Comparison of Ethinylestradiol and Nitrogen Removal in a Conventional and Simultaneous Nitrification-Denitrification Membrane Bioreactor

By

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Author's Declaration

I hereby declare that I am the sole author of this thesis. I understand that my thesis may be made electronically available to the public.

ABSTRACT

The purpose of this study was to compare ethinylestradiol (EE2) and nitrogen removal in a conventional membrane bioreactor (C-MBR) and a simultaneous nitrification-denitrification membrane bioreactor (SND-MBR). Two lab-scale MBRs were operated in parallel for over 450 days; various MBR operating parameters, as well as total nitrogen removal (TN) and estrogenic activity removal (EA) were measured. The SND-MBR was able to remove 59% of influent TN with an additional 21% removed via sludge wasting; the C-MBR had a TN removal efficiency of only 31%. There was no significant impact of SND processes on membrane fouling though the SND process was associated with higher concentrations of fouling indicators such as sCOD and TEPs. Particle size analysis showed that the SND-MBR mixed liquor had a volume weighted mean particle size of 146 ± 28 μm; the C-MBR had a smaller mean at 89 ± 2 μm. An investigation of microbial populations within the activated sludge (AS) revealed that process changes, such as low dissolved oxygen (DO) conditions, can affect the microbial populations within AS. In terms of estrogenic activity, the C-MBR and SND-MBR removed 57% and 58% of influent EA, respectively; there was no significant difference in their removal efficiencies. Biodegradation was the dominant removal mechanism for both reactors with K_{BIO} coefficients of 1.5 ± 0.6 and 1.6 ± 0.4 $days^{-1}$ for the C-MBR and the SND-MBR, respectively. Adsorption removed approximately 1% of influent EA in each reactor; the particle partitioning coefficient, K_D, was calculated to be 0.21 ± 0.07 L/(g MLSS) for the C-MBR and 0.27 ± 0.1 L/(g MLSS) for the SND-MBR. The findings of this thesis indicate that SND was able remove greater amounts of TN with no observable impact on EA reduction and membrane operations.

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ABBREVIATIONS AND NOMENCLATURE

AS:	Activated sludge
AOB	Ammonia oxidizing bacteria
BNR:	Biological nutrient removal
BOD:	Biological oxygen demand
CAS:	Conventional activated sludge system
CI	Confidence interval
C-MBR:	Conventional membrane bioreactor
COD:	Chemical oxygen demand
CPRG	Chlorophenol red-ß-D-galactopyranoside
DO:	Dissolved oxygen
E1:	Estrone
E2:	Estradiol
E2-eq:	Estrogenic activity of a solution as a relative concentration of E2
E3:	Estriol
EE2:	Ethinylestradiol
EA:	Estrogenic activity (measured as E2-eq)
EDC:	Endocrine disrupting compound
ESI:	Interface electrospray
E-Screen:	Estrogen screen
FISH:	Fluorescent in-situ hybridization
F/M:	Food to microorganism ratio
GC-MS:	Gas chromatography-mass spectrophotometry
GC-MS-MS:	Gas chromatography-tandem mass spectrometry
HRT:	Hydraulic retention time
K_D:	Particle partitioning coefficient
K_{BIO}	Biological degradation coefficient
K_{OW}:	Octanol water coefficient; used to estimate solubility
LC-MS-MS:	Liquid chromatography-tandem mass spectrometry

LOD:	Limit of detection
MBR:	Membrane bioreactor
MF:	Microfiltration
MLSS:	Mixed liquor suspended solids
MLVSS:	Mixed liquor volatile suspended solids
ng/L:	Nanograms per litre
N_2:	Nitrogen gas
NH_3:	Ammonia
NH_4:	Ammonium
NOB	Nitrite oxidizing bacteria
NO_2:	Nitrite
NO_3:	Nitrate
N_2O:	Nitrous oxide gas
p*Ka*:	Acid dissociation constant
PSI:	Pounds per square inch
sCOD:	Soluble chemical oxygen demand
SPE:	Solid phase extraction
Spp.	Species
SND:	Simultaneous nitrification-denitrification
SRT:	Solids retention time
SND-MBR:	Membrane bioreactor with simultaneous nitrification-denitrification
TEPS:	Transparent exopolymeric particles
TMP:	Transmembrane pressure
TN:	Total nitrogen
TOC:	Total organic carbon
TSS:	Total suspended solids
UF:	Ultrafiltration
WAS:	Waste activated sludge
WWTP:	Wastewater treatment Plant
YES:	Yeast estrogen screen

1 INTRODUCTION

1.1 Literature Review

1.1.1 Ethinylestradiol (EE2)

Endocrine disrupting compounds (EDCs) refer to a class of chemical substances that have the ability to disrupt the endocrine system in humans and animals. They are defined as an "exogenous substance or mixture that alters function(s) of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny or (sub)populations" by the World Health Organisation (WHO 2004). The US Environmental Protection Agency (USEPA) defines EDCs as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" ((USEPA) 1997). EDCs are in their simplest form natural or synthetic compounds that adversely interact with the hormonal system of living organisms. These interactions include direct damage to an endocrine organ, alteration of the function of an endocrine organ, interaction with receptors, or alteration of hormone metabolism (EC 2001).

Due to their relative complexity and heterogeneous nature, it is sometimes difficult to understand the exact cause and effect relationship between compounds and the endocrine system. Determining which compounds can be classified as endocrine disrupters is therefore complex and somewhat subjective (Canada 1999; Esperanza et al. 2004; Auriol et al. 2006). To further complicate the issue, there are few internationally recognized tests or risk-assessment procedures available that can be used to determine

whether a compound can act as an EDC (Groshart et al. 2000; EC 2001; WHO 2004). The Organization for Economic Co-Operation and Development (OECD) is, however, currently reviewing several procedures for testing and assessing endocrine disruption. It is hoped that these guidelines will provide some structure for determining which compound can be classified as an endocrine disrupter (OECD 2008).

Despite their complex and subjective nature, attempts have been made to identify priority compounds that are thought to disrupt the endocrine system. General classifications identify EDCs according to their origin, potency, and affect. In wastewater applications, focus has generally been limited to EDCs that are estrogen receptor agonists, meaning that they are compounds that have the ability to mimic endogenous estrogen (Snyder et al. 2001). Within this category, EDCs present in municipal wastewater are generally categorized according to their origin. Four main classes of agonist EDCs include natural steroidal estrogens, synthetic estrogens, phytoestrogens, and industrial chemicals (Auriol et al. 2006). Of these, it is believed that synthetic estrogens, such as ethinylestradiol (EE2) are the most potent in municipal wastewater (Tyler et al. 1998; Johnson et al. 2001; Auriol et al. 2006).

Ethinylestradiol (EE2) is a synthetic estrogen that is used to manufacture contraceptive medication (de Mes et al. 2005; Rasier et al. 2006). It is of especial concern in the wastewater industry because of its resistance to biological degradation (Ternes et al. 1999a; Cirja et al. 2007), its relatively strong estrogenic activity (EA) (Routledge et al. 1996; Johnson et al. 2001; Lai et al. 2002b; Kidd et al. 2007), and because of its tendency to adsorb onto solid particles (Layton et al. 2000; Liu et al. 2005; Cirja et al. 2008). While other estrogens are known to biologically degrade in wastewater treatment plants

(Ternes et al. 1999b; Andersen et al. 2003; Joss et al. 2004) EE2 is more resistant because the ethinyl group in position 17β, as seen in Figure 1, blocks oxidation making it less soluble and more stable in aqueous environments (Desbrow et al. 1998; Ternes et al. 1999b; Birkett et al. 2003; Ren et al. 2007).

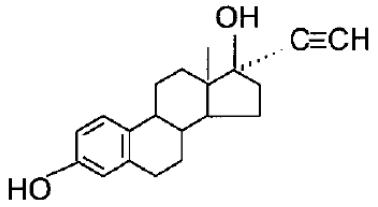


Figure 1. The chemical structure of EE2

This chemical structure also makes EE2 relatively insoluble and hydrophobic; it has a great affinity to solid particles and can readily adsorb onto their surface, more so than other estrogens (Layton et al. 2000; Yi et al. 2007b; Clouzot et al. 2010a). A summary of EE2's physicochemical properties can be found in Table 1.

Table 1. Summary of physico-chemical properties of estrogens

	Molecular Weight (g/mol)	Molecular Formula	Solubility (mg/L)	Log K_{ow}	p*Ka*	EA relative to E2 (E2-eq)
Estrone (E1)	270.37[3]	$C_{18}H_{22}O_2$	0.8[3] – 30[4]	2.45[2] - 3.43[3]	10.3[3] – 10.8[2]	0.38[6] 0.34 - 0.43[7] 0.01[8]
Estradiol (E2)	272.39[3]	$C_{18}H_{24}O_2$	3.85[3] – 13[2]	3.10[2] - 4.01[5]	10.2[3] – 10.7[2]	1
Estriol (E3)	288.4[3]	$C_{18}H_{24}O_3$	32[2] – 441[4]	2.13[2] – 3.43[5]	10.4[2]	0.024[6]
EE2	296.41[3]	$C_{20}H_{24}O_2$	4.8[2] – 116[4]	2.91[2] - 4.15[5]	10.21[3]	1.25[6] 1.5[9] 1.2[8]

[1]From (Cirja et al. 2008) citing (Hansch et al. 1995; Nakada et al. 2006)
[2]From (Campbell et al. 2006)
[3]From (Lee et al. 2008) citing (Holbrook et al. 2004; Shareef et al. 2006)

[4]From (Liu et al. 2009) citing (Tan 2006; NITE 2008)
[5]From (Auriol et al. 2006) citing (Ternes et al. 1999; Lai et al. 2000; Sayles 2001)
[6]From (Rutishauser et al. 2004)
[7]From (Thorpe et al. 2003)
[8]From (Yang 2009)
[9]From (Nishihara et al. 2000)

1.1.2 Environmental Impacts of EE2

EE2 is of growing concern in the wastewater industry because of its presence in wastewater influents and effluents. Relatively low concentrations (ng/L) have been known to interfere with hormonal balances and reproductive abilities of various aquatic species (Purdom et al. 1994; Desbrow et al. 1998; Ternes et al. 1999a). Male fish, for example, produce the female yolk hormone vitellogenin when exposed to estrogenic wastewater (Purdom et al. 1994; Jobling et al. 1998; Tyler et al. 1998). Due to their ability to interfere with animal hormone systems, it is thought that EE2 and other EDCs may be responsible for decreasing animal populations, changes in male to female sex ratios, behavior modification, and developmental abnormalities (Colborn et al. 1993; Solomon et al. 2000; Esperanza et al. 2004; Auriol et al. 2006). Identifying the exact impact a specific compound (i.e. EE2 versus other estrogens or estrogenic chemicals) has on the environment is difficult. Effects are often delayed and may not be obvious until an animal has reached full maturity. If impacts are manifested during the developmental stages of life they often vary, depending on the sex and age of the animal (Colborn et al. 1993).

While estrogenic effects have been noted in field or lab situations for vertebrates, and in insects, mollusks, and crustaceans, endocrine disruption appears to be particularly prevalent in aquatic environments (Matthiessen 2003). This is not surprising, as the primary sink for EDCs such as EE2 are groundwater, rivers, and lakes (Esperanza et al.

2004; Auriol et al. 2006). It is believed that some of the major contributors to these sinks are municipal wastewater effluents and agricultural runoff (Desbrow et al. 1998; Birkett et al. 2003; Hanselman et al. 2003; Auriol et al. 2006; Campbell et al. 2006; Lee et al. 2008).

In 1995, Sumpter et al. placed caged trout directly into the effluent of 28 sewage treatment plants (STP) throughout the United Kingdom and Wales. It was found that the male fish exhibited female characteristics that included the synthesis of a female, egg-yolk protein vitellogenin (Sumpter et al. 1995). Further study (Routledge et al. 1998) demonstrated that the vitellogenin could be induced with EE2 and βE2 at levels similar to that found in wastewater effluents. More recent work has also supported the concept that EDCs in wastewater effluents are harmful to wildlife. In 2004, the Environmental Protection agency tested 52 municipal wastewater treatment effluents from throughout the United States. Fathead minnows were exposed to the effluent for 24 hours; results found that in 10 of the effluents (21.7% of all tested), the male minnows had elevated vitellogenin levels (Lazorchak 2003).

In 2007, a seven-year study completed in northwestern Ontario, an experimental lake was spiked with EE2 in concentrations similar to those found in effluents. The goal of the study was to assess the effects of persistent, long-term, low concentration (5 -6 ng/L) exposure. Results found that the synthetic estrogen led to feminization of males through the production of vitellogenin and abnormal gonad development. Female species were also affected with prolonged production of vitellogenin. The most dramatic result, however, occurred in year two when the entire fish population collapsed to near-extinction due to an absence of viable, young fish. Reproductive failure continued for an

additional two years after EE2 additions had ceased, showing that even low concentrations can have potent and dramatic effects on fish populations (Kidd et al. 2007).

While there is good documentation that supports the theory that EDCs adversely affect wildlife there is very little data regarding their impact on the human population. In 2002 the World Health Organization issued a report stating that there is no direct cause and effect relationship between exposure to EDCs such as EE2 and human reproductive diseases/dysfunctions. They cautioned that this statement is true because of an absence of adequate exposure data and an inability to associate adverse effects to endocrine disruption. Latency issues and the relative complexity of the human endocrine system are to blame. They also cautioned that there is biological plausibility for EDC exposure to impact puberty, polycystic ovary syndrome, menopause, uterine fibroids, reduced sperm counts, time to pregnancy, and testicular cancer (WHO 2004). Further study is needed to understand the true risks to human health.

Other sources also support the World Health Organization's stance regarding EDCs. The Canadian Medical Association published an article in 2000 stating that there was room for caution and that some plausibility may exist regarding EDC exposure and its impacts on the human hormone systems, particularly at the early (fetal) stages of life. They also cautioned that improved monitoring of disease and exposure is required to further understand cause and effect relationships (Solomon et al. 2000). The European Commission has also supported such recommendations and have proposed that more research is warranted into the effect that EDCs may have on human health (EC 2001).

There is special concern regarding wastewater as human populations look to water reuse and recycling to supplement fresh water.

1.1.3 Estrogenic Activity (EA)

In wastewater, it is often desirable to determine the total estrogenic effect that a mixed solution, such as wastewater effluent, will have on an organism. The estrogen-screen (E-Screen) (Villalobos et al. 1995) and yeast-estrogen-screen (YES) test (Routledge et al. 1996) are particularly helpful because of their ability to indicate the overall EA of a complex solution (Campbell et al. 2006). The YES test is a bioassay that involves a genetically modified yeast strain. This strain has been altered to identify compounds that are able to interact with a human estrogen receptor. Depending on the EA of a compound, interaction will cause the cell to release the enzyme β-galactosidase which in turn metabolizes a chromogenic substrate that can be measured by absorbance (Routledge et al. 1996). The E-Screen method uses estrogen-sensitive breast-cancer cells by comparing cell yields between cultures treated with E2 and cultures treated with the compound(s) of interest (Villalobos et al. 1995).

Estrogenic activity measurements are generally based on the EA of E2 (E2-eq) and can be measured as a concentration, typically in ng/L (Yang et al. 2008). The E-Screen has a detection limit of approximately 0.27 ng/L E2-eq, while the lower limits of the YES test ranges from 0.3 to 30 ng/L E2-eq (Campbell et al. 2006). It is important to note that measurements of EA can only give the overall estrogenic affect and will not indicate the type or concentration of a specific EDC. Methods such as gas or liquid chromatography and mass spectrometry will give such information (Campbell et al. 2006). From an envirnmental perspective, overall EA values are more useful because they describe the overall affect that a particular solution has, in terms of its estrogenicity.

1.1.4 EE2 in Wastewater

The concentration of EE2 in wastewater are highly variable (Layton et al. 2000; Joss et al. 2006; Snyder et al. 2007; Hashimoto et al. 2009) and can depend on a number of factors such as rain events, diurnal curves, age demographics, and male to female ratios (Snyder et al. 2007). Contraception pills generally contain 35 μg of EE2. Approximately 22 – 50 % of the daily dose is excreted in urine while 30% is excreted in feces (de Mes et al. 2005). Table 2 gives general indications of relevant influent estrogen concentrations found in various studies. Influent concentrations of EE2 generally range from zero to 13 ng/L throughout North America, South America, and Europe (Baronti et al. 2000; Johnson et al. 2000; Birkett et al. 2003; Cargouet et al. 2004; Johnson et al. 2004; de Mes et al. 2005) though concentrations as high as 155 ng/L have been observed in raw wastewater (Cui et al. 2006; Pauwels et al. 2008). While EE2 influent concentrations appear to be lowest among the estrogens their removal from wastewater is difficult. It has the lowest biodegradability constant and highest estrogenic activity; its high log K_{ow} values show that EE2 has a tendency to adsorb onto biosolids; this can make it problematic for solid wastes treatment (Braga et al. 2005).

Table 2 Example of estrogen concentrations in wastewater influent and effluent

Sampling Site	Estrogen[1]								Analysis Method	Source
	E1		E2		E3		EE2			
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent		
France (AS with (de)nitrification & Upflow biofilers)	9.6-17.6	4.3 – 7.2	11.1-17.4	4.5 – 8.6	11.4-15.2	5 – 7.3	4.9-7.1	2.7 – 4.5	SPE[3]/GC-MS[4]	(Cargouet et al. 2004)
England	NA	< LOD[2] – 7.1	NA	< LOD - 25	NA	NA	<LOD	< LOD	SPE/GC-MS-MS[5]	(Fawell et al. 2001)
Italy	66		4 – 17	< LOD – 7	2 - 120	< LOD - 28	< LOD - 10	> LOD – 0.6	SPEL/LC-ESI[6]-MS-MS	(Johnson et al. 2000)
Italy	35.2 - 71	4.06 – 44.62	8.6 – 16.1	0.92 – 1.48	80	4	3	0.48 - 0.68	SPE/LC- MS-MS[7]	(Baronti et al. 2000)
Canada	NA	3 - 48	NA	6 - 64	NA	NA	NA	9 - 42	GC-MS-MS	(Ternes et al. 1999a)
Canada (AS)	19-78	1-96	2.4 - 26	0.2-15	NA	NA	NA	NA	GC-MS-MS	(Servos et al. 2005)
United States (AS, MBRs)		26		7	138 - 381	< 1 – 4.9	0 – 14.4	< LOD – 4.4	SPE/GC-MS-MS	(Drewes et al. 2005)
Austria	29 - 670	< 1	14 – 125	< 5	23 – 660	< 1	3 - 70	< 1	SPE/LC-MS-MS	(Clara et al. 2005b)

[1]Concentrations in ng/L
[2]LOD: limit of detection
[3]SPE: Solid Phase Extraction
[4]GC-MS: Gas chromatography-mass spectrophotometry
[5]GC-MS-MS: Gas chromatography-tandem mass spectrometry
[6]ESI: interface electrospray
[7]LC-MS-MS: liquid chromatography-tandem mass spectrometry

1.1.5 Removal Mechanisms of EE2 in WWTP

The main removal mechanisms of EE2 in WWTPs are thorough biological degradation and sorption to particles (Andersen et al. 2003; Larsen et al. 2004; Auriol et al. 2006; Koh et al. 2008). While the natural estrogens are primarily removed via biological degradation (Lai et al. 2000; Joss et al. 2004) there is some contention as to which mechanism dominates EE2 removal. Attempts to quantify how much of EE2 is removed via degradation, and how much is removed via sorption have also been somewhat ambiguous. Several articles suggest that because of EE2's low biodegredation constant and high K_{OW} value, sorption to biosolids is the primary removal mechanism. One study found removal efficiencies of 80% with sorption being the primary removal mechanism; 70% of all influent EE2 had adsorbed onto solid particles (Cirja et al. 2007). Research by Andersen et al (Andersen et al. 2005) also found that 70% of EE2 adsorbed onto solid particles in AS; this was similar to Layton (Layton et al. 2000) also found that approximately 80% of EE2 bound to sludge and was removed from the aqueous phase. Two recent studies by Clouzot et al had similar findings (Clouzot et al. 2010a; Clouzot et al. 2010b). The importance of sorption is further highlighted by an Australian study that found EE2 in waste activated sludge (WAS) despite having no detected traces of EE2 in wastewater influent. No biological degradation of EE2 was observed (Braga et al. 2005).

Other studies have, however, suggested that biological degradation is the primary removal mechanism for EE2, particularly in nitrifying conditions with high dissolved oxygen (DO) concentrations. Various studies yield different degradation rates though the following order of degradations have been noted:

$K_{BIO.E2} > K_{BIO.E3} > K_{BIO.E1} > K_{BIO.EE2}$ (Ren et al. 2007)

$K_{BIO.E2} > K_{BIO.E1} > K_{BIO.E3} > K_{BIO.EE2}$ (Shi et al. 2004)

The low degradation rate of EE2 can be attributed to the ethinyl group in position 17β, which blocks oxidation. EE2 generally degrades into unknown, non-estrogen compounds and/or carbon dioxide (Joss et al. 2004; de Mes et al. 2005).

Various studies have concluded that EE2 and EA reduction occur primarily via biological degradation. Yang et al. (2008) found that in a pilot-scale membrane bioreactor (MBR) system the majority of EA, approximately 85%, was biologically degraded and/or evaporated. While sorption did reduce the EA to a certain extent (9%) it was not a significant factor. Further study by the same author looked specifically at EE2 in MBRs. Again biological degradation was the primary removal factor, with 67% of influent EA being biodegraded, compared to 4% being removed via sludge wasting/sorption (Yang 2009). This is similar to Koerner et al. (2000) who examined EA in a conventional activated sludge (CAS) treatment plant in Germany. They found that overall EA was reduced by 90% in the treatment plant with only 3% being detected in the solid portions (Koerner et al. 2000). Another study investigating EE2 removal in AS found that there was no detectable EE2 bound to sludge; 85% of EE2 was removed through biodegradation (Joss et al. 2004). Further research by Andersen et al. confirmed this with 90% of influent EE2 being removed via aerobic biodegradation (Andersen et al. 2003).

1.1.6 Factors affecting EE2 removal and EA reduction in wastewater treatment

Wastewater treatment plants often vary in their ability to remove EE2 from wastewater, as seen in Table 2. There are a number of factors that will impact the ability of a WWTP to successfully treat EE2. Knowledge of such factors is important, especially as WWTPs are not specifically designed to reduce EA. The following is a discussion of such relevant parameters.

Influent concentrations

The concentration of EE2 in influents are highly variable (Layton et al. 2000; Joss et al. 2006; Snyder et al. 2007; Hashimoto et al. Article in Press, Corrected Proof 2008) and can depend on a number of factors such as rain events, diurnal curves, age demographics, and male to female ratios (Snyder et al. 2007). Generally EE2 is excreted into wastewater as inactive, conjugated compounds (typically glucuronides and sulfates) that have little to no EA (Belfroid et al. 1999; Panter et al. 1999; Birkett et al. 2003). In sewers and WWTPs the EE2 is de-conjugated into active forms by microbial activity and can become biologically available to living organisms (Panter et al. 1999; D'Ascenzo et al. 2003). Lyko et al. (2005) suggested that influent composition will impact which species comprise the activated sludge and the ability of activated sludge to reduce EA. Table 2 shows that the higher the influent concentration of EE2 the higher the effluent concentrations.

The solids retention time

The solids retention time (SRT) is the average amount of time that activated sludge remains in a WWTP system. It is believed to be the most critical parameter for activated-sludge design because of its ability to affect plant size and design, as well as performance (Tchobanoglous et al. 2003). While SRTs may vary according to design requirements, values will typically range anywhere from 3 to 12 days for conventional activated sludge treatment plants (CAS) (Tchobanoglous et al. 2003; Cirja et al. 2008). In membrane bioreactors (MBRs) SRTs have been known to be as high as 80 days, though typical values are usually between 25 to 30 days (Cicek et al. 1999; Johnson et al. 2004; Cirja et al. 2008). With respect to EDCs, the general consensus in literature is that

longer SRT values are associated with enhanced removal rates (Johnson et al. 2000; Vader et al. 2000; Johnson et al. 2001; Holbrook et al. 2002; Joss et al. 2004; Kreuzinger et al. 2004; Clara et al. 2004b; Johnson et al. 2005; Clara et al. 2005b; Johnson et al. 2007; Cirja et al. 2008).

The critical SRT for EE2 removal varies with some studies suggesting 5 to 10 days (Clara et al. 2005b; Cirja et al. 2008) and others recommending 10 to 15 days (Ivashechkin et al. 2004; Joss et al. 2004; Saino et al. 2004), though recommendations as high as 30 days have also been observed (Johnson et al. 2005) . These longer SRTs are considered important in EA reduction because they enhance biodegradation and sorption characteristics. They allow sufficient time for the development of diverse, slow-growing, complex bacteria that may be able to adapt and use complex substances, such as EE2 (Cicek et al. 1999; Vader et al. 2000; Birkett et al. 2003; Clara et al. 2005b; Cirja et al. 2008; Koh et al. 2008). They increase adsorbent characteristics in mixed liquor suspended solids (MLSS) by increasing diffusion time and influencing the biota and physical nature of floc particles (Johnson et al. 2000; Layton et al. 2000; Holbrook et al. 2002; Birkett et al. 2003; Cirja et al. 2008).

The hydraulic retention time

The hydraulic retention time (HRT) is the amount of time that wastewater remains in a WWTP. Typical HRT values will vary depending on the type of WWTP, effluent standards, and design requirements, generally ranging from 4 to 14 hours (Johnson et al. 2000; Tchobanoglous et al. 2003; Koh et al. 2008). Like the SRT, HRT can also have an impact on the ability of a WWTP to reduce EA, with longer HRTs attributed to increased biodegradation and adsorption rates in activated sludge from a variety of different

WWTPs (Svenson et al. 2003; CargouÎt et al. 2004; Johnson et al. 2005; Servos et al. 2005).

There have been different suggestions as to what range of HRT values would be considered influential in reducing EA in effluents. Kirk et al. (2002) compared five municipal WWTPs in the United Kingdom and their ability to reduce EA. For primary treatment they found that EA was only reduced in two of the plants when a high HRT (13 hours) was implemented. Svenson et al. (2003) compared 20 different WWTP across Sweden, representing different treatment and microbial processes. It was found that removal efficiencies in excess of 97% occurred when the HRT was greater than 12 hours in two of the WWTPs. Servos et al. (2005) compared 18 different WWTPs across Canada and found that longer HRTs (greater than 10 hours) were associated with high estrogen reduction (greater than 50%). Unlike Johnson et al. (2005), Servos et al. (2005) were unable to establish a statistically significant relationship between removal efficiencies and HRT, though the author cautioned that limited data sets and the diverse characteristics of the WWTPs may have affected the analysis (Servos et al. 2005).

Biomass

The ability of a microorganism community to utilize EE2 as a food source depends on a number of operating parameters. These parameters can be controlled and/or manipulated to enhance biodegradation. This has already been seen with high SRTs and HRTs, which promote complex, diverse, and resilient microbiological populations, as discussed previously.

Loading and food to microorganism ratios: The food to microorganism ratio (F/M) is a process parameter that indicates the amount of food available relative to the amount

biomass present in a WWTP. Typical values range from 0.4 g substrate g^{-1} $biomass^{-1}$ day^{-1} for extended aeration processes to 1 g substrate g^{-1} biomass day^{-1} for high rate processes (Tchobanoglous et al. 2003). In terms of EE2 removal it is generally believed that lower F/M ratios will correlate with enhanced biological degradation due to the reduction of competing and inhibiting substrates (Kreuzinger et al. 2004; Larsen et al. 2004; Clara et al. 2004b; Melin et al. 2006; Ren et al. 2007).

In 2004, Joss et al. published a study that examined the removal of E1, E2, and EE2 in various municipal WWTPs that were equipped for biological nutrient removal. It was observed that there were low degradation rates for E1 and E2 in the first compartments of the monitored reactors; sludge loading was an important factor because higher loads led to higher concentrations of competing substrates. Kreuzinger et al. (2004) found that high F/M ratios (0.5 kg BOD_5 kg^{-1} TSS^{-1} d^{-1}) were "not suitable" for removing micro-pollutants, including EE2. They suggested that a loading rate of 0.2-0.3 kg BOD_5 kg^{-1} TSS^{-1} d^{-1} be used because this range showed significant removal for most substances; further reduction in F/M ratios did not result in improved degradation rates. Melin et al. (2006) and Clara et al. (2004b) also observed that low sludge loading, when coupled with higher SRTs resulted in improved biological degradation.

Microbial communities: Different microbial communities can develop in AS depending on the type of treatment train. In recent years it has been noted that WWTPs that undergo nitrification could realize enhanced EE2 removal. Anderson et al. (2003) found 90% of EE2 could be removed via biological degradation in nitrifying activated sludge (AS). They concluded that biodegradation was the primary removal mechanism. This is supported by Shi et al. who found that EE2 degradation rates significantly

decreased when nitrification in AS was inhibited, though they allow that some degradation still occurred (Shi et al. 2004). When ammonia (NH_3) was used as the sole energy source, Vader et al. found that EE2 removal rates correlated with nitrification activity (Vader et al. 2000). This correlation was confirmed by another study which showed positive, linear correlation between EE2 biotransformation and nitrification rates (Yi et al. 2007b). More recently, Koh et al. compared removal rates and efficiencies between two CAS plants; one had nitrification/denitrification while the other had nitrification/denitrification with phosphorous removal. These authors found that biodegradation was the primary removal factor and that higher biological activity was observed in the nitrification/denitrification system (Koh et al. 2009).

In another study it was found that EE2 could be successfully metabolized (up to 87% when used as a sole carbon source) by *Sphingobacterium* sp. JCR5. This strain was isolated from the activated sludge of a WWTP treating the waste of an oral contraceptive manufacturing facility (Haiyan et al. 2007). Yoshimoto et al. (2004) was able to isolate four strains of *Rhodococcus* that specifically degrade estrogens using enriched activated sludge from a WWTP. One strain, *R. zopfii* was able to rapidly and completely degrade 100 mg of E2, E1, E3, and EE2, though it must be noted that such high concentrations are not generally observed in WWTP.

Influence of pH and organic matter

Two factors that will affect the ability of estrogens to adsorb onto particles are the presence of organic matter (Yamamoto et al. 2003; Clara et al. 2004b; Neale et al. 2009) and the pH of wastewater (Schaefer et al. 2002; Zhang et al. 2005; Melin et al. 2006; Cirja et al. 2008). The presence of organic matter may be advantageous because of its

contribution to sorption (Cicek 2002; Yamamoto et al. 2003; Holbrook et al. 2004). Literature reports that, generally, higher pH values (pH of approximately 9-10) are disadvantageous due to decreased sorption characteristics (Schaefer et al. 2002; Clara et al. 2004b; Urase et al. 2005). Clara et al. (2004b) investigated the adsorption of E2 and EE2 to activated and inactivated sludge; they found increasing solubility as the pH increased from 7 to 12. Approximately 30 to 50% of the initially adsorbed compounds desorbed at a pH of 10. Cirja et al. (2008) also noted that micro-pollutants, including estrogens, tended to desorb from sludge solids at a pH of 9. Neale et al. (2009) studied the interaction of E1 and E3 with environmentally relevant concentrations of humic and tannic acid at various pHs. It was found that when bulk organic matter was in non-dissociated form sorption was strongest; this corresponded with acidic conditions. In alkaline conditions bulk organics were dissociated and sorption of the hormones decreased considerably. Cirja et al. (2008) notes that adsorption and pH will have implications on sludge dewatering and conditioning with lime, which often results in alkaline conditions (Cirja et al. 2008). The implications of pH and adsorption may therefore be significant for solids treatment.

Temperature

Temperature is an important parameter for WWTPs and can affect reaction rates, biological treatment processes, and activation energies (Tchobanoglous et al. 2003; Price et al. 2004). Many attempts have been made to establish a link between EE2 removal and temperature. It has been suggested that temperature will affect the ability of biosolids to mineralize estrogens (Layton et al. 2000), impact the ability of specific slow-growing bacteria to degrade EDCs (Koh et al. 2008) and may impact nitrification rates, which may

in turn affect biodegradation (Vader et al. 2000; Koh et al. 2008). Warmer temperatures (i.e. 20°C) are considered to be more advantageous and appear to correlate with increased removal rates compared to extremely cold wastewater (i.e. 2°C) conditions (Ternes et al. 1999a; Baronti et al. 2000; Vader et al. 2000; Andersen et al. 2003; Cirja et al. 2008).

1.1.7 Nitrogen removal and its impact on EE2 in wastewater

Secondary treatment with nutrient removal is a method by which biodegradable organics, suspended solids, and nutrients (nitrogen and/or phosphorous) are removed from wastewater using activated sludge. According to Metcalf and Eddy (2003), the most common and crucial forms of nitrogen in wastewater are ammonia (NH_3), ammonium (NH_4), nitrogen gas (N_2), nitrite (NO_2) and nitrate (NO_3). Total nitrogen (TN) is reported as the sum of organic nitrogen, NH_3, NO_2^-, and NO_3^-, with the organic fraction being soluble and/or particulate forms of amino acids, amino sugars, and proteins.

For a typical biological nutrient removal (BNR) plant, nitrogen is eliminated in a two-step process. Fresh wastewater influent carries nitrogen in the form of urea and proteins, where the organic fraction is readily changed to NH_3 by bacteria. In the first step, known as nitrification, the NH_3 is oxidized in the presence of oxygen to NO_2^-. The unstable NO_2^- ion is readily oxidized to NO_3^-, which is considered the most oxidized form of nitrogen. Equation one describes the nitrification process:

Equation 1: $NH_3 + O_2 \rightarrow NO_2 + 3H^+ + 2e^-$

$NO_2 + H_2O \rightarrow NO_3 + 2H^+ + 2e^-$

In the second step, known as denitrification, NO_3 is ultimately reduced in a series of reactions to N_2 gas, as seen in Equation 2. The redox reaction is described in Equation 3.

Equation 2: $NO_3 \rightarrow NO_2 \rightarrow NO + N_2O \rightarrow N_2$

Equation 3: $2NO_3 + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$

This process generally occurs where oxygen, a powerful electron acceptor, is depleted; the bacteria utilize NO_3 as an alternative terminal electron acceptor. In denitrification the source of the electron donor is the chemical oxygen demand (COD) found in wastewater. In some instances, external carbon, such as methanol, is added to facilitate the denitrification process (Ferguson 1994; Tchobanoglous et al. 2003).

While attempting to understand removal mechanisms and identify bacteria responsible for biological degradation, researchers have observed that these systems can reduce appreciable amounts of EA (Svenson et al. 2003; Leusch et al. 2005; Clara et al. 2005b); further study has suggested that it is in the presence of nitrifying (Vader et al. 2000; Johnson et al. 2005) and denitrifying (Matsui et al. 2000 as observed by Andersen et al (2003); Fahrbach et al. 2006) bacteria that biological degradation is enhanced. Vader et al. (Vader et al. 2000) used batch experiments to investigate the ability of nitrifying activated sludge to degrade EE2. They found that degradation was complete after 6 days and that an unidentified metabolite was produced. Once nitrification was stopped biological degradation of EE2 also ceased. Andersen et al. (2003) investigated the fate of E1, E2, and EE2 at a municipal WWTP in Germany. Effluent concentrations were below the limit of detection (1 ng/L) with natural estrogens largely degraded (approximately 98%) in the denitrifying and aerated nitrifying tanks of the activated sludge system; EE2 was only degraded in the nitrifying tank (Andersen et al. 2003). Ren et al. (Ren et al. 2007) who noted that the degradation of E1, E2, and EE2 in nitrifying activated sludge was dominated by the co-metabolism of ammonia oxidizing bacteria. De

Gusseseme et al. supported these findings (De Gusseme et al. 2009) as does Clouzet et al (2010b). Another study found that when MBR sludge was acclimated for nitrification, EE2 removal rates increased from 88% to 99%; the primary removal mechanism was sorption but the increased removal in the acclimated sludge was due to biological degradation (Clouzot et al. 2010a).

While literature suggests that biological degradation will occur in nitrifying/denitrifying conditions, it is not clear if the nitrifying bacteria are solely responsible for the reduction in EA. One study (Shi et al. 2004) investigated the ability of nitrifying activated sludge and ammonia-oxidizing bacteria *Nitrosomonas europaea* to degrade E1, E2, E3, and EE2. It was found that the nitrifying activated sludge was able to follow first-order reaction kinetics to degrade the estrogens; E2 was the easiest to degrade via E1. When studying the ammonia-oxidizing bacteria, it was found that *N. europaea* was also able to degrade the estrogens but E1 was not found during the E2 degradation period; the authors suggested that other heterotrophic bacteria, as opposed to ammonia-oxidizing microorganisms, might cause degradation. Another study noted that while EE2 degradation decreased when nitrification was inhibited some biological removal was still observed (Yi et al. 2007b).

To summarize, the microbial community in activated sludge is diverse with many different genus', species, and strains that may contribute to biological degradation of natural and synthetic estrogens. Degradation rates appear to be significant in nitrifying/denitrifying conditions though it does not necessarily follow that nitrifying/denitrifying bacteria are solely responsible for EDC degradation. It is possible

that unidentified heterotrophic bacteria that thrive in similar conditions also contribute to enhanced removal.

Simultaneous Nitrification Denitrification (SND)

The requirement of oxygen in nitrification and its absence in denitrification has led to the design of WWTPs in which aerobic and anoxic phases exist either in time or in space. In the past, however, nitrogen removal has been observed in systems where denitrification was not specifically incorporated into treatment strategies. In 1987, an Iowa sequencing batch reactor was found to remove approximately 80% of the inorganic nitrogen, despite the fact that operating parameters did not specifically promote denitrification (Irvine et al. 1987). Other studies followed, which confirmed that denitrification could occur in systems where nitrification was also present (Bertanza 1997; Littleton et al. 2009). This has led to the term 'simultaneous nitrification/denitrification' (SND), a process in which nitrification and denitrification proceed concurrently.

There have been several attempts in literature to explain the SND phenomena. One theory, termed the biological theory (Holman et al. 2005), suggested that the heterotrophic bacteria thought responsible for denitrification were poorly understood, and that they could denitrify in aerobic environments. This has been found to be the case by several researchers for a variety of select species (Robertson et al. 1988; Drysdale et al. 1999; Pochana et al. 1999a). Likewise, some have suggested the existence of heterotrophic nitrifiers who are able to denitrify NO_2 and NO_3 (Robertson et al. 1988; Ferguson 1994; Jetten et al. 1997; Chiu et al. 2007). Others have suggested that

autotrophic denitrifiers could exist allowing for denitrification without additional carbon sources (Wiesmann 1994; Sliekers et al. 2002; Holman et al. 2005).

The 'physical theory' of SND is a second hypothesis that has become a more widely accepted explanation of SND processes. This theory maintains that nitrification and denitrification can occur simultaneously because of oxygen diffusion limitations within floc particles. When low concentrations of dissolved oxygen (DO) (i.e. < 2 mg/L) exist in wastewater the nitrifying bacteria, which exist in solution and on the surface of floccules, will still be able to convert available nitrogen to NO_3. The low DO will not, however, be able to penetrate the interior of the floc; this creates an oxygen-free zone where the denitrifiers are able to reduce the NO_3 to N_2 gas, thus completing nitrogen removal. Figure 2, adapted from Pochana et al. (1999b), illustrates this. Various experiments examining the impacts of floc sizes and dissolved oxygen have been performed, which support this theory (Pochana et al. 1999a; Pochana et al. 1999b; Tchobanoglous et al. 2003; Holman et al. 2005).

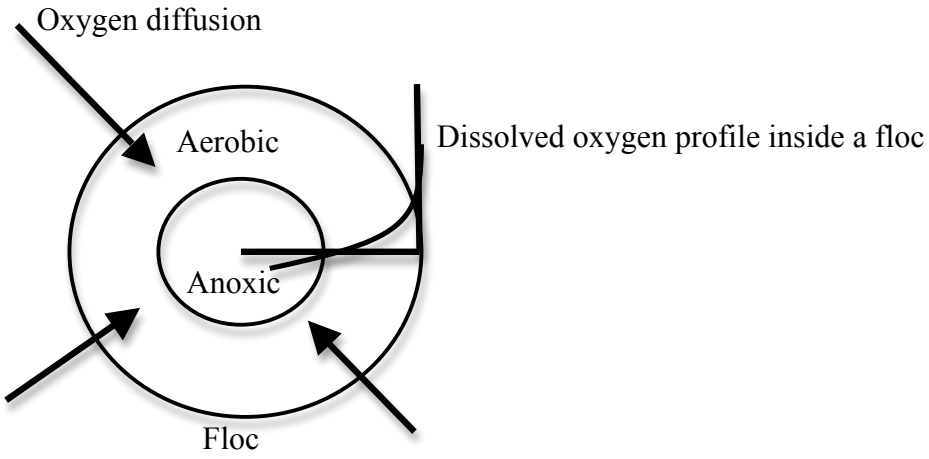


Figure 2. Aerobic and anoxic volume within floc particles (Adapted from (Pochana et al. 1999b)).

A process such as SND would be beneficial because of a reduction in aeration requirements; this would result in economical and energy savings (Annaka et al. 2006). The denitrification process could also provide the alkalinity that is required in nitrification, further reducing chemical costs (Li et al. 2007; Zhu et al. 2007). Such a process may also eliminate the need for separate anoxic/oxic zones in activated sludge systems; internal recycle pumping would be reduced offering more potential savings in design, construction, and maintenance (Tchobanoglous et al. 2003). Conventional activated sludge systems that have to meet new, more stringent nitrogen removal requirements may also find upgrades to SND processes relatively feasible and economically viable, even in cold weather situations (Eusebi et al. 2009; Littleton et al. 2009; McInnis et al. 2009).

Metcalf and Eddy caution, however, that nitrification and denitrification rates in such a system may be less than optimal. The lower DO concentrations would hamper nitrification, while substrate utilization may be difficult for denitrifiers who are limited to

internal anoxic zones in the floc. They note that longer detention times in WWTP would likely accommodate these reduced rates (Tchobanoglous et al. 2003). As with traditional nitrogen removal, there must be adequate carbon sources available for nitrogen to be completely converted to nitrogen gas (N_2) (Pochana et al. 1999b). Another study suggests that the process is fairly flexible. With proper monitoring and vigilance on the part of plant operators the SND process would be effective in certain large scale applications (McInnis et al. 2009).

The exact impact that SND would have on EE2 removal is not well understood. A comprehensive literature review could find no information on the impacts that SND would have on EE2 removal. It is known that anaerobic and anoxic conditions within AS are not conducive to EE2 reduction (Joss et al. 2004; Hashimoto et al. 2009) but when nitrification occurs, enhanced EE2 removal efficiencies are observed. It may be that the lower nitrification denitrification rates observed in SND systems would impact EE2 removal. The combined affect however, of anoxic conditions coupled with nitrification on EE2 concentrations is currently unknown in the wastewater industry. Such information would be useful, especially as concern for EE2 and the popularity of SND are both on the rise.

1.1.8 The role of membrane bioreactors in the treatment of EE2 and the reduction of EA

Membrane bioreactors (MBRs) are a hybrid technology involving biological treatment and membrane separation. Activated sludge and microfiltration (MF) or ultrafiltration (UF) membranes are combined and can be used for solid-liquid separation in industrial and municipal wastewater applications (Tchobanoglous et al. 2003; Yang et al. 2006). The solid-liquid separation mechanism is what differentiates MBRs from CAS

which rely on gravity settling in secondary clarifiers to remove biomass solids (Tchobanoglous et al. 2003; Cirja et al. 2008).

MBR systems are desirous because of their compact design, excellent effluent quality, and reliability (Manem et al. 1996; Melin et al. 2006). They allow for the independent control of solids and hydraulic retention times (SRT and HRT, respectively), have the ability to handle high MLSS loading, and are often associated with reduced sludge production (Cicek 2002; Melin et al. 2006). Removal of viruses and high molecular weight compounds are also possible in these systems though membrane fouling can be problematic for MBR treatment and removal of micropollutants, including estrogens (Dlugolecka 2007). Fouling control, such as aeration scouring, addition of coagulants, and regular maintenance can minimize these impacts ((AWWARF) 1996; Tchobanoglous et al. 2003).

MBRs are suitable for EE2 removal because of their high organic content in mixed liquor, complete retention of colloidal and particular matter and production of particle-free effluent (Cicek et al. 1999; Cicek 2002; Clara et al. 2005a), high concentrations of biomass, and long SRT (25 to 30 days compared to conventional treatment plant's 8 to 25) (Cicek et al. 1999; Cirja et al. 2008). Their smaller floc size, compared to conventional activated sludge systems is thought to contribute to improved mass-transfer conditions that may result in increased sorption of solids and pollutants, including EDCs such as EE2 (Yi et al. 2007a; Cirja et al. 2008). Biological degradation of estrogens may be enhanced in MBRs due to greater concentrations of free swimming bacteria (Cicek et al. 1999; Cirja et al. 2008) and adaptation of microorganisms for the degradation of micropollutants such as estrogens (Wintgens et al. 2002; Kreuzinger et al.

2004; Clara et al. 2005b). While the pore size of UF/MF membranes is 100 to 1000 times larger than that of estrogens (Larsen et al. 2004), some sources suggest that size exclusion due to the biofilm on the membrane surface may be possible (Urase et al. 2005; Khanal et al. 2006; Melin et al. 2006).

MBR systems have the ability to effectively treat high-strength, complex waste streams. Cirja et al. (Cirja et al. 2007) studied the ability of a lab-scale MBR system to treat artificial wastewater designed to mimic effluent from a contraceptives manufacturing facility. The system was seeded with sludge from a contraceptive plant and two different forms of radiolabelled EE2 were used to track degradation pathways. The authors reported removal rates above 80% for EE2, primarily through sorption onto suspended solids and solids attached to the reactor (approximately 70%) and through withdrawal of excess sludge (5%). Lee et al. (Lee et al. 2008) examined different membrane processes and their ability to reduce EA. They compared a MBR, nanofiltration, and reverse osmosis system for the purpose of water-reuse. Various parameters were measured including total organic carbon (TOC), COD, ammonia, nitrate, turbidity, and conductivity. Within the MBR system efficient removal rates were observed. A removal rate of 70% was found for EE2 (comparable to that found by (Cirja et al. 2007)).

1.1.9 Comparison of MBR and CAS systems with respect to EE2 removal

It has been hypothesized that MBRs may be able to remove EE2 to a greater extent compared to a CAS system. Indeed, research regarding EDC removal in MBR systems has often focused on comparing removal efficiencies in MBRs with other activated sludge systems. Comparisons of biomass through batch experiments have examined degradation rates of MBR and CAS sludge. Joss et al. (2004) performed batch

experiments on E1, E2, and EE2 with CAS and MBR sludge. They compared E1 and E2 degradation activities and found that MBR sludge removal was 2-3 times higher (SRT of 12 to 15 days). Analytical limitations could not, however, confirm the increased removal activity in a full-scale plant. In further works the authors suggested that the longer sludge age (or SRT) might have led to a more diverse microculture that enhanced activity; the smaller floc size in the MBR system is also thought to have contributed, though it was noted that higher inert matter accumulation in the MBR system did reduce the biological activity for other pharmaceutical compounds to below that found in the CAS plant (Joss et al. 2006). Yi et al. (Yi et al. 2007a) attempted to characterize the differences of biomass characteristics between CAS and MBR sludge. They found that the MBR's smaller floc size (10 μm compared to 120 μm) and greater particle partitioning led to increased sorption. In their experiment with EE2 the MBR sludge was found to have up to twice as much sorption capacity due to smaller floc size and greater particle partitioning coefficients (K_D values for CAS were 0.24-0.33 L/g compared to 0.33-0.57 L/g for MBR).

Holbrook et al. (Holbrook et al. 2002) examined the ability of a MBR pilot-scale plant to reduce EA. It was operated in parallel with a full scale conventional AS plant at an SRT of 20 – 25 days and an HRT of 8.5 hours; total EA was measured through a YES test. While results indicated that on average the MBR was able to reduce EA to a greater extent than the CAS (73% reduction in the MBR versus 60% in the CAS), the difference was not statistically significant. This was also found to be the case for Ivashechkin et al. (2004) who compared a conventional AS and MBR pilot plant by operating them in parallel with the same influent and sludge loading rate; both performed denitrification

and were operated at SRTs of 12 and 25 days. Results showed no observable difference in removal rates of EE2 in the CAS and MBR plant. Clara et al. (2004a) also found that removal rates for EE2 were similar between a CAS and MBR system (60-70%).

Other comparisons between CAS and MBRs have also found no significant difference between MBR and CAS when operated in similar conditions (Kreuzinger et al. 2004; Johnson et al. 2005; Weber et al. 2005; Clara et al. 2005a) While removal rates are generally comparable between the two systems, MBRs may lend themselves more easily to process configurations that are associated with enhanced EDC removal. Citing high biomass concentration and the ability to handle long SRTs while still maintaining small footprints, Clara et al. (2005a), concluded that MBR systems are a promising technology regarding the removal of micropollutants, including EE2. The overall removal efficiencies of the two systems may be comparable when operated in similar conditions, but the process configurations associated with MBRs may more favorable for EE2 removal. The high SRTs, flexible HRTs, and complete retention of solids are not always feasible in CAS systems particularly where design constraints are imposed (i.e. limited space, high population density, etc.).

1.1.10 MBRs and SND

MBR systems offer several specific advantages for SND. The complete retention of solids in MBRs allows for particulate free, high quality effluent; no nutrients associated with solid flocs are released into receiving waters (Cirja et al. 2008). They can also operate at high biomass concentrations and are able to easily retain slow growing microorganisms such as nitrifiers (De Silva et al. 1998). Their long detention times would also compensate for the reduced nitrification and denitrification rates (Tchobanoglous et al. 2003). These advantages make MBRs conducive to SND

applications. SND may also be a benefit to MBR applications by reducing aeration costs (Yoon et al. 2004; Annaka et al. 2006), though these savings may be negated by increased fouling rates (Arabi et al. 2009).

While mean floc size has been reported to be smaller for MBRs compared to conventional activated sludge (CAS) systems (Zhang et al. 1997; Henriques et al. 2005; Durante et al. 2006) oxygen transfer at high MLSS concentrations appears to be less (Krampe et al. 2003; Manser et al. 2005; Holakoo et al. 2007; Sarioglu et al. 2009). When comparing CAS and MBR flocs Munz et al. found that ammonia oxidizing bacteria (AOB) aggregates were only found on the surfaces of MBR flocs suggesting that anoxic zones may still exist within (Munz et al. 2008). A comprehensive review of literature has suggested that MBRs are able to conduct SND with varying degrees of success. Removal efficiencies range from as low as 33% to greater than 95% in lab, pilot, and full-scale operations (De Silva et al. 1998; Fatone et al. 2005; Holakoo et al. 2007; Chen et al. 2008; Fatone et al. 2008; Arabi et al. 2009; He et al. 2009; Sarioglu et al. 2009).

Arabi et al. (2009) compared two MBRs, one conventional and one with SND. While the application of SND correlated with increased floc size, fouling was significantly higher. Extracellular polymeric substances (EPS) were found to be 20% higher in the SND reactor; cake density on the membrane fibers was 60% higher and fouling rates were 47% greater (Arabi et al. 2009). In contrast Nagaoka et al. (1999) found that EPS degradation by intermittent aeration improved membrane operations (Nagaoka 1999). Other studies could not be found to verify/refute concerns regarding SND and membrane fouling, though one study found that denitrification was associated with higher fouling rates (Jang et al. 2006).

1.2 Research Needs

1.2.1 Removal mechanisms of EE2 in wastewater

It is well established in literature that sorption to solid particles and biological degradation are the two primary removal mechanisms of EE2. There is no clear consensus, however, on the extent to which mechanism is dominant. Some studies report that it is sorption, while others claim that is biological degradation that primarily removes EE2. There is therefore a need for more study to investigate the extent to which each removal method is effective. Such knowledge can help optimize the design and operation of wastewater treatment for the effective removal of EE2.

1.2.2 Simultaneous nitrification and denitrification and EE2

In the past few years there has been a large focus on EE2 removal in nitrifying environments. To the author's knowledge no literature can indicate what affect, if any, SND has on EE2 removal. It may be that the anoxic conditions, which are often associated with ineffective removal will hinder EA treatment. It could also be, however, that the nitrifying environments in the SND system will be able to realize enhanced removal rates. Such information is important as the harmful impacts of EE2 and EA are realized and as SND grows in popularity. Detailed evaluation is required if WWTP are able to remove one pollutant, be it nitrogen or EA, at the expense of the other.

1.2.3 MBRs and EE2 removal

Literature suggests that the longer SRT, HRT, and higher biomass concentration in MBRs should result in enhanced EE2 removal. Actual comparisons between MBRs and CAS are inconclusive; some find no difference whatsoever, while others observe minor increases in removal efficiencies. Further investigation of MBRs may help clarify the extent to which MBRs can treat EE2 and reduce EA.

1.3 Thesis Objectives

An attempt is made in this thesis to address some of the issues described in Section 1.2. The specific objectives of this Masters thesis are as follows:

Objective 1). Evaluate what impacts, if any, SND has on overall nitrogen removal, sludge characteristics and MBR operations

Objective 2). Assess what impacts, if any, SND has on EA reduction in an MBR treating wastewater

2 Methodology

2.1 Experimental set-up

Two bench-scale MBRs, each with a working volume of 3 liters, were operated in parallel with the same feed in similar experimental conditions (Figure 1). Both reactors were seeded with AS from a local WWTP; coarse bubble aeration was applied to the MBRs directly below the membrane for surface scouring and biomass growth. The membranes were submerged, hollow-fiber membranes manufactured by Korean Membrane Separation Ltd. Each had a working surface area of 0.08 m^2 and a pore size of 0.4 µm. Two high-strength magnetic stirrers provided mixing.

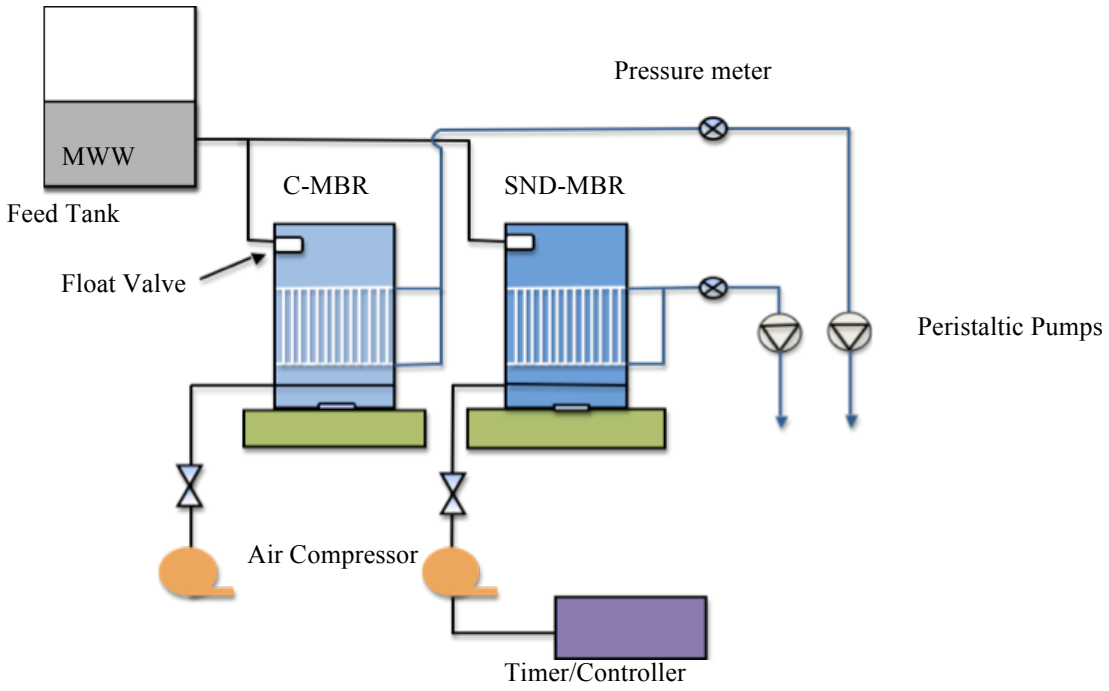


Figure 3: Schematic of the experimental setup

2.2 Operation of MBRs

Objective 1

Both MBRs were operated in constant flux mode via peristaltic pumps. Permeate was withdrawn from outside-to-inside hollow membrane fibers. To control fouling a cyclic pumping mode with 2.5 minutes on and 3.5 minutes off was used. Timers purchased from Fisher controlled the cyclical pumping and SND aeration. Both systems were fed simultaneously with identical synthetic wastewater by gravity; float-valves controlled the water level. The influent was characterized as having a chemical oxygen demand (COD) of 450 mg/L, and an average ammonia-N (NH_3-N) concentration of 20 mg/L. The exact feed composition can be seen in Appendix 7.1. Table 3 is a summary of the MBR operating parameters.

Table 3. Summary of MBR operating parameters

Parameter	Unit	Value
SRT	Days	25
HRT	Hours	12
Temperature	°C	20 – 22
TMP	PSI	0.5 – 5
Pore Size	μm	0.4

A solids retention time (SRT) of 25 days and a hydraulic retention time (HRT) of 12 hours were maintained in both reactors. The SRT was controlled by wasting a portion of the mixed liquor from each bioreactor on a daily basis. During the experimental period the trans-membrane pressure (TMP) was monitored daily. If the TMP was greater than 4 psi, or a noticeable decrease in flux was found, both membranes were cleaned with a 6% bleach solution. Temperature, DO, and pH were measured on a regular basis. Influent and effluent NH_3-N, NO_2^- and NO_3^- concentrations were measured several times per

week. Analysis of particle size, nitrous oxide gas (N_2O) concentration within the headspace, and characterization of microbial species was done periodically.

For the SND-MBR the DO was maintained by running an air compressor in on/off mode. Fifteen seconds of aeration, at 8 litres per minute, followed by five minutes of no aeration resulted in average DO of 0.71 mg/L; this resulted in a minimum and maximum DO value of 0 and 1.5 – 2 mg/L, respectively for each on/off cycle. The C-MBR had continuous aeration at approximately 4 liters per minute resulting in dissolved oxygen (DO) concentrations that were greater than 4 mg/L in the activated sludge (AS).

Objective 2

EE2, purchased by Sigma Aldrich, was dissolved in ethanol and then dosed into the feedstock to give a final influent concentration of 500 ng/L. This concentration was chosen based on the studies of Yang et al. (2009) and while it is higher than typical influent values, it is similar to or less than concentrations used in other studies (Vader et al. 2000; Holbrook et al. 2004; Cirja et al. 2007). Higher EE2 concentrations are generally utilized because typical wastewater values are at the limit of quantification and can be difficult to measure.

2.3 Analytical Methods

Objective 1

Mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sCOD, alkalinity, pH, and DO were all measured according to Standard Methods (APHA-AWWA-WEF 1998). NH_3-N, NO_2^-, and NO_3^- were measured according to Standard Methods (APHA-AWWA-WEF 1998) using a QuickChem 8500 flow-injection analyzer, manufactured by Lachat. Total nitrogen (TN) was considered to

be the sum of NH_3-N, NO_2^-, and NO_3^- concentrations. This assumption was validated by comparing the sum to TN concentrations using HACH® total nitrogen vials (0 – 25 mg/L range) and by HACH Digesdahl® Digestion. For effluent TN values, composite samples were collected over a half hour period, which corresponded with 5 aeration/no aeration events. Nitrous oxide gas (N_2O) in the ambient atmosphere and headspace of both reactors were measured according to Standard Methods (APHA-AWWA-WEF 1998) via gas chromatography using a Varian CP-3800 with an electron capture detector. Particle size analysis was conducted with laser diffraction using a Malvern Mastersizer 2000 particle size analyzer. Transparent exopolymeric particles (TEPS), the acidic fraction of polysaccharides, are known fouling indicators and were used to determine the degree of fouling in both MBRs. The procedure used was based on de la Torre et al. (2008); a step-by-step procedure can be found in the Appendix, Section 8.2.

Fluorescent in-situ hybridization (FISH) analysis was conducted to confirm/deny the presence of certain microbial species within the sludge. To quantify the nitrifiers, two groups of ammonia oxidizing bacteria (AOB) (*Nitrosospira spp*: NSV 443; *Nitrosomonas spp*: NSM 156) and nitrite oxidizing bacteria (NOB) (*Nitrospira*: NTSPA 662; *Nitrobacter spp*: NIT3) were selected for FISH analysis using oligonucleotide probes targeted for 16srRNA sequences. An additional probe was used to quantify the presence of a common SND denitrifier, *Paracoccus* (*Paracoccus spp:* PAR1457). Details of all probes are available at probeBase (Loy et al. 2003). The washing and hybridization method was conducted according to Armann et al. (1995); a Nikon Microscope Eclipse E400 with Image-Pro® Plus software was used to view the slides. A detailed procedure can be found in the Appendix, Section 8.3.

Objective 2

Powder forms of E2 and EE2 were purchased from Sgima Aldrich and were dissolved in ethanol to make 1g/L stock solutions. The E2 was dissolved in ethanol and used to create a standardized curve. The estrogenic activity of the feed, sludge, and effluent was measured according to Yang et al. (2009) who in turn based his method on Routledge et al. (1996). A detailed, step-by-step procedure can be found in the Appendix, Section 8.4.

In brief the feed, the liquid fraction of the sludge, the filtered solid portion of the sludge, and the effluent were extracted using cyclohexane. Samples were mixed with 10 mL cyclohexane and shaken for four hours. Afterwards two to three milliliters of cyclohexane were dried under nitrogen and reconstituted in ethanol. These samples were then placed on a 96-well optically flat bottom microtitre plate. Blank ethanol and E2 standards were also placed on the plate and allowed to dry to air. The plate was then filled with the estrogen-sensitive yeast and allowed to incubate for 72 hours. During this time the yeast releases an enzyme, β-galactosidase, in proportion to the amount of estrogen present. This metabolizes with chlorophenol red-ß-D-galactopyranoside (CPRG) causing the yellow solution to turn different shades of pink, red, or purple, depending on how much estrogen is present. Figure 4 illustrates this reaction. The colour changes were measured using a BioTek Microplate Reader with KC Juniour Software and related back to standard E2 concentrations as E2-eq.

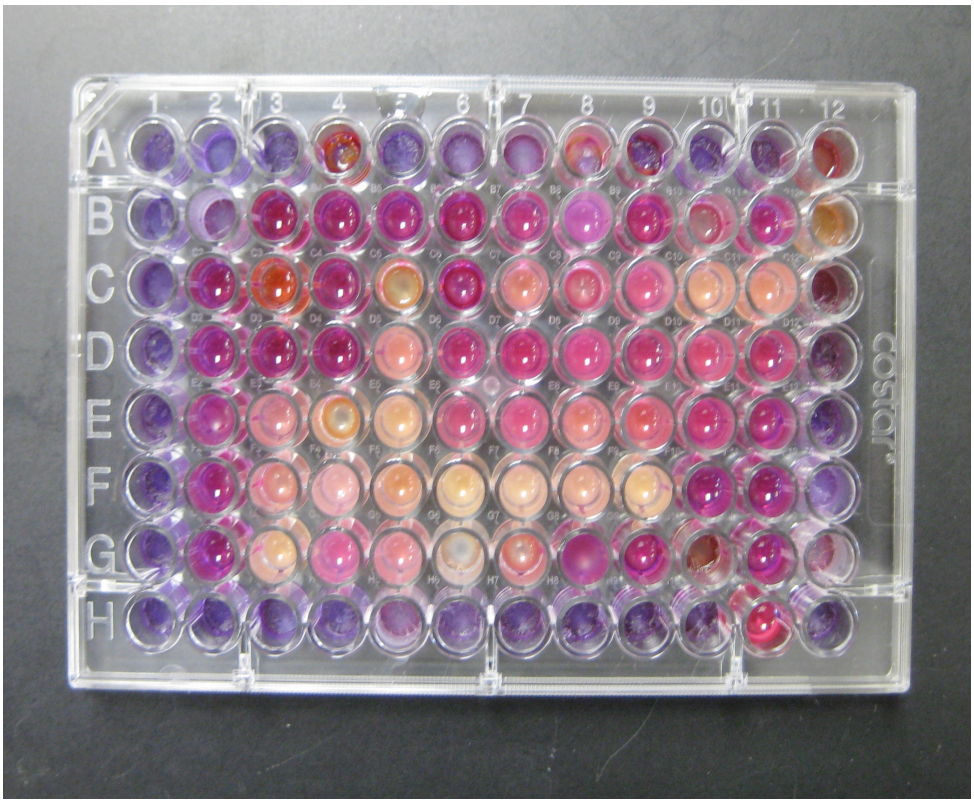


Figure 4. Microplate containing YES bioassay solution and estrogenic water/wastewater samples

3 Results and Discussion

Both reactors were seeded with AS from a local wastewater facility. After 60 days of start-up and acclimation, stable operation and consistent performance was achieved. Both reactors were then run in parallel for over one year. The running averages and standard deviations for various operating characteristics are listed in Table 4. Effluent COD concentrations for both MBRs resulted in removal efficiencies greater than 95%. The pH, alkalinity (data not shown), and MLVSS, were also similar in both reactors.

Table 4. The operating characteristics of the SND-MBR and C-MBR

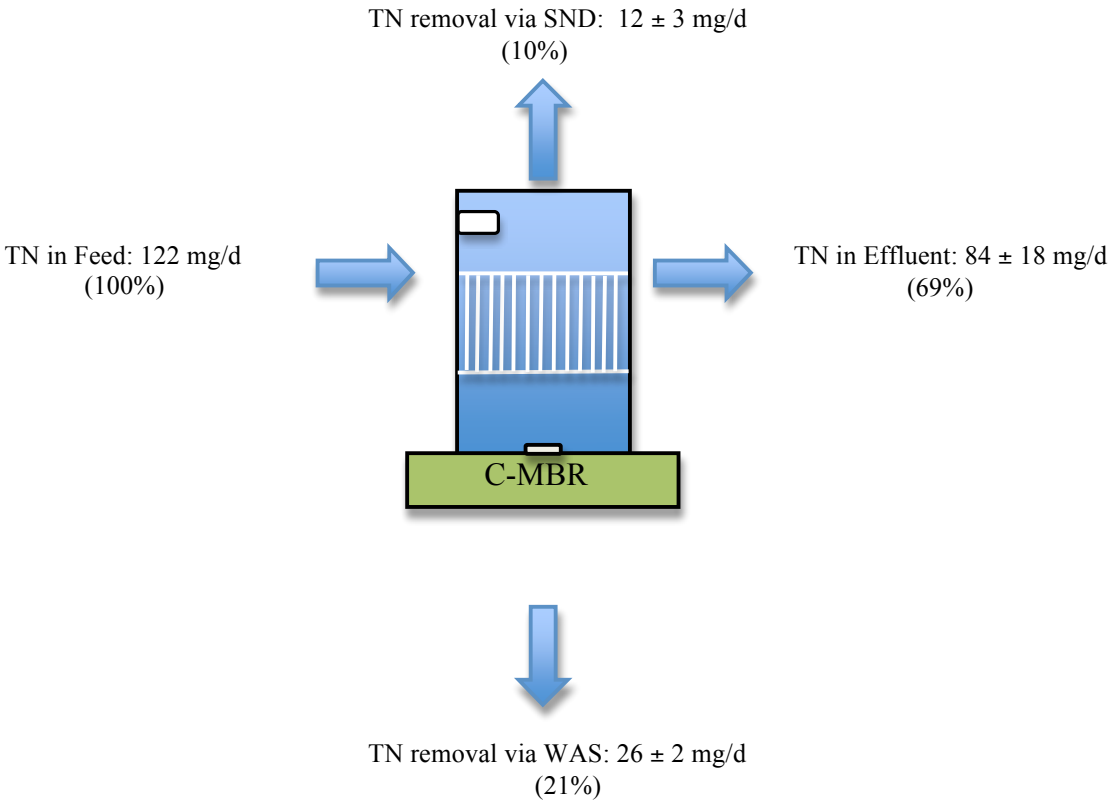
Value	C-MBR	SND-MBR
Dissolved Oxygen (mg/L)	> 4	0 – 1.5
pH	6.9 ± 0.3	6.6 ± 0.2
MLVSS (g/L)	3.9 ± 0.7	4.1 ± 0.9
COD removal (%)	97 ± 2%	97 ± 2%
sCOD (mg/L)	37 ± 20	64 ± 44
Effluent NH_4-N (mg/L)	0.2 ± 0.3	1.2 ± 1.8
Effluent NO_2 (mg/L)	0	0
Effluent NO_3 (mg/L)	13.9 ± 3	2.6 ± 4.6

3.1 Objective 1: Operating Characteristics and Nitrogen Removal

3.1.1 Nitrogen removal

After the stabilization period the SND-MBR was able to remove significant amounts of total nitrogen compared to the C-MBR, as seen in Figure 6. It was assumed that all unaccounted nitrogen was lost via denitrification. Average effluent TN in the SND-MBR was 4.0 ± 4.6 mg/L, with 1.2 ± 1.8 mg/L as NH_3-N; nitrogen existed primarily in the form of NO_3^- with no NO_2^- detected. The C-MBR, in contrast, had

average TN concentrations of 13.9 ± 3.1 mg/L as NO_3^-; little NH_3-N and no NO_2^- was detected. A mass balance for the C-MBR was able to account for 90% of influent TN with 69% of leaving the system as NO_3^- in the effluent; approximately 21% was wasted in the waste activated sludge (WAS). The SND-MBR mass balance was able to account for 41% of influent TN in the effluent and WAS, suggesting that the remaining 59% was removed via denitrification. Figure 5 illustrates these mass balances.



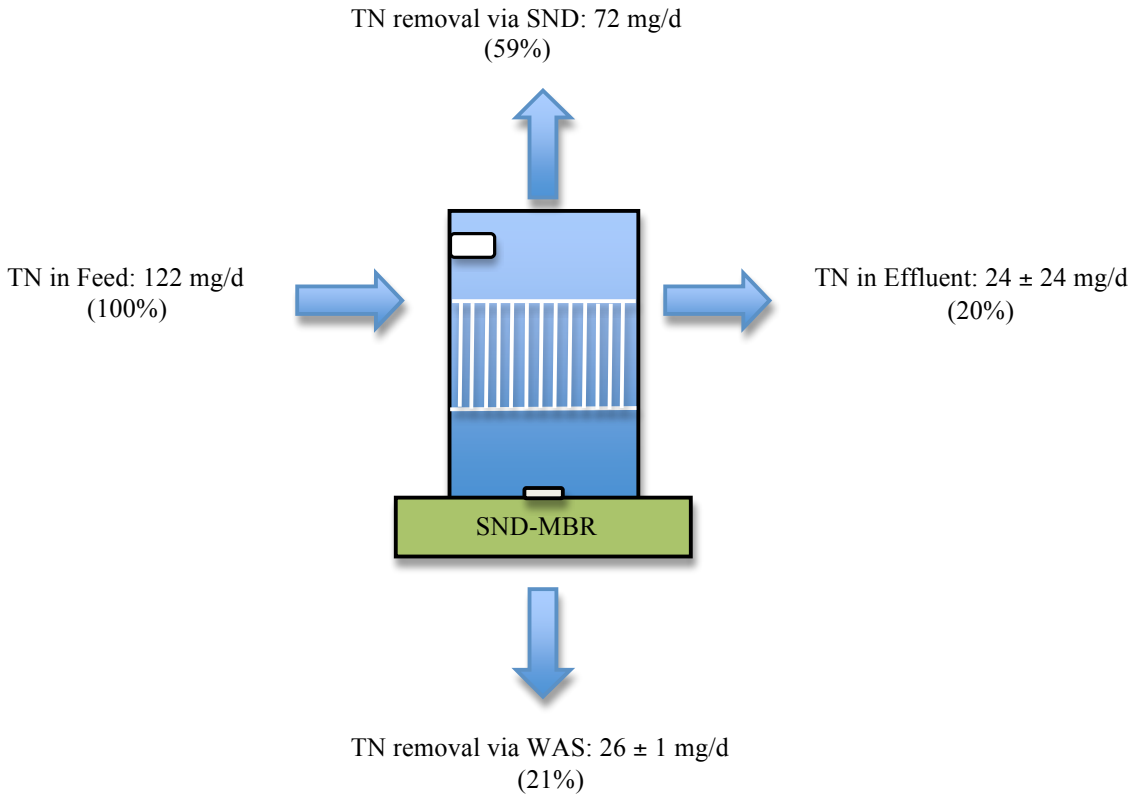


Figure 5: Total nitrogen mass balance for the SND-MBR and C-MBR

As seen in Figure 6, between day 128 and 200, denitrification in the SND-MBR was compromised with effluent TN concentrations doubling to 8.9 mg/L, primarily as NO_3^-. This loss was primarily responsible for the large standard deviation seen in Table 4. During this time, it was noted that there was significant foaming within the SND-MBR. The decrease in denitrification corresponded with an increase in sCOD (104 mg/L), as seen in Figure 7, and average DO concentration (approximately 2 mg/L, compared to 0.75 mg/L); MLVSS also showed a minor decrease during this time. While no significant statistical correlation could be calculated for these variables, the foaming and change in sCOD and DO during this period suggests a temporary reduction in microbial activity

within the system. The lysing of cells is a known cause of foaming in activated sludge systems (Jenkins et al. 2004) and is the likely cause of foaming within the SND-MBR.

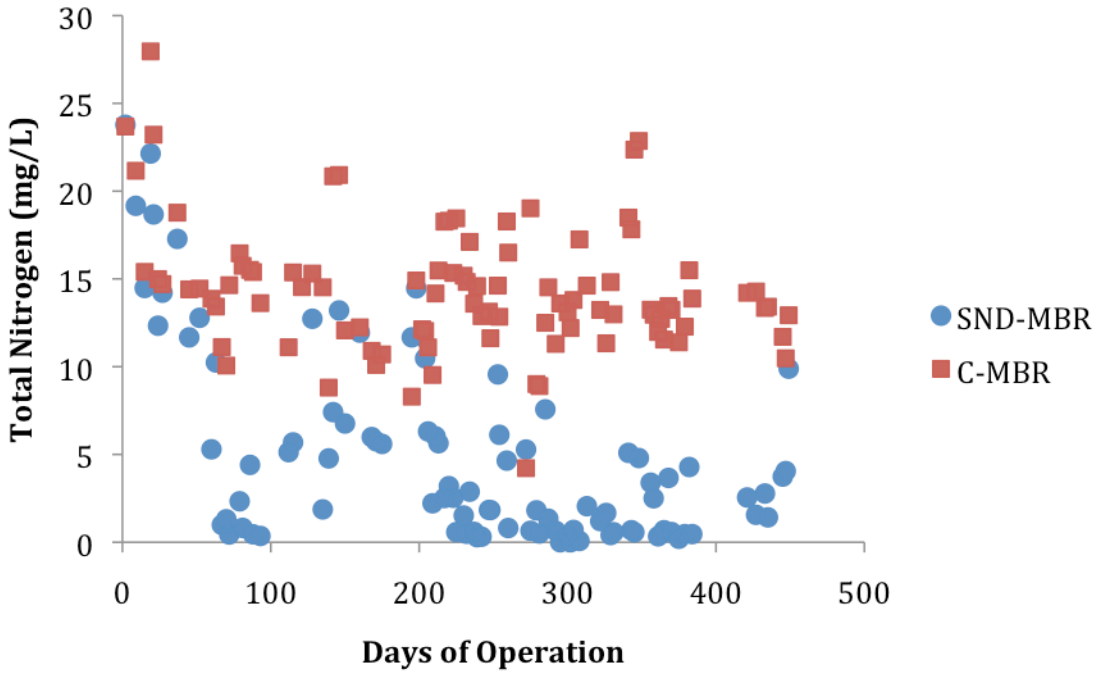


Figure 6. TN profile in the effluents of the SND-MBR and C-MBR

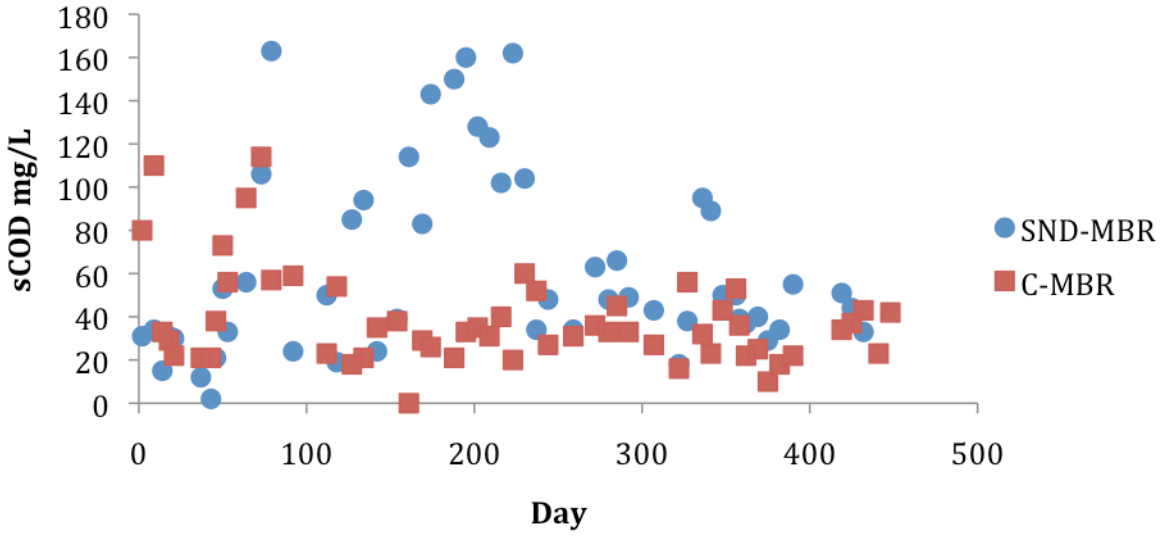


Figure 7. sCOD in the SND-MBR and C-MBR

TEPS during this period also had elevated values (i.e. 25 mg/L Xantham gum during foaming period, compared to 15 mg/L during non-foaming event) suggesting that cell die-off was the likely cause. A decrease in activity would cause a decrease in oxygen and substrate utilization and would likely explain the increase in sCOD and DO. The increase in DO would, in turn, hamper denitrification, which requires anaerobic environments in order to convert NO_3^- to nitrogen gas (N_2).

The removal efficiencies for the SND-MBR, seen in Figure 5, are similar to other findings. Chen et al. (Chen et al. 2008) examined submerged membrane bioreactors (MBRs) by running the system with four different DOs (0.6, 1.2, 3, and 5 mg/L). They found that a maximum TN removal of 90% occurred at a DO of 1.2 mg/L, similar to the 80% TN removal rate observed by the SND-MBR in this study. Chen's findings resulted in less than 2 mg/L total nitrogen (TN) in reactor filtrate (Chen et al. 2008), which is slightly less than the average of 4 mg/L observed in this study. Another study, conducted by Meng et al. (2008) also used a lab-scale MBR to study SND. Batch tests were conducted to determine that an optimal DO range of 0.75 – 1.0 mg/L would be most suitable; this range is in keeping with the average ambient DO of the SND-MBR of 0.75 mg/L. As in this study, the authors were not able to completely eliminate NH_3; their concentrations were greater than 1 mg/L when the DO was below 1.5 mg/L (Meng et al. 2008). Given that the maximum DO in the SND-MBR was 1.5 mg/L, an average NH_3 concentration of 1 mg/L is reasonable. He et al. (2009) also investigated the impact of DO on total nitrogen removal in an MBR system. Their findings were in keeping with Meng and Chen. After testing it was found that a DO concentration of 0.8 and 1.5 mg/L resulted in TN removal efficiencies of 87% and 78%, respectively.

In addition to measuring TN in the feed and effluent, gas samples were also taken from the headspace of the reactors to determine if N_2O gas was generated. Several studies (Fuerhacker et al. 2000; Zeng et al. 2003) have found that incomplete denitrification can produce N_2O, which is an intermediary product in denitrification processes. This can be problematic as N_2O is a potent greenhouse gas and could increase the overall carbon footprint of a wastewater treatment system. At all times the C-MBR showed no detectable amount of N_2O gas in its headspace. The SND-MBR did generate trace amounts of N_2O gas. Concentrations were measured prior to each aeration event, with no N_2O detected following aeration.

Assuming that the gas in the headspace was purged with ambient air during each aeration event, and that the concentration of gas detected just prior to each aeration event was the amount generated in the five-minute cycle, the amount of gas generated was calculated to be 59 μL per day. A mass balance indicates that this is approximately 0.2% of the total denitrified nitrogen. While such small quantities appear to be inconsequential for a lab-scale, three-liter MBR, further research would be needed to determine the exact implications on full scale, mega-liter systems.

3.1.2 Particle Size

In SND systems denitrification occurs primarily within the anoxic interior of flocculent particles. A larger particle size is desirable because it creates larger anoxic volume within the reactor where denitrification could occur (Holakoo et al. 2007). Floc size is also important because of its influence on MBR fouling, due to its ability to bloc membrane pores and release extracellular polymeric substances (EPS) upon floc breakup (Chang et al. 2002). Particle size analysis showed that the distribution of floc sizes within the SND-MBR is weighted towards the 100–1000 μm range with a volume-weighted

mean of 146 ± 28 μm. In contrast the C-MBR had a volume-weighted mean of 89 ± 2 μm; particle sizes were more evenly distributed. An example of the particle distrubtion can be seen in Figures A4 and A5 in the Appendix, Section 8.12.

It should be noted that the average floc size found in the SND-MBR is greater than typical MBR floc sizes, which are generally smaller than conventional activated sludge systems (10-100 μm and 100 – 500 μm for MBRs and CAS, respectively) (Zhang et al. 1997; Pochana et al. 1999b; Huang et al. 2001; Yi et al. 2007a; Cirja et al. 2008). While 146 μm is above average, similar sizes have been observed previously. Lee et al. (2003) found that particle sizes in the 50'th percentile ranged from 58 – 121 μm in size for their MBR. Another study (Lee et al. 2003) found MBR floc sizes as high as 280 μm. Various operating parameters are known to affect floc size and development. Zhang et al. found that in MBRs, larger floc sizes can correspond with higher MLSS concentrations (Zhang et al. 1997; Holakoo et al. 2007). Other studies have found that SRT will also affect floc size (Huang et al. 2001; Koh et al. 2008). Given that these operating parameters (i.e. MLVSS and SRT) were the same for both the C-MBR and the SND-MBR, they are not likely responsible for the discrepancy in floc size. The SND-MBR's larger floc size was likely caused by reduced shear stress on floc particles. The on/off aeration cycles resulted in less mixing and turbulence and, as a result, less shear stress, which is known to impact floc development in MBRs (Wisniewski et al. 1998).

The larger floc size in the SND-MBR may have certain implications for nitrogen and EE2 removal. SND requires an anoxic zone within the centre of sludge flocs where denitrification can occur. The larger anoxic volume found in larger flocs is therefore beneficial because there is more space available where NO_3 can be converted to nitrogen

gas. The greater floc size, however, may not be beneficial for EE2 removal. Generally a smaller floc size is thought to contribute to improved mass-transfer conditions, which results in increased sorption of solids and pollutants, such as EE2 (Yi et al. 2007a; Cirja et al. 2008).

3.1.3 TEPS, TMP, and Fouling

Membrane fouling continues to be problematic for MBR applications because of increased operation costs associated with membrane cleaning and replacement. Figure 8 shows the TMP profile for both the C-MBR and the SND-MBR. Fouling is defined as the point at which the TMP in one or both of the membranes was in excess of 4.5 PSI; the membrane was also said to be fouled if flow through the membrane was significantly reduced (i.e. 10% reduction in flow rates). During the first half of the experiment the SND-MBR was the first to foul. On Day 260 new membranes were installed in both the C-MBR and SND-MBR; performance of the SND-MBR had continually deteriorated and new membranes were required for stable flow through the system. Following this replacement a reverse trend can be seen, in which the C-MBR is the first to foul.

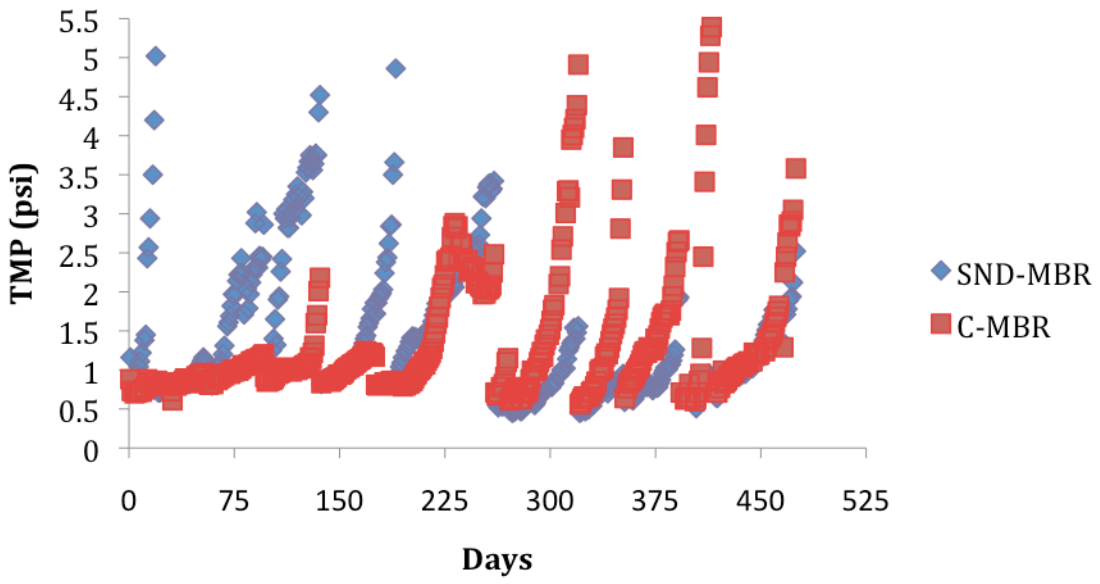


Figure 8 TMP profile of the SND-MBR and C-MBR

Various attempts were made to correlate TMP fouling rates with operating parameters such as sCOD and TEPS. TEPS are the acidic fraction of polysaccharides and can be easily measured using blue dye and related to standard concentrations of Xantham gum. Their sticky nature makes them a potentially useful tool as a fouling indicator (de la Torre et al. 2008). For the C-MBR significant correlation at a 99% confidence interval (CI) was found between TEPS and TMP ($r = 0.8292$, $p = 0.0016$) and TEPS and sCOD ($r = 0.8299$, $p < 0.001$); as the concentration of TEPS increased so did the TMP. The SND-MBR had no significant correlation, which was likely due to intermittent membrane scouring. A buildup of cake layer on the SND membrane surface masked the influence of both TEPS and sCOD, despite the fact that both TEPS and sCOD were elevated during the unstable denitrification period.

The reverse TMP trend for the SND-MBR and C-MBR is an interesting point that bears further discussion. The trend started to happen at Day 232. As discussed in the previous paragraphs, the C-MBR suggests that a relationship can exist between TEPS,

sCOD, and TMP. Generally TMP appears to increase as the sCOD and the TEPS increase. Examining Figure 7 one can see that sCOD in the SND-MBR is higher and more unstable during the first half of the experiment, the half where the SND-MBR tended to foul. TEPS were also found to be highest in the SND-MBR at this time. This would suggest that the TEPS and sCOD would be responsible for fouling the membrane. The first half of the experiment may have been an adjusting period for the SND-MBR where its AS was adjusting to a lower DO range.

This adjusting period could have stressed the microbes within the AS and caused them to release TEPS. This stress would suggest that the AS was not able to utilize substrates efficiently and may explain the higher sCOD values. Examining Figure 6, the TN effluent concentration, one can see that TN removal during the first half of the experiment was varied and unstable. As with sCOD, TN effluent removal improved and followed a steadier trend in the second half of the experiment. Once the microbes had adjusted to the low DO and were removing nitrogen stably, operations improved. It should be noted that while the reverse TMP trend seen in Figure 7 appears striking, there was no significant difference in the cleaning frequencies. The first set of membranes were cleaned every 37 ± 21 days on average; cleaning cycles were determined by the SND-MBR. The second set of membranes were cleaned every 43 ± 15 days; cleaning cycles were determined in this section by the C-MBR. While the acclimatization period for the SND-MBR may have had a slight impact on TMP and fouling, such affects appear minor and do not hinder overall operations. This is especially true once stable operation and nitrogen removal has been achieved.

3.1.4 Fluorescent in-situ hybridization analysis (FISH)

Attempts to identify specific bacteria within the C-MBR and SND-MBR were done for comparison purposes to determine if process changes in WWTP could alter microbial composition within the AS. For the purposes of this thesis, the FISH procedure was used to verify if specific bacteria were present in the activated sludge. Due to the inexactitude and limitations of the visualization software, only general conclusions could be made regarding relative population sizes. In-situ hybridization was used to determine if two ammonia oxidizing bacteria (AOB), *Nitrosomonas* spp. and *Nitrosospira* spp, were present in the AS. Probes were also used to determine if two nitrite oxidizing bacteria (NOB), *Nitrospira* spp. and *Nitrobacter*, species were present. These species were selected because of their nitrifying capabilities and prevalence in nitrifying sludge (Wagner et al. 1996; Mobarry et al. 1997). A bacteria associated with SND, *Paracoccus* spp., was also selected to determine if the SND-MBR could cultivate bacteria associated with SND.

Of all the species tested, the C-MBR is characterized as having primarily *Nitrospira* and *Nitrobacter* species within its AS. Occasional fluorescence occurred with the *Paracoccus* and *Nitrosospira* probes; no *Nitrosomonas* were ever detected. The SND-MBR, in contrast, had greatest fluorescence with the *Paracoccus* spp. (an example of which can be seen in Figure 9) and *Nitrosospira* spp. probes; there was only minor fluorescence with the *Nitrobacter* or *Nitrospira* probes and only on one occasion were very few *Nitrosomonas* highlighted.

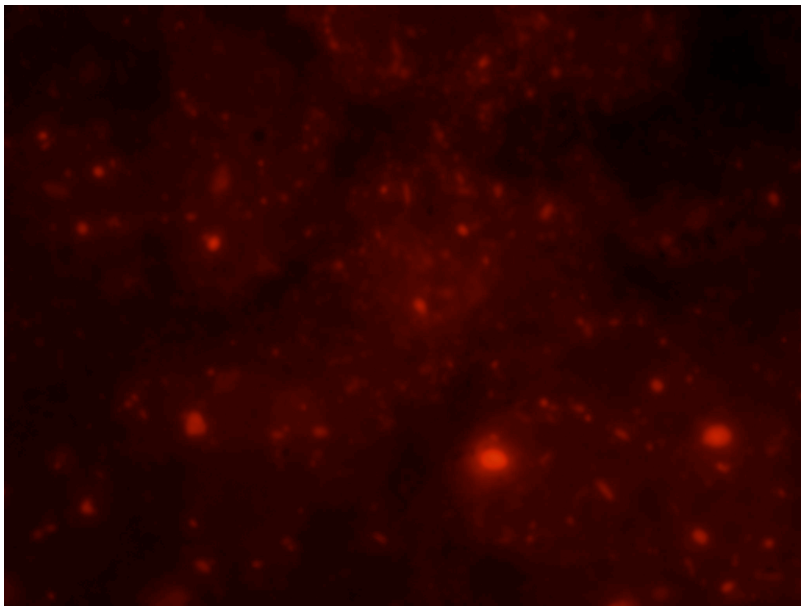


Figure 9 Fluorescing *Paracoccus* spp., as seen in bright red, in the AS of the SND-MBR

Various studies have attempted to identify specific bacteria in different types of AS. Most have focused on bacteria associated with nitrification. Juretschko et al. (1998) had similar bacterial composition to the C-MBR. They examined nitrifying sludge and determined that the dominant species were *Nitrosococcus* and *Nitrospira*; the two species were often found together in co-aggregates. *Nitrobacter* genes were also present in large amounts. Harms et al. found that in a municipal WWTP *Nitrospira* was a dominant NOB at 8.6% of the total bacterial population. Unlike the C-MBR, their dominant AOB was *Nitrosomonas* (Harms et al. 2002).

Further study by Juretschko et al. investigated an industrial, intermittently aerated nitrification-denitrification WWTP. Again *Nitrospira* was present in significant amounts, approximately 12% of the total biovolume (Juretschko et al. 2002). This is in contrast to the SND-MBR, which had only minor reactions with the *Nitrospira* probe. In a different study, Hibiya et al. (2003) used a membrane aerated biofilm reactor to characterize

microbes performing SND. These authors noted that, similar to the SND-MBR, no *Nitrobacter* or *Nitrospira* species were detected within the reactor; a lack of *Nitrobacter* was also reported by Meyer et al. (2005) though they did identify *Nitrosomonas* and *Nitrospira* in small clusters.

The presence of *Paracoccus* in the SND-MBR and its virtual absence in the C-MBR is of particular interest. Certain types of *Paracoccus* spp. are known anaerobic denitrifiers species that have the ability to continue denitrification in aerobic environments when the DO is low (Davies et al. 1989); as the DO increases, *Paracoccus denitrifican* is known to switch from N_2 production to NO_x production (Lloyd et al. 1987); if *Paracoccus denitirifican* is one of the *Paracoccus* species present it would explain the trace amounts of NO_x detected in the headspace of the SND-MBR. The probe used in these experiments, however, is not able to identify *Paracoccus denitrifican* specifically; while it is likely that this bacterium is present in the SND-MBR this conjecture could not be conclusively verified.

It should be noted that FISH analysis can only confirm the presence of probe-specific bacteria. Other bacteria associated with nitrification, denitrification and/or SND that were not tested could be present in the reactors and contribute to nitrogen removal. The results of this thesis do, however, indicate that the bacterial composition in the C-MBR and SND-MBR are different. This suggests that process changes in WWTPs, such as altering DO concentration, can affect the microbial populations within AS, giving certain species advantages over others.

3.2 Objective 2: EA Reduction

The YES test was used to verify the EA of the feed, and to determine the EA in the effluent, the liquid fraction of the WAS, and solid fraction of the WAS for both the SND-MBR and C-MBR. Samples were taken over a six-week period. As with the nitrogen mass balance, unaccounted EA was assumed to be lost via biodegradation; evaporation of EE2 was assumed to be negligible given its relatively low vapor pressure (Clara et al. 2005b; Cirja et al. 2008). Figure 10 shows the resulting EA of various fractions as a concentration in ng/L E2-eq (n=5). The EA of the feed was 755 ± 92 ng/L E2-eq. Using the theoretical EA equivalents listed in Table 1, this value corresponds with an EE2 concentration of approximately 503 – 629 ng/L as EE2. Using a T-test via PRISM statistical software to compare means it was found that there was no significant difference between the EA of the WAS solid fraction for the SND-MBR and C-MBR; the same can be said for the liquid fraction. The effluent EA of the SND-MBR and the C-MBR was 324 ± 23 and 336 ± 56 ng/L E2-eq, respectively. As with the WAS fractions, there was no statistically significant difference in EA between the effluents of the SND-MBR and C-MBR.

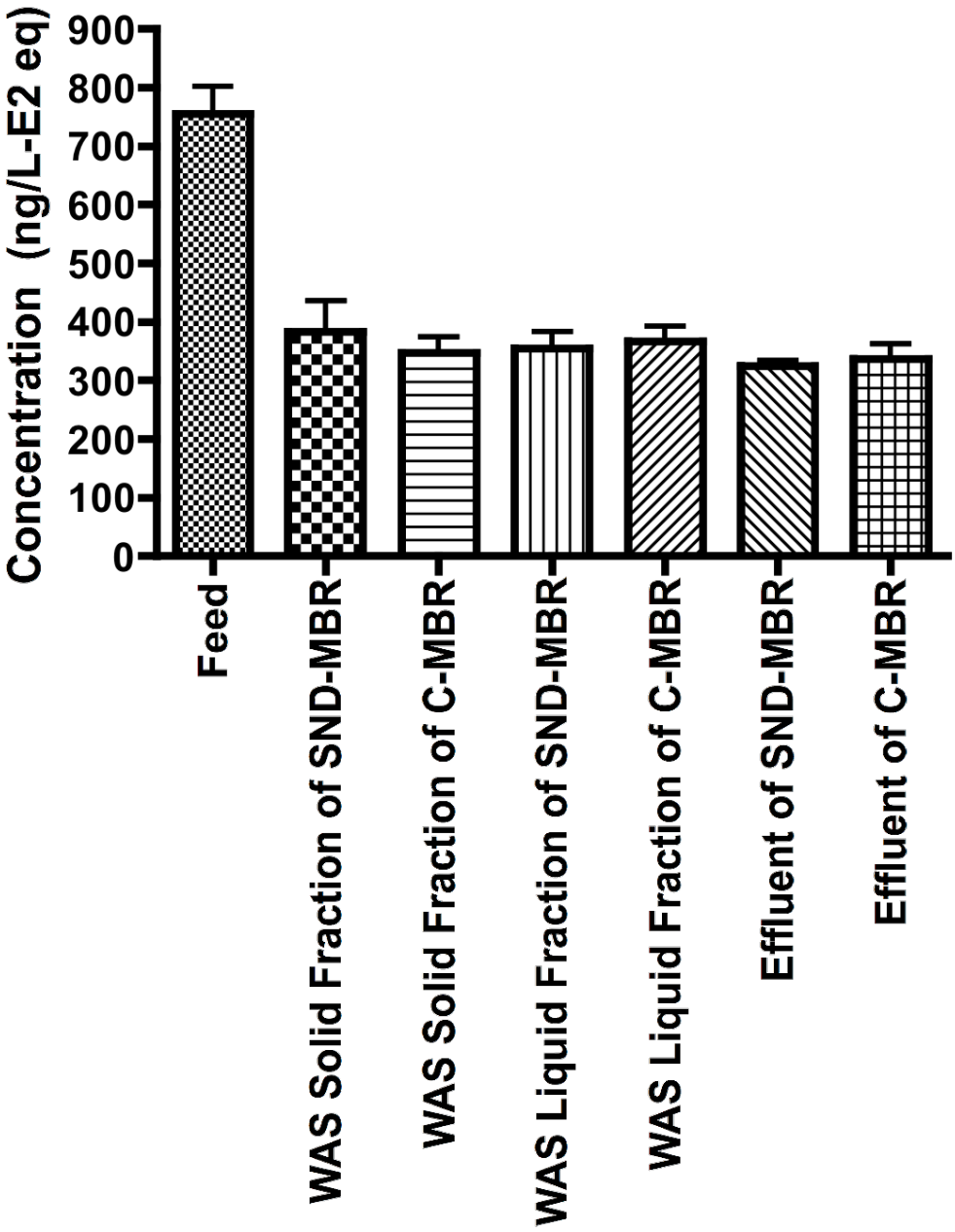
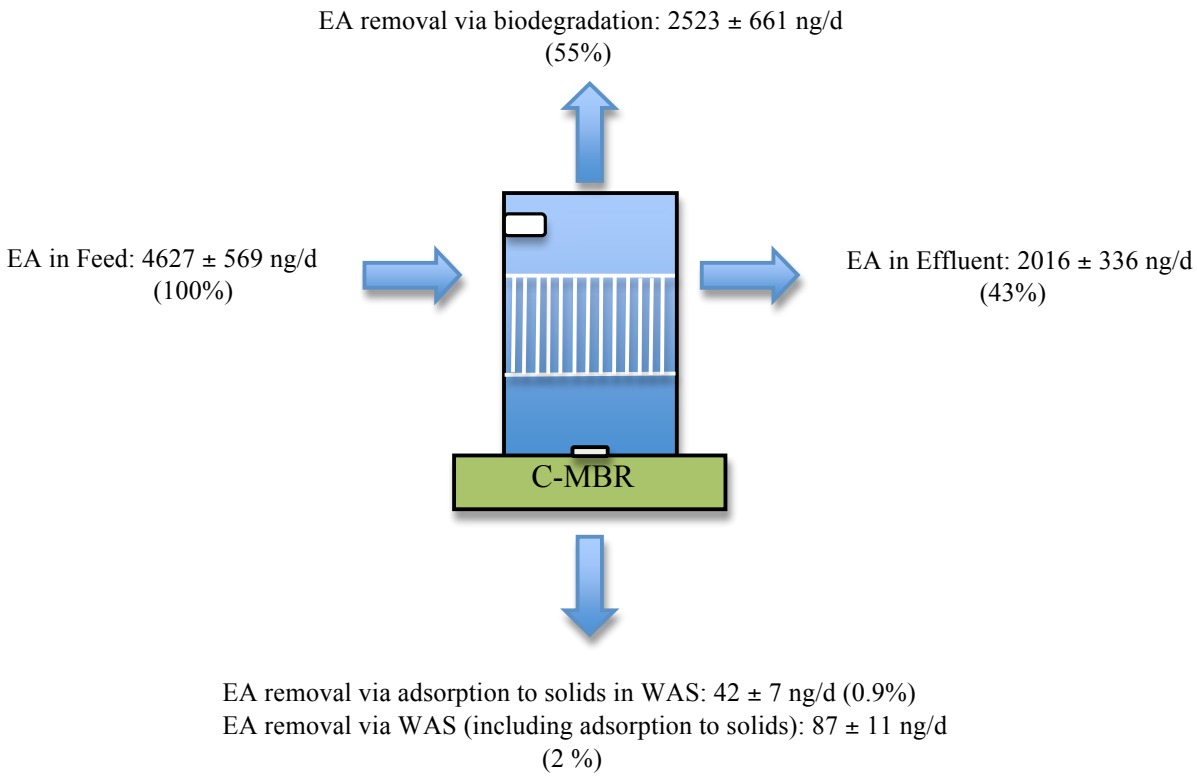


Figure 10 Estrogenic activity of various fractions within the SND-MBR and the C-MBR

Figure 11 illustrates the mass balance for both the C-MBR and the SND-MBR. The C-MBR had a total EA removal efficiency of 57%. The SND-MBR had a total EA removal efficiency of 58%. These results indicate that there was no significant difference between the two MBRs, suggesting that SND did not have any advantageous or adverse

affects on EA removal. The anoxic sludge did not appear to have the same affect on treatment that has been reported for anaerobic sludge (Joss et al. 2004; Hashimoto et al. 2009), and the mass balance indicates that for both MBRS 2% of the EA was removed via WAS; approximately half of this amount was associated with the solid particles within the WAS.

The solids extraction efficiency was 70% (Yang et al. 2008). It may be possible that a small portion of EA removal attributed to biodegradation could be EA that was not completely extracted from WAS solids. The large discrepancy between removal via adsorption to solids (0.9%) and removal via biodegradation (55% and 56% for the C-MBR and SND-MBR, respectively), though, indicates that in this study the primary removal mechanism is via biological degradation. Any possible discrepancy due to the extraction efficiency would not have a large impact on the mass balance, given the relatively small amount found adsorbed to the solid particles.



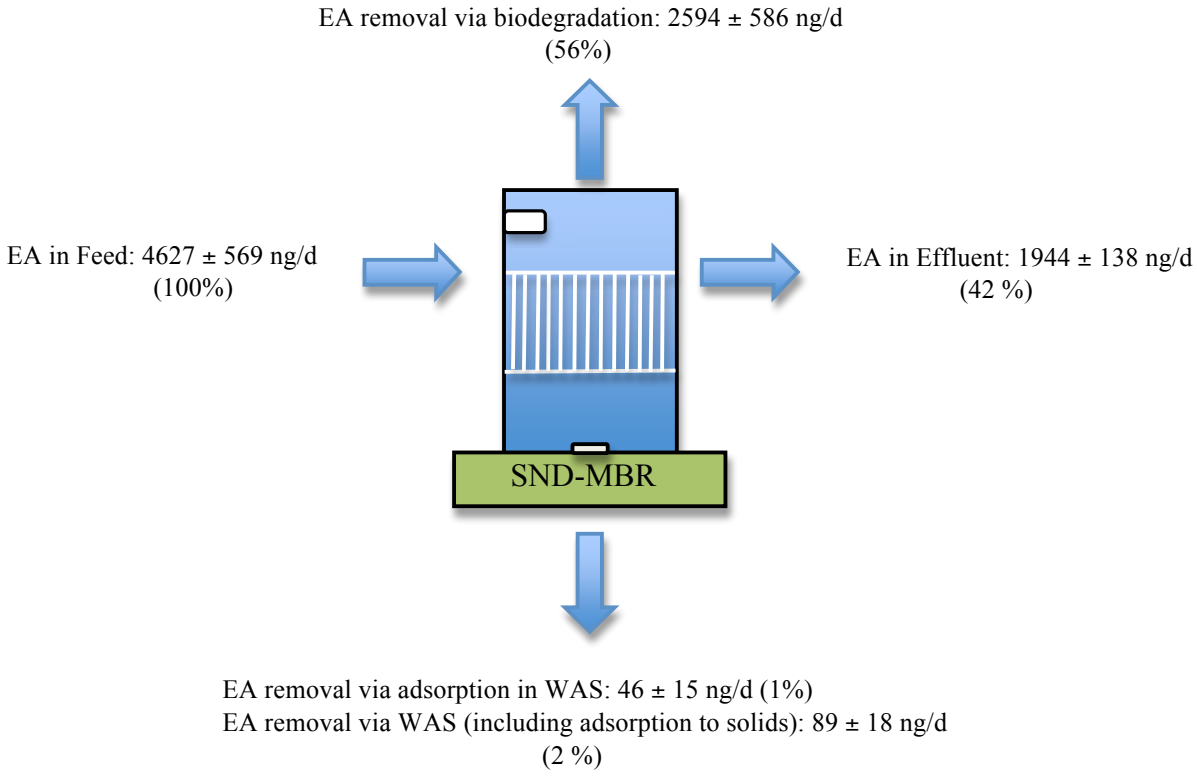


Figure 11. A mass balance of EA in the C-MBR and the SND-MBR

Various studies have discussed how full scale, pilot scale, and laboratory systems can treat EE2 in wastewater. The majority of these studies examine removal based on actual EE2 concentrations and not on EA reduction. Full scale and pilot scale WWTPs using real wastewater have been known to remove as little as 10% of influent EE2 (Ternes et al. 1999a) though typical removal rates are generally in the 60 – 80% range (Johnson et al. 2001; Esperanza et al. 2004; Clara et al. 2004a; Johnson et al. 2005; Cui et al. 2006). In these instances typical EE2 influent concentrations are generally between 1 – 10 ng/L as EE2 though values as high as 155 ng/L have been observed (Cui et al. 2006). Lab scale MBRs generally have similar ranges of removal efficiencies. Clara et al. used lab scale MBRs to investigate EE2 removal. A mass balance revealed that generally 60 – 80% of influent EE2 was removed (Clara et al. 2005b). A different study used a lab scale MBR to treat EE2 and found that 60-70% of EE2 was removed, though influent concentrations were relatively high at 100 μg/L (Urase et al. 2005).

Comparisons between the above mentioned studies and this study are difficult to make. The majority of those studies use chemical analyses, such as gas chromatography and mass spectrometry to measure actual EE2 concentrations. These methods, while useful, fail to take into account possible degradation byproducts, such as E2, which can still contribute to overall EA. A 90% reduction in EE2 may not necessarily result in a 90% reduction in EA. The wide range of influent concentrations used by researchers further complicates comparisons. Many studies using laboratory setups dose EE2 into wastewater at 100 – 250 μg/L concentrations (Ivashechkin et al. 2004; Urase et al. 2005; Cirja et al. 2007); this level is far greater than the 500 ng/L used in this study. Cirja et al., for example, dosed 100 μg/L into their MBR and found an 80% reduction in EE2 (Cirja

et al. 2007). This removal efficiency is greater than the removal efficiency found in the SND-MBR and C-MBR. Using the flow rates given in their study to calculate the daily effluent load, however, results in an average value of 160 μg/day as EE2, which is far greater than the 2 μg/day as E2-eq found in this study. This illustrates that higher removal efficiency does not necessarily indicate better treatment in terms of total EA reduction.

There are several studies however, that do use the YES assay to determine EA reduction in MBRs. Layton et al. found that in a full scale MBR only 40% of the EA of radiolabelled EE2 was mineralized; 20% of EA remained in the effluent, compared to 42% and 43% found in this study (Layton et al. 2000). In another study Yang et al. dosed 500 ng/L of EE2 into a three-litre MBR. A total removal efficiency of 71% was observed, with most of the influent EA (67%) being biologically degraded; only 4% was removed via sludge wasting. Operating conditions were similar to those found in this study but a slightly higher MLSS/MLVSS might explain his enhanced removal efficiency (Yang 2009).

Joss et al. found that EE2 biodegradation kinetics at low concentrations follow pseudo-first-order rates (Joss et al. 2004). Sorption kinetics of EE2 are known to follow the Freundlich Isotherm model (Clara et al. 2004b; Lee et al. 2009). Using the method described by Joss et al. (2004) and the results from Figure 10 the biological degradation constant (K_{BIO}) and the particle partitioning coefficient (K_D) was calculated for each reactor. The results, as well as other values found in literature, are summarized in Table 5; results from this study are shown italicized. Both the SND-MBR and C-MBR had similar coefficients. The C-MBR had a slightly lower K_{BIO} compared to the SND-MBR,

which is in keeping with the results from Figure 11. The values of both K_D and K_{BIO} for the C-MBR and SND-MBR, while on the lower end, are still in keeping with values reported in literature.

Table 5 Summary of the K_{BIO} and K_D values of the C-MBR and SND-MBR

Particle partitioning coefficient (K_D)		
Unit	**Value**	**Source**
L/(g MLSS)	*0.25 ± 0.07*	*C-MBR*
L/(g MLSS)	*0.31 ± 0.10*	*SND-MBR*
L/(g MLSS)	0.33 - 0.57	(Yi et al. 2007a)
L/(g MLSS)	0.28 - 0.35	(Ternes et al. 2004)
L/(g MLSS)	0.58 - 0.14	(Andersen et al. 2005)
L/(g MLSS)	1.43	(Yang 2009)
Biological degradation constant (K_{BIO})		
Unit	**Value**	**Source**
day^{-1}	*1.5 ± .6*	*C-MBR*
day^{-1}	*1.6 ± 0.4*	*SND-MBR*
day^{-1}	1.4 - 5.36	(Urase et al. 2005)
day^{-1}	1.5 - 6	(Joss et al. 2004)
day^{-1}	4.41	(Yang 2009)

3.3 Summary

3.3.1 Objective 1

The SND-MBR was able to remove 59% of influent TN via SND processes with an additional 21% removed via sludge wasting. The C-MBR, in contrast, had a TN removal efficiency of only 31%. There was no significant impact of SND processes on membrane fouling though the SND process was associated with higher concentrations of fouling indicators such as sCOD and TEPs. Particle size analysis showed that the SND-MBR had a volume weighted mean of 146 ± 28 μm; the C-MBR had a smaller mean at 89 ± 2 μm. An investigation of microbial populations within the AS revealed that process changes, such as low DO conditions, can affect the microbial populations within AS;

certain species may gain advantages over others depending on environmental conditions. This was the case in this study, where the SND-MBR encouraged the development of *Nitrsospira* and *Paracoccus* species; the C-MBR favoured the development of *Nitrospira* and *Nitrobacter* species.

3.3.2 Objective 2

The C-MBR and SND-MBR removed 57% and 58% of influent EA, respectively; there was no significant difference in their removal efficiencies. Biodegradation was the dominant removal mechanism for both reactors with K_{BIO} coefficients of 1.5 ± 0.6 and 1.6 ± 0.4 $days^{-1}$ for the C-MBR and the SND-MBR, respectively. Adsorption removed approximately 1% of influent EA in each reactor; the particle partitioning coefficient, K_D, was calculated to be 0.21 ± 0.07 L/(g MLSS) for the C-MBR and 0.27 ± 0.1 L/(g MLSS) for the SND=MBR.

4 Conclusion

4.1 Objective 1

The first objective of this thesis was to evaluate what, if any, impacts SND has on sludge characteristics, nitrogen removal, and MBR operations. The findings of this study show that both the C-MBR and SND-MBR had similar sludge characteristics and operating conditions. While the SND-MBR was associated with larger flocs and higher fouling indicators during unstable SND periods, this did not result in a noticeable detriment to important operating parameters such as TMP. Nitrogen removal was enhanced in the SND-MBR, indicating that SND processes could be an alternative form of nitrogen removal in the future.

4.2 Objective 2

The second objective of this thesis was to evaluate what impacts, if any, SND has on EA reduction in an MBR treating wastewater. The findings of this study indicate that the SND-MBR and C-MBR were both able to reduce EA to the same extent; K_{BIO} and K_D values were comparable in both systems. Biological degradation was the primary removal mechanism in both reactors with sorption playing a minor, secondary role.

5 Engineering Significance

The findings of this study have potential implications for how nitrogen and EE2 are removed from wastewater. In this report SND was found to have no significant impact on membrane operations, indicating that the two technologies can be combined in a novel way to treat wastewater. If SND and MBRs are able to satisfactorily work together savings could be realized by reducing aeration requirements for the activated sludge. MBRs are associated with higher operating and maintenance costs so reducing aeration requirements would be of benefit and make MBR technology more competitive in the future. The findings also imply that SND technology can be successfully applied to wastewater treatment processes and can remove significant amounts of nitrogen without constructing costly, separate anoxic/oxic zones. Wastewater treatment plants that need to upgrade for nitrogen removal may realize greater savings by utilizing SND over traditional nitrogen removal processes.

A large portion of this thesis focused on EE2 removal from the SND-MBRs. Findings indicated that removing nitrogen via SND processes did not have an affect on EA treatment. As the extent of the environmental impacts of EA are realized, it is important to evaluate which technologies are capable of successfully reducing EA. If, in the future, EA limits are mandated in wastewater treatment it appears that plants utilizing SND and/or SND-MBR technology will still be able to treat EA to similar extents as plants with conventional layouts.

6 Further Recommendations

6.1 Objective 1

The findings of this study are based on two MBRs that were operated for approximately 450 days; four membrane modules were used (two for each reactor). Further study is recommended to investigate the stability and robustness of SND processes. It may be that over a longer period of time the incidences of higher TEPs and sCOD values could hinder MBR operations. Repeated exposure to these foulants over a long period of time (i.e. years) may result in higher cleaning rates and greater incidences of membrane fouling. Preventative measures should also be investigated to ensure that the SND process is robust and can withstand the natural fluctuations of wastewater temperature, flow, and influent characteristics.

6.2 Objective 2

EA reduction was based on five data points collected over a six-week period. No information on EA reduction was available when sCOD and TEPs were high, or during the period when denitrification was compromised (during Days 128 – 200). Further study is recommended to investigate what impact, if any, these incidences have on EA reduction. While the biological degradation and sorption coefficients for the C-MBR and SND-MBR were similar, the SND-MBR did have slightly enhanced removal rates. Further study is recommended to determine if this is a result of variability within the data or if SND processes do have slightly enhanced removal rates.

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8 Appendix

8.1 Components of the synthetic wastewater

Table A1: Composition of synthetic wastewater; recipe obtained from Dr. Wenbo Yang (Yang 2009)

Component	Unit	Concentration
$FeCl_3$ $6H_2O$	mg/L	7.0
KH_2PO_4	mg/L	60
$NaHCO_3$	mg/L	200
$(NH_4)_2SO_4$	mg/L	120
$CaCl_2$ $2H_2O$	mg/L	12
$MgSO_4$	mg/L	38
Glucose	mg/L	400
Yeast Extract	mg/L	10
EE2	ng/L	500

8.2 Transparent Exopolymeric particles (TEP) Method

8.2.1 Materials:

Filter Paper Schleicher & Schuell Black Ribbon
Alcian Blue solution 1% in 3% acetic acid (Clin Tech LTD)
Natrium Acetate (NaAc) 99%
Acetic acid (Ac) 99.8%

8.2.2 Reagents

Preparation of Alcian Blue solution

- Prepare Alcian blue solution (1%) in 0.3% HAC solution by dissolving 1 gram Alcian Blue 8GX and 0.3 ml HAc in 100 mL flask; fill flask with deionized water

Preparation of acetate buffer solution (pH = 4, 0.2 M)

- 1 liter buffer solution by adding the following; dilute to 1 litre
  - 9.75 mL in Ac
  - 2.4526 g NaAC
- Adjust pH with HAc if necessary to get a pH of 4

Preparation of the Alcian Blue solution 0.055% (m/v)

- Put 5.5 ml of the alcian blue solution 1% in a 100 mL flask and make up the volume with the acetate buffer solution. Shake the flask always before use

8.2.3 Calibration and Protocol

Preparation of the calibration curve

1. Prepare a stock solution of 100 mg/L xanthan gum in acetate buffer solution
   a. This should be done day before and left on stir plate overnight
2. Prepare reference solutions from 1 to 20 mg/L xanthan gum in acetate buffer solution from the stock solution
3. Mix in a centrifuge tube:
   a. 5 mL of sample (sludge filtered through a filter paper Schleicher and Schuell Black Ribbon), 0.5 mL of the prepared alcian blue solution 0.055% (m/v) and 4.5 mL of acetate buffer solution
4. Vortex for 1 min
5. Centrifuge at 15300 rpm (23292 xg) for 10 minutes at 25C
6. Measure the absorbance of the supernatant at 602 nm DIRECTLY after centrifugation. The blank used is acetate buffer solution

Protocol for high concentrated samples (20-100 mg TEP/L)

- Dilute the sample 5:1 by taking only 1 mL of sample instead of 5 mL and add 4 mL more of acetate buffer solution in the centrifuge tube. The concentration will be then multiplied by 5 after measuring it with the spectrophotometer

Recommendations:

- It is recommended to use the alcian blue solution only for one month after its preparation, as the dyee aggregates and its absorbance decreases with time.
- The use of the stock solution of 1% Alcian blue is quite useful and avoids the dilution of the alcian blue in powder form
- The calibration curve will change depending on the alcian blue supplier and batch

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8.3 FISH Method

8.3.1 Media and Reagents

Washing 10% KOH solution:

1. Dissolve 20 g KOH in 200 mL of autoclaved de-ionized (DI) water or ethanol

Gelatin Solution

1. Dissolve 0.1 g gelatin and 0.01 g $KCr(SO_4)_2$ in 100 mL autoclaved DI water
2. Stir on hot plate for approximately 15 minutes; take care that solution does not rise above 60°C
3. Cool down

1X Phosphate Buffered Saline (PBS) solution:

1. Dissolve the following in 800 mL DI water
    a. 8 g NaCl
    b. 0.2 g KCl
    c. 1.44 g Na_2HPO_4
    d. 0.24 KH_2PO_4
2. Adjust pH to 7.4 with 10M HCl
3. Add DI to 1 L
4. Autoclave 20 min
5. Store at room temperature

Four percent paraformaldehyde fixative solution (PF)

1. Dissolve 2 g of paraformaldehyde in 50 mL of 1X PBS solution on hot plate; do not let solution rise above 60°C
2. Add 20 μL of 10N NaOH
3. Allow to cool and adjust pH to 7.2 using concentrated HCL
4. Filter with 0.2 μm cellulose filter
5. Store in 4°C fridge and use within 24 hours

Tris HCl solution (20 mM)

1. Dissolve 78.8 trishydroxymethyl aminomethane hydrochloride in 500 mL of DI water
2. Adjust to 7.2 pH with 10N NaOH
3. Store in fridge

DEPC treated DI water

1. Add o.5 mL of diethyl pyrocarbonate (DEPC) to 500 mL DI water
2. Seal with aluminum foil and stir overnight
3. Autoclave for 45 minutes, store at room temperature (can be stored indefinitely with foil intact)

Hybridization buffer (20 mL)

1. Start with 5 mL of 3.6M NaCl (final concentration will be 0.9 M)
2. Add X mL of formamide
    a. X amount specific to each probe and given as FA% by the supplier (i.e. for 30% probe take 0.3*20 = 6 mL formamaide)
3. Add 0.01 mL of 20% sodium dodecyl sulfate (SDS) to create 0.01% in final solution (SDS 20% purchased from BioRad laboratory #161-0418)
4. Add 0.4 mL Tris HCL solution
5. Fulfill solution to 20 mL with DEPC treated DI water
6. Store hybridization buffer at 46 °C

Washing buffer (WB)

1. Prepare in the same way as the hybridization buffer. Instead of using formamide, use adequate volume of 3.6 M NaCl (formamide is expensive and can be replaced with NaCl for washing procedure). Store at 46 °C

8.3.2 Procedure

The procedure is a four-day test; preparation of slides and hybridization are the limiting steps due to the time of reaction with overnight incubation. Preparation of probes, placing microbial material on slides, and hybridization has to be done within one day. The final preparation of slides and capturing of pictures should be done on the same day as well.

Day 1

1. Preparing gelatin coated slides
    a. Pour washing 10% KOH solution into autoclaved box and soak new slides for 1 hour
    b. Wash slides twice in autoclaved DI; do not touch the surface
    c. Dip each slide in the gelatin solution making sure to coat both sides. Shake and put in slide box to dry vertically. Store indefinitely in 4°C fridge

Day 2

1. Sampling and slide fixation
    a. Dilute 1 mL of actiated sludge with DI water
    b. Mix with syringe 50 times to break up flocks
    c. Transfer 3 mL of diluted and mixed sample to 15 mL centrifuge tube
    d. Add 9 mL 4% PF solution
    e. Shake well and leave for 45 – 60 minutes to fixate samples
    f. Resuspend sample with vortex
    g. Take 2 mL of resuspended sample and filter it with black filter on the vacuum station; filter some autoclave DI through filter afterwards to get rid of the PF (vacuum apparatus should be autoclaved before use)
    h. Sample will stay on filter; remove the filter carefully and stick the side with the sample to your slide. Press the Kimwipe and let stand for 10 minutes. Pull off filter carefully; cells will be transferred to gelatin coated slide
2. Preparation of probes (done in dark with red lamp)

a. Purchased probes must be stored in -80°C fridge; take them out 2 hours before test and shaw them in 4°C in ice (DO NOT EXPOSE TO LIGHT).
b. Use 12 μM concentration for each probe: i.e. 3.8 μL probe in 100 μL HB
c. Transfer to vial and vortex to mix
d. Smear 100 μL of probe/HB solution on slide (slide must be wet!)
e. Dab tissue paper/kim wipe with 0.9 NaCL and place in conical tube. Put smeared slide in tube on top of tissue paper. Cap loosely and incubate at 46°C overnight (at least six hours)

Day 3

1. Rinsing the slide/probe
    a. After incubation take slide from tube; ensure slide is still wet. Shake off excess liquid
    b. Rinse once with washing bufferusing a pipette and then immerse the whole slide in the WB solution. Place back in incubator for an additional 20 minutes
    c. If more than one probe is to be placed on each slide you have to repeat probe preparation; multiple probes can be used so long as they have the same washing buffer and different dyes to distinguish between them
2. DAPI staining
    a. Ensure DAPI concentration of 5 μg/mL
    b. Apply 50 μL of DAPI on the dry slide and smear it; let stand in darkness in room for 5 minutes
    c. Remove excess DAPI by shaking slide; rinse twice with autoclaved DI and let stand to dry
3. Slide Finishing
    a. Put 5 drops of mounting oil on slide and cover with slip, if desired.
    b. Place under microscope and observe: slide should be viewed and captured as soon as possible. Storage, if necessary, should be done in the dark at 4°C

8.4 Yeast Estrogen Screen (YES) Method

8.4.1 Media and Reagents

RINSE ALL GLASSWARE, SPATULAS, STIR BARS TWICE WITH ETHANOL AND LEAVE TO DRY

Minimal Medium (pH 7.1)
Add the following to 1 L Milli-Q water; place on heated stirrer to dissolve

13.61 g	KH_2PO_4
1.98 g	$(NH_4)_2SO_4$
4.2 g	KOH pellets
0.2 g	$MgSO_4$
1 ml	$Fe_2(S0_4)_3$ solution (40 mg/50 ml H_20)
50 mg	L-leucine
50 mg	L-histidine
50 mg	adenine
20 mg	L-arginine-HCl
20 mg	L-methionine
30 mg	L-tyrosine
30 mg	L-isoleucine
30 mg	L-lysine-HCl
25 mg	L-phenylalanine
100 mg	L-glutamic acid
150 mg	L-valine
375 mg	L-serine

Dispense 45 ml aliquots into glass bottles (125 ml×22).
Sterilize at 121˚C for 15 min, and store at room temperature.

D-(+)-Glucose
Prepare a 200 ml 20% w/v solution

1. Add 40 g glucose to 200 ml Milli-Q water.
2. Sterilise in 20 ml aliquots (60 ml glass bottles × 10) at 121˚C for 15 min.

Store at room temperature.

L-Aspartic Acid
Make a 100 ml stock solution of 4 mg/ml.

1. Add 0.4 g L-Aspartic Acid into 100 ml Milli-Q water.
2. Sterilise in 20 ml aliquots (60 ml glass bottles × 5) at 121˚C for 15 min.

Store at room temperature.

10X Concentrated Yeast Stocks

Day 1

1. Add the following to 45 mL minimal medium (final solution is called the 'growth medium)
    a. 5 mL glucose solution
    b. 1.25 mL L-aspartic acid solution
    c. 0.5 mL vitamin solution
    d. 0.4 mL L-threonine solution
    e. 125 μL copper (II) sulfate solution
2. Transfer to a sterile conical flask (final volume: 50 mL)
3. Add 125 μL of 10X concentrated yeast stock from cryogenic vial stored at -20°C
4. Incubate at 28°C in water bath for approximately 24 hours on an orbital shaker, or until turbid

Day 2

1. Prepare growth medium (see growth medium recipe)
2. Transfer to two conical flasks with final volume of 50 mL
3. Add 1 mL yeast from 24-hr culture to each flask
4. Incubate at 28°C in water bath for approximately 24 hours on an orbital shaker, or until turbid

Day 3

1. Transfer each 24-h culture to a sterile 50-mL centrifuge tube
2. Centrifuge at 4°C for 10 minutes at 2000 g.
3. Decant the supernatant and resuspend each culture in 5 mL of minimal medium with 15% glycerol
    a. Add 8 mL sterile glycerol to 45 mL minimal medium
4. Transfer 0.5 mL aliquots of 10X concentrated stock culture to 1.2-mL sterile cryovials (x20)
5. Store at -20°C for maximum of 4 months

Vitamin Solution

Add the following to 90 mL Milli-Q water

4 mg	thiamine
4 mg	pyridoxine
4 mg	pantothenic acid
20 mg	inositol
10 ml	biotin solution (2 mg/100 ml H_2O)

Sterilize by filtering through a 0.2-μm pore size disposable filter, in a laminar air flow cabinet. Filter into sterile glass bottles in 10 ml aliquots (20 ml glass bottles × 10). Store at 4˚C.

L-Threonine

Prepare a 50 ml solution of 24 mg/ml.
Add the following to 50 mL Milli-Q water

1.2 g L-Threonine

Sterilise in 10 ml aliquots (20 ml glass bottles × 5) at 121˚C for 10 min.
Store in fridge at 4˚C.

Copper (II) Sulfate

Prepare a 25 ml 3.2 mg/ml solution.
Add the following to 25 mL Milli-Q water

0.08 g $CuSO_4$

Sterilise by filtering through a 0.2-µm pore size filter, in a laminar flow cabinet. Filter into sterile glass bottles (20 ml × 5) in 5 ml aliquots.
Store at room temperature.

Chlorophenol red-ß-D-galactopyranoside (CPRG)

Make a 10 ml 10 mg/ml stock solution.
Add 100 mg CPRG into 10 ml MIlli-Q water.
Sterilise by filtering through a 0.2-µm pore size filter into sterile glass bottle (20 ml× 1) ,in a laminar flow cabinet.
Store in fridge at 4˚C.

Preparation of Standards

1. Prepare the 17ß-estradiol (E2) stock solution in absolute ethanol, at 50 µg/L
    a. Add 50 mg E2 in 10 ml absolute ethanol to make a 5 mg/ml solution.
    b. Add 0.1 ml 5 mg/ml solution in 9.9 ml absolute ethanol to make a 50 mg/l solution.
    c. Add 10 µl of 50 mg/l solution into 10 ml absolute ethanol to make a 50 µg/L solution.

Serially dilute solutions at concentrations of 5 ng/L to 50 µg/L

8.4.2 Assay Procedure

Solvent Extraction

1. Centrifuge influent and WAS at 10,000 g for 15 minutes
2. Decant supernatant and filter through .45 um GF/C filters
    a. Filters should be pre-burned in a 550 oven to remove EA
3. Collect 20 mL of filtered sample (for liquid fraction) and the solids left behind (for solid fraction)
    a. Add 10 mL cyclohexane to each fraction
    b. Shake for 4 hours on vertical shaker
4. Extract 5 mL of cyclohexane from each conical flask and dry down under N_2 stream
5. Reconstitute in 0.5 mL absolute ethanol for YES assay

Plating and yeast growth

Day 1

1. Prepare growth medium by adding the following to 45 mL minimal medium
    a. 5 mL glucose solution
    b. 1.25 mL L-aspartic acid solution
    c. 0.5 mL vitamin solution
    d. 0.4 mL L-threonine solution
    e. 125 µL copper (II) sulfate solution
2. Transfer to sterile conical flask (50 mL volume). Add 0.25 mL of 10X concentrated yeast stock from cryogenic vial
3. Incubate at 28°C for approximately 24 hours on an orbital shaker or until turbid

Day 2

1. Add 0.5 mL CPRG to 50 mL fresh growth medium
    a. Seed this with 2 mL yeast from 24-hr culture prepared day before
        i. For every 2.5 assay plates, prepare 50 mL assay medium
2. Transfer 10 µL water samples to a 96-well optically falt bottom microtitre plate
    a. Add 200 µL of seeded assay medium (containing CPRG and yeast) using a multichannel pipette

    ! Each plate should contain at least one row of blanks (solvent and assay medium only)
    ! Each assay should have a standard curve
3. Seal the plates with parafilm wrapping and shake vigorously for 2 minutes on a titre plate shaker
4. Incubate at 32°C in an incubator for 72 h

Day 5

1. Shake plates for 2 minutes and leave for approximately 1 hr to let yeast settle
2. Read the plates at an absorbance of 540 nm (optimum absorbance for CPRG 575 nm) and 620 nm (for turbidity) using a plate reader
3. If necessary leave plates at room temperature and read later

    ! To correct for turbidity apply the following equation to the data:

    Corrected value = ABS(540 nm) – [$ABS_{chem.}$(620 nm) – ABS_{blank} (620 nm)]

8.5 TN DATA Summary

Table A2 Nitrogen Data for the SND-MBR (R1) and the C-MBR (R2)

Day of Operation	NH4 (mg/L N)			NO2 (mg/L N)		
	Feed	R1	R2	Feed	R1	R2
		0.03	0.03		0.00	0.00
		0.00	0.23		0.10	0.06
		0.53	0.29		0.10	0.05
2	21.70	2.50	1.67	0.22	0.22	0.23
9	20.60	1.38	0.30	0.12	0.17	0.14
15	15.70	0.06	0.14	0.26	0.20	0.20
19	19.20	0.00	0.00	0.12	0.08	0.06
21	14.20	0.34	0.27	0.00	0.00	0.00
24	24.80	0.71	0.15	0.06	0.05	0.04
27		0.00	0.00	0.05	0.05	0.06
37	23.70	0.36	0.05	0.00	0.03	0.02
45	19.50	0.17	0.00	0.10	0.12	0.07
52	14.80	0.12	0.00	0.03	0.06	0.02
60	12.60	0.04	0.02	0.03	0.04	0.02
63	21.80	0.09	0.00	0.02	0.24	0.02
67	22.40	0.58	0.11	0.02	0.22	0.03
70	22.10	0.21	0.02	0.04	0.05	0.03
72	21.30	0.27	0.02	0.02	0.02	0.02
79	23.80	0.42	0.13	0.03	0.04	0.03
81	14.90	0.71	0.00	0.02	0.02	0.04
86	21.90	0.25	0.00	0.02	0.02	0.01
88	17.90	0.19	0.00	0.03	0.03	0.03
92	23.90	0.28	0.00	0.00	0.00	0.00
112	30.40	0.56	0.19	0.00	0.00	0.00
115	21.70	0.23	0.00	0.91	0.91	0.92
121	33.40	1.20	0.13	0.00	0.00	0.00
127	13.50	0.18	0.00	0.00	0.00	0.00
135	19.20	0.00	0.00	0.25	0.29	0.29
139	14.50	0.00	0.00	1.22	1.21	1.20
142	22.90	0.52	0.16	0.02	0.00	0.00
146	22.40	0.30	0.02	0.00	0.00	0.00
150	28.10	2.20	1.04	0.00	0.00	0.00
160	13.60	0.21	0.02	0.01	0.00	0.00
168	23.10	2.05	1.14	0.11	0.02	0.01
171		0.00	0.00		0.00	0.00
175	22.60	0.28	0.00	0.21	0.05	0.00
195	14.90	0.81	0.32	0.03	0.06	0.03
198	27.40	0.97	0.51	0.00	0.04	0.02

202	10.20	0.81	0.43	0.00	0.02	0.01
204	23.90	1.35	0.79	0.03	0.02	0.02
206	24.00	1.00	0.60			
209	24.00	2.18	0.25	0.01	0.00	0.00
211	25.30	6.03	1.01	0.02	0.00	0.01
213	24.70	5.06	1.07	0.04	0.02	0.02
217	22.40	2.51	0.16	0.00	0.00	0.00
220	22.20	2.73	0.49	0.00	0.06	0.21
223	13.00	2.30	0.09	1.26	0.23	0.14
225	20.00	0.58	0.30	0.00	0.00	0.00
230	20.70	0.00	0.00	0.00	0.12	0.02
232	23.10	0.44	0.23	0.00	0.00	0.00
234	10.50	0.38	0.14	0.36	0.28	0.06
237	22.00	0.56	0.04	0.00	0.03	0.03
239	23.50	0.29	0.08	0.00	0.00	0.00
241	22.50	0.31	0.12	0.00	0.00	0.00
244	9.82	0.07	0.00	0.00	0.00	0.00
246	24.00	0.46	0.20	0.07	0.06	0.04
248	23.00	1.79	0.22	0.23	0.00	0.00
253	21.90	9.56	0.32	0.02	0.00	0.00
254		6.09	0.00	0.00	0.00	0.02
259	24.10	0.42	0.14	0.02	0.02	0.01
261	12.30	0.36	0.00		0.00	0.00
272	11.70	1.12	0.00	0.00	0.00	0.00
275	21.80	0.35	0.12	0.00	0.00	0.00
279	21.80	1.70	0.14	0.00	0.00	0.00
281	16.40	0.40	0.20	0.00	0.00	0.00
285	16.70	7.57	0.00	0.00	0.00	0.00
287	23.20	1.34	0.02	0.00	0.00	0.00
292	19.40	0.65	0.00	0.00	0.00	0.00
295	21.40	0.00	0.00	0.00	0.00	0.00
299	21.10	0.36	0.00	0.00	0.00	0.00
301	19.20	0.00	0.00	0.00	0.00	0.00
303	20.80	0.00	0.00	0.00	0.00	0.00
307	14.30	0.00	0.00	0.10	0.04	0.05
313	20.60	1.97	0.00	0.03	0.00	0.02
322	21.40	0.46	0.13	0.04	0.06	0.05
326	14.00	1.55	0.40	0.10	0.12	0.10
329	21.50	0.31	0.05	0.09	0.09	0.10
331	22.00	0.55	0.23	0.04	0.01	0.03
334	48.50			0.11	0.07	1.17
336	33.10			0.05	0.03	0.06
338	22.10			0.31	0.06	0.04
341	22.60	4.10	0.29	0.09	0.96	0.11
343	21.10	0.54	0.20	0.12	0.11	0.07

345	21.90	0.40	0.74	0.05	0.03	0.48
348	14.10	4.64	0.23	0.14	0.10	0.11
356	21.70	3.24	0.13	0.04	0.09	0.01
358	19.00	1.70	1.61	0.00	0.81	0.00
361	19.30	0.26	0.03	0.00	0.00	0.00
363	18.50	0.32	0.05	0.02	0.02	0.00
365	20.10	0.51	0.12	0.02	0.00	0.00
368	13.50	3.49	0.00	0.00	0.00	0.00
370	20.90	0.30	0.03	0.12	0.13	0.00
375	20.30	0.19	0.00	0.04	0.00	0.00
379	21.00	0.31	0.06	0.08	0.03	0.03
382		4.21	0.10			
384	20.40	0.30	0.00	0.04	0.02	0.03
421	21.50	2.51	0.21	0.07	0.04	0.03
427	20.00	1.44	0.18	0.04	0.06	0.03
433	19.60	0.27	0.04	0.01	0.02	0.02
435	25.10	1.36	0.02	0.03	0.03	0.03
445		0.11	0.00		0.06	0.06
447	14.70	0.17	0.00	0.00	0.00	0.00
449		0.03	0.03		0.04	0.00

Day of Operation	NO3 (mg/L N)			Total N (mg/L N)		
	Feed	R1	R2	Feed	R1	R2
		26.20	37.70		26.23	37.73
		15.89	14.96		15.99	15.25
		11.92	14.31		12.54	14.66
2	0.00	21.06	21.78	21.92	23.78	23.68
9	0.00	17.62	20.72	20.72	19.16	21.16
15	0.00	14.23	15.07	15.96	14.48	15.40
19	0.00	22.06	27.90	19.32	22.14	27.96
21	0.05	18.33	22.94	14.25	18.67	23.21
24	0.00	11.58	14.79	24.86	12.33	14.99
27	0.00	14.16	14.65		14.22	14.71
37	0.04	16.89	18.71	23.74	17.28	18.77
45	0.00	11.38	14.33	19.60	11.67	14.40
52	0.00	12.61	14.43	14.83	12.78	14.45
60	0.08	5.21	13.84	12.70	5.29	13.87
63	0.13	9.91	13.40	21.95	10.24	13.42
67	0.11	0.19	10.98	22.53	0.99	11.12
70	0.09	1.05	10.01	22.23	1.31	10.06
72	0.08	0.16	14.60	21.41	0.44	14.64
79	0.13	1.87	16.30	23.96	2.33	16.46
81	0.11	0.09	15.70	15.03	0.82	15.74
86	0.02	4.14	15.50	21.93	4.41	15.52
88	0.02	0.24	15.36	17.95	0.45	15.39

92	0.08	0.08	13.62	23.98	0.36	13.62
112	0.41	4.57	10.92	30.81	5.13	11.11
115	0.00	4.54	14.45	22.61	5.69	15.37
121	0.05	27.70	14.40	33.45	28.90	14.53
127	0.04	12.55	15.31	13.54	12.73	15.31
135	1.42	1.58	14.23	22.96	1.87	14.52
139	0.00	3.57	7.61	15.72	4.78	8.81
142	0.05	6.89	20.69	22.96	7.41	20.85
146	0.02	12.90	20.90	22.42	13.20	20.92
150	0.21	4.56	11.03	28.31	6.76	12.07
160	0.00	11.72	12.22	13.61	11.94	12.24
168	0.00	3.92	9.75	23.21	5.99	10.90
171		5.75	10.10		5.75	10.10
175	0.00	5.27	10.70	22.81	5.60	10.70
195	0.07	10.80	7.94	15.00	11.67	8.29
198	0.04	13.46	14.38	27.45	14.47	14.91
202	0.05	10.88	11.69	10.25	11.71	12.13
204	0.11	9.11	11.23	24.04	10.48	12.04
206	0.08	5.31	10.50	24.08	6.31	11.10
209	0.04	0.04	9.28	24.05	2.22	9.53
211	0.00	0.00	13.15	25.32	6.03	14.17
213	0.00	0.56	14.39	24.74	5.64	15.48
217	0.06	0.01	18.10	22.46	2.52	18.26
220	0.06	0.41	17.62	22.26	3.20	18.32
223	0.00	0.00	15.11	14.26	2.53	15.34
225	0.00	0.00	18.15	20.00	0.58	18.45
230	0.00	1.40	15.16	20.70	1.52	15.18
232	0.07	0.04	14.60	23.17	0.48	14.83
234	0.00	2.22	16.91	10.86	2.88	17.11
237	0.00	0.00	13.52	22.00	0.59	13.59
239	0.00	0.00	14.52	23.50	0.29	14.59
241	0.00	0.00	12.76	22.50	0.31	12.88
244	0.02	0.72	15.72	9.84	0.79	15.72
246	0.04	1.32	12.90	24.12	1.84	13.14
248	0.03	0.04	11.40	23.26	1.83	11.62
253	0.00	0.00	14.30	21.92	9.56	14.62
254	0.00	0.03	12.82		6.12	12.84
259	0.06	4.21	18.12	24.17	4.65	18.27
261	0.00	0.45	16.50	12.30	0.81	16.50
272	3.41	4.16	4.22	15.11	5.28	4.22
275	0.39	0.31	18.90	22.19	0.65	19.02
279	0.15	0.12	8.87	21.95	1.82	9.01
281	0.11	0.10	8.70	16.51	0.50	8.90
285	0.00	0.00	12.50	16.70	7.57	12.50
287	0.00	0.00	14.50	23.20	1.34	14.52

292	0.00	0.00	11.30	19.40	0.65	11.30
295	0.00	0.00	13.60	21.40	0.00	13.60
299	0.00	0.00	13.10	21.10	0.36	13.10
301	0.03	0.00	12.20	19.23	0.00	12.20
303	3.69	0.70	13.80	24.49	0.70	13.80
307	0.08	0.03	17.20	14.48	0.07	17.25
313	0.13	0.08	14.60	20.77	2.05	14.62
322	0.06	0.69	13.05	21.50	1.21	13.23
326	0.01	0.00	10.83	14.11	1.67	11.33
329	0.02	0.01	14.67	21.61	0.41	14.82
331	0.00	0.01	12.73	22.04	0.57	12.98
334	0.64	0.00	9.32	49.25		
336	0.00	0.00	15.20	33.15		
338	0.00	0.00	22.00	22.41		
341	0.02	0.04	18.09	22.70	5.09	18.49
343	0.01	0.03	17.56	21.23	0.68	17.82
345	0.08	0.13	21.15	22.03	0.57	22.37
348	0.76	0.06	22.51	15.00	4.80	22.86
356	0.06	0.06	13.11	21.80	3.39	13.25
358	0.05	0.00	11.35	19.05	2.51	12.96
361	0.10	0.08	11.95	19.40	0.34	11.98
363	0.01	0.12	12.68	18.54	0.47	12.72
365	0.08	0.18	11.43	20.20	0.68	11.55
368	0.02	0.17	13.46	13.52	3.66	13.46
370	0.16	0.15	13.21	21.18	0.58	13.24
375	0.00	0.01	11.39	20.34	0.20	11.39
379	0.00	0.12	12.18	21.08	0.46	12.28
382		0.07	15.40		4.28	15.49
384	0.11	0.14	13.85	20.55	0.46	13.88
421	0.10	0.00	13.97	21.68	2.55	14.21
427	0.01	0.07	14.07	20.04	1.56	14.28
433	2.50	2.49	13.28	22.11	2.78	13.34
435	0.02	0.03	13.37	25.15	1.42	13.42
445		3.57	11.64		3.74	11.70
447	0.00	3.88	10.48	14.70	4.05	10.48
449		9.82	12.90		9.89	12.93

8.6 COD and sCOD Data

Table A3 Summary of COD and sCOD Data for the SND-MBR (R1) and the C-MBR (R2)

Day of Stable Operation	R1		R2	
	COD	sCOD	COD	sCOD
		115		116
	33	82	41	133
	26	81	11	40
	2	69	2	27
2	6	31	17	80
9	28	34	25	110
14	8	15	25	33
18	28	31	22	29
21	29	30	10	22
37		12	9	21
43	20	2	20	21
46	22	21	10	38
50	25	53	15	73
53	23	33	26	56
64	20	56	21	95
73	25	106	46	114
79	15	163	15	57
92	14	24	17	59
112	8	50	4	23
118	14	19	4	54
127	12	85	9	18
134	8	94	7	21
142	11	24	0	35
154	16	39	19	38
161	14	114	23	0
169	14	83	0	29
174	1	143	12	26
188	19	150	12	21
195	23	160	13	33
202	0	128	16	35
209	13	123	17	31
216	26	102	30	40
223	11	162	11	20
230	0	104	0	60
237	15	34	15	52
244	8	48	10	27
259	7	34	8	31

272	9	63	10	36
280	12	48	17	33
285	16	66	0	45
292	0	49	10	33
307	4	43	9	27
322	20	18	17	16
327	17	38	16	56
336	24	95	0	32
341	20	89	10	23
348	13	50	0	43
356	25	50	22	53
358	13	39	14	36
362	6	37	8	22
369	12	40	28	25
375	7	29	10	10
382	0	34	0	18
390	16	55	4	22
419	15	51	11	34
425	0	44	0	37
432	14	33	8	43
441	23	27	7	23
448	9	51	0	42

8.7 MLVSS Data

Table A4 Summary of MLVSS Data for the SND-MBR and C-MBR

Days of Stable Operation	SND-MBR	C-MBR
2	6.35	6.71
9	4.96	5.51
14	4.86	5.46
18	4.19	4.57
21	4.23	4.61
27	3.55	3.71
37	3.76	4.84
43	4.23	5.24
46	3.84	4.79
51	3.77	4.50
54	3.41	3.98
60	3.16	3.08
63	3.33	3.35
67	3.17	3.25
73	3.14	2.92
79	2.32	3.27
85	3.57	2.22
92	3.58	2.44
112	3.86	3.60
118	4.06	3.59
127	3.73	3.78
134	3.34	3.46
142	3.28	3.13
157	3.62	3.68
161	3.31	2.98
169	3.58	3.46
174	4.17	4.58
188	3.09	3.44
196	2.67	3.23
203	3.28	3.77
210	3.39	3.38
217	3.77	3.66
224	3.67	3.34
230	3.92	3.46
239	4.28	3.85
246	4.26	4.04
252	4.65	4.45
259	5.030	4.00
275	4.2	3.8

286	4.6	3.7
292	4.420	4.420
300	5.41	4.42
323	5.04	4.78
328	4.64	4.52
336	6.280	5.68
342	6.230	4.90
349	5.840	4.51
358	5.710	4.06
362	6.100	4.51
369	5.250	4.39
376	4.95	4.24
383	5.060	4.40
391	5.380	4.83
419	3.670	3.13
425	4.210	3.67
433		
441	3.59	3.42
448	3.05	2.56

8.8 TMP Data

Table A5: Summary of TMP Data for the SND-MBR and C-MBR

Day	SND	Control
1	1.16	0.88
2	0.84	0.73
4	0.8	0.7
5	0.85	0.71
6	0.96	0.71
7	1.04	0.72
8	1.11	0.72
9	1.22	0.72
11	1.38	0.75
12	1.45	0.78
13	2.43	0.88
14	2.57	0.84
15	2.94	0.84
17	3.5	0.84
18	4.2	0.84
19	5.02	0.85
20	0.8	0.86
21	0.71	0.8
22	0.71	0.8
23	0.72	0.8
25	0.73	0.81
24	0.74	0.82
25	0.75	0.83
26	0.76	0.83
27	0.81	0.84
28	0.77	0.84
29	0.75	0.82
30	0.7	0.73
31	0.68	0.61
32	0.84	0.82
33	0.82	0.83
34	0.79	0.8
35	0.83	0.82
36	0.82	0.84
37	0.8	0.83
38	0.82	0.84
39	0.84	0.86
40	0.87	0.89
42	0.82	0.86
43	0.82	0.83
44	0.85	0.88
45	0.86	0.88
46	0.88	0.87

47	0.94	0.88
48	0.97	0.92
50	1.03	0.94
51	1.08	0.94
52	1.12	0.94
53	1.16	0.96
54	1.13	0.96
57	0.81	0.81
58	0.83	0.83
59	0.8	0.82
60	0.84	0.82
61	0.9	0.87
63	0.96	0.87
64	1.03	0.89
65	1.07	0.9
66	1.11	0.91
67	1.2	0.94
68	1.31	0.93
70	1.56	0.96
71	1.61	0.95
72	1.69	0.95
73	1.82	0.97
74	1.97	1
75	1.95	0.98
77	2.14	1
78	2.22	0.99
79	2.24	1.01
80	2.43	1.02
81	2.24	1.06
82	1.71	1.05
84	2.04	1.06
85	1.79	1.07
86	1.97	1.08
87	2.12	1.11
88	2.22	1.11
89	2.3	1.12
90	2.88	1.13
91	3.02	1.14
92	2.31	1.14
93	2.46	1.15
94	2.41	1.16
95	2.45	1.18
96	2.86	1.2
97	1.05	0.91
98	0.98	0.85
99	1	0.85
100	1.09	0.86

102	1.25	0.87
103	1.4	0.89
104	1.65	0.92
105	1.32	0.95
106	1.9	0.95
107	1.94	0.96
108	2.26	0.97
109	2.42	0.99
110	3	1.03
111	2.94	1
112	3.06	1.01
113	2.82	0.99
114	2.82	0.99
115	2.95	0.99
116	3.02	0.98
117	3.18	1.02
118	3.05	1
119	3.25	1
120	3.35	1
121	3.21	1.03
123	2.98	1.02
124	3.28	1.04
125	3.2	1.06
126	3.53	1.05
127	3.59	1.08
128	3.67	1.09
129	3.75	1.16
130	3.68	1.14
131	3.56	1.22
132	3.64	1.31
133	3.77	1.6
134	3.75	1.7
135	4.3	2.01
136	4.52	2.18
137	0.82	0.83
138	0.84	0.87
139	0.89	0.85
140	0.89	0.85
141	0.89	0.85
142	0.89	0.86
143	0.89	0.86
144	0.89	0.85
145	0.9	0.87
146	0.9	0.88
147	0.9	0.9
148	0.93	0.91
149	0.94	0.94

150	0.94	0.94
151	0.95	0.94
152	0.96	0.95
153	1.04	0.97
154	1.05	1
155	1.06	1.01
156	1.09	1.03
157	1.09	1.03
158	1.08	1.05
159	1.1	1.07
160	1.12	1.06
161	1.13	1.09
162	1.16	1.1
163	1.19	1.12
164	1.2	1.13
165	1.25	1.14
166	1.28	1.16
167	1.34	1.19
168	1.4	1.22
169	1.44	1.24
170	1.55	1.22
171	1.6	1.22
172	1.66	1.21
173	1.78	1.22
174	1.7	1.2
175	1.86	1.17
176	1.72	0.81
177	1.86	0.8
178	1.92	0.8
179	1.95	0.81
180	1.98	0.8
181	2.03	0.81
182	2.24	0.84
183	2.38	0.83
184	2.44	0.83
185	2.62	0.84
186	2.84	0.85
187	2.86	0.86
188	3.5	0.85
189	3.66	0.85
190	4.86	0.86
191	0.89	0.82
192	0.96	0.82
193	1.04	0.81
194	1.09	0.8
195	1.16	0.79
197	1.24	0.8

199	1.3	0.82
200	1.36	0.83
201	1.41	0.85
202	1.43	0.86
203	1.41	0.89
204	1.42	0.93
205	1.39	0.95
206	1.4	0.99
207	1.41	1
208	1.43	1.02
209	1.43	1.05
210	1.47	1.1
211	1.44	1.07
212	1.44	1.12
213	1.44	1.15
214	1.46	1.18
215	1.53	1.22
216	1.62	1.27
217	1.7	1.36
218	1.77	1.47
219	1.85	1.59
220	1.87	1.67
221	1.92	1.81
222	2.04	1.92
223	2	2.05
224	2	2.09
225	2.01	2.21
226	2.03	2.39
227	1.98	2.41
228	1.98	2.39
229	2	2.44
230	2.08	2.7
231	2.11	2.84
232	2.06	2.88
233	2.23	2.81
234	2.27	2.84
235	2.26	2.43
236	2.28	2.62
237	2.42	2.6
238	2.38	2.39
240	2.53	2.41
241	2.42	2.41
242	2.41	2.26
243	2.42	2.28
244	2.57	2.28
245	2.56	2.32
246	2.52	2.3

247	2.49	2.11
248	2.57	2.13
249	2.61	2.11
250	2.73	2.08
251	2.94	2.06
252	3.22	1.97
254	3.2	1.98
255	3.34	2.05
256	3.38	2.03
257	3.39	2.05
258	3.29	2.08
259	3.32	2.25
260	3.42	2.48
261	0.64	0.71
262	0.55	0.68
263	0.52	0.71
265	0.55	0.78
266	0.53	0.78
267	0.56	0.82
268	0.6	0.91
269	0.61	1.1
270	0.64	1.15
271	0.48	0.61
272	0.48	0.62
273	0.45	0.64
274	0.46	0.7
275	0.47	0.74
276	0.49	0.76
277	0.51	0.78
278	0.49	0.63
279	0.47	0.63
280	0.48	0.63
281	0.53	0.7
282	0.54	0.7
283	0.54	0.71
284	0.55	0.72
285	0.59	0.78
286	0.68	0.88
287	0.72	0.99
288	0.6	0.98
289	0.55	0.98
290	0.58	0.99
291	0.64	1.02
292	0.64	1.1
293	0.68	1.15
294	0.69	1.23
295	0.7	1.17

296	0.74	1.32
297	0.74	1.39
299	0.79	1.45
300	0.8	1.52
301	0.78	1.63
302	0.84	1.77
303	0.83	1.83
306	0.93	2.1
307	0.94	2.2
308	1	2.54
309	1	2.71
311	1.02	3.01
312	1.14	3.29
313	1.25	3.3
314	1.29	3.21
315	1.34	3.95
316	1.37	4.01
317	1.54	4.1
318	1.4	4.21
319	1.48	4.39
320	1.56	4.91
321	0.45	0.56
322	0.46	0.59
323	0.48	0.63
324	0.54	0.66
325	0.47	0.65
326	0.49	0.66
327	0.49	0.66
328	0.52	0.66
329	0.54	0.71
330	0.54	0.73
332	0.61	0.79
333	0.67	0.85
335	0.7	0.98
336	0.72	1.01
337	0.76	1.01
338	0.73	1.02
339	0.74	1.17
340	0.76	1.21
341	0.7	1.2
342	0.77	1.28
343	0.81	1.37
344	0.86	1.46
345	0.86	1.53
346	0.81	1.59
347	0.85	1.67
348	0.8	1.77

349	0.81	1.92
350	0.9	2.81
351	0.92	3.31
352	0.95	3.85
353	0.59	0.64
355	0.66	0.77
356	0.66	0.83
357	0.66	0.86
358	0.62	0.87
359	0.61	0.89
360	0.63	0.93
361	0.67	0.97
362	0.71	1
363	0.72	1.03
364	0.73	1.07
365	0.75	1.12
366	0.77	1.19
367	0.8	1.23
368	0.82	1.24
369	0.78	1.22
370	0.81	1.29
371	0.81	1.29
372	0.81	1.26
373	0.74	1.25
374	0.76	1.27
375	0.78	1.4
376	0.77	1.34
377	0.78	1.44
378	0.83	1.53
379	0.87	1.61
380	0.95	1.67
381	0.99	1.7
382	1.03	1.72
383	1.01	1.7
384	1.01	1.71
385	1.05	1.7
386	1.07	1.76
387	1.12	1.94
388	1.19	2.08
389	1.26	2.32
390	1.96	2.51
391	1.9	2.64
392	1.93	2.66
393	0.68	0.71
396	0.64	0.62
397	0.64	0.65
398	0.63	0.62

399	0.78	0.82
400	0.62	0.64
403	0.59	0.6
404	0.51	0.65
405	0.61	0.71
406	0.77	0.86
407	0.85	0.95
408	0.82	1.28
409	0.84	2.45
410	0.86	3.41
411	0.93	4.01
412	0.91	4.62
413	0.95	4.94
414	0.89	5.28
415	0.91	5.39
416	0.92	5.88
417	0.89	7.42
418	0.65	0.71
419	0.64	0.71
421	0.7	0.77
422	0.74	0.86
423	0.8	0.99
424	0.79	0.83
425	0.8	0.85
426	0.8	0.87
427	0.83	0.91
428	0.84	0.92
429	0.85	0.94
430	0.89	0.96
431	0.9	0.98
432	0.91	1.01
433	0.91	1.03
434	0.91	1.04
435	0.92	1.04
436	0.93	1.04
437	0.96	1.06
438	0.95	1.08
439	0.95	1.07
440	0.98	1.09
441	0.99	1.11
444	1.02	1.11
445	1.08	1.22
446	1.1	1.18
447	1.12	1.2
448	1.15	1.19
449	1.23	1.2
452	1.38	1.24

453	1.45	1.24
454	1.51	1.29
455	1.58	1.38
457	1.59	1.42
458	1.68	1.47
459	1.77	1.51
460	1.78	1.61
461	1.73	1.68
462	1.74	1.75
463	1.78	1.82
466	1.35	1.29
467	1.75	2.25
468	1.71	2.45
469	1.78	2.63
470	1.89	2.86
471	1.92	2.84
472	1.94	2.9
473	2.12	3.05
475	2.52	3.58

8.9 TEPS Data

Table A6: Sumary of TEPs Data for the SND-MBR (first line) and the C-MBR (second line)

Date	Average	mg/L gum
01-Mar	0.509625	6.195595855
	0.556333333	3.775474957
03-Mar	0.532666667	5.001727116
	0.548	4.207253886
03-Mar	0.519666667	5.675302245
	0.533333333	4.967184801
12-Mar	0.425	10.58031088
	0.545333333	4.345423143
26-Mar	0.1592	25.84076433
	0.522	2.732484076
01-Apr	0.413	9.675159236
	0.42725	8.767515924
07-Apr	0.418666667	9.314225053
	0.439	8.01910828
08-Apr	0.437	8.146496815
	0.41	9.866242038

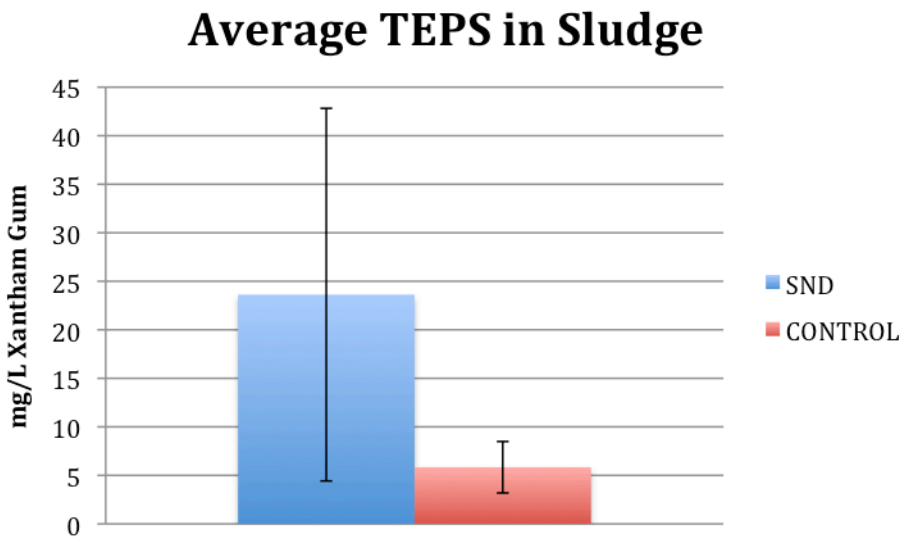


Figure A1 Average TEPs value in the AS of the SND-MBR and the C-MBR

8.10 FISH Analyses Photographs

Examples of FISH photographs. Due to poor camera resolution and inaccuracy of software particle counter, estimations were done directly on viewer; More photographs were taken then are currently displayed in this thesis. These photographs are available by request.

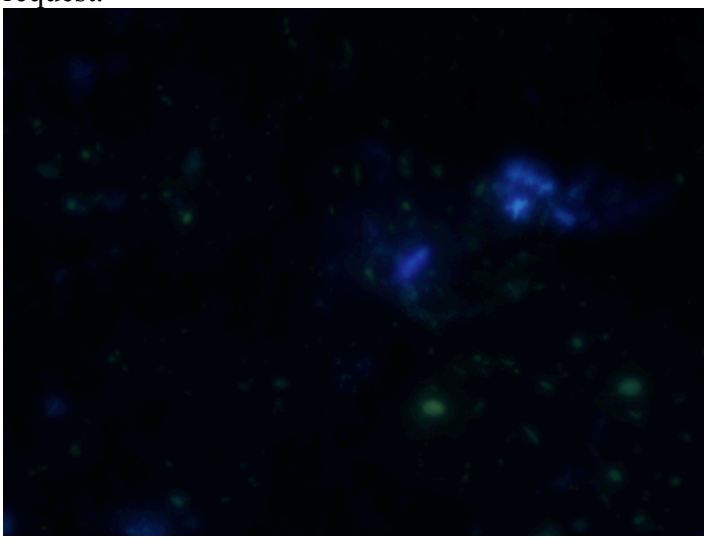


Figure A2 DAPI shot of SND reactor

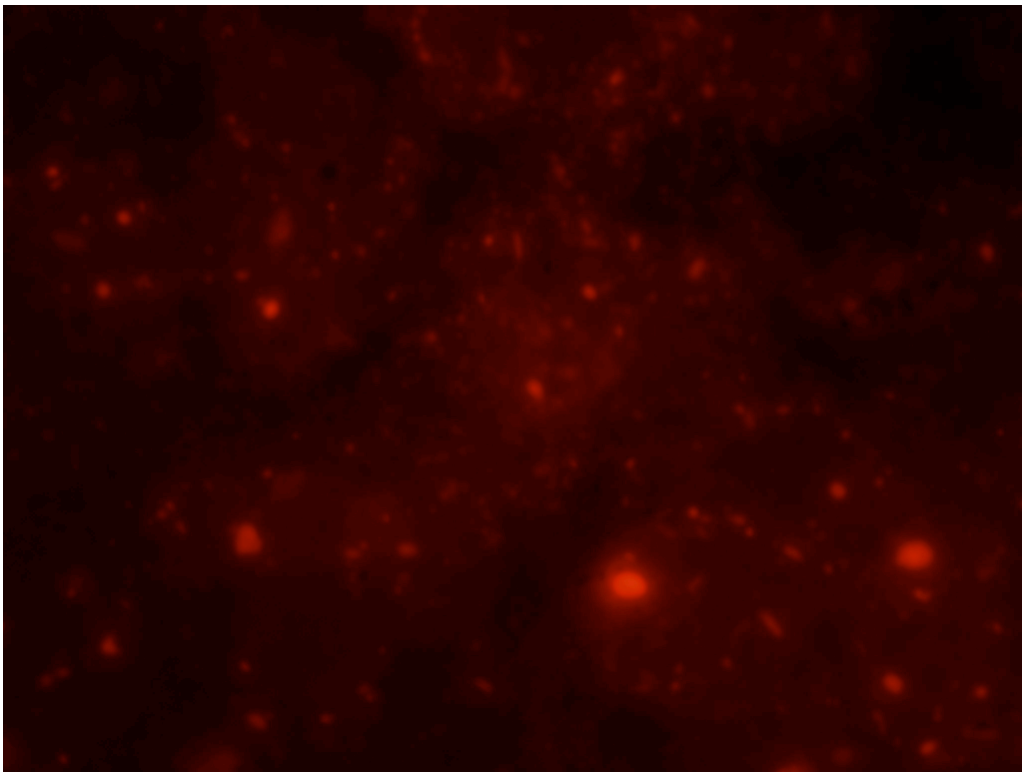


Figure A3 SND-Reactor, *Paracoccus* spp. probe

8.11 Gas Composition Data

Table A7: Summary of Nitrous Oxide gas concentration for the SND-MBR (R1) and the C-MBR (R2)

Sample	NO2 Concentraiton	Average
ATM	0.386310935	0.377944872
	0.37590437	
	0.371619313	
	0.397	0.385
	0.380	
	0.377	
	0.362	0.363
	0.365	
	0.361	
	0.389	0.383
	0.378	
BIOSYS ATM	0.378	0.377
BIOSYS ATM	0.403	
BIOSYS ATM	0.351	
R1 Blowing	0.454347133	0.423725014
	0.406511914	
	0.410315994	
	0.644944724	0.634606863
	0.762704454	
	0.49617141	
	0.434	0.466
	0.535	
	0.430	
	0.563	
R1 Middle	0.374723793	0.389765215
	0.410097369	
	0.384474482	
		0.441909123
	0.433638834	
	0.450179412	
R1 Before	0.508990358	0.578567995
	0.504395753	
	0.722317875	
	0.465	0.489
	0.482	
	0.518	
	0.404	0.384
	0.366	
	0.383	
	0.385	0.386

	0.378	
	0.396	
	BIOSYS R1	0.500
	BIOSYS R1	0.508
	BIOSYS R1	0.522
R1 After	0.392	0.362
	0.392	
	0.354	0.354
	0.360	
	0.348	
	0.353	0.343
	0.336	
	0.340	
R2	0.382463129	0.388424313
	0.379664725	
	0.403145084	
	0.357276496	0.386666654
	0.398949565	
	0.4037739	
	0.396	0.391
	0.383	
	0.394	
	0.383	0.383
	0.387	
	0.378	
	0.370	0.382
	0.384	
	0.391	

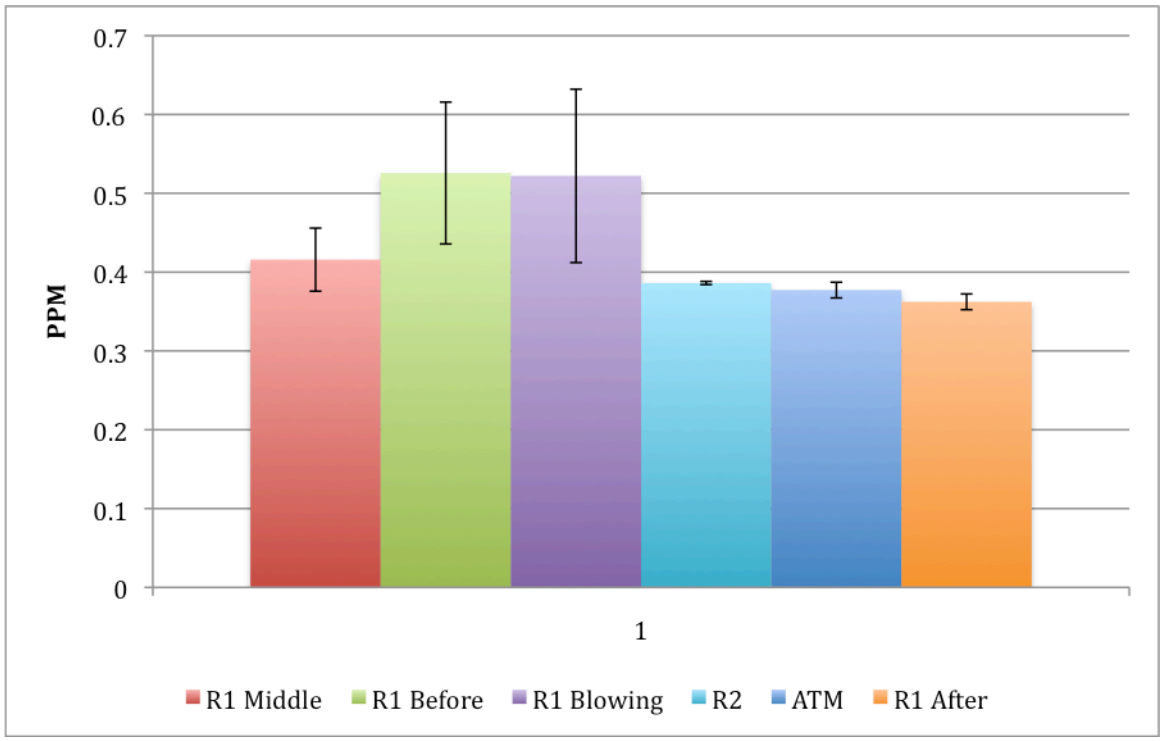
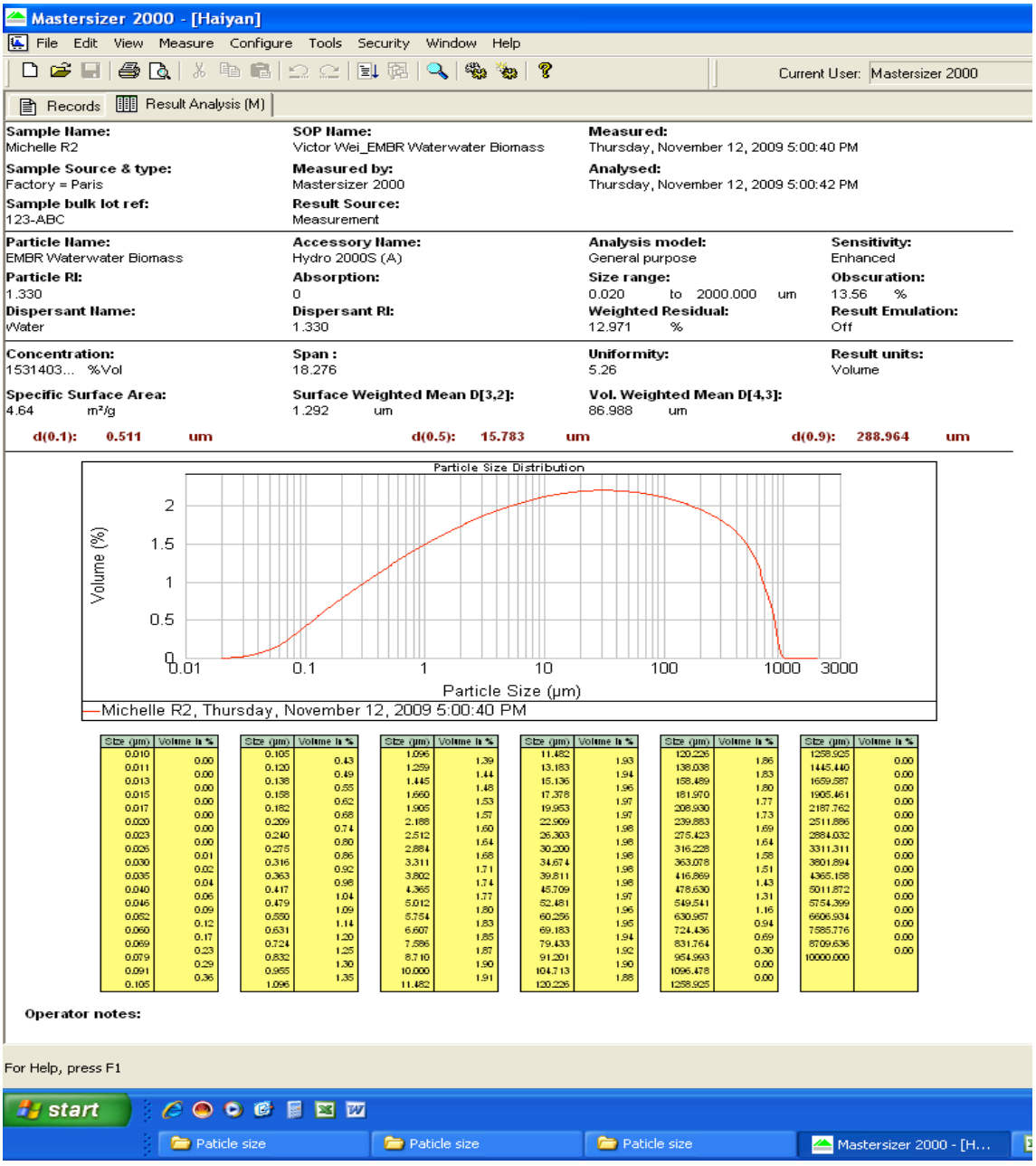


Figure A4 Concentrations of NO_2 in the headspace of the SND-MBR (R1) and the C-MBR (R2)

8.12 Particle Size Analysis Data

Particle size analyses was done three times during MBR Operation. Analysis was done off campus on a computer in one of the Smart Park facilities. The following are selected screenshots from that computer



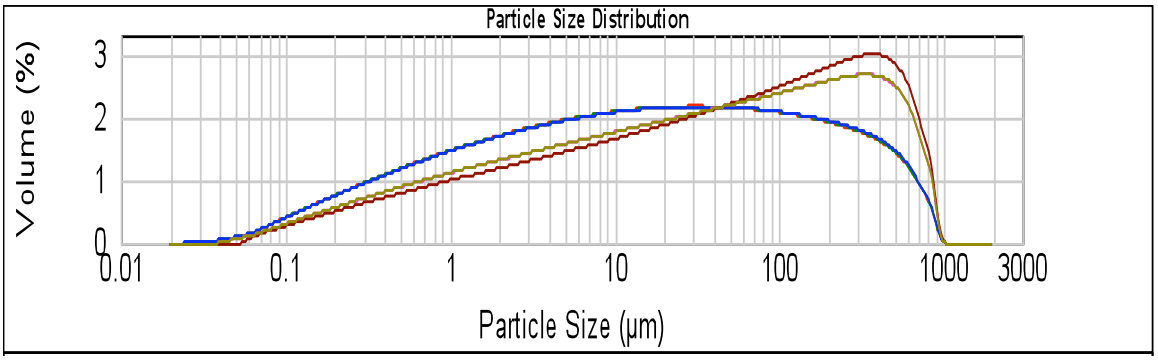


Figure A5 Screenshots from the particle size analysis in the SND-MBR and C-MBR; Lines seen in red (weighted in the 100-1000 µm range) are the SND-MBR; Lines in blue are the C-MBR

8.13 Summary of EA Concentrations

Table A8 Summary of EA Concentrations for the SND-MBR (labeled as 1) and the C-MBR (labeled as 2) in ng/L

Extraction No.	Feed	Solid 1	Solid 2	Liquid 1	Liquid 2	E1	E2
1	852	343	360	354	385	340	375
2	686	290	281	319	775	296	287
3	667	298	293	307	303	305	282
4	1270	576	431	330	351	329	413
5	818	409	369	463	426	351	325