., 2008; Carballa et al., 2007; Carballa et al., 2006; Joss et al., 2004b). Flux removal of E1 was 70%.

However, E2 concentration at the 1st RT increased from the feed concentration (6 μg kg^{-1} dw) and reached 14 μg kg^{-1} dw. The concentration of E2 at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 14 μg kg^{-1} dw, 22 μg kg^{-1} dw, 22 μg kg^{-1} dw, 22 μg kg^{-1} dw and 27 μg kg^{-1} dw, respectively. Overall, E2 indicated a significant increase in concentration after the 2nd retention time of mesophilic digestion and it was the dominant steroid estrogen at the 6th retention time as it was the case for E1. The accumulated mass of E2 at the end of the digestion period (6th retention time) was 0.5 μg d^{-1} and this reflected the negative flux removal (accumulation) of -325%. Interestingly, during the 3rd and 4th retention times, the respective concentrations of E1 and E2 remained constant. This suggested possible biological inter-conversion between E1 and E2. The inter-conversion between E1 and E2 under anaerobic conditions, has been suggested by (Czajka and Londry, 2006). The ΣE1+E2 remained constant after the 2nd retention time which is in argument with primary sludge and suggests that no biodegradation occurred in mixed sludge under mesophilic conditions. One possible explanation for this phenomenon is that mixed sludge lacks the suitable substrates and nutrients to promote the growth of

certain bacteria to degrade E1 and E2. The lack of E1 and E2 biodegradation in mixed sludge is in agreement with (de Mes et al., 2008; Czajka & Londry, 2006) but contrary to other workers (Carballa et al., 2006) who observed the removal of ΣE1+E2 with sludge adaptation, under mesophilic anaerobic conditions.

E3 reduced to 6.5 μg kg^{-1} dw from the feed concentration (8 μg kg^{-1} dw) at the 1st RT. For the remaining of the digestion period, the concentration of E3 indicated recalcitrance and remained at low levels (5 μg kg^{-1} dw). In terms of mass, E3 indicated an insignificant reduction at the end of the mesophilic digestion period and indicated 43% flux removal which was in agreement with primary sludge during the mesophilic digestion.

Regarding E1-3S, its concentration at the 1st RT was reduced (5 μg kg^{-1} dw) from the feed concentration (7 μg kg^{-1} dw) whilst for the remaining period of digestion its concentration remained unchanged. The change in concentration of -1.5 μg E1-3S kg^{-1} dw, which resulted from the difference between the effluent obtained from the 6th retention time and the influent levels lied within the associated standard error. The mass reduction of E1-3S at the end of the digestion period was negligible and was in agreement with primary sludge. Flux removal was 21%.

The concentration of EE2 at the 1st RT (6 μg kg^{-1} dw) was reduced from the feed concentration (10 μg kg^{-1} dw^{1}) whereas for the remaining of the digestion period and towards the last retention times, its concentration indicated an increase. However, this obscure increase was associated with large standard errors. The persistence of EE2 in mixed sludge was in agreement with its persistence during primary sludge digestion as well as with (de Mes et al., 2008) but in argument with (Carballa et al., 2006). However, in terms of flux removal, EE2 was removed only by 4%.

5.2.4. Mixed sludge (thermophilic digestion)

Mixed sludge thermophilic (15 days) digestates, for each retention time, are shown in (Figure 5-5). The sum of the concentration of all steroid estrogens (ΣEST) at the 1st retention time (RT) was 47 µg kg^{-1} dw. A reduction of 8 µg kg^{-1} dw from the feed concentration. Concentrations of ΣEST at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT were 40 µg kg^{-1} dw, 45 µg kg^{-1} dw, 44 µg kg^{-1} dw, 45 µg kg^{-1} dw and 46 µg kg^{-1} dw, respectively.

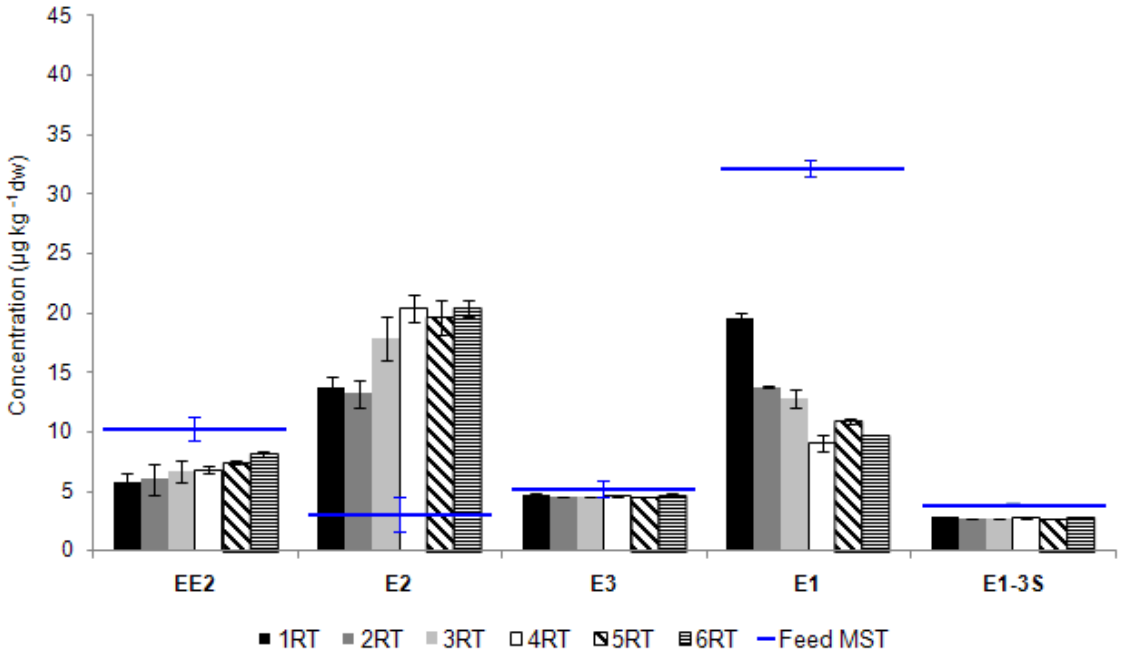


Figure 5-5 Mean (*n*=2) trends of steroid estrogen concentrations (µg kg^{-1} dw ± SE) per retention time (15 days) during thermophilic digestion (53°C ± 0.2°C) of mixed sludge (MST). The start concentration is displayed ⊣⊢.

During thermophilic digestion of mixed sludge, concentration of E1 (19.5 µg kg^{-1} dw) at the 1st RT, indicated a reduction from the feed concentration (32.3 µg kg^{-1} dw). Concentration of E1 at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 14 µg kg^{-1} dw, 13 µg kg^{-1} dw, 9 µg kg^{-1} dw, 11 µg kg^{-1} dw and 01 µg kg^{-1} dw, respectively. Overall, E1 indicated a progressive reduction in concentration during the entire period of

thermophilic digestion (Figure 5-5). At the 6th retention time E1 was not the dominant steroid estrogen as it was the case in mixed sludge under mesophilic conditions.

The change in concentration of -23 μg E1 kg^{-1} dw between the effluent obtained from the 6th retention time and the influent levels is indicative of the significant reduction potential of E1 in mixed sludge. Flux removal of E1 was 68%.

The overall biodegraded mass of E1 was 1.4 μg d^{-1} whereas 4.5 μg d^{-1} were converted to E2. This observation is not consistent with the mesophilic digestion of mixed sludge where more E1 was degraded than it was biotransformed to E2. However, the partial biodegradation of E1 was observed from the early stages of thermophilic digestion indicative of the presence of E1 degrading bacteria/enzymes.

The overall reduction of E1 to E2 in mixed sludge is in agreement with the literature (de Mes et al., 2008; Carballa et al., 2007; Carballa et al., 2006; Joss et al., 2004b) where these workers utilised activated sludge.

However, the concentration of E2 was increased at the 1st RT (14 μg kg^{-1} dw) from the feed concentration (3 μg kg^{-1} dw) as it was seen in the mesophilic digestion of mixed sludge. Overall, E2 indicated an increase in concentration, however, this increase was emphasised after the 2nd RT. At the 6th RT, E2 was the dominant steroid estrogen during the thermophilic digestion of mixed sludge. The accumulated mass of E2 at the end of the digestion period was 0.9 μg d^{-1} and this resulted to the negative flux removal (accumulation) of -621% as it was seen during the mesophilic digestion of mixed sludge.

During thermophilic digestion of mixed sludge, the ΣE1+E2 remained constant for the entire digestion period which is in close agreement with the mixed sludge mesophilic digestion but in contrary with the primary sludge digestion trials (mesophilic and thermophilic). This observation suggests that no biodegradation occurred in mixed sludge under thermophilic conditions. Since a similar trend of ΣE1+E2 was observed in the same sludge type during mesophilic digestion it could be assumed that mixed sludge lacks of the suitable substrates and nutrients to promote the growth of certain bacteria to

degrade both E1 and E2. In addition, certain chemical species originated from activated sludge, could have served as supplementary electron acceptors to promote the conversion of E2 to E1, which eventually equilibrated the biotransformation of E1 to E2. A variety of organic compounds, or the greater amount of carbonate CO_3^{2-} in mixed sludge due the higher total alkalinity compared to primary sludge, could have sustained the microbial conversion of E2 to E1.

However, the above observations are in agreement with (de Mes et al., 2008; Czajka & Londry, 2006) but contrary to other workers who (Carballa et al., 2006) observed the removal of ΣE1+E2 with sludge adaptation, under thermophilic anaerobic conditions.

The mass reduction of E3 in mixed sludge was negligible (0.01 μg d^{-1}) and accounted for 4% flux removal.

The concentration of E1-3S at the 1st RT (3 μg kg^{-1} dw) was reduced insignificantly from the feed concentration (4 μg kg^{-1} dw) and remained at low levels for the entire digestion period as it was seen in the mesophilic digestion. The reduction in concentration between the effluent obtained from the 6th retention time and the influent levels was within the associated standard error. The mass reduction of E1-3S at the end of the thermophilic digestion period was negligible (0.06 μg d^{-1}) which was in agreement with the mixed sludge mesophilic digestion and indicated 28% flux removal.

At the 1st RT, EE2 concentration (6 μg kg^{-1} dw) was reduced by 4 μg kg^{-1} dw from the feed concentration (10 μg kg^{-1} dw). For the remaining of the digestion period EE2 concentration indicated increase as it was the case with the mesophilic digestion of mixed sludge and similarly both the feed and the digestate concentrations were associated with large standard errors. The persistence of EE2 during the thermophilic digestion of mixed sludge was in agreement with the mesophilic digestion of the same sludge type as well as with its persistence during primary sludge digestion and other workers (de Mes et al., 2008). However, the persistence of EE2 during thermophilic digestion of mixed sludge is in contrary with (Carballa et al., 2006). Overall, the mass reduction of EE2 at the end of the thermophilic digestion period of mixed sludge was negligible (0.07 μg d^{-1}) and its flux removal was 15%.

5.2.5. Effect of digestion temperature on steroid estrogens

5.2.5.1. Primary sludge (mesophilic vs. thermophilic)

The efficiency between mesophilic and thermophilic digestion of primary sludge in terms of overall steroid estrogen removal (ΣEST) indicated that the mesophilic digesters demonstrated higher overall EST removal (ΣEST = 53%) compared to thermophilic digesters (ΣEST = 51%) despite their considerable faster rate of digestion (Figure 5-7).

The highest first order kinetic constants (*k*) for E1 in primary sludge digestion were observed during mesophilic digestion (k = 0.1161 d^{-1} at 35^{o}C) compared to thermophilic digestion (k = 0.0415 d^{-1} at 53^{o}C) (Table 5-2).

Both mesophilic and thermophilic bacteria indicated capability of E1 biodegradation as well as E1 biotransformation to E2 in the presence of primary sludge. Biodegradation of E1 (E1 removed – E2 formed), occurred with increasing digestion period under both digestion temperatures with different retention times. Moreover, biodegradation of E1 was more pronounced at thermophilic temperatures with 15 days retention time. Overall, the biodegradation trend of E1 indicated progressive sludge acclimation with time, particularly at the mesophilic temperature.

The highest flux rate of E1 biodegradation occurred by the mesophilic bacteria (-0.2402 μg d^{-1} RT^{-1}) regardless of the higher bacterial activity and the faster rate of digestion associated with thermophilic cultures (Gerardi, 2003; Metcalf and Eddy, 2003). Biodegradation flux rate of E1 in thermophilic digestion was lower by a factor of 3 compared to the mesophilic digestion. This phenomenon proposed that mesophilic E1 degrading bacteria in primary sludge were in plethora as opposed to thermophilic E1 degrading bacteria. There are two possible explanations for this phenomenon namely, the lack of the thermophilic bacterial diversity (limited E1 enzymes) due to their low growth rates and high endogenous death rates (Gerardi, 2003; Metcalf and Eddy, 2003) and in contrast the higher sludge retention time of the mesophilic cultures that promoted the establishment of E1 degrading bacteria.

Interestingly, the relationship between E2 formation and E1 removal rate ($E2_{formed}$ μg $E1_{removed}$ d^{-1}) indicated that this rate was faster (0.4463 μg $E2_{formed}$ μg $E1_{removed}$ d^{-1}) during thermophilic digestion despite the shorter sludge retention time, (please note, E1 removal does not refer to E1 (partial) biodegradation). However, this observation does not imply that more E2 was formed than it was degraded by thermophiles but rather emphasises the lack of thermophilic bacterial diversification and also their higher bacterial activity. This statement is based on the fact that the thermophiles were removing less E1 (flux removal) than the mesophiles (Figure 5-7).

Overall, more E1 was biodegraded than it was biotransformed to E2 during both temperature digestions.

Biotransformation of E1 to E2 is probably due to co-metabolite activity (non-growth linked reactions) as opposed to growth-linked reactions (metabolic reactions that commonly involved in energy or carbon sources for microbial growth), because steroid estrogens (like other micropollutants) are not present in high enough concentration to support substantial biomass growth (Yi & Harper, 2007; Yu et al., 2007). As co-metabolism requires a catalyst, then the higher rate of E2 formation per μg E1 removal d^{-1} in the thermophilic digesters could be possibly attributed to the higher digestion temperature.

Interestingly, the rate of formation of E2 (μg d^{-1} RT^{-1}) under thermophilic conditions was higher than it was under mesophilic conditions Table 5-1. Despite the growth and the non-growth linked reactions, this phenomenon could be explained by two possible explanations, namely the consequence of acclimation (more biodiversity) and the higher digestion temperature of the thermophiles, which could have resulted to the abundance of E1 enzymes required for its microbial conversion to E2. Similarly, the highest negative first order kinetic constants (k) for E2 were observed during mesophilic digestion (k = -0.0259 d^{-1} at 35°C) compared to the thermophilic digestion of primary sludge (k = -0.0145 d^{-1} at 53°C).

However, it has been demonstrated that ΣE1+E2 was decreasing over time in both temperature digestion trials by using primary sludge. Overall, the observed reduction of

E1 to E2 in primary sludge is consistent with the works of (de Mes et al., 2008; Carballa et al., 2006; Carballa et al., 2007). In addition, the persistence of E2 during both digestion temperatures is consistent with the general observations under anaerobic conditions (Ying et al., 2003; Lee & Liu, 2002) since it has been reported that E2 is degraded aerobically (D'Ascenzo et al., 2003a; Johnson & Sumpter, 2001b).

Whereas similar E2 flux removals were observed during mesophilic and thermophilic digestion of primary sludge, that was not the case for E1. This is in contrary to (Carballa et al., 2006) who observed similar ΣE1+E2 removal efficiencies of 85% ± 10% during mesophilic and thermophilic sludge digestion.

Higher E3 flux removals (45%) were obtained during mesophilic than the thermophilic digestion (17%) with kinetic constants (*k*) 0.0243 d^{-1} at 35ºC and 0.0205 d^{-1} at 53ºC, respectively. The persistence of E3 in primary sludge is consistent with the observations of (Czajka & Londry, 2006). These researchers observed the persistence of E3 during long batch incubation periods (over three years) (28ºC) of sludge (or lake sediment) under methanogenic conditions and by using different electron acceptors (nitrate, iron or sulphate) (Czajka & Londry, 2006).

E1-3S flux removals were slightly higher under mesophilic conditions (36%) than it was under thermophilic conditions (30%). First order kinetic constants (*k*) were similar in primary sludge i.e. 0.0161 d^{-1} at 35ºC and 0.0205 d^{-1} at 53ºC, respectively. However, no significant deconjugation of E1-3S was observed during anaerobic digestion of primary sludge and this suggests the limited abundance of arylsulphatase which is the enzyme involved in the hydrolysis of arylsulphate esters that has demonstrated some, deconjugation of E1-3S (Bandick & Dick, 1999). In fact, it is believed that E1-3S is aerobically degradable in the presence of activated sludge (Joss et al., 2004b). The deconjugation of E1-3S in mesophilic digestion accounted for 0.5 μg d^{-1} whereas in thermophilic digestion was 0.8 μg d^{-1}. Thermophilic anaerobic digestion achieved greater EE2 removals (44%) than mesophilic digestion (33%). The persistence of EE2 in primary sludge during mesophilic and thermophilic digestion is in agreement with (de Mes et al., 2008) but contrary to (Carballa et al., 2006). First order kinetic constants (*k*)

for EE2 in primary sludge during thermophilic digestion (k = 0.0519 d^{-1} 53^{o}C) were lower by a factor of 0.7 compared to the mesophilic digestion (k = 0.0701 days at 35^{o}C) (Table 5-2).

Generally, retention time had limited effect on E3, E1-3S and EE2 as it was demonstrated by their percentage distribution during the respective digestion periods.

Mesophilic digestion indicated higher rate on ΣEST removal per volatile solids (VS) removal (2.9 μg $\Sigma EST_{removal}$ g VS $_{removed}$ d^{-1}) than the thermophilic digesters (1.1 μg $\Sigma EST_{removal}$ g VS $_{removed}$ d^{-1}). However, the rate of VS removal under thermophilic conditions was 2.1 fold higher than mesophilic conditions and therefore this finding indicated that the mesophilic consortium was slightly better in terms of ΣEST removal per VS removal than the thermophiles.

Although mesophilic digesters removed 1.2 times more ΣESTs (μg d^{-1}) than the thermophilic digesters, this removal reflected the higher ΣEST loadings (μg d^{-1}) (Figure 5-7). Overall, the performance of the mesophilic digesters in terms of ΣEST removal was better compared to the thermophilic digestion of primary sludge.

5.2.5.2. Mixed sludge (mesophilic vs. thermophilic)

As it was seen in primary sludge, mesophilic digestion of mixed was more efficient for the overall steroid estrogen removal (ΣEST = 39%) than thermophilic digesters (ΣEST = 12%), despite their considerable faster rate of digestion (Figure 5-7).

The highest first order kinetic constants (*k*) for E1 in mixed sludge were observed during mesophilic digestion (k = 0.0811 d^{-1} at 35^{o}C) as opposed to the thermophilic digestion (k = 0.0459 d^{-1} 53^{o}C) (Table 5-2). Analogously, the same order from high to low was also observed in primary sludge.

The biodegradation rate of E1 in mixed sludge remained constant throughout the respective digestion periods and did not increase with time, as it was seen in primary sludge. The constant rates of E1 biodegradation throughout the digestion periods indicated restricted ability for E1 biodegradation i.e. partial biodegradation, when data are compared to primary sludge.

The constant trend of E1 biodegradation rate observed during mixed sludge digestion (particularly during the 15 days retention time), as opposed to an increasing trend, was probably attributed to the microbial conversion of E2 to E1.

It seemed that during mixed sludge digestion (regardless of temperature) the presence of electron acceptors other than those present in primary sludge promoted this conversion. On the other hand, another possible explanation to the constant E1 trend could be the lack of suitable substrates/nutrients to promote the growth of abundant bacteria/enzymes required to carry out specifically the biodegradation of E1. Interestingly, the removal of both E1 and E2 in mixed sludge seemed to plateau after the 2nd retention time which indicated faster acclimation of E1 and E2 by the mixed sludge consortium compared to the mesophiles.

Whilst the biodegradation rate of E1 during mesophilic digestion of mixed sludge was -0.0203 μg d^{-1} RT^{-1}, the same rate during thermophilic digestion indicated formation of

E1 (Table 5-1). This phenomenon could be attributed to the biological inter-conversion between E1 and E2 as has also been suggested by (Czajka & Londry, 2006).

The rate of E2 formation in relation to the rate of E1 removal by the mesophilic cultures was much greater than the rate in the thermophilic conditions i.e. 1.1842 μg $E2_{formed}$ μg $E1_{removed}$ d^{-1}, irrespective of the higher temperature. The higher rate by the mesophiles did not reflect the higher E1 influx in these digesters suggesting that the microbial conversion of E1 to E2 in thermophilic conditions was limited. The disappearance of E1 in the thermophilic trial was not contributed to the same degree to the formation of E2, as it did during mesophilic digestion, suggesting higher biodegradation potential of E1 under mesophilic conditions.

As it was seen in primary sludge, biotransformation of E1 to E2 was also manifested in the mixed sludge under mesophilic conditions. Similarly, biotransformation of E1 to E2 or the inter-conversion of E1 and E2 was probably due to co-metabolic activity as has been suggested by (Yi & Harper, 2007; Yu et al., 2007).

Overall, the opposing E1 and E2 trends observed during the mesophilic and thermophilic digestions could be probably explained by the different digestion temperatures and the associated retention times since both the substrate as well as all the measured physicochemical parameters, remained constant and at optimum levels throughout the respective digestion periods.

Interestingly, the rate of E2 formation (μg d^{-1} RT^{-1}) during thermophilic digestion was marginally higher than it was during mesophilic digestion (Table 5-1) indicative of the limitation of the conversion of E1 to E2 regardless of the digestion temperature and the retention time. Analogous negative first order kinetic constants (*k*) i.e. formation constants for E2 were observed i.e. k = -0.0511 d^{-1} at 53°C compared to k = -0.0255 d^{-1} at 35°C, respectively.

Since the sum of E1 and E2 during the different temperature digestions of mixed sludge indicated stability throughout the trials, this suggests that limited partial biodegradation occurred over time as opposed to the degree of the partial biodegradation observed in

primary sludge. This phenomenon further implied that irrespective of the retention time, mixed sludge lacks the suitable substrates and nutrients to promote the growth of certain bacteria capable to degrade E1 and E2 together. In addition, various electron acceptors and donors in mixed sludge could have promoted the inter-conversion of E1↔E2 and therefore restricted the overall removal. The lack of the partial biodegradation of E1 and E2 in mixed sludge is in agreement with (de Mes et al., 2008; Czajka & Londry, 2006) but contrary to other workers (Carballa et al., 2006) who observed the removal of ΣE1+E2 with sludge adaptation, under both mesophilic and thermophilic anaerobic conditions.

Whereas similar E1 flux removals were observed during the mesophilic and thermophilic digestion of mixed sludge, E2 flux removals were different which is in argument with (Carballa et al., 2006) who observed similar ΣE1+E2 removal efficiencies of 85% ± 10% during mesophilic and thermophilic mixed sludge digestion.

Much higher E3 flux removals (44%) were achieved by the mesophilic digesters than the thermophilic digesters (4%) and this was reflected on the first order kinetic constants (k) for mesophilic, k = 0.0226 d^{-1} at 35^{o}C and 0.0201 d^{-1} at 53^{o}C for the thermophilic digesters, respectively. Whereas the flux removals observed in mixed sludge are different to the primary sludge, higher E3 flux were achieved during mesophilic than thermophilic conditions in both sludge types. However, the persistence of E3 in mixed sludge is consistent with the long batch incubation experiments (over three years) (28ºC) by (Czajka & Londry, 2006).

Similarly, low E1-3S flux removals were observed during the different temperature digestion experiments of mixed sludge. Flux removals during mesophilic digestion were 21% whereas thermophilic digesters demonstrated 28% removals, possibly due to higher temperature. Irrespective of the flux removals, the deconjugation of E1-3S in mixed mesophilic sludge was 0.3 μg d^{-1} whereas 0.8 μg d^{-1} during thermophilic conditions. Interestingly the deconjugation of E1-3S was higher under thermophilic conditions than mesophilic conditions as it were the respective degradation constants (Table 5-2). This finding indicates the importance of temperature and it may further

imply that the arylsulphatase enzyme was present in both sludge types. Whereas it is believed that E1-3S is aerobically degradable (Joss et al., 2004b), there is supporting evidence that strictly anaerobic disulphate strains are capable of cleaving E1-3S (Johnson & Williams, 2004) and that this ability is believed to be associated with the use of sulphate as a terminal electron acceptor (Van Eldere et al., 1988). Thermophilic anaerobic digestion of mixed sludge demonstrated some low flux removals which is in agreement with (Johnson & Williams, 2004; Van Eldere et al., 1988).

Regarding EE2, only 5% was removed in mixed sludge during mesophilic digestion as opposed to 15% during thermophilic digestion. First order kinetic constants (*k*) were 0.0753 d^{-1} at 53^{o}C and 0.0021 d^{-1} at 35^{o}C. Overall, the persistence of EE2 in mixed sludge is in agreement with (de Mes et al., 2008) and consistent with the persistence in primary sludge, however, in contrary with the reported removals of (Carballa et al., 2006).

As it was observed in primary sludge, the retention time had little effect on the removal of E3, E1-3S and EE2 during mixed sludge digestion as it is demonstrated on their percentage distribution throughout the respective digestion periods.

Under mesophilic conditions, higher rate on ΣEST removal per volatile solids (VS) removal (1.2 μg $\Sigma EST_{removal}$ g $VS_{removed}$ d^{-1}) was observed than it was observed during thermophilic digestion (Table 5-1) of mixed sludge. Despite the fact that VS removal rate ($gVS_{removed}$ d^{-1}) in thermophilic digesters was 1.9 fold higher than it was in the mesophilic digesters, this finding indicated that mesophiles performed better in terms of ΣEST removal per VS removal than the thermophiles.

Mixed sludge mesophilic digesters were receiving 1 time more ΣEST (μg d^{-1}) than the thermophilic digesters, however the former digesters removed more ΣEST mass by a factor of 3.8 indicative of the much better performance of mesophilic over to thermophilic mixed sludge digesters in relation to ΣEST removals.

Table 5-1 Relationships between E1and E2 mass fluxes and biological rates.

	Mesophilic		Thermophilic	
	Primary sludge	Mixed sludge	Primary sludge	Mixed sludge
E1 removal rate μg d^{-1} RT^{-1}	-0.4111	-0.0870	-0.3826	-0.0713
r^2	0.88	0.52	0.90	0.76
E1 biodegradation rate μg d^{-1} RT^{-1}	0.2402	0.0203	0.1798	-0.0252
r^2	0.66	0.60	0.52	0.34
E2 formation rate μg d^{-1} RT^{-1}	0.1708	0.0667	0.2028	0.0965
r^2	0.96	0.84	0.93	0.84
μg $E2_{formed}$ μg $E1_{removed}$ d^{-1}	0.3517	0.4295	0.4463	1.1812
r^2	0.78	0.37	0.79	0.58
μg $E2_{formed}$ μg $E1_{degraded}$ d^{-1}	0.4276	0.1286	0.4615	-1.6571
r^2	0.53	0.59	0.51	0.82
μg $\Sigma EST_{removed}$ g VS $_{removed}$ d^{-1}	2.9	1.2	0.6 1.1	0.2
μg $\Sigma EST_{removed}$ g VS $_{content}^{-1}$	0.056	0.018	0.056	0.006

Note: Biodegradation refers to partial biodegradation i.e. microbial removal and not to mineralization.

Table 5-2 First order kinetic constants (*k*) for steroid estrogens in primary and mixed sludge during mesophilic and thermophilic anaerobic digestion trials.

	Mesophilic		Thermophilic	
	Primary sludge	Mixed sludge	Primary sludge	Mixed sludge
	k (per day)	*k* (per day)	*k* (per day)	*k* (per day)
E1	0.1161	0.0811	0.0415	0.0459
E2	-0.0259	-0.0255	-0.0145	-0.0511
E3	0.0243	0.0226	0.0205	0.0201
E1-3S	0.0161	0.0110	0.0205	0.0240
EE2	0.0701	0.0021	0.0519	0.0753

5.2.6. Effect of sludge type on steroid estrogens

5.2.6.1. Mesophilic digestion (primary sludge vs. mixed sludge)

Significantly, lower overall estrogen flux removal (ΣEST) was observed with the mixed sludge when compared to the primary sludge i.e. 39% and 53% during mesophilic digestion (Figure 5-7).

Overall, more E1 was biodegraded (partial biodegradation) (E1 removed – E2 formed) than it was biotransformed to E2, in the primary and mixed sludge mesophilic digestion trials, respectively. This finding indicated that both sludge types having 30 days retention time each were capable to carry out the biodegradation of E1. However, the biodegradation rate of E1 in primary sludge was 12 fold greater that of the mixed sludge (Table 5-1).

Deconjugation of E1-3S in mixed sludge was insignificant contributor to E1 and accounted only for 0.3 μg d^{-1} to the total E1. The partial biodegradation of E1 in primary sludge accounted for 14 μg d^{-1} whereas in mixed sludge it was 7 μg d^{-1}. When considering the influx of E1 in both sludge types, greater microbial conversion of E1 was achieved by the primary sludge as opposed to mixed sludge. Batch studies have indicated that E1 will not be completely removed in activated sludge (Johnson & Sumpter, 2001b) possibly because of the inter-conversion mechanism of E1↔E2. It therefore appears that the presence of SAS in the mixed sludge could explain the lower E1 removal.

As it was the case with the microbial removal of E1, by comparing the ratio of E1 biodegradation and E2 formation, the activity of each sludge type could be determined by inference. Primary sludge indicated greater biotransformation potential than mixed sludge (Table 5-3). The biotransformation in primary sludge accounted for 12 μg d^{-1} whereas in mixed sludge for 5 μg d^{-1}.

However, mixed sludge mesophilic consortium indicated a tendency to achieve a plateau between E1 and E2 faster than primary sludge. This phenomenon signifies that

the overall acclimation of mixed sludge for the whole E1↔E2 conversion was better than the primary sludge. This observation is based on the overall ability of mixed sludge to interconvert E1↔E2. A possible explanation for that phenomenon could be attributed to the presence of activated sludge (40% v/v) in mixed sludge. The aerobically treated activated sludge (oxidative process) could have promoted the conversion of E2 to E1. It is postulated that intrinsic bacteria/enzymes and/or electron acceptors in mixed sludge responsible for the conversion of E2 to E1 were over competed those bacteria/enzymes responsible for the reductive conversion of E1 to E2 and therefore a faster plateau was achieved in the mixed sludge compared to primary sludge.

Similar E3 flux removals were observed in primary (45%) and mixed sludges (44%). It should be noted that the concentration of E3 during the entire mesophilic trials was near or below the MDL for both sludge types. The ratio of the sum of E1+E2 and E3, which is the metabolic by-product, was higher at the 1st retention time compared to that at the 6th retention time in primary sludge. This demonstrated that E1 and E2 were removed and did not contribute to E3. In contrast, during mixed sludge digestion this ratio remained the same for the respective retention times which emphasised the inter-conversion of E1↔E2 and that these steroid estrogens were not contributed to E3 (Table 5-3).

On the other hand, the deconjugation of E1-3S to E1 was limited and similar for both sludge types and contributed by 0.5 μg d^{-1} and 0.3 μg d^{-1} in primary and mixed sludge, respectively. Overall, slightly higher flux removals were achieved in primary sludge (36%) than in the mixed sludge (21%).

Similarly, higher EE2 flux removals were observed in primary sludge (33%) than in mixed sludge (5%) that emphasised the importance of the substrate differences. Significantly higher EE2 removals (85%) were reported by (Carballa et al., 2006) during mesophilic (37^{o}C) mixed sludge (70% primary and 30% SAS) digestion. However, the reported removals accounted for the spiked EE2 concentrations plus the background feed concentrations. Regardless of the long retention time (30 days), the growth of appropriate bacteria capable of degrading EE2 in both sludge types was

limited. The results obtained from both sludge types are in agreement with (Koh, 2008) that the significant removal of EE2 occurs only under aerobic conditions and with (Vader et al., 2000) which have showed that the EE2 degradation capability of sludge was correlated with the nitrifying activity.

The rate of ΣEST removal per VS removal in primary sludge was higher (2.9 μg $\Sigma EST_{removal}$ g $VS_{removed}$ d^{-1}) compared to mixed sludge (1.2 μg $\Sigma EST_{removal}$ g $VS_{removed}$ d^{-1}), despite the fact that VS removal rates were similar between primary (1.07 g $VS_{removal}$ d^{-1}) and mixed sludge (0.98 g $VS_{removal}$ d^{-1}) digestions. This finding demonstrated the greater potency of the primary sludge substrate over the mixed sludge substrate.

A rough estimation of the removal efficiency of the biomass (the amount of organic matter present in VS) of the primary and mixed sludge during mesophilic digestion was evaluated by activity i.e. μg estrogen removed per gram of VS content per digester. The calculation was determined by taking the mass difference (M_{diff}) of the feed and the 6th retention time digestate and dividing it by the VS content in grams of the digesters. The calculation was performed for each sludge type. It should be noted that since volatile suspended solids were not measured, VS would represent in this case the closest approximation to biomass.

The biomass activity per microgram of total steroid estrogens, demonstrated that greater removals were obtained for ΣEST in primary sludge (0.056 μg g VS^{-1}) compared to mixed sludge (0.018 μg g VS^{-1}).

Although primary sludge digesters were receiving 1.9 times more steroid estrogens than mixed sludge digesters, flux removals of ΣEST during primary sludge digestion were 2.5 fold greater than the mixed sludge digestion which indicated the importance of the substrate.

However, in terms of concentration (μg kg^{-1} dw), primary sludge digesters demonstrated greater reduction of the concentration of total steroid estrogens (ΣEST = 102 μg kg^{-1} dw) compared to the mixed sludge digesters (ΣEST = 48 μg kg^{-1} dw) (Figure 5-6).

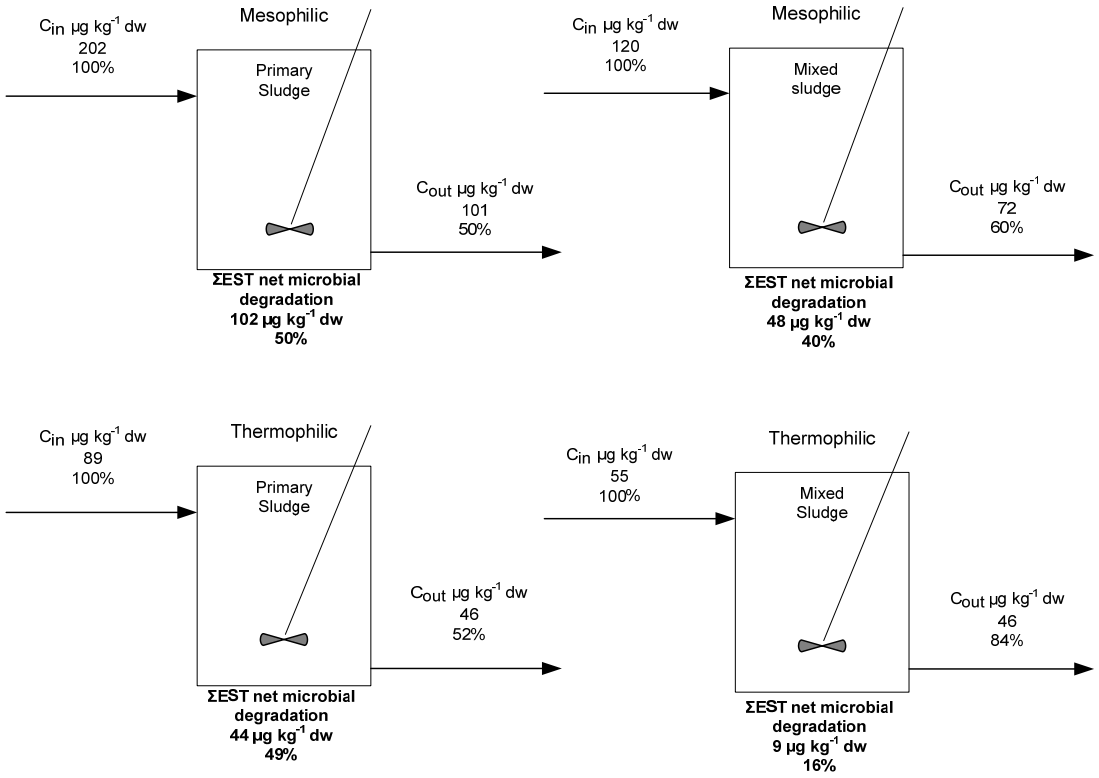


Figure 5-6 Concentrations (µg kg^{-1} dw) of steroid estrogens at the start and at the end of the anaerobic mesophilic and thermophilic digestion trials for both sludge types.

The efficiency of primary sludge in terms of ΣEST flux removal was better (53%) compared to mixed sludge (39%) (Figure 5-7). Although mixed sludge indicated the ability to interconvert E1↔E2, it demonstrated lower activity for the overall ΣEST removals and the lack of adequate suitable substrates/nutrients to promote the growth of certain microbes that biodegrade steroid estrogen. Moreover, this phenomenon could be attributed to the presence of dyspeptic substances in SAS (40% w/w in the mixed sludge) i.e. the extracellular polymeric substances (EPS) that pose physical barriers for organic compounds to be easily biodegraded (Baier & Schmidheiny, 1997; Bura et al., 1998), hence may have restricted the further degradation of E1.

It was demonstrated that the main route for E1 in primary sludge was partial biodegradation whilst biotransformation was the significant route in mixed sludge under mesophilic conditions. No complete biodegradation (mineralization) was observed for

the natural of synthetic steroid estrogens in the primary or mixed sludges during the mesophilic anaerobic digestion trials.

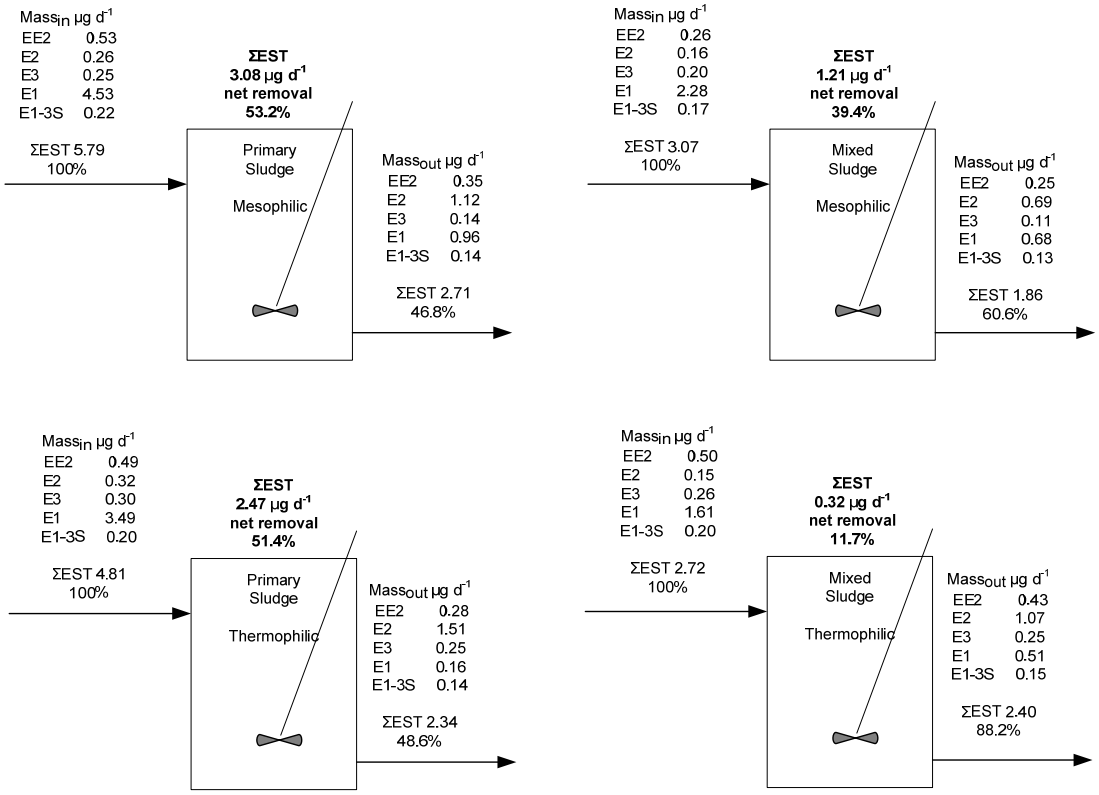


Figure 5-7 Mass flux (µg d^{-1}) for steroid estrogens at the start and at the end of the anaerobic mesophilic and thermophilic digestion trials for both sludge types.

Table 5-3 Ratio of steroid estrogens that demonstrates biodegradation and biotransformation.

	Mesophilic				Thermophilic				Phenomenon
	Primary sludge		Mixed sludge		Primary sludge		Mixed sludge		
	Start	6RT	Start	6RT	Start	6RT	Start	6RT	
$E1_{degraded}/E2_{formed}$	61	6	5	4	16	4	1	0.3	Biotransformation from E1 →E2
(E1+E2)/E3	19	15	12	12	13	7	7	6	Biodegradation from E1+E2 →E3
$E1/E1\text{-}3S_{degraded}$	37	21	25	18	15	6	6	5	Deconjugation E1-3S →E1

Note: Ratios were derived for each sludge type by obtaining the sum of each estrogen (μg d^{-1}) from each entire digestion period (6RT) divided by the respectively estrogens/combination of estrogens (μg d^{-1}). Values represent the total solids and aqueous phase combined.

5.2.6.2. Thermophilic digestion (primary sludge vs. mixed sludge)

As it was seen with the mesophilic digestion, significantly lower ΣEST flux removals were obtained during thermophilic digestion in mixed sludge compared to primary sludge i.e. 12% and 51%, respectively.

In thermophilic conditions, only the primary sludge demonstrated greater microbial removal (partial biodegradation) of E1 (E1 removed – E2 formed) than biotransformation to E2. In mixed sludge biotransformation was greater than E1 partial biodegradation. Nonetheless, both sludge types with 15 days retention time demonstrated the partial biodegradation of E1. In primary sludge, the microbial removal accounted for 13 μg d^{-1} whereas in mixed sludge the removal was much lower (1.4 μg d^{-1}). The amount of the removed E1 in each sludge type during the thermophilic trials was in accordance with the respective mesophilic trials i.e. primary sludge removed more E1 than the mixed sludge (regardless of digestion temperature). Besides the limited partial biodegradation of E1 in mixed sludge, the rate of removal was much lower than it was in primary sludge and in fact, it was the lowest rate among all digesters in both mesophilic and thermophilic trials. Generally, the removal rate of E1 in mixed sludge (regardless the digestion temperature) was lower than that in the primary sludges. Interestingly, the biodegradation rate of E1 demonstrated accumulation over time (Table 5-1) during the thermophilic digestion, suggestive of the formation of E1 in mixed sludge. Notably, the contribution of the deconjugated E1-3S to E1 in the mixed sludge was 0.8 μg d^{-1}, which considered significant when taking into account the overall microbial removal of E1 in the mixed sludge.

The biotransformation potential of E1 to E2 in primary sludge was much greater than that in the mixed sludge when considering the ratio of E1 biodegradation and E2 formation. On the other hand, the same ratio in the mixed sludge indicated that E2 was converted to E1 (Table 5-1). Whereas the biotransformation of E1 to E2 in primary sludge accounted for 9 μg d^{-1}, in mixed sludge the negative rate (-3.1 μg d^{-1}) indicated the reversed reaction.

The apparent microbial conversion of E2 to E1 in mixed sludge was consistent with the observed inter-conversion of E1↔E2 during the mesophilic trials in the mixed sludge. However, it appears that thermophilic conditions had favoured the inter-conversion mechanism of E1↔E2 and ultimately had adversely affected the activity of the removal of E1 in mixed sludge. The inter-conversion phenomenon of E1↔E2 in mixed sludge (regardless of the digestion temperature) has emphasised the previous hypothesis that the presence of activated sludge (40% w/w) in mixed sludge could be an accountable factor, despite the retention time.

As it was the case during the mesophilic trials, the ratio of the sum of E1+E2 and E3 in primary sludge demonstrated that the removal of E1 and E2 did not contribute to E3. During the thermophilic mixed sludge digestion, this ratio emphasised the inter-conversion of E1↔E2 hence, no contribution to E3 was observed (Table 5-3). The E3 flux removals were different between primary (17%) and mixed sludge (4%). E3 remained unchanged for the entire thermophilic digestion of mixed sludge.

Interestingly, the deconjugation of E1-3S was similar for both primary and mixed sludges in the thermophilic trials and its contribution to E1 accounted for 0.8 μg d^{-1} and 0.8 μg d^{-1}, respectively. The deconjugation in thermophilic trials was higher compared to the mesophilic trials. However, E1-3S flux removals were in accordance with the mesophilic trials i.e. greater flux removals in primary sludge (30%) than in the mixed sludge (28%), (36% and 21% in mesophilic conditions, respectively). Anaerobic disulphate strains are capable of cleaving E1-3S (Johnson & Williams, 2004) and this ability is believed to be associated with the use of sulphate as a terminal electron acceptor (Van Eldere et al., 1988). In addition, arylsulphatase enzyme has demonstrated some deconjugation of E1-3S (Bandick & Dick, 1999). Considering the findings of primary and mixed sludges and in conjunction with the literature, it can be hypothesized that the greater potential of primary sludge to deconjugate E1-3S was attributed to the greater abundance of the arylsulphatase enzyme compared to mixed sludge, which in turn indicated greater activity at the thermophilic temperature. If the above hypothesis is accountable, then a possible explanation for the limited arylsulphatase enzyme in mixed sludge is the fact that mixed sludge was partially treated (40% w/w of activated sludge

in mixed sludge) hence, the aerobic treatment could have resulted to the partial depletion of the E1-3S desulphating enzyme. It should be noted that the use of sulphate (SO_4^{-2}) as a terminal electron acceptor for the E1-3S deconjugation should not be considered appropriate since in all six digesters ORP ranged from -320 mV to -419 mV which indicates fermentation (methane production) rather than sulphate reduction (Bouwer & Zehnder, 1993; Gerardi, 2003). In addition, the activity of the biomass for the deconjugation of E1-3S (μg E1-3S removed per gram of VS content^{-1}) as described in section (5.2.6.1) was investigated. It was demonstrated that primary sludge exhibited greater potential in mesophilic conditions (0.0014 μg g VS^{-1}) compared to mixed sludge (0.0005 μg g VS^{-1}) and similarly greater activity was observed during thermophilic conditions in primary (0.0014 μg g VS^{-1}) and mixed sludge (0.0010 μg g VS^{-1}), respectively.

However, as it was seen in the mesophilic digestion, higher EE2 flux removals were observed in primary sludge (44%) compared to mixed sludge (15%). Higher removals were obtained in thermophilic digesters with 15 days retention time and this was also demonstrated by the activity (μg EE2 removed per gram of VS content^{-1}). The biomass activity in primary sludge during thermophilic digestion was greater (0.005 μg g VS^{-1}) than it was in mixed sludge (0.001 μg g VS^{-1}). Similarly, the activity of primary sludge for EE2 removal under mesophilic conditions was greater in primary sludge (0.003 μg g VS^{-1}) compared to the mixed sludge (0.0002 μg g VS^{-1}).

The rate of ΣEST removal per VS removal in primary sludge in thermophilic conditions was higher (1.1 μg $\Sigma EST_{removal}$ g VS $_{removed}$ d^{-1}) compared to mixed sludge (0.2 μg $\Sigma EST_{removal}$ g VS $_{removed}$ d^{-1}). despite the fact that VS removal rates were lower in mixed sludge (1.84 g VS $_{removal}$ d^{-1}) compared to primary sludge (2.24 g VS $_{removal}$ d^{-1}). This observation emphasised the greater potency of the primary sludge substrate to remove ΣEST compared to mixed sludge as it was seen in the mesophilic trials.

The activity of the biomass for the removal of ΣEST (μg ΣEST removed per gram of VS content^{-1}) demonstrated that primary sludge exhibited the greatest removal (0.056 μg g VS^{-1}) compared to mixed sludge (0.006 μg g VS^{-1}). Although the biomass removal

activity in primary sludge remained constant under both mesophilic and thermophilic conditions, the activity in mixed sludge was reduced in thermophilic conditions.

Primary sludge thermophilic digesters were receiving 1.8 times more steroid estrogens than the mixed sludge digesters, however at the end of the respective digestion trials flux removals of ΣEST in primary sludge were 7.8 fold greater compared to mixed sludge as it was demonstrated by the greater biomass activity of the primary sludge compared to mixed sludge. The low flux removals in mixed sludge reflected the reduction in concentration (ΣEST = 9 μg kg^{-1} dw) whereas the reduction in primary sludge account for 49% of the feed concentration i.e. ΣEST = 44 μg kg^{-1} dw).

Statistical analyses based on fluxes were performed to estimate the statistical inferences before and after mesophilic and thermophilic digestion. Anderson-Darling normality tests hypotheses were not violated ($p>0.1$) (Appendix III) and therefore Bartlett's and Levene's tests were performed to test the variances between the different temperature digestions. Statistical analysis demonstrated that the steroid estrogen mass entering the primary and mixed mesophilic digesters had similar variances that did not differ significantly ($p>0.05$) and the same hypothesis was true for the thermophilic trials ($p>0.05$). Further 2-sample t-tests ($t=3.78$, Degrees of Freedom (DF) = 2, $p>0.05$) indicated that the mean differences between the influxes for the primary and mixed sludge mesophilic digesters and equally among the respective thermophilic digesters ($t=7.94$, $DF=2$, $p>0.01$) were not differed significantly. Presumably, the observed similarities between the different sludge types within the respective digestion temperatures were due to the similar feed rates among the different digestion temperatures and were due to the large standard deviations (Appendix III). Because temperature and retention time between primary and mixed sludge mesophilic digestions were similar as it was the case for the respective thermophilic digestion trials, these results showed that differences could be narrowed down to the microbiology and may be independent of the overall estrogenic loadings.

The efficiency in terms of ΣEST removal in primary sludge was better (51%) compared to mixed sludge (12%) (Figure 5-7) which is consistent with the results obtained during

the mesophilic trials. Thermophilic digestion of mixed sludge indicated the conversion of E2 to E1 and demonstrated the lower biomass activity for the overall ΣEST removals compared to primary sludge. In addition, mixed thermophilic digestion demonstrated the lowest potential to remove ΣEST. As it was seen in mesophilic conditions, these phenomena were enhanced in the thermophilic trials. It therefore appears that temperature had affected the syntrophic microbial combinations that are common in methanogenic degradative processes (Schink, 1997) and resulted to different degree and extend of steroid estrogen conversion if each sludge type.

It was demonstrated that the main route for E1 in primary sludge was partial biodegradation whilst in mixed sludge the conversion of E2 to E1 predominated. These observations are in close argument with the mesophilic digestion trials. Similarly, no complete biodegradation (mineralization) was observed for the natural of synthetic steroid estrogens in the primary or mixed sludges during the mesophilic and thermophilic anaerobic digestion trials.

6. RESULTS: FATE OF NONYLPHENOL ETHOXYLATES DURING ANAEROBIC DIGESTION (SIX RETENTION TIMES)

6.1. Feed characterization for the primary and mixed sludges for mesophilic and thermophilic anaerobic digesters

Nonylphenol ethoxylates (nonylphenolics) were obtained from the combined (aqueous and solid phases) freeze-dried feed sludge samples; hence, the concentrations refer to the total amount of nonylphenolics present in each feed sample. Five individual samples (n=5) from each sludge type (primary or mixed) were extracted and analysed for nonylphenolics to determine the respective feed concentrations. The respective feed concentrations from each sludge type were then combined to produce an average value (arithmetic mean) representative for each type of feed i.e. primary or mixed feed. The feed concentrations represent the start concentrations or the influent. The individual concentrations from each digester are presented in Appendix IV.

6.1.1. Primary sludge

The background mean concentrations of nonylphenolics in the primary feed sludges over the two sampling periods are shown in Figure 6-1. The mean value of the summed carboxylated nonylphenolics (NPECs) dominated in primary sludge (13.3±26.5-0.04 mg kg^{-1} dw) of which the major carboxylated nonylphenolic analyte was NP_2EC (26.5 mg kg^{-1} dw). Short-chained nonylphenolics ($NP_{1\text{-}2}EOs$) mean concentration (8.6±2.1-15 mg kg^{-1} dw) was the second dominant species followed by the long-chained nonylphenolics ($NP_{3\text{-}12}EOs$) with a mean concentration (1.4±1.5-1.3 mg kg^{-1} dw). Nonylphenol mean concentration in primary sludge was (0.3±0.3-0.2 mg kg^{-1} dw).

The mean background concentration of ΣNPEOs for the mesophilic trials (first sampling period) was 1.8 times higher than the feed for the thermophilic trials. Mean concentration of ΣNPEOs for the mesophilic digestion (first sampling period) was

30.5±0.01-26.5 mg kg^{-1} dw whilst the mean concentration for the thermophilic digestion (second sampling period) was 16.7±0.01-15 mg kg^{-1} dw.

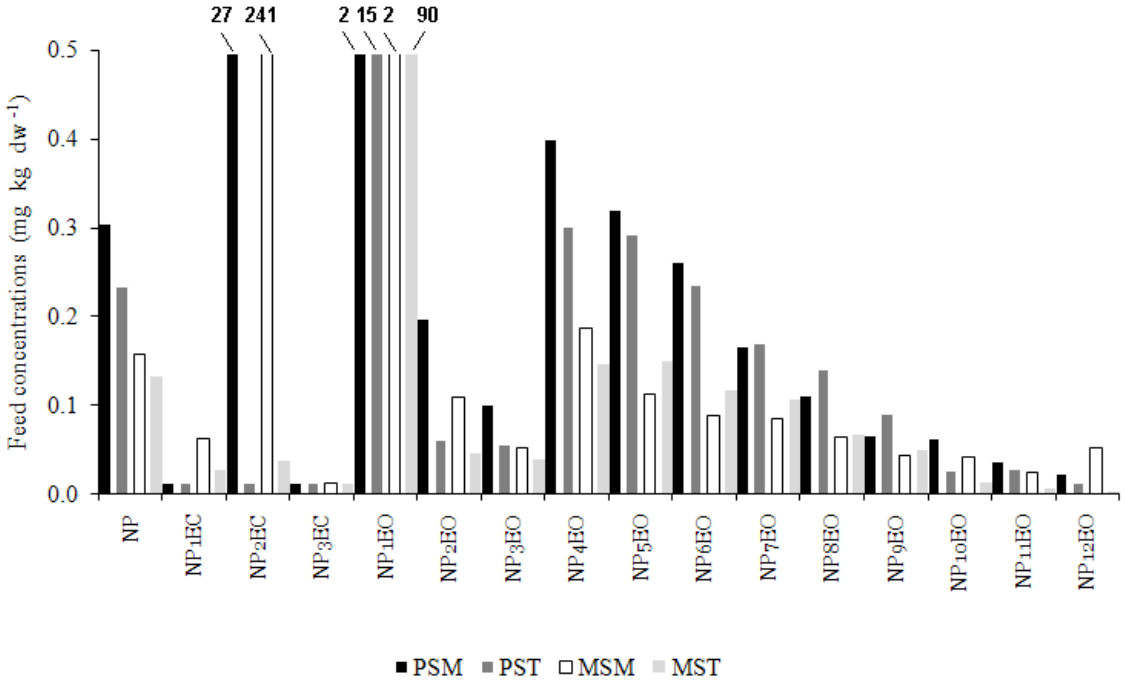


Figure 6-1 Mean (*n*=5) background nonylphenolic concentrations (mg kg^{-1} dw) for the mesophilic trials in the primary (PSM) and mixed feed sludges (MSM) and the thermophilic trials in primary (PST) and mixed feed sludges (MST), respectively.

The contribution of the dominant specie (NP_2EC) in primary feed sludge entering the mesophilic digesters was 87% of the sum of nonylphenolics (ΣNPEOs) whilst that in thermophilic feed was NP_1EO and contributed to 90% of the sum of nonylphenolics entering the thermophilic digesters (Figure 6-2).

The contribution of $NP_{3\text{-}12}EOs$ in the thermophilic feed was slightly higher (8%) than the contribution in the mesophilic feed (5%).

Nonylphenol mean concentrations were similar for the mesophilic (0.3 mg kg^{-1} dw) and thermophilic feeds (0.2 mg kg^{-1} dw) and its contribution was 0.01% for each type of feed, respectively (Figure 6-2).

Aerobic and anaerobic partial biodegradation and biotransformation of the alkylphenolic compounds was apparent in the primary sludge feeds for both mesophilic and thermophilic trials. As it was seen for the steroid estrogen trends, the two possible explanations for this phenomenon are the consequence of the biologically active of returned liquors or sludge returned to the head of the works and biodegradation/biotransformation in the sewerage system. The percentage distribution of alkylphenolics in primary sludge entering the mesophilic digesters was as follows: NPECs (87%)> $NP_{1-2}EOs$ (7%)> $NP_{3-12}EOs$ (5%)>NP (1%). For the thermophilic trials, the percentage distribution was as follows: $NP_{1-2}EOs$ (90.3%)> $NP_{3-12}EOs$ (8.1%)>NP (1.4%)> NPECs (0.2%).

6.1.2. Mixed Sludge

The background mean concentrations of nonylphenolics in the mixed feed sludges over the two sampling periods are shown in Figure 6-1. The dominant species in mixed sludge was NPECs with a mean concentration 120.8±241.5-0.08 mg kg^{-1} dw of which NP_2EC was the dominant carboxylated specie (241.5 mg kg^{-1} dw). The mean background concentration of $NP_{1-2}EOs$ in the mixed feed over the two sampling periods was 45.6±1.7-90 mg kg^{-1} dw whilst the mean for $NP_{3-12}EOs$ was 0.7±0.7-0.7 mg kg^{-1} dw over the two sampling periods. Mean concentration of NP was 0.1±0.2-0.1 mg kg^{-1} dw. The mean of ΣNPEOs in the feed mixed sludge for the first sampling period (mesophilic digestion) was 244±0.02-241.4 whilst for the second sampling period (thermophilic digestion) the mean concentration was 90.5±0.02-89.5 mg kg^{-1} dw.

Overall, the mean background concentration of ΣNPEOs in the mixed sludge for the mesophilic trials was 2.7 times higher than the feed for the thermophilic trials.

The contribution of NP_2EC in the feed for the mesophilic trial was 99% whilst for $NP_{1-2}EOs$ was 1%. The respective contributions of $NP_{3-12}EOs$ and NP in the feed sludge were less than 0.1% respectively (Figure 6-2). The contribution of $NP_{1-2}EOs$ in the feed for the thermophilic trials was 99% and circa 1% for the long-chained nonylphenolics whereas the contribution of NPECs and NP was below 0.1%, respectively.

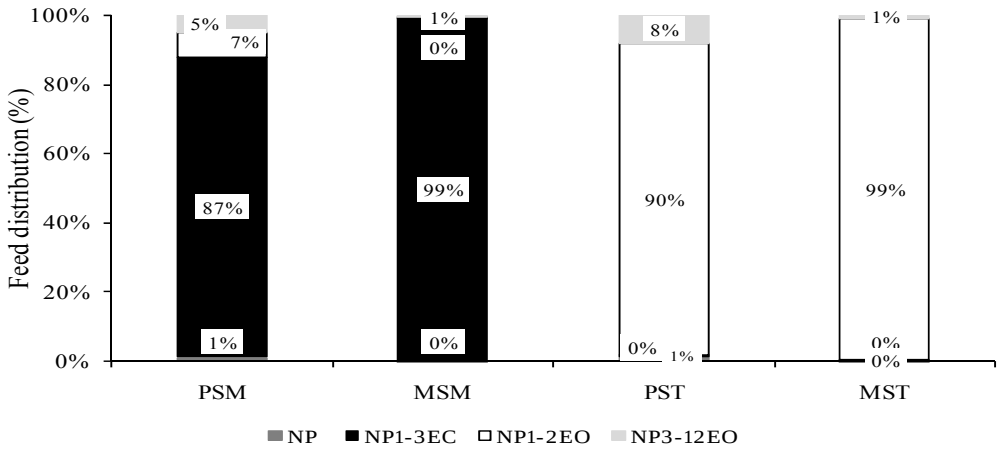


Figure 6-2 Mean (*n*=5) composition of nonylphenolics in the primary and mixed feeds for the mesophilic and thermophilic trials.

The percentage distribution of alkylphenolics in mixed sludge that was entering the mesophilic digesters was as follows: NPECs (99%)> $NP_{1-2}EOs$ (1%)> $NP_{3-12}EOs$ (0%)≈NP (0%). The distribution for the respective thermophilic trials was: $NP_{1-2}EOs$ (99%)> $NP_{3-12}EOs$ (1%)>NPECs (0%)≈NP (0%).

As it was seen in primary sludge, aerobic and anaerobic partial biodegradation and biotransformation of the alkylphenolic compounds was apparent in the mixed sludge for both temperature digestions. However, this is a typical trend for alkylphenolic compounds that have undergone some degree of biotransformation and biodegradation in mixed sludge. The biotransformation/biodegradation products of ΣNPEOs i.e. NPECs and $NP_{1-2}EOs$ were found in greater concentrations in mixed sludge than in primary sludge whilst higher levels of parent alkylphenolics i.e. $NP_{3-12}EOs$ were present in primary sludge. Mixed sludge contained 7 fold higher ΣNPEOs compared to primary sludge due to the higher concentration of metabolites in this sludge.

6.2. Trends for nonylphenol ethoxylates during anaerobic digestion of primary and mixed sludges under mesophilic and thermophilic conditions

This chapter reports the results from the mesophilic and thermophilic trials for nonylphenol ethoxylates over 180 and 90 days, respectively. The aim was to understand if there were any differences in terms of removals in relation to different sludge types i.e. primary or mixed (60:40 v/v, primary sludge: SAS) sludge during mesophilic and thermophilic digestion temperatures. The first (*H1*N) and second research hypotheses (*H2*N) were:

*H1*N_0: Mesophilic anaerobic digestion of nonylphenol ethoxylates by using mixed or primary sludge will not result to significantly different mass removal.

*H1*N_α: Mesophilic anaerobic digestion of nonylphenol ethoxylates by using mixed or primary sludge will result to significantly different mass removal.

*H2*N_0: Digestion temperature will not result to significant effect on nonylphenol ethoxylate removals.

*H2*N_α: Digestion temperature will result to significant effect on nonylphenol ethoxylate removals.

The objectives were to:

1. Examine the effect of primary and mixed sludge (60:40 v/v, primary sludge: SAS) on nonylphenol ethoxylate removals during anaerobic digestion.

2. Investigate the effects of mesophilic ($35^oC \pm 0.2^oC$) and thermophilic ($53^oC \pm 0.2^oC$) digestion on nonylphenol ethoxylate removals.

At the end of each retention time i.e. 30 days for the mesophilic and 15 days for thermophilic digestion, a composite sample from each individual digester was used for the quantification of alkylphenolics. The composite sample was comprised of all

digestates collected from each individual digester over a period of the last week of each retention time, respectively and were kept frozen (-26º ± 1.5ºC) until analysis. Each digestate (50 ml) was extracted and analysed twice for alkylphenolic concentrations to produce an average value (n=4), for mesophilic digesters and (n=2) for the thermophilic digesters representative for each type of digester i.e. primary or mixed sludge digester. All values reported represent the mean values of alkylphenolics from the primary or the mixed digesters, respectively. The individual concentrations from each separate digester are presented in Appendix IV. Effluent refers to digestate i.e. total solids and aqueous phase combined and similarly, influent refers to the feed i.e. total solids and aqueous phase combined.

6.2.1. Primary sludge (mesophilic digestion)

The concentrations of nonylphenolics obtained for each retention time from the mesophilic (30 days retention time) primary sludge digesters are shown in (Figure 6-3).

The sum of the concentration in primary sludge of all nonylphenolics i.e. $NP_{1\text{-}12}EO$, NP and NPEC (NPECs) at the end of the 1st retention time (RT) was 163 mg kg^{-1} dw which was increased from the feed concentration (30.5 mg kg^{-1} dw). The increase in concentration was mainly attributed to the carboxylated nonylphenolic species, in particular to NP_2EC and NP_3EC.

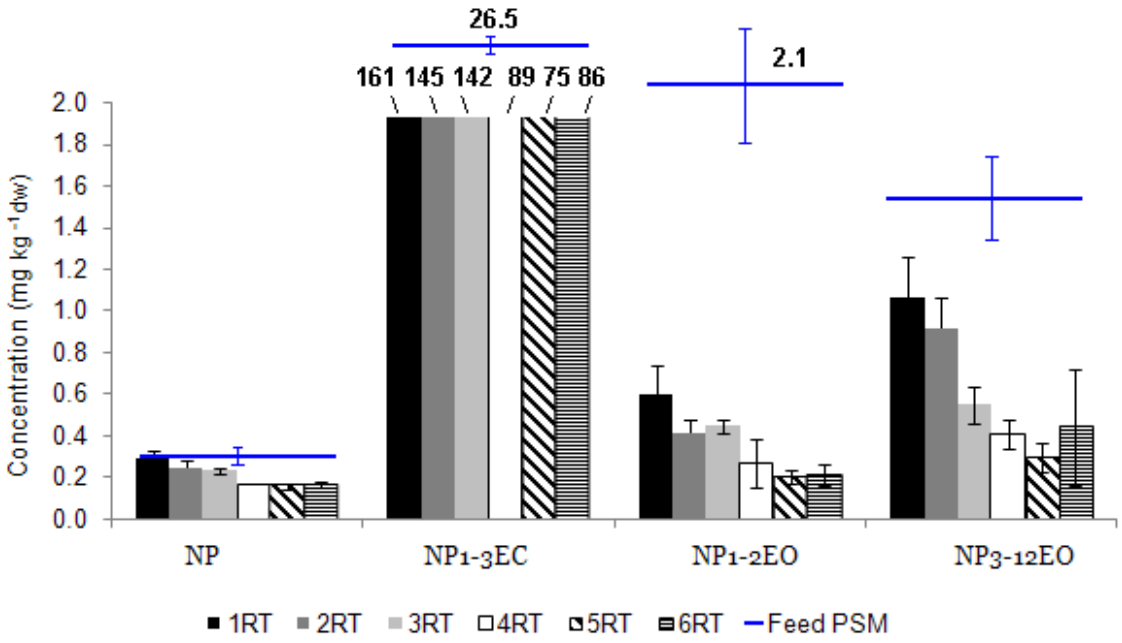


Figure 6-3 Mean (*n*=4) trends of nonylphenol ethoxylate concentrations (mg kg^{-1} dw ± SE) per retention time (30 days) during mesophilic digestion (35°C ± 0.2°C) of primary sludge. The start concentration is displayed as follows: ⊣⊢.

The concentration of ΣNPEOs at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 147 mg kg^{-1} dw, 143 mg kg^{-1} dw, 90 mg kg^{-1} dw, 76 mg kg^{-1} dw and 87 mg kg^{-1} dw, respectively.

However, the concentration of the long- chained nonylphenolics ($NP_{3-12}EOs$) at the 1st RT was 1.1 mg kg^{-1} dw whereas at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 0.9 mg kg^{-1} dw, 0.5 mg kg^{-1} dw, 0.4 mg kg^{-1} dw, 0.3 mg kg^{-1} dw and 0.4 ± 0.3 $^{-1}$, respectively. Although at the 6th retention time, $NP_{3-12}EOs$ concentration indicated an 1.5 fold increase from the concentration observed at the 5th retention time, this value was associated with a large standard error. Overall, $NP_{3-12}EOs$ indicated a progressive reduction in concentration during the entire period of mesophilic digestion.

Overall, the mass reduction of $NP_{3-12}EOs$ at the end (6th retention time) of the ($Mass_{out}$ – $Mass_{in}$) was M_{diff}=-0.05 mg d^{-1} indicated the partial biodegradation/biotransformation of $NP_{3-12}EOs$ during mesophilic anaerobic digestion of primary sludge

During the entire digestion period, the removed $NP_{3-12}EOs$ ($Mass_{in}$ – $Mass_{out}$) accounted for 0.23 mg d^{-1}. No contribution to NP_1EO or NP_2EO was observed from the breakdown of $NP_{3-12}EOs$ during the digestion period. If $NP_{3-12}EOs$ had been broken down to either of these metabolites then it would be expected an increase in their respective out fluxes unless these species were further biotransformed. In fact, NP_1EO, NP_2EO and NP decreased from the commencement of the mesophilic trial and were always decreasing during the digestion period. In contrast, the carboxylated nonylphenolics, in particular NP_2EC and NP_3EC increased at the 1st retention time from their respective influxes and then decreased over the entire mesophilic trial.

The mass difference (M_{diff}) of the sum of NP_2EC and NP_3EC for the entire digestion period ($Mass_{in}$ – $Mass_{out}$) was -21.3 mg $NP_{1-2}EC$ d^{-1}. The disappeared mass of $NP_{3-12}EOs$ was partially biotransformed to NP_2EC and NP_3EC onset the mesophilic digestion of primary sludge. There was no evidence for the direct contribution of $NP_{3-12}EOs$ to the short-chained metabolites ($NP_{1-2}EOs$) or to NP. Therefore, considering the biodegradation/biotransformation mechanism of the long chained nonylphenolics i.e. long chained NPEOs → short-chained NPEOs ↔ NPECs → NP (Renner, 1997; Montgomery-Brown & Reinhard, 2003; Schröder, 2001), the difference between the removed $NP_{3-12}EOs$ and the formed $NP_{1-2}ECs$ suggested that 21.03 mg d^{-1} of $NP_{3-12}EOs$ were possibly biotransformed to unmeasured metabolite species and/or biodegraded.

This peculiar observation implied that the formation of short-chained carboxylates occurred from the commencement of the experiment whilst de-carboxylation occurred with time and at non-constant rate.

Overall, $NP_{3\text{-}12}EOs$ indicated 72% flux removal. The reduction in concentration of $NP_{3\text{-}12}EOs$ during the anaerobic trials is consistent with the accepted theory that the breakdown of long-chained nonylphenolics proceeds fast by the stepwise removal mechanism of one ethylene glycol unit (Montgomery-Brown & Reinhard, 2003; Chiu et al., 2010). The high removal of $NP_{3\text{-}12}EOs$ is consistent with high removals obtained by (Zhang et al., 2008) from the spiked NPEO mixture in the continuous lab-scale UASB trials (pH= 7.1, ORP= −350mV).

Overall, $NP_{1\text{-}2}EOs$ indicated a progressive reduction in concentration during the entire period of mesophilic digestion (Figure 6-3) but their levels were not eliminated. The mass reduction of $NP_{1\text{-}2}EOs$ between the 6th RT digestate and the influx ($Mass_{out}$ − $Mass_{in}$) was M_{diff}=−0.1 mg d^{-1} which accounted for 90.4% mean flux removal in the primary sludge which was the highest flux removal among the nonylphenolics in primary sludge. The NP_2EO indicated the highest flux removal among the short-chained nonylphenolics.

It appeared that the breakdown of $NP_{1\text{-}2}EOs$ did not contribute to a significant degree to the NP because NP levels were always decreasing from the commencement of the digestion trial. The gradual disappearance of $NP_{1\text{-}2}EOs$ could be possibly explained by their biotransformation to NPECs and/or to NP. If all the $NP_{1\text{-}2}EOs$ had been biotransformed to $NP_{1\text{-}2}ECs$ then the maximum contribution from $NP_{1\text{-}2}EOs$ to $NP_{1\text{-}2}ECs$ would be the M_{diff} = 0.4 mg d^{-1}.

The results were not in close agreement with (Montgomery-Brown & Reinhard, 2003) that short-chained nonylphenolics persist under anaerobic conditions.

The concentration of the sum of the measured carboxylated nonylphenol ethoxylates (NPECs) at the 1st RT (161 mg kg^{-1} dw) was significantly increased from the feed concentration (26.5 mg kg^{-1} dw). After the 1st retention time, the concentration of

NPECs decreased with the digestion period and at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT the mean concentration was 145 mg kg^{-1} dw, 142 mg kg^{-1} dw, 89 mg kg^{-1} dw, 75 mg kg^{-1} dw and 86 mg kg^{-1} dw, respectively (Figure 6-3). The difference in concentration between the 6th retention time digestate and the start concentration indicated the. At the end of the mesophilic digestion period (6th retention time) a significant accumulated mass ($Mass_{out} - Mass_{in}$) accounted for M_{diff}= 2.4 mg d^{-1} which reflected the negative flux removal (accumulation) of -215%. The greatest accumulation was observed for NP_3EC (-7692%) and NP_2EC (-211%) whereas NP_1EC indicated removal (54.2%). It appeared that the breakdown of $NP_{3-12}EOs$ contributed to the formation of $NP_{2-3}EC$. The estimated total contribution of $NP_{1-12}EOs$ to $NP_{2-3}EC$ for the entire digestion period was 22 mg d^{-1}. The estimation of the contribution was calculated from the mass difference between the removed $NP_{1-12}EOs$ and the partially biodegraded $NP_{1-12}EOs$ ($NP_{1-12}EOs$ removed – $NP_{1-12}EOs$ 'partial biodegradation') since no evidence was observed for their contribution to NP.

However, the concentration of nonylphenol (NP) at the 1st RT remained at similar levels to the start levels i.e. 0.3 mg kg^{-1} dw. Concentration of NP at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 0.2 mg kg^{-1} dw, 0.2 mg kg^{-1} dw, 0.2 mg kg^{-1} dw, 0.2 mg kg^{-1} dw and 0.2 mg kg^{-1} dw, respectively. In general, NP decreased with time. Overall, NP indicated a reduction in concentration during the 2nd, 3rd and 4th retention times and then its concentration reached a plateau for the remaining period of the mesophilic digestion (Figure 6-3). No evidence was observed that the breakdown of the non-ionic nonylphenolics as well as the NPECs were contributed to NP. The change in concentration between the difference of the 6th retention time digestate and the start concentration was -0.14 mg NP kg dw^{-1}. The partial biodegradation of NP in terms of concentration is consistent with the results obtained by (Patureau et al., 2008) during continuous mesophilic anaerobic digestion but contrary to (Chang et al., 2005; Chang et al., 2004) who observed NP biodegradation (mineralization) in batch mesophilic anaerobic trials by using petrochemical sludge and river sediment with additional electron acceptors at pH =7. However, the mass reduction of NP at the end (6th retention time) of the mesophilic digestion period of primary sludge ($Mass_{out} - Mass_{in}$) was M_{diff}=−0.01 mg d^{-1}. The lowest flux removal of all the measured nonylphenolics was the

removal of NP (45%). The overall partial biodegradation of NP during the entire digestion period ($Mass_{in} - Mass_{out}$) accounted for 0.02 mg d^{-1}.

6.2.2. Primary sludge (thermophilic digestion)

The concentrations of nonylphenolics from the thermophilic digestion (15 days retention time) of primary sludge are shown in Figure 6-4. The sum of the concentration of all nonylphenolics (ΣNPEOs) measured at the 1st retention time (RT) was 16 mg kg^{-1} dw whereas the start concentration was 17 mg kg^{-1} dw. For the remaining of the retention times, the concentration of ΣNPEOs did not change significantly. For the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th retention times the concentration of ΣNPEOs was 18 mg kg^{-1} dw, 19 mg kg^{-1} dw, 17 mg kg^{-1} dw, 17 mg kg^{-1} dw and 17 mg kg^{-1} dw, respectively.

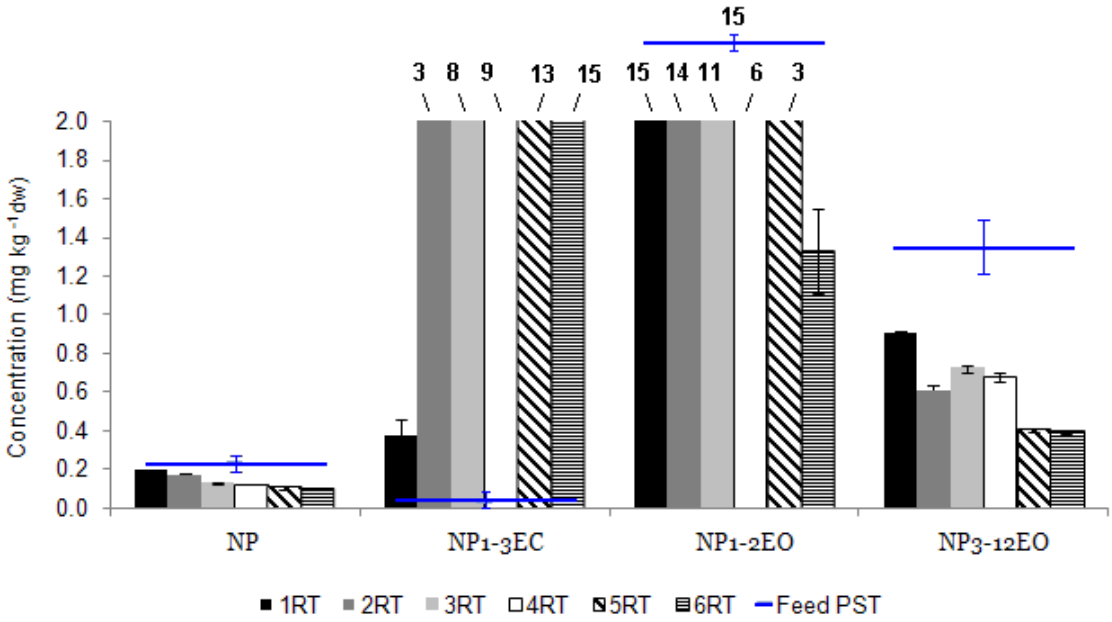


Figure 6-4 Mean (*n*=2) trends of nonylphenol ethoxylate concentrations (mg kg^{-1} dw± SE) per retention time (15 days) during thermophilic digestion (53°C ± 0.2°C) of primary sludge. The start concentration is displayed as follows: ⊣⊢.

Long chained nonylphenolics ($NP_{3-12}EOs$) were slightly reduced at the 1st RT (0.9 mg kg^{-1} dw) from the start concentration (1.3 mg kg^{-1} dw). During the entire thermophilic digestion of primary sludge (90 days), the concentration of $NP_{1-2}EOs$ did not reduce significantly. For example, the concentration at the 2nd RT, 3rd RT, 4th RT, 5th RT and

6th RT was 0.6 mg kg^{-1} dw, 0.7 mg kg^{-1} dw, 0.7 mg kg^{-1} dw, 0.4 mg kg^{-1} dw and 0.4 mg kg^{-1} dw, respectively. Interestingly, similar reduction in concentration i.e. 1.0 mg kg^{-1} dw was also observed during the mesophilic trial of primary sludge with similar start concentrations.

Overall, the mass reduction of $NP_{3-12}EOs$ at the end of the thermophilic digestion period (6th retention time) of primary sludge ($Mass_{out} - Mass_{in}$) was M_{diff}=-0.08 mg d^{-1} which resulted to 71% flux removal and it was similar to the mesophilic trial. Moreover, the $NP_{3-12}EOs$ removal is in agreement with the literature (Montgomery-Brown & Reinhard, 2003; Chiu et al., 2010; Zhang et al., 2008). Overall, the removal of $NP_{3-12}EOs$ during the entire thermophilic digestion was 0.36 mg d^{-1}. In contrary to the mesophilic digestion of primary sludge, the breakdown of $NP_{3-12}EOs$ contributed to the short-chained nonylphenolics and in particular to NP_1EO.

However, the concentration of short-chained nonylphenolics ($NP_{1-2}EOs$) at the 1st RT was slightly reduced (14.6 mg kg^{-1} dw) from the start concentration (15 mg kg^{-1} dw). The concentration for the consecutive retention times was also reduced (Figure 6-4) and at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT retention times the concentration levels were 14 mg kg^{-1} dw, 11 mg kg^{-1} dw, 6 mg kg^{-1} dw, 3 mg kg^{-1} dw and 1.3 mg kg^{-1} dw, respectively. However, the concentration of NP_1EO at the end of the digestion period was reduced by 14 mg kg^{-1} dw from the start concentration whereas NP_2EO concentration had increased by 1 mg kg^{-1} dw. In terms of flux, the overall mass difference between the 6th retention time and the influx ($Mass_{out} - Mass_{in}$) was M_{diff}=-0.1 mg $NP_{1-2}EOs$ d^{-1}. The highest flux removal among the short-chain nonylphenolics demonstrated by NP_2EO (80%) whereas significantly lower removal occurred for NP_1EO (2%). Overall, the mass difference of NP_1EO for the entire digestion period ($Mass_{in} - Mass_{out}$) was 3.4 mg d^{-1}. The removal of the short-chained nonylphenolics under thermophilic conditions was lower than it was during the mesophilic digestion. The persistence of NP_1EO during the thermophilic digestion of primary sludge is in accordance with (Montgomery-Brown & Reinhard, 2003) that short-chained nonylphenolics persist under anaerobic conditions.

The concentration of the sum of carboxylated nonylphenolics (NPECs) increased during the thermophilic digestion of primary sludge. Although the start concentration was 0.1 mg kg^{-1} dw the concentrations at the 1st RT, 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT were 0.4 mg kg^{-1} dw, 3 mg kg-^{1} dw, 8 mg kg^{-1} dw, 9 mg kg^{-1} dw, 13 mg kg^{-1} dw and 15 mg kg^{-1} dw, respectively (Figure 6-4). The change in concentration between the 6th retention time digestate and the start concentration accounted for 15 mg kg^{-1} dw and it was mainly attributed to NP_2EC. However, the mass difference between the 6th retention time digestate and the influx accounted for 0.11 mg $NP_{1\text{-}2}ECs$ d^{-1}. Nonylphenoxy carboxylate (NP_1EC) was formed directly after the commencement of the experiment and it was gradually removed at constant rate. However, the residual levels at the 6th retention time indicated its accumulation (-4684%). On the other hand, NP_2EC increased (-6078%) whilst NP_3EC resulted to an insignificant flux removal (0.3%). As it was the case during mesophilic digestion the results from the thermophilic digestion regarding NPECs are in argument consistent with (Schröder, 2001; Field & Reed, 1999; Lee et al., 1997).

Regarding nonylphenol (NP), its concentration at the 1st RT (0.2 mg kg^{-1} dw) reduced from the feed concentration (0.23 mg kg^{-1} dw) and continued to decrease with the digestion period. At each retention time i.e. 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT its concentration was 0.2 mg kg^{-1} dw, 0.1 mg kg^{-1} dw, 0.1 mg kg^{-1} dw, 0.1 mg kg^{-1} dw and 0.1 mg kg^{-1} dw, respectively. NP did not accumulate during the thermophilic digestion of primary sludge (Figure 6-4) which is in agreement with its reduction during mesophilic conditions. The concentration was reduced at the end of the digestion period (6th retention time) by -0.1 mg NP kg^{-1} dw from the start concentration . The reduced NP at the end of the thermophilic digestion period (6th retention time) ($Mass_{out} - Mass_{in}$) was M_{diff}=-0.01 mg d^{-1} and its flux removal (57%) was slightly greater compared to mesophilic conditions (45%). The overall partial biodegradation of NP during the entire digestion period ($Mass_{in} - Mass_{out}$) accounted for 0.05 mg d^{-1} and occurred over the entire digestion period as it was observed in the mesophilic trial. The reduction of the NP mass during the thermophilic digestion is consistent with (Patureau et al., 2008).

6.2.3. Mixed sludge (mesophilic digestion)

The concentration of nonylphenolics for each retention time during mixed sludge mesophilic (30 days retention time) digestion are shown in (Figure 6-5). The sum of the concentration of all nonylphenolics (ΣNPEOs) at the 1st retention time (RT) (118 mg kg^{-1} dw) decreased from the start concentration (244 mg kg^{-1} dw) which was mainly attributed to decrease in concentration of NPECs in particular that of NP_2EC. Overall, the concentrations of ΣNPEOs during the entire digestion period decreased from the initial concentration. At the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT, ΣNPEOs concentrations were 71 mg kg^{-1} dw, 44 mg kg^{-1} dw, 61 mg kg^{-1} dw, 63 mg kg^{-1} dw and 104 mg kg^{-1} dw, respectively.

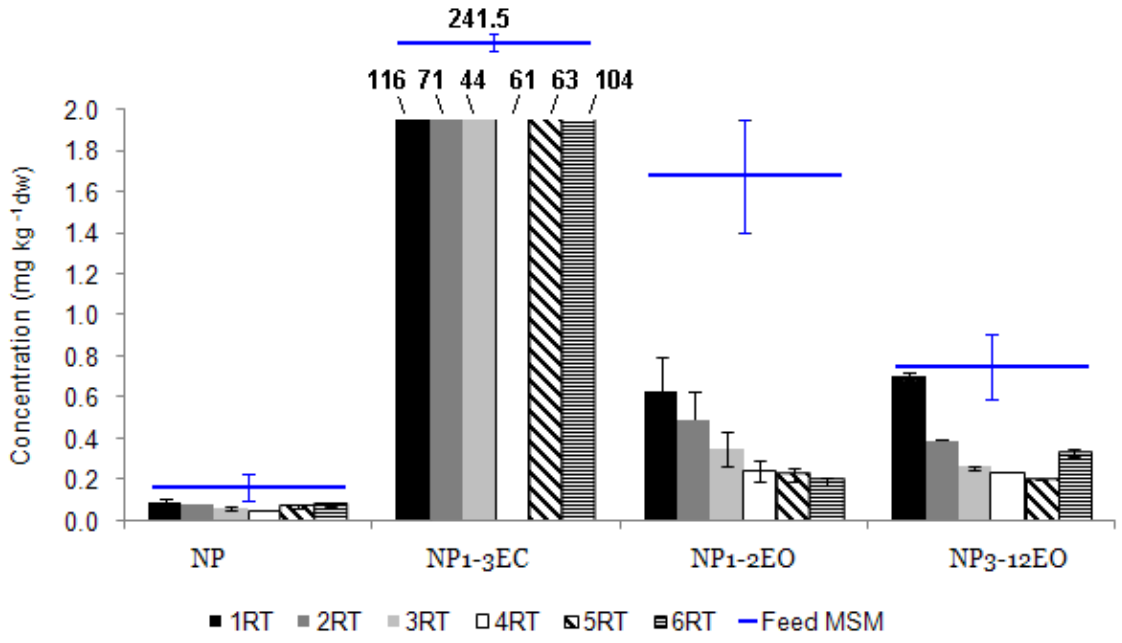


Figure 6-5 Mean (*n*=4) trends of nonylphenol ethoxylate concentrations (mg kg^{-1} dw ± SE) per retention time (30 days) during mesophilic digestion (35°C ± 0.2°C) of mixed sludge. The start concentration is displayed as follows: ⊣⊢.

However, the concentration of the long-chained nonylphenolics ($NP_{3-12}EOs$) in the digestates of the 1st RT (0.7 mg kg^{-1} dw) remained the same with the start concentration of $NP_{3-12}EOs$ (0.7 mg kg^{-1} dw). Nevertheless, post the 1st retention time the

concentration of $NP_{3-12}EOs$ decreased with time. At the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT, concentrations were 0.4 mg kg^{-1} dw, 0.3 mg kg^{-1} dw, 0.2 mg kg^{-1} dw, 0.2 mg kg^{-1} dw and 0.3 mg kg^{-1} dw, respectively. Overall, $NP_{3-12}EOs$ indicated reduction in concentration during the first five retention times and then an insignificant increase in concentration occurred at the 6th retention time. The increased concentration between the 5th retention time and the 6th retention time was 0.1 mg kg^{-1} dw (Figure 6-5). This increase in concentration could be a result of the breakdown of NPEOs having longer than 12 ethoxylate units that were presumably present in the primary sludge portion of the mixed sludge. Overall, the mass reduction at the 6th retention time ($Mass_{out} - Mass_{in}$) was M_{diff}=-0.02 mg d^{-1} and $NP_{3-12}EOs$ exhibited 57% flux removal. The reduction of $NP_{3-12}EOs$ during the mesophilic digestion of mixed sludge is in agreement with the removals obtained during primary sludge mesophilic digestion.

In addition, the results are consistent with the literature (Montgomery-Brown & Reinhard, 2003) and other studies (Zhang et al., 2008; Patureau et al., 2008). During the entire digestion period, the removed $NP_{3-12}EOs$ ($Mass_{in} - Mass_{out}$) accounted for 0.09 mg d^{-1}. There was no direct evidence that the breakdown of $NP_{3-12}EOs$ contributed to the short-chained nonylphenolics i.e. NP_1EO or NP_2EO, however, the concentration NP_2EC increased. The difference between the removed $NP_{3-12}EOs$ did not equilibrate with the metabolites which suggested that $NP_{3-12}EOs$ were degraded or that were biotransformed to unmeasured metabolite species.

An overall reduction in concentration was observed for $NP_{1-2}EOs$ with time during the mesophilic mixed sludge digestion. The concentrations reduced from the 1st RT (0.6 mg kg^{-1} dw) compared to the start concentrations 1.7 mg kg^{-1} dw. At the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT the concentrations were 0.5 mg kg^{-1} dw, 0.4 mg kg^{-1} dw, 0.2 mg kg^{-1} dw, 0.2 mg kg^{-1} dw and 0.2 mg kg^{-1} dw, respectively (Figure 6-5). A small difference between the concentration in the feed and that at the 6th retention time digestate was observed and accounted for 1.5 mg $NP_{1-2}EOs$ kg^{-1} dw. Overall, the mass reduction of $NP_{1-2}EOs$ at the end of the mesophilic digestion period of mixed sludge ($Mass_{out} - Mass_{in}$) was M_{diff}=-0.06 mg d^{-1} and $NP_{1-2}EOs$ removed by 88.7%. The greatest flux removal among the short-chained nonylphenolics demonstrated by NP_1EO

(90%) whilst NP_2EO removed by 73%. During the entire digestion period, the sum of the removed short-chained nonylphenolics was 0.31 mg d^{-1}. There was no clear evidence for the stoichiometric contribution of NP_1EO to NP_1EC or NP. As it was seen in primary sludge, the persistence of $NP_{1-2}EOs$ was equally occurred in the mixed sludge during 30 days anaerobic digestion. In addition, the results from the mixed sludge are in agreement with (Montgomery-Brown & Reinhard, 2003; Zhang et al., 2008).

Nevertheless, the concentration of NPECs decreased from the start concentration of 241.5 mg kg^{-1} dw to 118 mg kg^{-1} dw at the 1st RT. At the 2nd RT and 3rd RT the concentration decreased i.e.72 mg kg^{-1} dw and 44 mg kg^{-1} dw, respectively. For the remaining digestion period, the concentration of NPECs increased. At the 4th RT, 5th RT and 6th RT, NPECs concentration was 61 mg kg^{-1} dw, 64 mg kg^{-1} dw and 104 mg kg^{-1} dw, respectively (Figure 6-5). The change in concentration between the 6th RT digestate and the start concentration indicated a significant reduction in concentration of -138 mg kg^{-1} dw. The mass reduction of NPECs between the 6th RT and the influx ($Mass_{out}$ − $Mass_{in}$) was M_{diff}= -5.6 mg d^{-1}. However, the accumulation was mainly attributed to NP_2EC (M_{diff}=-5.6 mg d^{-1}). Overall flux removal for NPECs was 57.6% of which $NP_1EC$and NP_2EC removals accounted for 90% and 58%, respectively. The accumulation of NP_3EC resulted to a negative flux removal of -10312%. It is worth mentioning that the removed NP_3EO (5.5mg d^{-1}) was greater than the formed NP_3EC (2.4 mg d^{-1}) which implied the possible biotransformation of NP_3EO to NP_3EC. However, since the quantification of NP_3EC was based on the instrument's response for NP_1EC it is not possible to conclude for the accounted contribution of NP_3EO to NP_3EC. Nevertheless, the increase of NP_2EC and NP_3EC after the 4th retention time suggested the biotransformation of NPEOs to NPECs.

However, NP concentration at the 1st RT (0.09 mg kg^{-1} dw) reduced from the start concentration (0.23 mg kg^{-1} dw). The concentration of NP decreased until the fourth retention time i.e. 2nd RT (0.08 mg kg^{-1} dw), 3rd RT (0.06 mg kg^{-1} dw), 4th RT (0.05 mg kg^{-1} dw) and then increased at the 5th RT and 6th RT (0.07 mg kg^{-1} dw and 0.08 mg kg^{-1} dw, respectively), (Figure 6-5). Although NP concentration reduced from the start

concentration, this reduction was insignificant and accounted for 0.08 mg kg^{-1} dw between the start and the 6th retention time digestate. The overall biodegradation of NP during the entire digestion period of mixed sludge ($Mass_{out} - Mass_{in}$) was M_{diff}=-0.02 mg d^{-1}). Flux removal of NP was 51%. There was no clear evidence for the stoichiometric contribution of the short-chained or carboxylated nonylphenolics to NP. However, the observed increased patterns of NP_2EC and NP_3EC after the 3rd retention time were analogous with the increased NP after the 3rd retention time. The reduction of NP during mesophilic anaerobic digestion of mixed sludge is consistent with the observations of primary sludge as well as with the literature (Patureau et al., 2008; Chang et al., 2005; Chang et al., 2004).

6.2.4. Mixed sludge (thermophilic digestion)

Mixed sludge thermophilic (15 days retention time) digestates, from each retention time, are shown in (Figure 6-6). Overall, the sum of the concentration of ΣNPEOs at the 1st retention time (16 mg kg^{-1} dw) reduced from the start concentration 90.5 mg kg^{-1} dw. The concentration of ΣNPEOs at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT were 12 mg kg^{-1} dw, 11 mg kg^{-1} dw, 10 mg kg^{-1} dw, 9 mg kg^{-1} dw and 7 mg kg^{-1} dw, respectively.

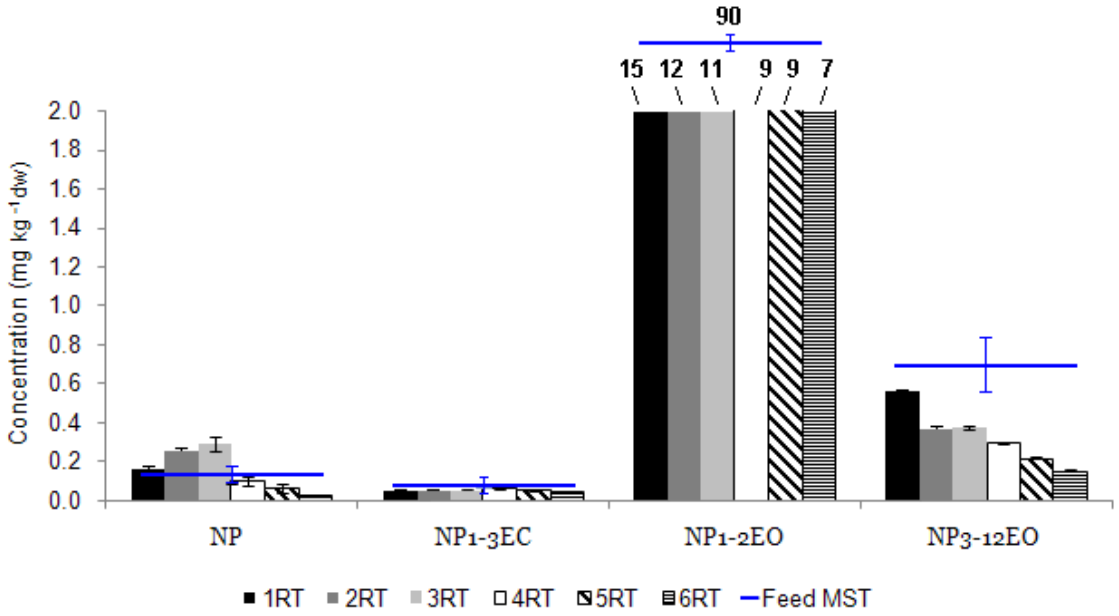


Figure 6-6 Mean (*n*=2) trends of nonylphenol ethoxylate concentrations (mg kg^{-1} dw ± SE) per retention time (15 days) during thermophilic digestion (53°C ± 0.2°C) of mixed sludge. The start concentration is displayed as follows: ⊣⊢.

Regarding the $NP_{3\text{-}12}EOs$ concentration, it slightly decreased at the 1st RT (0.6 mg kg^{-1} dw) compared to the start concentration (0.7 mg kg^{-1} dw). For the remaining of the digestion period i.e. at the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT, $NP_{3\text{-}12}EOs$ concentration was 0.4 mg kg^{-1} dw, 0.4 mg kg^{-1} dw, 0.3 mg kg^{-1} dw, 0.2 mg kg^{-1} dw and 0.2 mg kg^{-1} dw, respectively. Overall, $NP_{3\text{-}12}EOs$ indicated reduction in concentration during the entire period of thermophilic digestion (Figure 6-6). The mass reduction of $NP_{3\text{-}12}EOs$ between the 6th retention time and the influx ($Mass_{out}$ – $Mass_{in}$) was M_{diff}=-

0.05 mg d^{-1} indicated an overall 79% flux removal which was higher to what was seen (57%) during mesophilic digestion of mixed sludge. During the entire digestion period, the removed $NP_{3-12}EOs$ ($Mass_{in} - Mass_{out}$) accounted for 0.19 mg d^{-1} and as it was the case with the mesophilic digestion of mixed sludge there was no direct evidence for the contribution of $NP_{3-12}EOs$ to $NP_{1-2}EOs$ since the start levels on $NP_{1-2}EOs$ were too high. However, NP, NP_1EC and NP_2EC indicated overall accumulation in terms of flux. The reduction in concentration of $NP_{3-12}EOs$ during thermophilic digestion was consistent with mesophilic digestion of mixed sludge.

Short-chained nonylphenolics reduced during the entire digestion period from the 1st retention time. The start concentration was 90 mg kg^{-1} dw whereas the concentration at the 1st RT, 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT was 16 mg kg^{-1} dw, 12 mg kg^{-1} dw, 11 mg kg^{-1} dw, 10 mg kg^{-1} dw, 9 mg kg^{-1} dw and 7 mg kg^{-1} dw, respectively. The change in concentration between the 6th retention time digestate and the start levels was -74 mg $NP_{1-2}EOs$ kg^{-1} dw. Overall, the mass reduction of $NP_{1-2}EOs$ at the 6th retention time ($Mass_{out} - Mass_{in}$) was M_{diff}=-42.2 mg d^{-1} which resulted to 99.7% flux removal. Nonylphenol monoethoxylate was removed by 99.9% whereas NP_2EO was removed by 76%. During the entire digestion period, the sum of the removed short-chained nonylphenolics was 45.3 mg d^{-1}.

The residual $NP_{1-2}EOs$ concentrations after 90 days of anaerobic digestion are in close agreement with (Montgomery-Brown & Reinhard, 2003) that short-chained nonylphenolics persist under anaerobic conditions. Additionally, (Zhang et al., 2008) observed accumulation of $NP_{1-2}EOs$ in UASB effluents after the period of five months.

However, NPECs did not change significantly in concentration at the 1st retention time (0.06 mg kg^{-1} dw) compared to the start concentration (0.08 mg kg^{-1} dw). At the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT the concentration of NPECs was 0.06 mg kg^{-1} dw,0.06 mg kg^{-1} dw, 0.06 mg kg^{-1} dw, 0.06 mg kg^{-1} dw and 0.05 mg kg^{-1} dw, respectively. An insignificant difference (-0.03 mg NPECs kg^{-1} dw) was obtained between the 6th retention time digestate and the start concentration. Overall, the mass accumulation of NPECs at the end of the thermophilic digestion period of mixed sludge ($Mass_{out}$ –

$Mass_{in}$) was M_{diff}= -0.6 mg d^{-1} which resulted to a negative overall flux removal of -8922%. Only NP_3EC indicated removal (3%) whereas NP_2EC (-849%) and NP_1EC (-23389%) exhibited the highest accumulation. Although the accumulation of NP_1EC could not be balanced with the NP_1EO removal there was evidence that NP_1EO was contributed to NP_1EC.

However, regarding nonylphenol, its concentration at the 1st RT (0.2 mg kg^{-1} dw) increased from that at the start concentration (0.1 mg kg^{-1} dw). At the 2nd RT, 3rd RT, 4th RT, 5th RT and 6th RT the concentration was 0.3 mg kg^{-1} dw, 0.3 mg kg^{-1} dw, 0.1 mg kg^{-1} dw, 0.1 mg kg^{-1} dw and 0.03 mg kg^{-1} dw, respectively. Overall, NP indicated a progressive increase in concentration during the first three retention times and then decreased over the digestion period (Figure 6-6). The change in concentration of -0.1 mg kg^{-1} dw, which resulted from the difference between the 6th retention time digestate and the start concentration indicated its overall reduction irrespectively of the increasing trends during the first three retention times. The mass reduction of NP between the 6th RT and the influx ($Mass_{out}$ – $Mass_{in}$) was M_{diff}=-0.01 mg d^{-1}. In terms of flux removal, NP removed by 80.2%. The overall biodegradation of NP during the entire digestion period of mixed sludge ($Mass_{out}$ – $Mass_{in}$) was M_{diff}=-0.01 mg d^{-1}). Although the loss of NP_1EC could not be balanced for the increased NP, both compounds exhibited opposing trends during the first three retention times. However, the reduction of NP in thermophilic conditions was consistent with the mesophilic digestion of mixed sludge. In addition, the NP results were in contrary with (Patureau et al., 2008) and (Chang et al., 2005; Chang et al., 2004) during their batch thermophilic anaerobic trials by using different electron acceptors at pH 7.0.

6.2.5. Effect of digestion temperature on alkylphenol ethoxylates

6.2.5.1. Primary sludge (mesophilic vs. thermophilic)

The efficiency between mesophilic and thermophilic digestion of primary sludge in terms of the overall nonylphenolic (ΣNPEOs) removal was low. Thermophilic digestion indicated 0.8% flux removal for the total nonylphenolics i.e. ΣNPEOs whilst during mesophilic digestion the removal was negative i.e. formation of nonylphenolics occurred (-177%) despite their longer retention time (Figure 6-8).

High and similar flux removals of $NP_{3-12}EOs$ were achieved during both mesophilic (72%) and thermophilic digestion (71%). The rate of $NP_{3-12}EOs$ removal during thermophilic digestion was slightly higher (-0.007 mg d^{-1} RT^{-1}) than it was during mesophilic digestion (-0.006 mg d^{-1} RT^{-1}) which may indicate higher bacterial activity in these digesters (Table 6-1). However, thermophilic digesters were receiving 1.7 times higher $NP_{3-12}EOs$ loads. The high $NP_{3-12}EOs$ removals during both temperature digestions implied the existence of appropriate bacteria/enzymes for the attack of $NP_{3-12}EOs$. Furthermore, the results are consistent with the literature (Montgomery-Brown & Reinhard, 2003; Patureau et al., 2008).

Short-chained nonylphenolics in primary sludge did not accumulate under mesophilic conditions, however the persistence of NP_1EO was observed in the thermophilic digesters. The NP_1EO persistence could be partially explained by the higher $NP_{3-12}EOs$ loadings in these digesters, which resulted to $NP_{1-2}EOs$ that are typical metabolic products in anaerobic conditions (Brunner et al., 1988; Ejlertsson et al., 1999). Lipophilic compounds in the raw primary sludge such as fats, oils and greases could have served as significant adsorbent sites for the hydrophobic NP_1EO with a high octanol-water partitioning index (Log K_{ow} = 4.17) (Ahel & Giger, 1993a). Despite these reasons, shorter retention time could have restricted the bacterial growth and ultimately the biodiversity of the thermophiles as well as the development of specific enzymes who required for the initial attack of the lipophilic NP_1EO. Exoenzymes are responsible for the attack of lipophilic/insoluble substrates under anaerobic conditions (Dalton &

Stirling, 1982). It is therefore postulated that the appropriate exoenzymes required for the hydrolysis of the lipophilic NP_1EO were limited. If appropriate enzymes were present for the NP_1EO attack then the higher temperature during thermophilic conditions would have accelerated the rate of the specific biochemical reaction/ biotransformation. The synergy of the above parameters could have accounted for the persistence of NP_1EO in the thermophilic digestion of primary sludge. However, the combined removal for short-chained nonylphenolics during the thermophilic methanogenic conditions was limited (2.3%) due to the persistence of NP_1EO. In the same temperature digestion, NP_2EO exhibited higher flux removal (80%). The mesophilic digestion of primary sludge indicated the highest flux removals of NP_1EO and NP_2EO (>90%). The observed removals for NP_1EO and NP_2EO during both mesophilic and thermophilic digestions were higher than those presented by (Benabdallah El-Hadj, 2006) (48% and 71%, respectively) by using 100% SAS with 18 days SRT. First order degradation rates for NP_2EO were greater at mesophilic temperature compared to thermophilic digestion temperature. During thermophilic digestion these rates were negative, irrespective of sludge type, indicative for the formation of NP_2EO from the degradation of the longer nonylphenolic ethoxylates (Table 6-2). Regarding NP_1EO, degradation rates were greater during thermophilic digestion than they were during mesophilic digestion, presumably due to the higher digestion temperature.

The presence of the short-chained carboxylated nonylphenolics was observed in both temperature digestion trials. During mesophilic digestion, significantly high concentrations of NPECs, higher than the concentration in the feed sludge appeared in the digestates obtained from the 1st retention time (161 mg kg^{-1} dw) and then the concentrations gradually decreased with time. In contrary, during thermophilic digestion NPECs appeared from the 2nd retention time and gradually increased with time. Despite the higher NPECs loadings in mesophilic compared to thermophilic digesters, NPECs in the former digesters degraded over time. In addition, the fast biotransformation of $NP_{1-12}EOs$ to NPECs implied the occurrence of diversified mesophilic consortia in primary sludge capable for converting NPEOs to NPECs and in parallel the contaminant assimilation of NPECs. The abundance of electron acceptors/donors and the presence of

the appropriate enzymes to carry out the biotransformation of $NP_{1-12}EOs$ and in addition the successive assimilation of the carboxylated by-products could possibly lie in the long retention time and the dynamic mesophilic bacteria. The phenomenon of diversification of the mesophilic primary sludge was seen during the digestion of steroid estrogens in which higher removals were achieved by the mesophiles (53%) compared to the thermophiles (51%). However, the ability of the thermophiles to biotransform $NP_{1-12}EOs$ to NPECs implied acclimation of bacteria/enzymes to the specific species. The high influx of electron acceptors and donors in thermophilic digesters due to higher feed loadings could have contributed to the biotransformation. Despite the short retention time, and the various bacteriological concerns with thermophilic bacteria, the specific substrate demonstrated the ability to hydrolyse the non-ionic nonylphenolics, specifically the lypophilic species to more soluble substrates like NPECs. The apparent biotransformation of $NP_{1-12}EOs$ to NPECs by the methanogenic mesophilic and thermophilic bacteria in primary sludge could be a major co-metabolic mechanism of nonylphenolics because of the inherent bacteriology in this sludge type.

With respect to flux removals, mesophilic digesters demonstrated accumulation of the combined NPECs (-215%). Only NP_1EC indicated 54% removal with positive degradation rates (*k*) whilst both NP_2EC (-211%) and NP_3EC (-7692%) were accumulated under mesophilic conditions i.e. with negative (*k*) (Table 6-2). Significant mass difference between the digestate obtained at the 6th retention time and the feed (1.1 mg d^{-1}) occurred for NP_2EC (M_{diff}=2.3 mg d^{-1}) and that increase in mass accounted circa 3 fold. Overall, carboxylated species predominated in terms of concentration during the entire mesophilic digestion period as it is demonstrated on the percent distribution of each respective digestate. However, during thermophilic digestion, NPECs influx was less than 0.005 mg d^{-1}, lesser than the influx during mesophilic digestion. In contrast to the mesophilic digestion, only NP_3EC was removed (0.32%) during the thermophilic trial whereas both NP_1EC (-4685%) and NP_2EC (-6078%) were accumulated. Under thermophilic conditions, the evolution i.e. accumulation of NPECs occurred progressively for each retention time as it is demonstrated on the percent distribution of each respective digestate.

Based on the mass difference (M_{diff}) between the start (feed) and the 6th retention time digestate, NP was removed by 45% during mesophilic digestion and by 57% during thermophilic digestion of primary sludge. The associated standard errors (SE) of those removals were below 1%, respectively. The mean value (n=4) for NP flux removal (mg d^{-1}) over the entire mesophilic digestion was 33% whereas the mean value for the thermophilic digestion was 41%.

Besides the carboxylated nonylphenolics, NP exhibited the lowest removal during both digestion temperature experiments. Besides the physicochemical-structural relationships that characterise NP, the accumulation of this compound occurs inevitably from the build up of NPEOs metabolites as it has been demonstrated in many studies (Renner, 1997; Schröder, 2001; Ejlertsson et al., 1999; Brunner et al., 1988). However, the results obtained from both temperature digestions indicated that the partial biodegradation/ biotransformation of $NP_{3\text{-}12}EOs$ resulted predominantly to NPECs, with an exception of NP_1EO under thermophilic conditions and as a result, there was no contribution to NP. The results from the primary sludge digestions, irrespective of temperature, are in line with (Patureau et al., 2008; Ferguson & Brownawell, 2003; Chang et al., 2005; Chang et al., 2004; Chang et al., 2009).

First order degradation rates for NP in primary sludge were higher during thermophilic digestion (k = 0.0912 d^{-1} at 53^{o}C) compared to mesophilic digestion (k = 0.0248 d^{-1} at 35^{o}C) (Table 6-2). In general, NP removal was associated with the retention time for both temperature digestion trials which suggested bacterial adaptation and/or limited contribution from short-chained nonylphenolic metabolites. However, in terms of concentration, retention time seemed to have limited effect on NP.

Overall, mesophilic digesters indicated slightly higher rate on ΣNPEOs removal per volatile solids (VS) removal (28.4 mg $\Sigma NPEOs_{\ removal}$ g $VS_{\ removed}$ d^{-1}) than the thermophilic digesters (26.1 mg $\Sigma NPEOs_{\ removal}$ g $VS_{\ removed}$ d^{-1}). However, the rate of VS removal under thermophilic conditions was 2.1 fold higher than it was under mesophilic conditions, which implied that the mesophilic consortium was more efficient in terms of ΣNPEOs removal per VS removal than the thermophilic consortium.

Thermophilic digestion of primary sludge removed more ΣNPEOs than the primary sludge during the mesophilic digestion despite the similar ΣNPEOs loadings (1.27 and 1.35 mg d^{-1}, respectively) (Figure 6-8).

The concentration of ΣNPEOs in the mesophilic trial was increasing with digestion period due to the formation and the persistence of NPECs which were present at high concentrations in the feed. On the other hand, during thermophilic digestion of primary sludge the concentration of ΣNPEOs remained unchanged throughout the digestion period. It was evident that the low removals of ΣNPEOs in primary sludge irrespective of digestion period were mainly attributed to the carboxylated metabolites of nonylphenolics. The mesophilic digesters indicated formation of nonylphenolics (-177%) despite the longer retention time (30 days) compared to the thermophilic digestion (15 days) which indicated a slight removal (~1%). Longer retention times promote the growth of slow-growing methane-forming bacteria compared to shorter retention times. Although thermophilic methane-forming bacteria are limited in numbers, temperature seems to have a positive impact when considering the removal of nonylphenolics since biochemical reactions accelerate temperature. This finding implied

that the performance of the thermophilic digesters in terms of ΣNPEOs removal was better compared to mesophilic digestion. Nevertheless, the removals in primary sludge, irrespective of temperature digestion were insignificant.

6.2.5.2. Mixed sludge (mesophilic vs. thermophilic)

Mixed sludge mesophilic digesters were receiving 1.3 times more ΣNPEOs than the thermophilic digesters but they were removing 0.8 times less ΣNPEOs than the thermophilic digesters.

The $NP_{3\text{-}12}EOs$ flux removals were higher for the thermophilic (99.9%) digesters than it was for the mesophilic (88.7%) digestion of mixed sludge. Similarly, thermophilic digestion achieved higher removal rates -0.006 $NP_{3\text{-}12}EOs$ mg d^{-1} RT^{-1} than mesophilic digestion (-0.003 $NP_{3\text{-}12}EOs$ mg d^{-1} RT^{-1}) (Table 6-1). As it was seen in primary sludge digestion, during mixed sludge digestion the high $NP_{3\text{-}12}EOs$ removals were in line with the literature (Montgomery-Brown & Reinhard, 2003; Patureau et al., 2008) and suggested the degradability/biotransformation in the mixed sludge substrate at both mesophilic and thermophilic temperature.

No accumulation of $NP_{1\text{-}2}EOs$ was observed during mixed sludge digestion, irrespective of temperature. The concentrations of NP_1EO and NP_2EO decreased with time in both temperature digestions, which indicated acclimation to nonylphenolics in the presence of the mixed sludge consortium and implied the presence of appropriate bacteria/enzymes for their attack. Despite the higher $NP_{3\text{-}12}EOs$ loadings of the mesophilic digesters, no accumulation of $NP_{1\text{-}2}EOs$ was observed in these digesters. However, during both temperature digestions NP_2EO indicated lower flux removals than NP_1EO, which could be explained by the contribution of the longer-chained nonylphenolics. Nonylphenol mono ethoxylate (NP_1EO) was removed by 100% by the thermophiles whereas the removal by the mesophiles was lower (90%). Overall, thermophilic digestion achieved 100% removal of $NP_{1\text{-}2}EOs$ whereas lower removals were observed by the mesophilic digestion (89%) presumably to the higher $NP_{3\text{-}12}EOs$ loadings during mesophilic digestion. The faster biochemical reactions by the

thermophiles could have contributed to the rapid biotransformation and removal of $NP_{1-2}EOs$. Under thermophilic conditions, short-chained nonylphenolics, specifically NP_1EO was the predominant specie thorough the entire digestion period. The removals of $NP_{1-2}EOs$ during mixed sludge mesophilic digestion were much higher than those presented by (Benabdallah El-Hadj, 2006) (48% and 71%, respectively) by using 100% SAS with 18 days SRT possibly due to the different sludge types used.

Carboxylated nonylphenolics (NPECs) were present in the digested mixed sludge during both temperature digestions. Overall, during mesophilic digestion, NPECs indicated 58% flux removal. Nevertheless, post the 4th retention time NP_3EC increased and indicated an insignificant accumulation of 0.05 mg d^{-1}, which resulted to a large value of a negative flux removal (-10312%) and similar, NP_2EC increased post the 3rd retention time.

The above observations implied that although mixed sludge contained appropriate bacteria and enzymes to carry out the biotransformation of NPECs, their removal was restricted throughout the digestion period. This phenomenon could have occurred by the excessive contribution of NPEOs to $NP_{2-3}ECs$ after the 3rd or 4th retention times and/or by the delayed biotransformation/degradation of $NP_{2-3}ECs$. It is postulated that trapped enzymes in the dyspeptic extracellular polymeric substances (EPS), acclimated to this biotransformation during the activated sludge process, could have been released because of digestion and subsequently promoted the 'delayed' biotransformation of NPEOs to $NP_{2-3}ECs$. If this hypothesis is true, then the rate of NPEOs biotransformation to NPECs would have exceeded the rate of $NP_{2-3}ECs$ degradation. Due to their high concentrations, NPECs predominated in each respective digestate as it is demonstrated on the percent distribution.

However, despite the constant concentration of NPECs throughout the thermophilic digestion which remained at similar levels to those concentrations in the feed sludge (0.08 mg kg^{-1} dw), NPECs were indicated a negative flux removal (-8922%). Accumulation was observed for NP_1EC (-23389%) and NP_2EC (-849%) whilst NP_3EC was removed (2.3%). The overall NPECs accumulation during the thermophilic

digestion could be attributed to the high NPEOs loadings in these digesters. However, the presence of NPECs suggested that the appropriate bacteria/enzymes responsible for NPEOs biotransformation to NPECs were present in mixed sludge as it was seen in the same sludge type during the mesophilic digestion. Regardless of the high loadings, the persistence of NPECs during the 15 days retention time may point out that the thermophiles responsible for NPECs degradation/biotransformation could be slow-growing bacteria. However, since thermophilic bacteria are less abundant than mesophilic bacteria (Gerardi, 2003), NPECs persistence could equally be attributed to the limited biodiversity of the thermophiles.

Based on the mass difference (M_{diff}) between the start (feed) and the 6th retention time digestate, NP was removed by 51% during mesophilic digestion and by 80% during thermophilic digestion of mixed sludge. The associated standard errors (SE) of those removals were below 1%, respectively. However, the mean value (n=4) for NP flux removal (mg d^{-1}) obtained from each retention time during mesophilic digestion was 55% whereas the mean value for the thermophilic digestion indicated accumulation of NP (-11%). Nevertheless, since the six-retention time digestion period was evaluated and for comparison purposes, the M_{diff} values were used.

The higher $NP_{3-12}EOs$ loadings during mesophilic digestion may have accounted for the large differences of NP removal between the different temperature digestions. In addition, the higher temperature could have also initiated the enzymatic activity in these digesters. First order degradation rates for NP in mixed thermophilic sludge were the highest among the examined sludges i.e. (k = 0.3495 d^{-1} at 53^{o}C) compared to mesophilic digestion of mixed sludge (k = 0.0304 d^{-1} at 35^{o}C) (Table 6-2). There was no significant contribution from long-chained nonylphenolics and/or possibly from NPECs to NP and this phenomenon could have possibly accounted for the removal of NP in the mixed sludge in the different temperature digestion trials. During thermophilic digestion, NP accumulated during the first three retention times and then it was removed. This observation implied either the evolution of appropriate bacteria and/or bacterial acclimation i.e. lag period or the insignificant metabolic contribution of short-chained nonylphenolics or NPECs. The results from both temperature digestion

trials with mixed sludge are in line with (Patureau et al., 2008; Ferguson & Brownawell, 2003; Chang et al., 2005; Chang et al., 2004) that NP may undergo anaerobic degradation under certain conditions. There was no apparent association between NP removal and digestion period implied that bioavailability could be a limiting factor in this sludge.

Overall, the concentration of ΣNPEOs during thermophilic digestion decreased with digestion period, which indicated the possible effect of high temperature on the enzymatic activity. However, during mesophilic digestion the concentration of ΣNPEOs indicated an increasing trend with time. This observation implied that temperature may be more significant for ΣNPEOs degradation/biotransformation than longer retention time or higher biodiversity who is usually associated with the mesophilic bacteria (Metcalf and Eddy, 2003; Gerardi, 2003). Mesophiles demonstrated their capability for biotransformation whilst thermophilic bacteria indicated greater degradation potential of ΣNPEOs.

The efficiency in terms of ΣNPEOs flux removal between the different temperature digestions utilising mixed sludge indicated that thermophilic digestion was more efficient in removing alkylphenolics (92%) than mesophilic digestion (56%).

Table 6-1 Relationships of nonylphenol ethoxylate mass fluxes and biological rates.

	Mesophilic		Thermophilic	
	Primary sludge	Mixed sludge	Primary sludge	Mixed sludge
$NP_{3-12}EOs$ removal rate mg d^{-1} RT^{-1}	-0.006	-0.003	-0.007	-0.006
R^2	0.79	0.53	0.76	0.90
$NP_{1-2}EOs$ removal rate mg d^{-1} RT^{-1}	-0.003	-0.004	0.244	-0.009
R^2	0.88	0.92	0.98	0.18
NP removal rate mg d^{-1} RT^{-1}	-0.001	0.009	-0.002	-0.004
R^2	0.86	0.16	0.91	0.54
ΣNPEOs net removal rate mg d^{-1} RT^{-1}	-0.742	-0.097	-0.006	-0.123
R^2	0.85	0.02	0.02	0.93
mg $\Sigma NPEOs_{removed}$ g VS $_{removed}$ d^{-1}	2.1	-5.8	-0.005	-3.8
mg $\Sigma NPEOs_{removed}$ g VS $_{content}^{-1}$	0.041	-0.086	-0.000	-0.123

Note: Biodegradation refers to partial biodegradation i.e. microbial removal and not to mineralization.

Table 6-2 First order kinetic constants (*k*) for nonylphenol ethoxylates in primary and mixed sludge during mesophilic and thermophilic anaerobic digestion trials.

	Mesophilic		Thermophilic	
	Primary sludge	Mixed sludge	Primary sludge	Mixed sludge
	k (per day)	*k* (per day)	*k* (per day)	*k* (per day)
NP	0.0248	0.0304	0.0912	0.3495
NP_1EC	0.0035	0.1392	0.0007	0.0910
NP_2EC	-0.0274	0.0320	-0.0738	0.0030
NP_3EC	-0.0129	-0.0439	-0.0016	0.0000
NP_1EO	0.2969	0.2992	1.7958	0.8152
NP_2EO	0.4316	0.0917	-0.0352	-0.0417

6.2.6. Effect of sludge type on alkylphenol ethoxylates

6.2.6.1. Mesophilic digestion (primary sludge vs. mixed sludge)

Long-chained nonylphenolic flux removals were 72% for primary sludge and 57% for mixed sludge during 180 days of mesophilic anaerobic digestion (30 days retention time). A possible explanation for the differences is that primary sludge bacteria that receive the untreated stream of EDCs have developed the appropriate mechanisms for attacking the parent nonylphenolics. On the hand, the activated sludge content in mixed sludge is more adapted to 'treated' EDCs hence the slower assimilation of the parent nonylphenolics. The high $NP_{3\text{-}12}EOs$ removals of primary sludge are consistent with the literature (Zhang et al., 2008; Montgomery-Brown & Reinhard, 2003).

Short-chained nonylphenolics indicated similar flux removal for the primary (90%) and the mixed sludge (89%). Although $NP_{1\text{-}2}EOs$ regarded persistent metabolites due to their lipophilic properties (Ahel & Giger, 1993a), no accumulation of these species was observed in the different sludge digestions. In addition, their removal occurred with digestion time, which suggested acclimation. This phenomenon could be attributed to the long retention time in these digesters who promoted the growth of the slow growing bacteria which explains the increased biodiversity. Despite the higher $NP_{3\text{-}12}EOs$ loadings in primary sludge, $NP_{1\text{-}2}EOs$ removal rates were similar in both sludges which indicated the prevalence of appropriate conditions for the sustainability of vital bacteria/enzymes to attack $NP_{1\text{-}2}EOs$. The observed trends for the short-chained nonylphenolics during mesophilic digestion are in agreement with (Montgomery-Brown & Reinhard, 2003; Chiu et al., 2010; Zhang et al., 2008).

Mesophilic anaerobic digestion of primary and mixed sludge resulted to the formation of NPECs, which is consistent with (Schröder, 2001) and the observations of (Field & Reed, 1999) from real scale mesophilic anaerobic digesters. Because of the low concentrations of short-chained nonylphenolics throughout the respective digestion periods, microbial growth from these species could be difficult to be achieved and therefore a possible explanation for the presence of NPECs could be that of a co-metabolic activity. In

primary sludge, NPECs accumulated whilst in mixed sludge these species were reduced. In general, the maximum concentration of all nonylphenolics (ΣNPEOs) in primary and mixed sludge was centred at the carboxylated nonylphenolics (specifically NP_2EC) during the mesophilic digestion.

Nonylphenol exhibited greater reduction (partial biodegradation) in terms of flux removal in the mixed sludge (51%) compared to the primary sludge (45%) during mesophilic digestion. The lower NP removal in primary sludge could be due to the higher content of the lipophilic substances compared to the mixed sludge. The degradation/biotransformation of $NP_{1-12}EOs$ resulted to NPECs species and not to NP and this mechanism presumably restricted the build up of NP from NPEOs metabolites, as explained in section 6.2.5.1.

A rough estimation of the removal efficiency of the biomass (the amount of organic matter present in VS) of the primary and mixed sludge during mesophilic digestion was evaluated by activity i.e. mg nonylphenolics removed per gram of VS content in the digester. The calculation was determined by taking the mass difference (M_{diff}) of the feed and the 6th retention time digestate and dividing it by the VS content in grams of the digesters. The calculation was performed for each sludge type. Since volatile suspended solids were not measured, the VS should represent in this case the closest approximation to biomass.

The biomass activity per milligram of nonylphenolics indicated greater activity of mixed sludge biomass (0.086 mg ΣNPEOs g VS^{-1}) compared to that of the primary sludge (0.041 mg ΣNPEOs g VS^{-1}) (Table 6-3). However, higher (7.7x) ΣNPEOs loadings in mixed sludge may explain the ΣNPEOs (mg d^{-1}) removals as opposed to the formation observed in primary sludge. The observed elimination or removal of ΣNPEOs in mixed sludge cannot be interpreted in terms of ultimate biodegradation but rather as biotransformation into more persistent metabolites under the specific conditions.

In terms of concentration, mixed sludge digesters demonstrated reduction between the start concentration and the 6th retention time digestate. The concentration of ΣNPEOs at the 6th retention time was higher compared to the feed concentration in primary sludge (30.5 mg kg^{-1} dw) (Figure 6-7) due to formation of NPECs.

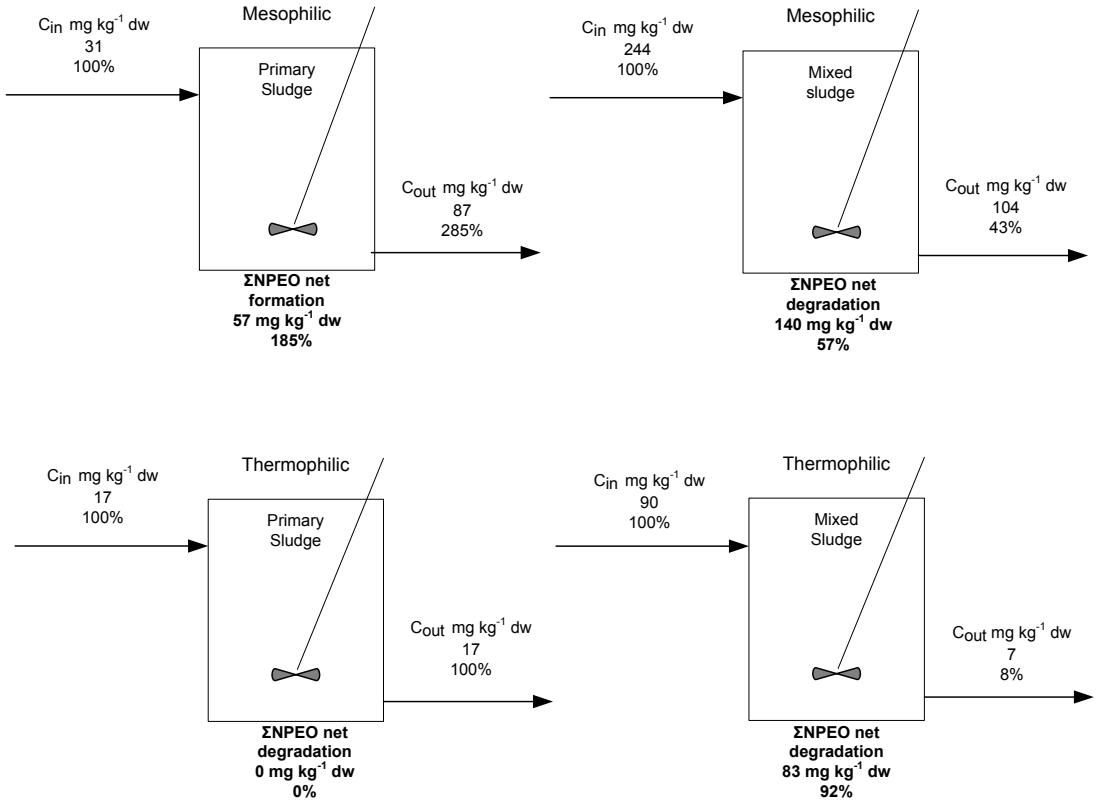


Figure 6-7 Concentrations (mg kg^{-1} dw) of nonylphenol ethoxylates at the start and at the end of the anaerobic mesophilic and thermophilic digestion trials for both sludge types.

Based on the concentrations observed at the end of the mesophilic digestion, the biomass of primary sludge indicated the potential to biotransform nonylphenolics to NPECs, whilst the activity of the mixed sludge biomass indicated greater potential for reducing NPEOs but less biotransformation potential. The indigenous acclimated enzymes to NPECs could possibly account for the observed degradation potential in mixed sludge whilst the lack of NPECs degrading enzymes in primary sludge favoured the co-enzymatic metabolism of NPECs.

Mixed sludge indicated 58% flux removal of ΣNPEOs whereas primary sludge indicated formation of nonylphenolics with emphasis the carboxylated species (Figure 6-8). The presence of activated sludge in mixed sludge could have accounted for the removals since activated sludge is more acclimated to EDCs through the returned liquors than primary sludge. The individual removal efficiencies for each alkylphenolic compound are presented in Appendix V.

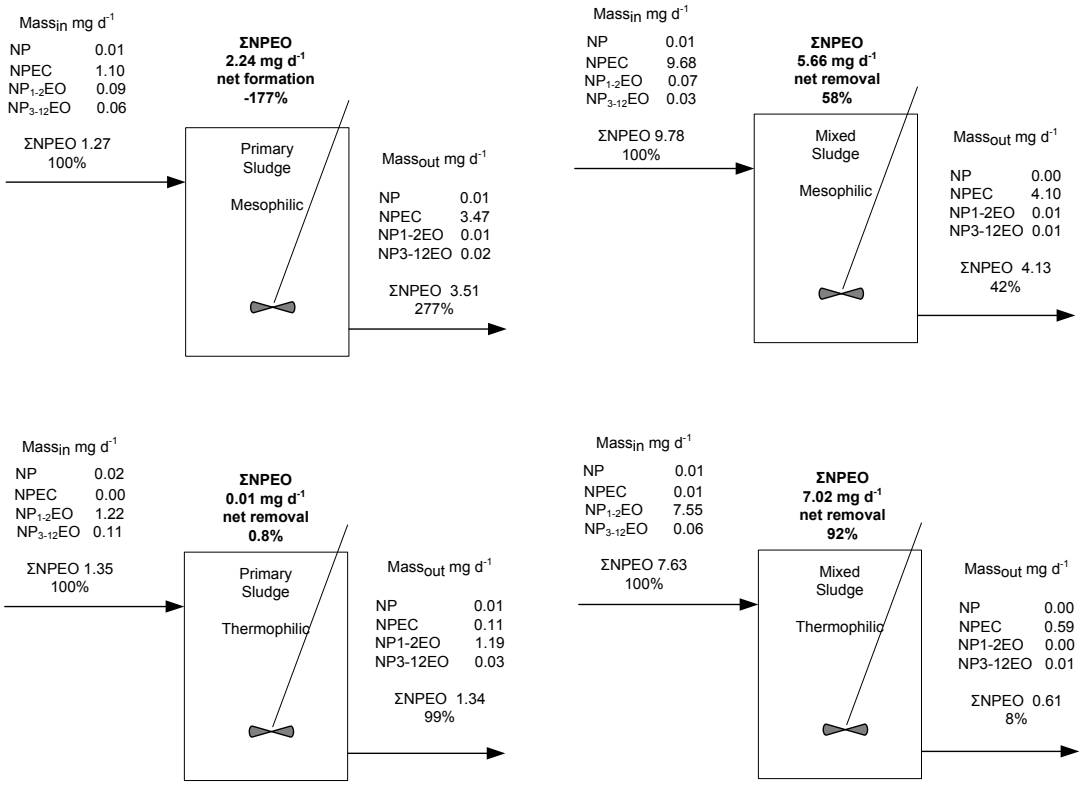


Figure 6-8 Mass flux (mg d^{-1}) for alkylphenol ethoxylates at the start and at the end of the anaerobic mesophilic and thermophilic digestion trials for both sludge types.

6.2.6.2. Thermophilic digestion (primary sludge vs. mixed sludge)

Removals of long-chained nonylphenolics during thermophilic digestion were higher than those obtained at mesophilic conditions despite the similar $NP_{3-12}EOs$ loadings. In particular, mixed sludge demonstrated 79% whilst primary sludge 71% $NP_{3-12}EOs$ removals which they achieved with increasing digestion period.

Mixed sludge demonstrated 99.9% $NP_{1-2}EOs$ flux removals whereas primary sludge indicated circa 2% removal because of the limited removal of NP_1EO due to the high content in feed sludge. Nevertheless, similar $NP_{1-2}EOs$ reducing trends were observed with increasing digestion period in thermophilic conditions suggesting acclimation. In contrary, during mesophilic digestion $NP_{1-2}EOs$ removals were greater in both sludge types, which indicated the importance of adequate sludge retention time. The least removed NP_1EO is in agreement with the literature (Ahel & Giger, 1993a) that the lipophilic properties of this specie makes it persistent in anaerobic environments.

Thermophilic digestion of primary sludge resulted to NPECs and specifically NP_2EC as it was also seen during the mesophilic digestion of primary sludge. The presence of NPECs during anaerobic digestion of primary sludge is also in agreement with (Schröder, 2001). In contrary, the absence of NPECs in mixed sludge digestion indicated that the formation of NPECs in this sludge under thermophilic conditions was not favoured. This finding supports the previous hypothesis that the biomass activity of mixed sludge has limited biotransformation potential compared to primary sludge. The presence of NPECs during thermophilic digestion of mixed sludge are not in agreement with (Benabdallah El-Hadj, 2006) who did not observe these species during the thermophilic anaerobic digestion of mixed sludge.

The flux removal of NP in mixed sludge (80%) was much higher compared to primary sludge (57%) during the thermophilic digestion. During the first three retention times, the increased trend of nonylphenol was occurred in parallel with the decreasing trend of $NP_{1-2}EOs$ whilst for the same digestion period, NPECs remained at low levels. Therefore, accumulation of NP could have resulted because of the metabolic biotransformation of NP_1EO and/or NP_1EC as explained in section 6.2.5.1. Post the 3rd

retention time, diffusion and bioavailability phenomena could have tentatively resulted to the reduction of NP. Interestingly, during the reduction period of NP, VFAs were increased suggesting a change in the microbial community, nevertheless no significant correlation was observed. The nonylphenol trends observed during primary and mixed thermophilic digestion are in argument with the results obtained during mesophilic digestion and equally are consistent with (Patureau et al., 2008; Chang et al., 2005; Chang et al., 2004).

The biomass activity per milligram of nonylphenolics in mixed sludge was significant (0.123 mg ΣNPEOs g VS^{-1}) as opposed to primary sludge (0.0002 mg ΣNPEOs g VS^{-1}) (Table 6-3). The biomass activity (based on VS) of mixed sludge was the highest of all sludges examined at mesophilic and thermophilic temperatures. However, mixed sludge was receiving 5.6 times higher ΣNPEOs loadings compared to primary sludge at thermophilic conditions (Figure 6-8).

As it was the case with steroid estrogens, statistical analyses were performed to estimate the statistical inferences before and after mesophilic and thermophilic digestion. Normality tests (Anderson-Darling) hypotheses were not violated ($p>0.1$) (Appendix III) and the variances between the different temperature digestions by using Bartlett's and Levene's tests were investigated. Statistical analysis confirmed that the nonylphenolic mass entering the primary and mixed thermophilic digesters had similar variances that did not differ significantly ($p>0.05$) and the same hypothesis was true for the mesophilic trials ($p>0.05$) as it was seen with steroid estrogens. Two-sample t-tests ($t=1.19$, $DF=7$, $p>0.05$) indicated that the mean differences between the influxes (mg d^{-1}) for the primary and mixed sludge in mesophilic digesters did not differ significantly. Similarly, primary and mixed sludge influxes were not significantly different ($t=2.65$, $DF=5$, $p>0.01$) during thermophilic digestion (Appendix III). Presumably, the observed similarities between the different sludge types within the respective digestion temperatures were due to the similar feed rates among the different digestion temperatures and due to the large standard deviations (Appendix III). Because temperature and retention time between primary and mixed sludge mesophilic digestions were similar as it was the case for the respective thermophilic digestion trials,

these results showed that differences could be narrowed down to the microbiology and may be independent of the overall nonylphenolic loadings as it was seen with steroid estrogens.

At the end of the thermophilic digestion of primary sludge, ΣNPEOs concentration remained the same as the feed concentration, whereas during mixed sludge digestion, the reduction in concentration at the 6th retention time from the feed concentration was 83 mg kg^{-1} dw. Similar observations were observed during mesophilic digestion implying that mixed sludge has greater potential for reducing NPEOs than primary sludge bacteria. Irrespective of digestion temperature, the acclimated portion of mixed sludge during the activated sludge process could have accounted for this difference. The efficiencies in terms of ΣNPEOs removals between primary (1%) and mixed sludge (92%) during thermophilic digestion indicate that mixed sludge demonstrates greater potency for the digestion and the significant reduction of the measured ΣNPEOs metabolites. The individual removal efficiencies for each individual alkylphenolic compound are presented in Appendix V.

As it was the case during mesophilic digestion, the observed elimination of ΣNPEOs during thermophilic digestion cannot be interpreted in terms of ultimate nonylphenolic biodegradation but as biotransformation into more persistent metabolites such as NPECs and the non-measured species as described in the literature review.

Table 6-3 Ratio of nonylphenolics that demonstrates biodegradation and biotransformation.

	Mesophilic				Thermophilic				Phenomenon
	Primary sludge		Mixed sludge		Primary sludge		Mixed sludge		
	Start	6RT	Start	6RT	Start	6RT	Start	6RT	
$NP_{3\text{-}12}EOs/NP_{1\text{-}2}EOs$	1.8	2.1	1.1	1.7	2.5	0.0	12.5	4.5	Biotransformation
$NP_{3\text{-}12}EOs/NP_{1\text{-}2}EOs+NP$	1.2	1.2	1.0	1.2	1.6	0.0	2.6	2.5	Biotransformation
$NP_{3\text{-}12}EOs/NPECs$	0.07	0.05	0.01	0.00	0.06	0.30	0.04	0.02	Biotransformation
$NP_{3\text{-}12}EOs/NP_{1\text{-}2}EC+NP$	0.007	0.005	0.01	0.00	0.06	0.30	0.04	0.02	Biotransformation
$NP_{1\text{-}2}EOs/NP_{1\text{-}2}EC$	0.004	0.002	0.01	0.00	0.02	11.12	0.003	0.005	Biotransformation
NP_1EO/NP	1.7	1.2	6.5	2.1	1.7	147.0	0.2	0.8	Biotransformation
$NP_{1\text{-}12}EOs+NPECs/NP$	563	515	1333	1343	80	164	94	269	Biodegradation to NP

Note: Ratios were derived for each sludge type by obtaining the sum of each nonylphenolic (mg d^{-1}) from each entire digestion period (6RT) divided by the respectively nonylphenolic/combination of nonylphenolic (mg d^{-1}). Values represent the total solids and aqueous phase combined.

7. RESULTS: FATE OF NONYLPHENOL ETHOXYLATES UNDER HIGH RECEIVING CONCENTRATIONS

After the completion of the mesophilic anaerobic digestion trials (six retention times i.e. 180 days) (chapter 6), a second experiment was carried out which involved higher feed concentrations (shock loading) of non-ionic alkylphenolics ($NP_{1-12}EOs$). The rational of this experiment was to understand whether the biomass would be able to biodegrade the additional NPEOs and whether the spiked NPEOs would have an effect on the biodegradation process or the anaerobic digestion process. Real scale anaerobic digesters may receive different NPEOs loadings due to the variability of EDCs in the influent of STWs as it was observed through the EDC-research group (Koh, 2008; Koh et al., 2009). The shock loading experiment was therefore carried out to investigate the overall capacity of anaerobic digestion to manage higher NPEOs loadings. The research hypothesis ($H1ND_0$) was:

$H1ND_0$: Primary or mixed sludge biomass would not be able to degrade the additional NPEOs loadings within one retention time.

$H1ND_\alpha$: Primary or mixed sludge biomass would be able to degrade the additional NPEOs loadings within one retention time.

The objective of the 'shock' loading experiment was:

1. Examine the effect of higher NPEOs loadings.

2. Identify whether mesophilic anaerobic digestion of mixed sludge (60:40 v/v, primary sludge: SAS) could demonstrate greater capacity for alkylphenolic removals than primary sludge digestion, when digesters receive higher concentrations of non-ionic nonylphenolics than the concentrations they were receiving continuously for 180 days of digestion (chapter 6).

Both type of feed sludges i.e. primary and mixed sludge were dosed with non-ionic alkylphenolics $NP_{1-12}EOs$ at a level circa 7 – 9 times higher their respective background

concentrations prior to feeding. The experiment was carried out at mesophilic temperatures as described in chapter 6.

This chapter examines the experimental description (section 7.1), performance of the anaerobic digesters during the shock loading experiment (section 7.2), feed characterization in terms of the respective dosed concentrations (section 7.3) and the trends observed in the primary and mixed sludge mesophilic digesters (section 7.4).

7.1. Experimental description

The description of the shock loading experiment was presented in section 3.3. Dosing concentrations were according to the respective total solids contents of the respective primary or mixed sludge mesophilic digesters. The dosing solution aimed to increase the concentration of the 'parent' $NP_{1\text{-}12}EOs$ in each feed sludge by 10 times. However, due to inconsistencies of the stock solutions and losses such as adsorption, digesters received lower concentrations. Dosing onto primary feed sludge resulted to 7.8x higher (28.7 mg kg^{-1} dw) the background concentration of $NP_{1\text{-}12}EOs$ and 9.0x higher (21.8 mg kg^{-1} dw) the background $NP_{1\text{-}12}EOs$ concentration in mixed sludge. However in terms of mass, primary sludge received 4.1x higher $NP_{1\text{-}12}EOs$ and mixed sludge 5.1x higher $NP_{1\text{-}12}EOs$ than what the digesters were receiving prior to spiking.

7.2. Anaerobic digesters performance during high receiving concentrations of nonylphenolics

The same type and number of digesters used during the six retention time trials were also used for the high loading experiment, hence four (1.5L) mesophilic ($35^{o}C \pm 0.2^{o}C$) anaerobic digesters were utilised in this study of which two digesters were fed with dosed primary sludge (n=2) and two digesters were fed with dosed mixed sludge (n=2). The feed sludges used in this experiment were identical to those sludges used during the six retention time experiments. The nominal mesophilic digesters retention times were 30 days for both mesophilic trials.

As it is shown on Table 7-1 the digesters did not appear to be adversely affected in terms of performance under 7.8x and 9.0x higher NPEOs loadings of the background concentrations. The measured physicochemical parameters remained at similar levels to those levels measured at the 6th retention time (6RT) during the 180 days of digestion (chapter 4).

Table 7-1 Feed sludge characteristics, anaerobic digestion performance and digestate quality during the 30 days NPEOs loading trial. NPEOs concentrations at 7.8x and 9.0x higher the background levels of primary and mixed sludges, respectively.

	Mesophilic	
Feed sludges	Primary sludge	Mixed sludge
TS (g l^{-1})	51.1±3.7	57.1±4.3
VS (g l^{-1})	36.5±2.6	44.0±3.0
VFA (mg acetic acid l^{-1})	1314±68	1592±44
Operational conditions		
T (^{o}C)	35±0.2	35±0.2
SRT (d)	30	30
OLR (kg VS m^{-3} d^{-1})	1.3±0.1	1.5±0.1
TS (g l^{-1})	24.8±2.6	37.1±1.9
VS (g l^{-1})	17.2±1.9	26.3±3.2
pH	7.3±0.1	7.5±0.1
ORP (mV)	-345.1±21.8	-374.9±15.2
VFA (mg acetic acid l^{-1})	71.3±5.1	122.7±12.9
Total alkalinity (mg l^{-1})	2437±42	5364±68
Biogas		
Daily production (l d^{-1})	0.8±0.1	0.8±0.1
GRP (m^3 m^{-3} d^{-1})	0.51±0.0	0.51±0.0
SGP (m^3 CH_4 kg $VS^{-1}_{removed}$)	0.6±0.1	0.7±0.1
Removal efficiencies (%)		
VS	52.9±6.9	40.2±2.0
TS	51.7±7.2	35.0±4.8

Mean values and standard error (*n*=4); TS: total solids; VS: volatile solids; VFA: volatile fatty acids; T: temperature; SRT: solids retention time; OLR: organic loading rate; ORP: oxidation-reduction potential; GPR: gas production rate; SGP: specific gas production.

7.2.1. Anaerobic digesters stability

Before the commencement of the experiment, both types of digesters were running continuously for 270 days (nine retention times) of which 90 days were the initial stabilization period (three retention times).

Overall, mean pH values for primary and mixed sludge digesters were within the marginal pH range (i.e. pH= 7.2 – 7.7) (Gerardi, 2003). Primary sludge pH (7.3 ± 0.02) was slightly lower than in the mixed sludge digesters (7.5 ± 0.02). During digestion of both spiked primary and mixed sludges, pH values indicated a peculiar increasing tendency from the commencement of the experiment (specifically at day 2).The increased pH trend during the dosed sludge digestions is inconsistent with the high and stable alkalinities measured in both types of digesters. However, the pH values obtained were associated with the same magnitude of error (standard error) as it was the case during the six retention time experiment. The highest pH value measured during primary sludge digestion was 7.5 ± 0.03 at day 14 whereas the highest pH value during the mixed sludge digestion was 7.7 ± 0.07 at days eight and nine, respectively.

Volatile fatty acid (VFAs) content remained at 71 mg acetic acid l^{-1} and 123 mg acetic acid l^{-1} i.e. at similar levels to those levels before spiking for primary and mixed sludge digestion, respectively. Volatile fatty acid contents during both primary and mixed sludge digestion were within the optimum levels (50-500 mg acetic acid l^{-1}) according to (Gerardi, 2003; Metcalf and Eddy, 2003) and similar to levels observed during the six retention time experiment Table 4-1.

Measured total alkalinities ($CaCO_3$) in all digesters were above 2000 mg l^{-1} and remained at similar levels without any buffer additions. Alkalinities were stable throughout the experiment in both, primary and mixed sludge digesters and their stability is inconsistent with the pH alterations observed. However, the relatively high levels of alkalinities in all digesters were indicative of their respective good buffering capacities and the establishment of anaerobic fermentation (Gerardi, 2003) as it was the case during the six retention time trials. Mixed sludge digestion exhibited higher

alkalinities (5364 mg l^{-1}) than primary sludge digestion (2437 mg l^{-1}) throughout the entire period of digestion. Total alkalinity values for both types of dosed sludges were within the optimum (1500-3000 mg l^{-1}) or accepted values for methane-forming bacteria (Gerardi, 2003; Metcalf and Eddy, 2003).

Redox potential was stable during the entire experimental period for both types of digesters and it was always less than -300 mV, which is required for the proper activity of methane-forming bacteria (Gerardi, 2003). Redox values for the dosed primary and mixed sludges were within the optimum range (-200 to -400 mV) for both survival and substrate degradation (Gerardi, 2003)).

7.2.2. Anaerobic digesters efficiency

Organic loading rates remained at the same levels as they were during the six-retention time experiment (1.3 ± 0.1 kg VS m^{-3} d^{-1}) for primary sludge and (1.5 ± 0.1 kg VS m^{-3} d^{-1}) for mixed sludge.

Volatile solids removals were greater during primary sludge digestion (circa 53 % ± 0.9%) than during mixed sludge digestion (circa 40% ± 0.5%). As it was the case during the six retention time experiment i.e. 53.5% and 40% for the primary and mixed sludge digesters, in that experiment volatile solids removals were in accordance with typical or better VS removals under mesophilic conditions (40-50%) (Metcalf and Eddy, 2003; CIWEM, 1996).

Total solid removals were higher during the dosed primary sludge digestion (51.7% ± 0.6) than it was the non-dosed primary sludge (47%). Similarly, higher TS removals during dosing were observed (35.4% ± 0.8) compared to non-dosed mixed sludge digestion. Total solids removals were according to typical values or better (30-35%) (CIWEM, 1996) and the removals were consistent during the entire period of high receiving nonylphenolic concentrations.

Methane content during the dosed primary (79%) and mixed sludge (80%) digestions respectively remained at similar levels to those levels measured at the 6th retention time in primary (78%) and mixed sludge (79%), respectively. Dosing did not affect the

anaerobic digestion efficiency and the slightly higher methane levels were attributed to the variability of the measurements. In general, methane content was higher than the typical methane values (65-70%) (Gerardi, 2003; CIWEM, 1996; Metcalf and Eddy, 2003) indicative that of good methanogenic activity. In particular, methane content during the dosed primary sludge anaerobic digestion was 78% ± 0.6 whereas in dosed mixed sludge anaerobic digestion methane was 79% ± 0.5. Specific gas production of methane (m^3 CH_4 kg $VS^{-1}_{removed}$) was similar for the dosed primary (0.6 m^3 CH_4 kg $VS^{-1}_{removed}$) and the dosed mixed sludge (0.7 m^3 CH_4 kg $VS^{-1}_{removed}$) digestions, respectively Table 7-1.

Biogas remained at similar levels to those levels observed during the six retention time experiment i.e. circa 0.9 L day^{-1} for primary digesters and 0.8 L day^{-1} for mixed sludge digesters. Gas production rates during the dose primary and mixed sludge digestions were similar at 0.51 m^3 m^{-3} d^{-1}.

7.3. Feed characterization of primary and mixed sludges dosed with nonylphenolics

Quantification of the background dosed concentrations in primary and mixed feeds was completed once, at the start of the experiment by a triplicate extraction (*n*=3) for each feed type. The individual concentrations from each feed type are presented in Appendix IV.

7.3.1. Dosed primary sludge

Mean background concentration of $NP_{3\text{-}12}EOs$ in dosed primary sludge was 24.9 ± 24.7-25.3 mg kg^{-1} dw whereas the mean concentration of $NP_{1\text{-}2}EOs$ was 3.8 ± 3.2-4.5 mg kg^{-1} dw (Figure 7-1). Mean concentration of NPECs was 10.5 ± 9.9-10.9 mg kg^{-1} dw of which NP_2EC predominated (99.8%) whereas NP mean concentration was 0.26 ± 0.3-0.2 mg kg^{-1} dw. Overall, mean ΣNPEOs concentration in the dosed primary feed sludge was 39.4 ± 38.1-40.2 mg kg^{-1} dw. The concentration (mg kg^{-1} dw) of non-ionic nonylphenolics $NP_{1\text{-}12}EOs$ in the dosed primary feed sludge was 7.8x higher the concentration of $NP_{1\text{-}12}EOs$ in the non-dosed primary feed (section 6.1.1).

7.3.2. Dosed mixed sludge

The mean background concentration of $NP_{3-12}EOs$ in the mixed sludge after dosing was 18.3 ± 17.9-18.8 mg kg^{-1} dw. Short-chained nonylphenolic mean concentration was 3.5 ± 2.7-4.5 mg kg^{-1} dw (Figure 7-1). The mean concentrations of NPECs was 254.3 ± 208-297.8 mg kg^{-1} dw and NP_2EC predominated (99.9%). Mean concentration of nonylphenol in the dosed mixed sludge was 0.24 ± 0.1-0.4 mg kg^{-1} dw. Overall, mean ΣNPEOs concentration in the dosed mixed feed sludge was 276.3 ± 231.6-318.8 mg kg^{-1} dw. The concentration of non-ionic nonylphenolics $NP_{1-12}EOs$ in the dosed primary feed sludge was 9.0x higher the concentration (mg kg^{-1} dw) of $NP_{1-12}EOs$ in the non-dosed mixed feed (Figure 7-1).

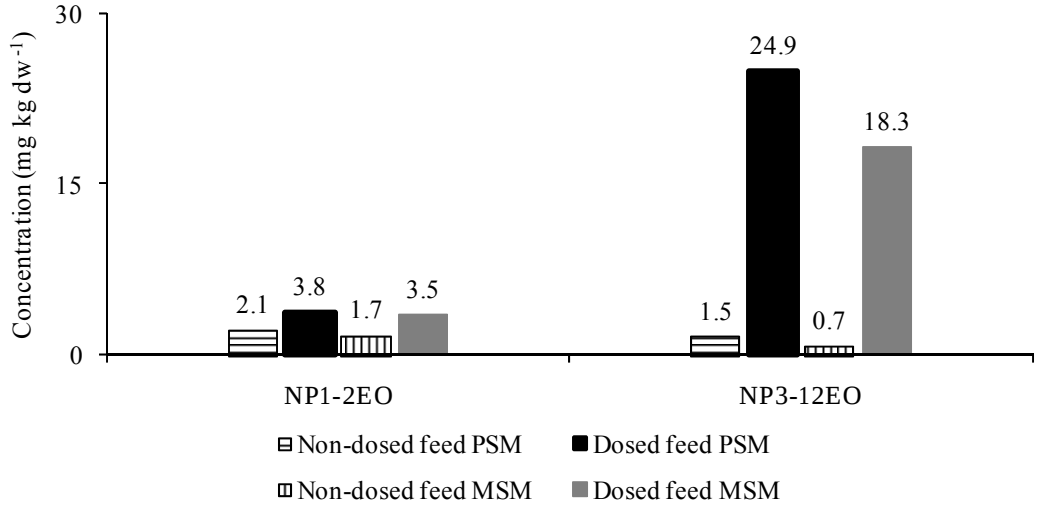


Figure 7-1 Effect of dosing on non-ionic nonylphenolics (*n*=3).

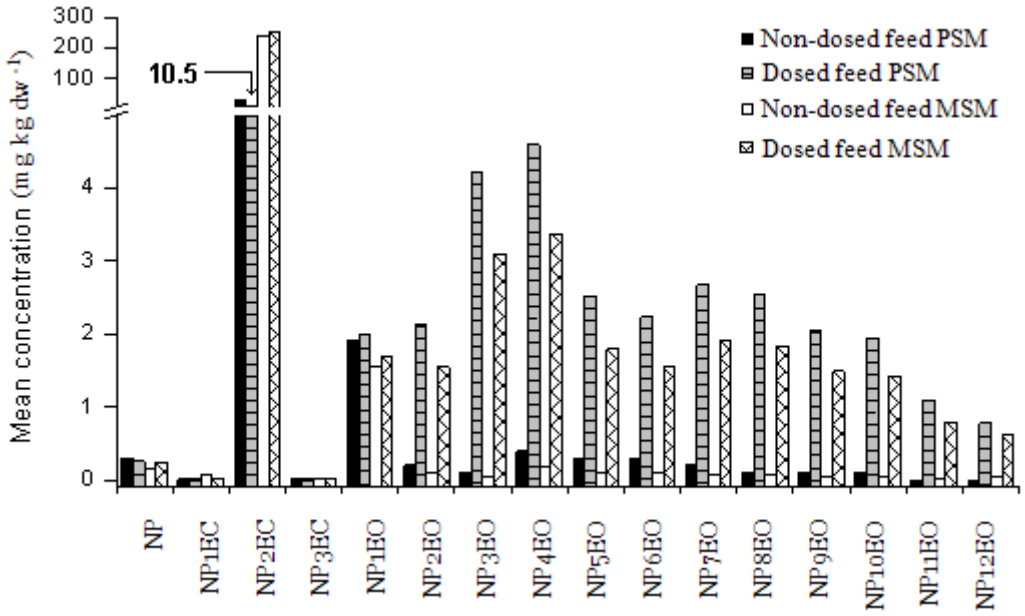


Figure 7-2 Mean values of dosed (*n*=3) and non-dosed (*n*=5) nonylphenolic concentrations in the primary (PSM) and mixed sludge (PSM) feeds, respectively.

7.4. Tends for nonylphenolics during high receiving concentrations in primary and mixed sludges for mesophilic anaerobic digesters

One digestate was collected from each digester on days (1, 3, 5, 7, 10, 20 and 30), extracted and analysed for nonylphenolic concentrations to produce an average value (n=2) representative for the primary (n=2) and mixed sludge (n=2) digesters at each time period. The individual concentrations from each digester are presented in Appendix IV.

7.4.1. Primary sludge (mesophilic digestion)

The NPEOs concentrations obtained on days 1,3,5,7,10,20 and 30 during primary sludge digestion are shown in Figure 7-3.

The sum of the concentration of ΣNPEOs at day 1 was 20.4 mg kg^{-1} dw, which was decreased from the start (dosed feed) concentration (39.4 mg kg^{-1} dw). The concentration of ΣNPEOs at days 3, 5, 7 and 10 was 14.3 mg kg^{-1} dw, 4.4 mg kg^{-1} dw, 2.2 mg kg^{-1} dw, 3 mg kg^{-1} dw, whereas the concentration of ΣNPEOs at days 20 and 30 was below 0.01 mg kg^{-1} dw, respectively. Similarly high removals of spiked long-chained nonylphenolics (>92%) reported by (Zhang et al., 2008) during their UASB study involved activated sludge.

Long chained nonylphenolics ($NP_{3-12}EOs$) were immediately reduced after dosing to 1 mg kg^{-1} dw and after 7 days were further reduced to concentrations close or below the method detection limit (MDL). This phenomenon indicated a lag phase of 7 days. The overall reduction in concentration between the digestate obtained at day 30 and the feed concentration (24.9 mg kg^{-1} dw) was equal to the feed concentration. The same observation was seen for the mass flux difference (M_{diff}=-1.01 mg d^{-1}), which indicated that all the mass of $NP_{3-12}EOs$ entered the digesters was degraded within one retention time Figure 6-3. No inhibitory effect was observed to the primary sludge acclimated bacteria, as it was shown from the methane content of biogas. The complete removal of

$NP_{3\text{-}12}EOs$ is not consisted with the six retention time experiment where the digesters receiving 7.7x less $NP_{1\text{-}12}EOs$ (mg d^{-1}).

Despite the large standard errors associated with $NP_{1\text{-}2}EOs$ on days 3 and 5, $NP_{1\text{-}2}EOs$ where reduced from the start concentration (4 mg kg^{-1} dw) within 7 days and no accumulation was observed. This result is consistent with (Minamiyama et al., 2006) who observed the same lag phase for the spiked NP_1EO during their continuous mesophilic anaerobic digestion of mixed sludge. The concentration increase at day 10 (0.03 mg kg^{-1} dw) could be attributed to the disappearance of $NP_{3\text{-}12}EOs$ post day 7. However, the depletion of $NP_{1\text{-}2}EOs$ concentration observed in the digestates obtained post day 10 were not in contrary with the persistence of $NP_{1\text{-}2}EOs$ when primary digesters were receiving 7.8x less (in terms of concentration) $NP_{1\text{-}12}EOs$. As it was seen with $NP_{3\text{-}12}EOs$ the lag phase of 7 days was also observed for $NP_{1\text{-}2}EOs$.

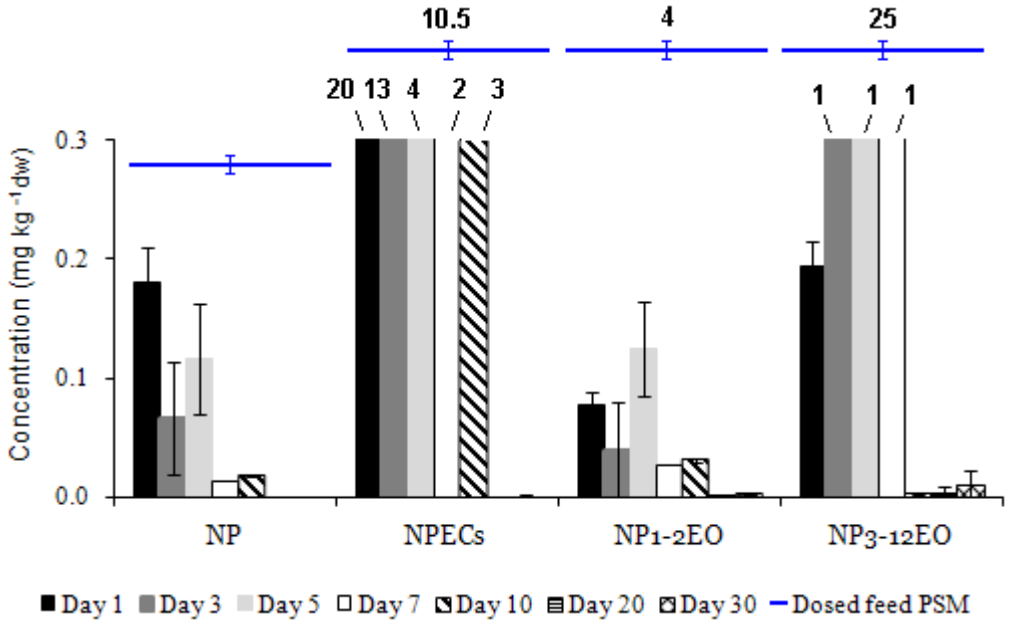


Figure 7-3 Mean (*n*=2) trends of nonylphenol ethoxylate concentrations (mg kg^{-1} dw ± SE) during mesophilic digestion (35°C ± 0.2°C) of primary sludge (PSM) where the $NP_{1\text{-}12}EOs$ feed concentration (spiked, where applicable) ⊣⊢ has been 7.8x higher from the non-dosed background concentration of $NP_{1\text{-}12}EOs$ (4 mg kg^{-1} dw) in the primary sludge feed.

The NPECs, particularly NP_2EC, in the digestates obtained on days 1 and 3 increased in concentration compared to the receiving concentrations due to biodegradation/biotransformation of $NP_{1-12}EOs$. Overall, post day 3, NPECs indicated a gradual reduction in concentration over time. The digestates obtained on days 7 and 10 were associated with a large standard error (±0.2 mg kg^{-1} dw). Carboxylated nonylphenolics were absent from the digestates obtained on days 20 and 30 suggesting similar lag phase to that observed for $NP_{1-12}EOs$.

Although under lower receiving concentrations NPECs indicated persistence during 180 days of digestion, these analytes were completely depleted at higher $NP_{1-12}EOs$ loadings.

Despite the higher loadings, NP concentration remained at similar levels to those levels observed during the 180 days digestion with 7.8x less $NP_{1-12}EOs$ loadings. Nonylphenol was not detected in the digestates obtained at days 20 and 30 whereas the concentration at days 7 and 10 were close to the MDL (0.011 mg kg^{-1} dw). Similar lag phase to NPECs was observed for NP. Interestingly, the trends for both classes of lipophilic analytes, NP and $NP_{1-2}EOs$ were similar. The overall reduction of NP in terms of concentration during the dosed primary sludge digestion was consistent with the trend observed under lower $NP_{3-12}EOs$ loadings during the 180 days digestion trial. This finding validates the previous results obtained with regards the non-accumulation of NP during the mesophilic anaerobic digestion of primary sludge. It is therefore plausible to assume that appropriate enzymes, were in abundance in the primary sludge bacterial community since even under high $NP_{3-12}EOs$ loadings (17. times higher than the six retention times experiment), NP was depleted. Finally, the decreasing NP trend is consistent with the results obtained by (Patureau et al., 2008). Interestingly, the disappearance of NP in the digestates obtained on days 20 and 30 occurred at pH 7.0, and this result is consistent with (Chang et al., 2005; Chang et al., 2004). Hence, this observation could be associated with the pH changes during the dosed digestion trial.

7.4.2. Mixed sludge (mesophilic digestion)

The ΣNPEOs concentrations obtained on days 1,3,5,7,10,20 and 30 during mixed sludge digestion are shown in Figure 7-4.

The sum of the concentration of ΣNPEOs at day 1 was 30.7 mg kg^{-1} dw, which was decreased from the dosed feed concentration (276.3 mg kg^{-1} dw). The concentration of ΣNPEOs at days 3, 5, 7 and 10 was 33 mg kg^{-1} dw, 4.1 mg kg^{-1} dw, 4.3 mg kg^{-1} dw, 4.2 mg kg^{-1} dw, whereas the concentration of ΣNPEOs at day 10 and 20 was below 0.02 mg kg^{-1} dw, respectively. The high removals were in line with those observed in the spiked primary sludge as well as those reported by (Zhang et al., 2008).

Long chained nonylphenolics ($NP_{3-12}EOs$) were reduced after the commencement of the experiment (i.e. day 1) to 1 mg kg^{-1} dw whereas their maximum removal was observed at day 10. The concentration of $NP_{3-12}EOs$ at days 10, 20 and 30 was below 0.02 mg kg^{-1} dw whilst a large standard error (0.01 mg kg^{-1} dw) was associated with the results from day 30. Despite the low concentrations, the results from mixed sludge did not clearly indicate a 7-day lag phase when data are compared to primary sludge. This finding suggested a competitive inhibition effect with other compounds being more accessible to be used as carbon sources. Overall, the mass of $NP_{3-12}EOs$ removed at the end of the dosing experiment (30 days) was 0.75 mg d^{-1}. Despite the fact that $NP_{3-12}EOs$ loadings (mg d^{-1}) were 25x higher than the 180 days digestion trial, higher $NP_{3-12}EOs$ removals occurred within one retention time during higher loadings.

From day 1, short-chained nonylphenolics were reduced from the start concentrations (3.5 mg kg^{-1} dw) and the levels remained low until day 10. Furthermore, the samplers obtained on days 20 and 30 were below the MDL. No accumulation of $NP_{1-2}EOs$ was observed during the high receiving concentrations in mixed sludge. The maximum removal was observed after 10 days of digestion and this finding suggested the degradation/transformation of $NP_{1-2}EOs$ and possibly a lag phase of 10 days which is consistent with the results obtained from primary sludge and those from (Minamiyama et al., 2006). The removed mass of $NP_{1-2}EOs$ at the end of the dosing experiment (30

days) was 0.14 mg d^{-1}, which was in balance with the $NP_{1-2}EOs$ loadings. The depletion of $NP_{1-2}EOs$ post day 10 of anaerobic digestion of mixed sludge was in close agreement with the results obtained from primary sludge under high receiving $NP_{1-12}EOs$ concentrations. However, the results are inconsistent with the non-dosed mixed sludge digestion.

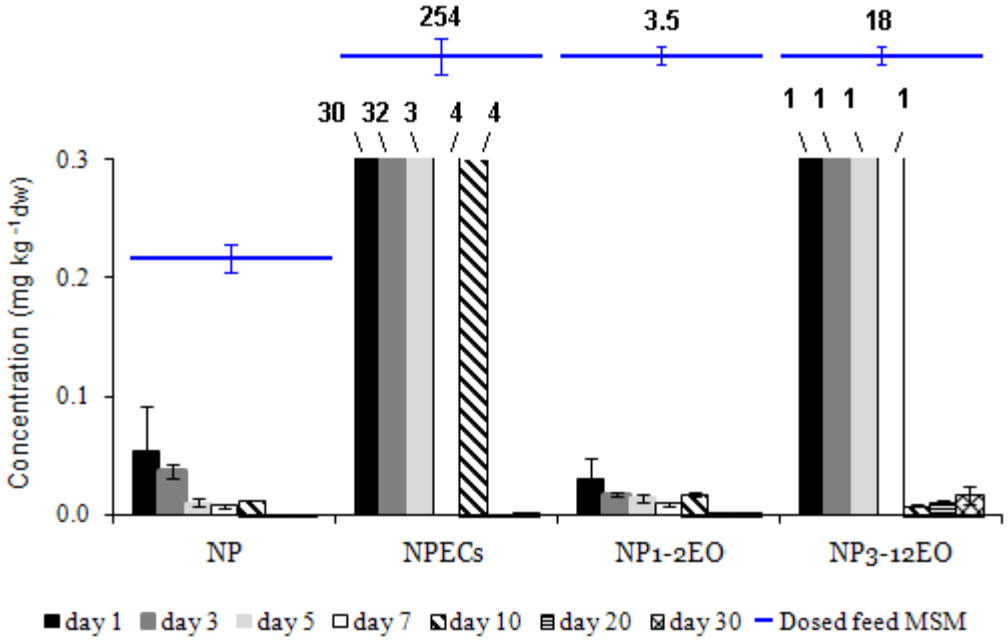


Figure 7-4 Mean (n=2) trends of nonylphenol ethoxylate concentrations (mg kg^{-1} dw ± SE) during mesophilic digestion (35°C ± 0.2°C) of mixed sludge (MSM) where the $NP_{1-12}EOs$ feed concentration (spiked, where applicable) ⊣⊢ has been 9.0x higher from the non-dosed background concentration of $NP_{1-12}EOs$ (2.4 mg kg^{-1} dw) in the mixed sludge feed. ⊣⊢.

Carboxylated nonylphenolics indicated a significant decrease in concentration after dosing irrespective of the high start (feed) concentrations of 254 mg NPECs kg^{-1} dw. Post day 3 concentrations were further reduced and NPECs were absent (below MDL) at days 20 and 30 as it was the case with $NP_{1-2}EOs$. In addition, similar trend was observed during the dosed primary sludge digestion. The maximum removal was observed after 10 days of digestion which indicated a lag phase of 10 days as it was seen with $NP_{1-2}EOs$. The overall decrease in concentration was in accordance with the

non-dosed mixed sludge digestion trials indicative of the presence of NPEC-degrading bacteria and enzymes in mixed sludge. The M_{diff} between the start and the 30 day digestate was 10.4 mg d^{-1} which was equilibrated with the influx on NPECs, in particular NP_2EC which suggested complete removal.

Nonylphenol did not accumulate during the dosed mixed sludge digestion. The concentrations at days 1 and 3 were below 0.06 mg kg^{-1} dw whereas at days 5, 7 and 10, NP concentration was very close to MDL (0.011 mg kg^{-1} dw). Nonylphenol was not detected above the MDL in the digestates obtained at days 20 and 30 (Figure 7-4) which suggested a lag phase of 10 days as it was observe for NPECs. In addition, the absence of NP at days 20 and 30 was in line with the result obtained during the dosed primary sludge. Despite the dissimilar physicochemical properties between NP and NPECs, the disappearance of NP followed the disappearing trends of $NP_{1\text{-}2}EOs$ and NPECs suggesting that both non-ionic and carboxylated species were bioavailable. The overall reduction of NP was consistent with the reduced NP concentrations observed during the non-dosed mixed sludge digestion trial. The low levels and the absence of NP in samples taken on days 20 and 30 validates the results obtained during the non-dosed mixed sludge trial and indicates that NP was not accumulated during the anaerobic digestion of mixed sludge. This result indicated abundant bacterial community and the absence of enzymatic inhibition in mixed sludge even under high $NP_{3\text{-}12}EOs$ loadings (9.0 times higher than the six retention times experiment). The results were in agreement with primary sludge and the recent reports that NP can be partially degraded anaerobically (Patureau et al., 2008). However, the depletion of NP during the dosed mixed sludge trial was observed at elevated pH=7.4 compared to the value of pH=7.0 reported by (Chang et al., 2005; Chang et al., 2004).

7.5. Effect of sludge type on alkylphenol ethoxylates

7.5.1. Primary sludge (dosed vs. non-dosed)

Although in terms of concentration, primary sludge digesters were receiving 7.8x higher ΣNPEOs, in terms of mass (mg d^{-1}), dosed primary sludge digesters, were receiving

1.3x more ΣNPEOs than the non-dosed primary sludge and removed 1.4x times more ΣNPEOs mass than the non-dosed digesters (180 days) (Figure 6-3).

Long-chained nonylphenolic loadings during the dosed trial were 17 times higher than the non-dosed trial and $NP_{3-12}EOs$ removals were 100% and 72%, respectively. This finding suggests that both bacteria and enzymes required for the biodegradation/biotransformation of long-chained nonylphenolics were in abundance in primary sludge and at high $NP_{1-12}EOs$ receiving concentrations primary sludge achieved 100% removal of $NP_{3-12}EOs$. In addition, the high receiving nonylphenolic concentrations did not affect the degrading ability of the primary sludge during mesophilic digestion. Similarly, greater than 92% removals were observed in a UASB study by (Zhang et al., 2008) who reported that degradation of NPEOs do not require sludge acclimation during anaerobic or aerobic systems.

The mass of $NP_{1-2}EOs$ entering the dosed primary mesophilic digesters (0.15 mg d^{-1}) was removed by 99.9% at the end of the digestion period however; NP_2EO indicated 100% flux removal. Although $NP_{1-2}EOs$ indicated persistence under lower receiving concentrations (90.4%), during high receiving concentrations $NP_{1-2}EOs$ flux removals were 99.9%. Since the samples analysed represented both the aqueous and the solid phases of a sample it is not possible to conclude whether $NP_{1-2}EOs$ were partitioned and to what extent to the solids after dosing. Nevertheless, the depleted pool of the lipophilic species in the whole digested sludge sample revealed their absence. Furthermore, the depletion of the lipophilic species suggested that the activity of the biomass to assimilate and/or biotransform these species was not hampered due the high NPEOs loadings nor due the presence of other similar to NPEOs molecules i.e. octylphenol ethoxylates (OPEOs).

Therefore, there was no competitive inhibition effect because of competitive sorption for the short-chained nonylphenolics as it was observed in lab-scale activated sludge trials between NPEOs and polybrominated diphenyl ether (PBDE) (Langford et al., 2007).

Regarding the carboxylated nonylphenolics, in particular NP_2EC was removed by 100% in dosed primary sludge. However, influxes for NP_1EC and NP_3EC were below the MDL (0.012 mg kg^{-1}dw), respectively. With regards the non-dosed primary digesters, which were receiving circa 2.6 times more NPECs, they indicated negative removals (accumulation) after 180 days of digestion. However, only NP_1EC exhibited flux removal (54%).

Nonylphenol influx was 0.01 mg d^{-1} and it was removed by 100% under high receiving $NP_{1-12}EOs$ concentrations. However, during the non-dosed primary sludge trial and with similar NP influx (0.01 mg d^{-1}), nonylphenol indicated circa 45% flux removal while these digesters were receiving circa 4.2 times lower $NP_{1-12}EOs$ influx, than the dosed primary digesters. Despite the higher NPEOs loadings, the degradation of nonylphenolics did not result to $NP_{1-2}EOs$ or to NP as it was seen during 180 days of digestion of non-dosed primary sludge. Because of this phenomenon, nonylphenol was depleted and disappeared from the samples taken on days 20 and 30. The disappearance of NP indicated that the acclimated biomass of primary sludge to NP in synergy with the plethora of appropriate enzymes resulted to its depletion. However, other phenolic NP-like compounds could have inhibited the sorption of NP due to competitive bio-sorption and this phenomenon ultimately resulted to NP bioavailability.

In terms of ΣNPEOs removal per VS removal, the dosed primary sludge digesters removed -1.5 mg $\Sigma NPEOs_{removal}$ g $VS_{removed}$ d^{-1} as opposed to the non-dosed primary sludge digesters that indicated formation (2.1 mg $\Sigma NPEOs_{removal}$ g $VS_{removed}$ d^{-1}) (Table 6-1). It should be noted that VS removal rates during both trials (primary sludge) were similar. Dosed digesters were more efficient in removing alkylphenolics (ΣNPEOs = 100%) than the non-dosed mesophilic digesters who indicated formation i.e. -177% (Figure 6-8). The biomass activity (based on VS) per milligram of nonylphenolics during high receiving concentrations indicated removal (-0.029 mg ΣNPEOs g VS^{-1}) as opposed to the non-dosed digesters (0.041 mg ΣNPEOs g VS^{-1}). This difference reflected the higher loadings of the dosed trial and suggested the ability of the primary sludge biomass to manage higher receiving concentrations of $NP_{1-12}EOs$. This

phenomenon could be explained because of the adequate acclimation period that resulted to the adaptation of the biomass to nonylphenolics and their complete disappearance within one retention time. In terms of concentration, ΣNPEOs levels at day 30 were less than 0.015 mg kg^{-1} dw compared to 87 mg kg^{-1} dw during non-dosed primary sludge digestion. Although, during the 180 days digestion of primary sludge the biomass indicated great potential for biotransformation of nonylphenolics to NPECs, the same biomass under shock loading conditions degraded the acidic metabolites presumably due to adaptation and utilization of the carbon source.

7.5.2. Mixed sludge (dosed vs. non-dosed)

In terms of mass, dosed mixed sludge digesters, were receiving 11.3 mg d^{-1} corresponding to 1.2 fold greater influx compared to non-dosed mixed mesophilic trial. Similarly the dosed mixed sludge removed 1.9x more ΣNPEOs than the non-dosed mixed sludge during the same temperature digestion (Figure 7-5). Overall $NP_{3-12}EOs$ mass removals at the end of the dosing experiment (30 days) were 0.45 mg d^{-1} and accounted for 100% flux removal which are in accordance with the reported value obtained from a study involved a UASB (Zhang et al., 2008).

The mass of $NP_{1-2}EOs$ entering the dosed mixed sludge mesophilic digesters (0.14 mg d^{-1}) was removed by 100% at the end of the digestion period however; NP_1EO indicated 99.9% flux removal. However, the non-dosed mixed sludge indicated 88.7% flux removal for $NP_{1-2}EOs$ whilst NP_1EO exhibited the greatest flux removal (89.8%). As it was seen in the dosed primary sludge, no accumulation of the short-chained nonylphenolics occurred in the acclimated mixed sludge suggesting the abundance of appropriate enzymes and bacteria that were possibly biotransformed/biodegraded the high influx of carbon arose from the nonylphenolics. As it was the case with the dosed primary sludge the activity of the biomass to assimilate and/or biotransform $NP_{1-2}EOs$ was not hindered because of either the high NPEOs loadings and/or the presence of similar hydrophobic compounds nor presumably due to the presence EPS in mixed sludge. Because of the absence of $NP_{1-2}EOs$ it was concluded that competitive bio-sorption was not observed in mixed sludge as it was the case for primary sludge which

in contrary with (Langford et al., 2007) who reported during the effect of competitive bio-sorption between NPEOs and PBDE during their aerobic lab-scale activated sludge trials.

Regarding the carboxylated nonylphenolics, only NP_2EC indicated an influx of 10.4 mg d^{-1} and it was removed by 100%, however, NP_1EC and NP_3EC influxes were below the MDL (0.012 mg kg^{-1} dw), respectively. The absence of $NP_{1+3}ECs$ and their presence during the dosed and the non-dosed fed sludges was attributed to the large standard errors associated with their quantification. Non-dosed mixed digesters were receiving circa 1.1 times less NPECs than the dosed mixed sludge trial; however, the overall NPECs flux removal was 58% after 180 days of digestion and in addition, only NP_1EC and NP_3EC exhibited removals whilst NP_2EC accumulated.

Nonylphenol influx was 0.01 mg d^{-1} and it was removed by 100% in the dosed mixed sludge. However, during the non-dosed mixed sludge, NP influx was circa 1.6 times less than the dosed mixed digesters and indicated 51% flux removal. During the dosed mixed sludge digestion, degradation of nonylphenolics did not result to the measured metabolites i.e. $NP_{1-2}EOs$ or NP as it was seen with the non-dosed mixed sludge. This phenomenon in conjunction with the abundance of adapted bacteria and enzymes as it was seen during the non-spiked (6 retention time trials) aided the depletion of NP from the samples taken on days 20 and 30. The disappearance of NP from the solid and the aqueous phases of the samples indicated that the adapted biomass of mixed sludge to NP resulted to its assimilation. The description was given for the dosed primary sludge could also applied for the mixed sludge that the presence of similar compounds to NP could have inhibited the bio-sorption of NP and ultimately increased NP bioavailability.

Statistical analysis was performed to investigate differences between the dosed and non-dosed sludges. Normality tests (Anderson-Darling) hypotheses were not violated ($p>0.1$) (Appendix III) and directional paired t-tests were used to estimate whether the mesophilic dosed primary sludge digesters were removing greater mass of NPEOs than the removed NPEOs mass (tested mean = 2.240 mg d^{-1}) from the non-dosed primary sludge biomass. The same test was carried for the mixed sludge (tested mean = 5.658

mg d^{-1}). The directional paired t-tests' respective hypotheses were accepted for primary sludge ($t= -1147.52$, $p>0.05$) and for mixed sludge ($t= -168.20$, $p>0.05$), that the dosed digesters removed greater mass of NPEOs than the non-dosed digesters which is interpreted that additional mass of NPEOs was removed by the dosed digesters.

However, in terms of ΣNPEOs removal per VS removal, the dosed mixed sludge digesters removed -11.5 mg $\Sigma NPEOs_{removal}$ g $VS_{removed}$ d^{-1} as opposed to -5.8 mg $\Sigma NPEOs_{removal}$ g $VS_{removed}$ d^{-1} demonstrated by the non-dosed mixed sludge with similar VS removals. Dosed mixed sludge was more efficient in removing alkylphenolics (ΣNPEOs = 100%) than the non-dosed mixed sludge (ΣNPEOs = 58%) (Figure 6-8). The biomass activity (based on VS) per milligram of nonylphenolics under high receiving concentrations was much higher (-0.1713 mg ΣNPEOs g VS^{-1}) compared to the activity of the non-dosed mixed sludge digesters (-0.086 mg ΣNPEOs g VS^{-1}). This difference reflected the higher loadings of the dosed trial and suggested the ability of the mixed sludge biomass to manage higher receiving concentrations of $NP_{1-12}EOs$. The greater removals achieved during the shock loading trial suggested that the acclimated biomass of mixed sludge indicated increased activity with increasing NPEOs concentrations. Finally, in terms of concentration, ΣNPEOs levels at day 30 were less than 0.020 mg kg^{-1} dw compared to 104 mg kg^{-1} dw during the non-dosed mixed sludge digestion. The ability of the mixed sludge biomass to degrade NPEOs, as it was seen during the non-dosed trial, increased with increasing NPEOs concentrations.

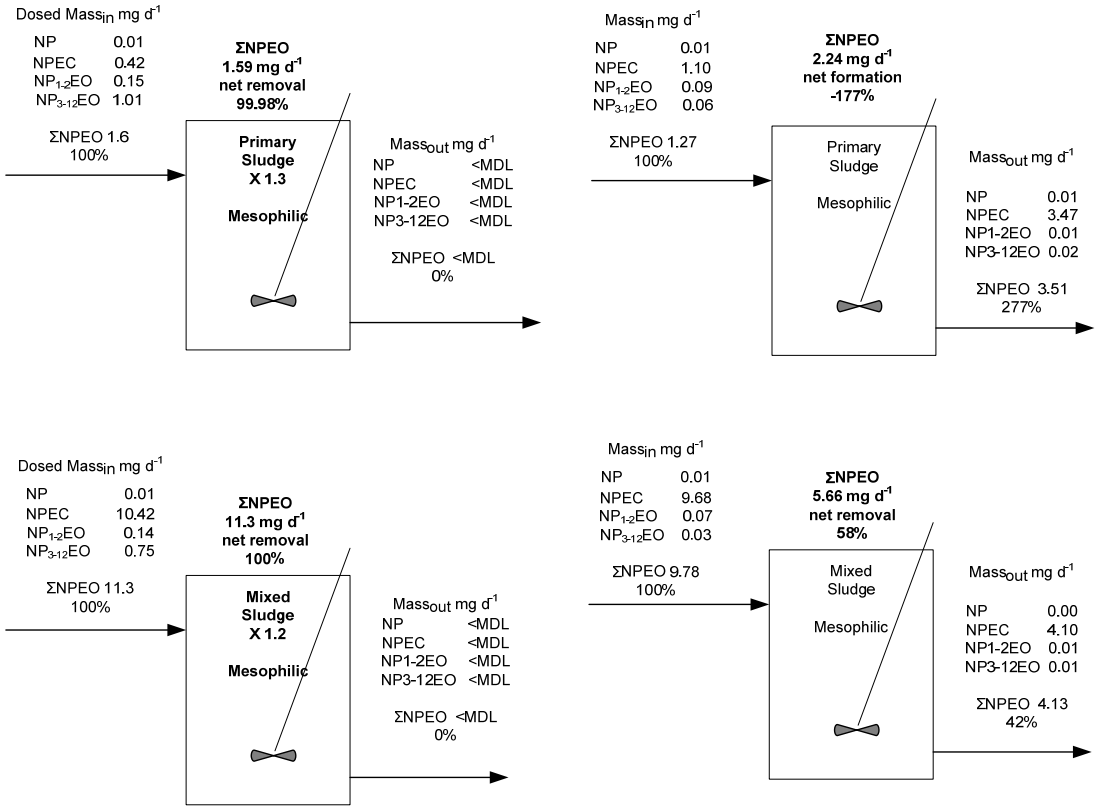


Figure 7-5 Mass flux for nonylphenolics during dosed (shock loading) and non-dosed primary and mixed sludge anaerobic digestion at mesophilic ($35^{o}C \pm 0.2^{o}C$) conditions.

8. DISCUSSION

In this lab-scale study, semi-continuous anaerobic digestion of steroid estrogens and nonylphenol ethoxylates has been examined. Primary and mixed sludge (60:40 v/v, primary sludge: SAS) has been used to investigate the removal of selected EDCs in mesophilic ($35^oC \pm 0.2^oC$) and thermophilic ($53^oC \pm 0.2^oC$) temperatures.

Statistical confidence of data

Feed sludges were obtained from one STWs at two different occasions, firstly (April 2007) for the mesophilic and secondly (April 2008) for the thermophilic digestion trials, respectively. The mesophilic trials involved duplicate digesters of primary (n=2) and mixed sludge (n=2) whilst the thermophilic digestion involved one primary (n=1) and one mixed sludge (n=1) digester, respectively. Three individual feed sludge samples (n=3) were extracted for the quantification of steroid estrogens from each respective sludge type and five (n=5) individual feed samples were extracted for the quantification of nonylphenol ethoxylates. Two samples were extracted from each individual digester (n=2) to determine the concentration of the selected EDCs in the digested sludges. The mass of the selected EDCs was quantified from the whole sludge sample comprised of both the solid and the aqueous phase. Quality control involved deuterated steroid estrogens and low or high spikes for both steroid estrogens and nonylphenol ethoxylates.

8.1. Start-up and operation of anaerobic digesters

8.1.1. Mesophilic vs. thermophilic digestion

Results from the different temperature digestion trials using primary or mixed sludge showed the stability of each digestion process during the entire digestion periods. The pH remained within the optimum range (6.8 – 7.2) in primary sludge irrespective of the temperature digestion whilst in mixed sludge the pH was within the marginal range (7.2 – 7.6), (Metcalf and Eddy, 2003). In addition, oxidation-reduction potential in all digesters was always lower than -320 mV, which favoured the survival of anaerobes

and the enzymatic activity in all anaerobic digesters at this pH ranges irrespective of temperature (Gerardi, 2003; Perry, 1979). Higher organic loading rates in thermophilic digesters resulted to higher gas production rates compared to mesophilic digesters however, the latter showed better or equal specific gas production rates indicative of high bacterial activity in these digesters.

8.2. Occurrence of steroid estrogens in primary and mixed sludges

Irrespective of the sampling period, mixed sludge contained lower ΣEST concentrations compared to primary sludge and this is because 40% of the mixed sludge had undergone aerobic treatment and some degree of biotransformation/biodegradation. It has been shown that activated sludge (secondary biological treatment) is the key process behind the ability of some STWs to remove most if not all estrogenic activity (Pickering & Sumpter, 2003). In addition, studies have showed that although some biological activity exists during primary sedimentation (Koh, 2008), ΣEST removals in primary sludge are mostly negative (Servos et al., 2005b; Johnson et al., 2005) and estrogen activity based on *in vitro* assays had actually increased after primary sedimentation (Kirk et al., 2002; Matsui et al., 2000). Similar trends for natural and synthetic steroid estrogens observed in both primary and mixed feed sludges possibly due to the recycled biological sludge to the head of the works and/or biodegradation/biotransformation in the sewerage system. E1-3S was the least dominant steroid estrogen in primary sludge that could explain the domination of E1 in this sludge via E1-3S deconjugation and/or from other conjugated species i.e. glucuronides which were not measured. Similarly, E1 dominated in the mixed sludge, presumably due to the partial oxidation of E2 within the activated sludge process, as has also been observed by (Carballa et al., 2004a).

8.3. Occurrence of nonylphenol ethoxylates in primary and mixed sludges

Feed sludges obtained from the different sampling occasions showed that primary sludge contained lower ΣNPEOs than mixed sludge. The partial biotransformation of

abundant complex mixtures of ethoxy homologues and alkyl isomers of nonylphenolic species within the activated sludge could have possibly accounted for the higher concentration of ΣNPEOs in mixed sludge since these mixtures enter STWs (European Commission, 2003, 2002; Knepper et al., 2003; Petrović et al., 2002). Higher concentration of metabolic products (NPECs and $NP_{1-2}EOs$) was observed in the mixed sludge compared to primary sludge whilst higher levels of parent alkylphenolic compounds were observed within the primary sludge. Such a phenomenon is typical because mixed sludge has undergone partial biodegradation/biotransformation during the activated sludge treatment. The similar ΣNPEOs trends observed within the primary and the mixed feed sludges, irrespective of the sampling occasion, suggested that partial biodegradation/ biotransformation occurred in both sludge types. Similarly to the explanation given for steroid estrogens, this phenomenon could be attributed to returned biological sludge entering the head of the works and/or biodegradation/ biotransformation in the sewerage system. This explanation is consistent with the literature where partial biodegradation (aerobic/anaerobic transformation) has been observed for nonylphenolics before the influent reached the STWs as well as the presence of short-chained nonylphenolics despite not been found in commercial formulations (Ahel et al., 1994a). The presence of short-chained nonylphenolics in the primary effluents examined from the 40 full-scale STWs, has also been reported by (Fujita et al., 2000b) that further justifies the biodegradation/biotransformation within the sewerage system.

8.4. Effect of sludge type on EDCs removal during mesophilic and thermophilic anaerobic digestion

8.4.1. Steroid estrogens

Removal (or deconjugation) of E1-3S during the anaerobic digestion experiments was limited, however E1-3S contributed to E1 (which is estrogenic) in all digesters and this finding is consistent with aerobic studies which demonstrate that E1-3S is aerobically degradable during activated sludge (Joss et al., 2004b; D'Ascenzo et al., 2003a). The overall removal of E1-3S from this study averaged 29% for all digesters, which is

lowered by a factor of circa 2 when compared to the reported removals from six activated sludge plants (64%) in Rome (D'Ascenzo et al., 2003a). Removals based at the 6^{th} retention time (μg d^{-1} in the feed - μg d^{-1} in the digestate obtained at the 6^{th} retention time) were in the order (from high to low): mesophilic (primary sludge) > thermophilic (primary sludge) > thermophilic (mixed sludge) > mesophilic (mixed sludge) i.e. 36% > 30% > 28% > 21%, respectively (Table 8-1). Currently there are no reports for the fate of E1-3S during anaerobic digestion. First order kinetic constants (*k*) for E1-3S obtained from this study were similar during the different anaerobic trials. In particular, mixed thermophilic sludge indicated the greatest degradation constant for E1-3S (0.0240 d^{-1} at 53°C whereas in primary sludge the rate was 0.0205 d^{-1} at 53°C). Mesophilic digestion demonstrated lower degradation constants compared to thermophilic digestion i.e. 0.0161 d^{-1} at 35°C and 0.0110 d^{-1} at 35°C for the primary and mixed sludge digestion, respectively (Table 5-2). The ability of disulphate strains to cleave E1-3S (Johnson & Williams, 2004) is associated with the use of sulphate as electron acceptor (Van Eldere et al., 1988). However, the use of sulphate (SO_4^{2-}) as a terminal electron acceptor may be considered inappropriate in the current study because redox ranged from -320 mV to -419 mV which is indicative of fermentation (methanogenesis) rather than sulphate reduction (Bouwer & Zehnder, 1993; Gerardi, 2003). A more appropriate explanation for the degradation of E1-3S in this study could lie in the presence of the arylsulphatase enzyme in all sludges studied which has demonstrated deconjugation of E1-3S and resulted to E1 as the by-product (Bandick & Dick, 1999). Since lower removals (based on the 6^{th} retention time) were achieved in mixed sludge, it is possible to say that the activity of the arylsulphatase enzyme was reduced in mixed sludge possibly because this sludge had been partially treated (40% w/w of activated sludge in mixed sludge) prior to anaerobic digestion. Deconjugation (or removal) of E1-3S during the entire anaerobic digestion study (all six retention time times) contributed to E1 (Figure 8-1) and varied (from high to low): mesophilic (primary sludge) 0.5 μg d^{-1} ≈ mesophilic (mixed sludge) 0.5 μg d^{-1} > thermophilic (mixed sludge) 0.4 μg d^{-1} > thermophilic (primary sludge) 0.2 μg d^{-1}, irrespective of the similar E1-3S influx for all digesters (0.2 μg d^{-1}). When considering deconjugation of E1-3S and its total contribution to E1 the mesophilic conditions seem to favour this

activity. Considering that the enzymatic activity of arylsulphatase enzyme should increase with increase in temperature (Gerardi, 2003) a possible reason for the slower activity of arylsulphatase in thermophilic digestion could be due to the shorter retention time of these digesters compared to the mesophilic digesters.

During this study a fraction of the removed E1 Figure 8-1 was reduced to E2 and another fraction was degraded in both primary and mixed sludges irrespective of digestion temperature according to the mass balance. The degraded fraction of E1 predominated over the reduction of E1 to E2 (see below) for the majority of the anaerobic trials. Overall, degradation during the entire anaerobic digestion study (all six retention time times) occurred with the order of efficiency (from high to low) being: mesophilic (primary sludge) > thermophilic (primary sludge) > mesophilic (mixed sludge) > thermophilic (mixed sludge) for 14.4 μg d^{-1}, 12.5 μg d^{-1}, 6.9 μg d^{-1} and 1.3 μg d^{-1}, respectively (Figure 8-1). The degraded fraction of the removed E1 who represents 100% accounted for 86%, 78%, 78% and 22%, respectively. Based on first order kinetic calculation under continuous addition, first order kinetic or degradation constants (*k*) for E1 ranged from 0.1161 d^{-1} at 35^{o}C to 0.0415 d^{-1} at 53^{o}C in primary sludge (Table 5-2). Irrespective of sludge type and start concentration, degradation constants (*k*) were higher during mesophilic digestion (35^{o}C) with longer retention times. In general, degradation constants for E1 were the greatest among the examined steroid estrogens during mesophilic digestion. It has been reported that degradation of E1 takes place under all redox conditions but at significantly different rates (Joss et al., 2004b). Biodegradation rates (*k*) for E1 in batch experiments with 12 days retention time ranged from 162 l/gSS d^{-1}, 30 l/gSS d^{-1} and 10 l/gSS d^{-1} for aerobic, anoxic and anaerobic conditions, respectively. Biodegradation rates indicate an increase by a factor of between 3 and 5 in the transition from anaerobic to anoxic (nitrate available) as well as between anoxic and aerobic (Joss et al., 2004b) suggesting that aerobic biodegradation of E1 is faster compared to anaerobic degradation. In another study, first order degradation rates of E1 during aerobic batch experiments (20^{o}C) containing diluted slurry of activated sludge obtained from real sewage treatment works ranged from 1.0080 d^{-1} to 11.5200 d^{-1} (calculated) with E1 starting concentration of 1000 μg l^{-1} and 1 μg l^{-1}, respectively (Ternes et al., 1999a). In the current study, E1 degradation constants

(*k*) ranged from 0.0415 d^{-1} to 0.1161 d^{-1} for primary sludge at 53°C and 35°C, respectively (Table 5-2) with 35 μg l^{-1} and 91 μg l^{-1} E1 feed concentrations, respectively. Considering the high E1 start concentration in (Ternes et al., 1999a) study i.e. 1000 μg l^{-1}, degradation rates in this study were significantly lower compared to aerobic studies suggesting that initial concentration of steroid estrogens may be more significant than digestion temperature. Furthermore, the lower degradation constants in this study compared to aerobic studies are in line with (Joss et al., 2004b; de Mes et al., 2005).

In general, overall degradation of E1 i.e. during the entire digestion periods, reflected the amount of the removed E1 for each digester. Therefore, irrespective of digestion temperature and sludge type, primary sludge (mesophilic) that was receiving higher loads of E1 (4.5 μg d^{-1}) resulted in higher E1 degradation i.e. 14.4 μg d^{-1} than the thermophilic mixed sludge (1.3 μg d^{-1}) which was receiving the lowest E1 (1.6 μg d^{-1}) (Figure 8-1). In addition these digesters were also subjected to similar E1 contributions from the deconjugated E1-3S. Whether specific bacteria or enzymes present in sludge or whether temperature digestion is the key for the significant degradation of E1 during anaerobic digestion it is yet not certain.

E1 was reduced to E2 which has a 5-10 folder higher estrogenic activity than E1 (Johnson & Sumpter, 2001b). The conversion of E1 to E2 i.e. contribution of E1 to E2 during the entire anaerobic digestion study (all six retention times) occurred in all digesters in the order (from high to low) being: thermophilic (mixed sludge) 4.5 μg d^{-1} > thermophilic (primary sludge) 3.5 μg d^{-1} > mesophilic (primary sludge) 2.4 μg d^{-1} > mesophilic (mixed sludge) 2.0 μg d^{-1} (Figure 8-1). The fraction of the formed E2 from the removed E1 who represented 100% was in the order (from high to low) being: thermophilic (mixed sludge) 78% > thermophilic (primary sludge) 22% ≈ mesophilic (mixed sludge) 22% > mesophilic (primary sludge) 14%. The differences in the conversion of E1 to E2 for each digester reflected the different E1 influx as well as the removed E1. Therefore despite the different sludge types and digestion temperatures it was evident that all sewage sludge had developed E1 reducing properties. This finding is important and confirms the reported results that E1 is converted to E2 during

anaerobic digestion. Therefore, based on first order calculation under continuous addition, first order kinetic constants (*k*) for E2 were negative and ranged from -0.0145 d^{-1} to -0.0511 d^{-1} for primary and mixed sludge at 53^{o}C (i.e. thermophilic) and -0.0259 d^{-1} to -0.0255 d^{-1} for primary and mixed sludge at 35^{o}C (i.e. mesophilic) trials, respectively (Table 5-2). Biodegradation rates (*k*) for E2 in batch experiments with 12 days retention time ranged from 350 l/gSS d^{-1}, 460 l/gSS d^{-1} and 175 l/gSS d^{-1} for aerobic, anoxic and anaerobic conditions, respectively (Joss et al., 2004b). Biodegradation rates indicate an increase by a factor of 2 in the transition from aerobic to anaerobic. This observation suggests that aerobic biodegradation of E2 is faster compared to anaerobic 'degradation' since under anaerobic conditions E2 is formed from E1.

The overall conversion of E1 to E2 has been reported to occur under anaerobic conditions in lab scale studies (batch and/or continuous) (de Mes et al., 2008; Carballa et al., 2007; Carballa et al., 2006; Joss et al., 2004b) (see Figure 2-3) and our results are consistent with these authors. It has been reported that the extent of this conversion depends on the inoculum (de Mes et al., 2008). The results from this study showed that the conversion of E1 to E2 was greater compared to digested pig manure and granular UASB sludge (industrial origin) (de Mes et al., 2008). This difference could therefore lie to the different sludge types, the different inoculums used and the diverse digestion temperatures.

Nevertheless, (Czajka & Londry, 2006) consistently observed the reverse conversion i.e. the production of E1 in E2 spiked sediments in nitrate-iron-sulphate and methanogenic reducing conditions in batch lab scale anaerobic reactors but no details were provided regarding pH or redox potential. Despite this observation, the authors reported that the reverse conversion i.e. E2 to E1 under anaerobic conditions is unlikely to provide energy to the cells involved and that E2 is possibly used as an electron donor (Czajka & Londry, 2006).

Although the conversion of E2 to E1 is unlikely to provide energy to cells during anaerobic digestion (Czajka & Londry, 2006), the reduction of E1 to E2 could be

mediated by hydrogen or other organic electron donors as it the case for instance with some aromatic and chlorinated organic compounds (Gerritse et al., 1999; Holliger & Schraa, 1994; Lovley & Lonergan, 1990). The conversion of E1 to E2 is possibly a co-metabolic activity because steroid estrogens (like other micropollutants) are not present in high enough concentration in the anaerobic sludge to support substantial biomass growth (Yi & Harper, 2007; Yu et al., 2007).

Flux removals for ΣE1+E2 during mesophilic digestion for primary (57%) and mixed sludge (44%) were higher to those obtained during thermophilic digestion i.e. 56% and 10 %, respectively. In general these removals were much lower compared to those reported by (Carballa et al., 2006) i.e. 85% at mesophilic (37°C) and 95% at thermophilic (55°C) conditions in mixed sludge (70:30 v/v, primary sludge: activated sludge), respectively. The lower removals observed in our study and (Carballa et al., 2006) study could be explained through the different substrates used. However, it should be noted that the calculation used to produce the high ΣE1+E2 removals in their study involved spiked concentrations of steroid estrogens (4-400 μg l^{-1}) (Carballa et al., 2006) which may be responsible for the high reported removals in mixed sludge.

Mesophilic digestion was more efficient for E3 removal (primary 45%, mixed 44%) than the thermophilic digestion (primary 17%, mixed 4%). The similar E3 removals between the primary and mixed sludge subjected to mesophilic digestion indicated that both sludge types accommodate E3 degrading bacteria/enzymes and that their performance is favoured during mesophilic digestion when compared to thermophilic digestion. Flux removals reflected E3 kinetic constants in the order (from high to low) being: mesophilic (35^{o}C) (primary sludge) 0.0243 d^{-1} > mesophilic (35^{o}C) (mixed sludge) 0.0226 d^{-1} > thermophilic (53^{o}C) (primary sludge) 0.0205 d^{-1} > thermophilic (53^{o}C) (mixed sludge) 0.0201 d^{-1}, respectively (Table 5-2). Thermophilic digestion demonstrated the lowest E3 degradation rates possibly due to the shortest retention time. Currently there are no published E3 degradation constants from aerobic or anaerobic studies for comparison purposes. However, field data from aerobic studies have reported 95% removal during activated sludge treatment (Baronti et al., 2000) suggesting a) that E3 degradation constants are expected to be much greater than those

reported herein and b) that strict anaerobic (methanogenic) conditions do not favour the elimination of E3 despite the fact that E3 is less estrogenic than EE2, E2 and E1. The activity of the biomass which was calculated as the amount of organic matter present in VS of primary sludge was more efficient for E3 removal than the activity of the mixed sludge. Despite the similarities of E3 influx among the primary and the mixed sludges at both digestion temperatures, thermophilic digesters obstructed the removal of E3. Concentration of E3 in the respective sludges at both mesophilic and thermophilic trials were near or below the MDL (4.5 μg kg^{-1} dw). In addition, no contribution of ΣE1+E2 to E3 was observed during the different digestion temperatures as shown in Table 5-3. No investigation for E3 by-products were examined in this research and currently there are no published studies for the degradation of E3 in strict anaerobic (methanogenic) conditions. It has been postulated however, that E3 would have similar pathway to E1 (Koh, 2008). Based on this information, degradation of E3 could have been initiated via hydroxylation or ring cleavage possibly during a co-metabolic process.

In general, higher EE2 removals were achieved in primary sludge compared to mixed sludge at both digestion temperatures which emphasised the importance of the primary sludge substrate for EE2 removal. In particular, removals for EE2 in primary sludge at 53^{o}C were 44% and at 35^{o}C were 33% as opposed to mixed sludge with 15% and 5%, removals respectively. Researchers did not observe any EE2 degradation in spiked sewage sludge obtained from a real scale STWs or lake sediment over the period of three years during a lab scale batch anaerobic experiment (Czajka & Londry, 2006). On the other hand, significantly higher removal was observed in spiked mixed sludge (70% primary and 30% SAS) and UASB sludge during mesophilic (85%) and thermophilic (75%) anaerobic digestion trials (Carballa et al., 2006). The contradictory reported removals in lab scale anaerobic conditions are consistent with the large differences reported from real scale STWs (Koh, 2008; Johnson & Sumpter, 2001b; Andersen et al., 2005) implying that the breakdown of one of the most potent steroid estrogen compounds investigated in this study is possibly highly dependant on the substrate besides redox conditions as the results suggest. Although EE2 metabolites were not investigated in this research it was proposed that EE2 is initially oxidised to E1

following ring opening oxidation reactions on ring B (Haiyan et al., 2007). First order kinetic constants (*k*) for EE2 ranged from 0.0519 d^{-1} to 0.0753 d^{-1} for primary and mixed sludge during thermophilic digestion (53^{o}C), respectively whereas mesophilic digestion (35^{o}C) resulted to lower degradation constants during primary sludge (0.0701 d^{-1}) and even lower during mixed sludge digestion (0.0021 d^{-1}) compared to the thermophilic mixed sludge digestion (Table 5-2). Biodegradation rates (*k*) for EE2 in batch membrane bioreactors with 30 days retention time ranged from 6 l/gSS d^{-1}, 3 l/gSS d^{-1} and 1.5 l/gSS d^{-1} for aerobic, anoxic and anaerobic conditions, respectively. It was reported that EE2 removal efficiency depends on the redox conditions with maximum removal rate occurring under aerobic conditions (Joss et al., 2004b) as it was the case with E1. Degradation rate for the anaerobic batch reactor did not deviate significantly from the abiotic control values. i.e. k =1 l/gSS d^{-1} according to (Joss et al., 2004b).

Based on their mass fluxes in micrograms per day during mesophilic digestion primary sludge achieved higher degradation and removal of steroid estrogens (53%) than the mixed sludge (39%). Analogously, higher removals in primary sludge (51%) than in the mixed sludge (12%) occurred during thermophilic digestion. A rough estimation of the removal efficiency of the biomass (the amount of organic matter present in VS) of the primary and mixed sludge was evaluated by activity i.e. µg estrogen removed per gram of VS content per digester. Given that the activity of the biomass of the primary and mixed sludge digestions was significantly ($p<0.05$) different for the mesophilic trials and similarly as it was the activity of the biomass for the respective sludges at thermophilic conditions ($p<0.05$) (Appendix III), it was evident that the biomass activity of primary sludge had a higher efficiency for biodegrading steroid estrogens than the mixed sludge, irrespective of digestion temperature (Table 5.1).

The initial research hypothesis ($H1\mathrm{E}_0$)) (see section 5.2) was therefore violated (for mesophilic, t=33.23, DF=2, $p<0.05$, and thermophilic, t=42.16, DF=1, $p<0.05$) in favour of the alternative hypothesis ($H1\mathrm{E}_\alpha$) which states; primary and mixed sludge digestion, irrespective of temperature, resulted in significantly different steroid estrogen removal

(based at the 6^{th} retention time (μg d^{-1} in the feed - μg d^{-1} in the digestate obtained at the 6^{th} retention time).

Table 8-1 Mass balance for steroid estrogens during mesophilic (35^{o}C) and thermophilic (53^{o}C) digestion of primary and mixed sludges.

	Flux (µg d^{-1})											
	Mesophilic digestion						**Thermophilic digestion**					
	Primary sludge			**Mixed sludge**			**Primary sludge**			**Mixed sludge**		
Steroid estrogens	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %
E1	4.53	0.96	78.80	2.28	0.68	70.32	3.49	0.16	95.51	1.61	0.51	68.22
E2	0.26	1.12	-323.56	0.16	0.69	-325.08	0.32	1.51	-367.38	0.15	1.07	-621.33
E3	0.25	0.14	45.40	0.20	0.11	43.55	0.30	0.25	16.64	0.26	0.25	4.08
E1-3S	0.22	0.14	36.36	0.17	0.13	21.06	0.20	0.14	30.31	0.20	0.15	27.88
EE2	0.53	0.35	32.71	0.26	0.25	4.55	0.49	0.28	43.56	0.50	0.43	14.82
Natural	**5.26**	**2.36**	**55.26**	**2.81**	**1.61**	**42.61**	**4.32**	**2.06**	**52.25**	**2.22**	**1.97**	**10.99**
ΣE1+E2	**4.79**	**2.08**	**56.58**	**2.44**	**1.37**	**43.85**	**3.81**	**1.67**	**56.16**	**1.76**	**1.58**	**10.23**
ΣESTs	**5.79**	**2.71**	**53.21**	**3.07**	**1.86**	**39.40**	**4.81**	**2.34**	**51.36**	**2.72**	**2.40**	**11.70**

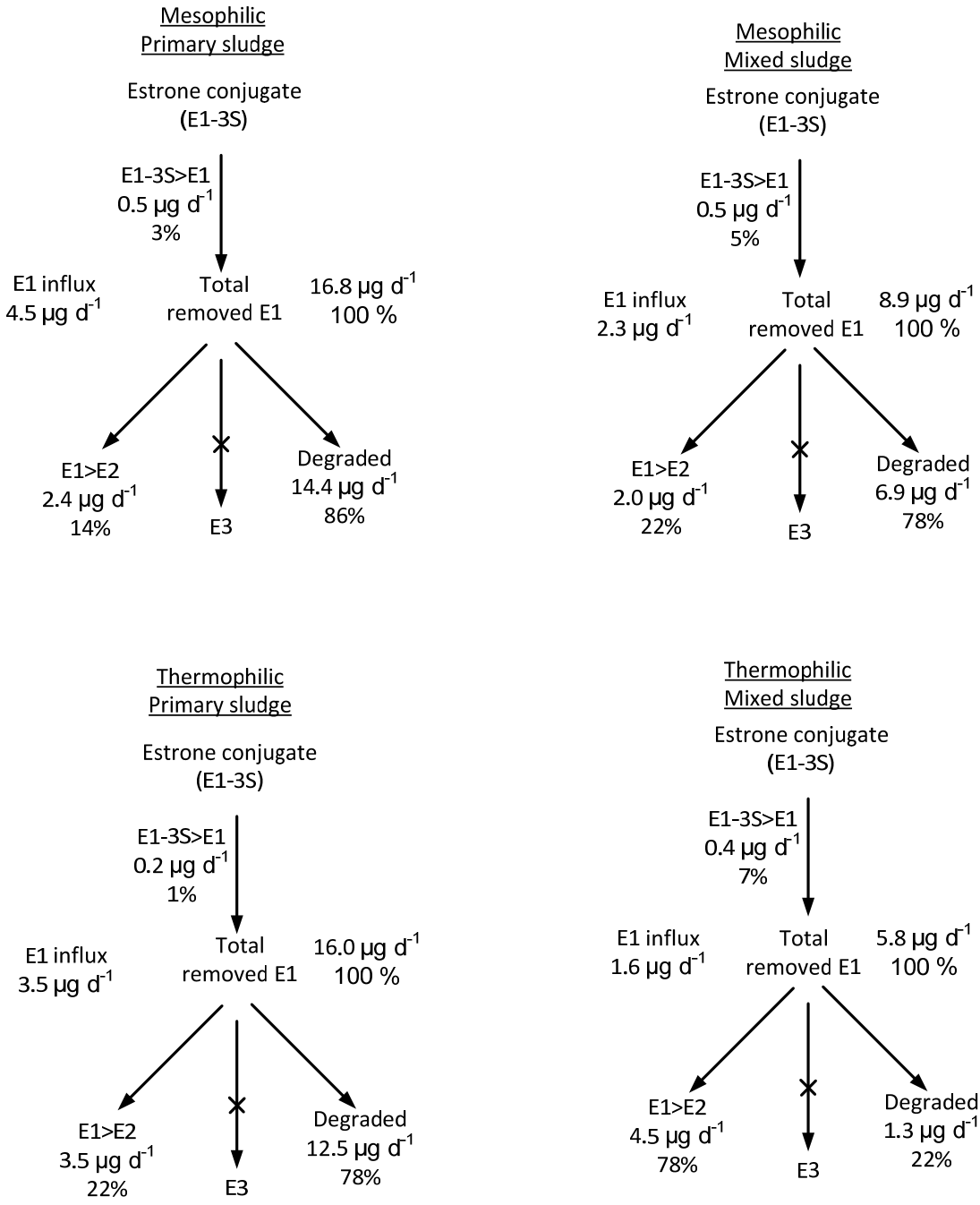


Figure 8-1 Mass balance for steroid estrogens during mesophilic and thermophilic anaerobic digestion of primary and mixed sludge. Values in (µg d^{-1}) represent the total mass loss or transfer. Percentages represent the mass loss or transfer fraction of the removed E1 mass who represents 100%.

8.4.2. Nonylphenol ethoxylates

Digestion of nonylphenolics (ΣNPEOs) resulted in high and similar long-chained nonylphenolic ($NP_{3-12}EOs$) removals (mg d^{-1}) in the presence of primary sludge during mesophilic (72%) and thermophilic (71%) digestion at the end of the digestion period i.e. at the sixth retention time. Thermophilic digestion of mixed sludge on the other hand achieved the highest $NP_{3-12}EOs$ removals (79%) among all examined sludges whilst the same sludge, during mesophilic digestion achieved the lowest $NP_{3-12}EOs$ removals (57%). Considering the results obtained from the mesophilic and thermophilic trials, retention time and temperature did not demonstrate a significant effect on the removal of the parent compounds with an exception of mixed sludge at mesophilic temperatures. Nevertheless, at higher $NP_{3-12}EOs$ loadings, a lag phase of 7 days was observed in primary and mixed sludge during mesophilic digestion with higher removals (100% for both sludges respectively) than those obtained during the non-spiked (non-dosed) mesophilic trials. These results indicated that both sludge types irrespective of digestion temperature were equally acclimated during the digestion period since biodegradation and/or biotransformation of $NP_{3-12}EOs$ occurred with time. The high $NP_{3-12}EOs$ removals obtained from this study are consistent with (Zhang et al., 2008).

The results indicated that long retention time (30 days) promoted the growth of appropriate bacteria for the biotransformation of the lipophilic short-chained nonylphenolics ($NP_{1-2}EOs$) in both primary and mixed sludge. Removal of $NP_{1-2}EOs$ occurred with digestion time and suggested acclimation. Higher kinetic constants (*k*) for NP_1EO were observed during thermophilic digestion i.e. 1.7958 d^{-1} and 0.8152 d^{-1} for primary and mixed sludge, respectively due to higher digestion temperature compared to mesophilic digestion (Table 6-2) and the current results are consistent with (Chang et al., 2004) who also observed higher *k* values for NP_1EO at 50^{o}C compared to 30^{o}C digestion temperatures. The current results are in line with the (Benabdallah El-Hadj, 2006) study who also observed sludge acclimation with 100% SAS digestion during mesophilic (35°C) and thermophilic (55°C) anaerobic digestion but not in agreement

with (Zhang et al., 2008) who observed the accumulation of $NP_{1\text{-}2}EOs$ in the UASB effluents (aqueous phase) after the period of five months.

Nevertheless, at higher receiving concentrations, similar lag phase of circa 7 days was observed for $NP_{1\text{-}2}EOs$ as it was the case for the long-chained nonylphenolics at higher loading conditions. The results from the dosed (spiked) experiment were in line with other studies who reported similar lag phase for the spiked NP_1EO (5 mg l^{-1}) during mesophilic anaerobic digestion (28 days retention time) of mixed sludge (thickened primary and SAS) (Minamiyama et al., 2006). On the other hand, (Zhang et al., 2008) by using the same reference material as (Minamiyama et al., 2006), observed the persistence of $NP_{1\text{-}2}EOs$ in the UASB effluents after spiking (23 μmol l^{-1}) activated sludge with (NP_nEOs, average EO=9). Interestingly, (Zhang et al., 2008) investigated the effect of bacterial number change with spiking during their anaerobic trial by using denaturing gradient gel electrophoresis (DGGE) and reported that spiking resulted to significant decrease in bacterial species; however adaptation to high concentrations occurred with time. Although in the current study bacterial changes were not examined, the observed 7-days lag phase suggests besides possible sludge structural changes, that biomass composition/activity or speciation changes could have occurred. However, no inhibitory effect was observed as a result of spiking for both sludge types since no changes occurred in methane, biogas production rate and VFAs concentration, therefore it is plausible to suggest that changes related to enzymatic activity as opposed to microcosm. $NP_{1\text{-}2}EOs$ flux removals were 99.9% for the dosed primary sludge and 100% for the dosed mixed type, respectively.

Nevertheless, during the six retention time trials, at thermophilic conditions with shorter retention time the lipophilic NP_1EO persisted in primary sludge but not in the mixed sludge. It was speculated that specific exoenzymes responsible for attacking lipophilic compounds (Dalton & Stirling, 1982) such as NP_1EO were not produced in adequate numbers by appropriate bacteria in primary sludge possibly due to the short digestion cycle. In addition to the limited numbers of enzymes the untreated primary sludge substrate (fats, grease) could have also served as significant adsorbent sites for NP_1EO with a high octanol-water partitioning index (Log K_{ow} = 4.17) (Ahel & Giger, 1993a).

The persistence of the lipophilic NP_1EO is in agreement with (Montgomery-Brown & Reinhard, 2003) that parent nonylphenolics persist under anaerobic conditions. Although under thermophilic conditions, $NP_{1-2}EOs$ flux removal in primary sludge was 2.3%, in the mixed sludge the removal was higher 99.9%. The observed removals for NP_1EO and NP_2EO during both mesophilic and thermophilic digestions were higher than those presented by (Benabdallah El-Hadj, 2006) (48% and 71%, respectively) by using 100% SAS with 18 days SRT, which indicated the importance of substrate. Overall, the disappearance of $NP_{1-2}EOs$ could have contributed to the formation of NPECs and/or to NP.

Although $NP_{1-2}EOs$ did not accumulate during the mesophilic trials, formation and persistence of carboxylated nonylphenolics (NPECs) occurred in primary sludge irrespective of a) digestion temperature b) retention time c) overall nonylphenolic influx and d) organic loading rate, directly after the commencement of the anaerobic trials. This important finding indicated the occurrence of carboxylated nonylphenolics in methanogenic conditions. The presence of carboxylated nonylphenolics could be due to co-metabolic transformation reactions since anaerobic metabolism requires a sequence of oxidation-reduction reactions for the energy yields (Gerardi, 2003; Dalton & Stirling, 1982). A variety of organic molecules, CO_2 or metals could have been used as the carrier electron acceptors to produce co-metabolically carboxylated homologues. This hypothesis is in agreement with (Hayashi et al., 2005) that NPECs may be generated co-metabolically with NPEOs in the presence of organic matter in aerobic biodegradation tests. In addition, irrespective of digestion temperature of the primary sludge, NP_1EC and NP_2EC indicated negative degradation rates (k) (Table 6-2) i.e. formation.

The formation of NPECs was the principal co-metabolic biotransformation/ biodegradation mechanism of $NP_{1-12}EOs$ in primary sludge possibly due to the rich intrinsic bacteria/enzymes as it was evident during the steroid estrogen digestion. Flux removals were therefore negative during mesophilic (-215%) and thermophilic (-3591%) digestion of primary sludge. The presence of short-chained NPECs and the concomitant disappearance of $NP_{3-12}EOs$ are in line with (Schröder, 2001) that used different STWs biocoenosis at 20^oC and -380mV in lab scale anaerobic trials. Similarly,

(Field & Reed, 1999) reported the presence of NPECs at circa 123 mg kg^{-1} dw by analysing real scale anaerobically digested sewage sludge (mesophilic). In addition, they reported that mesophilic anaerobic sludge digestion (real scale) results in the more rapid degradation of the *para* isomers relative to *ortho* isomers (Field & Reed, 1999). On the other hand, (Lee et al., 1997) reported considerably lower (up to 38 mg kg^{-1} dw) NPECs concentrations from real scale mesophilic anaerobic digesters. Although NPEOs loadings in the (Field & Reed, 1999; Lee et al., 1997) studies were not available for direct comparison, an implication of the (Field & Reed, 1999) study is that different anaerobic substrates may result to different isomeric NPECs distributions which in turn may direct their formation or bioavailability. The implication of the (Field & Reed, 1999) study could further explain the dissimilar NPECs trends observed between the mesophilic and thermophilic anaerobic trials. In contrary, (Lu et al., 2008; Lu et al., 2007) did not report any NPECs above 0.4 μg l^{-1} in the effluents obtained from biodegradation assays by using STWs inocula or river sediment under sulphate, nitrate or iron reducing conditions. Furthermore, (Ball et al., 1989a) reported the presence and persistence over 190 days of anaerobic digestion of long-chained carboxylated octylphenol ethoxylates (OPECs) in lab scale anaerobic trials. Although NPECs were observed in the mixed sludge during mesophilic digestion, the acidic species were reduced and removed by 58% (based on flux) over time and proposed that bacteria were acclimated to these species. In addition, there was an insignificant formation of NPECs in the mixed sludge during thermophilic digestion which resulted to high negative flux removal (-8921%) due to the low influx of NPECs. The observed trends of NPECs in mixed sludge irrespective of temperature digestion were explained by of the presence of acclimated enzymes and facultative bacteria that were able to utilise NPECs as co-substrate during a co-metabolic activity. Albeit the shorter sludge retention time of the thermophilic digestion, temperature could have also initiated this process. The differences between primary and mixed sludge could have been attributed to the presence of lipophilic compounds in the untreated primary sludge. The lipophilic compounds could have served as significant adsorbent sites for NPECs which are associated with high octanol-water partitioning index (Log K_{ow} >5.53) (Nielsen et al., 2000). The ability of the methanogenic consortia to have hydrolysed the non-ionic

nonylphenolics (presumably the lypophilic species) to more soluble substrates like NPECs for further assimilation is plausible since carboxylation increases both the solubility and persistence of alkylphenol ethoxylates (Ahel et al., 1994d).

Interestingly, acidic nonylphenolic species indicated a lag phase of 10 days in both primary and mixed mesophilic sludges at high $NP_{3-12}EOs$ loadings i.e. 1.01 mg d^{-1} and 0.75 mg d^{-1}, respectively. Their depletion occurred after the 7-days lag phase of $NP_{3-12}EOs$ and this observation is consistent with the non-dosed trials that NPECs were more persistent than $NP_{1-2}EOs$ in primary sludge.. The persistence of NPECs within the 10 days lag phase could be explained because of the higher Log $K_{ow} \geq 5.53$ as opposed to lower Log $K_{ow} \leq 4.21$ of $NP_{1-2}EOs$. Under lower receiving concentrations, formation of NPECs was the principal co-metabolic biotransformation/biodegradation mechanism of $NP_{1-12}EOs$ in primary sludge. Nevertheless, NPECs were absent from the samples after 10 days of digestion during the dosing experiment but they were still present at appreciable levels at lower $NP_{3-12}EOs$ loadings. This observation suggested a) that the high non-ionic surfactant concentrations had a significant effect on sludge structural characteristics which lead to desorption of NPECs hence, increased their bioavailability, b) acceleration of enzymatic activity for NPECs attack as a result of co-metabolism, due to more available carbon, which resulted to their depletion within one digestion cycle i.e. retention time.

Nonylphenol (NP) did not accumulate in primary or mixed sludge during mesophilic or thermophilic anaerobic digestion (six retention times experiments). Therefore, lower flux removals were achieved during mesophilic digestion in the primary (45%±1%) and mixed sludge (51%±1%) compared to thermophilic digestion (57%±1% and 80%±1%, respectively). This finding suggested that temperature was more significant than retention time, which is consistent with the observations of (Tanghe et al., 1998) that NP removal rates were varied as a function of temperature during their lab scale aerobic studies by using activated sludge. Based on first order calculation under continuous addition, kinetic constants (k) for NP ranged from 0.3495 d^{-1} to 0.0304 d^{-1} in mixed sludge during thermophilic (53^{o}C) and mesophilic (35^{o}C) anaerobic digestion (Table 6-2). The kinetics (k) in primary sludge ranged from 0.0912 d^{-1} to 0.0248 d^{-1} during

thermophilic (53^{o}C) and mesophilic (35^{o}C) anaerobic digestion. Lower NP removal and associated kinetic constants at 35^{o}C have also been reported by (Chang et al., 2005) (currently the only study) when mesophilic digestion was compared to thermophilic digestion in primary and petrochemical sludge.

The low NP removals observed in this study are in agreement with (Benabdallah El-Hadj, 2006) who explained that NP is more recalcitrant than other nonylphenolics because of the hydrophobic characteristics (Log K_{ow} = 4.48) (Ahel & Giger, 1993a) which complicates the bioavailability to anaerobes and thus their bioassimilation (Angelidaki et al., 2000; Ejlertsson et al., 1999; Ahel et al., 1994a). The differences between primary and mixed sludge could be therefore attributed to the higher content of lipophilic compounds in primary sludge that hampered NP degradation due to adsorption. The partial biodegradation of NP was attributed to the fact that there was no contribution of NPEOs metabolites to NP since biotransformation of the parent species resulted to NPECs with an exception of NP_1EO in thermophilic primary sludge and not to NP. This phenomenon had possibly served as a barrier to the build up or accumulation of NP from the supplementary metabolic influxes, which resulted to a limited degradation (partial biodegradation) of NP but not to its mineralization.

Nonetheless, when mesophilic digesters were receiving higher $NP_{1-12}EOs$ loadings, nonylphenol indicated a lag phase of 10 days, as it was the case with NPECs and then it was depleted from both aqueous and solids phases in each respective digestate. There was no significant contribution from NPECs or from the short-chained non-ionic nonylphenolics i.e. $NP_{1-2}EOs$ to NP during the 10 days digestion period neither in the primary nor in the mixed sludge. Because of the absence of nonylphenol and the material balance between the parent ($NP_{3-12}EOs$) and the short-chained non-ionic and carboxylated (NPECs) metabolites, flux removal on NP was 100% in both primary and mixed sludge digesters (see Figure 7-5).

The results from the current study (six retention times and the spiked experiments) are in support with the growing but small evidences that NP are able to undergo partial degradation under various anaerobic conditions (Patureau et al., 2008; Ferguson &

Brownawell, 2003; Chang et al., 2005; Chang et al., 2004; Chang et al., 2009) (see Figure 2-4). Although, under aerobic conditions, many studies have unequivocally demonstrated that certain bacteria are capable for NP biodegradation (Tanghe et al., 1999; Soares et al., 2003a; Soares et al., 2003b; Corvini et al., 2004; Gabriel et al., 2005a; Gabriel et al., 2005b), under anaerobic conditions degradation of NP has recently been proposed by (Chang et al., 2005; Chang et al., 2004). The batch studies by using petrochemical or sewage sludge and river sediment have indicated degradation of NP in the order of (from high to low) sulphate reducing conditions > methanogenic conditions > nitrate reducing conditions at pH = 7, and ca. -400mV. The authors proposed that sulphate reducing bacteria would constitute a major microbial component for the anaerobic degradation of NP (NP was virtually the sole carbon source) but the methanogens and eubacteria microbial populations would also be involved. The digestion of nonylphenolics in the current study occurred in methanogenic conditions with similar physicochemical parameters (pH and redox ranged between 7.1-7.6 and -320 to -419 mV respectively). Since no microbiological investigations were carried out in the current research the presence of eubacteria cannot be confirmed or excluded. Nevertheless, there are some uncertainties regarding the reported biodegradation of NP in (Chang et al., 2005; Chang et al., 2004) study. These uncertainties are a) the samples were obtained from the aqueous phase rather than the solid phase, b) no explanations were given for the accumulated NP in the petrochemical sludge which accounted circa 15% and c) the studies were carried out by using either spiked NP or NP_1EO, therefore the contribution of NPEOs metabolites to NP was virtually zero. In the current study, all analytes were quantified from the whole sludge sample including both the aqueous and the solid phase of digested sludge. In addition, NPEOs influxes were much higher compared to the spiked NP or NP_1EO influx of (Chang et al., 2005; Chang et al., 2004). Although during the six retention time study, biodegradation (mineralisation) was not observed, partial biodegradation of NP was evident. At high receiving concentrations, NP was eliminated. The high organic matter content in primary sludge (72% of the TS) could possibly explain the lower NP removals compared to the higher removals observed by (Chang et al., 2005; Chang et al., 2004). However, the important outcome when comparing both lab scale studies is that accumulation of NP in strict anaerobic

conditions does not necessarily occur if the contribution of nonylphenolic metabolites is insignificant or minimum. This observation suggests that metabolic influx as well as biodegradation pathway of nonylphenolics may be significant factors when considering degradation of NP. This statement is further supported from the current knowledge of the large number of aerobic studies i.e. activated sludge where biodegradation/biotransformation of NPEOs leads to $NP_{1-2}EOs$ and NPECs whilst accumulation of NP is insignificant (Montgomery-Brown & Reinhard, 2003; Ahel et al., 1994a; Renner, 1997; Koh, 2008). However, more recently, (Patureau et al., 2008) reported that the removed $NP_{1-2}EOs$ did not equilibrate stoichiometrically with the accumulation of NP during lab scale mesophilic anaerobic digestion (20 days retention time) of mixed sludge (50:50, v/v, primary sludge: SAS) which was treated with ozone prior to digestion. Because of this phenomenon, the authors concluded that NP had been degraded during their anaerobic studies in both aqueous and solid fractions of digested sludge whilst NPECs were absent. Despite the similar OLR between study and the current study, no further data were available for direct comparison. From the literature and the current research it seems that substrate play an important role for the fate and biodegradation pathway of NPEOs in strict anaerobic conditions.

In the current study, adequate stabilization, sludge acclimation, digestion stability in synergy with the microbial biodiversity and the fact the metabolites did not contribute to NP have accounted for the observed nonylphenolic removals. The stability of the anaerobic digesters as well as the accepted range of physicochemical parameters required for efficient anaerobic digestion implied that the acid-forming and methane-forming bacteria were in balance. For example, it has been reported that NP solubility (in water) increases at alkaline pH (p*K*a estimated between 10 and 12) due to its deprotonation, which then becomes desorbed, and more bioavailable (Ivashechkin et al., 2004; Höllrigl-Rosta et al., 2003). In addition, limitations for NP degradation include its adsorption to humic acid in the solvated organic matter through nonspecific lipophilic interactions (Höllrigl-Rosta et al., 2003) that are regulated by diffusion and are not completely reversible (Düring et al., 2002). The pH during the six retention time experiments lied between the optimum ranges for anaerobic digestion which may further increase its bioavailability. During shock loading, pH increased from day 2 in

the different sludge treatments. The increased pH during the shock loading experiment could have accounted for most of NP degradation in synergy with the factors mentioned earlier.

Based on their mass balance (mg d^{-1}), mixed sludge as opposed to primary sludge, indicated the highest removals of the parent nonylphenol ethoxylates. In particular, mesophilic digestion of mixed sludge did promote the removal of the by-products including the carboxylated species as well as the parent compounds. According to the mass balance, 58% removal or degradation was achieved in these digesters (see Figure 6-8). The thermophilic digestion of mixed sludge achieved the highest removal of the parent nonylphenolics (92%), however, the formation of carboxylated nonylphenolics did occur in these digesters. Primary sludge, irrespective of the temperature digestion did not demonstrate any significant removal of nonylphenolics. During the thermophilic digestion their removal in primary sludge was 0.8% whilst during mesophilic digestion their removal was negative (-177%), meaning that primary sludge promoted the formation of the nonylphenolic by-products.

At higher nonylphenolic loadings the mass balance (mg d^{-1}) indicated degradation of parent and the by-products of nonylphenolics in both sludges. In particular, degradation of nonylphenolics in primary sludge was 99.8% whilst in mixed sludge degradation was 100% (see Figure 7-5).

A rough estimation of the removal efficiency of the biomass (the amount of organic matter present in VS) of primary and mixed sludges was evaluated by activity i.e. mg of nonylphenolics removed per gram of VS content per digester. Given that the activity of the biomass of the primary and mixed sludge digestions (six retention time experiments) was significantly ($p<0.05$) different for the mesophilic trials as it was the activity of the biomass for the respective sludges during thermophilic digestion ($p<0.05$) (Appendix III), it was evident that the biomass activity of mixed sludge was more efficient for biodegrading nonylphenolics than primary sludge, irrespective of digestion temperature Table 6-1.

The initial research hypothesis ($H1N_0$) (see section 6.2) was therefore violated (t=44.39, DF=1, $p<0.05$ for the mesophilic and t=97.90, DF=1, $p<0.05$ for the thermophilic) in favour of the alternative hypothesis ($H1N_\alpha$) that primary and mixed sludge digestion irrespective of temperature, result in significantly different nonylphenolic removals (based at the 6th retention time (mg d^{-1} in the feed - mg d^{-1} in the digestate obtained at the 6th retention time). Removals refer to the parent nonylphenolics and not to their by-products.

At higher receiving concentrations i.e. 29 mg kg^{-1} dw of $NP_{1-12}EOs$ for primary sludge and 22 mg d^{-1} of $NP_{1-12}EOs$ in mixed sludge, both sludge types were equally efficient in removing nonylphenolics since there was more available carbon in the systems. Therefore, the initial research hypothesis ($H1ND_0$) (see section 7) is not valid and instead the alternative hypothesis ($H1ND_\alpha$) was found to be feasible since the 30 days old biomass of the dosed digesters removed the additional NPEOs mass.

PNEC values for both steroid estrogens and nonylphenolics refereeing to the aquatic organisms with units mass per volume i.e. ng l^{-1} (Environment Agency, 2000). Despite PNEC of NP for aquatic organisms being 330 ng l^{-1} (European Commission, 2002), the PNEC for soil which has been calculated from the no observed effect concentration (NOEC) for the most sensitive species of three trophic levels i.e. earthworms (*Apporectodea calignosa*) is 0.3 mg kg wet weight^{-1}. The summed concentrations of $NP+NP_{1-2}EOs$ after the mesophilic and thermophilic treatments of primary sludge ranged between 0.4 - 0.3 mg kg^{-1} dw and 2.1 - 0.8 mg kg^{-1} dw whilst for mixed sludge concentrations were 0.2 mg kg^{-1} dw and between 7.2 – 7.3 mg kg^{-1} dw, respectively. The values obtained from the current study were much lower than those presented by (Benabdallah El-Hadj, 2006) during mesophilic (1343 mg kg^{-1} dw) and thermophilic (1104 mg kg^{-1} dw) lab scale studies by using mixed sludge. Therefore, the maximum NPEOs sludge content ($NP+NP_{1-2}EOs$) proposed in the 3rd draft presented to the European Commission (European Commission, 1986) for its use as land conditioner (50 mg $NP+NP_{1-2}EOs$ kg^{-1} dw) was not exceeded by the mesophilic or thermophilic anaerobic digestion of primary or mixed sludge. According to (Harrison et al., 2006) a single application of sludge into the soil would be diluted approximately 100-fold,

however higher concentrations are expected on the soils surface if the treated sludge is not incorporated into the soil. Although it has been reported than microbial degradation by indigenous soil organisms can significantly reduce the risk of environmental contamination by alkylphenols (nonylphenolics and octylphenolics) (Hawrelak et al., 1999), concentration of metabolites could increase if sludge recycling rate exceeds that of biodegradation/biotransformation rate. Results from a sewage sludge amended soil containing significant concentrations of alkylphenols indicated that over the period of 121 days concentration of the metabolite nonylphenol diminished to <0.19 mg kg^{-1} dw from day 90 (WTI, 1998). Digested sludge application rate or sludge recycling and rainfall would therefore most likely determine the concentration of the specific EDCs in soil and their degree of leachate. Since nonylphenolics are associated with higher Log K_{ow} values than steroid estrogens it is expected that nonylphenolics would be more persistent to the terrestrial compartments and less prawn to leach.

8.4.3. Possible solutions from future work

Source separation for steroid estrogens

A significant finding during this research was that E1 was reduced to E2 which has twice the estrogenic potency of E1 (Table 2-1). A certain fraction (33%) of E1-3S could potentially become deconjugated in primary sludge and contribute to E1 which could be potentially reduced to E2. Although biodegradation of E1 was more pronounced than reduction of E1 to E2, primary sludge exhibited greater potential for the degradation of both E1 and E2.

Since the major pathway for the presence of these steroid estrogens in STWs and sludge is human urinary excretion, the reduction of the concentration of steroid estrogens in sewage by urine separation directly at the lavatory may be one of the solutions (Ternes & Joss, 2007) that is potentially useful for concentrating micropollutants before they arrive at the STWs. There remain many challenges and questions with the implementation of source separation of urine for the removal of steroid estrogens (Ternes & Joss, 2007). One of the most important aspects to take into consideration is the removal of estrogens from the concentrated stream before discharging.

Regulation on NPEOs use

Parent and metabolic products of nonylphenolic surfactants enter large scale anaerobic digesters due to their presence in the influents of STWs because of the widespread usage of commercial and industrial applications. With the implementation of the new European Community Regulation on chemicals and their safe use (EC 1907/2006), Registration, Evaluation, Authorization and Restriction of Chemical Substances (REACH) in June 2007, a decline in the use of NPEO in the UK is expected, hence less in the aquatic and terrestrial environment, since most will be replaced by alcohol ethoxylates.

Advance Technology

Although advanced process treatments such as ozone treatment, electron beam irradiation, advanced oxidation processes have been suggested to remove EDCs, these processes will result in large financial costs and increased energy consumption and carbon dioxide emissions (Jones et al., 2007). Membranes on the other hand, besides their drawbacks serve a physical separation and do not destroy or transform EDCs to harmless products. They will improve the quality of effluent but they will increase the sludge product and inevitably the EDC loadings to anaerobic digesters since they will contribute to EDC-concentrated sludge. An environmentally sustainable solution would therefore be to consider optimizing current STWs i.e. increased SRT/HRT and anaerobic digesters operating parameters i.e. acclimation, inoculation with appropriate bacteria, stable digestion temperature, constant feed substrate by utilising large buffering tanks, increased retention times and process stability.

9. CONCLUSIONS

- Mixed feed sludges contained lower steroid estrogens than primary feed sludges, irrespective of the sampling periods. It is postulated that this could related to the aerobic treatment of activated sludge present in mixed sludge. Primary feed sludges contained lower NPEOs than mixed feed sludges, irrespective of the sampling periods because of the presence of metabolites which were related to the aerobic treatment of activated sludge present in mixed sludge.

- Primary and mixed sludge had developed E1 reducing properties irrespective of digestion temperature.

- Degradation of E1 predominates over the reduction of E1 to E2 for the majority of sludge types irrespective of digestion temperature. It is postulated that the observed reduction of E1 to E2 could be related to hydrogen or other organic electron donors and that this biotransformation is mediated co-metabolically.

- Mesophilic anaerobic digestion promotes the removal of E3 irrespective of sludge type compared to thermophilic digestion. No significant contribution of ΣE1+E2 to E3 observed in the different sludges irrespective of temperature digestion. It seems that strict anaerobic bacteria do not favour E3 elimination.

- E1-3S deconjugation is limited during both mesophilic and thermophilic anaerobic digestion in the presence of primary and mixed sludge. However, deconjugation of E1-3S was greater in primary sludge and this process is presumably associated with the presence of arylsulphatase enzyme.

- Higher EE2 flux removals were achieved in primary sludge compared to mixed sludge irrespective of digestion temperature. The removal of EE2 proposed that substrate, redox and temperature appear to play important role for the synthetic estrogen. However, strict anaerobic digestion do not favour significant removal of EE2.

- Flux removal of $NP_{3-12}EOs$ in mixed sludge ranges from 57% to 79% during mesophilic and thermophilic digestion whereas in primary sludge removal ranges from 72% to 71%, respectively. Higher loadings of parent NPEOs resulted to a lag phase of 7 days before the complete disappearance of $NP_{3-12}EOs$ (100% flux removal, respectively)

- No accumulation of $NP_{1-2}EOs$ was observed in mixed sludge irrespective of temperature digestion during the six retention time trials. It appeared that the long retention time promoted the growth of appropriate bacteria for $NP_{1-2}EOs$ assimilation/biotransformation. At higher loadings, a lag phase of 7 days occurred before the complete disappearance of $NP_{1-2}EOs$ in both sludge types as it was observed for $NP_{3-12}EOs$.

- Accumulation of NP_1EOs was observed in primary sludge with shorter retention time at thermophilic temperature but not in mixed sludge. It was concluded that due to the shorter retention time, bacteria responsible for attacking NP_1EOs did not produce the appropriate specific exoenzymes which are required for the attack of lipophilic compounds (Dalton & Stirling, 1982) such as NP_1EO. In addition, fats and grease in the untreated primary sludge may have contributed to the adsorption of the lipophilic NP_1EO.

- Formation of NPECs was the principal biotransformation/biodegradation mechanism of $NP_{1-12}EOs$ in primary sludge, irrespective of digestion temperature. It was hypothesised that this process was a result of co-metabolism with $NP_{1-12}EOs$ in the presence of appropriate bacteria/enzymes because NPECs occurred from the first retention time after the commencement of the trials. When primary sludge is compared to mixed sludge, lipophilic compounds in the former sludge could have possibly accounted for the persistence of NPECs. Hydrolysis of the lypophilic nonylphenolics to more soluble substrates is considered a plausible biotransformation pathway since carboxylation increases solubility and persistence (Ahel et al., 1994d).

- Irrespective of digestion temperature NPECs in mixed sludge reduced presumably because of the presence of acclimated bacteria/enzymes that were able to

utilise NPECs as co-substrate during a co-metabolic activity. Higher digestion temperature could have also initiated this process irrespective of the lower retention time. No evidence was provided for the effect of retention time.

- At higher loadings of parent NPEOs, NPECs exhibited greater persistence (lag phase of 10 days) than the lipophilic $NP_{1\text{-}2}EOs$. The explanation for this phenomenon could be attributed to the higher affinity of NPECs for organic material than that for $NP_{1\text{-}2}EOs$ i.e. Log K_{ow}.

- NPECs were completely degraded (100% flux removal, respectively) after the 10 day lag phase during the higher loadings of parent NPEOs. It was hypothesised that a) high non-ionic surfactant concentrations significantly affected the sludge structural characteristics that lead to the desorption of NPECs and their consecutive assimilation and b) increased rate of co-metabolism occurred ($NP_{1\text{-}12}EOs \rightarrow NPECs$) due to the increased loading rate of $NP_{3\text{-}12}EOs$.

- Nonylphenol was partially degraded in both sludge types. Thermophilic digestion resulted to higher NP flux removals for primary (57%±1%) and mixed sludge (80%±1%) than mesophilic digestion (45%±1%, 51%±1%, respectively). Digestion temperature and possibly pH were more significant than retention time for the removal of NP. However, higher loadings of parent NPEOs resulted to the complete removal of NP in both sludge types (100% flux removal, respectively).

- There was no significant contribution of $NP_{1\text{-}2}EOs$ or NPECs to NP during the six retention time trials and this phenomenon resulted to the partial degradation of NP under strict anaerobic conditions. However, at higher receiving loadings of parent NPEOs, NP was biodegraded (mineralization) and that was evident from the mass balance between the influx and the out flux. The pH could have possibly accounted for this result.

- It appeared that accumulation of NP during strict anaerobic digestion of primary and mixed sludge depends whether nonylphenolic metabolites contribute to NP or not.

- Mixed sludge enhanced the microbial degradation and removal of nonylphenol ethoxylates including most of the quantified metabolites (Figure 6-8) over mixed sludge irrespective of temperature digestion due to the higher biomass activity (based on VS) which may be independent of the overall estrogenic loadings. Mesophilic digestion irrespective of sludge type, favoured degradation and removal of steroid estrogens (Figure 5-7) compared to thermophilic digestion due to the higher biomass activity (based on VS).

- Anaerobic digestion of primary or mixed sludge at mesophilic and thermophilic temperatures resulted to lower (NP+$NP_{1-2}EOs$) concentrations than the proposed concentration in the 3rd draft of 50 mg NP+$NP_{1-2}EOs$ kg dw^{-1} presented to the European Commission (European Commission, 1986) for its use as land conditioner.

- Considering the results from the current study and the available literature it appears that degradation of NPEOs in strict anaerobic and aerobic environments, do not differ significantly.

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10. APPENDICES

10.1 Appendix I

10.1.1 Temperature conversion (mesophilic to thermophilic)

The initial strategy followed for the preparation of the thermophilic seed involved $5^0 \pm 0.2$ºC temperature increase every 7 days. After an elapsed period of approximately one retention time the temperature remained constant at ($45^0 \pm 0.2$ºC) for approximately 2 retention times for the pH stabilisation and the recovery and establishment of the methanogenic bacteria. After this period had elapsed, the change in temperature was gradually raised by $2^0 \pm 0.2$ºC for every 7 days of digestion. These increments were proven to be successful as it is shown in (Figure 10-1, Figure 10-2 and Figure 10-3). The total period required for the preparation of the successful thermophilic seed from a mesophilic seed lasted approximately 4 months.

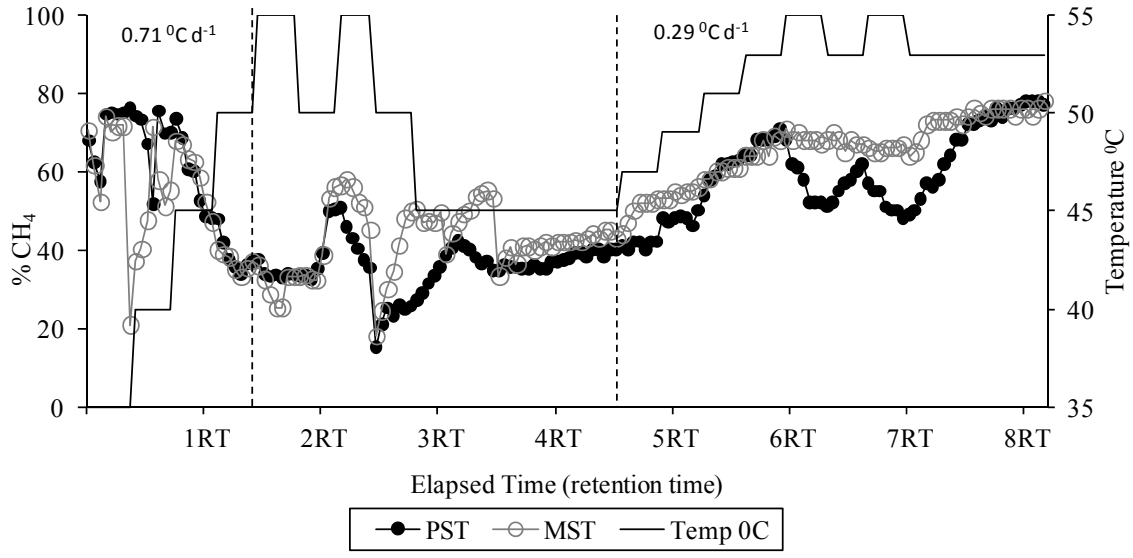

Figure 10-1 Methane content and the change in temperature during the preparation of the thermophilic seed.

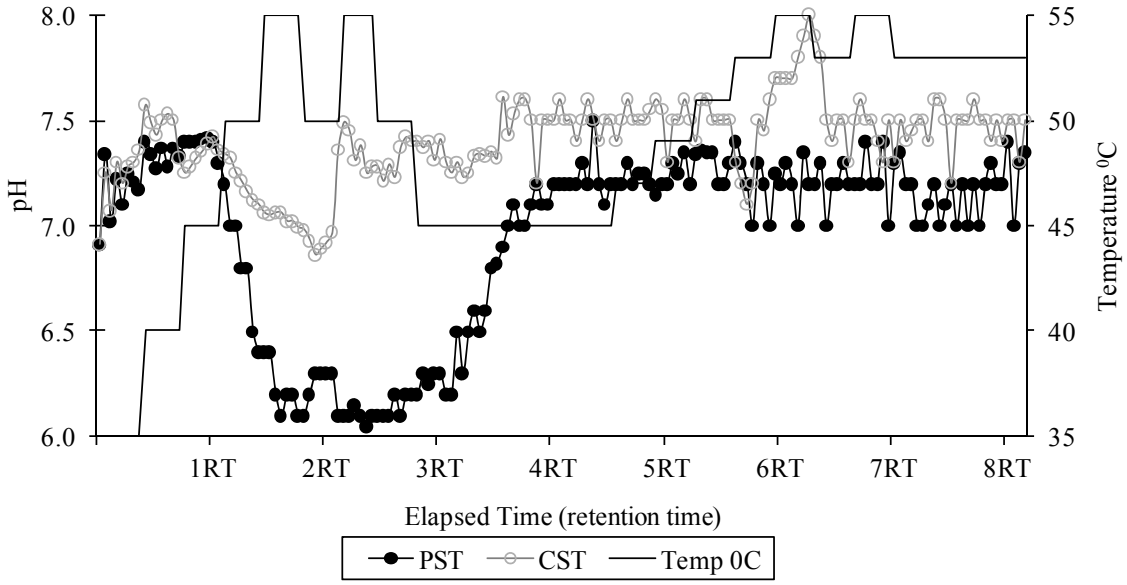

Figure 10-2 pH levels and the change in temperature during the preparation of the thermophilic seed.

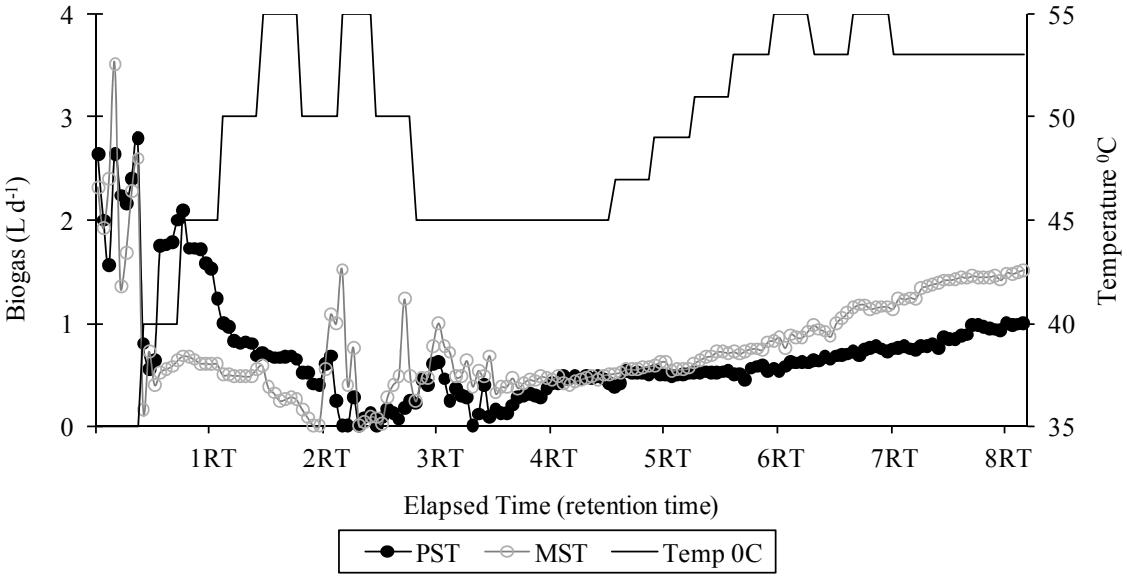

Figure 10-3 Biogas production and the change in temperature during the preparation of the thermophilic seed.

10.2 Appendix II

10.2.1 Eudiometer operation

During non sampling/feeding conditions, the acid reservoir line to the graduated cylinder (V1) and the vent valve (V3) were remained closed whereas the biogas line from the digester to the graduated cylinder (V2) was open. After biogas collection/analysis, sampling and feeding, the biogas collection apparatus was prepared and set up for the next day accordingly. This was achieved by raising the acid level in the graduated cylinder so as to release the biogas to the fume cupboard. This step was necessary in order to obtain accurate biogas measurements the following day and avoid biogas cross contamination from one day to another. The acid level in the graduated cylinder was raised by closing the biogas line (V2) followed by the opening of the line from the acid reservoir to the graduated cylinder (V1) followed by the opening of the vent valve (V3) in that order. By closing the valve (V2) the biogas was not returned to the digester but it was rather released to the fume cupboard. When the acid level in the graduated cylinder was higher than that in the balancing reservoir, the line from the acid reservoir to the graduated cylinder (V2) was closed. After the acid level in both graduated cylinder and balancing reservoir were the same i.e. the overflow port was dry, the vent (V3) was closed and the biogas line (V2) was opened, subjecting in that way the digesters' gaseous environment to the atmospheric pressure. The new acid level in the graduated cylinder was then recorded and it was subsequently subtracted from the next day's level allowing in that way to calculate the level of acid drop. This method of measurement compared to that of the weighing of the acid displacement has advantages such as no individual tanks are required neither analytical balance.

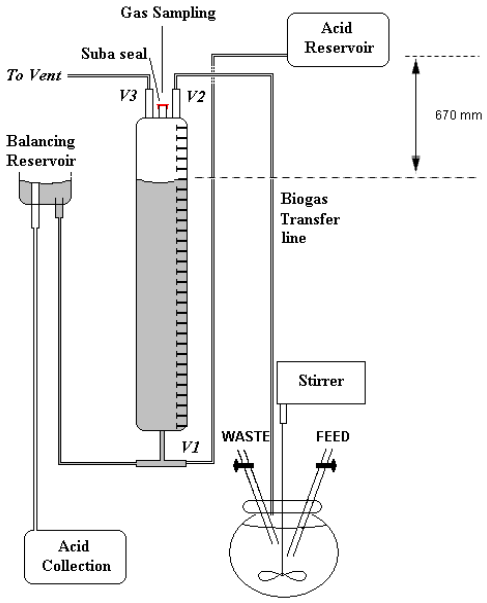

Figure 10-4 Eudiometer apparatus

10.3 Appendix III

10.3.1 Sampling/feeding

Pilot sampling and feeding procedures were developed in order to meet the student's physical impairment and project's requirements. This involved the development of a manual method to sample and feed each digester without using the piston motion of the syringes. This condition became a requirement after the permanent damage of two waste lines, because of the use of a piston during the trial experiments.

The developed method was based on the principle of the difference in hydrostatic pressure between the acid level in the acid reservoir and that in the graduated cylinder (Figure 10-4). After laborious experiments it was observed that 670mm of hydrostatic difference between the two liquid levels was adequate to raise the acid level in the graduated cylinder by 20mm. This increase in acid height was achieved by closing the lower port of the balancing reservoir in such a way to break its connection with the graduated cylinder so no acid replacement could further occur. Then the connection between the acid reservoir and the graduated cylinder was established, in that order. The 20mm raise of acid level within the graduated cylinder exerted a hydrostatic pressure to the graduated cylinder's head space which in turn, this pressure was transferred in the head space of the digester's sludge surface that led to increased pressure within the digester. By opening the sampling line valve, after connecting a syringe barrel to that line, sludge was withdrawn out of the digester (as much as 155ml) and was collected into the syringe; as the internal pressure equilibrated with the atmospheric pressure (provided that the sampling line was immersed in the sludge). After the collection of appropriate sludge volume (50ml for the mesophilic and 100 ml for the thermophilic digesters) the waste line valve was closed and the digester's pressure was dropped (vacuum). After sampling, the V1 valve was closed and the balancing reservoir was exposed back to the atmospheric pressure. A syringe barrel on the feed line was then loaded with the appropriate volume of sludge and the valve of the feed line was then opened. Sludge was then introduced into the digester as the internal negative pressure (vacuum) equilibrated with the atmospheric pressure.

10.4 Appendix IV

10.4.1 Statistical analysis for the mesophilic and thermophilic anaerobic digesters

Table 10-1 Two-Sample T-Test and CI: VS g l^{-1} MS, VS g l^{-1} PS

Two-sample T for VS g/l MS vs VS g/l PS - Mesophilic				
	N	Mean	StDev	SE Mean
MSM VS feed g/L	6	44.04	3.20	1.3
PSM VS feed g/L	6	36.52	2.81	1.1
Difference = mu (MSM VS feed g/L) - mu (PSM VS feed g/L)				
Estimate for difference: 7.52167				
95% CI for difference: (3.58630, 11.45704)				
T-Test of difference = 0 (vs not =): T-Value = 4.32 P-Value = 0.002 DF = 9				
Two-sample T for VS g/l MS vs VS g/l PS - Thermophilic				
	N	Mean	StDev	SE Mean
MST VS feed g/L	6	38.12	0.00408	0.0017
PST VS feed g/L	6	29.22	0.00408	0.0017
Difference = mu (MST VS feed g/L) - mu (PSM VS feed g/L)				
Estimate for difference: 8.89000				
95% CI for difference: (8.88475, 8.89525)				
T-Test of difference = 0(vs not =):T-Value = 3771.71 P-Value = 0.000 DF = 10				

Key: Testing differences between VS in primary and mixed sludge feeds.

Table 10-2 Paired T-Test and CI: PSM1 VS, PSM2 VS (% Removals)

Paired T for PS1M VS% - PS2M VS% - MESOPHILIC				
	N	Mean	StDev	SE Mean
PS1M VS%	6	46.3200	7.5378	3.0773
PS2M VS%	6	45.2483	6.3134	2.5774
Difference	6	1.07167	2.16981	0.88582
95% CI for mean difference: (-1.20541, 3.34875)				
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.21 P-Value = 0.280				

Key: Testing differences between VS % removals within primary digesters.

Table 10-3 Paired T-Test and CI: MSM1 VS, MSM2 VS (% Removals)

Paired T for MS1M VS% - MS2M VS% - MESOPHILIC				
	N	Mean	StDev	SE Mean
MS1M VS%	6	39.7067	2.6351	1.0758
MS2M VS%	6	39.3350	1.5021	0.6132

Difference 6 0.371667 1.206373 0.492500
95% CI for mean difference: (-0.894345, 1.637678)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.75 P-Value = 0.484

Key: Testing differences between VS % removals within mixed digesters.

Table 10-4 Testing correlations among the PSM digesters variables at the 0.05 significance level - Mesophilic

Results for: PSM TOTAL
Correlations: pH PSM 1, pH PSM 2
Pearson correlation of pH PS 1 and pH PS 2 = 0.636
P-Value = 0.174 Rejected at 5%
Correlations: PSM 1 CH4%, PSM 2 CH4%
Pearson correlation of PS1 CH4 and PS 2 CH4 = 0.849
P-Value = 0.033
Correlations: PSM 1 BIO (L/d), PSM 2 BIO (L/d),
Pearson correlation of PS 1 BIO and PS 2 BIO = 0.465
P-Value = 0.352 Rejected at 5%
Correlations: PSM 1 VFAs (mg/L), PSM 2 VFAs (mg/L)
Pearson correlation of PS 1 VFAs and PS 2 VFAs = -0.560
P-Value = 0.248 Rejected at 5%
Correlations: PSM 1 ORP, PSM 2 ORP
Pearson correlation of PS 1 REDOX and PS 2 REDOX = 0.914
P-Value = 0.011
Correlations: PSM 1 TS % REM, PSM 2 TS % REM
Pearson correlation of PS 1 TS and PS 2 TS = 0.946
P-Value = 0.004
Correlations: PSM 1 VS % REM, PSM 2 VS % REM
Pearson correlation of PS 1 VS and PS 2 VS = 0.968
P-Value = 0.002

Table 10-5 Two-Sample T-Test and CI: TS g l^{-1} PSM, TS g l^{-1} MSM

Two-sample T for MSM TS feed g/L vs PSM TS feed g/L - Mesophilic

	N	Mean	StDev	SE Mean
MSM TS feed g/L	6	57.07	4.34	1.8
PSM TS feed g/L	6	51.15	3.69	1.5
Difference = mu (MSM TS feed g/L) - mu (PSM TS feed g/L)				
Estimate for difference: 5.92500				
95% CI for difference: (0.65874, 11.19126)				
T-Test of difference = 0 (vs not =): T-Value = 2.55 P-Value = 0.031 DF = 9				
Two-sample T for TS g/l MS vs TS g/l PS - Thermophilic				
	N	Mean	StDev	SE Mean
MST TS feed g/L	6	49.68	0.00408	0.0017
PST TS feed g/L	6	39.49	0.00408	0.0017
Difference = mu (MST TS feed g/L) - mu (PST TS feed g/L)				
Estimate for difference: 10.1900				
95% CI for difference: (10.1847, 10.1953)				
T-Test of difference = 0(vs not =): T-Value = 4323.25 P-Value = 0.000 DF = 10				

Key: Testing differences between TS in primary and mixed sludge feeds.

Table 10-6 Two-Sample T-Test and CI: TS g l^{-1} PST, TS g l^{-1} PSM

Two-sample T for PST TS feed g/L vs PSM TS feed g/L				
	N	Mean	StDev	SE Mean
PST TS feed g/L	6	39.50	0.004	0.002
PSM TS feed g/L	6	51.15	3.69	1.5
Difference = mu (PST TS feed g/L) - mu (PSM TS feed g/L)				
Estimate for difference: -11.6550				
95% CI for difference: (-15.5310, -7.7790)				
T-Test of difference = 0 (vs not =): T-Value = -7.73 P-Value = 0.001 DF = 5				

Key: Testing differences between TS in the primary feed sludges of mesophilic and thermophilic digesters.

Table 10-7 Two-Sample T-Test and CI: VS g l^{-1} PST, VS g l^{-1} PSM

Two-sample T for PST VS feed g/L vs PSM VS feed g/L				
	N	Mean	StDev	SE Mean
PST VS feed g/L	6	29.22	0.004	0.002
PSM VS feed g/L	6	36.52	2.81	1.1
Difference = mu (PST VS feed g/L) - mu (PSM VS feed g/L)				
Estimate for difference: -7.29333				
95% CI for difference: (-10.24513, -4.34154)				
T-Test of difference = 0 (vs not =): T-Value = -6.35 P-Value = 0.001 DF = 5				

Key: Testing differences between VS in the primary feed sludges of mesophilic and thermophilic digesters.

Table 10-8 Two-Sample T-Test and CI: TS g l^{-1} MST, TS g l^{-1} MSM

Two-sample T for CST TS feed g/L vs CSM TS feed g/L				
	N	Mean	StDev	SE Mean
MST TS feed g/L	6	49.69	0.00408	0.0017
MSM TS feed g/L	6	57.07	4.34	1.8
Difference = mu (CST TS feed g/L) - mu (CSM TS feed g/L)				
Estimate for difference: -7.39000				
95% CI for difference: (-11.94942, -2.83058)				
T-Test of difference = 0 (vs not =): T-Value = -4.17 P-Value = 0.009 DF = 5				

Key: Testing differences between TS in the mixed feed sludges of mesophilic and thermophilic digesters.

Table 10-9 Two-Sample T-Test and CI: VS g l^{-1} MST, VS g l^{-1} MSM

Two-sample T for CST VS feed g/L vs CSM VS feed g/L				
	N	Mean	StDev	SE Mean
MST VS feed g/L	6	38.11167	0.00408	0.0017
MSM VS feed g/L	6	44.04	3.20	1.3
Difference = mu (CST VS feed g/L) - mu (CSM VS feed g/L)				
Estimate for difference: -5.92500				
95% CI for difference: (-9.28432, -2.56568)				
T-Test of difference = 0 (vs not =): T-Value = -4.53 P-Value = 0.006 DF = 5				

Key: Testing differences between VS in the mixed feed sludges of mesophilic and thermophilic digesters.

Table 10-10 Paired T-Test and CI: PSM1 TS, PSM2 TS (% Removals)

Paired T for PS1M TS% - PS2M TS% - MESOPHILIC				
	N	Mean	StDev	SE Mean
PS1M TS%	6	42.5933	8.9406	3.6500
PS2M TS%	6	42.2200	8.2901	3.3844
Difference	6	0.373333	2.913133	1.189282
95% CI for mean difference: (-2.683813, 3.430479)				
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.31 P-Value = 0.766				

Key: Testing differences between TS % removals within primary digesters.

Table 10-11 Paired T-Test and CI: MST1 TS, MST2 TS (% Removals)

Paired T for MS1M TS% - MS2M TS% - THERMOPHILIC				
	N	Mean	StDev	SE Mean
MST1 TS%	6	33.7500	4.8938	1.9979
MST2 TS%	6	33.6633	4.4450	1.8147

Difference 6 0.086667 1.608399 0.656626
95% CI for mean difference: (-1.601244, 1.774578)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.13 P-Value = 0.900

Key: Testing differences between TS % removals within mixed digesters.

Table 10-12 Testing correlations among the MSM digester's variables at the 0.05 significance level - Mesophilic

Results for: MSM TOTAL
Correlations: MS 1 pH, MS 2 pH
Pearson correlation of MS 1 pH and MS 2 pH = 0.994
P-Value = 0.000
Correlations: MS 1 CH4 %, MS 2 CH4 %
Pearson correlation of MS 1 CH4 and MS 2 CH4 = 0.979
P-Value = 0.001
Correlations: MS 1 BIO (L/d), MS 2 BIO (L/d)
Pearson correlation of MS 1 BIO and MS 2 BIO = 0.900
P-Value = 0.014
Correlations: MS 1 VFAs (mg/L), MS 2 VFAs (mg/L)
Pearson correlation of MS 1 VFAs and MS 2 VFAs = 0.818
P-Value = 0.046
Correlations: PS 1 REDOX, PS 2 REDOX
Pearson correlation of PS 1 REDOX and PS 2 REDOX = 0.998
P-Value = 0.000
Correlations: MS 1 VS % REM, MS 2 VS % REM
Pearson correlation of MS 1 VS and MS 2 VS = 0.878
P-Value = 0.022
Correlations: MS 1 TS % REM, MS 2 TS % REM
Pearson correlation of MS 1 TS and MS 2 TS = 0.945
P-Value = 0.004

10.4.2 Statistical analysis for steroid estrogens

Table 10-13 Descriptive Statistics: ΣEST PSM, ΣEST MSM, ΣEST PST, ΣEST MST

Variable	N	N*	Mean	SE Mean	StDev	Variance	CoefVar	Minimum	Q1
Σ EST PSM	6	0	120.1	10.5	25.7	657.9	21.35	100.8	102.9
Σ EST MSM	6	0	70.78	3.01	7.38	54.49	10.43	61.20	66.45
Σ EST PST	6	0	45.80	4.03	9.88	97.64	21.57	36.90	37.50
Σ EST MST	6	0	70.78	3.01	7.38	54.49	10.43	61.20	66.45

Variable	Median	Q3	Maximum	Range
Σ EST PSM	108.6	140.9	167.1	66.3
Σ EST MSM	69.60	75.28	83.60	22.40
Σ EST PST	42.05	57.43	59.30	22.40
Σ EST MST	69.60	75.28	83.60	22.40

Table 10-14 Normality tests (α=0.10)

Total EST Influx µg d^{-1}	P-Value
PSM	0.606
MSM	0.352
PST	0.609
PST	0.125

Table 10-15 Test for Equal Variances: PSM influx, MSM influx

Test for Equal Variances: PSM µg/d, MSM µg/d				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
PSM FL_1	3	0.581791	1.21788	10.8589
MSM FL_1	3	0.123928	0.25942	2.3131
F-Test (normal distribution)				
Test statistic = 22.04, p-value = 0.087				
Levene's Test (any continuous distribution)				
Test statistic = 2.26, p-value = 0.207				

Key: Testing differences between PSM and MSM influxes.

Table 10-16 Test for Equal Variances: PST influx, MST influx

Test for Equal Variances: PST FL_1, MST FL_1				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
PST FL_1	3	0.043082	0.090185	0.80411
MST FL_1	3	0.102494	0.214554	1.91301

F-Test (normal distribution)
Test statistic = 0.18, p-value = 0.300
Levene's Test (any continuous distribution)
Test statistic = 0.40, p-value = 0.560

Key: Testing differences between PST and MST influxes.

Table 10-17 Two-Sample T-Test and CI: PSM μg d^{-1}, MSM, μg d^{-1}

Two-sample T for PSM FL_1 vs MSM FL_1				
	N	Mean	StDev	SE Mean
PSM FL_1	3	5.79	1.22	0.70
MSM FL_1	3	3.070	0.259	0.15
Difference = mu (PSM FL_1) - mu (MSM FL_1)				
Estimate for difference: 2.71667				
95% CI for difference: (-0.37659, 5.80993)				
T-Test of difference = 0 (vs not =): T-Value = 3.78 P-Value = 0.063 DF = 2				

Key: Testing differences between PSM and MSM influxes.

Table 10-18 Two-Sample T-Test and CI: PST μg d^{-1}, MST, μg d^{-1}

Two-sample T for PST FL_1 vs MST FL_1				
	N	Mean	StDev	SE Mean
PST FL_1	3	4.810	0.171	0.099
MST FL_1	3	2.717	0.423	0.24
Difference = mu (PST FL_1) - mu (MST FL_1)				
Estimate for difference: 2.09333				
99% CI for difference: (-0.52272, 4.70939)				
T-Test of difference = 0 (vs not =): T-Value = 7.94 P-Value = 0.015 DF = 2				

Key: Testing differences between PST and MST influxes.

Table 10-19 Test for Equal Variances: PSM influx, PST influx

Test for Equal Variances: PSM FL_1, PST FL_1				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
PSM FL_1	3	0.581791	1.21788	10.8589
PST FL_1	3	0.081631	0.17088	1.5236
F-Test (normal distribution)				
Test statistic = 50.80, p-value = 0.039				
Levene's Test (any continuous distribution)				
Test statistic = 2.78, p-value = 0.171				

Key: Testing differences between PSM and PST influxes.

Table 10-20 Test for Equal Variances: MSM influx, MST influx

Test for Equal Variances: MSM FL_1, MST FL_1				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
MSM FL_1	3	0.123928	0.259422	2.31307
MST FL_1	3	0.202242	0.423360	3.77477
F-Test (normal distribution)				
Test statistic = 0.38, p-value = 0.546				
Levene's Test (any continuous distribution)				
Test statistic = 0.14, p-value = 0.730				

Key: Testing differences between MSM and MST influxes.

Table 10-21 Two-Sample T-Test and CI: PSM μg d^{-1}, PST, μg d^{-1}

Two-sample T for PSM FL_1 vs PST FL_1						
	N	Mean	StDev	SE Mean		
PSM FL_1	3	5.79	1.22	0.70		
PST FL_1	3	4.810	0.171	0.099		
Difference = mu (PSM FL_1) - mu (PST FL_1)						
Estimate for difference: 0.976667						
95% CI for difference: (-2.078351, 4.031685)						
T-Test of difference = 0 (vs not =): T-Value = 1.38 P-Value = 0.303 DF = 2						

Key: Testing differences between PSM and PST influxes.

Table 10-22 Two-Sample T-Test and CI: MSM μg d^{-1}, MST, μg d^{-1}

Two-sample T for MSM FL_1 vs MST FL_1						
	N	Mean	StDev	SE Mean		
MSM FL_1	3	3.070	0.259	0.15		
MST FL_1	3	2.717	0.423	0.24		
Difference = mu (MSM FL_1) - mu (MST FL_1)						
Estimate for difference: 0.353333						
95% CI for difference: (-0.558968, 1.265635)						
T-Test of difference = 0 (vs not =): T-Value = 1.23 P-Value = 0.306 DF = 3						

Key: Testing differences between MSM and MST influxes.

Table 10-23 Two-Sample T-Test and CI: PSM μg d^{-1}, MSM, μg d^{-1}

Two-sample T for PSM biomass vs MSM µg/d removed				
	N	Mean	StDev	SE Mean
PSM µg/d removed	2	3.0800	0.0566	0.040
MSM µg/d removed	2	1.2000	0.0566	0.040
Difference = mu (PSM biomass) - mu (MSM µg/d removed)				
Estimate for difference: 1.88000				
95% CI for difference: (1.63661, 2.12339)				
T-Test of difference = 0 (vs not =): T-Value = 33.23 P-Value = 0.001 DF = 2				

Key: Testing differences between PSM and MSM µg d^{-1} removals.

Table 10-24 Two-Sample T-Test and CI: PST µg d^{-1}, MST, µg d^{-1}

Two-sample T for PST µg/d removed vs MST µg/d removed				
	N	Mean	StDev	SE Mean
PST µg/d removed	2	2.4700	0.0141	0.010
MST µg/d removed	2	0.3200	0.0707	0.050
Difference = mu (PST µg/d removed) - mu (MST µg/d removed)				
Estimate for difference: 2.15000				
95% CI for difference: (1.50211, 2.79789)				
T-Test of difference = 0 (vs not =): T-Value = 42.16 P-Value = 0.015 DF = 1				

Key: Testing differences between PST and MST µg d^{-1} removals.

Table 10-25 Two-Sample T-Test and CI: PSM µg d^{-1}, PST, µg d^{-1}

Two-sample T for PSM µg/d removed vs PST µg/d removed				
	N	Mean	StDev	SE Mean
PSM µg/d removed	2	3.0800	0.0566	0.040
PST µg/d removed	2	2.4700	0.0141	0.010
Difference = mu (PSM µg/d removed) - mu (PST µg/d removed)				
Estimate for difference: 0.610000				
95% CI for difference: (0.086110, 1.133890)				
T-Test of difference = 0 (vs not =): T-Value = 14.79 P-Value = 0.043 DF = 1				

Key: Testing differences between PSM and PST µg d^{-1} removals.

Table 10-26 Two-Sample T-Test and CI: MSM µg d^{-1}, MST, µg d^{-1}

Two-sample T for MSM µg/d removed vs MST µg/d removed				
	N	Mean	StDev	SE Mean
MSM µg/d removed	2	1.2000	0.0566	0.040
MST µg/d removed	2	0.3200	0.0707	0.050
Difference = mu (MSM µg/d removed) - mu (MST µg/d removed)				
Estimate for difference: 0.880000				
95% CI for difference: (0.066406, 1.693594)				
T-Test of difference = 0 (vs not =): T-Value = 13.74 P-Value = 0.046 DF = 1				

Key: Testing differences between MSM and MST μg d^{-1} removals.

10.4.3 Statistical analysis for nonylphenol ethoxylates

Table 10-27 Normality tests (α=0.10)

Total NPEOs Influx mg d^{-1}	P-Value
PSM	0.483
MSM	0.496
PST	0.893
PST	0.247

Table 10-28 Test for Equal Variances: PSM influx, MSM influx

Test for Equal Variances: PSM NPE mg/d, MSM NPE mg/d				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
PSM NPE FL	5	4.29038	7.66342	26.5120
MSM NPE FL	5	2.98316	5.32848	18.4341
F-Test (normal distribution)				
Test statistic = 2.07, p-value = 0.499				
Levene's Test (any continuous distribution)				
Test statistic = 0.29, p-value = 0.603				

Key: Testing differences between PSM and MSM influxes.

Table 10-29 Test for Equal Variances: PST influx, MST influx

Test for Equal Variances: PST NPE mg/d, MST NPE mg/d				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
PST NPE FL	5	2.82552	5.04691	17.4600
MST NPE FL	4	3.81220	7.25364	34.3531
F-Test (normal distribution)				
Test statistic = 0.48, p-value = 0.495				
Levene's Test (any continuous distribution)				
Test statistic = 1.04, p-value = 0.341				

Key: Testing differences between PST and MST influxes.

Table 10-30 Two-Sample T-Test and CI: PSM mg d^{-1}, MSM, mg d^{-1}

Two-sample T for PSM NPE FL vs MSM NPE FL				
	N	Mean	StDev	SE Mean
PSM NPE FL	5	33.91	7.66	3.4
MSM NPE FL	5	29.12	5.33	2.4
Difference = mu (PSM NPE FL) - mu (MSM NPE FL)				
Estimate for difference: 4.78400				
95% CI for difference: (-5.08647, 14.65447)				
T-Test of difference = 0 (vs not =): T-Value = 1.15 P-Value = 0.289 DF = 7				

Key: Testing differences between PSM and MSM influxes.

Table 10-31 Two-Sample T-Test and CI: PST mg d^{-1}, MST, mg d^{-1}

Two-sample T for PST NPE FL vs MST NPE FL				
	N	Mean	StDev	SE Mean
PST NPE FL	5	59.82	5.05	2.3
MST NPE FL	4	46.26	7.25	3.6
Difference = mu (PST NPE FL) - mu (MST NPE FL)				
Estimate for difference: 13.5615				
99% CI for difference: (-3.6629, 30.7859)				
T-Test of difference = 0 (vs not =): T-Value = 3.17 P-Value = 0.025 DF = 5				

Key: Testing differences between PST and MST influxes.

Table 10-32 Test for Equal Variances: PSM influx, PST influx

Test for Equal Variances: PSM NPE FL, PST NPE FL				
99% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
PSM NPE FL	5	3.78193	7.66342	40.2687
PST NPE FL	5	2.49067	5.04691	26.5198
F-Test (normal distribution)				
Test statistic = 2.31, p-value = 0.438				
Levene's Test (any continuous distribution)				
Test statistic = 0.30, p-value = 0.597				

Key: Testing differences between PSM and PST influxes.

Table 10-33 Test for Equal Variances: MSM influx, MST influx

Test for Equal Variances: MSM NPE FL, MST NPE FL				
95% Bonferroni confidence intervals for standard deviations				
	N	Lower	StDev	Upper
MSM NPE FL	5	2.98316	5.32848	18.4341
MST NPE FL	4	3.81220	7.25364	34.3531
F-Test (normal distribution)				
Test statistic = 0.54, p-value = 0.556				
Levene's Test (any continuous distribution)				

Test statistic = 0.78, p-value = 0.406

Key: Testing differences between MSM and MST influxes.

Table 10-34 Two-Sample T-Test and CI: PSM mg d^{-1}, PST, mg d^{-1}

Two-sample T for PSM NPE FL vs PST NPE FL				
	N	Mean	StDev	SE Mean
PSM NPE FL	5	33.91	7.66	3.4
PST NPE FL	5	59.82	5.05	2.3
Difference = mu (PSM NPE FL) - mu (PST NPE FL)				
Estimate for difference: -25.9160				
95% CI for difference: (-35.9572, -15.8748)				
T-Test of difference = 0 (vs not =): T-Value = -6.32 P-Value = 0.001 DF = 6				

Key: Testing differences between PSM and PST influxes.

Table 10-35 Two-Sample T-Test and CI: MSM mg d^{-1}, MST, mg d^{-1}

Two-sample T for MSM NPE FL vs MST NPE FL				
	N	Mean	StDev	SE Mean
MSM NPE FL	5	29.12	5.33	2.4
MST NPE FL	4	46.26	7.25	3.6
Difference = mu (MSM NPE FL) - mu (MST NPE FL)				
Estimate for difference: -17.1385				
95% CI for difference: (-28.2939, -5.9831)				
T-Test of difference = 0 (vs not =): T-Value = -3.95 P-Value = 0.011 DF = 5				

Key: Testing differences between MSM and MST influxes.

Table 10-36 Two-Sample T-Test and CI: PSM mg d^{-1}, MSM, mg d^{-1}

Two-sample T for PSM NPE mg/d removed vs MSM NPE mg/d removed				
	N	Mean	StDev	SE Mean
PSM NPE mg/d rem	2	30.4000	0.0566	0.040
MSM NPE mg/d rem	2	24.995	0.163	0.11
Difference = mu (PSM NPE mg/d removed) - mu (MSM NPE mg/d removed)				
Estimate for difference: 5.40500				
95% CI for difference: (3.85792, 6.95208)				
T-Test of difference = 0 (vs not =): T-Value = 44.39 P-Value = 0.014 DF = 1				

Key: Testing differences between PSM and MSM mg d^{-1} removals.

Table 10-37 Two-Sample T-Test and CI: PST mg d^{-1}, MST, mg d^{-1}

Two-sample T for PST NPE mg/d removed vs MST NPE mg/d removed

	N	Mean	StDev	SE Mean
PST NPE mg/d rem	2	57.135	0.163	0.12
MST NPE mg/d rem	2	45.0450	0.0636	0.045
Difference = mu (PST NPE mg/d removed) - mu (MST NPE mg/d removed)				
Estimate for difference: 12.0900				
95% CI for difference: (10.5209, 13.6591)				
T-Test of difference = 0 (vs not =): T-Value = 97.90 P-Value = 0.007 DF = 1				

Key: Testing differences between PST and MST mg d^{-1} removals.

Table 10-38 Two-Sample T-Test and CI: PSM mg d^{-1}, PST, mg d^{-1}

Two-sample T for PSM NPE mg/d removed vs PST NPE mg/d removed				
	N	Mean	StDev	SE Mean
PSM NPE mg/d rem	2	30.4000	0.0566	0.040
PST NPE mg/d rem	2	57.135	0.163	0.12
Difference = mu (PSM NPE mg/d removed) - mu (PST NPE mg/d removed)				
Estimate for difference: -26.7350				
95% CI for difference: (-28.2821, -25.1879)				
T-Test of difference = 0 (vs not =): T-Value = -219.57 P-Value = 0.003 DF = 1				

Key: Testing differences between PSM and PST mg d^{-1} removals.

Table 10-39 Two-Sample T-Test and CI: MSM mg d^{-1}, MST, mg d^{-1}

Two-sample T for MSM NPE mg/d removed vs MST NPE mg/d removed				
	N	Mean	StDev	SE Mean
MSM NPE mg/d rem	2	24.995	0.163	0.11
MST NPE mg/d rem	2	45.0450	0.0636	0.045
Difference = mu (MSM NPE mg/d removed) - mu (MST NPE mg/d removed)				
Estimate for difference: -20.0500				
95% CI for difference: (-21.6191, -18.4809)				
T-Test of difference = 0 (vs not =): T-Value = -162.36 P-Value = 0.004 DF = 1				

Key: Testing differences between MSM and MST mg d^{-1} removals.

10.4.4 Statistical analysis for nonylphenol ethoxylates (dosed feed)

Table 10-40 Normality tests (α=0.10)

Total NPEOs Influx mg d^{-1}	P-Value
PSM dosed	0.618
MSM dosed	0.348

10.5 Appendix V

10-41 Method detection limits for steroid estrogens in sludge.

Steroid estrogens	MDL ng g^{-1} Sludge (µg kg^{-1} dw) *n*=3
E1	2.1
E2	4.9
E3	4.5
EE2	5.3
E1-3S	2.6

10-42 Method detection limits for nonylphenolics in sludge.

Nonylphenolics	MDL ng g^{-1} Sludge (µg Kg^{-1}) *n*=3 Based on spike at 0.125LS and 1.25HS µg g^{-1} on sludge
NP_1EO	6
NP_2EO	12
NP_3EO	0.5
NP_4EO	7.5
NP_5EO	10
NP_6EO	13
NP_7EO	4
NP_8EO	4
NP_9EO	11
$NP_{10}EO$	10
$NP_{11}EO$	4.5
$NP_{12}EO$	5.5
NP_1EC	12
NP_2EC	NA
NP_3EC	NA
NP	11

Table 10-43 Background (feed) concentrations for steroid estrogens (µg kg^{-1} dw).

PSM	**EE2**	**E2**	**E3**	**E1**	**E1-3S**
a	18.4	9.2	8.9	158.3	7.6
b	14.1	8.3	9.0	132.6	7.5
c	22.6	10.2	8.9	183.9	7.7
mean	18.36	9.25	8.91	158.28	7.63

SD	4.25	0.96	0.04	25.66	0.10
RSD%	23.17	10.33	0.48	16.21	1.26
SE	2.46	0.55	0.02	14.82	0.06
MSM	**EE2**	**E2**	**E3**	**E1**	**E1-3S**
a	10.04	6.25	7.89	89.01	6.82
b	9.38	5.91	13.55	81.13	5.69
c	10.99	6.89	1.63	97.39	7.35
mean	10.14	6.35	7.69	89.17	6.62
SD	0.81	0.50	5.96	8.13	0.85
RSD%	7.98	7.84	77.58	9.12	12.85
SE	0.47	0.29	3.44	4.70	0.49
PST	**EE2**	**E2**	**E3**	**E1**	**E1-3S**
a	9.80	5.98	6.60	64.33	3.70
b	7.91	4.94	4.84	61.92	3.94
c	9.76	7.02	5.47	66.75	3.51
mean	9.15	5.98	5.64	64.33	3.72
SD	1.08	1.04	0.89	2.42	0.22
RSD%	11.82	17.41	15.80	3.76	5.80
SE	0.62	0.60	0.51	1.40	0.12
MST	**EE2**	**E2**	**E3**	**E1**	**E1-3S**
a	10.85	2.98	5.99	32.33	3.85
b	9.80	2.56	5.22	30.19	4.20
c	9.75	3.41	4.56	34.47	4.22
mean	10.13	2.98	5.26	32.33	4.09
SD	0.62	0.43	0.71	2.14	0.21
RSD%	6.11	14.33	13.57	6.61	5.14
SE	0.36	0.25	0.41	1.23	0.12

Table 10-44 Steroid estrogen concentrations (μg kg^{-1} dw) from individual samples of the primary sludge mesophilic (six retention times) digesters (PSM).

	PSM	**EE2**	**E2**	**E3**	**E1**	**E1-3S**
PSM 1	a	12.8	18.0	6.3	139.6	5.7
	b	17.1	15.9	6.8	139.5	4.7
PSM 2	c	14.82	8.45	4.67	127.2	5.72
	d	7.92	5.29	6.49	115.42	5.98
1RT	mean	13.16	11.90	6.08	130.44	5.52
	SD	3.89	6.01	0.96	11.59	0.54
	RSD%	29.60	50.54	15.75	8.88	9.77
	SE	1.95	3.01	0.48	5.79	0.27
PSM 1	a	10.8	21.9	5.6	67.1	5.1
	b	12.2	19.5	5.2	87.0	5.2
PSM 2	c	14.24	12.5	5.46	108.89	4.26
	d	13.83	9.1	6.12	107.57	6.67
2RT	mean	12.77	15.76	5.61	92.64	5.31
	SD	1.58	5.98	0.37	19.74	1.00
	RSD%	12.38	37.92	6.65	21.31	18.80
	SE	0.79	2.99	0.19	9.87	0.50
PSM 1	a	15.4	27.4	4.9	59.2	3.2
	b	9.8	28.9	4.9	62.3	6.4
PSM 2	c	14.87	22.45	5.38	65.78	4.89
	d	10.74	19.65	5.45	72.56	5.12
3RT	mean	12.69	24.60	5.16	64.97	4.89
	SD	2.85	4.30	0.29	5.72	1.30
	RSD%	22.43	17.48	5.70	8.81	26.52
	SE	1.42	2.15	0.15	2.86	0.65
PSM 1	a	14.2	23.6	5.5	61.4	4.4
	b	11.3	30.7	6.0	61.7	5.1
PSM 2	c	14.52	24.65	4.89	52.14	5.28
	d	12.94	22.44	5.89	41.6	5.98
4RT	mean	13.24	25.35	5.58	54.21	5.19

	PSM	EE2	E2	E3	E1	E1-3S
	SD	1.48	3.68	0.50	9.50	0.65
	RSD%	11.17	14.52	9.01	17.53	12.60
	SE	0.74	1.84	0.25	4.75	0.33
PSM 1	a	12.7	28.7	4.7	26.7	5.2
	b	12.7	28.7	4.7	26.7	5.2
PSM 2	c	11.14	38.75	5.69	47.46	6.14
	d	15.86	38.76	6.15	44.86	6.45
5RT	mean	13.09	33.73	5.30	36.41	5.75
	SD	1.99	5.81	0.74	11.31	0.64
	RSD%	15.17	17.21	14.05	31.07	11.22
	SE	0.99	2.90	0.37	5.66	0.32
PSM 1	a	14.5	31.4	4.5	22.1	5.0
	b	10.8	46.9	5.5	58.9	5.0
PSM 2	c	14.56	42.15	5.12	32.77	4.78
	d	12.89	46.04	5.48	29.14	5.78
6RT	mean	13.19	41.62	5.15	35.74	5.14
	SD	1.77	7.12	0.47	16.06	0.44
	RSD%	13.42	17.11	9.07	44.94	8.54
	SE	0.88	3.56	0.23	8.03	0.22

Table 10-45 Steroid estrogen concentrations (μg kg^{-1} dw) from individual samples of the primary sludge thermophilic (six retention times) digesters (PST).

	PST	EE2	E2	E3	E1	E1-3S
	a	5.5	7.7	5.1	39.7	2.8
	b	5.4	8.5	4.8	38.5	2.8
1RT	mean	5.45	8.08	4.93	39.07	2.80
	SD	0.09	0.61	0.18	0.83	0.04
	RSD%	1.62	7.53	3.59	2.14	1.52
	SE	0.06	0.43	0.13	0.59	0.03
	a	5.5	14.8	5.1	28.8	2.7
	b	5.5	15.2	4.8	30.5	2.8
2RT	mean	5.48	14.99	4.95	29.63	2.74
	SD	0.03	0.23	0.26	1.20	0.04
	RSD%	0.52	1.56	5.29	4.03	1.42
	SE	0.02	0.16	0.18	0.84	0.03
	a	5.4	11.2	5.0	13.5	2.7
	b	5.4	12.0	4.8	15.1	2.8
3RT	mean	5.36	11.55	4.90	14.28	2.74
	SD	0.01	0.57	0.11	1.11	0.09
	RSD%	0.13	4.93	2.17	7.78	3.23
	SE	0.00	0.40	0.08	0.78	0.06
	a	5.3	18.8	4.6	5.3	2.8
	b	5.4	20.1	4.6	6.1	2.8
4RT	mean	5.38	19.45	4.64	5.67	2.79
	SD	0.06	0.92	0.00	0.57	0.05
	RSD%	1.12	4.73	0.00	9.98	1.78
	SE	0.04	0.65	0.00	0.40	0.04
	a	5.3	26.5	4.8	4.2	2.8
	b	5.6	20.6	4.8	4.7	2.7
5RT	mean	5.45	23.53	4.78	4.44	2.74
	SD	0.15	4.20	0.00	0.35	0.02
	RSD%	2.79	17.83	0.00	7.88	0.64
	SE	0.11	2.97	0.00	0.25	0.01
	a	5.4	29.5	5.1	2.8	2.7
	b	5.5	29.6	4.8	3.3	2.8
6RT	mean	5.46	29.53	4.95	3.06	2.75
	SD	0.06	0.07	0.23	0.31	0.01
	RSD%	1.04	0.25	4.64	10.06	0.39
	SE	0.04	0.05	0.16	0.22	0.01

Table 10-46 Steroid estrogen concentrations (μg kg^{-1} dw) from individual samples of the mixed sludge mesophilic (six retention times) digesters (MSM).

	MSM	EE2	E2	E3	E1	E1-3S
MSM 1	a	6.9	14.5	6.2	58.7	4.8
	b	8.9	18.3	7.5	50.6	6.2
MSM 2	c	4.45	11.23	5.68	49.45	3.68
	d	4.78	12.69	6.61	52.65	5.98
1RT	mean	6.25	14.18	6.51	52.86	5.17
	SD	2.06	3.06	0.78	4.14	1.18
	RSD%	33.02	21.59	11.96	7.83	22.80
	SE	1.03	1.53	0.39	2.07	0.59
MSM 1	a	7.2	14.5	6.1	36.3	5.1
	b	8.8	14.7	4.8	34.3	5.4
MSM 2	c	4.68	12.68	5.11	31.25	4.56
	d	4.12	12.65	6.14	32.68	6.48
2RT	mean	6.22	13.62	5.53	33.63	5.40
	SD	2.21	1.11	0.70	2.17	0.80
	RSD%	35.59	8.15	12.68	6.46	14.89
	SE	1.11	0.56	0.35	1.09	0.40
MSM 1	a	9.3	14.7	3.9	28.2	4.3
	b	6.8	22.2	5.6	33.0	5.5
MSM 2	c	5.98	21.28	4.69	24.14	5.48
	d	5.45	29.68	6.22	26.45	6.12
3RT	mean	6.86	21.96	5.10	27.94	5.35
	SD	1.68	6.15	1.01	3.76	0.74
	RSD%	24.52	27.99	19.82	13.44	13.84
	SE	0.84	3.07	0.51	1.88	0.37
MSM 1	a	7.4	18.3	5.6	29.0	4.9
	b	10.3	19.5	5.8	31.6	4.9
MSM 2	c	6.15	20.56	5.15	21.35	5.42
	d	6.78	28.75	5.89	22.91	4.68
4RT	mean	7.64	21.78	5.62	26.22	4.96
	SD	1.82	4.74	0.33	4.88	0.32
	RSD%	23.85	21.75	5.92	18.62	6.43
	SE	0.91	2.37	0.17	2.44	0.16
MSM 1	a	10.3	18.9	4.2	33.6	5.1
	b	11.3	23.8	4.3	32.7	4.9
MSM 2	c	7.15	22.11	4.57	26.68	5.12
	d	7.42	23.78	5.21	36.56	5.08
5RT	mean	9.04	22.14	4.57	32.37	5.05
	SD	2.07	2.31	0.45	4.14	0.12
	RSD%	22.88	10.45	9.92	12.79	2.29
	SE	1.03	1.16	0.23	2.07	0.06
MSM 1	a	10.5	25.9	4.9	28.2	5.5
	b	10.2	26.6	4.7	25.7	4.8
MSM 2	c	8.19	25.68	4.5324	25.42	4.97
	d	9.16	27.86	4.6748	24.69	5.24
6RT	mean	9.51	26.51	4.71	25.99	5.13
	SD	1.05	0.99	0.15	1.52	0.31
	RSD%	11.00	3.74	3.21	5.83	6.00
	SE	0.52	0.50	0.08	0.76	0.15

Table 10-47 Steroid estrogen concentrations (μg kg^{-1} dw) from individual samples of the mixed sludge thermophilic (six retention times) digesters (MST).

	MST	EE2	E2	E3	E1	E1-3S
	a	5.1	12.8	4.6	19.1	2.8
	b	6.5	14.7	4.8	20.0	2.8
1RT	mean	5.79	13.75	4.70	19.55	2.81
	SD	0.98	1.34	0.14	0.57	0.03
	RSD%	16.92	9.72	3.01	2.93	1.13
	SE	0.69	0.94	0.10	0.40	0.02
	a	7.3	14.3	4.6	13.8	2.7
	b	4.7	12.1	4.6	13.8	2.7
2RT	mean	6.01	13.21	4.59	13.79	2.71
	SD	1.82	1.55	0.01	0.05	0.00
	RSD%	30.31	11.75	0.31	0.33	0.13
	SE	1.29	1.10	0.01	0.03	0.00
	a	7.7	19.8	4.6	12.1	2.7
	b	5.7	16.0	4.5	13.6	2.7
3RT	mean	6.70	17.88	4.53	12.84	2.74
	SD	1.39	2.65	0.04	1.07	0.00
	RSD%	20.70	14.81	0.94	8.37	0.13
	SE	0.98	1.87	0.03	0.76	0.00
	a	7.2	21.5	4.6	9.7	2.8
	b	6.5	19.3	4.6	8.3	2.8
4RT	mean	6.82	20.40	4.62	9.01	2.81
	SD	0.50	1.54	0.03	0.97	0.05
	RSD%	7.36	7.56	0.61	10.75	1.76
	SE	0.35	1.09	0.02	0.69	0.03
	a	7.6	21.2	4.6	11.1	2.7
	b	7.3	18.2	4.5	10.7	2.7
5RT	mean	7.44	19.69	4.53	10.93	2.69
	SD	0.22	2.07	0.04	0.30	0.01
	RSD%	2.95	10.51	0.94	2.72	0.39
	SE	0.16	1.46	0.03	0.21	0.01
	a	8.4	21.1	4.6	9.8	2.8
	b	7.9	19.7	4.8	9.7	2.8
6RT	mean	8.14	20.38	4.70	9.74	2.82
	SD	0.37	1.02	0.13	0.04	0.02
	RSD%	4.56	5.00	2.86	0.40	0.88
	SE	0.26	0.72	0.09	0.03	0.02

Table 10-48 Background (feed) concentrations of nonylphenol ethoxylates (μg kg^{-1} dw), (six retention times).

PSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	NP_2EO	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
a	337.22	14.11	10364.37	12.20	2168.27	269.31	119.01	219.35	261.16	269.15	198.58	119.47	55.19	64.53	34.35	26.32
b	384.25	12.59	12469.27	12.00	1138.35	174.43	97.35	492.32	397.64	238.47	134.60	97.61	64.12	62.16	31.35	23.16
c	227.60	14.01	10568.24	12.40	1935.52	202.34	84.27	468.66	275.45	264.49	142.70	107.13	82.18	53.16	42.65	22.15
d	363.71	12.00	89527.51	12.00	2974.81	224.36	141.22	657.97	428.33	283.30	206.23	159.21	57.36	77.15	29.35	24.35
e	209.51	12.00	9643.90	12.00	1530.62	112.26	55.50	157.21	232.26	244.54	144.30	71.92	70.26	51.68	46.48	17.47
mean	**304.46**	**12.94**	**26514.66**	**12.12**	**1949.51**	**196.54**	**99.47**	**399.10**	**318.97**	**259.99**	**165.28**	**111.07**	**65.82**	**61.74**	**36.83**	**22.69**
SD	80.43	1.05	35240.73	0.18	695.12	58.52	32.76	206.98	87.90	18.36	34.20	32.09	10.89	10.25	7.40	3.31
RSD%	26.42	8.11	132.91	1.47	35.66	29.77	32.94	51.86	27.56	7.06	20.69	28.89	16.55	16.60	20.10	14.57
SE	35.97	0.47	15760.13	0.08	310.87	26.17	14.65	92.56	39.31	8.21	15.29	14.35	4.87	4.58	3.31	1.48
MSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	NP_2EO	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
a	385.49	107.40	201264.64	12.00	2259.20	114.79	49.23	22.16	77.16	95.39	52.65	36.61	53.48	42.35	22.42	54.18
b	202.35	39.32	289544.28	12.16	987.14	127.61	87.49	422.15	212.66	110.72	76.65	94.68	43.66	49.39	19.37	52.18
c	24.49	75.64	243165.42	12.12	1956.35	72.50	29.35	175.41	47.42	64.17	114.68	56.17	36.49	36.48	34.27	47.19
d	125.71	62.17	250327.51	12.27	1614.81	174.64	63.49	249.32	167.99	94.18	111.62	46.53	47.15	47.36	27.61	51.62
e	47.51	25.97	222943.90	12.00	1009.62	58.43	28.35	65.68	59.35	76.90	68.69	83.19	31.49	32.32	16.16	56.16
mean	**157.11**	**62.10**	**241449.15**	**12.11**	**1565.43**	**109.59**	**51.58**	**186.94**	**112.91**	**88.27**	**84.86**	**63.43**	**42.45**	**41.58**	**23.97**	**52.27**
SD	145.59	31.86	32985.42	0.12	565.66	46.31	24.85	159.06	73.18	18.03	27.26	24.62	8.67	7.19	7.14	3.35
RSD%	92.67	51.30	13.66	0.95	36.13	42.25	48.18	85.09	64.81	20.42	32.12	38.81	20.43	17.29	29.80	6.42
SE	65.11	14.25	14751.53	0.05	252.97	20.71	11.11	71.13	32.73	8.06	12.19	11.01	3.88	3.22	3.19	1.50
PST	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	NP_2EO	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
a	198.89	12.00	12.00	12.00	15889.40	32.31	20.36	404.64	398.18	232.76	190.39	137.98	79.35	21.19	25.20	10.16
b	145.18	12.00	12.00	12.00	14236.62	117.16	86.37	189.16	306.18	229.66	162.78	157.58	84.72	22.16	20.35	9.19
c	368.35	12.40	12.00	12.00	14987.16	30.36	78.69	348.19	187.69	239.66	157.20	124.53	102.48	34.17	37.15	12.35
d	306.27	12.00	12.00	12.00	14522.16	28.57	38.67	435.64	282.17	246.17	188.84	160.20	82.99	29.31	25.17	14.56
e	142.65	12.27	12.00	12.00	15270.56	96.64	54.73	120.65	285.85	226.17	147.69	118.77	95.69	20.82	34.16	12.16
mean	**232.27**	**12.13**	**12.00**	**12.00**	**14981.18**	**61.01**	**55.77**	**299.66**	**292.01**	**234.88**	**169.38**	**139.81**	**89.05**	**25.53**	**28.41**	**11.68**
SD	100.90	0.19	0.00	0.00	646.86	42.54	27.43	137.95	74.99	8.03	19.25	18.78	9.67	5.94	6.99	2.09
RSD%	43.44	1.55	0.00	0.00	4.32	69.72	49.18	46.04	25.68	3.42	11.37	13.43	10.86	23.29	24.60	17.92
SE	45.12	0.08	0.00	0.00	289.28	19.02	12.27	61.69	33.54	3.59	8.61	8.40	4.33	2.66	3.12	0.94
MST	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	NP_2EO	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
a	50.20	27.12	42.41	12.00	99412.90	34.51	49.64	127.84	102.18	91.50	145.62	52.16	50.17	11.61	5.14	3.18
b	194.20	32.12	45.20	12.00	87469.47	67.16	32.43	168.42	207.16	148.68	96.26	93.22	56.87	14.55	8.17	6.50
c	209.20	14.15	38.35	12.00	102689.47	57.15	48.42	196.15	194.84	126.19	107.19	74.32	52.30	17.20	9.53	4.17
d	77.88	40.10	23.16	12.00	68558.35	30.29	25.78	94.35	96.65	104.67	80.50	47.14	41.20	9.65	5.43	2.68
mean	**132.87**	**28.37**	**37.28**	**12.00**	**89532.55**	**47.28**	**39.07**	**146.69**	**150.21**	**117.76**	**107.39**	**66.71**	**50.13**	**13.25**	**7.07**	**4.13**
SD	80.51	10.89	9.83	0.00	15436.96	17.75	11.83	44.77	58.91	25.09	27.74	21.25	6.58	3.31	2.13	1.69

RSD%	60.60	38.37	26.36	0.00	17.24	37.53	30.28	30.52	39.22	21.30	25.83	31.86	13.13	25.00	30.19	40.99
SE	40.26	5.44	4.91	0.00	7718.48	8.87	5.91	22.39	29.45	12.54	13.87	10.63	3.29	1.66	1.07	0.85

Table 10-49 Nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from individual samples of the primary sludge mesophilic (six retention times) digester 1 (PSM-1).

	PSM 1	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
	a	336.86	24.06	179222.81	27466.31	842.16	132.34	50.43	169.89	171.86	189.94	141.73	88.37	50.66	27.38	12.36	17.81
	b	147.35	12.28	75196.46	3732.81	306.97	24.56	50.94	173.06	136.16	154.75	120.58	86.91	46.43	22.25	23.63	21.36
1RT	mean	242.11	18.17	127209.64	15599.56	574.57	78.45	50.68	171.48	154.01	172.35	131.15	87.64	48.54	24.81	17.99	19.59
	SD	134.01	8.33	73557.74	16782.12	378.43	76.21	0.36	2.24	25.25	24.88	14.95	1.03	2.99	3.63	7.97	2.51
	RSD%	55.35	45.85	57.82	107.58	65.86	97.15	0.72	1.31	16.39	14.44	11.40	1.18	6.15	14.63	44.29	12.81
	SE	94.76	5.89	52013.17	11866.75	267.59	53.89	0.26	1.58	17.85	17.59	10.57	0.73	2.11	2.57	5.63	1.77
	a	122.91	12.29	84906.59	786.63	454.77	24.58	54.69	228.14	205.79	214.00	144.79	95.11	46.90	24.63	11.94	12.38
	b	274.31	12.47	159276.81	498.75	274.31	24.94	43.39	161.64	189.51	203.96	160.50	107.78	60.88	31.95	15.18	15.72
2RT	mean	198.61	12.38	122091.70	642.69	364.54	24.76	49.04	194.89	197.65	208.98	152.65	101.45	53.89	28.29	13.56	14.05
	SD	107.06	0.13	52587.69	203.56	127.60	0.25	7.98	47.02	11.51	7.10	11.10	8.96	9.88	5.17	2.29	2.36
	RSD%	53.90	1.02	43.07	31.67	35.00	1.02	16.28	24.13	5.82	3.40	7.27	8.84	18.33	18.29	16.89	16.83
	SE	75.70	0.09	37185.11	143.94	90.23	0.18	5.65	33.25	8.14	5.02	7.85	6.34	6.99	3.66	1.62	1.67
	a	185.83	12.39	121258.67	1288.40	507.93	24.78	34.41	132.33	119.30	103.44	71.98	45.85	25.02	15.30	9.37	9.39
	b	305.92	12.24	142241.80	281.45	256.98	36.71	39.49	104.82	110.17	84.18	55.34	36.43	17.07	10.85	6.69	2.50
3RT	mean	245.88	12.31	131750.24	784.93	382.45	30.74	36.95	118.58	114.73	93.81	63.66	41.14	21.04	13.08	8.03	5.94
	SD	84.92	0.11	14837.31	712.03	177.45	8.44	3.59	19.46	6.46	13.62	11.77	6.66	5.62	3.15	1.90	4.87
	RSD%	34.54	0.87	11.26	90.71	46.40	27.45	9.72	16.41	5.63	14.52	18.48	16.19	26.72	24.06	23.62	81.94
	SE	60.05	0.08	10491.56	503.48	125.48	5.97	2.54	13.76	4.57	9.63	8.32	4.71	3.98	2.22	1.34	3.44
	a	184.28	12.00	92469.29	12.00	208.85	12.29	17.93	82.53	90.87	71.31	54.31	36.90	18.32	10.36	4.40	2.50
	b	160.02	12.00	81006.89	307.73	73.86	12.31	25.47	101.84	117.17	108.29	84.50	51.64	26.25	16.76	7.58	3.59
4RT	mean	172.15	12.00	86738.09	159.87	141.35	12.30	21.70	92.18	104.02	89.80	69.40	44.27	22.28	13.56	5.99	3.05
	SD	17.15	0.00	8105.14	209.11	95.45	0.02	5.33	13.65	18.60	26.15	21.35	10.42	5.61	4.53	2.25	0.77
	RSD%	9.96	0.00	9.34	130.81	67.53	0.14	24.58	14.81	17.88	29.12	30.76	23.55	25.17	33.41	37.52	25.36
	SE	12.13	0.00	5731.20	147.87	67.49	0.01	3.77	9.65	13.15	18.49	15.10	7.37	3.96	3.20	1.59	0.55
	a	160.09	12.00	79068.84	12.00	160.09	13.34	15.46	69.23	96.57	85.68	65.83	45.44	21.63	14.14	6.13	3.00
	b	189.49	12.00	85560.89	896.92	151.59	12.63	14.23	57.47	65.22	54.68	39.83	25.40	14.24	7.62	3.71	2.50
5RT	mean	174.79	12.00	82314.86	454.46	155.84	12.99	14.85	63.35	80.90	70.18	52.83	35.42	17.94	10.88	4.92	2.75
	SD	20.79	0.00	4590.57	625.73	6.01	0.50	0.87	8.31	22.17	21.92	18.39	14.17	5.22	4.62	1.70	0.35
	RSD%	11.90	0.00	5.58	137.69	3.85	3.85	5.88	13.12	27.40	31.23	34.80	40.00	29.11	42.42	34.64	12.76
	SE	14.70	0.00	3246.03	442.46	4.25	0.35	0.62	5.88	15.67	15.50	13.00	10.02	3.69	3.26	1.21	0.25
	a	148.29	12.00	85059.32	877.41	234.80	12.36	14.70	73.80	94.47	82.17	61.78	42.72	19.38	12.11	4.28	2.74
	b	164.31	12.00	88056.12	1036.40	252.78	12.64	17.48	127.79	218.45	215.32	185.50	131.25	64.94	34.96	15.04	23.70

6RT	mean	156.30	12.00	86557.72	956.91	243.79	12.50	16.09	100.79	156.46	148.75	123.64	86.98	42.16	23.53	9.66	13.22
	SD	11.32	0.00	2119.06	112.42	12.71	0.20	1.96	38.18	87.67	94.15	87.49	62.60	32.21	16.16	7.61	14.82
	RSD%	7.24	0.00	2.45	11.75	5.22	1.59	12.18	37.88	56.03	63.29	70.76	71.97	76.41	68.67	78.79	112.12
	SE	8.01	0.00	1498.40	79.50	8.99	0.14	1.39	27.00	61.99	66.57	61.86	44.27	22.78	11.43	5.38	10.48

Table 10-50 Nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from individual samples of the primary sludge mesophilic (six retention times) digester 2 (PSM-2).

	PSM 2	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
	a	562.58	44.50	145321.47	10251.44	520.12	107.43	84.20	207.45	233.51	207.89	185.67	107.59	101.02	35.17	21.72	24.11
	b	97.12	62.84	195761.01	7434.51	246.88	216.78	107.89	106.78	285.20	243.78	248.79	158.92	89.23	10.14	12.44	18.64
1RT	mean	329.85	53.67	170541.24	8842.98	383.50	162.11	96.05	157.12	259.36	225.84	217.23	133.26	95.13	22.66	17.08	21.38
	SD	329.13	12.97	35666.14	1991.87	193.21	77.32	16.75	71.18	36.55	25.38	44.63	36.30	8.34	17.70	6.56	3.87
	RSD%	99.78	24.16	20.91	22.52	50.38	47.70	17.44	45.31	14.09	11.24	20.55	27.24	8.76	78.12	38.42	18.10
	SE	232.73	9.17	25219.77	1408.47	136.62	54.67	11.85	50.33	25.84	17.95	31.56	25.67	5.90	12.52	4.64	2.74
	a	348.44	33.04	153847.56	9598.27	517.85	84.66	80.54	142.27	145.32	178.45	104.58	82.45	87.25	21.14	15.45	16.76
	b	212.66	48.76	165289.56	6582.14	128.75	141.34	68.78	87.52	124.89	121.41	107.41	119.40	71.44	10.11	12.08	7.47
2RT	mean	280.55	40.90	159568.56	8090.21	323.30	113.00	74.66	114.90	135.11	149.93	106.00	100.93	79.35	15.63	13.77	12.12
	SD	96.01	11.12	8090.72	2132.73	275.14	40.08	8.32	38.71	14.45	40.33	2.00	26.13	11.18	7.80	2.38	6.57
	RSD%	34.22	27.18	5.07	26.36	85.10	35.47	11.14	33.70	10.69	26.90	1.89	25.89	14.09	49.92	17.31	54.22
	SE	67.89	7.86	5721.00	1508.07	194.55	28.34	5.88	27.38	10.22	28.52	1.41	18.48	7.90	5.52	1.68	4.65
	a	265.35	28.53	156324.56	6543.23	458.45	68.75	51.36	112.68	109.54	152.04	84.21	54.78	64.49	12.24	10.60	2.50
	b	168.25	29.41	133568.74	5423.28	321.87	107.45	23.16	54.78	89.55	112.53	61.08	86.11	54.96	6.21	2.10	5.42
3RT	mean	216.80	28.97	144946.65	5983.26	390.16	88.10	37.26	83.73	99.55	132.29	72.65	70.45	59.73	9.23	6.35	3.96
	SD	68.66	0.62	16090.79	791.92	96.58	27.37	19.94	40.94	14.14	27.94	16.36	22.15	6.74	4.26	6.01	2.06
	RSD%	31.67	2.15	11.10	13.24	24.75	31.06	53.52	48.90	14.20	21.12	22.51	31.45	11.28	46.22	94.65	52.14
	SE	48.55	0.44	11377.91	559.98	68.29	19.35	14.10	28.95	10.00	19.76	11.57	15.67	4.77	3.02	4.25	1.46
	a	207.81	15.62	108456.56	5485.12	397.24	42.15	23.88	62.34	68.75	69.41	63.44	42.12	22.40	7.06	2.50	2.50
	b	131.25	14.38	65314.61	3597.89	268.45	65.48	12.35	48.56	36.81	94.68	44.72	64.23	32.12	4.00	0.50	2.50
4RT	mean	169.53	15.00	86885.59	4541.51	332.85	53.82	18.12	55.45	52.78	82.05	54.08	53.18	27.26	5.53	1.50	2.50
	SD	54.14	0.88	30505.97	1334.47	91.07	16.50	8.15	9.74	22.58	17.87	13.24	15.63	6.87	2.16	1.41	0.00
	RSD%	31.93	5.85	35.11	29.38	27.36	30.65	45.01	17.57	42.79	21.78	24.48	29.40	25.21	39.13	94.28	0.00
	SE	38.28	0.62	21570.98	943.62	64.39	11.67	5.77	6.89	15.97	12.64	9.36	11.06	4.86	1.53	1.00	0.00
	a	172.35	12.60	84562.36	3265.48	229.65	18.24	17.32	28.12	52.14	89.53	34.53	37.14	12.07	4.00	0.50	2.50
	b	114.35	12.68	46471.26	1989.56	178.24	44.68	9.36	34.80	22.70	67.12	16.30	19.45	10.22	4.00	0.50	2.50
5RT	mean	143.35	12.64	65516.81	2627.52	203.95	31.46	13.34	31.46	37.42	78.33	25.42	28.30	11.15	4.00	0.50	2.50
	SD	41.01	0.06	26934.47	902.21	36.35	18.70	5.63	4.72	20.82	15.85	12.89	12.51	1.31	0.00	0.00	0.00
	RSD%	28.61	0.45	41.11	34.34	17.82	59.43	42.19	15.01	55.63	20.23	50.72	44.21	11.74	0.00	0.00	0.00
	SE	29.00	0.04	19045.55	637.96	25.71	13.22	3.98	3.34	14.72	11.21	9.12	8.84	0.93	0.00	0.00	0.00

	a	164.52	12.00	63524.19	1062.53	124.53	12.00	12.19	12.45	38.47	63.28	30.16	16.45	10.00	6.24	0.50	2.50
	b	198.53	12.00	104345.71	875.66	168.74	21.54	6.52	22.04	10.62	28.75	5.21	11.19	32.30	4.00	0.50	2.50
6RT	mean	181.53	12.00	83934.95	969.10	146.64	16.77	9.36	17.25	24.55	46.02	17.69	13.82	21.15	5.12	0.50	2.50
	SD	24.05	0.00	28865.17	132.14	31.26	6.75	4.01	6.78	19.69	24.42	17.64	3.72	15.77	1.58	0.00	0.00
	RSD%	13.25	0.00	34.39	13.64	21.32	40.23	42.86	39.32	80.23	53.06	99.76	26.91	74.56	30.94	0.00	0.00
	SE	17.01	0.00	20410.76	93.43	22.11	4.77	2.84	4.80	13.93	17.27	12.48	2.63	11.15	1.12	0.00	0.00

Table 10-51 Mean nonylphenol ethoxylate concentrations (μg kg^{-1} dw) in primary sludge mesophilic (six retention times) digesters (PSM).

	PSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
1RT	mean	285.98	35.92	148875.44	12221.27	479.03	120.28	73.36	164.30	206.68	199.09	174.19	110.45	71.83	23.73	17.54	20.48
	SD	62.05	25.10	30640.07	4777.63	135.10	59.15	32.08	10.15	74.49	37.82	60.87	32.26	32.94	1.53	0.64	1.26
	RSD%	21.70	69.88	20.58	39.09	28.20	49.18	43.72	6.18	36.04	19.00	34.94	29.21	45.85	6.43	3.67	6.17
	SE	43.87	17.75	21665.80	3378.29	95.53	41.83	22.68	7.18	52.67	26.74	43.04	22.81	23.29	1.08	0.46	0.89
2RT	mean	239.58	26.64	140830.13	4366.45	343.92	68.88	61.85	154.89	166.38	179.46	129.32	101.19	66.62	21.96	13.66	13.08
	SD	57.94	20.17	26500.14	5266.19	29.16	62.40	18.12	56.56	44.23	41.76	32.99	0.37	18.00	8.96	0.15	1.37
	RSD%	24.18	75.70	18.82	120.61	8.48	90.59	29.29	36.52	26.58	23.27	25.51	0.36	27.02	40.79	1.06	10.45
	SE	40.97	14.26	18738.43	3723.76	20.62	44.12	12.81	40.00	31.27	29.53	23.33	0.26	12.73	6.33	0.10	0.97
3RT	mean	231.34	20.64	138348.44	3384.09	386.31	59.42	37.10	101.15	107.14	113.05	68.15	55.79	40.38	11.15	7.19	4.95
	SD	20.56	11.78	9331.27	3675.77	5.45	40.56	0.22	24.64	10.74	27.21	6.36	20.72	27.35	2.72	1.19	1.40
	RSD%	8.89	57.06	6.74	108.62	1.41	68.25	0.60	24.36	10.02	24.07	9.33	37.14	67.73	24.42	16.49	28.33
	SE	14.54	8.33	6598.21	2599.16	3.85	28.68	0.16	17.42	7.59	19.24	4.49	14.65	19.34	1.93	0.84	0.99
4RT	mean	170.84	13.50	86811.84	2350.69	237.10	33.06	19.91	73.82	78.40	85.92	61.74	48.72	24.77	9.55	3.74	2.77
	SD	1.85	2.12	104.29	3098.29	135.41	29.36	2.53	25.97	36.23	5.48	10.83	6.30	3.52	5.68	3.17	0.39
	RSD%	1.08	15.71	0.12	131.80	57.11	88.81	12.73	35.19	46.22	6.38	17.55	12.93	14.21	59.49	84.76	13.93
	SE	1.31	1.50	73.75	2190.82	95.75	20.76	1.79	18.37	25.62	3.88	7.66	4.45	2.49	4.02	2.24	0.27
5RT	mean	159.07	12.32	73915.84	1540.99	179.89	22.22	14.09	47.41	59.16	74.25	39.12	31.86	14.54	7.44	2.71	2.62
	SD	22.23	0.45	11878.02	1536.59	34.02	13.06	1.07	22.55	30.74	5.76	19.39	5.04	4.80	4.86	3.13	0.18
	RSD%	13.97	3.67	16.07	99.71	18.91	58.78	7.56	47.57	51.97	7.75	49.55	15.81	33.03	65.39	115.33	6.68
	SE	15.72	0.32	8399.03	1086.53	24.05	9.24	0.75	15.95	21.74	4.07	13.71	3.56	3.40	3.44	2.21	0.12
6RT	mean	168.91	12.00	85246.33	963.00	195.21	14.63	12.72	59.02	90.50	97.38	70.66	50.40	31.65	14.33	5.08	7.86
	SD	17.84	0.00	1854.58	8.62	68.70	3.02	4.76	59.08	93.28	72.64	74.92	51.73	14.86	13.02	6.47	7.58
	RSD%	10.56	0.00	2.18	0.90	35.19	20.64	37.43	100.10	103.07	74.60	106.03	102.64	46.93	90.88	127.50	96.44
	SE	12.61	0.00	1311.38	6.09	48.58	2.14	3.37	41.77	65.96	51.37	52.98	36.58	10.50	9.21	4.58	5.36

Table 10-52 Nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from individual samples of the mixed sludge mesophilic (six retention times) digester 1 (MSM-1).

	MSM1	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
	a	86.81	12.40	86483.13	1302.08	235.62	24.80	44.03	189.69	185.16	214.89	154.14	95.15	53.74	26.64	11.27	15.05
	b	123.64	12.36	172910.48	6058.36	247.28	24.73	32.41	117.19	99.59	112.70	138.18	52.60	32.23	15.99	9.38	6.55
1RT	mean	105.22	12.38	129696.81	3680.22	241.45	24.76	38.22	153.44	142.37	163.80	146.16	73.87	42.99	21.32	10.33	10.80
	SD	26.05	0.03	61113.37	3363.19	8.25	0.05	8.22	51.26	60.51	72.26	11.29	30.09	15.21	7.53	1.33	6.02
	RSD%	24.75	0.21	47.12	91.39	3.42	0.21	21.50	33.41	42.50	44.11	7.72	40.73	35.37	35.32	12.91	55.71
	SE	18.42	0.02	43213.67	2378.14	5.83	0.04	5.81	36.25	42.79	51.09	7.98	21.28	10.75	5.32	0.94	4.25
	a	49.50	12.00	51930.69	569.31	173.27	12.38	22.45	66.88	43.83	56.29	37.38	26.55	16.05	10.00	4.50	5.50
	b	111.33	12.37	137085.60	3067.79	185.55	24.74	25.04	76.73	66.33	75.24	48.82	36.56	19.78	10.95	5.79	5.50
2RT	mean	80.42	12.19	94508.15	1818.55	179.41	18.56	23.75	71.81	55.08	65.76	43.10	31.55	17.91	10.47	5.15	5.50
	SD	43.72	0.26	60213.61	1766.69	8.69	8.74	1.83	6.96	15.91	13.40	8.10	7.08	2.64	0.67	0.92	0.00
	RSD%	54.36	2.15	63.71	97.15	4.84	47.11	7.70	9.70	28.89	20.37	18.78	22.43	14.73	6.40	17.78	0.00
	SE	30.91	0.19	42577.45	1249.24	6.14	6.18	1.29	4.92	11.25	9.47	5.72	5.00	1.87	0.47	0.65	0.00
	a	48.31	12.00	19951.69	1280.19	157.00	12.08	15.51	38.58	23.54	30.60	3.82	12.29	7.36	10.00	4.50	5.50
	b	60.98	12.00	32060.98	1353.66	170.73	24.39	21.53	58.36	39.02	45.68	21.18	22.04	11.75	10.00	4.50	5.50
3RT	mean	54.64	12.00	26006.33	1316.93	163.87	18.23	18.52	48.47	31.28	38.14	12.50	17.17	9.56	10.00	4.50	5.50
	SD	8.96	0.00	8562.56	51.95	9.71	8.71	4.26	13.99	10.95	10.66	12.28	6.89	3.10	0.00	0.00	0.00
	RSD%	16.39	0.00	32.92	3.94	5.92	47.75	22.98	28.86	35.00	27.95	98.22	40.15	32.47	0.00	0.00	0.00
	SE	6.33	0.00	6054.64	36.73	6.86	6.16	3.01	9.89	7.74	7.54	8.68	4.87	2.19	0.00	0.00	0.00
	a	37.07	12.00	26124.57	679.68	135.94	12.36	9.58	36.87	31.64	36.98	29.21	18.43	11.59	10.00	4.50	5.50
	b	60.86	12.00	43147.52	2823.76	109.54	24.34	24.36	72.98	65.75	57.31	42.70	25.46	14.60	10.00	4.50	5.50
4RT	mean	48.97	12.00	34636.04	1751.72	122.74	18.35	16.97	54.92	48.70	47.14	35.96	21.94	13.09	10.00	4.50	5.50
	SD	16.82	0.00	12037.04	1516.09	18.66	8.47	10.45	25.54	24.12	14.38	9.53	4.97	2.13	0.00	0.00	0.00
	RSD%	34.35	0.00	34.75	86.55	15.21	46.18	61.57	46.50	49.53	30.49	26.51	22.67	16.25	0.00	0.00	0.00
	SE	11.89	0.00	8511.47	1072.04	13.20	5.99	7.39	18.06	17.05	10.16	6.74	3.52	1.50	0.00	0.00	0.00
	a	90.77	12.00	65300.83	2321.06	168.57	25.93	24.76	62.45	42.86	42.27	30.63	21.87	12.12	10.00	4.50	5.50
	b	60.68	12.00	54308.25	279.13	109.22	12.14	17.39	41.08	33.53	25.71	16.08	11.75	11.00	10.00	4.50	5.50
5RT	mean	75.72	12.00	59804.54	1300.09	138.90	19.03	21.08	51.76	38.19	33.99	23.36	16.81	11.56	10.00	4.50	5.50
	SD	21.28	0.00	7772.93	1443.86	41.96	9.76	5.21	15.11	6.59	11.71	10.28	7.15	0.79	0.00	0.00	0.00
	RSD%	28.10	0.00	13.00	111.06	30.21	51.26	24.73	29.19	17.26	34.45	44.03	42.55	6.84	0.00	0.00	0.00
	SE	15.04	0.00	5496.29	1020.97	29.67	6.90	3.69	10.69	4.66	8.28	7.27	5.06	0.56	0.00	0.00	0.00
	a	89.97	12.00	132107.97	2802.06	167.10	25.71	27.07	61.81	44.88	37.73	25.26	18.80	10.00	10.00	4.50	5.50
	b	75.45	12.00	66788.23	1559.36	88.03	25.15	28.56	117.36	170.24	151.56	119.56	75.42	37.04	14.85	7.19	6.44
6RT	mean	82.71	12.00	99448.10	2180.71	127.56	25.43	27.81	89.58	107.56	94.65	72.41	47.11	23.52	12.43	5.85	5.97
	SD	10.27	0.00	46188.03	878.72	55.91	0.39	1.05	39.28	88.64	80.49	66.68	40.03	19.12	3.43	1.90	0.67
	RSD%	12.41	0.00	46.44	40.30	43.83	1.55	3.78	43.84	82.41	85.05	92.08	84.99	81.28	27.62	32.55	11.17

	SE	7.26	0.00	32659.87	621.35	39.53	0.28	0.74	27.77	62.68	56.92	47.15	28.31	13.52	2.43	1.35	0.47

Table 10-53 Nonylphenol ethoxylate concentrations (µg kg^{-1} dw) from individual samples of the mixed sludge mesophilic (six retention times) digester 2 (MSM-2).

	MSM2	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
	a	64.68	32.62	65229.95	1182.17	1052.48	69.16	35.11	168.50	98.17	66.18	64.95	52.37	26.98	34.94	11.68	42.20
	b	75.35	18.20	129341.62	2214.42	719.50	107.94	46.39	142.39	76.32	78.16	51.38	62.19	37.17	21.28	15.32	59.13
1RT	mean	70.02	25.41	97285.78	1698.29	885.99	88.55	40.75	155.44	87.24	72.17	58.17	57.28	32.08	28.11	13.50	50.66
	SD	7.54	10.20	45333.80	729.91	235.46	27.42	7.97	18.46	15.45	8.47	9.59	6.94	7.20	9.66	2.57	11.97
	RSD%	10.77	40.13	46.60	42.98	26.58	30.96	19.56	11.88	17.71	11.74	16.50	12.12	22.46	34.37	19.05	23.63
	SE	5.33	7.21	32055.83	516.13	166.49	19.39	5.64	13.06	10.93	5.99	6.78	4.91	5.09	6.83	1.82	8.47
	a	57.32	17.62	62127.18	943.18	875.20	79.16	22.69	142.17	57.17	41.12	49.17	46.05	20.39	20.65	9.17	23.87
	b	93.20	12.99	26189.32	1185.61	529.19	67.62	31.06	138.15	42.29	51.62	36.22	51.20	22.17	16.38	11.22	42.78
2RT	mean	75.26	15.30	44158.25	1064.40	702.19	73.39	26.88	140.16	49.73	46.37	42.69	48.63	21.28	18.51	10.19	33.33
	SD	25.37	3.28	25411.91	171.42	244.67	8.17	5.92	2.84	10.52	7.42	9.16	3.64	1.26	3.02	1.45	13.37
	RSD%	33.71	21.40	57.55	16.11	34.84	11.13	22.03	2.02	21.16	16.01	21.45	7.49	5.90	16.31	14.21	40.12
	SE	17.94	2.32	17968.93	121.22	173.01	5.77	4.19	2.01	7.44	5.25	6.48	2.57	0.89	2.14	1.02	9.45
	a	53.35	12.00	34094.17	567.18	548.61	35.33	14.68	120.13	71.28	26.93	31.68	31.06	16.68	14.17	5.19	17.25
	b	76.16	12.00	84159.67	789.17	389.16	47.62	26.18	79.17	34.16	22.15	21.02	42.17	19.16	12.34	7.32	24.17
3RT	mean	64.75	12.00	59126.92	678.18	468.89	41.47	20.43	99.65	52.72	24.54	26.35	36.61	17.92	13.25	6.25	20.71
	SD	16.13	0.00	35401.66	156.97	112.75	8.69	8.13	28.97	26.25	3.38	7.54	7.86	1.75	1.29	1.51	4.90
	RSD%	24.91	0.00	59.87	23.15	24.05	20.96	39.79	29.07	49.79	13.76	28.62	21.46	9.78	9.75	24.09	23.64
	SE	11.40	0.00	25032.75	110.99	79.73	6.15	5.75	20.48	18.56	2.39	5.33	5.56	1.24	0.91	1.06	3.46
	a	51.95	12.00	42189.36	414.17	307.19	42.17	12.10	79.16	45.32	18.35	20.17	20.62	11.68	9.65	2.19	6.19
	b	46.87	12.00	127492.74	527.20	307.65	34.17	22.79	42.61	20.17	19.25	17.15	36.19	16.14	10.61	4.12	11.02
4RT	mean	49.41	12.00	84841.05	470.68	307.42	38.17	17.45	60.89	32.74	18.80	18.66	28.40	13.91	10.13	3.16	8.61
	SD	3.59	0.00	60318.60	79.92	0.33	5.65	7.56	25.84	17.78	0.64	2.14	11.01	3.15	0.68	1.37	3.41
	RSD%	7.27	0.00	71.10	16.98	0.11	14.81	43.35	42.44	54.31	3.39	11.44	38.76	22.64	6.70	43.30	39.63
	SE	2.54	0.00	42651.69	56.52	0.23	4.00	5.35	18.27	12.57	0.45	1.51	7.79	2.23	0.48	0.97	2.41
	a	62.50	12.00	52614.68	378.20	243.69	45.20	10.98	52.64	39.35	16.43	12.92	28.94	11.23	6.16	1.75	5.24
	b	62.16	12.00	76497.69	489.98	258.16	39.19	20.19	31.62	14.29	24.62	26.15	26.20	12.15	7.62	3.80	5.32
5RT	mean	62.33	12.00	64556.19	434.09	250.92	42.19	15.59	42.13	26.82	20.52	19.53	27.57	11.69	6.89	2.77	5.28
	SD	0.24	0.00	16887.84	79.05	10.24	4.25	6.51	14.87	17.72	5.79	9.36	1.94	0.66	1.03	1.44	0.06
	RSD%	0.38	0.00	26.16	18.21	4.08	10.08	41.76	35.28	66.08	28.23	47.91	7.03	5.62	14.94	52.10	1.10
	SE	0.17	0.00	11941.51	55.89	7.24	3.01	4.60	10.51	12.53	4.10	6.62	1.37	0.46	0.73	1.02	0.04
	a	60.89	12.00	66183.74	289.36	207.20	35.20	12.68	38.17	27.17	19.38	14.12	31.09	11.69	5.17	2.68	3.94
	b	84.19	12.00	145178.20	504.38	179.24	31.97	17.35	24.36	12.04	38.16	39.65	20.98	12.70	6.19	1.75	4.12
6RT	mean	72.54	12.00	105680.97	396.87	193.22	33.58	15.02	31.26	19.61	28.77	26.89	26.04	12.19	5.68	2.22	4.03

	SD	16.48	0.00	55857.52	152.04	19.77	2.28	3.30	9.76	10.70	13.28	18.05	7.14	0.71	0.72	0.66	0.13
	RSD%	22.71	0.00	52.85	38.31	10.23	6.79	21.96	31.23	54.56	46.16	67.13	27.44	5.85	12.71	29.74	3.11
	SE	11.65	0.00	39497.23	107.51	13.98	1.61	2.33	6.90	7.56	9.39	12.76	5.05	0.50	0.51	0.47	0.09

Table 10-54 Mean nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from the mixed sludge mesophilic (six retention times) digesters (MSM).

	MSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
1RT	mean	87.62	18.90	113491.30	2689.26	563.72	56.66	39.49	154.44	114.81	117.99	102.16	65.58	37.53	24.71	11.91	30.73
	SD	24.90	9.21	22918.06	1401.43	455.76	45.11	1.79	1.42	38.98	64.79	62.22	11.73	7.72	4.80	2.24	28.19
	RSD%	28.41	48.74	20.19	52.11	80.85	79.61	4.52	0.92	33.96	54.91	60.90	17.89	20.56	19.43	18.83	91.72
	SE	17.60	6.51	16205.51	990.96	322.27	31.89	1.26	1.00	27.57	45.81	44.00	8.30	5.46	3.40	1.59	19.93
2RT	mean	77.84	13.74	69333.20	1441.47	440.80	45.97	25.31	105.98	52.40	56.07	42.90	40.09	19.60	14.49	7.67	19.41
	SD	3.65	2.21	35602.75	533.26	369.66	38.77	2.21	48.33	3.78	13.72	0.29	12.07	2.38	5.68	3.57	19.68
	RSD%	4.69	16.04	51.35	36.99	83.86	84.33	8.74	45.61	7.22	24.46	0.67	30.11	12.15	39.22	46.51	101.36
	SE	2.58	1.56	25174.95	377.07	261.39	27.42	1.56	34.18	2.68	9.70	0.20	8.54	1.68	4.02	2.52	13.91
3RT	mean	59.70	12.00	42566.63	997.55	316.38	29.85	19.48	74.06	42.00	31.34	19.43	26.89	13.74	11.63	5.38	13.10
	SD	7.15	0.00	23419.79	451.66	215.68	16.43	1.35	36.19	15.16	9.62	9.79	13.75	5.92	2.30	1.24	10.75
	RSD%	11.98	0.00	55.02	45.28	68.17	55.04	6.95	48.87	36.11	30.68	50.40	51.13	43.07	19.79	23.04	82.06
	SE	5.06	0.00	16560.29	319.37	152.51	11.62	0.96	25.59	10.72	6.80	6.92	9.72	4.18	1.63	0.88	7.60
4RT	mean	49.19	12.00	59738.54	1111.20	215.08	28.26	17.21	57.91	40.72	32.97	27.31	25.17	13.50	10.07	3.83	7.05
	SD	0.31	0.00	35500.30	905.83	130.59	14.02	0.34	4.22	11.28	20.04	12.23	4.57	0.58	0.10	0.95	2.20
	RSD%	0.64	0.00	59.43	81.52	60.72	49.59	1.96	7.28	27.71	60.80	44.79	18.15	4.29	0.94	24.83	31.14
	SE	0.22	0.00	25102.50	640.52	92.34	9.91	0.24	2.98	7.98	14.17	8.65	3.23	0.41	0.07	0.67	1.55
5RT	mean	69.03	12.00	62180.37	867.09	194.91	30.61	18.33	46.95	32.51	27.26	21.44	22.19	11.62	8.44	3.64	5.39
	SD	9.47	0.00	3359.92	612.36	79.22	16.38	3.88	6.81	8.04	9.52	2.70	7.61	0.09	2.20	1.22	0.16
	RSD%	13.72	0.00	5.40	70.62	40.64	53.49	21.18	14.50	24.75	34.94	12.61	34.28	0.80	26.06	33.56	2.91
	SE	6.70	0.00	2375.82	433.00	56.01	11.58	2.75	4.82	5.69	6.73	1.91	5.38	0.07	1.56	0.86	0.11
6RT	mean	77.63	12.00	102564.53	1288.79	160.39	29.51	21.42	60.42	63.58	61.71	49.65	36.57	17.86	9.05	4.03	5.00
	SD	7.19	0.00	4407.30	1261.36	46.43	5.77	9.05	41.24	62.19	46.58	32.19	14.90	8.01	4.77	2.57	1.37
	RSD%	9.26	0.00	4.30	97.87	28.95	19.54	42.26	68.25	97.82	75.48	64.84	40.74	44.85	52.71	63.62	27.43
	SE	5.09	0.00	3116.44	891.92	32.83	4.08	6.40	29.16	43.98	32.94	22.76	10.54	5.66	3.37	1.81	0.97

Table 10-55 Mean nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from the primary sludge thermophilic (six retention times) digester (PST).

	PST	NP	NP_1EC	NP_2EC	NP_3EC	NP_1EO	NP_2EO	NP_3EO	NP_4EO	NP_5EO	NP_6EO	NP_7EO	NP_8EO	NP_9EO	$NP_{10}EO$	$NP_{11}EO$	$NP_{12}EO$
	a	202.6	12.0	92.4	12.0	13669.6	142.4	50.4	163.5	161.3	178.4	104.4	50.9	27.3	11.0	16.5	11.1
	b	193.2	13.6	600.6	13.4	15291.8	110.7	33.3	267.6	197.2	198.4	120.2	105.6	60.7	19.3	16.3	10.5
1RT	mean	197.88	12.78	346.50	12.68	14480.67	126.52	41.84	215.52	179.24	188.42	112.29	78.24	43.98	15.16	16.38	10.82
	SD	6.63	1.10	359.37	0.96	1147.08	22.42	12.12	73.60	25.35	14.12	11.18	38.72	23.59	5.85	0.12	0.46
	RSD%	3.35	8.61	103.71	7.57	7.92	17.72	28.97	34.15	14.14	7.49	9.96	49.49	53.64	38.56	0.73	4.24
	SE	4.69	0.78	254.11	0.68	811.11	15.85	8.57	52.04	17.93	9.99	7.91	27.38	16.68	4.13	0.08	0.32
	a	181.3	12.0	4329.7	12.0	12396.3	110.6	4.7	97.1	20.2	98.1	26.6	19.0	18.4	33.2	34.5	10.8
	b	160.6	15.7	919.8	12.2	15515.8	244.9	34.4	224.7	144.8	157.6	96.4	102.2	48.7	14.3	15.6	6.7
2RT	mean	170.94	13.83	2624.75	12.10	13956.02	177.78	19.59	160.90	82.51	127.83	61.52	60.57	33.55	23.74	25.06	8.74
	SD	14.59	2.58	2411.21	0.14	2205.84	94.99	20.99	90.23	88.13	42.11	49.33	58.81	21.39	13.41	13.42	2.95
	RSD%	8.53	18.69	91.86	1.12	15.81	53.43	107.14	56.08	106.81	32.94	80.19	97.10	63.75	56.47	53.55	33.73
	SE	10.32	1.83	1704.98	0.10	1559.76	67.17	14.84	63.80	62.32	29.78	34.88	41.59	15.12	9.48	9.49	2.09
	a	132.9	12.0	5008.1	12.0	10281.4	245.6	13.0	354.0	54.1	218.1	64.9	28.4	35.2	34.4	23.6	10.0
	b	126.2	12.5	10519.0	12.0	10480.9	599.2	40.1	181.6	90.1	109.9	66.8	54.9	37.4	9.2	13.3	5.5
3RT	mean	129.56	12.24	7763.55	12.00	10381.15	422.39	26.53	267.79	72.11	164.01	65.81	41.64	36.31	21.79	18.44	7.79
	SD	4.71	0.34	3896.77	0.00	141.09	250.00	19.14	121.87	25.48	76.53	1.36	18.73	1.57	17.87	7.28	3.19
	RSD%	3.64	2.74	50.19	0.00	1.36	59.19	72.13	45.51	35.34	46.66	2.06	44.97	4.32	82.02	39.46	40.88
	SE	3.33	0.24	2755.43	0.00	99.77	176.78	13.53	86.17	18.02	54.11	0.96	13.24	1.11	12.63	5.14	2.25
	a	121.0	12.0	7663.1	12.0	6244.3	606.3	37.2	434.9	50.0	191.4	79.5	37.5	37.0	18.0	7.7	6.4
	b	122.0	12.0	10764.4	12.0	4958.8	1159.4	41.5	141.4	62.5	67.8	58.6	32.0	32.4	12.7	9.3	3.7
4RT	mean	121.51	12.00	9213.76	12.00	5601.55	882.86	39.38	288.16	56.25	129.59	69.05	34.72	34.68	15.31	8.49	5.00
	SD	0.77	0.00	2192.94	0.00	908.92	391.10	3.07	207.56	8.89	87.40	14.73	3.91	3.23	3.74	1.09	1.91
	RSD%	0.63	0.00	23.80	0.00	16.23	44.30	7.79	72.03	15.80	67.44	21.32	11.26	9.33	24.43	12.89	38.15
	SE	0.54	0.00	1550.65	0.00	642.70	276.55	2.17	146.77	6.29	61.80	10.41	2.76	2.29	2.64	0.77	1.35
	a	113.6	12.0	11398.3	12.0	2107.3	859.6	51.3	116.1	35.9	114.4	15.4	20.3	31.2	16.9	13.0	4.2
	b	98.5	12.0	14894.6	12.0	1584.4	1218.0	42.3	167.3	35.7	50.9	36.6	22.0	22.6	8.3	6.2	2.8
5RT	mean	106.05	12.00	13146.43	12.00	1845.84	1038.77	46.76	141.74	35.81	82.64	26.00	21.14	26.88	12.61	9.64	3.52
	SD	10.61	0.00	2472.22	0.00	369.79	253.44	6.36	36.20	0.09	44.90	14.93	1.24	6.11	6.11	4.81	0.96
	RSD%	10.00	0.00	18.81	0.00	20.03	24.40	13.61	25.54	0.26	54.33	57.41	5.84	22.72	48.47	49.89	27.35
	SE	7.50	0.00	1748.12	0.00	261.48	179.21	4.50	25.60	0.07	31.75	10.56	0.87	4.32	4.32	3.40	0.68
	a	98.8	12.0	14557.4	12.0	490.9	198.5	36.4	162.5	29.0	90.9	30.0	25.5	35.4	18.2	21.2	2.5
	b	102.1	12.0	14913.9	12.3	672.1	1296.3	38.2	164.1	20.5	40.4	29.8	15.6	16.7	6.0	3.8	2.5
6RT	mean	100.45	12.00	14735.65	12.14	581.51	747.38	37.29	163.32	24.78	65.62	29.91	20.51	26.03	12.09	12.48	2.48
	SD	2.39	0.00	252.06	0.20	128.10	776.31	1.24	1.14	6.01	35.71	0.18	6.99	13.26	8.62	12.31	0.04
	RSD%	2.38	0.00	1.71	1.65	22.03	103.87	3.33	0.70	24.25	54.42	0.60	34.10	50.94	71.30	98.63	1.43

	SE	1.69	0.00	178.23	0.14	90.58	548.93	0.88	0.81	4.25	25.25	0.13	4.95	9.38	6.09	8.70	0.02

Table 10-56 Nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from individual samples of the mixed sludge thermophilic (six retention times) digester (MST).

	MST	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
	a	181.6	12.0	26.8	12.0	13979.1	63.6	13.7	67.3	114.2	110.6	82.1	48.9	62.6	14.6	10.4	2.5
	b	157.1	12.0	39.2	12.0	16446.4	64.6	27.2	161.7	113.5	99.3	75.2	61.7	40.6	12.3	5.8	2.5
1RT	mean	169.34	12.00	32.99	12.00	15212.76	64.11	20.44	114.52	113.89	104.91	78.64	55.27	51.61	13.44	8.12	2.50
	SD	17.29	0.00	8.74	0.00	1744.68	0.65	9.51	66.71	0.48	7.99	4.92	9.04	15.52	1.64	3.22	0.00
	RSD%	10.21	0.00	26.50	0.00	11.47	1.02	46.52	58.25	0.42	7.62	6.26	16.36	30.08	12.18	39.68	0.16
	SE	12.23	0.00	6.18	0.00	1233.68	0.46	6.72	47.17	0.34	5.65	3.48	6.39	10.98	1.16	2.28	0.00
	a	253.8	12.0	23.6	12.0	11775.6	132.3	8.2	76.8	10.0	46.5	22.4	8.9	11.0	14.6	0.5	2.5
	b	271.0	12.0	40.6	12.0	11043.2	107.2	23.0	202.0	88.7	86.5	55.7	47.3	22.1	10.6	5.7	3.1
2RT	mean	262.44	12.00	32.13	12.00	11409.42	119.76	15.61	139.37	49.34	66.54	39.02	28.09	16.55	12.60	3.11	2.78
	SD	12.15	0.00	12.01	0.00	517.84	17.70	10.46	88.52	55.63	28.28	23.54	27.16	7.85	2.78	3.70	0.39
	RSD%	4.63	0.00	37.36	0.00	4.54	14.78	67.01	63.52	112.76	42.50	60.32	96.66	47.44	22.06	118.71	14.15
	SE	8.59	0.00	8.49	0.00	366.17	12.51	7.40	62.60	39.34	20.00	16.64	19.20	5.55	1.97	2.61	0.28
	a	257.6	12.0	30.1	12.0	10138.4	621.3	2.7	153.2	25.1	76.5	28.0	13.2	14.3	14.1	0.5	2.5
	b	328.9	12.0	42.1	12.0	10092.3	357.1	18.4	182.1	62.1	71.8	38.3	23.2	14.4	8.4	4.8	2.9
3RT	mean	293.24	12.00	36.06	12.00	10115.36	489.17	10.55	167.65	43.65	74.11	33.12	18.21	14.37	11.27	2.63	2.71
	SD	50.43	0.00	8.50	0.00	32.63	186.84	11.17	20.38	26.16	3.31	7.28	7.11	0.03	4.02	3.01	0.30
	RSD%	17.20	0.00	23.57	0.00	0.32	38.19	105.83	12.16	59.95	4.47	21.97	39.05	0.24	35.70	114.55	11.00
	SE	35.66	0.00	6.01	0.00	23.08	132.11	7.90	14.41	18.50	2.34	5.15	5.03	0.02	2.84	2.13	0.21
	a	81.1	12.0	40.8	12.0	7696.2	731.1	8.1	135.9	11.9	47.2	11.7	9.0	12.6	14.3	0.5	2.5
	b	124.7	12.0	35.3	12.0	8881.2	874.7	15.5	147.2	43.4	66.8	30.9	16.3	10.3	11.2	5.6	2.5
4RT	mean	102.89	12.00	38.04	12.00	8288.67	802.89	11.80	141.53	27.65	56.99	21.33	12.64	11.46	12.71	3.03	2.50
	SD	30.88	0.00	3.90	0.00	837.92	101.49	5.22	8.00	22.26	13.82	13.59	5.15	1.58	2.19	3.58	0.00
	RSD%	30.01	0.00	10.26	0.00	10.11	12.64	44.25	5.65	80.52	24.24	63.73	40.75	13.76	17.20	118.10	0.00
	SE	21.84	0.00	2.76	0.00	592.50	71.76	3.69	5.66	15.74	9.77	9.61	3.64	1.12	1.55	2.53	0.00
	a	44.1	12.0	29.7	12.0	7991.0	542.2	8.4	69.6	10.0	60.8	31.0	13.0	12.9	9.0	0.5	2.5
	b	90.8	12.0	36.4	12.0	8195.9	364.3	11.6	111.7	18.6	20.5	21.8	14.3	10.0	9.8	4.6	3.1
5RT	mean	67.42	12.00	33.06	12.00	8093.44	453.23	9.96	90.62	14.32	40.65	26.41	13.66	11.43	9.39	2.53	2.80
	SD	33.03	0.00	4.71	0.00	144.86	125.77	2.28	29.77	6.11	28.55	6.46	0.90	2.03	0.52	2.87	0.42
	RSD%	48.99	0.00	14.25	0.00	1.79	27.75	22.88	32.85	42.67	70.23	24.44	6.62	17.73	5.51	113.50	14.94
	SE	23.36	0.00	3.33	0.00	102.43	88.93	1.61	21.05	4.32	20.19	4.57	0.64	1.43	0.37	2.03	0.30
	a	25.0	12.0	19.5	12.0	6864.9	449.0	7.1	47.1	10.0	34.5	26.4	7.1	11.1	10.0	4.7	2.5
	b	30.3	12.0	26.4	12.0	6858.4	279.1	9.7	51.2	11.6	30.9	16.1	10.7	10.0	7.6	3.6	2.5
6RT	mean	27.65	12.00	22.95	12.00	6861.66	364.04	8.36	49.13	10.79	32.72	21.29	8.90	10.55	8.78	4.15	2.50

	SD	3.75	0.00	4.88	0.00	4.61	120.11	1.85	2.89	1.12	2.50	7.28	2.60	0.78	1.72	0.73	0.00
	RSD%	13.55	0.00	21.25	0.00	0.07	32.99	22.14	5.88	10.38	7.65	34.19	29.18	7.41	19.58	17.63	0.00
	SE	2.65	0.00	3.45	0.00	3.26	84.93	1.31	2.04	0.79	1.77	5.15	1.84	0.55	1.22	0.52	0.00

Table 10-57 Dosed background (feed) concentrations of nonylphenol ethoxylates (μg kg^{-1} dw).

PSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
a	338.35	12.00	10622.82	12.68	2264.73	2198.56	4266.90	4542.39	2507.72	2244.09	2701.52	2566.57	2050.44	1955.71	1097.97	792.95
b	228.64	12.00	10848.29	12.78	2042.14	1595.97	3704.14	4570.85	2920.55	3074.59	2404.49	2417.16	2597.82	1949.41	857.50	801.46
c	210.65	12.00	9902.36	12.48	1657.55	1556.65	3685.53	4282.25	2900.21	3085.10	2428.94	2371.78	2497.06	1937.77	833.41	753.63
mean	**259.21**	**12.00**	**10457.83**	**12.64**	**1988.14**	**1783.73**	**3885.53**	**4465.16**	**2776.16**	**2801.26**	**2511.65**	**2451.84**	**2381.77**	**1947.63**	**929.63**	**782.68**
SD	69.12	0.00	494.08	0.15	307.17	359.79	330.41	159.05	232.70	482.55	164.89	101.92	291.33	9.10	146.29	25.52
RSD%	26.67	0.00	4.72	1.21	15.45	20.17	8.50	3.56	8.38	17.23	6.57	4.16	12.23	0.47	15.74	3.26
SE	39.91	0.00	285.26	0.09	177.35	207.73	190.76	91.83	134.35	278.60	95.20	58.84	168.20	5.25	84.46	14.73
MSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
a	386.07	12.00	207973.42	12.32	2331.46	2132.61	2330.17	3170.25	1711.42	2459.71	1881.47	1815.40	1495.04	1415.35	1350.96	1167.78
b	203.38	12.00	297796.29	12.51	1081.64	1591.41	3124.41	3596.18	1883.98	1580.07	1935.12	1866.06	1422.22	1418.69	769.80	543.20
c	126.29	12.00	257036.29	12.59	1700.05	1619.91	3098.55	3421.50	1824.49	1557.97	1955.26	1825.32	1438.68	1420.37	783.60	579.70
mean	**238.58**	**12.00**	**254268.67**	**12.47**	**1704.38**	**1781.31**	**2851.04**	**3395.97**	**1806.63**	**1865.92**	**1923.95**	**1835.59**	**1451.98**	**1418.14**	**968.12**	**763.56**
SD	133.42	0.00	44975.35	0.14	624.92	304.57	451.28	214.11	87.65	514.35	38.14	26.84	38.19	2.55	331.62	350.54
RSD%	55.92	0.00	17.69	1.12	36.67	17.10	15.83	6.30	4.85	27.57	1.98	1.46	2.63	0.18	34.25	45.91
SE	77.03	0.00	25966.53	0.08	360.80	175.84	260.55	123.62	50.61	296.96	22.02	15.50	22.05	1.47	191.46	202.38

Table 10-58 Mean nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from the primary sludge mesophilic (dosed experiment) digester (PSM).

	PSM	**NP**	**NP_1EC**	**NP_2EC**	**NP_3EC**	**NP_1EO**	**NP_2EO**	**NP_3EO**	**NP_4EO**	**NP_5EO**	**NP_6EO**	**NP_7EO**	**NP_8EO**	**NP_9EO**	**$NP_{10}EO$**	**$NP_{11}EO$**	**$NP_{12}EO$**
	a	209.16	2.55	16730.13	1860.04	83.42	3.82	6.11	32.72	54.36	40.78	34.50	23.49	7.31	4.87	8.91	7.71
	b	152.27	2.51	19401.24	1884.04	66.44	2.51	3.73	22.35	39.31	30.41	22.08	17.37	5.92	2.72	1.96	8.76
day1	mean	180.72	2.53	18065.69	1872.04	74.93	3.16	4.92	27.53	46.84	35.60	28.29	20.43	6.61	3.80	5.43	8.23
	SD	40.23	0.03	1888.76	16.97	12.00	0.93	1.68	7.33	10.64	7.34	8.79	4.33	0.99	1.52	4.92	0.74
	RSD %	22.26	1.14	10.45	0.91	16.02	29.37	34.25	26.63	22.71	20.61	31.06	21.19	14.91	39.95	90.53	9.04
	SE	28.45	0.02	1335.55	12.00	8.49	0.66	1.19	5.18	7.52	5.19	6.21	3.06	0.70	1.07	3.48	0.53
	a	56.79	1.28	11697.03	73.51	32.55	1.92	23.07	89.30	140.99	122.30	107.06	72.33	33.07	21.27	5.15	19.01

	PSM	NP	NP_1EC	NP_2EC	NP_3EC	NP_1EO	NP_2EO	NP_3EO	NP_4EO	NP_5EO	NP_6EO	NP_7EO	NP_8EO	NP_9EO	$NP_{10}EO$	$NP_{11}EO$	$NP_{12}EO$
	b	74.39	1.32	14681.47	159.02	41.44	2.63	30.76	218.60	438.03	391.22	352.82	242.95	109.33	54.62	23.17	0.00
Day3	mean	25.35	0.58	673.00	6.45	12.25	15.02	17.72	15.91	15.12	12.90	18.63	12.13	7.80	20.51	47.00	4.78
	SD	4.37	0.79	936.99	7.84	5.33	20.31	23.38	15.16	10.74	10.91	17.57	12.82	10.05	27.49	61.55	6.02
	RSD %	17.25	136.46	139.22	121.56	43.46	135.23	131.92	95.33	71.05	84.55	94.27	105.72	128.79	134.03	130.96	125.86
	SE	3.09	0.56	662.55	5.55	3.77	14.36	16.53	10.72	7.60	7.71	12.42	9.07	7.10	19.44	43.53	4.25
	a	162.92	3.02	3957.70	145.67	156.82	7.17	16.56	87.80	124.82	107.93	80.53	62.45	29.68	17.97	7.67	19.84
	b	68.84	1.81	2969.31	109.06	80.68	4.20	18.33	81.24	96.21	82.34	62.61	45.70	18.97	13.01	4.20	11.10
Day5	mean	10.17	68.51	400.89	63.55	23.61	74.80	74.22	53.03	39.32	46.13	53.35	57.39	67.95	76.73	87.24	65.06
	SD	10.01	96.09	370.05	82.03	28.07	85.47	81.59	59.83	44.87	54.33	57.88	68.34	86.04	81.03	61.82	85.99
	RSD %	98.42	140.27	92.31	129.08	118.87	114.27	109.93	112.82	114.11	117.78	108.49	119.08	126.63	105.60	70.86	132.17
	SE	7.08	67.95	261.66	58.00	19.85	60.44	57.69	42.30	31.73	38.42	40.92	48.33	60.84	57.29	43.72	60.80
	a	14.46	1.26	1543.27	10.04	25.78	1.26	11.53	69.51	101.98	95.75	79.84	55.53	27.84	15.61	4.33	15.93
	b	13.90	1.21	1574.70	7.86	25.39	1.21	22.83	144.21	209.88	209.17	174.56	116.66	65.75	36.34	15.10	35.41
4RT	mean	52.75	104.11	176.98	93.54	69.36	87.35	83.81	77.56	72.92	78.10	74.71	83.70	93.74	81.44	57.29	96.49
Day7	SD	64.59	51.14	119.75	50.26	70.02	38.07	36.93	49.87	58.25	56.12	47.78	50.03	46.52	34.15	19.20	50.47
	RSD %	122.44	49.12	67.66	53.73	100.95	43.58	44.07	64.29	79.88	71.85	63.95	59.77	49.63	41.94	33.51	52.30
	SE	45.67	36.16	84.68	35.54	49.51	26.92	26.12	35.26	41.19	39.68	33.78	35.38	32.90	24.15	13.57	35.69
	a	18.59	1.28	3101.17	13.49	31.38	1.28	0.00	2.38	0.47	0.00	0.00	0.00	0.00	0.00	0.01	0.00
	b	16.98	1.13	2714.29	11.91	27.18	1.13	0.00	1.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Day10	mean	84.06	42.64	76.17	44.63	75.23	35.25	35.09	49.78	60.54	55.77	48.87	47.58	41.26	33.04	23.54	43.99
	SD	54.28	9.16	12.03	12.86	36.37	11.78	12.69	20.53	27.36	22.75	21.33	17.25	11.83	12.58	14.10	11.75
	RSD %	64.58	21.49	15.80	28.82	48.35	33.42	36.17	41.24	45.20	40.80	43.65	36.26	28.68	38.06	59.88	26.71
	SE	38.38	6.48	8.51	9.10	25.72	8.33	8.98	14.52	19.35	16.09	15.08	12.20	8.37	8.89	9.97	8.31
	a	0.01	0.00	0.00	0.00	1.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.02	0.00	0.15	0.00	0.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.15
Day20	mean	51.48	13.99	12.15	18.96	37.04	20.88	22.58	27.88	32.27	28.44	29.37	24.23	18.52	23.48	34.92	17.51
	SD	18.52	10.61	5.15	13.95	16.00	17.74	19.23	18.90	18.28	17.47	20.20	17.01	14.36	20.62	35.29	13.01
	RSD %	35.98	75.90	42.41	73.57	43.21	84.99	85.19	67.79	56.64	61.43	68.79	70.22	77.53	87.85	101.06	74.31
	SE	13.10	7.51	3.64	9.86	11.32	12.55	13.60	13.36	12.93	12.35	14.28	12.03	10.15	14.58	24.95	9.20
	a	0.02	0.00	0.00	0.00	1.99	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	b	0.03	0.00	1.13	0.00	3.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	18.77
Day30	mean	24.54	41.70	23.03	41.72	27.26	48.77	49.39	40.57	34.78	36.89	41.53	41.13	43.84	51.22	63.01	41.76
	SD	16.18	48.36	27.41	45.05	22.55	51.23	50.62	38.48	30.91	34.70	38.54	41.15	47.64	51.81	53.81	46.04
	RSD %	65.94	115.97	119.04	107.99	82.72	105.04	102.49	94.85	88.87	94.06	92.78	100.05	108.67	101.15	85.41	110.26

	PSM	NP	NP_1EC	NP_2EC	NP_3EC	NP_1EO	NP_2EO	NP_3EO	NP_4EO	NP_5EO	NP_6EO	NP_7EO	NP_8EO	NP_9EO	$NP_{10}EO$	$NP_{11}EO$	$NP_{12}EO$
	SE	11.44	34.20	19.38	31.85	15.95	36.22	35.80	27.21	21.86	24.54	27.25	29.10	33.69	36.63	38.05	32.55

Table 10-59 Mean nonylphenol ethoxylate concentrations (μg kg^{-1} dw) from the mixed sludge mesophilic (dosed experiment) digester (MSM).

	MSM	NP	NP_1EC	NP_2EC	NP_3EC	NP_1EO	NP_2EO	NP_3EO	NP_4EO	NP_5EO	NP_6EO	NP_7EO	NP_8EO	NP_9EO	$NP_{10}EO$	$NP_{11}EO$	$NP_{12}EO$
	a	92.18	1.90	25436.42	7457.65	36.52	10.90	36.89	194.49	185.25	118.90	73.55	45.95	17.66	7.44	5.30	7.53
	b	17.12	1.18	26052.78	1174.91	11.23	2.94	25.98	74.77	61.94	51.13	37.77	28.87	10.84	5.85	17.73	24.20
day1	mean	54.65	1.54	25744.60	4316.28	23.87	6.92	31.43	134.63	123.60	85.02	55.66	37.41	14.25	6.65	11.51	15.86
	SD	53.07	0.51	435.83	4442.57	17.88	5.63	7.72	84.66	87.20	47.92	25.30	12.08	4.82	1.13	8.79	11.79
	RSD%	97.12	32.96	1.69	102.93	74.89	81.28	24.55	62.88	70.55	56.36	45.46	32.29	33.86	16.94	76.33	74.29
	SE	37.53	0.36	308.18	3141.37	12.64	3.98	5.46	59.86	61.66	33.88	17.89	8.54	3.41	0.80	6.21	8.33
	a	30.44	1.27	16459.77	718.15	12.08	3.82	53.97	171.69	172.11	149.88	119.58	84.89	37.10	18.78	9.18	14.55
	b	43.50	1.34	45757.91	932.02	14.71	4.69	72.92	270.27	337.43	307.71	242.61	172.36	81.83	39.46	0.00	14.07
Day 3	mean	67.32	16.66	154.94	1622.15	43.76	42.63	15.00	61.37	66.10	45.12	31.68	20.42	18.63	8.87	41.27	41.31
	SD	42.13	23.05	216.72	2148.50	44.01	54.66	13.50	2.13	6.29	15.90	19.50	16.79	21.53	11.42	49.58	46.64
	RSD%	62.58	138.37	139.88	132.45	100.57	128.22	89.99	3.48	9.51	35.23	61.54	82.26	115.53	128.73	120.13	112.89
	SE	29.79	16.30	153.24	1519.22	31.12	38.65	9.54	1.51	4.45	11.24	13.79	11.87	15.22	8.07	35.06	32.98
	a	7.29	0.00	1089.06	29.08	8.50	2.43	58.27	189.13	160.72	138.94	107.65	74.15	35.27	17.53	6.35	7.29
	b	13.61	0.62	5226.09	45.76	13.62	4.33	68.15	180.89	150.22	117.85	89.79	67.34	30.30	13.86	4.84	13.59
Day 5	mean	46.19	77.33	146.56	825.83	65.85	83.43	49.77	2.49	6.98	23.23	37.66	47.07	65.38	68.40	77.59	72.94
	SD	23.19	86.32	9.45	980.60	49.11	63.34	56.88	1.39	3.58	16.96	33.77	49.77	70.93	85.31	60.15	56.51
	RSD%	50.20	111.62	6.45	118.74	74.58	75.91	114.30	55.83	51.33	73.01	89.66	105.74	108.49	124.73	77.52	77.48
	SE	16.40	61.03	6.68	693.39	34.72	44.79	40.22	0.98	2.53	11.99	23.88	35.19	50.15	60.33	42.54	39.96
	a	6.20	0.63	1777.76	44.09	6.22	1.86	31.77	151.36	114.61	121.13	99.60	71.15	29.79	20.52	11.16	18.72
	b	9.44	1.26	5231.38	46.69	8.81	2.52	29.33	148.90	133.20	141.39	128.20	88.09	49.17	28.48	11.43	0.00
4RT	mean	33.30	86.33	6.57	406.06	54.65	60.35	77.26	28.40	26.93	42.50	56.77	70.47	79.32	92.53	60.03	58.72
Day 7	SD	23.90	35.77	0.17	406.34	28.18	22.01	52.38	38.78	34.50	43.14	46.51	49.89	41.25	45.54	24.74	26.53
	RSD%	71.79	41.43	2.52	100.07	51.57	36.47	67.80	136.52	128.12	101.51	81.94	70.80	52.00	49.22	41.21	45.18
	SE	16.90	25.29	0.12	287.32	19.93	15.56	37.04	27.42	24.40	30.51	32.89	35.27	29.17	32.20	17.49	18.76
	a	11.95	0.00	4292.18	60.63	12.61	3.14	0.00	2.00	0.00	0.00	0.00	0.00	0.64	0.00	0.00	0.00
	b	13.02	0.00	3980.76	33.12	16.13	3.71	0.00	1.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	24.19

	MSM	NP	NP_1EC	NP_2EC	NP_3EC	NP_1EO	NP_2EO	NP_3EO	NP_4EO	NP_5EO	NP_6EO	NP_7EO	NP_8EO	NP_9EO	$NP_{10}EO$	$NP_{11}EO$	$NP_{12}EO$
Day 10	mean	44.34	33.36	1.32	193.70	35.75	26.02	52.42	81.97	76.26	66.01	57.41	53.03	40.59	40.71	29.35	31.97
	SD	38.81	11.41	1.70	132.41	22.37	14.78	21.75	77.15	73.34	50.21	34.68	25.12	16.15	12.03	16.77	18.68
	RSD%	87.52	34.21	128.87	68.36	62.58	56.82	41.49	94.11	96.18	76.06	60.41	47.36	39.78	29.56	57.14	58.44
	SE	27.44	8.07	1.20	93.63	15.82	10.45	15.38	54.55	51.86	35.50	24.52	17.76	11.42	8.51	11.86	13.21
	a	0.01	0.00	0.13	0.00	1.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.75	8.13
	b	0.01	0.00	0.37	0.00	0.48	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.34	14.58
Day 20	mean	57.48	21.14	65.04	80.99	39.20	33.64	28.44	74.33	74.02	55.78	42.46	32.56	25.60	19.03	34.50	35.83
	SD	42.48	18.48	90.28	17.87	33.07	32.79	18.47	27.98	31.34	28.68	25.37	20.93	20.06	14.88	32.02	31.98
	RSD%	73.90	87.43	138.81	22.06	84.35	97.47	64.94	37.64	42.33	51.42	59.75	64.28	78.35	78.20	92.80	89.27
	SE	30.04	13.07	63.83	12.63	23.38	23.19	13.06	19.78	22.16	20.28	17.94	14.80	14.18	10.52	22.64	22.61
	a	0.02	0.00	1.63	0.00	2.58	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.43	12.73
	b	0.03	0.00	1.03	0.00	0.81	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.48
Day 30	mean	51.97	50.25	101.32	17.35	53.87	60.33	39.00	28.71	32.25	35.85	38.85	39.54	46.27	44.36	57.72	55.94
	SD	31.02	52.58	53.01	6.67	43.11	52.53	36.68	12.62	14.27	22.02	29.56	34.99	45.37	47.85	49.61	47.13
	RSD%	59.68	104.64	52.32	38.42	80.03	87.07	94.07	43.98	44.25	61.41	76.11	88.49	98.07	107.87	85.95	84.25
	SE	21.93	37.18	37.49	4.71	30.48	37.14	25.94	8.93	10.09	15.57	20.90	24.74	32.08	33.84	35.08	33.33

10.6 Appendix VI

10.6.1 Mass balance for steroid estrogens

Table 10-60 Mass balance for steroid estrogens during mesophilic (35^{o}C) and thermophilic (53^{o}C) digestion of primary and mixed sludges.

	Flux (µg d^{-1})											
	Mesophilic digestion						**Thermophilic digestion**					
	Primary sludge			**Mixed sludge**			**Primary sludge**			**Mixed sludge**		
Steroid estrogens	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %
E1	4.53	0.96	78.80	2.28	0.68	70.32	3.49	0.16	95.51	1.61	0.51	68.22
E2	0.26	1.12	-323.56	0.16	0.69	-325.08	0.32	1.51	-367.38	0.15	1.07	-621.33
E3	0.25	0.14	45.40	0.20	0.11	43.55	0.30	0.25	16.64	0.26	0.25	4.08
E1-3S	0.22	0.14	36.36	0.17	0.13	21.06	0.20	0.14	30.31	0.20	0.15	27.88
EE2	0.53	0.35	32.71	0.26	0.25	4.55	0.49	0.28	43.56	0.50	0.43	14.82
Natural	**5.26**	**2.36**	**55.26**	**2.81**	**1.61**	**42.61**	**4.32**	**2.06**	**52.25**	**2.22**	**1.97**	**10.99**
ΣE1+E2	**4.79**	**2.08**	**56.58**	**2.44**	**1.37**	**43.85**	**3.81**	**1.67**	**56.16**	**1.76**	**1.58**	**10.23**
ΣESTs	**5.79**	**2.71**	**53.21**	**3.07**	**1.86**	**39.40**	**4.81**	**2.34**	**51.36**	**2.72**	**2.40**	**11.70**

10.6.1 Mass balance for nonylphenol ethoxylates

Table 10-61 Mass balance for nonylphenol ethoxylates during mesophilic digestion of primary and mixed sludges.

	Flux (mg d^{-1})					
	Primary digesters			**Mixed digesters**		
Alkylphenolic compounds	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %
			NPEOs			
NP_1EO	0.08	0.01	90.16	0.06	0.01	89.83
NP_2EO	0.01	0.00	92.60	0.00	0.00	73.05
NP_3EO	1.72	0.00	99.97	0.91	0.00	99.91
NP_4EO	7.56	0.00	99.97	3.95	0.00	99.94
NP_5EO	7.11	0.00	99.95	3.66	0.00	99.93
NP_6EO	6.09	0.00	99.94	3.26	0.00	99.92
NP_7EO	4.05	0.00	99.93	2.71	0.00	99.93
NP_8EO	2.78	0.00	99.93	1.99	0.00	99.93
NP_9EO	1.17	0.00	99.89	0.77	0.00	99.91
$NP_{10}EO$	1.11	0.00	99.95	0.76	0.00	99.96
$NP_{11}EO$	0.62	0.00	99.97	0.40	0.00	99.97
$NP_{12}EO$	0.49	0.00	99.94	0.96	0.00	99.98
$NP_{1-2}EO$	**0.09**	**0.01**	**90.38**	**0.07**	**0.01**	**88.74**
$NP_{3-12}EO$	**32.70**	**0.02**	**99.95**	**19.37**	**0.01**	**99.93**
$NP_{1-12}EO$	**32.79**	**0.03**	**99.92**	**19.44**	**0.02**	**99.89**
			NPEC			
NP_1EC	0.00	0.00	54.19	0.00	0.00	90.31
NP_2EC	1.10	3.44	-211.33	9.68	4.05	58.13
NP_3EC	0.00	0.04	-7692.09	0.00	0.05	-10312.28
NPEC	**1.10**	**3.47**	**-214.56**	**9.68**	**4.10**	**57.62**
			NP			
NP	**0.01**	**0.01**	**45.43**	**0.01**	**0.00**	**51.03**
ΣNPEO	**33.91**	**3.51**	**89.66**	**29.12**	**4.13**	**85.83**

Table 10-62 Mass balance for nonylphenol ethoxylates during thermophilic digestion of primary and mixed sludges.

	Flux (mg d^{-1})					
	Primary digesters			**Mixed digesters**		
Alkylphenolic compounds	In-flux	Out-flux 6th RT	Removal %	In-flux	Out-flux 6th RT	Removal %
			NPEO			
NP_1EO	1.22	1.19	1.98	7.55	0.00	99.98
NP_2EO	0.00	0.00	80.18	0.00	0.00	75.68
NP_3EO	2.00	0.00	99.85	1.50	0.00	99.95
NP_4EO	11.70	0.01	99.89	6.97	0.00	99.94
NP_5EO	13.22	0.00	99.98	8.87	0.00	99.99
NP_6EO	11.31	0.01	99.95	7.67	0.00	99.97
NP_7EO	8.20	0.00	99.97	6.31	0.00	99.97
NP_8EO	6.46	0.00	99.97	4.20	0.00	99.98
NP_9EO	3.08	0.00	99.93	1.87	0.00	99.95
$NP_{10}EO$	1.02	0.00	99.90	0.64	0.00	99.89
$NP_{11}EO$	0.97	0.00	99.90	0.28	0.00	99.88
$NP_{12}EO$	0.62	0.00	99.97	0.38	0.00	99.95
$NP_{1-2}EO$	**1.22**	**1.19**	**2.30**	**7.55**	**0.00**	**99.96**
$NP_{3-12}EO$	**58.58**	**0.03**	**99.95**	**38.70**	**0.01**	**99.97**
$NP_{1-12}EO$	**59.80**	**1.23**	**97.95**	**46.25**	**0.02**	**99.97**
			NPEC			
NP_1EC	0.00	0.05	-4684.47	0.00	0.56	-23389.46
NP_2EC	0.00	0.06	-6077.73	0.00	0.03	-848.50
NP_3EC	0.00	0.00	0.32	0.00	0.00	3.13
$NP_{1-3}EC$	**0.00**	**0.11**	**-3591.44**	**0.00**	**0.59**	**-8921.60**
			NP			
NP	0.02	0.01	**57.00**	0.01	0.00	**80.21**
ΣNPEO	**59.82**	**1.34**	**97.76**	**46.27**	**0.61**	**98.68**