Impact of Biological Nutrient Removal Process Operating and Design Conditions on the Removal of Micropollutants from Wastewater

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The efficacy of three different wastewater treatment configurations, conventional activated sludge (CAS), nitrifying activated sludge (NAS) and biological nutrient removal (BNR) for removal of selected micropollutants from authentic wastewater was investigated. The processes were also characterized based on their proficiency to reduce the estrogenic activity of the influent wastewater using the *in-vitro* recombinant yeast assay. The selected micropollutants (MPs) covered a broad spectrum of therapeutic classes, i.e non-prescription analgesic (Ibuprofen (IBU)), anti-convulsant/epileptic (meprobamate (MEP) and carbamazepine (CBZ), lipid lowering drug (Gemfibrozil (GEM)), antibiotic (trimethoprim (TMP) and sulfamethoxazole (SMX)), steroid hormone (androstenedione (ADR)), estrogen (estrone (E1)) and estrogenic compounds (nonyl phenol (NP) and bisphenol A (BPA)). The removal efficiency of TMP improved with the complexity of the three treatment process configurations. IBU, ADR, SMX, NP, E1 and BPA had moderate to high removals (> 65%) while CBZ and MEP remained recalcitrant in the three treatment process configurations. The removal of GEM was better in the NAS than in BNR and CAS treatment configurations. The YES assay analyses showed an improvement in estrogenicity removal in the BNR and NAS treatment configurations as compared to the CAS treatment configuration. Comparing the estrogenic responses from the three treatment configurations, the removal efficiencies followed the order of BNR = NAS > CAS and all were greater than 81%.

The removal of estrogenicity in a University of Cape Town- biological nutrient removal (UCT-BNR) wastewater treatment processes was investigated using pilot and bench scale systems, batch experiments and mathematical modeling. In the pilot BNR process, 96 ± 5% of the estrogenicity exerted by the EDCs in the wastewater was removed by the treatment process. The degradation efficiencies in the anaerobic, anoxic and aerobic zones of the pilot BNR bioreactor were 11± 9%, 18 ± 2% and 93 ± 10% respectively. In order to further understand the performance of the BNR process in the removal of EDCs from wastewater, a bench scale BNR process was operated with synthetic wastewater dosed with E1 and E2. The removal of estrogenicity in the bench scale system (95 ± 5%) was comparable to the pilot BNR process and the degradation efficiencies were estimated to be 8± 0.8%, 38 ± 4% and 85 ± 22% in the anaerobic, anoxic and aerobic zones. A biotransformation model developed to predict the fate of E1 and E2 in batch tests using the sludge from the BNR process was calibrated using the data from the experiments. The biotransformation rate constants for the transformation of E2 to E1 were estimated as 71 ± 1.5, 31 ± 3.3 and 1 ± 0.9 $L.gCOD^{-1}d^{-1}$ for the aerobic, anoxic and anaerobic batch tests respectively while the corresponding biotransformation rate constants for the transformation of E1 were estimated to be 7.3 ± 1.0, 3 ± 2.0, and 0.85 ± 0.6 $L.gCOD^{-1}d^{-1}$. A steady state mass balance model formulated to describe the interactions between E2 and E1 in BNR activated sludge reasonably described the fate of E1 and E2 in the BNR process.

A combination of pilot scale biological nutrient removal (BNR) process, batch experiments and modeling exercises were employed to investigate the removal and biotransformation of trimethoprim (TMP) in a BNR activated sludge. The concentrations of the active microbial groups- ammonia oxidizing bacteria (AOB), ordinary heterotrophic organism (OHO) and polyphosphate accumulating organism (PAO) in the BNR bioreactor were estimated to be 40, 780 and 2710 g COD/m^3 respectively. TMP was biotransformed in all the redox zones of the BNR bioreactor. The TMP biotransformation efficiencies in the anaerobic, anoxic and aerobic sections were 13 ± 12%, 17 ± 10% and 24 ± 4% respectively. Batch tests with and without nitrification inhibition showed that AOB played a role in the biotransformation of TMP in BNR activated sludge. A pseudo first order model that incorporated the contributions of PAO, OHO and AOB to the overall biodegradation of TMP was found to describe the biodegradation of TMP in batch tests with and without nitrification inhibition. The estimated biotransformation rate constant with respect to PAO, OHO and AOB were 0.32 ± 0.06, 0.58 ± 0.06 and 13.7 ± 0.06 L/gCOD/d respectively. This model showed that PAOs, OHOs and AOBs contributed towards the biotransformation of TMP in BNR activated sludge with the trend AOBs = PAOs > OHOs.

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Dedication

To my wife Sileola Ogunlaja (Abebi) and my children, Daniel Ireoluwa Ogunlaja (Sunshine) and Victoria Aanuoluwa Ogunlaja (Angel) who endured the hard times, supported and encouraged me throughout my program.

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Nomenclature

A^2O	Anaerobic/Anoxic/Aerobic
AOB	Ammonia Oxidizing Bacteria
AR	Anoxic Recycle
AN	Anaerobic
AX	Anoxic
AO	Aerobic
BOD	Biochemical Oxygen Demand
BNR	Biological Nutrient Removal
CAS	Conventional Activated Sludge
Ca	Calcium
CSTR	Continuously Stirred Tank Reactor
CFSTR	Continuous Flow Stirred Tank Reactor
COD	Chemical oxygen demand
DO	Dissolved Oxygen
DPAO	Denitrifying Phosphorus Accumulating Organism
EBPR	Enhanced Biological Phosphorus Removal
EDC	Endocrine Disrupting Compounds
HRT	Hydraulic Residence Time
IMLR	Internal Mixed Liquor Recycle

MBR	Membrane Biological Reactor
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MP	Micropollutant
N	Nitrogen
NAS	Nitrifying Activated Sludge
NH_3	Ammonia
NO_3	Nitrate
NO_2	Nitrite
NR	Nitrified Recycle
O_2	Oxygen
OHO	Ordinary Heterotrophic Organism
P	Phosphorus
PhAc	Pharmaceutically Active compounds
PAO	Phosphorous Accumulating Organism
PHA	poly-β-hydroxyalkanoates
PHB	Poly-β-hydroxybutyrate
PolyP	Polyphosphate
PO_4-P	Orthophosphate
RAS	Return Activated Sludge

rbCOD	Readily Biodegradable COD
SCFA	Short Chain Fatty Acid
SRT	Solids Residence Time
TAN	Total Ammonia Nitrogen
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphorous
TrOC	Trace Organic Compounds
TSS	Total Suspended Solids
UCT	University of Cape Town
VFA	Volatile Fatty Acid
VIP	Virginia Initiative Process
VSS	Volatile Suspended Solids
GC	Gas Chromatography
LC-MS	Liquid Chromatography Mass Spectrometer
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant

List of Chemical Compounds

IBU	Ibuprofen
MEP	Meprobamate
CBZ	Carbamazepine
GEM	Gemfibrozil
TMP	Trimethoprim
SMX	Sulfamethoxazole
ADR	Androstenedione
E1	Estrone
E2	17-β-Estradiol
NP	Nonyl-phenol
BPA	Bisphenol-A
EE2	Ethinylestradiol

Chapter 1 Introduction

1.1 Background

Micropollutants (MPs) in wastewater have been reported as early as the 1950's (Stumm-Zollinger and Fair, 1965). However, recent interest of the presence of these compounds in the aquatic environment (Heberer, 2002; Kolpin et al., 2002; Joss et al., 2005) was borne out of the advancement in analytical technologies available to detect trace levels of these compounds in different environmental matrices and the potential ecotoxicological effects of these compounds on aquatic organisms. This has led to the detection of MPs in wastewater treatment plants (WWTPs) effluents, surface waters, ground water, sediments and soil in Europe (Heberer, 2002; Carballa et al., 2004) North America (Kolpin et al., 2002) and Asia (Nakada et al., 2006). These prior studies have recommended further investigation into the occurrence, fate, effect and attenuation of MPs in the environment.

Micropollutants such as human and veterinary pharmaceuticals and their metabolites, natural and synthetic endocrine disrupting compounds are usually excreted via urine and feces or inappropriately disposed in the septic system and can subsequently enter into the aquatic environment through municipal wastewater treatment plant effluents (Daughton and Ternes, 1999). Appendix A

presents a summary of the sources and distribution of pharmaceuticals in the environment.

Wastewater treatment plant effluents have been identified as an important point of discharge for MPs into the aquatic environment (Koplin et al., 2002; Joss et al., 2004; Clara et al., 2005a) because WWTPs are not typically designed to remove MPs. The limited removals of MPs that have been reported from wastewater treatment processes are considered to be due to cometabolism because the concentrations at which the MPs exist in the WWTP influent are too low to sustain microbial growth or metabolic activities like conventional soluble organic substrates. The complex chemical structure of some MPs also enhances their persistence and recalcitrance during wastewater treatment.

The concern about MPs has been accentuated by reports of gonad and reproductive abnormalities in various trophic levels of aquatic organisms at very low concentrations (Purdom et al., 1994; Gagne and Blaise, 1998; Fent et al., 2006). Thereafter, several questions have been raised concerning the chemical persistence, microbial resistance and synergistic effects of the cocktail of MPs present in the effluents of WWTP.

Tertiary wastewater treatment technologies such as advanced oxidation process (using UV, hydrogen peroxide or ozone) and membrane technologies (using MBR, nano-filtration and reverse osmosis) have been reported to deliver

higher removal of MPs from wastewater (Ternes et al., 2003) as compared to conventional treatment methods. However, there are concerns of producing more toxic oxidation by-products or metabolites during the use of advanced oxidation process to treat wastewater (Magdeburg et al., 2012; Maletz et al., 2013). In addition, these tertiary treatment processes are energy intensive, resulting in increased operational cost to the plant owners. Therefore, the optimization of the operation of existing biological wastewater treatment processes to remove MPs is desirable.

It is hypothesized that advanced biological wastewater treatment process such as BNR process could be optimized to deliver higher removal efficiencies of MPs as compared to conventional activated sludge treatment processes. The BNR processes develops a complex microbial consortia in unique reactor configurations to remove COD, nitrogen and phosphorus from wastewater. The important fundamental metabolic processes that occur in a typical BNR process include; nitrification, denitrification and biological phosphorus removal. These processes are mediated by diverse microorganisms that exists under different redox conditions and these organisms may cometabolically degrade MPs along with the utilization of macropollutants as growth substrates. Hence, the BNR process has the potential to degrade MPs in wastewater.

The microbial populations that exist in a BNR process vary, but based on the metabolic processes mediated by these organisms, three major groups can be identified, namely ammonia oxidizing bacteria (AOBs), ordinary heterotrophic organisms (OHOs) and phosphorus accumulating organism (PAOs). The AOBs mediates nitrification reaction while utilizing ammonia for growth and cellular maintenance, the OHOs mediate a wide range of processes including COD removal, hydrolysis, ammonification (conversion of organic nitrogen to ammonia), fermentation in anaerobic zones, and so on. The PAOs are responsible for the biological phosphorus removal from the process. Hence, the PAOs, OHOs and AOBs collectively co-exist in BNR activated sludge but function differently depending on the prevailing redox condition (Tchobanoglous et al., 2003).

The role of the microbial communities in the biotransformation of MPs in activated sludge systems has been previously treated as a "black box", where all the microbial communities are lumped together in one single term as mixed liquor suspended solid (MLSS) or mixed liquor volatile suspended solid (MLVSS) (Cowan et al., 1993-WWTreat; Monteith et al., 1995-Toxchem™; Plotz et al., 2010-WEST®). This approach has been employed because of the difficulty in determining the active fractions of the various biomass groups, thus it is usually practiced in the modeling of the biotransformation of MPs in activated sludge system. The disadvantage of using MLSS or MLVSS for predicting the

biotransformation kinetics of MPs is that MLSS or MLVSS contains both active and inactive fractions of the biomass. While the inactive fraction does not contribute to the biotransformation of MPs, the active biomass is dependent on the process operating and design conditions (Layton et al., 2000). The dynamics of the microorganisms present in an activated sludge varies depending on the operating condition and configuration of the bioreactor and the ability of an activated sludge to degrade MPs strongly depends on the presence of appropriate microbial population (Tchobanoglous et al., 2003). Therefore, the use of the active biomass concentration rather than MLSS concentration should give a better description of the biotransformation kinetics of MPs in activated sludge systems.

There are very few reports on the contribution of heterotrophic organisms to the biotransformation of MPs in activated sludge. Previous studies have identified some of the microorganisms responsible for MPs' biotransformation in activated sludge systems (Shi et al., 2004; Gaulke et al., 2008; Khunjar et al., 2011), although this issue still remains debatable. These studies suggest that heterotrophic organisms co-contribute or predominantly contribute to the biotransformation of some MPs in activated sludge systems (Gaulke et al., 2008; Khunjar et al., 2011; Majewsky et al., 2011). However, no study has specifically

investigated the role of PAOs, OHOs and AOBs in the biotransformation of MPs in BNR activated sludge systems.

The presence of different redox conditions is important to the operation of a BNR process because the alternating redox zones proliferate PAOs for biological phosphorus removal. Previous studies have shown that combined anaerobic/anoxic/aerobic processes may create favorable conditions for the removal of MPs in WWTPs (Li et al., 2010; Joss et al., 2004; Dytzak et al., 2008). Biodegradation of MPs like E1, E2 and EE2 has been found to depend on redox conditions, for example, a configuration of anaerobic/aerobic/anoxic yielded 18% overall removal of E2 while a sequence of anoxic/anoxic/aerobic and anaerobic/anoxic/aerobic yielded overall removal of 80% and 97.6% of E2 respectively. These results suggest the possibility of a relationship between the redox zones in a BNR process and the removal of MPs as well as the importance of the zone sequencing in a BNR system because of the variability in the removal efficiencies in the different redox zones and with different redox zone arrangements in a multi-redox zone system. However, the data in literature on which this premise was based is limited. Therefore, further study is required to elucidate the contribution of redox conditions to the removal of MPs in BNR wastewater treatment processes.

Assessing the biological effects of MPs on the flora and fauna of the aquatic ecosystem is an indispensable tool for conducting detailed and appropriate risk assessment of MPs in the environment. Unlike chemical analyses that can provide a quantitative measure of the compounds present in a sample, biological analyses such as *in vitro* bioassays can provide a qualitative and quantitative measure of the estrogenic potential or estrogenicity of all the endocrine disrupting compounds (EDCs) that are present in an effluent (Leusch et al., 2010). Previous studies have investigated the removal of MPs in wastewater using different wastewater treatment technologies, but it has not been conclusively established whether an improved removal of MPs will translate into a reduction in the biological effects or estrogenicity. Hence, the investigation of the removal of MPs in a wastewater treatment process in relation to the degree of the estrogenic response in the effluents will provide a holistic approach in determining the performance of the treatment process.

A comprehensive review conducted by Pomies et al (2013) on modeling of MPs in biological wastewater treatment recommended the need for further research on the effects of oxidation-reduction conditions on biodegradation of MPs, the need for authors to clearly specify the operating conditions that guides the domain of validity of their models and the estimation of the active biomass fraction that mediates biodegradation or biotransformation of MPs. Therefore,

there is the need to elucidate the role of PAO, OHO and AOB in the removal of MPs from wastewater, investigate the effects of redox conditions on the biotransformation of MPs in activated sludge systems and ultimately assess the treated effluent quality with respect to the removal of estrogenicity.

1.2 Scope and Objectives

The overall scope of this study was to assess the impact of BNR process design and operating conditions on the removal of micropollutants from wastewater. The specific objectives of the research conducted over the course of this study were to:

- Compare the performance of different activated sludge process configurations in terms of the removal of MPs and estrogenicity.
- Assess the removal of endocrine disrupting compounds in BNR wastewater treatment process.
- Elucidate the effects of redox conditions on the removal of MPs in a BNR process.
- Evaluate the role of the active microbial groups in a BNR process bioreactor in the biodegradation of micropollutants.

A portion of this research work was carried out at the Wastewater Technology Center (Science and Technology branch of Environment Canada), in

Burlington, Ontario. Existing pilot scale activated sludge treatment processes were employed in this research work while bench-scale BNR and batch tests were designed and operated in the University of Waterloo laboratory. The Wastewater Technology Center was suitable for this research study because the pilot plants had access to authentic wastewater from the Burlington Skyway WWTP and the center provided the logistics and technical support for the operation of the pilot plants.

1.3 Thesis Outline

This thesis is divided into six chapters, references and seven appendices. Chapter one presents the introduction to the problem under investigation, the scope and objectives of the research work. Chapter two contains the literature review on micropollutants, BNR process, BNR process configurations, operating conditions necessary for biological phosphorus removal in BNR process, activated sludge microorganisms involved in MPs degradation, MPs removal mechanisms, MPs removal from wastewater and endocrine disrupting compounds in sewage treatment plant. Chapters three, four and five are presented in paper format investigating the specified objectives. Chapter six contains the conclusions of the research, recommendations and suggestions for future research.

Chapter 2 Literature Review

This chapter reviews the important processes and the mediating organisms in a typical BNR system. The background information required to understand the operation of a typical BNR process was reviewed in this section. MP removal mechanisms and the role of the bacterial community in the removal of MPs were reviewed. Endocrine disrupting compounds in wastewater and the available biological analytical techniques were reviewed to determine the appropriate bioassay for qualitative and quantitative analysis.

2.1 Micropollutants

Micropollutants (MPs) can be broadly defined as any synthetic or naturally occurring chemical that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and or human health effects (USEPA, 2013) . These pollutants are usually organic compounds that persist in the environment because they do not biodegrade. The term MPs is believed to envelope a wide array of compounds which includes but not limited to human and veterinary MPs and their metabolites, personal care products - detergent, cosmetics, fragrance,

natural and synthetic hormones, fire retardants, surfactants, plasticizers, anti-microbial compounds, endocrine disrupting compounds, ultraviolet absorbing compounds, domestic products and recently nanotechnology residuals. These compounds are typically detected at low concentrations, usually in the range of nanogram per liter or microgram per liter, hence the name “Micropollutants”. Other terms that are commonly used to refer to these compounds are “xenobiotic compounds” because most of these compounds are of anthropogenic origin; “emerging contaminants” because of the recent technological advancement in analytical methods that are sufficiently sensitive to detect the MPs at environmentally relevant trace concentrations ;“Trace organic compounds” because of their detectable low concentrations in different environmental matrices; “Persistent Organic Compounds” because of their non-biodegradable nature in the environment.

2.2 Biological Nutrient Removal Processes in Wastewater Treatment

Biological nutrient removal (BNR) processes are advanced biological wastewater treatment processes for the removal of COD, nitrogen and phosphorus from wastewater. Despite the complexity of their design and operation, they have favor among researchers and operators as an economic and

effective method of treating municipal wastewater. Some of the benefits of BNR systems (Randall et al., 1992) are:

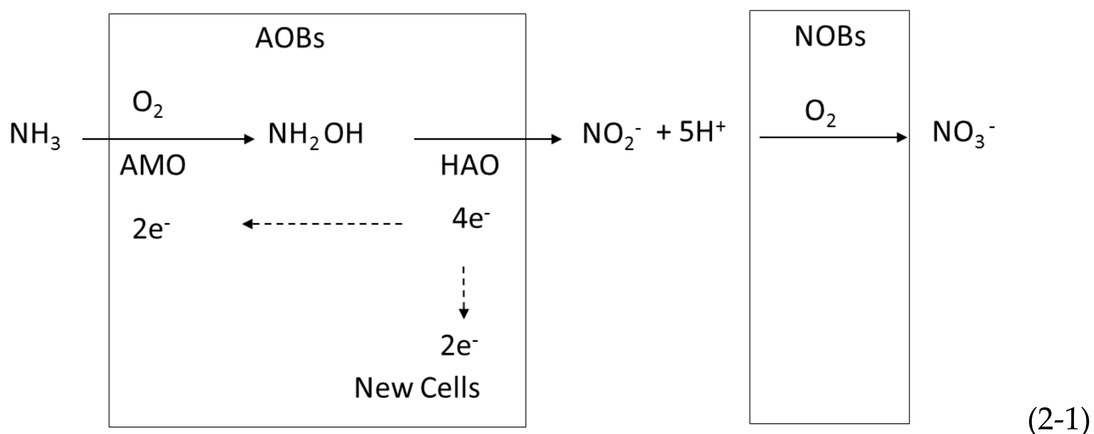
- Reduction or elimination of chemical addition for phosphorus removal
- Decrease in oxygen requirements as compared to nitrifying activated sludge
- Decrease in sludge production as compared to conventional activated sludge
- Recovery of alkalinity by the denitrification process (denitrification produces alkalinity)
- Reduction in filamentous growth and improvement in sludge settleability.

A typical BNR system is an adaptation of a basic activated sludge system that involves: 1) a slurry of microorganisms, 2) suspended solid recycle, 3) quiescent sedimentation and 4) solid retention time (SRT) control. In addition, the bioreactor of a BNR is divided into different redox zones; anaerobic (AN), anoxic (AX) and aerobic (AO), and typically includes mixed liquor recirculation. These zones are defined in terms of the electron acceptor that is utilized. Oxygen is the electron acceptor in aerobic zone, nitrate-N is the electron acceptor in anoxic zone and neither oxygen nor nitrate-N is present in the anaerobic zone. The uniqueness of the BNR system is the presence of the alternating redox condition that enhances the cultivation of different microbial species. The

anaerobic zone is essential for phosphorus removal, the anoxic zone is necessary for nitrogen removal and the aerobic zone is a pivotal component of all BNR systems (Grady et al., 1999).

The biological nutrient removal (BNR) process involves the utilization of three different biochemical processes for the removal of nutrients (N, P) in a wastewater treatment facility. These three key processes are nitrification, denitrification (both processes are termed biological nitrogen removal) and biological phosphorus removal.

Nitrification is a two-step process that involves two obligate aerobic bacteria consortia namely, Ammonia Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB). Ammonia is initially oxidized to hydroxylamine, a reaction that is catalyzed by ammonia monooxidase (AMO). Hydroxylamine is then oxidized to nitrite, catalyzed by hydroxylamine oxidoreductase (HAO). This process is summarized in Equation 2-1.



(2-1)

Nitrification converts nitrogen from a reduced form (ammonia) to an oxidized form (nitrate). It is not in itself a nitrogen removal mechanism, therefore; denitrification is employed after nitrification to achieve complete total nitrogen removal.

Denitrification involves the utilization of readily biodegradable organic matter (rbOM) by specific heterotrophic bacteria under anoxic conditions to reduce nitrate to nitrogen gas as shown in equation 2-3;

$$NO_2^- + rBOM \rightarrow N_2(g) + CO_2 + H_2O + OH^- + New\ cells \quad (2\text{-}3)$$

Biological phosphorus removal is achieved by cycling the mixed liquor between anaerobic and aerobic conditions to cultivate phosphorus accumulating organisms (PAOs) e.g *Accumulibacter phosphatis* (Gu et al., 2008), which are then removed through excess sludge removal. Figure 2-1 simplifies the events that occur in a typical BNR bioreactor with respect to poly-p organism (PAOs) and other non-poly-p accumulating organisms; ordinary heterotrophic organism (OHO) and ammonia oxidizing bacteria (AOB).

- In the anaerobic stage, PAOs do not grow because they cannot utilize or degrade the fermentative compounds in an anaerobic condition, but they convert short chain fatty acids (SCFAs) to intracellular energy rich carbon polymers, poly-β-hydroxybutyrate (PHB). The fatty acids (particularly acetate and propionate) are produced through the anaerobic activity of fermentative bacteria (anaerobic OHOs) or supplied externally by chemical addition. The polymerization of fatty acids requires an expenditure of cellular energy by the PAO. This energy is obtained from the breakdown of intracellular poly-phosphates to release orthophosphates from the PAO into the bulk solution of the anaerobic tank. As a result of the release of orthophosphate from the poly-P bacteria, the anaerobic tank contains two pools of phosphorus; the phosphorus in the influent wastewater (feed phosphorus) and the released phosphorus by the poly-P bacteria. Magnesium and potassium ions are concurrently released to the anaerobic tank along with phosphate (WEF, 2005). Previous studies have shown that anaerobic phosphorus release is a precursor to effective aerobic phosphorus uptake by the PAOs (Barker and Dold, 1996).

- Under anoxic condition, where nitrate (NO_3) become available, it is believed that OHOs continues to mediate hydrolysis of particulate organic substrates while a fraction of the PAOs that is capable of utilizing nitrate as an electron acceptor (denitrification) oxidizes previously stored PHBs to obtain energy for growth and maintenance requirements (Barker and Dold 1996). However, the anoxic phosphorus uptake per intracellular PHB oxidized is less efficient when compared to aerobic phosphorus uptake and phosphorous uptake per unit PHB oxidized seems to occur simultaneously with phosphorus release per PHB storage when SCFAs are available under anoxic condition.
- In the aerobic zone, where sufficient external electron acceptors (NO_3 and O_2) become available, PAOs oxidize the previously stored PHBs to obtain energy for growth and maintenance requirements. PHB in PAOs serves two important functions. First, it helps the bacteria to grow and rebuild polyphosphates by taking up soluble phosphate. Second, PHB along with polyphosphates help aerobic poly-P bacteria to survive in an anaerobic condition. Concurrently, the poly-P bacteria restore intracellular energy reserves through absorption of

orthophosphate to form intracellular polyphosphates granules or volutins.

The energy obtained from the degraded PHB is high enough for the PAOs to absorb not only the released phosphorus but also a significant quantity of feed phosphorus. Phosphorus removal is achieved when the bacteria (sludge) are wasted from the secondary clarifier. Thus a low effluent phosphorus concentration is achieved from the BNR reactor (WEF, 2005). Sludge that is not wasted is returned to the anaerobic tank where the BNR process is repeated. By exposing the poly-P bacteria to alternating anaerobic and aerobic conditions, the poly-P bacteria are stressed and take up phosphorus in excess of normal cellular requirements.

Table 2-1 summarizes the processes occurring and the mediating organisms in the different zones of a typical BNR system. From Table 2-1, it is quite clear that there are different processes that proceed in the different zones and the organisms responsible for these processes could either be heterotrophic or autotrophic. These organisms are present in all of the zones due to the internal recycles but growth and metabolic activity is however dependent on the redox conditions of the zones.

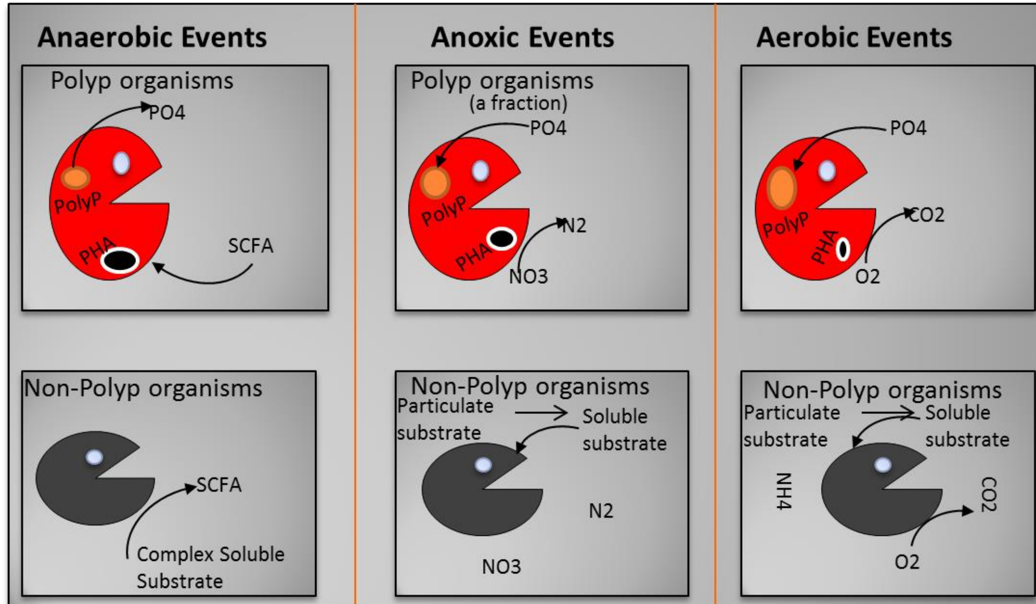


Figure 2-1. Events in a typical BNR process bioreactor (adapted from EnviroSim 2008).

Table 2-1. BNR process reactions and mediating organisms

Zone	**Process**	**Mediating Organism**
Anaerobic	Phosphorus release and PHB storage	Heterotrophs (PAOs)
	Fermentation: Complex organics converted to VFAs.	Heterotrophs (non-PAOs)
Pre-anoxic	Denitrification: Nitrate to nitrogen gas via the: -Use of influent substrate-BOD removal, and	Heterotrophs (non-PAOs)
	-Use of stored substrate (PHB) – phosphorus uptake	Heterotrophs (DPAOs)
Post Anoxic (if provided)	Denitrification: Nitrate to nitrogen gas via the: -Use of cellular substrate (endogenous reactions), or -Use of methanol	Heterotrophs (non-PAOs)
Aerobic	BOD removal	Heterotrophs (non-PAOs)
	Ammonification: Organic nitrogen to ammonia nitrogen	Heterotrophs (non-PAOs)
	Nitrification: Ammonia nitrogen to nitrate nitrogen	Autotrophs (*AOB* & *NOB*)
	PHB degradation and excess phosphorus uptake	Heterotrophs (PAOs)

Source: Jeyanayagam (2005).

2.2.1 Summary of BNR Processes in Wastewater Treatment

Important fundamental processes that occur in a typical BNR system include 1) Nitrification, 2) denitrification and 3) biological phosphorus removal. Even though other processes like hydrolysis, fermentation and ammonification also occur in a typical BNR process, the former three directly relate to the existence of the different redox zones present in a BNR process. The microorganisms that mediate these processes are ammonia oxidizing organisms (*Nitrosomonas and Nitrobacter*), denitrifying bacteria and phosphorus accumulating organism all existing in the activated sludge but functioning differently depending upon the redox zones (anaerobic/anoxic/aerobic), assuming all other operating conditions are appropriate

BNR systems can be configured in various designs with differing zone sequence. The type of configuration employed may have an effect on the degradation or removal of micropollutants in wastewater treatment. The next section examines the various BNR basin configurations that have been explored for the treatment of wastewater.

2.3 BNR Process Configurations

Various combinations and modifications of the BNR system have been developed to meet economic and regulatory demands that have been placed upon wastewater treatment. BNR process configuration can be designed to

achieve either total nitrogen (TN) removal or both TN and total phosphorus (TP) based on the incorporation of either two of the three redox conditions (anoxic/aerobic) or a combination of the three (anaerobic/anoxic/aerobic) (Jeyanayagam, 2005). There are a variety of configurations of BNR systems and the selection of a configuration depends on the influent characteristics, effluent limits and desired operating conditions (WEF, 2005). Although this list is not exhaustive, it presents common BNR system configurations, their process diagrams and brief descriptions. Detailed descriptions of the BNR process configurations have been reported by WEF (2005).

1) Modified Ludzack-Ettinger (MLE) Process – continuous-flow suspended-growth process with an initial anoxic stage followed by an aerobic stage, to enable denitrifying bacteria first access to the influent substrate. An increase in internal mixed liquor recycle (IMLR) rate increases the rate of denitrification, but a rate beyond 400% of influent, provides no added benefits (WEF, 2005). The absence of an anaerobic zone prevents MLE from being suitable phosphorus removal. Figure 2-2 presents the flow schematic of the MLE process.

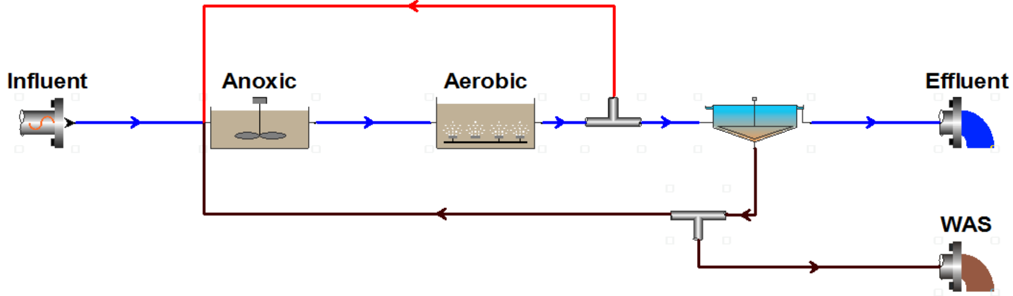


Figure 2-2. Flow schematic of MLE process.

2) Phoredox (A^2O) Process – MLE process preceded by an initial anaerobic stage; used to remove both TN and TP. Here the introduction of the anaerobic zone before the anoxic and aerobic zone enables the cyclic pattern required for proliferation of PAOs (phosphorus removal) and the IMLR from aerobic to anoxic zone enables nitrogen removal, although the system is sensitive to nitrate and dissolved oxygen recycle to the anaerobic zone. Figure 2-3 presents the flow schematic of the A^2O process.

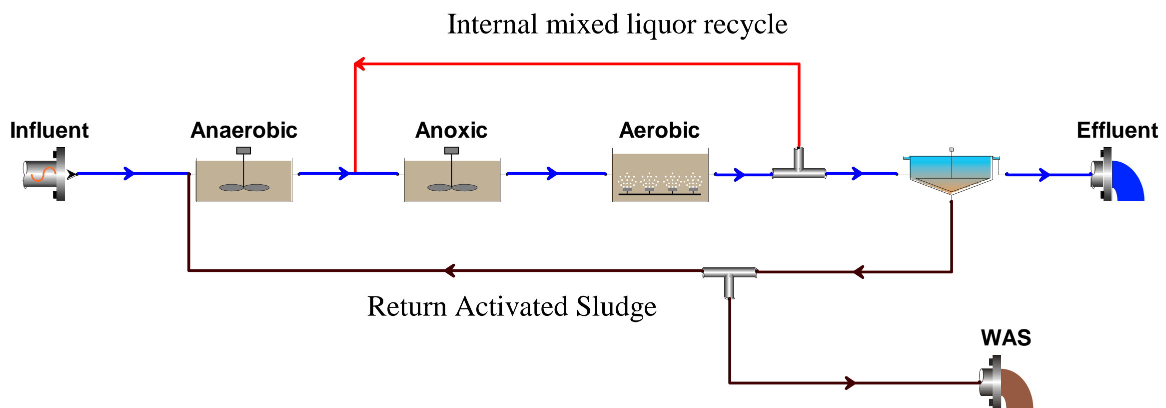


Figure 2-3. Flow schematic of A^2O process.

3) Bardenpho Process (Four-Stage) – continuous-flow suspended-growth process with alternating anoxic/aerobic/anoxic/aerobic stages. Excellent for total nitrogen removal but no capability for phosphorus removal due to the absence of an anaerobic zone, a selector for PAOs. Figure 2-4 presents the flow schematic of Bardenpho process.

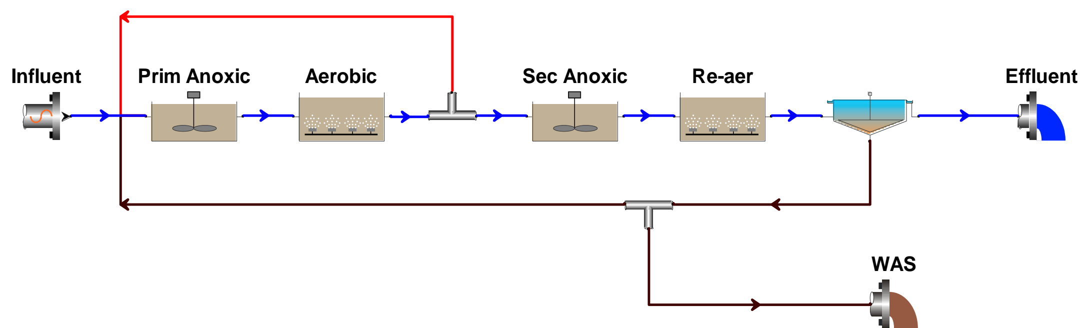


Figure 2-4. Flow schematic of Bardenpho process.

4) Modified Bardenpho Process – Bardenpho process with addition of an initial anaerobic zone, a selector for PAOs enrichment in the sludge. It provides excellent nitrogen removal and good phosphorus removal. Figure 2-5 shows the flow schematic of modified Bardenpho process.

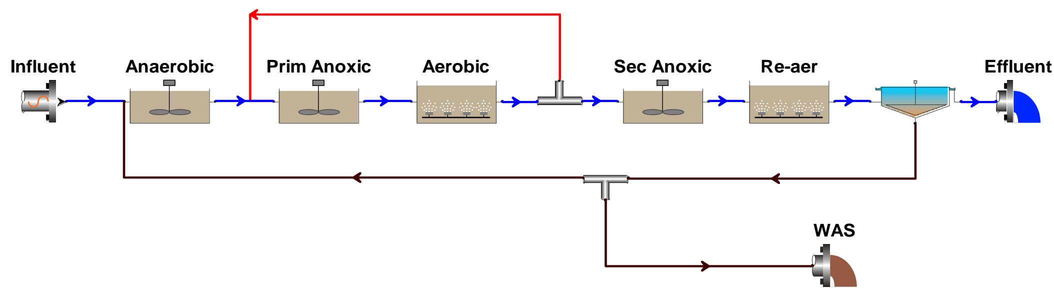


Figure 2-5. Flow schematic of modified Bardenpho process.

5) Virginia Initiative Process (UCT-BNR) – A variation of the A^2O configuration with the addition of an internal mixed liquor recycle from the anoxic zone to the anaerobic zone and the RAS returned to the anoxic zone rather than the anaerobic zone. This configuration minimizes the adverse effects of the nitrate return to the anaerobic zone. Here PAOs are given the selective advantage of full access to all available readily biodegradable organic matter or acetate. It is good for both total nitrogen and total phosphorus removal. Figure 2-6 shows the flow schematic of a UCT-BNR process.

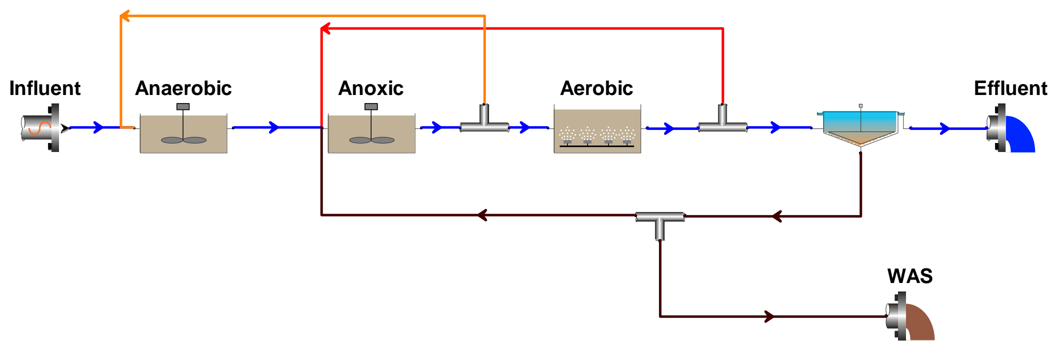


Figure 2-6. Flow schematic of UCT-BNR process.

6) Modified University of Cape Town (UCT) Process – To further minimize the effects of the nitrate recycle to the anaerobic zone from the anoxic zone, a second anoxic stage is introduced, where the internal nitrate recycle is returned. The first anoxic zone provides the mixed liquor recycle to the anaerobic zone, and under appropriate design and operating conditions, no nitrates are returned to the anaerobic zone. It provides good nitrogen removal and excellent phosphorus removal. Figure 2-7 shows the flow schematic of a modified UCT process.

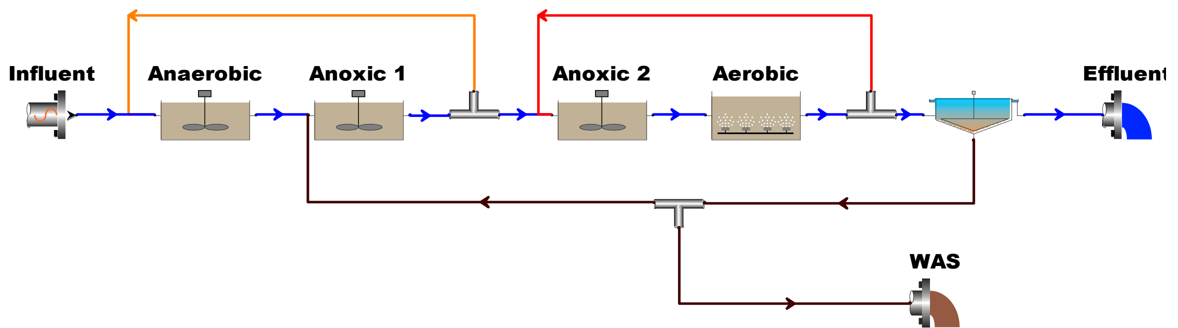


Figure 2-7. Flow schematic of modified UCT process.

7) Step Feed Process – alternating anoxic and aerobic stages, influent flow is split to several feed locations and the recycle sludge stream is sent to the beginning of the process; used to remove TN. Figure 2-8 shows the flow schematic diagram of step feed process.

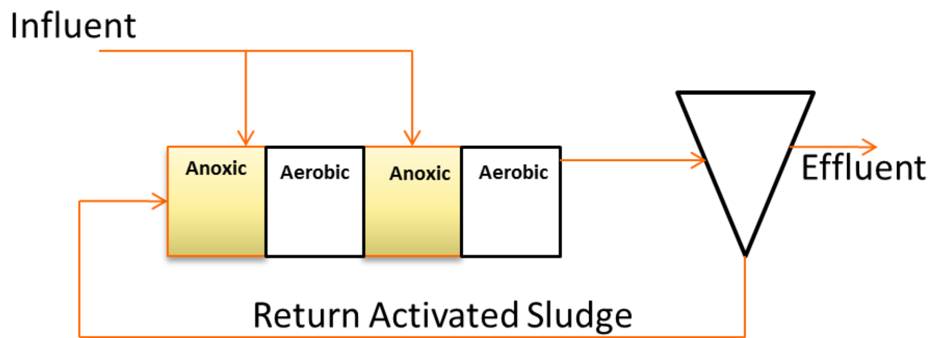


Figure 2-8. Schematic of Step Feed process.

8) Sequencing Batch Reactor (SBR) Process – SBRs are fill-and-draw reactors that operate sequentially through the different redox conditions by adjusting the mixing and aeration. The anaerobic/anoxic/aerobic

progression is necessary for removal of phosphorus and total nitrogen. Because of the fill-and-draw nature of SBRs, it is necessary to remove the nitrates remaining from the previous aerobic cycle before anaerobic conditions can be established, thus the typical treatment progression becomes anoxic/anaerobic/aerobic as depicted in the Figure 2-9.

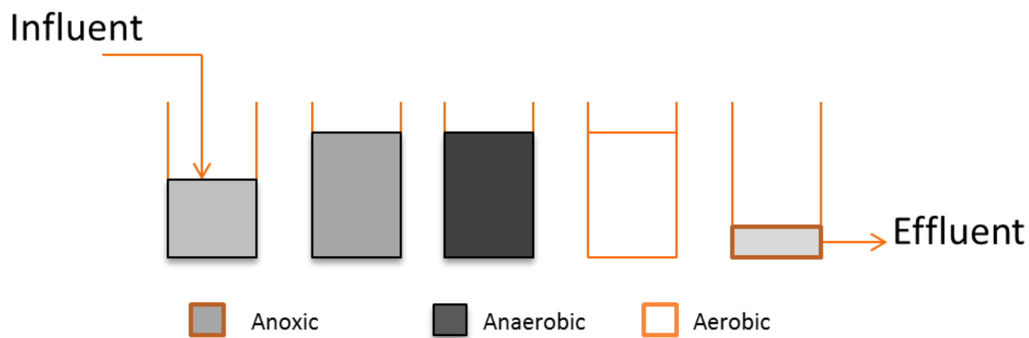


Figure 2-9. Schematic of Sequencing Batch Reactor.

9) Oxidation Ditch – continuous-flow process using looped channels to create time sequenced anoxic, aerobic, and anaerobic zones, cycling pattern essential for the proliferation of PAOs in the activated sludge. It provides both total nitrogen and total phosphorus removal. Figure 2-10 shows flow schematic of the oxidation ditch process.

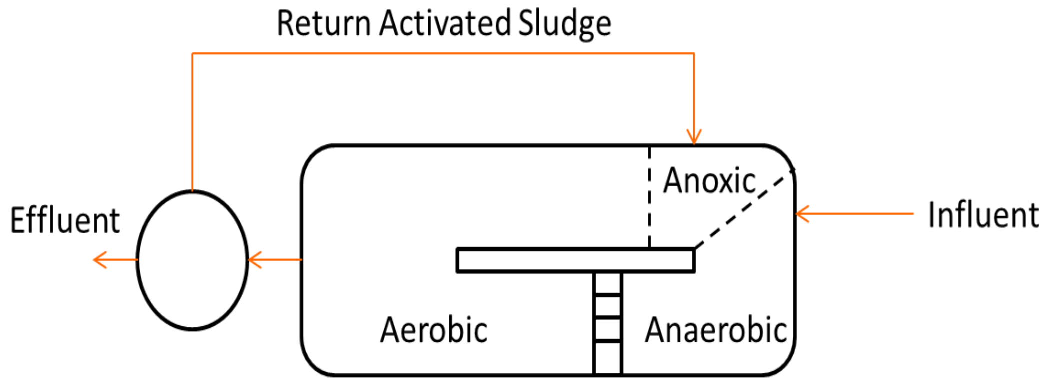


Figure 2-10. Schematic of Oxidation Ditch process.

2.3.1 Summary on BNR Process Configurations

Various configurations or basin arrangements of the BNR system have been developed to achieve efficient nutrient removal. The use of three zones, anaerobic/anoxic/aerobic is common in the configurations with exceptions that employ only two of the three. Two major features differentiate one configuration from the other; 1) zone sequencing and 2) recycle stream locations. The proliferation and enrichment of PAOs in a BNR bioreactor requires the selection of an appropriate BNR configuration. This is essential so as to ensure a stable reactor performance in terms of meeting the effluent quality while simultaneously enriching the population of PAOs in the bioreactor. The UCT-BNR process was selected for the research study because the process is amenable to changes in operating conditions and the configuration of the reactor supports proliferation of PAOs while maintaining good effluent quality.

It has been proposed that subdivision of the reactor volume into a cascade of compartments, significantly improves the removal of biodegradable MPs compared to a single mixed reactor (Joss et al., 2006), suggesting that, different configurations of BNR systems may provide different MP removal. Therefore, the estimation of the biotransformation constants of MPs under different redox condition would provide a platform that could be extrapolated to other BNR configurations. The next section examines the operating conditions for ensuring efficient biological phosphorus removal in a typical BNR process.

2.4 Biological Phosphorus Removal in BNR Process

The previous section identified the importance of selecting a BNR configuration that includes sequencing of anaerobic, anoxic and aerobic zones, creating the appropriate conditions for the enrichment of PAOs. In addition to having proper zone sequencing, appropriate design and optimum operating conditions such as optimum recycle ratio, influent carbon to phosphorus ratio and solids retention time must be employed so as to sustain the redox conditions and ultimately establish a healthy biomass in the reactors (WEF, 2005). Therefore, an understanding of the factors that affect the performance and stability of the BNR process is needed to effectively design, maintain, control and optimize the performance of the process. This section summarizes the design and operating

conditions required for operating a BNR system for enhanced biological phosphorus removal.

PAOs are organisms capable of storing phosphate as intracellular polyphosphate, leading to phosphorus (P) removal from the bulk liquid phase via PAO cell removal in the waste activated sludge. Unlike most other microorganisms, PAOs can take up carbon sources such as volatile fatty acids (VFAs) under anaerobic conditions, and store them as intracellular carbon polymers, called poly-β-hydroxyalkanoates (PHAs). The energy for this anabolic build-up is generated by the cleavage of polyphosphate leading to the release of phosphate from the cell. Reducing power is also required for PHA formation, which is produced largely through the glycolysis of internally stored glycogen (Mino et al., 1998). Aerobically, PAOs use their stored PHA as the energy source for biomass growth, glycogen replenishment, P uptake and polyphosphate storage. Net P removal from the wastewater is achieved through the removal of waste activated sludge containing high polyphosphate content. While the majority of P removal from the BNR process is often achieved through anaerobic–aerobic cycling, anaerobic–anoxic operation also allows P removal to occur, due to the ability of some PAOs (i.e. denitrifying PAOs or DPAOs) to use nitrate or nitrite instead of oxygen as electron acceptors and, therefore, perform P uptake and denitrification simultaneously. Maximizing the fraction of P removal

in the anoxic zone can reduce process operational costs, due to savings in aeration as well as in the amount of carbon sources needed for denitrification.

The design of any BNR process configuration to optimize biological phosphorus removal requires that the microbial reaction proceed in favor of the PAO population (WEF, 2005). There are a number of operational conditions that affect PAO development and growth in a BNR activated sludge. These conditions include:

- VFA potential of the influent stream (Jonsson et al., 1996; Mulkerrins et al., 2004)
- SRT/HRT (Shao et al., 1992; Smoulder et al., 1995)
- Temperature (Chang and Park, 2008; Whang and Park, 2001)
- pH (Filipe et al., 2001; Mulkerrins et al., 2004)
- Dissolved O_2 (Jonsson et al., 1996)
- Internal Mixed Liquor Recycle Rate (Jonsson et al., 1996)

2.4.1 VFA Potential of the Influent Stream

The performance of biological phosphorus removal processes has been reported to depend on the composition of the readily biodegradable organic matter (acetates) in the influent (Comeau et al., 1986). These acids may be in the feed, produced through the fermentation of municipal wastewater or can be added as a commercial or waste product. As previously mentioned, PAOs

transforms short chain VFAs (acetate and propionic) into intracellular PHAs. Therefore, a high concentration of VFAs in the influent stream will result in their rapid phosphorus uptake by the PAOs (Copp, 1998). Jonsson et al. (1996) indicated that 14 mg of VFA-potential is required to remove 1 mg of phosphorus. VFA-potential was defined as the concentration of VFA in a fermented sample of wastewater. Abu-Ghararah and Randall (1991) reported similar VFA-potential values within the range of 10 to 20 mg VFA/$P_{removed}$.

Comeau et al. (1996) indicated that a critical factor essential for optimizing phosphorus removal in a BNR system is the amount of VFA in the influent stream. Barnard and Steichen (2006) reported that 7 – 9 mg of VFA was needed to remove 1 mg of phosphorus, while Oldham et al. (1994) used VFAs to achieve effluent phosphorus level as low as 0.2 – 0.3 mg/L. Typically, to achieve phosphorus removal to an effluent concentration less than 1.0mg/L, the COD: TP ratio should be 40 or more (U.S. EPA, 2009).

Another group of organisms, glycogen accumulating organisms (GAOs) e.g *Competibacter* and *Defluviicoccus*, also have the ability to take up acetate in the anaerobic zone, not by using energy in phosphate bonds but by using stored glycogen as the energy source (Kong et al., 2006). The VFA are stored as a complex carbohydrate containing polyhydroxyvalerate (PHV), instead of PHB formed with poly-phosphorus as the energy source. Under certain conditions,

such as high temperatures over 28°C, high SRT, low pH in the aerobic zone or longer anaerobic HRT, GAOs may out-compete PAOs for the VFAs, which would result in less or no release of phosphorus in the anaerobic zone. This in turn will result in fewer fractions of PAOs in the biomass and ultimately deterioration of the BNR system (Filipe et al., 2001; Saunders et al., 2003).

Oehmen et al. (2005) indicated that PAO can have a competitive advantage over GAO when the VFA consists of roughly equal proportions of acetic and propionic acid as substrate. This is because PAOs are able to switch to propionate much more quickly and effectively than GAOs. Therefore to stimulate optimal growth of PAOs, the strategy is to feed the BNR system with an equal amount of acetic acid and propionic acid (Oehmen et al., 2005; Bott et al., 2007).

2.4.2 SRT/HRT

The SRT represents the average length of time a particulate constituent stays in a bioreactor. HRT determines the contact time between the solution phase that contains the substrate and nutrients and the biomass, which is made up of the micro-organisms scavenging food materials for growth. Phosphorus release and uptake rates in anaerobic and aerobic zones must be considered in selecting the overall system and individual zone HRT values. The HRT value can be controlled by appropriate flow adjustments at constant volume.

The effect of SRT on the BNR process has been widely investigated (Shao et al., 1992; Smoulder et al., 1995). An increase in SRT causes an increase in the concentration of ordinary heterotrophs that produce VFAs in the anaerobic zone and therefore proliferation of PAOs and subsequent P-removal may be enhanced in processes with low VFAs in the feed. However, the SRT increase could lead to reduction in P-removal due to reduced rate of sludge wastage.

BNR systems can operate at SRT values greater than 3 days. However, at SRT values greater than 4 days and at temperature greater than 15°C, nitrification will become active and nitrates should be denitrified. As the SRT is increased to a level where endogenous reactions become significant, secondary release of phosphorus may lead to decreased performance at constant feed VFA and COD values (WEF, 2005). Although the performance of other heterotrophic reactions rely more on the system sludge age, in BNR, performance cannot be defined solely based on the SRT and HRT. The feed COD: P ratio shapes the microbial composition of the BNR sludge and the effluent levels that can be attained (WEF, 2005).

Previous studies have shown that the ratio of HRT in the anaerobic zone to the HRT in the aerobic zone is important for the optimal operation of a BNR system. Sufficient time should be allowed for the formation of VFAs and storage of intracellular PHAs in the anaerobic zone. If the time is too short, phosphorus

uptake in the aerobic zone will be lower than achievable because insufficient PHAs were stored in the anaerobic zone. Neethling et al. (2005) reported a ratio of between 3 and 4 for aerobic HRT to anaerobic HRT led to optimal performance of a BNR system.

Grady et al. (1999) suggested that at temperatures above 15°C, it may be very difficult to operate at an aerobic SRT sufficiently high to allow PAOs to grow without also populating nitrifying bacteria. Under such circumstances, the UCT-BNR process has distinct advantages.

2.4.3 Temperature

High temperature can have an adverse effect on phosphorus removal. Whang and Park (2006) and Lopez-Vazquez et al. (2007) reported that temperatures lower than 20°C could favor PAO over GAO, resulting in a stable BNR process, while the opposite could occur at temperature greater than 20°C. Bott et al. (2007) reported predominance of GAOs in a BNR operated at temperatures greater than 28°C.

Full and pilot scale studies have shown that BNR can be affected by low temperature. PAOs outcompete GAOs at temperatures as low as 5°C, the GAOs practically disappeared in the 5°C reactor (Erdal et al. (2002). However, fermentation in the collection system will decrease with decrease in temperature,

thus leading to insufficient VFA in the feed, thereby interfering with overall phosphorus removal.

2.4.4 Effect of pH

Many studies have shown that a higher ambient pH in enriched PAO sludges has resulted in a higher anaerobic P release (Smolders et al., 1994; Liu et al., 1996; Bond et al., 1999; Filipe et al., 2001). Smolders et al. (1994) found that the ratio of anaerobic P release to acetate uptake varied linearly from 0.25 to 0.75 P-mol/C-mol when pH rose from 5.5 to 8.5. The reason for this variation was explained as follows: under the assumption that the internal pH of the cell is kept constant, there is an increased pH gradient and a corresponding increase in electrical potential difference across the cell membrane at a high ambient pH. Therefore, more energy is needed for acetate transport through the membrane when external pH is high. This increased energy is generated through an increase in polyphosphate degradation.

However, the acetate uptake, glycogen degradation and PHA accumulation rates of PAOs have been shown in batch tests to be independent of pH over the range 6.5–8.0 (Filipe et al., 2001), indicating that the higher energy requirements to take up acetate does not negatively affect their ability to metabolize VFA. Aerobically, a series of batch tests has shown that P uptake, PHA utilization and biomass growth were all inhibited by a low pH (6.5),

suggesting that a higher aerobic pH (7–7.5) would be more beneficial for PAOs (Filipe et al., 2001).

It has been postulated that an anaerobic pH of 7.25 is a critical point, whereby GAOs are able to anaerobically take up VFA faster than PAOs at pH values below 7.25, and PAOs take up acetate faster above this pH value (Filipe et al., 2001). However, Liu et al. (1996) indicated an optimum pH of 6.8 ± 0.7 for anaerobic acetate metabolism (acetate uptake rate coupled with P-release rate). An improved level of P removal was observed when the anaerobic pH set point was increased from 6.8 to 7.25 (Filipe et al., 2001). Other studies have also shown higher P removal when the anaerobic and/or aerobic pH level was increased (from pH 7 to 7.5–8.5) (Bond et al., 1998; Serafim et al., 2002). The reason for the improved performance was hypothesized to be a shift in the microbial competition from GAOs to PAOs.

2.4.5 Dissolved Oxygen (DO)

A BNR process needs to satisfy different oxygen demands from the different bacterial populations present in the system. BNR systems designed for COD removal and nitrification typically require DO levels greater than 2 mg/L (Louzeiro et al., 2002). In a BNR system, the anaerobic zone must be kept devoid of oxygen (0 - 0.2 g/L O_2) as the presence of oxidizing substances such as oxygen and nitrate interfere with the efficiency of the BPR process.

The presence of low levels of oxygen or other oxidizing agents affects the redox potential and thus negatively impacts on the rate of phosphate release. (Shehab et al., 1996). Maintaining an oxygen concentration of between 3.0 and 4.0 mg/l in the aerobic zone has been recommended (Shehab et al., 1996). It has also been reported that, for successful BNR, a DO concentration 3.0 - 4.0 mg/l is essential. Brdjanovic et al. (1998) revealed that excess aeration can have a negative impact on the BPR process as cessation of P-uptake occurs due to depletion of poly-hydroxy-butyrate (PHB) in an over aerated process.

The presence of nitrate in the anaerobic zone has also been reported to affect the BPR process (Shehab et al., 1996). Residual nitrate in the anaerobic phase results in consumption of rbCOD by denitrifiers, thus decreasing the availability of organic matter for PAOs.

2.4.6 Internal Mixed Liquor Recycle Flows (IMLR)

Typical internal recycle flows employed in BNR processes include:

- Recycle from aerobic zone to anoxic zone (nitrified mixed liquor recirculation-NR)
- Recycle from anoxic zone to anaerobic zone (anoxic mixed liquor recirculation-AR), and

A low NR flow limits the amount of DO and nitrate available for the PAOs to uptake phosphorus and could lead to secondary release of phosphorus.

Increasing the NR recycle flow will reduce the retention time in the anoxic zone and increase the amount of nitrates and dissolved oxygen in the anoxic zone. This will stop secondary release of phosphorus thus reducing the mass of released phosphorus that must be taken up in the aeration basin. One of the major factors influencing the occurrence of DPAO and associated anoxic P uptake appears to be the nitrate load into the anoxic reactor, i.e, the nitrate load should be large enough or exceed the denitrification potential of ordinary heterotrophic organisms (OHO), i.e non-PAOs in the anoxic reactor to stimulate DPAO in the system (Hu et al., 2002). If the nitrate load into the anoxic reactor is less than the denitrification potential of OHO, the OHO will outcompete PAO for the use of the limited nitrate, while if the nitrate load exceeds the denitrification potential of OHO, the PAO will utilize the "excess" nitrate and thus develop in the system (Hu et al., 2002).

The AR rate should be established such that oxygen and nitrates are not recycled to the anaerobic zone. The presence of oxygen and nitrate in the anaerobic zone inhibits fermentation of the soluble organics to acetate thereby starving the PAOs. Also, denitrifying bacteria and OHOs will compete with PAOs for rbCOD/VFA substrate, reducing the selective advantage for the PAOs in the zone. This inadvertently interferes with the efficiency of biological phosphorus removal.

In the UCT process, an increase in the anoxic mixed liquor recycle (AR) provides for increased organic utilization in the anaerobic stage. In this configuration the AR contains soluble BOD but little or no nitrate, thereby providing optimal conditions for fermentation and VFA uptake in the anaerobic zone. Because the mixed liquor rather than the RAS is recycled to the anaerobic zone, the MLSS concentration in the zone is lower than the MLSS concentration in the remainder of the bioreactor. Therefore, a longer anaerobic zone HRT (1 to 2 hrs) is needed to achieve the desired SRTs. The anoxic recycle (AR) and the nitrified mixed liquor recirculation rates (NR) are typically two times the process influent flow rates (Grady et al., 1999).

Phosphorus may also be released when there is a low nitrate concentration in the mixed liquor to the final clarifier. When a deep sludge blanket develops in the final clarifier, nitrates will be denitrified, clarifier becomes anaerobic and hence phosphorus can be released. This release may not affect the effluent phosphorus but may return a large portion of the released phosphorus back to the anoxic/aerobic zone, where there may not be enough VFA for uptake of this additional released phosphorus (WEF, 2005).

Typical operating parameters used in the design of various BNR systems are shown in Table 2-2. The operating parameters vary depending on the BNR configuration. The BNR system selected for investigating MPs removal should be

able to accommodate the removal of conventional wastewater pollutants (COD, N, P) as well as the removal of MPs. As previously stated, the UCT-BNR design was selected for this study because it satisfies the requirements for the research work.

Table 2-2. Typical design parameters for commonly used BNR processes

Process/ Design Parameters	SRT, d	MLSS, mg/L	Anaerobic HRT, h	Anoxic HRT, h	Aerobic HRT, h	RAS, % of Influent	IMLR, % of Influent
UCT	10-25	3000-4000	1-2	2-4	4-12	80-100	100-300 (aero) 200-400(anoxic)
A^2O	5-25	3000-4000	0.5-1.5	0.5-1	4-6	25-100	100-400
A/O	2-5	3000-4000	0.5-1.5	-	1-3	25-100	
PhoStrip	5-20	1000-3000	8-12		4-10	50-100	10-20
SBR	20-40	3000-4000	1.5-3	1-3	2-4		
UCT-BNR	5-10	2000-4000	1-2	1-2	4-6	80-100	100-200(anoxic) 100-300(aero)
Modified Bardenpho	10-20	3000-4000	0.5-1.5	1-3 (1st Stage) 2-4 (2^{nd}Stage)	4-12 (1^{st}Stage) 0.5-1 (2^{nd}Stage)	50-100	200-400
General Design Considerations: Aerobic zone DO > 2 mg/L, pH > 6.5							

Adapted from Tchobanoglous et al., 2003.

2.4.7 Summary of Biological Phosphorus Removal in BNR Process

One of the objectives of this research work was to investigation the role of PAOs in the removal of MPs in wastewater. PAO enrichment in a typical BNR system requires careful selection and operation of the system to achieve desired

objectives, given the fact that BNR operation is more onerous to maintain than conventional activated sludge system.

Factors that may affect the proliferation of PAOs in the anaerobic basin of a BNR include temperature, SRT, and pH. The selected experimental SRT was expected to favor PAOs over GAOs in the pilot plant because the operating SRT (18-20 days) was beyond the range that could affect the competition of PAOs and GAOs (3 to 5 days; Whang and Park, 2001). pH has been found to be one the key factors that affect the metabolic rates and competition between PAOs and GAOs (Filipe et al., 2001). Acetate uptake rate by both GAOs and PAOs has been shown to increase with pH up to 6.5, but while it starts to decrease at pH 7.5 for GAOs, it remains nearly constant from 7.0 to 8.5, and starts to decrease at pH 8.5 for PAOs (Filipe et al., 2001; Schuler and Jenkins, 2002). Therefore, the operating conditions of a BNR process should be carefully monitored and maintained in order to proliferate PAOs for enhanced biological phosphorus removal.

2.5 Micropollutant Removal Mechanisms in Activated Sludge Systems

Sorption to solids (dependent on solid–water distribution coefficient), biodegradation (aerobic/anoxic/anaerobic), volatilization (influenced by Henry's Law constants) and chemical transformation (hydrolysis, acid base, photolysis, and precipitation) are possible mechanisms of removal of MPs. However, the

former two are typically considered as the most significant for many non-volatile MPs and personal care products (PPCP) (Ternes et al., 2004). In this section, the fundamental mechanisms (sorption and biodegradation) that impact MPs in activated sludge systems are reviewed.

2.5.1 Sorption

The process by which chemicals become associated with the solid phase is generally referred to as sorption. The attachment of the molecule to a two-dimensional surface is termed adsorption while molecular penetration into a three dimensional matrix is called absorption (Schwarzenbach et al., 2003). In wastewater treatment, sorption onto reactor biomass can be an important removal mechanism depending on the propensity of MPs to partition to the sludge. The sorption potential of a given compound is largely dependent on the distribution coefficient, K_d. The distribution coefficient is the ratio between the concentration of a compound in the sorbed phase and the liquid phase. Under equilibrium conditions, the concentration sorbed onto sludge (X) is assumed to be proportional to the concentration in solution (S) (Ternes et al., 2004) :

$$K_d = \frac{X_{part}}{S} = \frac{X}{X_{SS}.S} \qquad (2\text{-}4)$$

Where,

X= concentration sorbed onto sludge, per unit reactor volume [$\mu g.L^{-1}$]

X_{part}= concentration sorbed onto sludge, per amount of sludge dry matter [$\mu g.g_{SS}^{-1}$]

K_d= solid-water distribution coefficient [$L.g_{SS}^{-1}$]

X_{ss}= suspended concentration in raw wastewater or production of suspended solids in primary and/or secondary treatment per L of wastewater [$g_{SS}.L^{-1}$]

S= dissolved concentration [$\mu g.L^{-1}$]

The total concentration (C) of a compound in a solution is given as C=X+S and hence under equilibrium conditions,

$$C = S.\,(1+X_{ss}.K_d) \qquad (2\text{-}5)$$

In a situation when K_d cannot be determined experimentally, Dobbs et al. (1989) presented an expression to estimate K_d of a substance from its K_{ow}. This expression has been reported to be valid for determining the sorption of compounds in wastewater.

$$\text{Log } K_d = 1.14 + 0.58 \text{ Log } K_{ow} \qquad (2\text{-}6)$$

The K_{ow} (octanol-water partition coefficient) is defined as the ratio of the concentration of a chemical in octanol to the concentration of the chemical in water at equilibrium in a two phase water/octanol system at a specified temperature.

Table 2-3 shows solid-liquid partitioning coefficient, K_d values reported by different studies suggesting the existence of variability between secondary sludge which ultimately affects sorption. However, removal by sorption in municipal WWTP is typically considered to be negligible (<10%) for compounds with log K_d values ≤ 2.5 $L.kgSS^{-1}$ (Joss et al., 2006; Ternes et al., 2004). Most hydrophilic MPs have log K_d values ≤ 2.5 $L.kgSS^{-1}$. Therefore, removal efficiencies observed for most hydrophilic MPs in WWTP are attributed to biodegradation and not sorption (Ternes et al., 2004). Based on this premise, this study will be conducted with an assumption that sorption does not contribute significantly to the removal of the target MPs, since their log K_d values are ≤ 2.5 $L.kgSS^{-1}$.

Table 2-3. K_d of selected Micropollutants in contact with secondary sludge

Compound	log K_d (Secondary sludge) $L.kgSS^{-1}$	pKa	References
Carbamazepine	1.4 0.09 1.8	<1,13.9	Jones et al., 2002 Ternes et al., 2004 Urase and Kikuta, 2005
Trimethoprim	2.3 0.28		Gobel et al., 2005 Batt et al., 2006
Estrone	3 2.9		Joss et al., 2004 Carballa et al., 2007
17-β-Estradiol	2.8		Clara et al., 2004
17-α-Ethinylestradiol	2.5		Ternes et al., 2004
Gemfibrozil	1.9	4.8	Urase and Kikuta, 2005
Ibuprofen	2.7 0.85 1.9	4.5-5.2	Jones et al., 2002 Ternes et al., 2004 Urase and Kikuta, 2005
Sulfamethoxazole	2.4	1.8/5.6	Gobel et al., 2005

2.5.2 Biodegradation of Micropollutants

Biodegradation is considered the most important elimination process for MPs in activated sludge (Quintana et al. 2005). This process can occur through direct metabolism or cometabolism. Direct metabolism involves microbial use of MPs as primary or secondary carbon and energy sources while cometabolic transformation involves microbial energy gain from non-limiting biodegradable organic compounds (Volodymyr, 2011), while indirectly transforming MPs. Another facet of cometabolism, called ‘‘commensalism,’’ involves sequential transformation of a substance by a group of microbial species with some species of the community specialized in early and other in later stages of biotransformation (Volodymyr, 2011).

Various studies have examined MP removal by biodegradation in many different systems, including WWTPs, membrane bioreactors (MBRs), sequencing batch reactors (SBRs), and constructed wetlands. Some of these studies focus solely on biodegradation as a removal process (Layton et al., 2000; Quintana et al., 2005; Yu et al., 2006) , while others examined overall removal due to a combination of different processes including biodegradation (Andersen et al., 2003; Carballa et al., 2005; Gobel et al., 2007). These studies have expanded the knowledge base regarding the fate of MPs in various treatment systems (Lab scale, pilot scale and WWTP). However, there exists a wide variation in removal efficiencies across therapeutic class, treatment processes, and even among separate studies for the same individual compounds (Onesios et al., 2009) as shown in Table 2-4. This variability prevents the generalization of compound behavior in engineered or natural systems thus making the characterization of the fate and risks associated with MPs in the environment challenging. However, these studies are useful as they provide insight into the potential for biodegradation or biotransformation of MPs in the environment. The results shown in Table 2-4 provide an overview of the removal efficiency of different processes where biodegradation or biotransformation has been assumed to be the predominant removal mechanism.

The differences in removal of the compounds have been attributed to various factors (Xue et al., 2010; Onesios et al., 2009; Joss et al., 2006) including; the use of different definitions of removal by various studies investigating the removal of MPs, the physical and chemical properties of the MPs, the degree of biodegradation or biotransformation in different studies (operating condition of the system used), type of biological treatment employed, redox conditions of the reactors, initial MP concentrations, primary substrate concentrations, incubation times for batch experiments, and source or type and concentration of microbial inoculums. Therefore there is the need for more research to critically assess the effects of these factors on the removal of MPs and to investigate the optimization potential of these factors in improving the removal of MPs in treatment systems.

It is noted that the disappearance of the parent compound of MPs cannot be solely considered synonymous with complete biodegradation. The loss of the parent compound indicates biotransformation to an unknown degree, and not necessarily mineralization. Without monitoring of metabolites or mineralization end products, the extent or degree of biodegradation cannot be accurately determined. In order to determine the actual biodegradability of MPs, detailed biodegradation studies, such as mineralization experiments and biodegradation pathway studies, are employed (Onesios et al., 2009). Therefore, it is

recommended that proper nomenclature should be encouraged among researchers to avoid confusion or report variability.

Table 2-4. Micropollutant removal attributed to biodegradation/biotransformation

Compound	Removal efficiency	System Studied	Reference	comments
Anticonvulsant				
Carbamazepine	0 (M), 0(T)	Anaerobic digester, pilot scale	Carballa et al. (2006)	Anaerobic digester: Pilot scale, mesophilic (M) and thermophilic (T) conditions, 4–400 μg L^{-1} PPCP
Hormone				
EE2	<1	MBR, lab scale	Cirja et al. (2007)	MBR: Lab scale, % mineralization reported, 100 μg L^{-1} PPCP
	85 ± 5 (M), 75 ± 15 (T)	Anaerobic digester, pilot scale	Carballa et al. (2006)	Anaerobic digester: Pilot scale, mesophilic (M) and thermophilic (T) conditions, 4–400 μg L^{-1} PPCP
	~ 100[a], 20.2 ± 11[b]	Batch	[a]Vader et al. (2000); [b]Layton et al. (2000)	Batch: Nitrifying activated sludge inoculum, 6 d incubation, 50 μg L^{-1} PPCP; 72 μg L^{-1} PPCP, 24 h incubation, WWTP biosolids inoculum; % mineralization presented
Lipid regulator and statin				
Gemfibrozil	>99	Batch	Yu et al. (2006)	WWTP: 24 h composite influent and effluent samples
				Batch: Activated sludge inoculum, 50 d incubation, 1, 10, and 50 μg L^{-1} PPCP
NSAID				
Ibuprofen	40 ± 15(M), 73 ± 9(T)	Anaerobic digester, pilot scale	Carballa et al. (2006)	Anaerobic digester: Pilot scale, mesophilic (M) and thermophilic (T) conditions, 4–400 μg L^{-1} PPCP

Table 2.4 continued. Micropollutant removal attributed to biodegradation/biotransformation

Compound	Removal efficiency	System Studied	Reference	comments
	64-70[1]	Lab columns	Zwiener and Frimmel (2003)	WWTP: Pilot scale, activated sludge inoculum, 10 μg L^{-1} PPCP + 30 mg L^{-1} acetone;
	17-21[2]			Lab column: Activated sludge inoculum, 10 μg L^{-1} + 35 mg L^{-1} acetone, (1)oxic, (2)anoxic
	97- >99	Batch	Buser et al.(1999)	WWTP: 24 h flow proportional samples, influent to biological stage and treated effluent sampled; Batch: WWTP influent inoculum and activated sludge, incubated for 8 h
	ND[1], 100[2]		Quintana et al. (2005)	WWTP: 24 h composite samples from WWTP MBR, mean removals reported; Batch: sludge inoculum, mean removals by transformation reported, 28 d incubation; (1)20 mg L^{-1}, PPCP as sole carbon source, (2)5 mg L^{-1} PPCP and 50 mg L^{-1} milk
	>99		Yu et al. (2006)	WWTP: 24 h composite influent and effluent samples Batch: Activated sludge inoculum, 50 d incubation, 1, 10, and 50 μg L^{-1} PPCP
Antibiotic				
Sulfamethoxazole	99 ± 1(M), 99 ± 1(T)	Anaerobic digester, pilot scale	Carballa et al. (2006)	Anaerobic digester: Pilot scale, mesophilic (M) and thermophilic (T) conditions, 4–400 μg L^{-1} PPCP

Adapted from Onesois et al. (2009)

2.5.3 Micropollutant Biodegradation Models

Several models have been postulated to fit the biodegradation data for MPs in a biological reactor. Table 2-5 shows model equations that have been reported for modeling biodegradation of MPs. The table shows the common use of a pseudo-first order formulation which is dependent on the liquid phase concentration of the MPs and the mixed liquor biomass concentration, "X". Different models have used different values for "X". For example using the mixed liquor suspended solids (MLSS) concentration as the value of X in the model would lead to a linear increase in the removal rate from a system with a low value of MLSS to a system with a high value of MLSS without considering the sludge activity.

Some studies have used the volatile suspended solid (VSS) concentration for the value of "X" in their attempt to account for the active biomass concentration but there are concerns involved in the use of VSS as an estimate of the active bacterial population because it does not differentiate between active and inactive biomass. In fact, other protozoa and metazoan may contribute to the volatile content of the solid. It is not unreasonable to assume that not all the organic fraction of the biomass contributes to substrate degradation, only the active portion of the organic fraction does. Therefore the use of viable biomass concentration is more appropriate.

Furthermore, it is also reasonable to assume that there are different groups of viable biomass that participate in the biodegradation of MPs in the activated sludge. For example, some studies have suggested that nitrifying bacteria and heterotrophic bacteria could be responsible for MP biotransformation or co-metabolic transformation (Shi et al., 2004; Gaulke et al., 2008; Khunjar et al., 2011). Therefore, the model developed for this study incorporates the concentration of specific groups of heterotrophs; poly-phosphate accumulating organisms (PAOs, X_{PAO}) and ordinary heterotrophic organisms (OHOs, X_{OHO}) rather than the concentration of the total organic fraction of the biomass, X_{vss}.

Table 2-5. Biodegradation models for micropollutants removal

Process	Model	Biodegradation rate equation	References
1a	1st order	K_mC	Zhao et al., 2008
1b	1st and pseudo order	$K_mC(1+K_dX_v)$	Gang et al., 2007
2	Two 1st order	$K_mC + K_m.X_v$	Cowan et al., 1993
3a	Pseudo-1st order	K_mX_vC	Schwarzenbach et al., 2003
3b	Pseudo-1st order	K_mX_TC	Joss et al., 2006
3c	Pseudo-1st order	K_mX_HC	Jacobsen et al., 1996
3d	Pseudo-1st order	K_mX_cC	Lee et al., 1998
4	Monod Model	$K_mC/(C+K_s)$	Marfil-Vega et al., 2010

Source: adapted from Banihashemi and Drote (2011)

K_m-biodegradation rate coefficient; C-concentration of dissolved Micropollutants; X_v-volatile suspended solid concentration (mg/L); X_T-total concentration of solids (mg/L); X_H-concentration of heterotrophy biomass (mg/L); X_c-concentration of specific Micropollutants degrader (mg/L); K_s-half velocity constant for soluble degradable substrate (ML^{-3}) and K_1-inhibition constant.

2.5.4 Summary of Micropollutant Removal Mechanisms in Activated Sludge System

Biodegradation has been suggested as the most important removal mechanism for MPs, especially for compounds with Log $K_d \leq 2.5$ Lkg_{SS}^{-1}. Micropollutants degradation by biomass in the activated sludge process is considered to be achieved through co-metabolic degradation due to the low concentrations at which the MPs exist in wastewater. A pseudo first order kinetic model is the most commonly used model to describe the degradation of MPs in wastewater. A modified form of this model was used in this research.

2.6 Review of Microorganisms involved in Micropollutant Degradation

The source and type of the microbiological populations is an important factor in the removal of MPs, be it biotransformation, biodegradation or mineralization. This was demonstrated by Layton et al. (2000) when they reported that the removal efficiency of E2 in biosolids obtained from municipal plants was 80% greater than removal efficiency found in biosolids obtained from industrial plants.

Several previous studies investigating the removal mechanism of MPs in wastewater treatment have attributed their degradation to the presence of autotrophic ammonia oxidizing bacteria in nitrifying activated sludge (Clara et al., 2005b). Although an isolated culture of heterotrophic bacteria has been

grown under a high concentration of estrogens as primary substrate for carbon and energy (Shi et al., 2002; Haiyan et al., 2007), there has been very few reports on the role of heterotrophs in the removal of MPs at the concentrations typical of WWTPs.

A few recent studies (Table 2-6), have reported that heterotrophs play a role in the overall degradation of MPs.

The important findings from these reports are;

- Both autotrophic and heterotrophic organisms contribute to the removal of MPs from wastewater.
- The relationships between autotrophic organisms and heterotrophic organisms in MP degradation in wastewater could lead to commensalism (Khunjar et al., 2011). Commensalism refers to the sequential transformation of a substance by a group of microbial species with some species of the community specialized in early and other in later stages of biotransformation. It is a relationship between biotic elements in which only one element benefits. (Volodymyr, 2011).
- There are a variety of heterotrophic organisms that are involved in MP degradation but no report has investigated the role of PAOs.

- Co-metabolic enzymes produced by heterotrophic bacteria seem to cover broader range than ammonia oxidizing bacteria's enzyme (Khunjar et al., 2011).
- There is insufficient information in the literature to conclusively link either autotrophic or heterotrophic organisms to MP degradation in wastewater treatment.

Table 2-6. Summary of reports on role of microorganisms in MP removal from wastewater

Compound	Degradation Environment	Dominant Organisms/ Biomass involved in degradation	Comments	Reference
EE2,TMP	Ammonia Oxidizing Bacteria (AOB) Culture	AOB biotransformed EE2 but not TMP, AOB biotransformed EE2 better than heterotrophs	High degree of transformation (≤ 1mg/L) was observed for EE2 and TMP in the presence of heterotrophic dioxygenase enzyme. AOB and heterotrophs may cooperatively enhance the efficiency of EE2 removal.	Khunjar et al., 2011
	Heterotrophic culture devoid of nitrifiers	Heterotrophs mineralized EE2, biotransforms TMP and mineralized EE2-derived metabolites generated by AOBs		
EE2	Batch test with enriched Autotrophic Ammonia Oxidizer	AOB	Strong relationship between nitrification and co metabolic EE2 degradation. Suggested heterotrophic organisms might play a role but not investigated.	Yi and Harper (2007)
EE2	Batch test with AOB	*N. europaea & Nitrospira multiformis*	Results suggested EE2 removal at concentrations found in WWTP is not due to co metabolic degradation by AOB or abiotic nitration, but most likely due to heterotrophic bacteria.	Gaulke et al., 2008
EE2	Batch Test at low and High SRT	Autotrophs and Heterotrophs	Suggested Heterotrophs are capable of PhAc degradation at low and high SRT	Gaulke et al., 2009
E1, E2, EE2	Batch Test with a culture of AOB	*N. europaea*	Suggested there are other organisms involved in Estrogen degradation apart from AOB	Shi et al., 2004

Table 2-6 (continued). Summary of reports on role of microorganisms in MP removal from wastewater.

Compound	Degradation Environment	Dominant Organisms/ Biomass involved in degradation	Comments	Reference
CAF,CBZ, DCF,SMX, PCT	Batch Test with heterotrophs	Heterotrophs	Reported faster degradation of PhAc in the presence of high concentration of heterotrophs and low SRT	Majewsky et al., 2011
4-NP, TCS, BPA	Batch experiment using sludge from 3, 10 and 20 d SRT	Autotrophic and Heterotrophic Org.	High removal rate (>90% were reported for the compounds at the 3 SRTs, suggesting heterotrophs also play a significant role in Micropollutants degradation	Stasinakis et al., 2010
Various EDCs and PhAcs	Pilot scale experiment with NAS, CAS, BNR	Autotrophic and Heterotrophic Org.	BNR achieved better removal of EDC and PhA/cs than other methods suggesting the involvement of other heterotrophs in the removal of Micropollutants.	Ogunlaja et al., (2013)
E1, E2, E3, EE2	Batch test with isolated Heterotrophic bacteria	*Rhodococcus erythropolis*	Reported rapid degradation of estrogen	Yoshimoto et al., 2004
		Sphingobacterium sp.	The microorganism grew on EE2 (30mgL^{-1}) as the sole carbon and energy source (87% metabolized) within 10 days and 30ºC	Haiyan et al., 2007

CAF-caffeine, CBZ-carbamazepine, DCF-Diclofenac, SMX-Sulfamethoxazole, BPA-Bisphenol A, 4-NP-4 Nonyl phenol,TCS-Triclosan, PhAc-pharmaceutically active compounds, TMP-Trimethroprim, E1- Estrone, E2- Estradiol, EE2- Ethinylestradiol, PCT- Paraceutamol.

Research into novel methods of isolating and culturing MP degrading inocula found in WWTP and introducing them in bulk concentration into the biological reactors of wastewater treatment plants could potentially reduce the concentrations and estrogenic activities of MP released into the environment. However, isolation of the microbial species in the activated sludge of a BNR process is not a trivial task.

Currently, investigation of the microbial ecology in BNR systems has relied on molecular based techniques such as fluorescence in situ hybridization (FISH) or PCR-based methods (Seviour et al., 2003; Oehmen et al., 2010). BNR performance, kinetics and metabolic characterization has typically been based on measurements of the substrates and products using enrichment mixed cultures due to the unsuccessful attempts to obtain any PAO isolates. As a result, BNR population characterization has been limited to the species whose phylogenetic information has been obtained.

However, it is recognized that the phylogenetic diversity of PAOs in full-scale BNR systems are likely larger than those known (Gu et al., 2008; Seviour et al., 2003). On the other hand, phylogenetic identification and quantification do not necessarily reflect cell metabolic state and their activities and so, linking the

BNR performance activities with the relative population composition/structure has been quite difficult.

Advanced techniques such as FISH combined with microautoradiography are useful to identify and understand the *in vivo* physiology, diversity and activity of functional microbial groups in biological processes (Nielsen et al., 1999), but again, this method can reveal organic substrate assimilation and phosphate uptake by only those bacteria that can be targeted by available probes (Kong et al., 2004). Recently, flow cytometry was used as a novel fluorescent staining technique that allows reliable quantification of PAOs and polyphosphate accumulation dynamics revealing growth activities of not-yet cultivable bacteria in BNR (Gunther et al., 2009). However, these methods are very laborious, time consuming and require specialized technical knowhow. Therefore, quantification of active biomass species would not be carried out during this study but their abundance will be monitored through their metabolic activities in the BNR process.

2.6.1 Summary of Microorganisms involved in Micropollutant Degradation

Microorganisms that co metabolize or commensalize MP in wastewater treatment plants are diverse but can be grouped as either autotrophic or

heterotrophic organisms. Previous attention has been given to autotrophic organisms as the dominant contributors to MP degradation with little insight into the likely contribution of heterotrophic organisms. Currently, there is no consensus on the type of active organism responsible for the biotransformation of MPs in activated sludge systems. Hence, further study is required to elucidate the active microorganisms involved in the biotransformation of the MPs in wastewater. It is believed that the current study is the first to investigate the role of PAOs, OHOs, and AOB in the removal of MPs in a BNR activated sludge treatment configuration.

2.7 Influence of Redox Conditions on Micropollutant Removal

The removal mechanisms for conventional contaminants under different redox conditions are well understood and have been efficiently employed in most full-scale WWTPs. The impact of redox conditions on the removal of MPs in WWTPs is however still unclear.

The relatively few studies that have investigated the influence of redox condition on MP removal from wastewater are summarized in Table 2-7. Many of the studies were conducted using synthetic wastewater in batch experiments and the sludge samples were either taken from an existing full scale wastewater treatment plant or a lab scale continuous system. Typically the MPs were spiked

into the reactor and the degradation rate in each redox zone or the overall removal from the system was monitored. Some of the findings from the literature review summarized in Table 2-7 are itemized below;

- There has been more research on estrogen (E1, E2, and EE2) removal under different redox condition than other MPs. Apart from estrogens, few studies have been conducted to investigate the removal of MPs under anaerobic conditions.
- Due to the scarce research data describing removal of MPs under different redox condition, it is difficult to make broad comparisons or generalizations.
- With the exception of a few studies, most reports that describe the effect of different redox condition on MP removal, have reported only the overall removal without considering the contribution of each zone.
- MP removal seems to be affected by the different redox conditions, some positively, with better removal along the reactor like Ibuprofen, Roxithromycin, Estrone, E1+E2, Estradiol (E2) and Ethinylestradiol (EE2), some negatively, with worse removal along the reactor like Carbamazepine, Citalopram, Triclosan and Sulfametazine and some in between like Sulfamethoxazole and Sulfadiazine.

- Different redox conditions do not seem to have an effect on the adsorption of MPs to activated sludge biomass (log K_d).
- Except for E1 and E2, there is no consistency in the reported values from the various studies of the removal of compounds under different redox conditions.
- Aerobic and anoxic zones seem to be the dominant zones that contribute to the removal of MPs from wastewater. Except for SDZ, SMR and SMX, the greatest removals of MPs were observed under aerobic conditions.

Since most of the literature reviewed investigated the removal of estrogens under different redox conditions, listed below are some important findings in relation to estrogen degradation;

- E1 could be degraded under all redox condition but at different rates.
- E2 could be degraded under all redox condition but at different rates.
- E1, E2, E3 and EE2 could be significantly degraded under anaerobic digestion.
- Overall removal of E1 and E2 does not seem to depend on the redox zone sequence but similar conclusions cannot be drawn for EE2.

Table 2-7. Summary of influence of redox condition on Micropollutant removal

			Removal % due to Transformation during batch experiment				
Compound	**Redox Comb.**	**Log K_d (L $kgss^{-1}$)**	**Ao**	**Ax**	**An**	**Overall removal**	**Reference**
		(An/Ax/Ao)					
4-NP	An/Ax/Ao	4.6/4.9/5.1				75.6	Xue et al. (2010)
4-Op	An/Ax/Ao	4.9/4.5/4.2				79.3	Xue et al. (2010)
AHTN	Ao/Ax		99.9	82.9			Levine et al. (2006)
BPA	An/Ax/Ao	4.6/5.1/5.4				>90	Xue et al. (2010)
Bromoform	Ao/Ax		93	96			Levine et al. (2006)
Caffeine	An/Ax/Ao	2.5/2.8/2.9				>90	Xue et al. (2010)
Caffeine	Ao/Ax		90	-68.2			Levine et al. (2006)
CBZ	Ao/Ax		68.6	21.4			Levine et al. (2006)
CBZ	Ao/Ax	0.1	6±12	1±10			Suarez et al (2010)
CBZ	An/Ax/Ao	3.6/3.8/3.0				<20	Xue et al. (2010)
CTL	Ao/Ax	2	60±17	44±9			Suarez et al (2010)

			Removal % due to Transformation during batch experiment				
Compound	**Redox Comb.**	**Log K_d (L $kgss^{-1}$)**	**Ao**	**Ax**	**An**	**Overall removal**	**Reference**
		(An/Ax/Ao)					
DZP	Ao/Ax	1.3	17±11	16±17			Suarez et al (2010)
E1	An/Ax/Ao	3±2				96±1	Joss et al. (2004)
E1	An/Ao/Ax		99	0	70	99	Pholchan et al. (2008)
E1	An/Ao		90		75	100	Pholchan et al. (2008)
E1	An/Ax/Ao	4.3/4.5/4.7				>90	Xue et al. (2010)
E1	Ao/Ax		83.8	9.6			Levine et al. (2006)
E1 + E2	Ao/Ax		99±0	72±2			Suarez et al (2010)
E1 + E2	Ax/Ax/Ao		96.5	-96.6/85.4		97.8	Anderson et al.(2003)
E2	An/Ax/Ao	2.8±1.3/2.7±1.1	97±0.1	37±1	10±0.5	99.99± 0.0002	Li et al (2010)
E2	Ax/Ao		94	85			Dytzak et al (2008)
E2	An/Ax/Ao	2.4±2.2				>97	Joss et al. (2004)
E2	An/Ax/Ao	4.4/4.5/4.7				>90	Xue et al. (2010)
E2	An/Ao		100		70	100	Pholchan et al. (2008)
E2	An/Ao/Ax		99	0	90	100	Pholchan et al. (2008)
17α-E2	An/Ax/Ao	4.8/4.6/5.3				>90	Xue et al. (2010)
E3	An/Ax/Ao	2/1/1.5				>90	Xue et al. (2010)

			Removal % due to Transformation during batch experiment				
Compound	**Redox Comb.**	**Log K_d (L $kgss^{-1}$)**	**Ao**	**Ax**	**An**	**Overall removal**	**Reference**
		(An/Ax/Ao)					
EE2	Ax/Ax/Ao		71.4	-14.3/-42.9		80	Andersen et al.(2003)
EE2	An/Ax/Ao	4.1/5.3/5.2				97.6	Xue et al. (2010)
EE2	Ao/Ax	2.5	87±11	20±10			Suarez et al (2010)
EE2	An/Ax/Ao	2.8±0.95/2.8±0.85 /2.8±0.5	27±2	10±0.9	n.r	79.13±0.77	Li et al (2010)
EE2	Ax/Ao		22	5			Dytzak et al (2008)
EE2	An/Ax/Ao	2.5±1.6				94±2	Joss et al. (2004)
EE2	An/Ao		-58		0	0	Pholchan et al. (2008)
EE2	An digester				89.6	41.9	Esperanze et al. (2006)
EE2	An/Ao/Ax		45	30	-98	18	Pholchan et al. (2008)
ERY	Ao/Ax	2.2	89±2	20±10			Suarez et al (2010)
Fluoxetin	Ao/Ax	0.7	92±3	88±15			Suarez et al (2010)
HHCB	An/Ax/Ao	3.8/3.5/3.6				59	Xue et al. (2010)
Ibuprofen	Ao/Ax	0.9	95±4	37±26			Suarez et al (2010)
METOP	An/Ax/Ao	1.3/1/1					Xue et al. (2010)

			Removal % due to Transformation during batch experiment				
Compound	**Redox Comb.**	**Log K_d (L $kgss^{-1}$)**	**Ao**	**Ax**	**An**	**Overall removal**	**Reference**
		(An/Ax/Ao)					
ROX	Ao/Ax	2.2	91±0	15±17			Suarez et al (2010)
SDZ	Ax/An/Ao		41	49	-27		Hong et al. (2008)
SMR	Ax/An/Ao		0	73	47		Hong et al. (2008)
SMX	Ao/Ax	2.4	22±5	n.a			Suarez et al (2010)
SMX	Ax/An/Ao		40	43	-89		Hong et al. (2008)
SPD	Ax/An/Ao		33	24	-91		Hong et al. (2008)
TMP	Ao/Ax	2.3	14±10	n.a			Suarez et al (2010)
Triclosan	Ao/Ax		11.8	-17.7			Levine et al. (2006)

SMX; Sulfamethoxazole, CBZ;Carbamazepine, TMP;Trimethroprim, DZP;Diazepam, NPX; Naproxen, TCS; Triclosan, ERY;Erythromycin, CRL; Citalopram, ROX; Roxithromycin, 4-NP; 4Nonyl phenol, 4-OP; 4 Octyl phenol, BPA; Bisphenol A, E1; Estrone, E2; Estradiol, EE2; Ethinylestradiol, DCF; Diclofenac, METP; Metoprolol, CAF; Caffeine, SDZ; Sulfadiazine, SPD; Sulfapyridine, SMR; Sulfamerazine, IBU;Ibuprofen, BRMF;Bromoform,
n.d-non detect; n.a- not analysed; n.r- not recorded. An-Anaerobic; Ax-Anoxic; Ao- Aerobic.

- Oxidation of E2 to E1 was observed under aerobic and anoxic conditions (Dytczak et al., 2008).
- Reduction of E1 to E2 could occur under anaerobic condition (Joss et al., 2004).
- EE2 seems to be significantly degraded under aerobic conditions.
- EE2 is more resistant to degradation than E1 and E2.
- Slight EE2 degradation could occur under anoxic condition (Dytczak et al., 2008; Li et al., 2011 and Suarez et al., 2010).

Based on these findings it is clear that there is need for more information on the effects of redox conditions on the removal of MPs from wastewater.

For contaminant fate modeling, estimation of the values of biodegradation rate constants of the MPs is essential in order to assess the kinetics of the MPs in the environment. Suarez et al. (2010) reported faster degradation under aerobic condition for over 90% of the investigated MPs compared to the anoxic condition. Table 2-8 shows the reported biodegradation rate constants under different redox conditions and treatment conditions for the target MPs. It is obvious from Table 2-8 that the different redox zones contributed to the overall degradation of the MPs with the aerobic zone contributing the largest percentage. It also shows that few reports exist that present the biodegradation rate coefficients of MPs with respect to varying redox condition. Therefore it is

recommended that a comprehensive data base be generated to present the biodegradation rate coefficient of various MPs of environmental concern with respect to varying redox conditions since these chemicals has the tendency of existing under different redox conditions in the natural environment.

Table 2-8. Pseudo first order biodegradation rate coefficient (k_b) ($Lg_{ss}^{-1}d^{-1}$) of selected micropollutants during biological wastewater treatement

MP	log K_{ow}	AO	AX	AN	CAS	MBR	Reference
CBZ	2.45	<0.06	<0.03				Suarez et al., 2010
E1	3.13	162 ± 25	30 ± 10	10 ± 1			Joss et al., 2004
		32/77					Lust and Stensel, 2011
		32/66					Lust and Stensel, 2011
GEM	4.77				6.4-9.6	0.5-1.8	Joss et al., 2006
					2.26 ± 1.35		Urase and Kikuta, 2005
IBU	3.97	20	1.5				Suarez et al., 2010
					21-35	9.0-22	Joss et al., 2006
					1.33		Abegglen et al., 2009
					10.75 ± 13.08		Suarez et al., 2010
					3.04 ± 1.59		Urase and Kikuta, 2005
					15.38 ± 14.95		Clara et al., 2005a
SMX	0.89	0.3	n.a				Suarez et al., 2010
					5.9-7.6	3.2-5.0	Joss et al., 2006
					0.2 ± 0.01		Abegglen et al., 2009
TMP	0.91				0.15		Suarez et al., 2010
					0.22		Abegglen et al., 2009
					0.56 ± 0.46		Xue et al., 2010

AO-Aerobic; AX-Anoxic, AN- Anaerobic

2.7.1 Summary of Influence of Redox Condition on Micropollutant Removal

Previous studies have investigated the effects of redox condition on the removal of MPs from wastewater. Most of these studies have focused on estrogens, with very few investigations of other MPs. The degradation of E1, E2 and EE2 has been found to depend on redox condition but the overall removal of E1 and E2, yielded consistent results (> 96%) regardless of the sequence of the redox condition while EE2 removal seems to be affected by the redox zone sequencing, for example, a configuration of anaerobic/aerobic/anoxic yielded 18% overall removal while a sequence of anoxic/anoxic/aerobic and anaerobic/anoxic/aerobic yielded overall removal of 80% and 97.6% respectively.

Many researchers have also indicated that the aerobic zone contributed the largest removal of the three redox zones investigated for MP removal. Although the transformation of MPs has been reported to occur under anoxic and anaerobic conditions, the studies are few and there is considerable variability between studies. The proposed work will seek to address this knowledge gap and hence one of the objectives of this study is to investigate the effects of redox condition on the removal of MPs from wastewater. Investigating the effects of redox conditions on the removal and biodegradation of MPs will give vital information required to optimize the efficiency of the treatment method employed.

2.8 Endocrine Disrupting Compounds in Wastewater Treatment Plant Effluents

Endocrine disrupting compounds (EDCs) are defined by their ability to mimic or interfere with the mechanisms that govern the biosynthesis, transport or availability, and metabolism of hormones (Lister et al., 2001). The Canadian Environmental Protection Act of 1999, article 3, subsection 43 defined "hormone disrupting substance" also known as EDC as "a substance that has the ability to disrupt the synthesis, secretion, transport, binding action or elimination of hormones in an organism, or its progeny, that are responsible for the maintenance of homeostasis, reproduction, development or behavior of an organism".

Steroid sex hormones and related synthetic compounds such as those used in contraceptive pills, have been shown to be present in the aquatic environment, mainly as a result of inefficient removal in sewage treatment plants. The concentrations of these compounds, although very low, at ng/L, are sufficient to induce estrogenic responses and alter the normal reproduction and development of wildlife and human endocrine system.

A number of previous studies have investigated the concentrations of EDCs in treated wastewater effluents (Ternes, 1998; Jones et al., 2002; Kolpin et al., 2002) and some studies have investigated the ecotoxicological effects of EDCs in treated wastewater (Cleuvers, 2003; Koh et al., 2009). In these studies, the

acute toxicity in the environment was found to be very rare except during spills. However, there are concerns that chronic or synergistic effects may exist due to their persistence in the environment (Fent et al., 2006).

Although various studies have been conducted to investigate the ecotoxicological effects of wastewater effluents on the environment, it is still not clear whether the cost that would be required to upgrade existing WWTPs to accommodate EDCs removal along with conventional pollutants removal would eventually reduce the intrinsic effects of the EDCs on the environment. The investigation of the relationship between the removal of EDCs from wastewater and the reduction of effects when discharged into the environment is challenging because there are various mechanism by which these chemicals may exert effects on organisms (e.g., interference with various receptors which affect enzymatic processes and antagonistic effects of mixture of EDCs). In addition, it is difficult to link EDC concentrations and biological effects in the environment because of the weak correlation between intrinsic gene expression and physiological effects on organisms after exposure. One of the objectives of this study is to compare treated effluents that are generated under different treatment configurations with respect to MPs and estrogenicity removal.

2.8.1 Bioassays for Detection of EDCs

One of the methods used to detect EDCs in the environment is the use of biologically based assays (Chang et al., 2009). They usually provide either qualitative or quantitative responses especially in the presence of multiple EDCs. The use of mass-based analytical methods to quantify EDCs has provided accurate and precise measurements. However, these methods are limited in their ability to describe overall estrogenic effects, such as the synergistic or anti-estrogenic influences in the presence of multiple EDCs (Liu et al., 2010). Therefore, biological assays are useful methods in studying the overall effects of EDCs as well as the identification of other xenobiotic compounds that exhibit endocrine disrupting behaviors similar to EDCs.

Several mechanisms have been employed in biological assays that are used to measure EDCs and these include; cell proliferation, luciferase induction, ligand binding, vitellogenin induction, or antigen–antibody interactions.

- Cell proliferation utilizes the estimation of cell growth and reproduction in different samples, while ligand binding quantifies the number of specific estrogen binding sites (Soto et al., 2004; Hamers et al., 2008).
- Luciferase induction measures the amount of luciferase induced from estrogen receptors and response elements with luminescence

after cell lysing and the addition of luciferin (Legler et al., 2002; Michallet-Ferrier et al., 2004).

- Vitellogenin induction quantifies the amount of vitellogenin in the plasma of female fish liver after extraction, which is secreted as a response to estrogens. In addition, the production of vitellogenin in male fish can be seen as an indication of endocrine disruption (Jimenez, 1997; Saaristo et al., 2009).
- Antigen–antibody interactions use the principle of immunoassays based upon the non-covalent binding of antigen to antibodies (Gascón et al., 1997; Alyea and Watson, 2009).

Biological assays could either be "*in vivo*" or "*in vitro*". "*In vivo*" assays utilize the endocrine disruption process in amphibians, fishes, birds, and insects in order to monitor the EDCs in aquatic environments. The responses in the organisms are determined by deformities, reproductive deficiencies, egg and offspring development, and serum protein production, such as vitellogenin (Chang et al., 2009). Many "*In vivo*" assays have been used to investigate the effects of EDCs on fishes, some of the species used include rainbow trout *(Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), sheephead minnow (*Cyprinodon variegates*), and zebrafish (*Brachydanio rerio*) to mention but a few (Fenner-Crisp et al., 2000; Folmar et al., 2000; Legler et al., 2002; Micael et al.,

2007; Warner and Jenkins, 2007; Soares et al., 2008). In addition, the populations of wild leopard frogs (*Rana pipiens*) have been known to be particularly sensitive to the exposure of EDCs, based on a study of their gonadal abnormalities (Hayes et al., 2002; Opitz et al., 2006).

An advantage of using "*In vivo*" assays in the detection of EDCs is the ability to quantify the actual effects of EDCs on a target species as well as the usage of the species as a representative biological indicator in their natural habitats. In addition, the method may provide a cumulative estrogenic effect caused by exposure to a mixture of EDCs in a given environment. The major disadvantage of this method is associated with the deficiency of a specific organism response to certain EDCs (Chang et al., 2009).

The "*in vitro*" assays can be categorized into three types (Kinnberg, 2003):

1) Estrogen receptor (ER) competitive ligand binding assays: measure the binding affinity of a chemical to ERs

2) Cell proliferation assays: measure the increase in the number of target cells during the exponential phase of proliferation (i.e. E-screen assay) and

3) Reporter gene assays: measure ER binding-dependent transcriptional and translational activity (i.e. ER-CALUX assay, MVLN cell assay, Yeast estrogen screen (YES) assay).

The aforementioned *in-vitro* assays use a protein expression system, representing the estrogen response formed or stimulated by a dimer that is produced from the binding between the estrogen and the estrogen receptors. Luciferase, quantified with a luminometer after cell lysis and β-galactosidase, measured with a spectrophotometer, using a back-calculation from the amount of colored products after the enzyme-catalyzed reaction process has been completed, are some examples of these types of response proteins (Chang et al., 2009). Out of all the methods available for measuring the estrogenic activity of water samples, the yeast assay has been reported to be fast, sensitive and reliable. Therefore, the YES assay method was used to estimate the estrogenicity of the pilot plant effluents in relation to the different operating conditions.

2.9 Summary of Literature Review

A detailed understanding of the active microbial groups involved in the removal of MPs from BNR wastewater treatment process will enhance the ability to design treatment methods which would lead to a reduced load and ultimately reduced effects of MPs on the environment. Presently, there is no general consensus among researchers on the role of various active microbial groups in activated sludge with respect to the removal of MPs from wastewater. Some studies have suggested that nitrifying organisms contributed significantly to biodegradation while other studies suggested that heterotrophs played a

dominant role in the biotransformation of MPs in wastewater. Other studies have suggested that nitrifying and heterotrophic organisms work together as co-contributors to biotransform MPs. These inconsistencies impede the development of a robust model for the removal of MPs in activated sludge wastewater treatment processes. Hence, further study is required to elucidate the role of active microbial groups in the biotransformation on MPs.

Previous studies have shown that high removals of some MPs can be achieved through biological wastewater treatment processes operated under different redox conditions. However, these studies are few and the variability between studies prevents the generalization of conclusions. Therefore, the investigation of the removal of MPs under different redox conditions will contribute to the general data base of knowledge while presenting the opportunity for optimization of existing biological wastewater treatment processes.

The YES assay is a useful biological tool for detecting EDCs present in different matrices in the environment. This method is appropriate for estimating qualitative or quantitative estrogenic responses of environmental samples. It is unique in its mode of operation because it quantifies the overall estrogenic effects (synergistic or anti-estrogenic) induced by the WWTP effluents. However, the

YES assay has limitations in that it can produce false negative results due to the presence of antagonistic compounds or toxicants in the extracted samples.

Chapter 3 Impact of Activated Sludge Process Configuration on Removal of Micropollutants and Estrogenicity

A version of this chapter was published as the paper: Ogunlaja, O. O.; Parker, J. W.; Metcalfe, C.; Seto, P. (2013) "Impact of activated sludge process configuration on removal of Micropollutants and estrogenicity" Proceedings of the 86th Annual Water Environment Federation Technical Exposition and Conference (CD-ROM), Illinois, Chicago, Oct. 5-9. Water Environment Federation: Alexandria, Virginia.

Other collaborative publications involving the study in this chapter are;

1) Pileggi V., M. Ogunlaja, X. Chen, J. W. Parker, P. Yang, S. Kleywegt, N. Feisthauer, S. Tabe, J. Schroeder, T. Fletcher and P. Seto (2013). Comparison of effluent conventional and microcontaminant chemistry in three pilot wastewater treatment processes during winter and summer simulated conditions, Proceedings of the 86th Water Environment Federation Technical Exposition and Conference, Chicago, IL.
2) Wojnarowicz, P., O. Ogunlaja, C. Xia, W. Parker, C. Helbing. (2013). Impact of Wastewater Treatment Configuration and Seasonal Conditions on Thyroid Hormone Disruption and Stress Effects in *Rana catesebeiana* Tailfin. *Enviro. Sci. Technol*., 47(23), 13840-13847.
3) Parker W.J., V. Pileggi, P. Seto, X. Chen, M. Ogunlaja, G. Van Der Kraak, J. Parrott. (2014). Impact of Activated Sludge Configuration and Operating Conditions on In Vitro and In Vivo Responses and Trace Organic Compound Removal. *Sci. of Total Environ*., 490,360-369.

Summary of contributions to aforementioned publications;

- Conducted daily checks and measurements to maintain the smooth operations of the pilot plants.

- Collected and prepared samples before other chemical quantifications.
- Conducted YES assay analysis on collected samples.
- Prepared samples before sending to laboratories for chemical analysis
- Collated conventional pollutants and chemical analysis results for ease of comparison between treatment trains.
- Participated in writing a portion of article #2.

3.1 Introduction

Wastewater treatment plant (WWTP) effluents have been identified as the primary route of entry for micropollutants (MPs) into the aquatic environment (Joss et al., 2004). The concern with respect to MPs in the environment results from the potential deleterious effects of these chemicals on the aquatic ecosystem. These chemicals have been shown to cause developmental and reproductive abnormalities in various trophic levels of organisms when present at very low concentrations (Purdom et al., 1994; Fent et al., 2006; Parrott and Blunt, 2005, Parrott et al., 2009) and their occurrence has been measured in both effluent and surface water in North America (Kolpin et al., 2002, Metcalfe et al., 2003; Lishman et al., 2006).

Wastewater treatment plants are generally designed to remove conventional pollutants such as nitrogen, phosphorus, BOD and TSS and the limited removal of MPs from WWTPs is generally fortuitous. A multi-level approach with layers of different removal mechanisms (sorption, biodegradation, size exclusion, oxidation, etc.) has been suggested for improvement in the removal of MPs from wastewater (Koh et al., 2009; Qian et al., 2011). However, there is still uncertainty as to whether upgrading wastewater treatment for enhanced conventional pollutant removal (i.e. nitrification,

denitrification, enhanced biological phosphorous removal) will yield improved removal of MPs.

Assessing the biological effects of MPs on the flora and fauna of the aquatic ecosystem is an indispensable tool required to conduct detailed and appropriate risk assessment of MPs in the environment. Unlike chemical analyses that can provide a quantitative measure of the compounds present in a sample, biological analyses such as *in vitro* bioassays can provide a qualitative and quantitative measure of the net endocrine-disrupting potential of all the endocrine-active substances that are present in an effluent (Leusch et al., 2010). Studies that have compared the removal of micropollutants and the potential for endocrine disruption in the effluents between different wastewater treatment technologies are few. Specifically, it has not been established whether improved removal of MPs will translate into a reduction in the biological effects on an aquatic ecosystem.

In this study, the removal of 10 MPs classified according to EU directive 93/67/EEC as either toxic (gemfibrozil, ibuprofen, trimethoprim), harmful (carbamazepine), non-toxic (sulfamethoxazole, trimethoprim), estrogenic (estrone, bisphenol-A and nonyl-phenol) or androgenic (androstendione) in three different wastewater treatment technologies (conventional activated sludge (CAS), nitrifying activated sludge (NAS) and biological nutrient removal (BNR))

was investigated. The assessment was conducted at steady state in pilot scale processes treating authentic municipal wastewater as an influent. In addition, the potential biological effects of the effluents were assessed using a recombinant yeast estrogen screen (YES) assay to quantify their net estrogenicity. The reduction of estrogenic activity was estimated by calculating E2-Equivalent concentration (E2-Eq) in the effluents of the treatment trains. It was hypothesized that as the sophistication of the treatment methods increased from CAS to BNR, there would be an improvement in MP removal and reduction in estrogenic activity in the effluents.

3.2 Methodology

3.2.1 Process Description

Three pilot wastewater treatment plants located at the Environment Canada Wastewater Technology Centre (WTC) in Burlington, Ontario were employed for this study. The WTC receives raw municipal wastewater from Burlington Skyway Wastewater treatment plant. Schematics of the pilot plants are shown in Figures 3-1 and 3-2. The conventional activated sludge (CAS), nitrifying activated sludge (NAS) and biological nutrient removal (BNR) pilots were fed from a common primary clarifier that received the raw wastewater. The activated sludge reactors were segmented into six cells (60 L each) to simulate

pseudo-plug flow. Coarse bubble aerators were used for aeration and mixing (in a cyclic pattern; high air flow at 40 Lpm and low air flow at 10 Lpm) in the CAS, NAS and the aerated zones of the BNR. The operating and design conditions are presented in Table 3-1. An insulated water jacket with automatic temperature controller was installed around the bioreactors to control the temperature of the bioreactors. Di-potassium phosphate (K_2HPO_4) (11 g/L at the rate of 14.4 L/d) and sodium bicarbonate ($NaHCO_3$) (22 g/L at the rate of 14.4 L/d) were added to the primary effluent before entering into the reactors. This was done to ensure the system was not phosphorus limited and to provide a pH buffer for the system.

The CAS pilot was a single sludge aerobic system operated at sludge retention time (SRT) of 3 days to facilitate BOD removal without nitrification. The NAS was a single sludge aerobic system operated at SRT of 10 days to facilitate BOD removal and nitrification. The longer SRT enabled the proliferation of autotrophic nitrifying bacteria which mediates nitrification reactions. The BNR process consisted of anaerobic, anoxic and aerobic zones and was operated at a SRT of 20 days. This mode of operation was designed to achieve nitrification, denitrification and enhanced biological phosphorous removal. To achieve an appropriate range of COD/P ratio that would support a healthy BNR activated sludge, the clarified influent wastewater stream to the

BNR bioreactor was augmented with an exogenous source of readily biodegradable organic substrate (150 mg/L of acetate).

The pilot plants were operated for over 365 days with monitoring for over 180 days to ascertain stable operation in terms of biomass and effluent characteristics so as to enable a comparison among the three treatment trains. Plant performance was ascertained by monitoring SRT, temperature, COD, NH_3-N, TP, soluble PO_4-P, NO_3-N, TSS, VSS and pH.

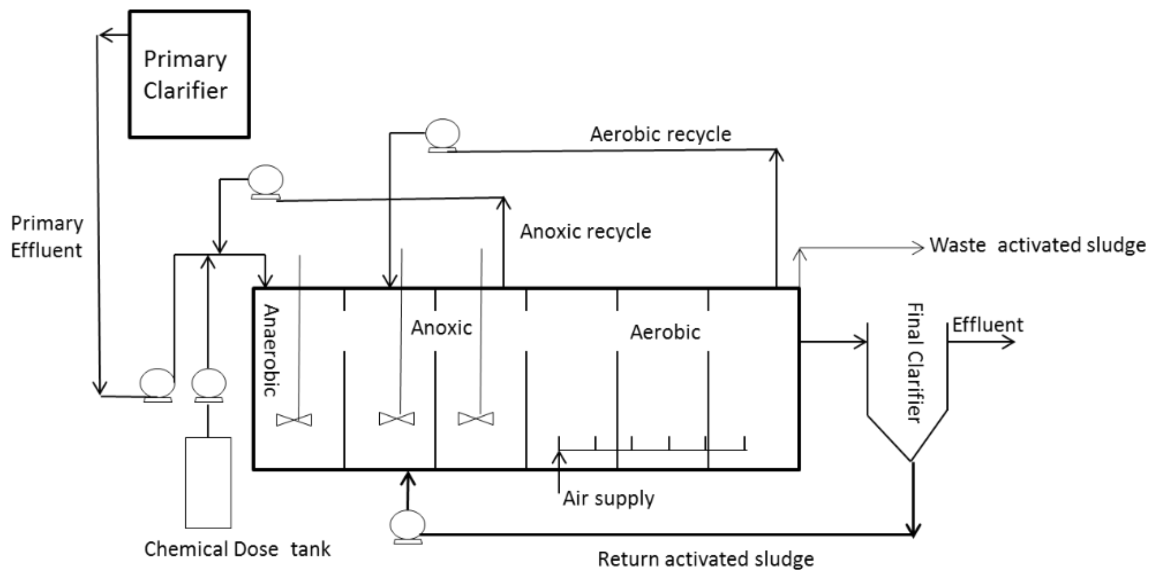


Figure 3-1. Flow diagram of the pilot-scale BNR treatment process.

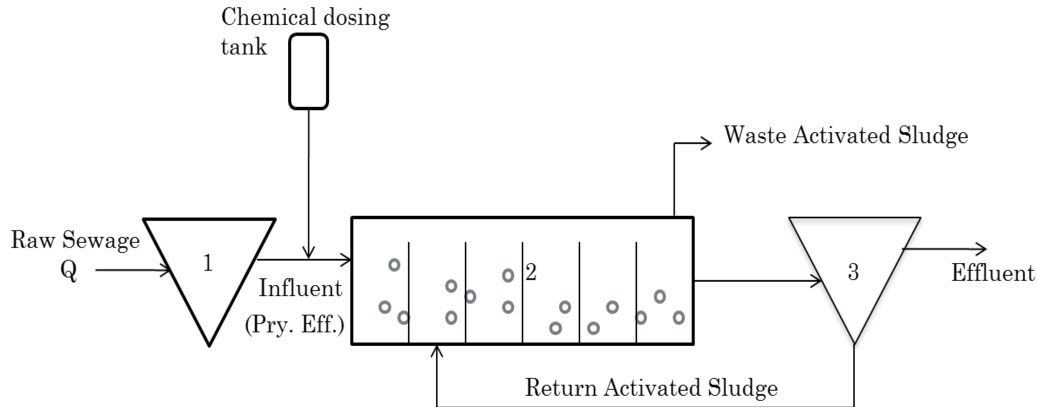


Figure 3-2. Flow diagram of the pilot-scale CAS and NAS treatment process. 1. Primary clarifier; 2. Aerobic bioreactor; 3. Final clarifier.

Table 3-1. BNR, NAS and CAS operating and design information

	Size/description	**Unit**
Flow rate (Q)	1282	L/d
Primary settler	Area = 0.46	m^2
	Depth = 1.56	m
Activated sludge	Pass volume = 6 X 60	L
	Depth = 1.28	m
Dissolved Oxygen	CAS-DO = 2-2.5 NAS-DO = 4-4.5 BNR DO(aerobic) = 4-5 DO(anoxic) = 1-2.5 DO(anaerobic) = 0-0.2	mg/L
Final settling tank	Area = 0.204	m^2
	Depth = 1.4	m
	Underflow = 962	L/d
Waste flow	CAS = 107 NAS = 34 BNR = 18	L/d
CAS-BNR Internal recirc. split factor	Aerobic = 2Q Anoxic = Q	L/d
SRT	CAS = 3 NAS = 10 BNR = 20	days
HRT	Average nominal = 7	h
Temperature	18 ± 2	^oC

3.2.2 Sample Collection and Extraction

A total of 5 sampling campaigns were conducted over three months during the steady state plant operation (July 2011 – Sept. 2011). The influent samples were collected after it had passed through primary clarification while the effluent from each train was collected at the outlet of the secondary clarifier. Eight hour composite grab samples were also collected from the anaerobic, anoxic and aerobic zones of the bioreactor during the same time as when the 24 h composite samples were collected. To reduce the influence of diurnal changes, 24 h composite samples of the influent and effluent were collected in 10 L stainless steel canisters using flow proportional (150 mL / 30mins) refrigerated auto samplers (HACH Company, Loveland USA).

Immediately after sample collection, the samples were centrifuged at 4000 rpm for 5 minutes and the supernatant filtered through 1.5 μm glass microfiber filters (Whatman, Toronto, Ontario, Canada). The filtered samples were split into two sub-samples each with a volume of 120 mL. The sub-samples were acidified to pH 3 and stored in amber glass bottles at $4^{o}C$ until they were extracted and analyzed. One set of sub-samples was extracted using the subsequently described multi-residue solid phase extraction technique (SPE) before shipping to Trent University for chemical quantification using LC/MS-MS. The second set of samples was extracted using the same procedure as the first set but without

spiking with deuterated compounds and processed for the yeast estrogen screen (YES) assay.

The samples were also analyzed for conventional wastewater parameters (as per Standard Methods for the Examination of Water and Wastewater (Eaton, 2005)) including, chemical oxygen demand (COD), biochemical oxygen demand (BOD), total Kjeldahl nitrogen (TKN), ammonia, nitrate, total suspended solids (TSS), alkalinity and pH.

3.2.3 Solid Phase Extraction

A multi-residue extraction procedure was used for extraction of the compounds from the water samples (Li et al., 2010) prior to the MP analysis and the YES assays. The samples extracted for MP analysis were initially spiked with surrogate standard solution (100 µL of 0.5 ppm) of the selected compounds prepared in methanol before the extraction. The samples extracted for characterization by the YES assay did not include the spiked standards. The SPE process is summarized in Figure 3-3.

In the extractions, Visiprep solid-phase extraction (SPE) vacuum manifolds coupled with Oasis® MAX SPE 6 mL (500 mg) SPE cartridges were used to concentrate the filtered samples (sample pH was initially adjusted to 8 by adding 0.1M NaOH). The HLB tubes were conditioned with 6 mL of methanol, 6 mL 0.1M NaOH in Milli-Q water, and 6 mL of Milli-Q water, taking

care not to let the cartridge go dry. The filtered wastewater sample was loaded onto the cartridge at a flow rate of approximately 5 mL/min to ensure good recovery and the sample bottles were rinsed with 10 mL of pH 8 distilled water. After loading, the cartridges were rinsed with 2 mL of pH 8 water and dried under vacuum for 10-20 minutes. The compounds were then eluted from the cartridges with 2 mL of MeOH at a flow rate of about 1 mL/min and rinsed thrice with 3 mL of 2 % formic acid in methanol at a flow rate of 1 mL/min, waiting six minutes between each elution. After elution, the cartridge was aspirated to dryness for about 10 min under vacuum. The eluted fractions were collected in amber glass tubes and dried under a gentle stream of nitrogen gas (N_2). The dried residue was dissolved in 0.4 mL of methanol and stored at 4°C before using it for YES assay or LC-MS-MS analysis.

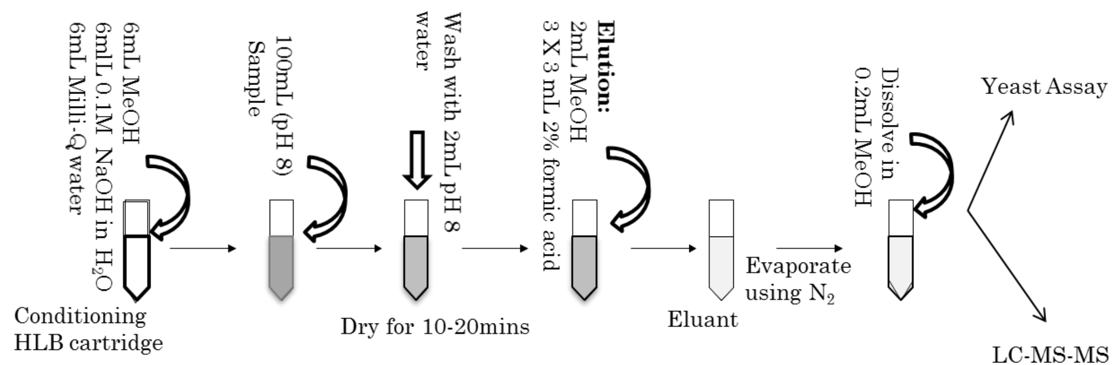


Figure 3-3. Summary of multi-residue extraction procedure.

3.2.4 Biological Testing

The estrogenic activity of the extracted samples was evaluated using the recombinant yeast screen for estrogenicity bioassay (YES) according to the methods developed by Routledge and Sumpter (1996) with adjustments described by Citulski and Farahbakhsh, (2010). A recombinant yeast strain (*Saccharomyces cerevisiase*) containing the human estrogen receptor was used for the assay. The strain contains an expression plasmid carrying the lac-Z reporter gene. When the cells are incubated for 3 days at 32°C in the presence of estrogenic compounds, the lac-Z product, β-galactosidase, is secreted into the medium and causes the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), to turn from yellow to red.

In the YES assay, the samples were initially evaporated to dryness under N_2, after which the evaporated residue was re-dissolved in 400 μL of ethanol. The sample was then transferred to a vial which was stored at 4°C. A 100 μL aliquot of each E2 sample was transferred to a 96 well micro titer plate (the dilution plate) and a dilution series was produced (using 100 μL of absolute ethanol). Standard curves for each test were prepared from positive-control rows of 17-β-estradiol (E2) in ethanol, diluted from 10 nM to $5x10^{-3}$ nM nominal concentration. The dilution factor in each titre plate was 2 times with ethanol across the 12 wells to produce a final dilution of 1/2048 (i.e 2^{11} times dilution).

A 10 μL aliquot of each dilution was transferred to a new micro titer plate (the assay plate) in a laminar air flow bench. The assay plate contained one row of standard solutions (17β-estradiol as positive control), one row of blank samples (absolute ethanol as negative control) and three rows of serially diluted environmental samples (80 μL of sample extract diluted with 80 μL of ethanol). The assay plates were allowed to dry in the laminar air flow bench after which 200 μL of combined growth medium/CPRG/yeast mixture were added. Subsequently, the assay plates were incubated at 32°C with the shaker tray set to 150 rpm for 72 hours to promote suspension of the yeast cells and constant mixing with the assay medium.

The absorbance (AB) at 540 nm and 620 nm was determined using a micro titer plate reader (Sunrise Basic TECAN). In order to adjust the absorbance of chlorophenol red at 540 nm for the extent of yeast growth (turbidity), the absorbance measurements for E2 standards and environmental samples were transformed as per equation 3-1 (Fent et al., 2006):

$$\text{Adj. Absorbance} = AB_{540nm}\,(\text{sample}) - [AB_{620nm}\,(\text{sample}) - AB_{620nm}\,(\text{blank, average})] \quad 3\text{-}1$$

The dose response curve for the standards and environmental samples were calculated using GraphPad Prism 6 (v. 6.02) that employed a four-

parameter sigmoidal Hill equation to calculate the EC-50. To obtain the estrogenicity values for the extracted sample, the logarithm of the extraction dilution that yield a 50% response was computed from the fitted curve. The E2-Eq was calculated as the ratio between the amount of E2 in the incubation well at EC50 in the standard curve and the equivalent volume at EC50 as per equation 3-2.

$$\mathrm{E2} - Eq\ (\frac{ng}{L}) = \left(\frac{E2\ EC50(\ ng/L)}{EC50\ dilution\ factor*0.4}\right) \qquad 3\text{-}2$$

The value 0.4 accounted for the air dried 80 μL sample extract applied to the titer well and reconstituted with 200 μL of the growth medium containing yeast cells (80/200 = 0.4). The method detection limit based on E2 standard was determined as 1 ng/L.

3.2.5 Target MPs

The micropollutants investigated in this study are presented along with their physicochemical characteristics in Table 3-2. The target compounds included a broad range of substances including acidic, basic and neutral drugs and estrogenic compounds and were selected on the basis of their detection frequency in WWTP effluents (Metcalfe et al., 2003; Lishman et al., 2006) and the

ability to detect low concentrations (ng/L) using LC-MS/MS (Miao and Metcalfe, 2002)

3.2.6 Statistical Analysis

The conventional and chemical data were analyzed for outliers using the Grubb's test. The regressions used to construct the YES assay response curves for the samples were compared to the response curves of estradiol (E2) standard using the F-test. To check the yeast growth absorbance (AB620 nm) for the presence of toxic effects resulting from the wastewater extracts, the yeast growth was compared to the average turbidity (± 3 standard deviations) of 12 ethanol only negative-control wells that were incubated in the same plate as the samples. Samples that had yeast growth with turbidity values below the average minus 3 standard deviations of the negative control were removed from the dose-response curve analysis. The final conditioned data were employed to generate the dose-response curve for the estimation of the EC50. A paired-t-test was employed to compare the MPs' concentrations and the E2-Eq concentrations in the influent and pilots' effluent streams at the 95% confidence level using Microsoft excel 2013.

Table 3-2. Physicochemical properties of selected micropollutants

Micropollutants	Molecular mass (g/mol)	Water solubility @ 25°C (mg/L) - Dissolving	Henry's law constant @ 25°C(atm m3 /mol)- Evaporation	(Log K_{ow})- Bioconcentration	(K_d)* L/Kg_{SS}	Biological or Clinical effects
Ibuprofen	206.29	21 (moderate)	1.5E-07 (moderate)	3.97 (moderate)	<30	Non-prescription analgesic
Meprobamate	218.25	4700 (high)	1.8E-10 (high)	0.7 (low)	<30-190	Anti-convulsant
Nonyl phenol	220.35	7 (low)	3.4E-05 (low)	5.76 (high)	7k-13k[c]	Estrogenic
Bisphenol A	228.29	120 (moderate)	1E-11 (high)	3.32 (moderate)	314-502	Estrogenic
Carbamazepine	236.27	17.7 (moderate)	1.08E-10 (high)	2.45 (low)	36-65	Anti-epileptic
Gemfibrozil	250.33	10.9 (moderate)	1.19E-08 (high)	4.77 (high)	30-45	Cholesterol lowering Drug
Sulfamethoxazole	253.28	610 @ 37°C (moderate)	6.42E-13 (high)	0.89 (low)	200-400	Antibiotic
Estrone	270.37	30 (moderate)	3.8E-10 (high)	3.13 (moderate)	607-645	Estrogen
Androstenedione	286.41	57.8 (moderate)	3.68E-08 (high)	2.75 (moderate)	134-174	Steroid hormone
Trimethoprim	290.32	400 (moderate)	2.39E-14 (high)	0.91 (low)	119-251	Antibiotic

Physprop Database. http://www.syrres.com/esc/physdemo.htm *Stevens-Garmon et al., 2011; [c] Xue et al.,2010

3.3 Results and Discussions

3.3.1 Conventional Parameters

The pilot plants were monitored with respect to the removal of conventional wastewater pollutants such as biochemical oxygen demand (BOD) and nitrogen species. This data was employed to establish whether the treatment plants were operating within normally established ranges and to provide insight into the types of microbial metabolisms (i.e. aerobic heterotrophic growth, anoxic heterotrophic growth, aerobic autotrophic growth) that were active in the bioreactors. The measured responses are presented in Table 3-3.

Considering the inherent variability of processes treating raw municipal wastewater, the effluents from the pilot plants as indicated in Table 3-3, were relatively consistent with time. Carbonaceous BOD_5 was consistently removed in all pilot plants and most final concentrations were less than 10 mg/L. This was considered indicative of good removal of biodegradable organic matter. The BNR and NAS processes produced the lowest effluent BOD concentrations while the effluent concentrations from the CAS process were slightly elevated (average of 13.3 mg/L).

Based upon the operating conditions that were employed in this study it was expected that the effluents from the CAS pilot would have higher concentrations of TKN and TAN and low concentrations of NO_3-N and NO_2-N as compared to NAS and BNR pilot effluents. From Table 3-3 it can be observed that the CAS pilot effluent NO_3-N concentrations were consistently low and the effluent TKN and TAN concentrations remained elevated. The NAS pilot was expected to have low concentrations of TKN and TAN and elevated concentrations of NO_3-N in the effluent. From Table 3-3 it can be seen that these patterns were consistently observed. Hence, it was concluded that the CAS pilot was not nitrifying while the NAS pilot was nitrifying effectively. The BNR pilot was expected to have low effluent concentrations of PO_4-P, TKN, TAN, NO_3-N and NO_2-N. A review of Table 3-3 indicated that these patterns were consistently observed. Hence, it was concluded that the BNR process was effectively nitrifying, denitrifying and biologically removing phosphorus. Collectively, it was concluded that the pilots were achieving levels of treatment that are typical of their respective operations at technical scale (Tchobanoglous et al., 2003).

Table 3-3. Influent and effluent concentrations of conventional responses (mg/L) (Mean (S.D))

	TAN	NO_3-N	NO_2-N	TKN	$cBOD_5$	PO_4-P
Influent	20.9(6.6)	0.62(0.9)	0.16(0.23)	27.1(10.4)	79.8(56.4)	5.15(1.6)
CAS	21.1(6.4)	1.7(4)	0.4(0.6)	21.4(5.7)	13.3(10.1)	5.38(1.4)
NAS	0.076(0.039)	22(2.6)	0.013(0.018)	1.2(0.3)	3.5(1.7)	5.24(1.4)
BNR	1.3(2.1)	5.4(2.2)	0.3(0.4)	2.4(2.3)	6.4(6)	0.19(0.4)

3.3.2 Micropollutant Removal during Treatment

All 10 compounds were detected in the influent wastewater. The observed average influent concentrations were generally lower than previously reported studies (Lishman et al., 2006, Lajeunesse et al., 2012). The influent data was conditioned by using Grubb's test to determine any outliers which were removed before conducting other statistical analysis. There were some overall variability associated with the influent concentrations of the selected compounds with a median relative standard deviation (RSD) of 25% across all influent samples for the five sampling campaigns. The variability was primarily attributed to temporal changes in the influent concentrations.

The effluents from the three pilots were compared to examine whether any of the pilot out performed each other by examining the responses of 10 representative MPs. The effluent data was conditioned by using Grubb's test to

determine any outliers which were removed before conducting other statistical analysis. A paired-t-test was employed to compare the mean concentrations of the MPs in the influent to the mean concentrations of the MPs in the effluent streams from CAS, NAS and BNR treatment configurations. A paired-t-test was also used to compare the mean concentrations of the MPs in the BNR to the mean concentrations of the MPs in the effluent from NAS and CAS treatment configuration. This analysis was conducted in order to determine the statistical significance of the differences in the mean concentrations between the influent and the effluent streams from the treatment configurations. Hence, Table 3-4 summarizes the results from the statistical analysis.

Table 3-4 shows the p-values of the paired t-tests that compared the mean concentrations in the streams from the pilot plants. The results of the paired-t-test showed a statistically significant difference between the mean concentrations in the influent and CAS, NAS and BNR effluents for IBU, CBZ, GEM, ADR, E1, NP and BPA. There was no statistically significant difference between the mean concentrations in the influent and CAS, NAS and BNR for MEP. The result showed that the mean concentrations in the influent and the NAS effluent for GEM were statistically significant. The mean of the difference in the

concentrations of TMP in the influent and in the BNR effluents were also statistically significant. In addition, there were no statistical difference between CAS and BNR effluents or NAS and BNR effluents at the 95% confidence level for all the investigated compounds except for TMP. These results show that majority of the responses of the MPs in the influent and the effluent from the CAS, NAS and BNR treatment configurations were significant. Hence, the data were employed for further technical comparisons among the three treatment configurations.

Table 3-4. Statistical significant ($p \leq 0.05$) testing results – P values

Comparison	**IBU**	**MEP**	**GEM**	**CBZ**	**TMP**	**SMZ**	**ADR**	**E1**	**NP**	**BPA**
IN-CAS	0.002	0.252	0.425	0.025	0.219	0.020	0.006	0.038	0.042	0.004
IN-NAS	0.002	0.429	0.001	0.006	0.201	0.003	0.005	0.017	0.035	0.01
IN-BNR	0.002	0.402	0.091	0.043	0.008	0.067	0.005	0.018	0.036	0.004
BNR-CAS	0.07	0.64	0.23	0.62	0.03	0.62	0.13	0.06	0.24	0.08
BNR-NAS	0.09	0.99	0.08	0.41	0.03	0.15	0.35	0.72	0.19	0.19

IN-influent

Box plots and averages of the concentrations of the compounds in the influent and effluents from CAS, NAS and BNR are presented in Figures 3-4, 3-5, and 3-6. These plots show that 5 MPs (IBU, ADR, E1, NP and BPA) had comparable effluent concentrations for CAS, NAS and BNR. Two MPs (CBZ, MEP) had effluent concentrations equal or higher than the influent concentrations for all the treatment processes. TMP, GEM and SMX effluent

concentrations followed the trend of BNR < NAS < CAS, NAS < BNR < CAS and NAS < CAS = BNR respectively.

The removal efficiencies of each compound across the treatment processes were calculated as the percent difference in concentration from influent to effluent. The term removal used in this study described the loss of the target compound and did not necessarily imply mineralization. The removal of the target compound could have proceeded via different mechanisms including chemical and physical transformation, biodegradation or biotransformation and sorption. The MPs removal efficiencies across the three treatment processes are presented in Figure 3-7. It is apparent from Figure 3-7 that 6 MPs (IBU, ADR, SMX, E1, NP and BPA) were consistently removed with removal efficiency greater than 65% across the three treatment processes while no removal was observed for 2 MPs (CBZ and MEP) regardless of the treatment process utilized.

The moderate to high removal efficiencies for the IBU, ADR, SMX, E1, NP and BPA were consistent with the removal efficiencies previously reported in the literature (USEPA 2010). Treatment processes with SRTs ranging from 2 – 68 days in a lab scale and 22- 82 days in WWTP pilot scale were reported to have IBU removals ranging from 80% to 100% (Onesios et al., 2009). The average

reported removal efficiency for BPA in a variety of treatment processes and SRTs was reported to be 83% (Melcer and Klecka, 2011). Clara et al. (2005) reported BPA and IBU average removals of 82 to 98% at SRTs as low as 2 days in an activated sludge system. Reported values for ADR, NP and E1 removal efficiency in various aerobic activated sludge systems and SRTs have ranged from 98 to 100% with an average of 99%. These results suggest that the removals of IBU, ADR, SMX, E1, NP and BPA in the CAS, NAS and BNR were independent of the process configuration or system's SRT.

The apparent negative removals for CBZ and MEP (Figure 3-7) reflected the increased effluent concentrations of CBZ and MEP from the treatment processes as compared to the influent concentrations (Figures 3-4D and 3-5E). This suggests that CBZ and MEP were not removed by any of the treatment processes. This may have resulted from de-conjugation of conjugated versions of the parent compounds by the action of the microorganisms during the treatment process (Lindqvist et al., 2005). Gobel et al. (2007) suggested that the increase in effluent concentration of some MPs as compared to their influent concentration could be due to the encasing of the influent MPs in fecal particles leading to an apparent increase in concentration during treatment as the fecal particles are

degraded. Previous studies have shown that recalcitrant compounds like CBZ rarely sorb or degrade in a variety of wastewater treatment processes (Ternes et al. 2004; Clara et al. 2005a). The recalcitrant behavior of CBZ and MEP could be a useful characteristic in applications where they might be employed as an anthropogenic tracer.

Figure 3-7 shows an increase in the removal of TMP as the treatment process complexity progressed from a simple non-nitrifying CAS to a complex BNR configuration. Longer solids retention times (SRTs) accommodate slower growing bacteria like nitrifiers and can also support the proliferation of a wider range of bacteria species. The presence of a broad array of bacteria likely allows for a wider range for biotransformation of TMP thereby leading to improved removal at longer SRTs (CAS-3 days, NAS-10 days and BNR- 20 days). Correlations between treatment efficiency and SRT have previously been established (Clara et al., 2005a) and an SRT longer than 10 days has been suggested as being more effective in removing micropollutants such as estrogens (Carballa et al., 2007). Gobel et al., 2007 investigated the removal of MPs by activated sludge and showed an increase in the removal of TMP from 50% to 90% when SRTs were increased from 16 ± 2 to 60 days. Therefore, the increased

removal efficiency of TMP in the BNR could be due to microbial diversity and SRT of the process as compared to the CAS and NAS treatment configurations.

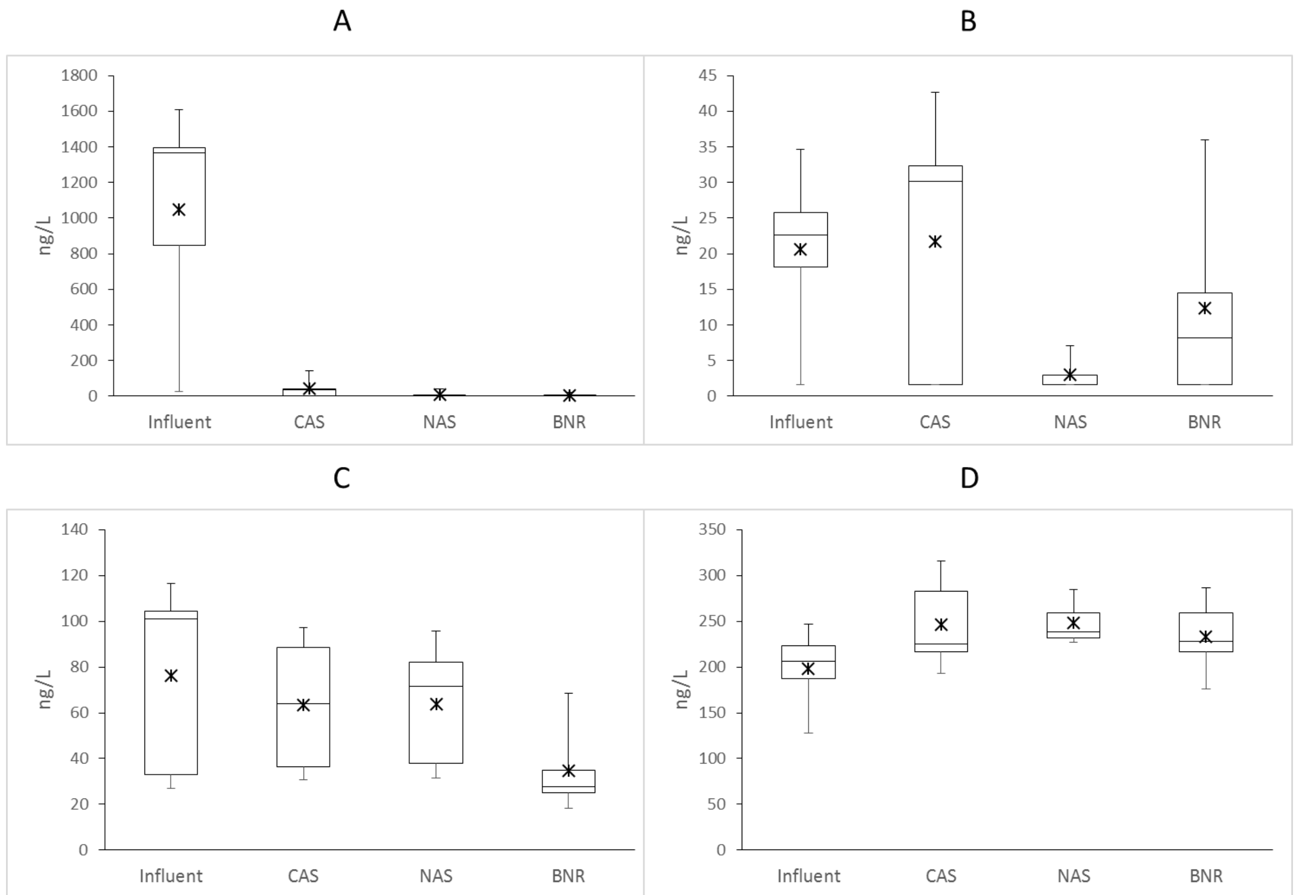


Figure 3-4. Box plot and average of influent and effluents concentrations from CAS, NAS and BNR treatment A- Ibuprofen, B- Gemfibrozil, C- Trimethoprim, D- Carbamazepine.

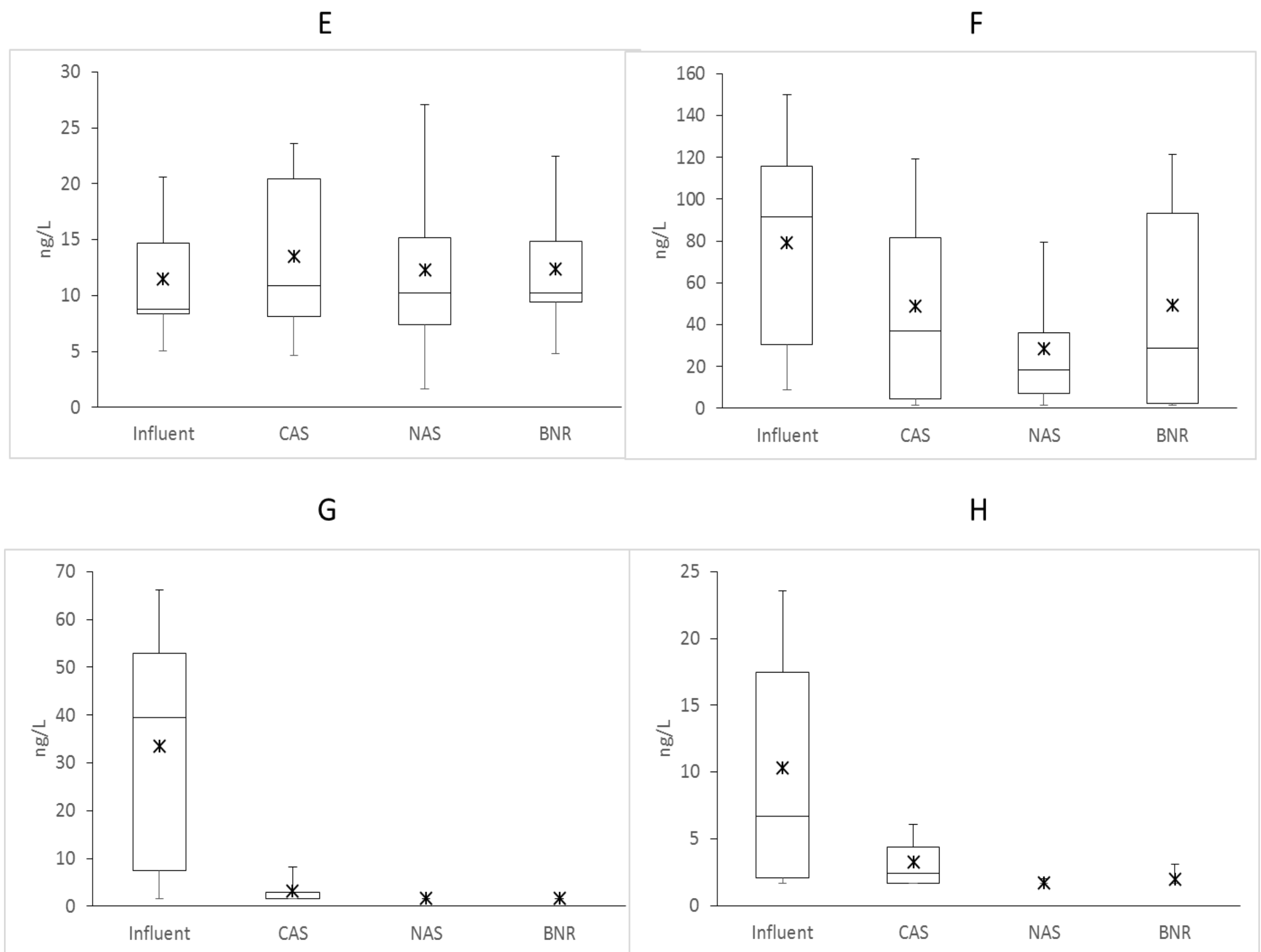


Figure 3-5. Box plot and average of influent and effluents concentrations from CAS, NAS and BNR treatment. E- Meprobomate, F- Sulfamethoxazole, G-Androstedione, H-Estrone.

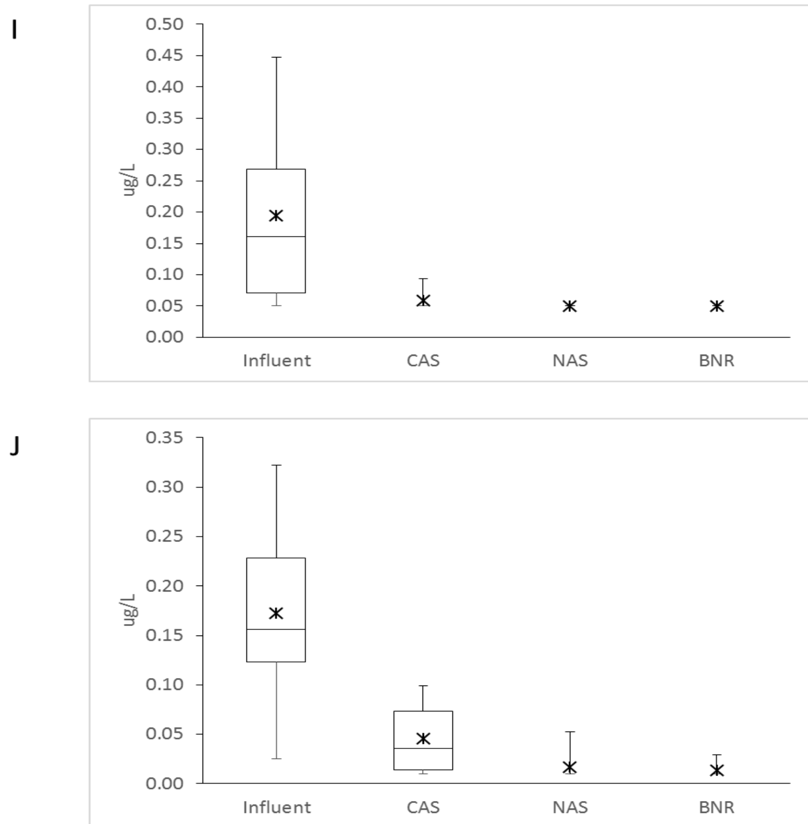


Figure 3-6. Box plot and average of influent and effluents concentrations from CAS, NAS and BNR treatment. I- Nonyl-phenol, J- Bisphenol-A.

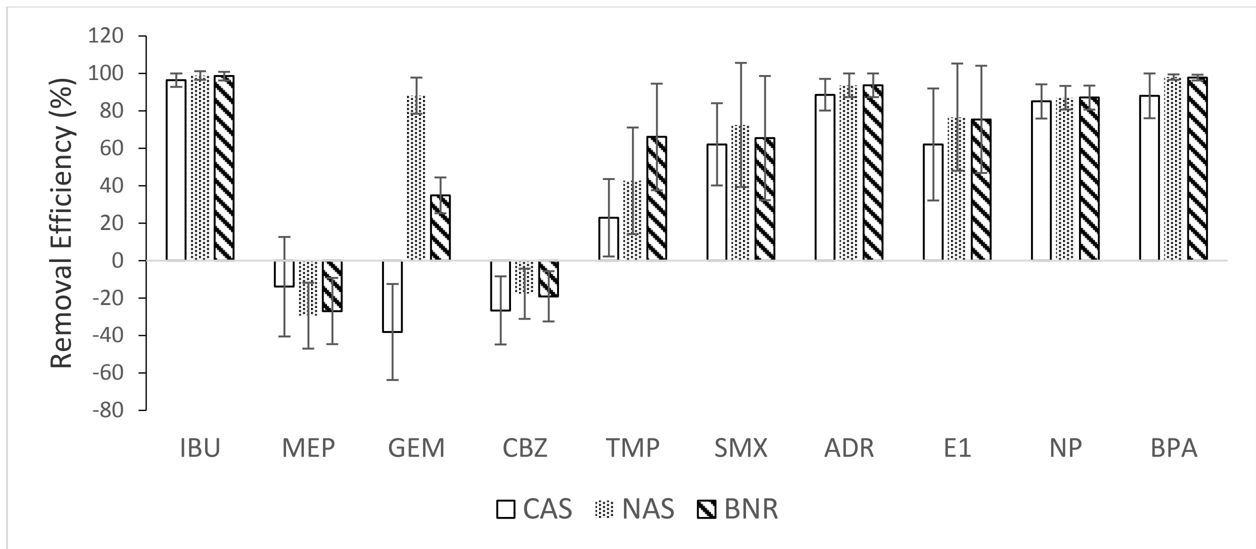


Figure 3-7. Micropollutant removal efficiencies in pilots.

3.3.3 Micropollutant Removal in BNR Treatment Configuration

The bioreactors of the CAS and NAS pilots were operated as single aerobic reactors but the BNR bioreactor was divided into three different zones operated at different redox conditions. Therefore, it was expected that the different zones would contribute differently to the biotransformation of the MPs in the BNR treatment configuration. This expectation was based on the fact that the amount of energy that is captured by the microorganisms in aerobic conditions is usually higher than the energy captured in anoxic and anaerobic conditions (Tchobanoglous et al., 2003). This bioenergy could be instrumental to the biotransformation of the MPs in the BNR bioreactor. Hence, the contribution

of the redox conditions to the removal of the MPs that were removed in the BNR treatment configuration was assessed.

The MPs concentrations in the influent and the intermediate stages of the BNR bioreactor are presented in Figures 3-8 and 3-9. It is apparent from Figures 3-8 and 3-9 that the concentrations of the MPs decreased through each stage of the bioreactor, which suggested that each of the zones contributed to the overall MPs' removal. However, during the operation of the pilot BNR process, mixed liquors were recycled from the aerobic zone to the anoxic zone and from the anoxic zone to the anaerobic zone. The returned activated sludge was also recycled from the final clarifier to the anoxic zone of the bioreactor. These recycle flows can have dilution effects on the pilot plant influent at different points of the treatment system. Therefore, a set of mass balances were employed to characterize the fate of the MPs that were removed in the pilot BNR treatment configuration.

In the mass balances, the biotransformation efficiencies of the MPs in each zone were calculated as the difference between the mass flow entering and leaving the zone, divided by the mass flow entering the zone. The difference between the mass inflow and outflow in the aqueous phase across each zone of

the bioreactor was assumed to be due to microbial biotransformation within the zone of the bioreactor. Figure 3-10 shows a representative schematic of the mass flows employed for the MPs that were removed in the BNR treatment configuration. The biotransformation efficiencies of the MPs in the anaerobic, anoxic and aerobic sections of the BNR bioreactor are presented in Table 3-5. Table 3-5 shows that the biotransformation efficiencies in the bioreactor of the BNR process increased from the anaerobic to the anoxic and aerobic zone for IBU and TMP while only the aerobic zone degraded SMX, ADR, E1 and GEM. Hence, it was concluded that out of the 6 MPs that were removed in the BNR treatment configuration, only IBU and TMP were biotransformed in all the redox zones of the BNR bioreactor at different percentages while the other 4 MPs were biotransformed only in the aerobic zone.

The energy that is captured by the microorganisms in aerobic conditions is higher than the energy captured in anoxic and anaerobic conditions (Tchobanoglous et al, 2003). This difference in energy could explain why the aerobic zone had the highest and in some cases only biodegradation efficiency among the three stages for the pilot BNR treatment configuration. The high biotransformation efficiencies in the aerobic zones as compared to the anoxic and

aerobic zones suggests the potential for significant removal of MPs in aerobic environments. It was concluded that the aerobic environment is an important condition for significant biotransformation and removal of MPs in wastewater treatment. However, further study is recommended to investigate ways of improving the removal of MPs in anoxic and anaerobic environments.

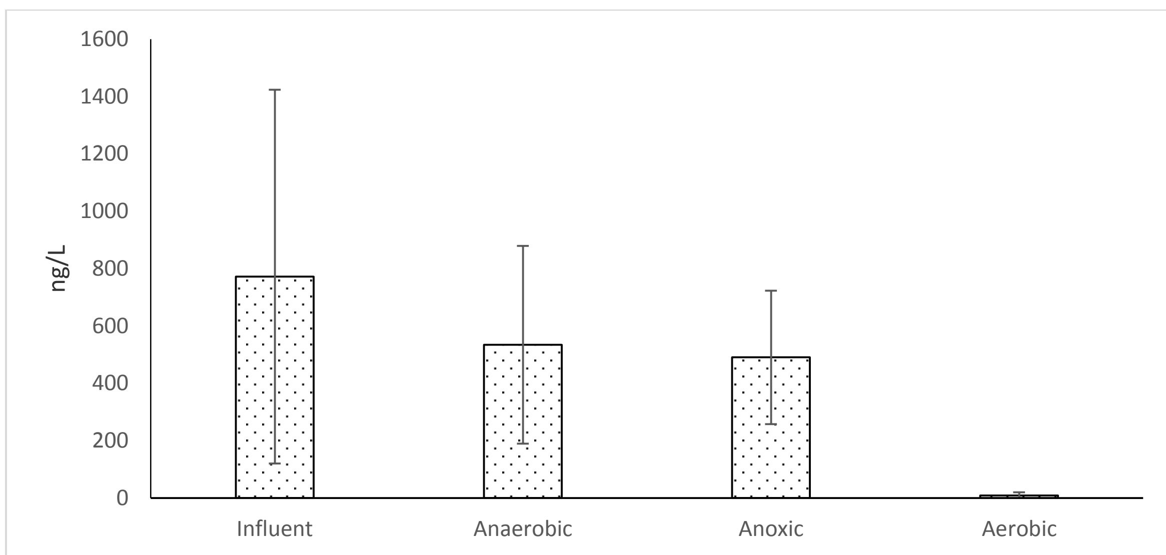


Figure 3-8. Ibuprofen concentrations in the influent and stages of BNR process (deviation bar represents standard deviation of measurements (n = 5)).

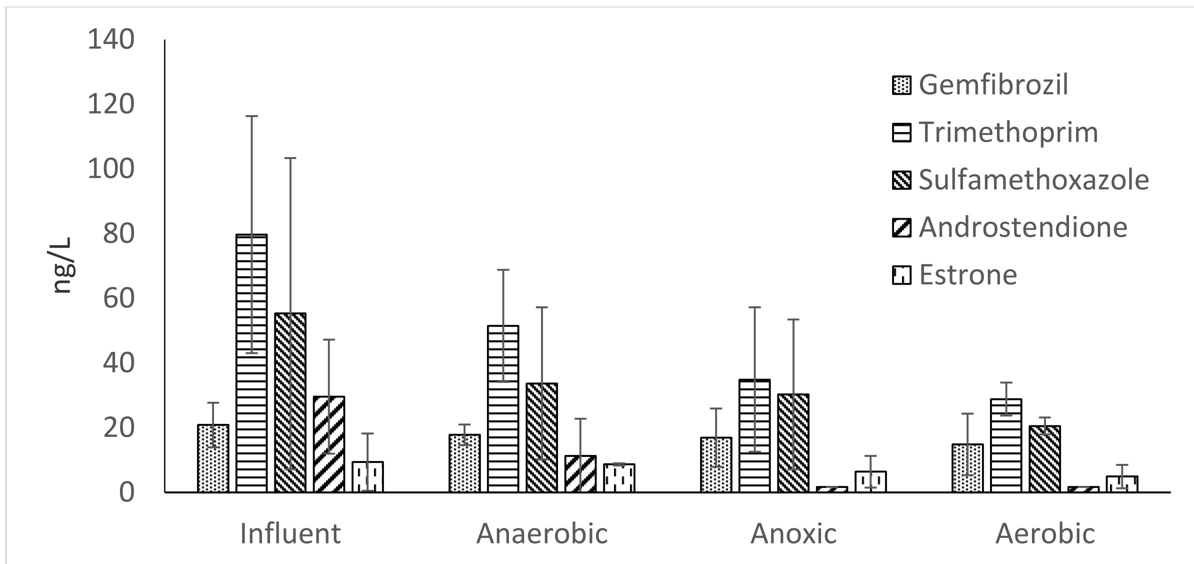


Figure 3-9. Micropollutant concentrations in the influent and stages of BNR process (deviation bar represents standard deviation of measurements (n = 5)).

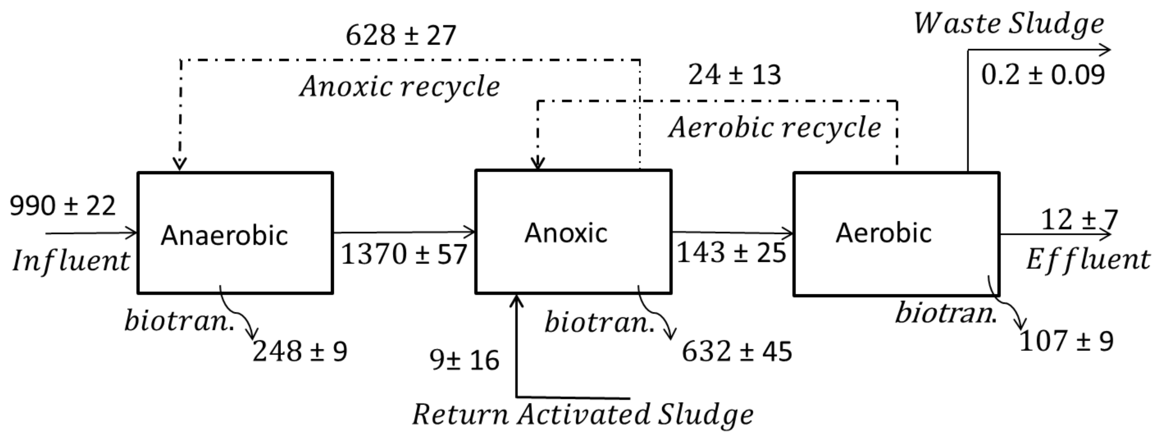


Figure 3-10. Mass balances of Ibuprofen in BNR pilot plant. (mass flow rate in μg/d).

Table 3-5. Biotransformation efficiency of MPs in BNR bioreactor

	Biodegradation efficiency (%)

Micropollutant	Anaerobic	Anoxic	Aerobic
Ibuprofen	15 ± 1	45 ± 1	83 ± 5
Trimethoprim	13 ± 12	17 ± 10	24 ± 4
Sulfamethoxazole	n.d	n.d	48 ± 11
Androstendione	n.d	n.d	100 ± 14
Estrone	n.d	n.d	95 ± 0.2
Gemfibrozil	n.d	n.d	67 ± 23

n.d – not degraded.

3.3.4 Biological Activity

Due to constraints on chemical analysis, it is unlikely that the suite of chemicals selected for characterization will account for the entire range of endocrine disrupting compounds (EDCs) present in the effluent. Hence, chemical quantification may not present a holistic picture of the potential for endocrine-disrupting activity of the effluents. In fact, differences in endocrine-active potency amongst different chemical species, or possible antagonism or synergism of chemical mixtures cannot be accounted for by chemical analysis. Therefore, the application of *in-vitro* bioassays to determine the estrogenicity of environmental samples (Leusch et al., 2006; Fernandez et al., 2009) has been developed and were examined in this study.

The YES assay was employed to assess the performance of the treatment processes in terms of estrogenicity removal. The YES assay E2-Eq values from the

treatment processes are summarized in Figure 3-11. The influent estrogenic equivalence (E2-Eq) ranged from 32 to 44 ng/L with an average concentration of 37.6 ± 5 ng/L and relative standard deviation (RSD) of 14% across all influent samples. The effluent E2-Eq values ranged from 4.2 - 8.5 ng/L for the CAS, 0.6 - 8.6 ng/L for the NAS and 0.2 - 2.1 ng/L for the BNR. The average effluent concentrations for the three processes were 6.3, 4.7 and 0.84 ng/L for the CAS, NAS and BNR processes respectively.

A paired-t-test was used to compare the E2-Eq in influent to the E2-Eq in the three effluent streams from CAS, NAS and BNR treatment configuration. The results of the paired-t-test showed a statistical difference between the influent and CAS, NAS and BNR effluents ($p < 0.05$). The BNR effluent values were significantly lower than the CAS ($p = 0.006$) but not significantly lower than the NAS effluent values. Further statistical analysis could not be employed to discriminate between the means of the data set because of the relatively limited number of available data. The removal efficiencies calculated as the percent difference between the influent and effluent E2-Eq concentrations across each treatment process were 84 ± 3 %, 89 ± 10 % and 98 ± 2 % for CAS, NAS and BNR respectively. The results show that the BNR and the NAS performed better than

the CAS in terms of estrogenicity removal. In general, the trend in the removal of estrogenicity by the process configurations were consistent with the removal of E1, NP and BPA that were previously presented.

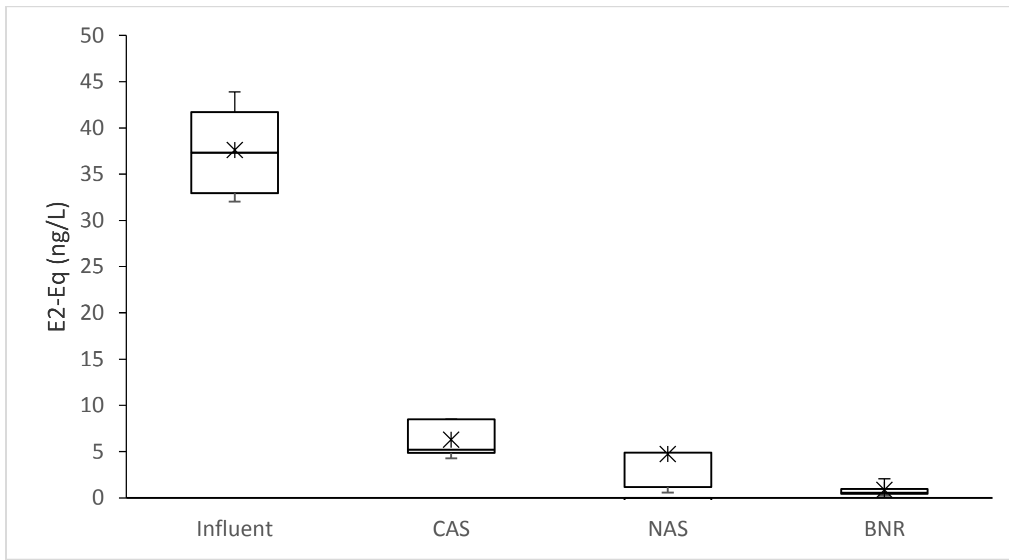


Figure 3-11. Box plot comparing the E2-Eq responses of influent and effluents from CAS, NAS and BNR (n = 4-5).

Conclusions

A comparative study of the performance of 3 different wastewater treatment processes was conducted. The concentrations of 10 MPs including acidic, basic and neutral compounds were determined in the influent and the effluent of CAS, NAS and BNR treatment configurations. The estrogenic activity of the influent and effluent from each of the treatment configuration was also analyzed using the YES assay. The removal efficiency of TMP improved with the

complexity of the three treatment processes configurations and SRTs. IBU, ADR, SMX, NP, E1 and BPA had moderate to high removals (> 65%) while CBZ and MEP remained recalcitrant in the three treatment process configurations. The removal of GEM was better in the NAS than in the BNR and CAS treatment configurations. The fact that only TMP showed an increase in removal as the complexity of the treatment configuration increased from CAS to NAS and to BNR suggests further study to assess the behavior of TMP in BNR systems. The YES assay analyses showed an improvement in estrogenicity removal in the BNR and NAS treatment configurations as compared to the CAS treatment configuration. In general, similar trend was observed among the treatment processes in terms of the removal of MPs and estrogenicity. However, it is important to note that the similarity in the trends in MP and estrogenicity removals does not imply that the reduction in estrogenicity across the treatment trains was as a result of the MPs removed.

Chapter 4 Assessment of the Removal of Estrogenicity in Biological Nutrient Removal Wastewater Treatment Processes

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4.1 Introduction

Effluents from wastewater treatment plants (WWTPs) have been shown to contain a mix of various endocrine disrupting compounds (EDCs) that could induce physiological effects either individually or synergistically on aquatic organisms (Desbrow et al., 1998; Sumpter, 1998; Nakada et al., 2004; Vajda et al., 2011; Wojnarowicz, et al., 2013; Parker et al., 2014). The USEPA has defined EDCs as exogenous agents that interfere 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. Some of the effects of EDCs on aquatic organisms include reduced reproductive capacity and vitellogenin production (precursor of egg yolk protein) in male fish, decreased female fish fertility and survival of juveniles,

reduced fish egg fertilization and thyroid hormone disruption in tadpoles (Purdom et al., 1994; Jobling and Sumpter, 1993, Jobling et al., 1998; Andersen et al., 2003; Vajda et al., 2011; Wojnarowicz, et al., 2013; Parker et al., 2014).

Synthetic and natural EDCs enter sewer systems through human and animal excretions (Combalbert and Hernandez-Raquet, 2010). Stringent policies could be formulated by regulatory agencies to attenuate the risks associated with EDCs in the environment. However, the anthropogenic release of these substances into the environment is difficult to control because some of these compounds are naturally produced in the human or animal body. For example, the natural estrogens, E1 and E2 excreted by pregnant women could be as high as 600–940 and 170–330 μg/day/person respectively (Johnson et al., 2000). Hence, the removal of EDCs in wastewater treatment processes will be required for attenuating their release into the aquatic environment.

Biological nutrient removal (BNR) wastewater treatment processes are advanced configurations that provide carbon, nitrogen and phosphorus removal. The removal and biodegradation of macropollutants in BNR processes is well documented but the fate of EDCs that are prevalent in wastewater in these processes is less well understood. Previous studies that have investigated the

removal of EDCs in BNR wastewater treatment processes have reported greater than 90% removal efficiencies of the compounds (Koh et al., 2009; Li et al., 2011). However, it is still unclear whether the high removal of EDCs in BNR treatment processes can be translated into a high reduction in estrogenic responses from the systems. A recent study that compared the removal of estrogenicity in conventional activated sludge (CAS), nitrifying activated sludge (NAS) and biological nutrient removal (BNR) processes showed greater than 80% estrogenicity removal in all processes with the highest removal in the BNR treatment process (Ogunlaja et al. 2013). However, the impact of the different stages of treatment on estrogenicity reduction was not examined in detail.

The quantification of the estrogenic potency of EDCs in WWTPs is not trivial because EDCs exist as a cocktail in WWTPs influents and effluents. The potential for synergistic action of the mixture of EDCs in wastewater has challenged previous attempts to relate calculated EDC concentrations with measured estrogenicity in WWTPs (Petrovic et al., 2004). Therefore, in order to give a holistic assessment of the estrogenicity of a WWTP effluent, previous studies have employed *in vitro* bioassays to augment chemical measurements (Servos et al., 2005; Wu et al., 2011; Parker et al., 2014).

In general, there have been few studies that have employed bioassays to investigate estrogen biodegradation in BNR activated sludge. Previous studies that have investigated the biodegradation of EDCs in activated sludge systems have monitored the disappearance of the compounds using chemical techniques without an understanding of the estrogenicity associated with the disappearance of the compounds (Joss et al., 2004; Dytczak et al., 2008). However, it has been demonstrated in other process configurations that the disappearance of estrogenic compounds does not necessarily eliminate estrogenicity. For example, the transformation of 17β-estradiol (E2) in activated sludge processes was reported to involve E2 oxidation to estrone (E1), another estrogenic compound (Ternes et al., 1999; Shi et al., 2004; Dytczak et al., 2008). There is the potential for differing conversions between estrogenic compounds in different redox conditions (Joss et al., 2004; Czajka and Londry, 2006; Dytczak et al., 2008). Hence, bioassay could be an important tool for characterizing the impact of redox on estrogenicity.

This study employed the YES assay technique to investigate the removal and biotransformation of EDCs in BNR activated sludge. In the current study, E1 and E2 were evaluated as target EDCs because several studies have shown that

E1 and E2 constitute a substantial fraction of the dominant estrogens found in the effluents of WWTPs (Nakada et al., 2004; Aerni et al., 2004; Fernandez et al., 2007; Muller et al., 2008). Specifically, this study employed the recombinant yeast screen to 1) investigate the removal of estrogenicity in BNR processes operated with both authentic and synthetic wastewater, 2) estimate the biotransformation rate constants for E1 and E2 in aerobic, anoxic and anaerobic batch reactors, and 3) investigate the transformation kinetics between E2 and E1 under anaerobic, anoxic and aerobic conditions.

4.2 Approach

A combination of pilot and bench scale tests, batch experiments and mathematical modeling was employed to assess the removal and biotransformation of EDCs in BNR activated sludge processes. The experimental approach employed a pilot scale BNR process fed with authentic municipal wastewater and a bench scale BNR process fed with synthetic wastewater that was dosed with EDCs. The objective of operating the pilot scale BNR was to investigate the removal and biotransformation of EDCs in each treatment zone of a BNR treatment process. The goal of operating the bench scale BNR system was to investigate the removal kinetics of the natural estrogens, E1 and E2 and their

associated estrogenicity under controlled conditions. In addition, a series of batch experiments were conducted to investigate the transformation between E1 and E2 under aerobic, anoxic and anaerobic conditions in order to provide further insight into the removal and biodegradation of the compounds in BNR processes.

4.2.1 Pilot Scale BNR Wastewater Treatment Process

A detailed description of the UCT-BNR pilot plant was described elsewhere (Ogunlaja et al, 2013). The operating and design conditions are summarized in Table 4-1. The pilot UCT-BNR was operated on authentic municipal wastewater that was augmented with sodium bicarbonate (22 g/L at the rate of 14.4L/d) to provide alkalinity, di-potassium phosphate (11 g/L at the rate of 14.4 L/d) as phosphorus source and sodium acetate (34.36 g/L at the rate of 14.4 L/d) to enhance the proliferation of PAOs. The resultant influent concentration of COD, alkalinity and total phosphorus were 367 ± 48 mg/L, 268 ± 21 mg/L and 11 ± 7 mg/L respectively. The pH range of the authentic wastewater entering the bioreactor was 7.5 – 8.2. The bioreactor was partitioned into six 60L cells to simulate pseudo plug flow (Figure 4-1). The first cell was operated as an anaerobic zone, the next two cells were operated as anoxic zones and the last

three cells were operated as aerobic zones. Coarse bubble aerators were used for aeration and mixing in the aerobic section of the bioreactor while mechanical mixers were used in the non-aerated zones. The solids residence time (SRT) was maintained by wasting mixed liquor from the last aerobic section of the bioreactor. The temperature was controlled by an insulated water jacket that was wrapped around the bioreactor, primary and final clarifier.

Table 4-1. Pilot BNR operating and design conditions

Unit	Size/description	Unit
Flow rate	1.3	m^3/d
Primary clarifier	Area = 0.46	m^2
	Depth = 1.56	m
Bioreactor	Volume = 0.36	m^3
	Depth = 1.28	m
	DO(aerobic) = 4-5	g/m^3
	DO(anoxic) = 1-2.5	g/m^3
	DO(anaerobic) = 0-0.2	g/m^3
Final clarifier	Area = 0.204	m^2
	Depth = 1.4	m
Recycle rate	Aerobic = 2.6	m^3/d
	Anoxic = 1.3	m^3/d
SRT	20	d
Aerobic SRT	10	d
RAS flow rate	0.9	m^3/d
Waste rate	0.018	m^3/d
HRT	7	h
Temperature	18 ± 2	^oC

RAS-return activated sludge,SRT-solid residence time,HRT-hydraulic retension time

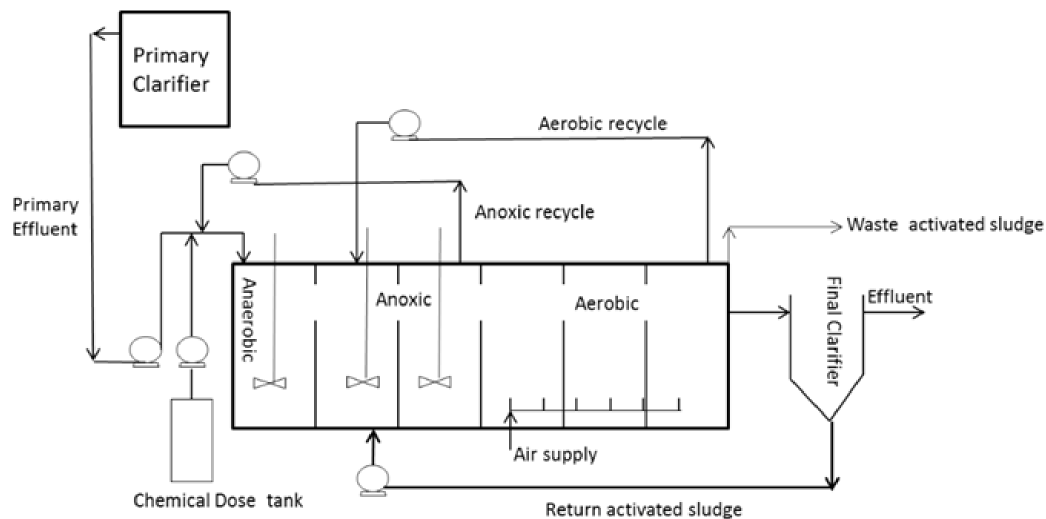

Figure 4-1. Flow schematic of pilot BNR activated sludge treatment system.

4.2.2 Bench Scale BNR Wastewater Treatment Process

The bench scale UCT-BNR process consisted of 3 - 10 L coupled reactors made of acrylic plastic and a 25 L final clarifier for solid –liquid separation (Figure 4-2). The aerobic reactor was mixed and aerated with fine bubble aerators while the anaerobic and anoxic reactors were mechanically mixed. The influent flow to the system was maintained at 0.086 ± 0.01 m^3/ day and the HRT was 5 hours. The return activated sludge was operated at 75% of the influent flow rate and the internal recycle ratios were 200% and 100% of the influent flow rate for the aerobic and anoxic recycles respectively. Approximately 0.9 ± 0.1 L/d of sludge was wasted from the aerobic zone of the bioreactor to maintain a total SRT of 20 days. The DO in the aerobic zone was maintained at 5-7 mg/L and the

temperature of the system was maintained at 20 ± 2°C. The operational parameters of the bench scale BNR process were consistent with typical operational parameters of commonly used BNR processes (WEF, 2005).

The bioreactors were inoculated with mixed liquor collected from the return activated sludge stream of a full scale BNR WWTP in southern Ontario. The system was fed with a synthetic wastewater containing carbon source, nitrogen source, phosphate buffer and micronutrients. The constituents of the synthetic wastewater and their target concentrations are presented in Table 4-2. The system was initially maintained without addition of E1 and E2 into the influent stream for one SRT to allow acclimatization of the biomass to the synthetic feed. Subsequently, a volume of E2 and E1 dissolved in water was dosed into the synthetic wastewater with the objective of achieving a target concentration of 100 ng/L for E1 and E2 to acclimatize the biomass to the estrogenic compounds. These dosed concentrations were higher than those typically observed in Canadian wastewater (Servos et al., 2005). However, this concentration enabled measurement of the estrogenicity in the samples, considering the sample matrix. After two months of operation, steady state was achieved and the aerobic bioreactor mixed liquor with an MLVSS concentration

of 3817 ± 150 g MLVSS/ m^3 was employed for the batch tests.

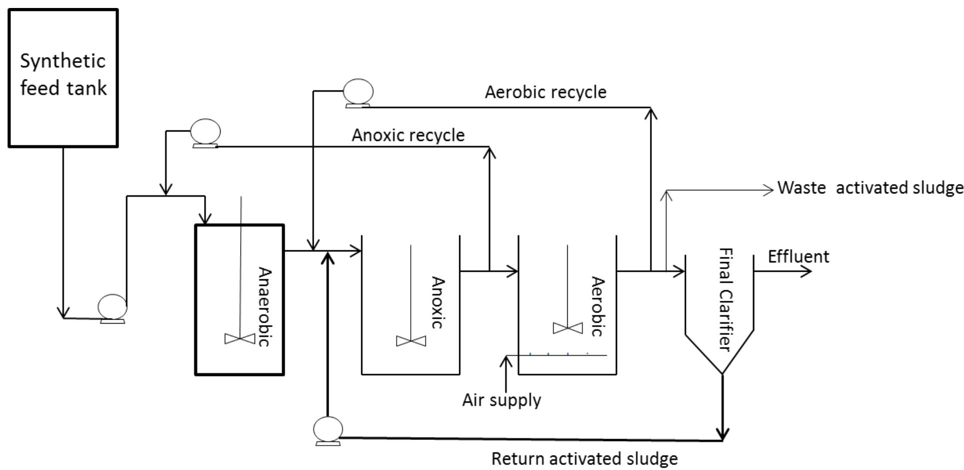

Figure 4-2. Flow schematic of the bench scale BNR system.

Table 4-2. Composition of influent synthetic wastewater

Function	Name	Molecular Formula	Target feed concentration	Unit
Carbon source	Sodium acetate	$NaCH_3CO_2$	475	g COD/ m^3
Nitrogen source	Ammonium chloride	NH_4Cl	15	g N/ m^3
Phosphorus source	Monopotassium phosphate	KH_2PO_4	10	g P/ m^3
Alkalinity	Sodium bicarbonate	$NaHCO_3$	167	g/ m^3
Micronutrients	Calcium chloride	$CaCl_2 \cdot 2H_2O$	250	g/ m^3
	Magnesium sulfate	$MnSO_4 \cdot H_2O$	180	mg/ m^3
	Copper sulfate	$CuSO_4 \cdot 5H_2O$	35	mg/ m^3
	Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	130	mg/ m^3
	Iron (II) sulfate	$FeSO_4 \cdot 7H_2O$	390	mg/ m^3
	Cobalt chloride	$CoCl_3 \cdot 6H_2O$	34	mg/ m^3

4.2.3 Monitoring and Sampling–Pilot and Bench Scale BNR System

The pilot plant was operated for over 12 months with consistent monitoring of the conventional and operational parameters for over 6 months. The bench scale setup was operated for 3 months with consistent monitoring for one month. Performance was assessed by monitoring chemical oxygen demand (COD), Total Kjeldahl Nitrogen (TKN), ammonia (NH_3), nitrite (NO_2), nitrate (NO_3), phosphate (PO_4), total suspended solid (TSS) and volatile suspended solid (VSS).

Twenty four hour composite samples of the pilot plant influent and effluent were collected in pre washed stainless steel containers, three times a week for two weeks using a refrigerated autosampler for estrogenicity analysis. Eight hour composite grab samples (sampled every 2 hours for eight hours and mixed together) were also collected thrice a week for two weeks from the anaerobic, anoxic and aerobic zones of the bioreactor. Five hour composite samples were collected from the bench scale influent and effluent streams in prewashed amber glass bottles thrice a week for 2 weeks for estrogenicity analysis. In addition, grab samples with a volume of 40 mL were collected thrice

a week for two weeks from the mixed liquors in the anaerobic, anoxic and aerobic reactors. The samples were centrifuged at 4000 rpm for 5 minutes and the supernatant filtered through 1.5 μm glass microfiber filters (Whatman, Toronto, Ontario, Canada). The filtered samples for the pilot plant were adjusted to pH 8 with 0.1M NaOH and stored in amber bottles in preparation for solid phase extraction while the bench scale samples were extracted immediately.

4.2.4 Batch Experiments

The mixed liquor from the aeration zone of the bench scale UCT-BNR was employed as the innoculum in a series of batch tests that were conducted to investigate the biotransformation of E2 and E1 under anaerobic, anoxic and aerobic conditions using the YES assay method. The batch experiments were conducted as described by Shi et al., 2004. The batch experiments were performed using a 1L Erlenmeyer flask placed on a magnetic stirrer and wrapped with aluminium foil to prevent photodegradation of the EDCs. In the aerobic experiment, the mixed liquor was mechanically stirred and air was supplied through air pumps to maintain a dissolved oxygen (DO) concentration of approximately 2 – 7 mg/L. The mixed liquor was mechanically stirred in the

anaerobic and anoxic experiments without aeration. The reactors were maintained at a temperature of 20 ± 2°C and pH range of 7.2 - 8.5.

A solution of either E2 or E1 in ethanol solution was added to the sterilized erlenmeyer flask to achieve a target concentration of 0.1g/m^3. After evaporation of the solvent, approximately 125 mL of mixed liquor from the BNR process was diluted with an appropriate volume of synthetic wastewater to achieve a mixed liqour volatile suspended solid (MLVSS) concentration of 255 gMLVSS/m^3. The batch experiments were conducted once and duplicate samples collected at every time interval The concentrations of the chemicals added into the batch reactors to facilitate biomass growth throughout the duration of the biotransformation tests are presented in Table 4-3.

Table 4-3. Feed composition for batch tests

Function	Molecular Formula	Anaerobic	Anoxic	Aerobic	Unit
Carbon source	$NaCH_3CO_2$	305	330	375	gCOD/m^3
Phosphorus source	KH_2PO_4	20	15	20	g P/m^3
Nitrogen source	NH_4Cl	n/a	n/a	14	g N/m^3
Nitrate source	KNO_3	n/a	72	n/a	g N/m^3

n/a-not added

4.2.5 Batch Experiment Monitoring and Sampling

The concentrations of E2, E1 (as indicated by the YES test) and conventional parameters in the mixed liquor in the batch reactors were analyzed to assess the removal of estrogenicity and conventional pollutants. From each batch reactor, 40 mL of mixed liquor samples were collected in prewashed amber bottles before the addition of E1 and E2 to the reactors in order to quantify background concentrations. Samples of the reactors' mixed liquor were then collected 15 minutes after dosing and subsequently every four hours for three days. The collected samples were centrifuged at 4000 rpm for 5 minutes and the supernatant liquids were filtered using 1.5 μm glass fiber filters (Whatman 934/AH). A volume of 10 mL was analyzed for conventional pollutants after filtering and the remaining 30 mL was immediately analyzed using SPE analysis. All parameters were analyzed in duplicates.

4.2.6 Sample Extraction

Solid phase extraction (SPE) is the most common extraction method used to concentrate EDCs in environmental samples and hence the method of Li et al., (2010) was employed to extract the EDCs from the wastewater samples in this study.

4.2.6.1 Solid Phase Extraction

100 ml of the filtered samples were passed through 6cc/500 mg OASIS HLB cartridges (Waters, USA) using Visiprep solid-phase extraction (SPE) vacuum manifolds and a vacuum pump. The cartridges were initially conditioned sequentially with 5 mL of methanol and 5 mL of Milli-Q water before loading the samples onto the cartridge. After extraction, the cartridges were rinsed with 5mL of water and 5 mL of hexane before being dried under vacuum for 5 minutes. Two 5-mL aliquots of ethyl acetate at an approximate flow rate of 1mL/min were subsequently used to elute the samples into 10 mL borosilicate collection tubes. The eluate was dried under a gentle stream of nitrogen gas and vortex-mixed with 500 μL of methanol. The final samples were transferred into amber bottles and stored at $4^{o}C$ until the YES assay procedure was conducted.

4.2.6.2 Extraction Recovery

A volume of 100 mL of mixed liquor from the anaerobic, anoxic and aerobic sections of the WWTP that was the source of the inoculum for the bench scale BNR was dosed with 30 μg/L of E2 to determine the sample extraction recovery. 100 mL of milli-Q water was also dosed with the same concentration of

E2 to investigate the possible effect of sample matrix on the extraction procedure. The wastewater samples were centrifuged at 5000 rpm for 5 minutes and the supernatant liquid were filtered using 1.5 μm glass fiber filter before solid phase extraction and YES assays were conducted.

4.2.7 Chemical Analysis

Conventional wastewater parameters including chemical oxygen demand (COD), total suspended solids (TSS), total Kjeldahl nitrogen (TKN), total phosphorus, ammonia-nitrogen, nitrate-nitrogen, total suspended solid (TSS) and volatile suspended solid (VSS) were analyzed according to *Standard Methods* (Eaton., 2005).

4.2.8 YES Assay

One of the widely used *in vitro* bioassays for the determination of estrogenicity in environmental samples is the recombinant yeast estrogen screen (YES) (Fent et al., 2006; Leusch et al., 2010; Citulski and Farahbakhsh, 2012). The recombinant yeast strain contains yeast cells that has been integrated with the DNA of human estrogen receptors (hER). The YES assay provides a qualitative and quantitative measure of the endocrine-disrupting potential of all EDCs present in WWTP effluent (Leusch et al., 2010). Hence, this method has proved to

be a useful tool in determining the combined estrogenic effects of the mixture of EDCs in complex environmental matrices such as WWTPs effluents.

A sample of concentrated human estrogen-receptor (hER) transfected recombinant yeast stock was provided by Prof. C. Metcalfe, (Trent University, Peterborough, ON). The YES assays were conducted as described by Routledge and Sumpter (1996) with modifications as reported by Citulski and Farahbakhsh, (2010). The bioassays were conducted in a sterilized laminar air flow cabinet to minimize aerosol formation and contamination. Each 96 well optically flat-bottom microtiter plate contained one row of 100 μL of serially diluted positive control of E2 (10 nM to 5×10^{-3} nM) in triplicates, one row of ethanol as negative control and three rows of serially diluted 80 μL extracted wastewater samples. A volume of 200 μL of combined growth medium/CPRG/yeast mixture was added to the dried wells of the plates. The plates were sealed with parafilm to prevent drying of the well solution in the incubator and shaken at 150 rpm and 32°C for 72 hours. The yeast growth in the plates was measured using a micro titer plate reader (Sunrise Basic TECAN) at an absorbance of 620 nm to measure turbidity and 540 nm to detect color change of the assay medium.

4.2.8.1 Data Processing

The absorbance measurements for E2 standards and wastewater samples were adjusted as per Fent et al (2006) (Equation 4-1) in order to adjust the absorbance of chlorophenol red at 620 nm for the extent of yeast growth (turbidity).

Adjusted Absorbance = AB540nm (sample) – [AB 620nm (sample) – AB 620nm (blank, average)] 4-1

Where,

AB540nm (sample) = sample absorbance at 540 nm
AB 620nm (sample) = sample absorbance at 620 nm
AB 620nm (blank, average) = average of blank absorbance at 620 nm.

The dose response curve for the standards and environmental samples were input to GraphPad Prism 6 (v. 6.02) that employs a four-parameter sigmoidal Hill equation to calculate the EC-50. To obtain the estrogenicity values for the extracted sample, the logarithm of the extraction dilution that yielded a 50% response was computed from the fitted standard curve. The estrogenicity of the samples were expressed as 17β-estradiol equivalents (E2-Eq), or the equivalent concentration of E2 that would have to be present in the wastewater

sample to achieve a response of the same magnitude. The E2-Eq was calculated as the ratio between the amount of E2 in the incubation well at EC50 in the standard curve and the equivalent volume at EC50 as follows:

$$E2 - Eq\ (\frac{ng}{L}) = \left(\frac{E2\ EC50(\ ng/L)}{EC50\ dilution\ factor*0.4}\right) \quad 4\text{-}2$$

The value of 0.4 employed in equation 2 accounted for dilution when the air dried 80µL wastewater sample extract applied to the titer well was reconstituted with 200µL of the growth medium containing yeast cells (80/200 = 0.4). The method detection limit for the assay was determined to be 1 ng/L.

4.2.8.2 Determination of Relative Potency between E1 and E2-Eq (y)

In order to determine the relative estrogenic response of E1 as E2-Eq, E1 stock solution was added to a volume of 100 mL of the mixed liquor used for the batch tests to achieve concentrations in the range of 100-2000 ng/L. The dosed mixed liquor samples were centrifuged at 5000 rpm for 5 minutes and the supernatant liquids were filtered using 1.5 µm glass fiber filter before SPE and YES assays were conducted.

4.2.9 Statistical Analysis

Outliers in replicate measurements were detected using Grubb's test. The regressions used to construct the YES assay response curves for the samples were compared to the response curves of estradiol (E2) standard using the F-test. To check the yeast growth absorbance (AB620nm) for the presence of toxic effects resulting from the wastewater extracts, the yeast growth was compared to the average turbidity (±3 standard deviations) of 12 ethanol only negative-control wells that were incubated in the same plate as the samples. Samples that had yeast growth with turbidity values below the average minus 3 standard deviations of the negative control were removed from the dose-response curve analysis. The final conditioned data were employed to generate the dose-response curve for the estimation of the EC50.

4.3 Results and Discussions

As previously described, this study integrated results from pilot and bench scale BNR processes and batch tests to obtain an improved understanding of the fate of EDCs in BNR processes. The UCT-BNR pilot and bench scale systems were monitored with respect to the removal of conventional wastewater pollutants and estrogenicity to establish the performance of the treatment

processes. Samples collected from the batch reactors were analyzed for conventional pollutants to monitor the progress of typical metabolic processes and to estimate the biodegradation and biotransformation kinetics of E1 and E2 in BNR activated sludge. The biotransformation and biodegradation rate constants for E1 and E2 were estimated using pseudo first order kinetic expressions.

4.3.1 Performance of Pilot and Bench Scale BNR System – Conventional Pollutants

The conventional pollutants of the pilot and bench scale BNR processes were analyzed to determine whether the processes were functioning within typical operating ranges of a BNR process. The influent and effluent concentrations of the conventional contaminants from the pilot and bench scale BNR processes are presented in Table 4-4. As expected, low effluent concentrations were observed for COD, TKN, NH_4, NO_3, and TP. The results indicate that the treatment systems were effectively nitrifying, denitrifying and biologically removing phosphorus during the sampling period. The performance of both BNR systems was consistent with typical operation of BNR systems (Jeyanayagam, 2005). Hence, it was assumed that the biomass contained

representative quantities of active ordinary heterotrophic organisms (OHOs), nitrifiers and polyphosphate accumulating organisms (PAOs).

Table 4-4. Conventional pollutants responses in BNR processes (Mean ± SD.)

	Pilot Scale (mg/L)		Bench Scale (mg/L)	
Parameter	Influent	Effluent	Influent	Effluent
COD	367 ± 48	33 ± 12	447 ± 150	23 ±13
TKN	25 ± 12	2.3 ± 0.2	20 ± 10	3.2 ± 0.1
NH_4-N	19 ± 8	0.04 ± 0.03	15 ± 4	0.03 ± 0.01
NO_3-N	0.54 ± 0.2	3 ± 2	0.61 ± 0.24	4 ± 2
TP	11 ± 7	3 ± 2	10 ± 2	1± 0.3
MLSS (gCOD/L)	6.8 ± 0.9		7.2 ± 0.3	

4.3.2 Evaluation of Extraction Recovery

The extraction recoveries of the EDCs in the wastewater samples were analyzed to assess the performance of the solid phase extraction procedure employed in extracting the EDCs in the treated wastewater. The results of the tests with known concentrations of E2 are presented in Table 4-5. The Table shows that the extraction procedure gave a high recovery of E2 from the wastewater samples and milli-Q water. As expected, the milli-Q water had the highest recovery. The comparable recoveries observed in the mixed liquor

samples and the milli-Q sample were deemed to indicate that the mixed liquor suspended solids did not adsorb the dosed E2 and the solid phase extraction method extracted most of the dosed E2. Hence, the solid phase extraction procedure used in this study was deemed appropriate to quantify the estrogenicity in the treated wastewater samples.

Table 4-5. Solid phase extraction recovery of E2

Sample	Recovery %
Anaerobic mixed liquor	90 ± 20
Anoxic mixed liquor	87.5 ± 11
Aerobic mixed liquor	86.4 ± 6
Milli Q water	98.8 ± 13

4.3.3 Estrogenicity Removal in Pilot and Bench Scale BNR

The estrogenicity was assessed at different points during the operation of the pilot and bench scale BNR process in order to analyze and compare the performance of the processes in terms of estrogenicity removal. The responses reported as E2-Equivalent (E2-Eq) concentrations in the influent, effluent, and the interstages of both systems are presented in Figure 4-3.The deviation bars in the figure represent the standard deviation of the measurements (n = 6). It is apparent from the figure that the estrogenicity concentration decreased through each successive stage of both BNR processes. The overall estrogenicity removal

efficiency was calculated as the difference between the E2-Eq concentrations in the influent stream into the bioreactor and that of the effluent exiting the bioreactor, divided by the E2-Eq concentration in the influent stream into the bioreactor. Thus, the overall estrogenicity removal efficiencies for the pilot and bench scale BNR processes were 96 ± 5% and 95 ± 5% respectively. Hence, significant estrogenicity removal was observed in the pilot and the bench scale BNR processes. This observation was consistent with previous studies that reported removal of estrogens in BNR wastewater treatment systems (Joss et al., 2004; Wu et al., 2011, Li et al., 2011). The consistency of the high estrogenicity removal in this study with the results of previous studies that have reported high removal of EDCs based on chemical measurements suggests that a high removal of EDCs in BNR process could be interpreted as a high removal of estrogenicity. It was concluded that both pilot and bench scale BNR processes were performing equivalently with respect to overall estrogenicity removal.

Mass balances were conducted to account for the effects of the recycle streams on the observed estrogenicity removal efficiencies. The mass balances around the bioreactors for both pilot and bench scale BNR are presented in Figures 4-4 and 4-5 respectively. The degradation efficiency of EDCs in each zone

was calculated as the difference between mass flow entering and leaving the zone, divided by the total mass flow entering the zone. The difference between the mass inflow and outflow for each redox zone was assumed to be due to biodegradation within the zone of the bioreactor. Previous studies have shown that sorption typically accounts for less than 10% of the estrogen removal from wastewater (Andersen et al., 2003; Joss et al., 2004; Ternes et al., 1999). Hence, the degradation efficiencies in the anaerobic, anoxic and aerobic reactors were calculated as 11± 9%, 18 ± 2%, 93 ± 10% and 8± 0.8%, 38 ± 4%, 85 ± 22% in the pilot and bench scale reactors respectively. Generally, these results show that similar degradation efficiencies were observed in corresponding redox zones of the pilot and bench scale processes. Therefore, it was concluded that comparable degradation of estrogenic compounds occurred in the individual zones of both BNR processes, albeit at different percentages.

Similar estrogenicity degradation efficiencies were observed along the bioreactors for both systems despite the fact that the pilot plant BNR was fed with authentic wastewater with presumably a mix of estrogens and the bench scale BNR was operated with synthetic wastewater with only two estrogens. Previous studies have shown that approximately 95% of the estrogenic activity in

treated effluents was due to the presence of the natural estrogens while other estrogenic compounds such as alkyl-phenol contributed less than 5% of the estrogenicity in WWTPs effluents (Matsui et al., 2000; Aerni et al., 2004; Muller et al., 2008). The results suggest that the majority of the estrogenic activity in the authentic wastewater was contributed by estrogens similar to the ones added to the synthetic wastewater.

4.3.4 Interstage Comparison of Estrogenicity Removal in Pilot and Bench Scale BNR

The analysis of the mass balance in the BNR processes enabled an assessment of the importance of the redox conditions towards the estrogenicity removal. It also enabled a comparison between each redox zone in order to assess which zone could be optimized to improve overall process performance. The ratio of the degradation efficiencies in the aerobic, anoxic and anaerobic zones for both pilot and bench scale BNR process were 8:2:1 and 11:5:1 respectively. The observed high degradation efficiencies in the aerobic zones of the pilot and the bench scale BNR process was consistent with previous studies that reported high degradation of EDCs as monitored by chemical concentrations (Joss et al., 2004; Dytczak et al., 2008). Under aerobic conditions, heterotrophic

organisms have the capability to produce oxygenase enzymes that catalyze the direct incorporation of oxygen molecule into the molecule of the organic compounds. This oxygenase reaction weakens the ring structure in the synthetic compounds which makes it accessible for subsequent oxidation steps and more water soluble (Rittman and McCarty, 2001). Thus, the high reductions of estrogenicity in the aerobic zone could be due to the combination of the redox condition and the bacteria activity on the estrogenic compounds.

The performance of the anoxic zones was compared to that of the respective aerobic zones of the BNR processes. The degradation efficiencies of EDCs in the anoxic zones of the pilot and bench scale BNR processes were lower than that of the aerobic zones. This result was consistent with previous studies that reported biodegradation of EDCs or removal of estrogenic activities in the anoxic zones of BNR processes (Jurgens et al., 2002 and Lee and Liu, 2002; Joss et al., 2004; Wu et al., 2011). The lower biodegradation efficiency in the anoxic zones as compared with the aerobic zones could be as a result of the lower energy available to the biomass in the zones due to the utilization of nitrate as electron acceptor instead of oxygen (Tchobanoglous et al., 2003). Hence, it was concluded

that estrogenicity removal can occur in anoxic zone of a BNR process, but at a lower efficiency when compared to the estrogenicity removal in the aerobic zone.

The performance of the anaerobic zones were compared with the respective aerobic and anoxic zones of the BNR processes. The degradation efficiencies of EDCs in the anaerobic zones of the pilot and bench scale BNR processes were the lowest out of the three zones. The lowest biodegradation efficiency in the anaerobic zones as compared with the anoxic and aerobic zones could be as a result of the low energy available to the biomass in the anaerobic zones (Tchobanoglous et al., 2003). The comparable and low biodegradation efficiencies in the anaerobic zones of both the pilot and bench scale BNR processes suggest that only modest removals of estrogenicity occur in the anaerobic zones of BNR processes.

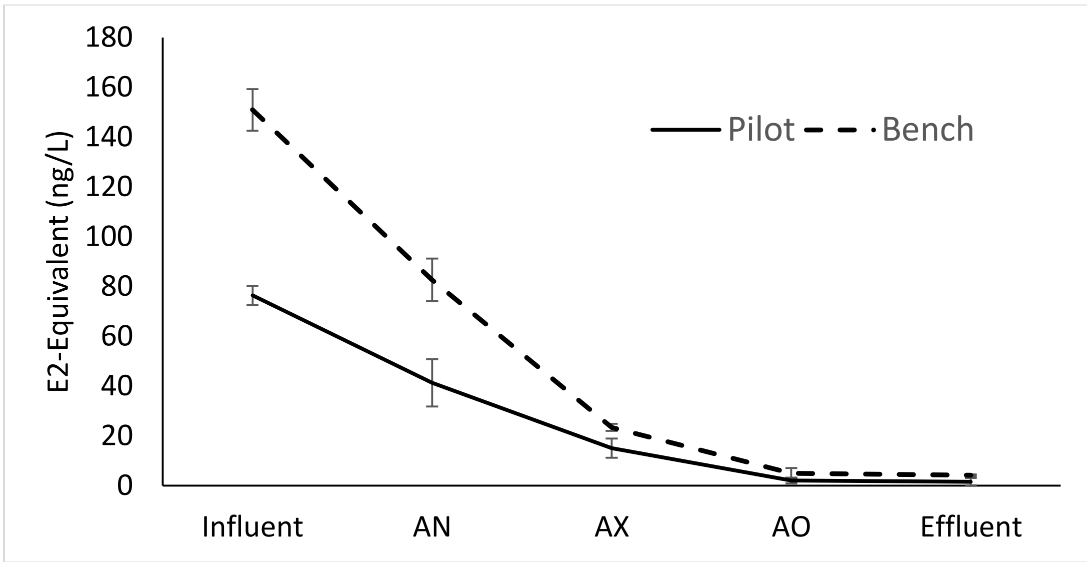

Figure 4-3. E2-Equivalent profiles along pilot and bench scale BNR bioreactors. AN-Anaerobic, AX-Anoxic, AO-Aerobic.

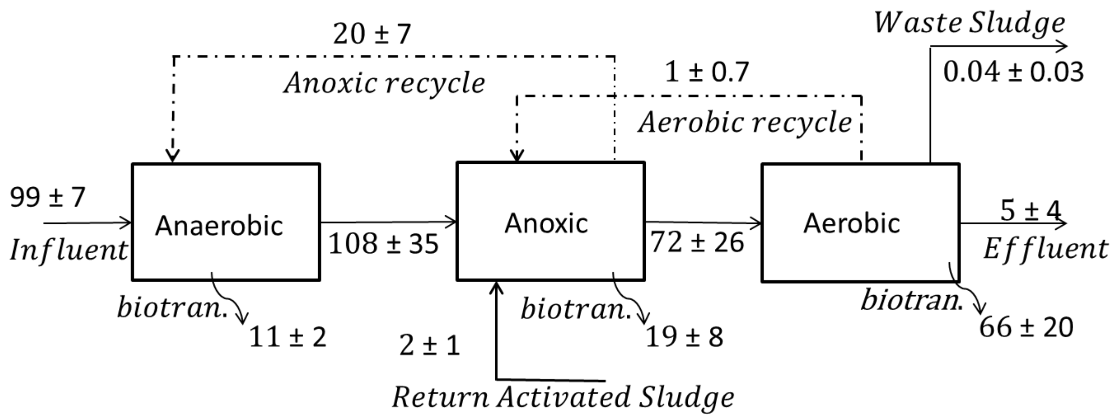

Figure 4-4. Mass balances (μg/d) of estrogenicity around pilot BNR bioreactor.

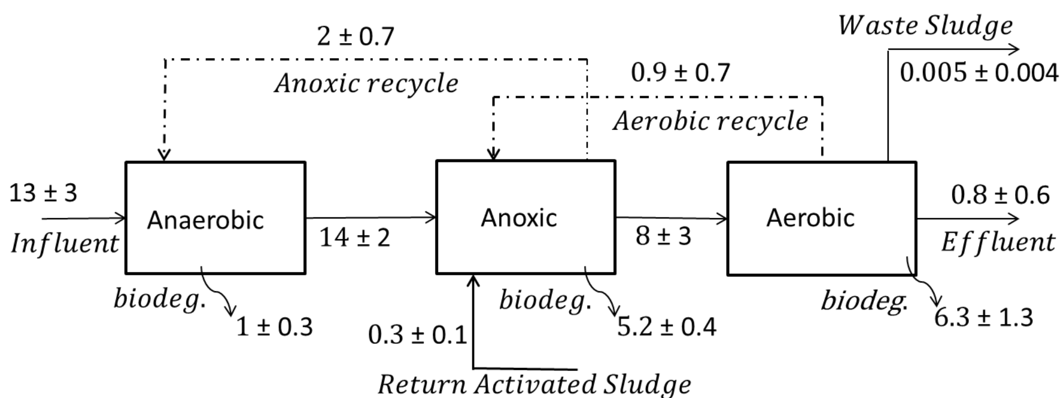

Figure 4-5. Mass balances (μg/d) of estrogenicity around bench scale BNR bioreactor.

4.3.5 Biotransformation of E2 and E1 in Batch Tests

A series of batch tests were conducted under similar redox conditions as the stages of the BNR process in order to further understand the biotransformation kinetics of E1 and E2 under the different redox conditions. The profiles of E2 equivalents in each batch test dosed with either E1 or E2 are presented in Figure 4-6. It is apparent from the Figure that the estrogenicity in all the reactors decreased with time, although at different rates.

The E2-Eq in reactors dosed with either E1 or E2 decreased at the fastest rate under aerobic conditions, followed by anoxic conditions and the slowest rate was observed under anaerobic conditions. These results were consistent with the previously described degradation efficiencies of the EDCs in the stages of the

pilot and bench scale BNR processes where the highest degradation occurred in the aerobic zones, followed by the anoxic zones and lastly the anaerobic zones. Therefore, it was concluded that biotransformation of E1 and E2 in the aerobic, anoxic and anaerobic batch reactors leads to a reduction in estrogenic responses in the reactors, albeit at different rates in the order of aerobic > anoxic> anaerobic.

Figure 4-6 shows that the E2 equivalence of the aerobic and anoxic reactors dosed with E2 decreased quickly during the first 4.25 hours of the tests. Previous studies have shown that the transformation or oxidation of E2 to E1 can occur under both aerobic and anoxic conditions (Ternes et al., 1999; Shi et al., 2004; Dytczak et al., 2008). This transformation reaction has also been previously reported to be rapid and usually occur early in batch experiments (Onda et al., 2002). It was not possible to separately monitor the biotransformation of E2 to E1 using the YES assay technique because the method measures the total estrogenic response elicited by both E1 and E2 at every point interval. However, since the relative estrogenic potency of E2 is significantly higher than that of E1 (Svenson et al., 2003; Tanaka et al., 2001), a significant reduction in E2 in the reactors would lead to a significant reduction in the total estrogenic response. Hence, the observed sharp decline in estrogenicity within the first approximately 4.25 hours

of the tests suggested the transformation of E2 to E1 in the aerobic and anoxic reactors.

From Figure 4-6 it can be seen that the E2-Eq of both E1 and E2 dosed reactors declined at the same rate in the anoxic and aerobic reactors after approximately 16 hours of reaction and this trend remained the same throughout the duration of the experiment. The E2-Eq in the E2 dosed reactors were the combined estrogenicity elicited by the residual E2 and the formed metabolite, E1 while the E2-Eq in the E1 dosed reactors only measured the estrogenicity due to residual E1 during the experiment. Hence the results support the previous observation that transformation of E2 to E1 occurred in the early stages of the experiments with E2 dosed reactors and that after approximately 16 hours, the majority of the estrogenicity was contributed by E1. Hence, these results suggest an accumulation of E1 in the E2 dosed reactors before further degradation as the experiment proceeded.

Previous studies have reported that biotransformation of E2 proceeded through initial formation of E1 and subsequent formation of metabolites or mineralization in anoxic and aerobic conditions (Ternes et al., 1999; Onda et al., 2003; Shi et al., 2004; Dytczak et al., 2008). However, similar conclusions were not

reported for biotransformation of E1 and E2 under anaerobic conditions. The reduction of E1 to E2 has been reported in anaerobic batch tests that were conducted with activated sludge taken from an anaerobic/anoxic/aerobic MBR pilot plant (Joss et al., 2004), an oxidation ditch (Mes et al., 2008) and a lab scale anaerobic/aerobic SBR (Lust and Stensel, 2011). On the contrary, other studies have reported oxidation of E2 to E1 under various anaerobic conditions ((Jürgens et al., 2002; Lee and Liu, 2002; Czajka and Londry, 2006). Hence, this study used bioassays to assess the biotransformation kinetics between E1 and E2 under anaerobic conditions.

Based on the bioassay method employed in this study, it was initially expected that the E2-Eq in the anaerobic reactor dosed with E2 would decrease to confirm the oxidation of E2 to E1 and increase in the E1 dosed anaerobic reactor to confirm the conversion of E1 to E2. This expectation was based on the fact that the estrogenic potency ratio of E2 to E2-Eq was 1:1 and E1 to E2-Eq was 1: 0.56. The results showed that the E2-Eq decreased in the anaerobic reactors dosed with either E1 or E2. The decline in the E2-Eq in the E1 dosed anaerobic reactor was faster than that of the E2 dosed reactor (Figure 4-6). This result implied that E2 was converted to E1 in the E2 dosed reactor and that degradation of E1

produced other metabolites than E2 in the E1 dosed reactor. This result was consistent with the previously observed reduction in estrogenicity in the anaerobic zones of the bench scale BNR, which was the source of the mixed liquor for the batch tests. Hence, it was concluded that the conversion of E1 and E2 to less estrogenic metabolites occurred in the anaerobic batch tests, with E1 degrading at a faster rate than E2.

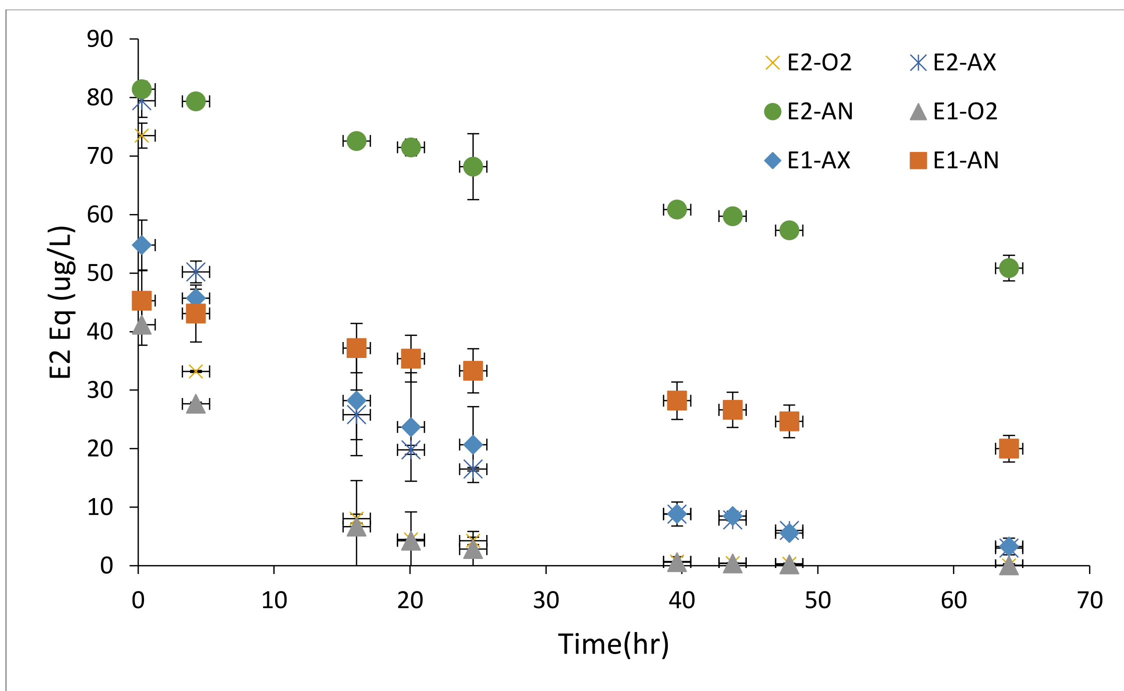

Figure 4-6. E2 equivalent profiles for batch tests dosed with E1 or E2. O2-aerobic, AX- anoxic, AN-anaerobic.

4.3.6 Modeling of Biotransformation of E1 and E2 in Batch Tests

Mathematical modeling was employed to further understand the biotransformation kinetics of E2 and E1 in terms of their estrogenicity response under anaerobic, anoxic and aerobic conditions. The models commonly used to predict biodegradation kinetics of EDCs in wastewater treatment typically employ either first order (Zhao et al., 2008) or pseudo first order expressions (Schwarzenbach et al., 2003; Joss et al., 2006). Therefore, a pseudo first order expression was employed to characterize the biotransformation kinetics of E2 and E1 in the aerobic, anoxic and anaerobic batch tests (Equation 4-3).

$$r_i = -k_b X S_i \qquad \text{4-3}$$

Where,

r_i = rate of biotransformation of compound i in batch reactor [$\mu g\ L^{-1} hr^{-1}$]
k_b = biotransformation rate constant [$L\ gCOD^{-1} hr^{-1}$]
X= MLVSS concentration [$gCOD\ L^{-1}$]
S_i= E2Eq due to E1 [$\mu g\ L^{-1}$]

The use of the YES assay technique to describe the behavior of E1 and E2 in batch tests was novel to this study. However, to employ the results of this study for modeling the biotransformation of E2 in the batch tests it was necessary

to address the degradation of E2 to E1, which is also an estrogenic compound, and the subsequent degradation of E1 (Ternes et al., 1999; Shi et al., 2004; Dytczak et al., 2008; Hashimoto and Murakami, 2009) (Equation 4-4). The measured E2-Eq values in the E2 dosed batch tests were recognized as the total estrogenic response elicited by E1 and E2 (Equation 4-5).

$$E2 \xrightarrow{k_{b,1}} E1 \xrightarrow{k_{b,2}} unknown \qquad 4\text{-}4$$

Where,

$k_{b,1}$ = biotransformation rate constant for the conversion of E2 to E1 [L $gCOD^{-1}hr^{-1}$]

$k_{b,2}$ = biotransformation rate constant for the degradation of E1 [L $gCOD^{-1}hr^{-1}$]

$$S'_{E2,t} = S_{E2,t} + yS_{E1,t} \qquad @\ t = 0,\ S_{E1,t} = 0 \qquad 4\text{-}5$$

Where,

$S'_{E2,t}$ = total E2-Eq at time t [$\mu g.L^{-1}$]

$S_{E2,t}$ = E2 concentration at time t [$\mu g.L^{-1}$]

$S_{E1,t}$ = E1 concentration at time t [$\mu g.L^{-1}$]

y = relative potency between E1 and E2-Eq

Equation 4-5 describes the E2-Eq concentrations in terms of summative effects of E1 and E2 in the E2 dosed reactors. The relative potency (y) was

employed to convert the E1 concentration to E2-Eq. This conversion was not required for E2 as the relative potency of E2 to E2-Eq is 1:1 (Svenson et al., 2003; Rutishauser et al., 2004). The measured E2-Eq in the E2 dosed batch tests was employed directly in the modelling of E2 in the reactors.

The mass balance on E1 in the E2 dosed batch reactors is presented in Equation 4-6, and includes the production of E1 from E2 and the subsequent biotransformation of E1

$$\frac{dS_{E1}}{dt} = \mu k_{b,1} X S_{E2} - k_{b,2} X S_{E1} \qquad 4\text{-}6$$

Where,

μ = stoichiometric conversion between E2 and E1

X= MLVSS concentration [g COD L^{-1}]

S_{E1}=soluble concentration of E1 [μg.L^{-1}]

S_{E2}=soluble concentration of E2 [μg.L^{-1}]

$k_{b,1}$ = biotransformation rate constant for the conversion of E2 to E1 [L $gCOD^{-1}hr^{-1}$]

$k_{b,2}$ = biotransformation rate constant for the conversion of E1 to metabolites [L $gCOD^{-1}hr^{-1}$]

Assuming a 1:1 mole ratio between E2 and E1, solving for E1 in equation 4-6 and substituting into equation 4-5 yields equation 4-7.

$$S'_{E2,t} = S_{E2,t} + y\left(S_{E2,t}k_1\left(\frac{e^{-k_1Xt}}{k_2-k_1} + \frac{e^{-k_2Xt}}{k_1-k_2}\right)\right) \qquad 4\text{-}7$$

The biotransformation rate constants k_2 and k_1 were estimated by solving equations 4-3 and 4-7 for each redox condition using an integral least square method that minimized the sum of squares of the residuals between the predicted and measured E2-Eq in the E1 and E2 dosed batch tests respectively.

4.3.7 Relative Potency between E1 and E2-Eq (y)

The estrogenic potency of E1 relative to E2 was determined based on the tests at known concentrations of E1 and measured E2 Eq. Figure 4-7 shows the relationship between E1 concentrations and measured E2 Eq. It is apparent from the plot that there was a linear relationship between the measured E2-Eq and the E1 concentrations as indicated by the r^2 value and residuals. The estrogenic potency of E1 was obtained from the slope of the linear fit and was estimated to be 0.56 ngE2/ngE1. This value was consistent with previously reported relative potencies between E2-Eq and E1 as measured by YES assays (Rutishauser et al., 2004; Muller et al, 2008). These previous studies reported relative potencies between E1 and E2-Eq in the range of 0.35 - 0.7. Hence, the estimated value of

0.56 was assumed to be reasonable and was employed as the relative potency between E1 and E2-Eq in this study.

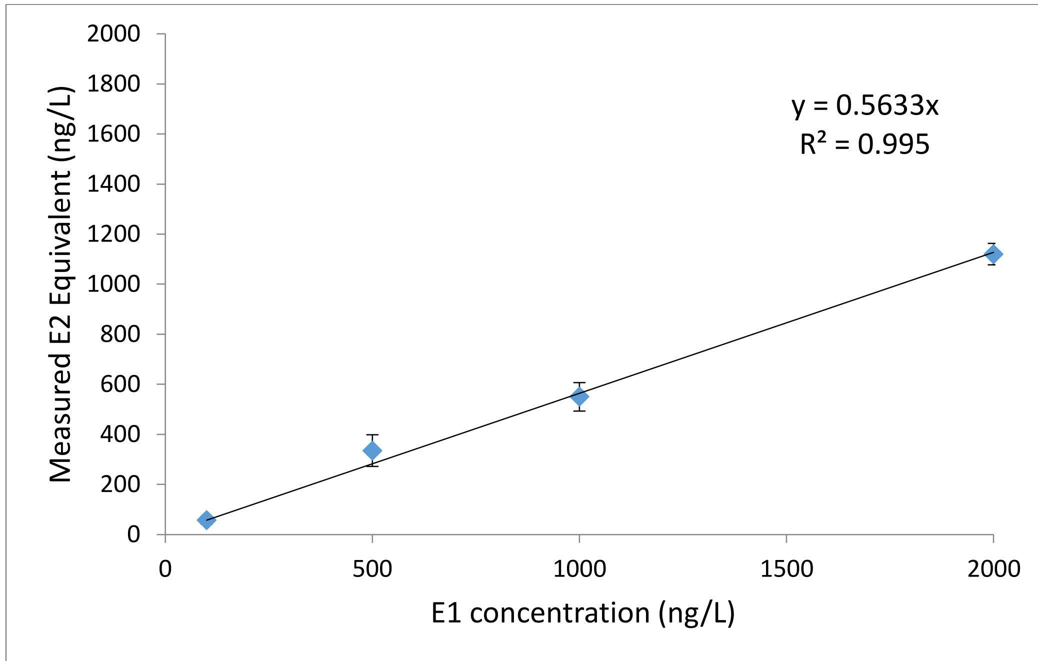

Figure 4-7. Correlation between E1 concentrations and E2 Equivalents as measured by YES assay.

4.3.8 Modeling Results

The integral least squares method was employed to estimate the biotransformation rate constants (k_b) for E1 and E2 in the aerobic, anoxic and anaerobic batch tests. Figure 4-8 presents the results of the model fitting for the measured E2-Eq in each of the reactors dosed with E1 and E2 while Table 4-6 presents the estimated parameter values and model performance evaluators. The

model performance evaluators, r^2 and NSE were deemed to be within a satisfactory performance rating range and hence it was concluded that there was a good fit between the predicted and measured E2-Eq in each of the reactors. From Table 4-6, it can be seen that the k_b values for both E2 and E1, decreased from the aerobic, to anoxic and anaerobic reactors. The trend in the k_b values among the three redox conditions was consistent with the previously described observations on the biodegradation efficiencies and removals from the different redox zones in the BNR processes and batch reactors. Hence, it was concluded that the biotransformation of E1 and E2 followed pseudo first order kinetics in the anaerobic, anoxic and aerobic batch tests.

The confidence intervals of the estimated parameter values were calculated in order to assess the uncertainty in the parameter values. The estimated k_b values along with their 95% confidence intervals are presented in Table 4-6. The confidence intervals of the estimated parameter values were less than 14%, 67% and 90% of the estimated values in the aerobic, anoxic and anaerobic zones respectively. Considering the uncertainty associated with analyzing estrogens in wastewater, this degree of uncertainty in the model parameter values was deemed to be acceptable.

The biotransformation of E2 through the intermediate metabolite, E1 was assumed to proceed as depicted in equation 4-4. Table 4-6 shows that the k_1 values in the aerobic and anoxic reactors were approximately an order of magnitude higher than the k_2 values in the corresponding test conditions. By contrast, the k_1 and $k_2$values in the anaerobic reactor were not statistically different. Thus, these results suggests that the biotransformation of E1 was the slower step in the two step reaction occurring in the aerobic and anoxic batch tests while under anaerobic conditions the rates of biotransformation of E2 to E1 and the biodegradation of E1 were similar.

Table 4-6. Model Performance and biotransformation rate constants with 95% confidence intervals

	Aerobic			Anoxic			Anaerobic		
k_b	$L.gCOD^{-1}d^{-1}$	r^2	NSE	$L.gCOD^{-1}d^{-1}$	r^2	NSE	$L.gCOD^{-1}d^{-1}$	r^2	NSE
k_1	71 ± 1.5	0.901	0.998	31 ± 3.3	0.972	0.998	1 ± 0.9	0.998	0.998
k_2	7.3 ± 1.0	0.959	0.998	3 ± 2.0	0.967	0.998	0.85 ± 0.6	0.998	0.998

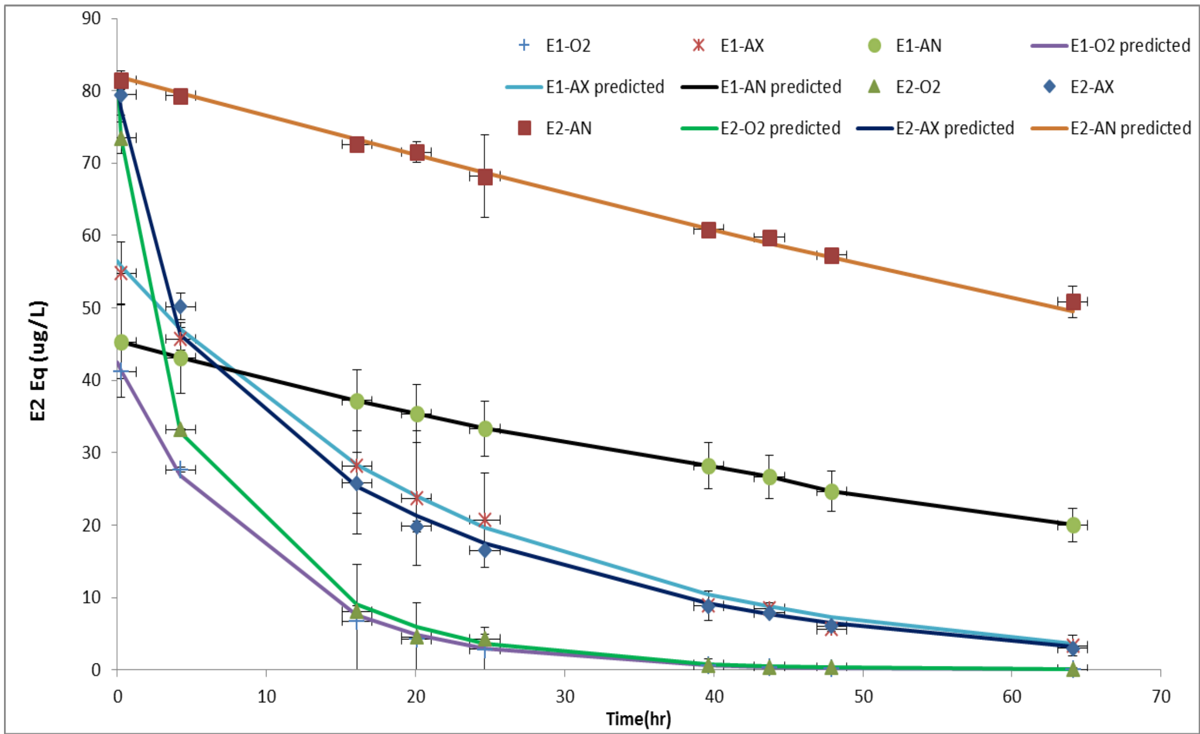

Figure 4-8. Measured and predicted E2-Eq in E1 and E2 dosed batch tests (aerobic (AO), anoxic (AX) and anaerobic (AN)).

4.3.9 Biotransformation Dynamics between E2 and E1 With Respect to E2-Eq

The calibrated model was employed to simulate the behaviors of E1 and E2 in the E2 dosed batch tests. This exercise was undertaken so as to elucidate the dynamics of the transformation between E2 and E1 in each of the batch reactors. Figures 4-9, 4-10 and 4-11 depict the results of the simulations of aerobic, anoxic and anaerobic batch reactors respectively and include the measured E2-Eq concentrations. It is apparent from the Figures that the

measured E2-Eq concentrations in all the reactors were described well by the simulated E2 + yE1 curves. The simulated E1 values were greater than the E2 + yE1 and E2-Eq responses in the aerobic and anoxic tests, but lower in the anaerobic tests. However, the predicted E2 concentrations were consistently lower than E2 + yE1 and E2-Eq in all three reactors. In the aerobic and anoxic tests (Figure 4-9 and 4-10), there was a rapid decline in E2 which led to an accumulation of E1 followed by subsequent degradation of E1. However, this pattern was different in the anaerobic test due to the much slower rates of transformation of E2 and degradation of E1 (Figure 4-11). The simulations provided useful insight into the dynamic responses of E2 and E1 in the batch tests that could not be directly elucidated from the YES responses.

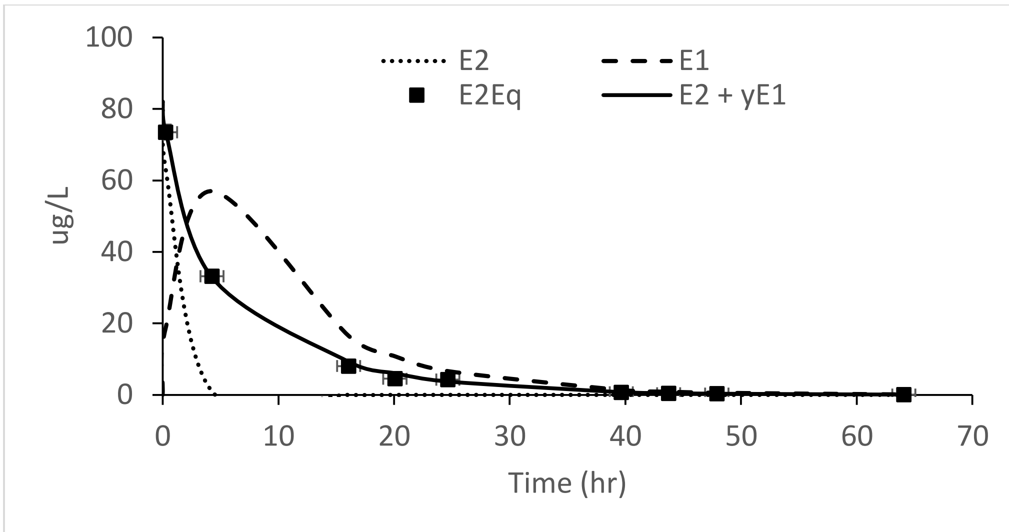

Figure 4-9. Simulated and observed estrogen concentration vs time in E2 dosed aerobic batch tests.

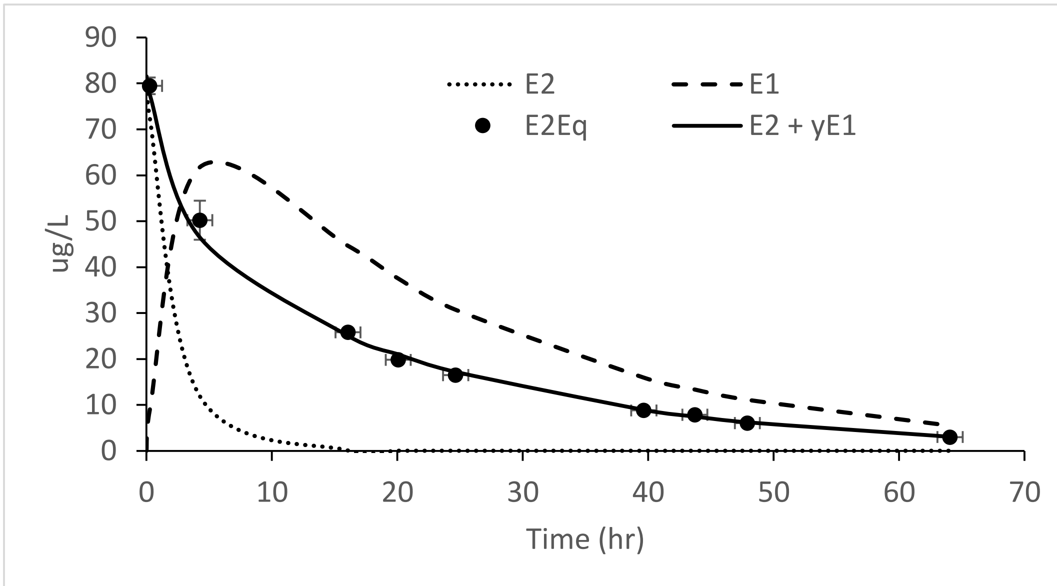

Figure 4-10. Simulated and measured estrogen concentrations vs time curve in E2 dosed anoxic batch tests.

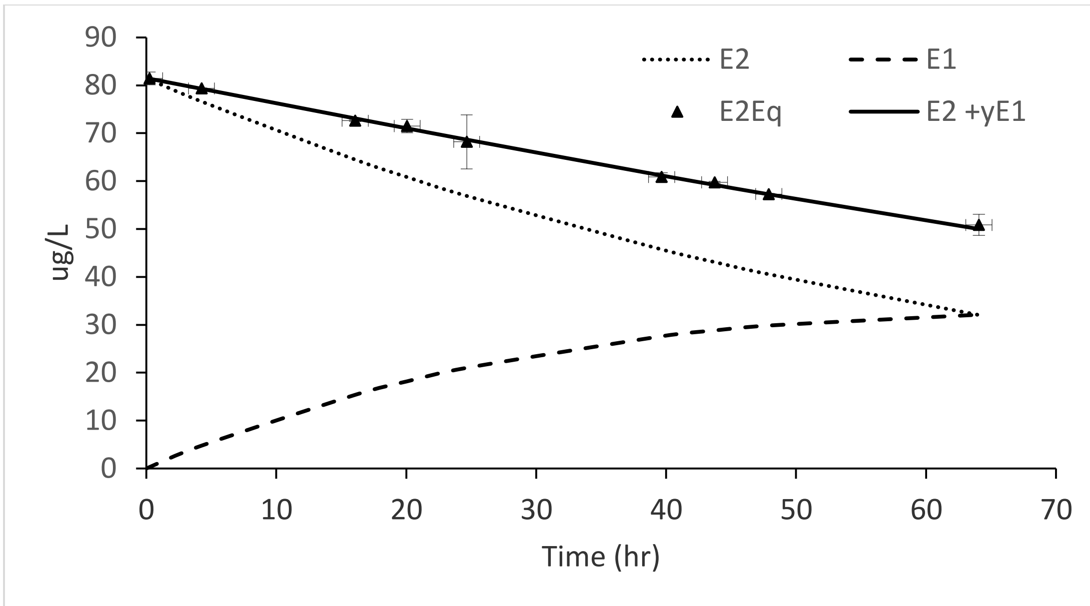

Figure 4-11. Simulated and measured estrogen concentrations vs time in E2 dosed anaerobic batch tests.

4.3.10 Model Verification

The biotransformation rate constants estimated from the bench scale testing were employed in a mass balance model to assess the behavior of E1 and E2, measured as E2-Eq along the stages of the bench scale BNR bioreactor. The model verification was conducted using the configuration and operating data that were employed in the bench scale BNR process, which was the source of the mixed liquor for the batch tests. Figure 4-12 shows the flows and concentrations employed to develop the mass balance models of the bench scale BNR process.

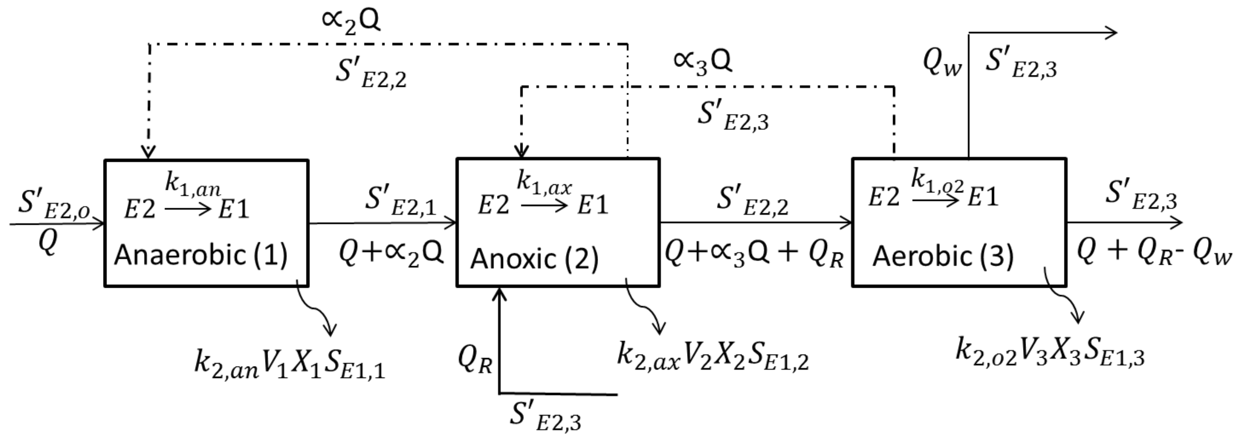

Figure 4-12. Flow and mass balance schematic for prediction of E2-Eq in BNR bioreactor.

The steady state mass balances for E2 and E1 in the anaerobic, anoxic and aerobic reactors are presented in equations 4-10-4-15.

Anaerobic

$$QS_{E2,0} - (Q + \alpha_2 Q)S_{E2,1} + \alpha_2 QS_{E2,2} - k_{1,an}S_{E2,1}X_1V_1 = 0 \quad \text{4-10}$$

$$QS_{E1,0} - (Q + \alpha_2 Q)S_{E1,1} + \alpha_2 QS_{E1,2} + k_{1,an}S_{E2,1}X_1V_1 - k_{2,an}S_{E1,1}X_1V_1 = 0 \quad \text{4-11}$$

Anoxic

$$(Q + \propto_2 Q)S_{E2,1} - (Q + \propto_3 Q + Q_R)S_{E2,2} - \propto_2 QS_{E2,2} + \propto_3 QS_{E2,3} + Q_R S_{E2,3} -$$

$$k_{1,ax}S_{E2,2}X_2V_2 = 0 \quad \text{4-12}$$

$$(Q + \propto_2 Q)S_{E1,1} - (Q + \propto_3 Q + Q_R)S_{E1,2} - \propto_2 QS_{E1,2} + \propto_3 QS_{E1,3} + Q_R S_{E1,3} + k_{1,ax}S_{E2,2}X_2V_2 - k_{2,ax}S_{E1,2}X_2V_2 = 0 \quad \text{4-13}$$

Aerobic

$$(Q + \propto_3 Q + Q_R)S_{E2,2} - (Q + Q_R - Q_w)S_{E2,3} - \propto_3 QS_{E2,3} - Q_w S_{E2,3} - k_{1,o2}S_{E2,3}X_3V_3 = 0 \quad \text{4-14}$$

$$(Q + \propto_3 Q + Q_R)S_{E1,2} - (Q + Q_R - Q_w)S_{E1,3} - \propto_3 QS_{E1,3} - Q_w S_{E1,3} + k_{1,o2}S_{E2,3}X_3V_3 - k_{2,o2}S_{E1,3}X_3V_3 = 0 \quad \text{4-15}$$

Where,

Q = influent flow rate

$S_{E1,0}$ = influent E1 concentration

$S_{E2,0}$ = influent E2 concentration

$\propto_2$ = anoxic recycle ratio with respect to influent flow rate

$\propto_3$ = nitrified recycle ratio with respect to influent flow rate

$k_{1,\mathrm{an}}$ = anaerobic zone biotransformation rate constant for E2 to E1

$k_{1,\mathrm{ax}}$ = anoxic zone biotransformation rate constant for E2 to E1

$k_{1,\mathrm{o2}}$ = aerobic zone biotransformation rate constant for E2 to E1

$k_{2,\mathrm{an}}$ = anaerobic zone biodegradation rate constant for E1

$k_{2,\mathrm{ax}}$ = anoxic zone biodegradation rate constant for E1

$k_{2,\mathrm{o2}}$ = aerobic zone biodegradation rate constant for E1

Q_w = wasted activated sludge flow rate

Q_R = returned activated sludge flow rate

V_1 = anaerobic zone volume

V_2 = anoxic zone volume

V_3 = aerobic zone volume

X_1 = anaerobic zone MLVSS concentration

X_2 = anoxic zone MLVSS concentration

X_3 = aerobic zone MLVSS concentration

y = relative potency of E1 with respect to E2-Eq,

The equations incorporated the inflow and outflow of both E1 and E2 and described the degradation of E2 through the intermediate metabolite E1 in each of the redox zones. The six equations were solved simultaneously, using the 'Inverse' and 'MMult' matrices functions in Excel, to determine the $S_{E1}$and S_{E2} in each of the redox zones. The relative potency of E1 that was previously estimated as 0.56 was then employed to convert the predicted E1 concentrations to E2-Eq,

to determine the E2-Eq in each of the redox zones ($S'_{E2,1}$, $S'_{E2,2}$ and $S'_{E2,3}$). Thus, the predicted E2-Eq along with the measured E2-Eq are presented in Figure 4-13.

Figure 4-13 shows the plot of predicted E2, yE1, E2 +yE1 and the measured E2-Eq in the BNR bench scale process. It is apparent from the plot that the predicted E2 +yE1 values reasonably described the measured E2-Eq in the BNR processes with slight deviation in the anoxic and aerobic zones. Figure 4-13 also shows that both the measured E2-Eq and the predicted E2 +yE1 concentrations decreased along the treatment zones of the BNR bioreactor. The plot shows that majority of the predicted E2 degraded along the bioreactor reaching a complete removal by the time the mixed liquor reached the aerobic zone. In contrast, the E1 remained in the system with a slight decrease in the anaerobic zone. The simulated behavior of E2 and yE1 in the bench scale BNR process was consistent with the simulated behavior of E2 and yE1 in the batch tests. In all, there was a good agreement between the measured E2-Eq and the predicted E2 + yE1 concentrations along the stages of the bench scale BNR process. The results were deemed to verify the model formulation describing the interactions between E2 and E1 and the estimated biotransformation rate constants reasonably estimated from the batch tests.

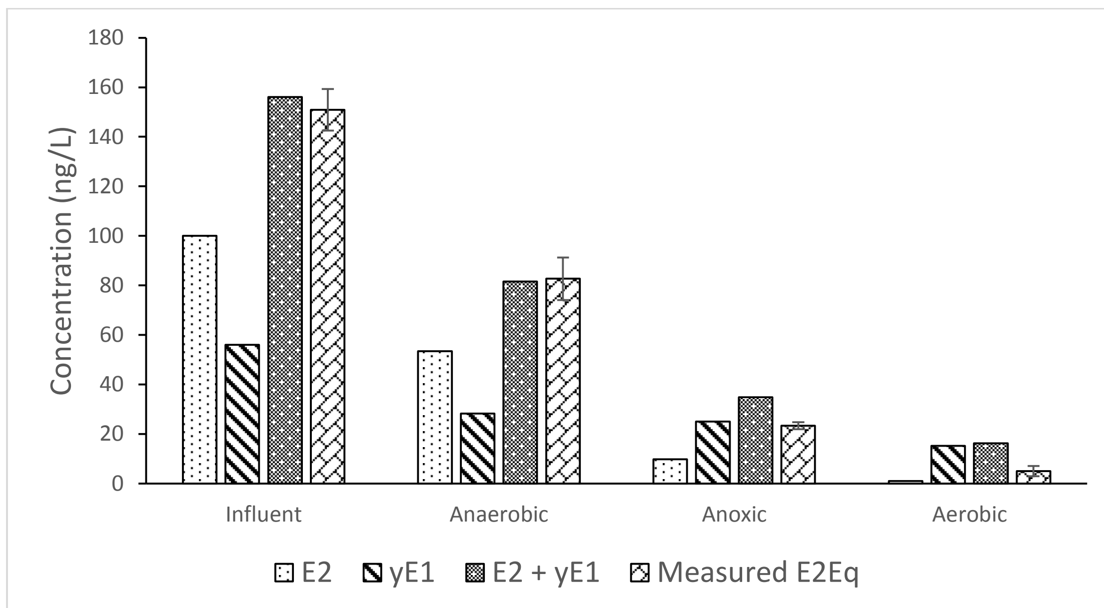

Figure 4-13. Measured E2-Eq and predicted E2, yE1 and E2 + yE1 in the influent and along the bioreactor in bench scale BNR process.

Conclusions

The estrogenicity in two BNR wastewater treatment processes was measured using a recombinant yeast screen assay. The removal and biodegradation of EDCs in a pilot BNR process fed with authentic wastewater was investigated and 96 ± 5% of the estrogenicity exerted by the EDCs in the wastewater was removed by the treatment process. The degradation efficiencies in the anaerobic, anoxic and aerobic zones of the bioreactor were calculated as 11± 9%, 18 ± 2% and 93 ± 10% respectively. In order to further understand the

performance of the BNR process in the removal of EDCs from wastewater, a bench scale BNR process was operated with synthetic wastewater dosed with E1 and E2. The removal of estrogenicity in the bench scale system (95 ± 5%) was comparable to the pilot BNR process and the degradation efficiencies were estimated to be 8± 0.8%, 38 ± 4% and 85 ± 22% in the anaerobic, anoxic and aerobic zones respectively. Both pilot and bench scale BNR processes showed that the aerobic zone was key to the biodegradation of EDCs. The biodegradation of EDCs in the bioreactors of the pilot and bench scale BNR processes followed the trend of aerobic >anoxic > anaerobic. The results from the pilot and bench scale BNR processes showed that BNR processes can effectively biotransform EDCs in wastewater.

Batch experiments were conducted to estimate the biotransformation kinetics of E1 and E2 in BNR activated sludge under different redox conditions. The behavior of E1 and E2 in each of the batch tests was consistent with the observed biodegradation efficiencies in the BNR process that was the source of the sludge for the batch tests. The biotransformation rate constants for the transformation of E2 to E1 were estimated as 71 ± 1.5, 31 ± 3.3 and 1 ± 0.9 $L.gCOD^{-1}d^{-1}$ for the aerobic, anoxic and anaerobic batch tests respectively while

the biotransformation rate constants for the degradation of E1 were estimated to be 7.3 ± 1.0, 3 ± 2.0, and 0.85 ± 0.6 $L.gCOD^{-1}d^{-1}$ for aerobic, anoxic and anaerobic batch tests respectively. The biotransformation of E1 was the slower step in the two step reaction occurring in the aerobic and anoxic batch tests while under anaerobic conditions there was a balance between the biotransformation of E2 to E1 and the biodegradation of E1.

A comparison between the simulated E2 + yE1 values and measured E2-Eq along the bioreactor of the bench scale BNR process revealed that the calibrated model parameters effectively predicted the steady state E2-Eq along the bioreactor of the BNR process, which was the source of the sludge for the batch tests. Hence, it was concluded that the calibrated model can be employed to predict the E2-Eq concentrations in BNR treatment processes.

Chapter 5 Biotransformation of Trimethoprim in Biological Nutrient Removal Treatment System – The Role of Active Microbial Groups

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5.1 Introduction

The removal of trace organic compounds (TrOCs) in wastewater treatment plants (WWTPs) has been an area of research for the past decade due to the potential risks associated with the undesirable effects of TrOCs on the ecosystem and human health (Heberer, 2002, Jones et al., 2004; Fent et al., 2006). Many of these compounds are either recalcitrant or partially removed during sewage treatment because the WWTPs were not designed to remove them (Ternes et al., 2003; Joss et al., 2004; Clara et al., 2005a). Thus, effluents from WWTPs have been identified as a primary source of TrOCs in the environment (Kolpin et al., 2002; Ternes et al., 2004; Lishman et al., 2006). Consequently, the optimization of WWTPs could play a critical role in attenuating the discharge of TrOCs into the environment (Onesios et al., 2009; Pomies et al., 2013).

WWTPs that are configured for biological nutrient removal (BNR) have been reported to have higher removal efficiencies of TrOCs along with macropollutants when compared to conventional activated sludge treatment systems (Clara et al., 2005a, Kimura et al., 2007, Ogunlaja et al., 2013). These previous studies have investigated the effects of WWTP design and operating conditions on the removal of TrOCs from wastewater. Some of the parameters that have been investigated included hydraulic retention time (HRT), solids retention time (SRT), biomass type, type and presence of growth substrate, temperature, pH, structure and physico-chemical properties of the TrOCs (Clara et al., 2005a; Cirja et al., 2007; Helbling et al., 2012). Despite all these studies, it is still unclear why BNR systems are more effective in removing TrOCs when compared to other conventional activated sludge systems.

Biotransformation or biodegradation has been identified as a major removal mechanism for attenuating the discharge of TrOCs. One of the plausible biotransformation pathways for TrOCs in activated sludge systems is the degradation of TrOCs by specialized microbial groups within the mixed liquor. A few studies have attempted to identify these organisms in order to elucidate the roles they play in the biotransformation of TrOCs (Shi et al., 2004; Batt et al.,

2006; Khunjar et al., 2011). However, there is no agreement in the literature on the type of active biomass (ammonia-oxidizing bacteria (AOB) and heterotrophic organisms) that is responsible for the biodegradation of TrOCs in activated sludge systems (Batt et al., 2006; Khunjar et al., 2011). Hence, further study is required to investigate the role of active biomass groups in the biotransformation of TrOCs.

Trimethoprim, one of the prevalent TrOCs found in wastewater, is a hydrophilic synthetic antibiotic usually prescribed for treatment of chest or urinary tract infections (Nolan et al., 1989; Batt et al., 2006). TMP is prevalent in the effluent of wastewater treatment plants (Kolpin et al., 2002) due to its poor biodegradability (Kummerer et al., 2000) and hydrophilic nature (Aga, 2008). Trimethoprim concentrations as high as 0.53 μg/L has been reported in the effluents of wastewater treatment plant in the United (Batt et al., 2005; Glassmeyer et al., 2005). There are limited reports on the removal and biodegradation of TMP in BNR treatment processes, hence the investigation of the removal and biotransformation of TMP in a BNR activated sludge systems was the focus of this paper.

Models of TrOC fate can be usefully employed to analyze test data for the purpose of obtaining an improved understanding of the processes involved in TrOC removal. Biotransformation modeling of TrOCs in activated sludge systems has usually been described by either first order (Byrns, 2001; Hashimoto and Murakami, 2009) or pseudo first order kinetics (Maurer et al., 2007; Wick et al., 2009). These kinetic expressions include one kinetic parameter, the dissolved TrOC concentration and the biomass concentration. In previous biodegradation studies, the biomass concentration has been approximated by the total or volatile suspended solid concentration (Joss et al., 2005; Gaulke et al., 2009). A weakness of this approach is that it does not consider the influence of growth substrates on the biotransformation kinetics of the TrOCs. TrOCs are present in minute concentrations in activated sludge systems and cannot sustain microbial growth. Usually, co-substrates like soluble organics or ammonia are necessary to serve as growth substrates (Pomies et al., 2013) for the active biomass that is presumably responsible for the biotransformation of TrOCs. Hence, the integration of active biomass fractions in biotransformation models may provide an improved description of the biotransformation of TrOCs in wastewater.

The objectives of this study were to 1) estimate the fractions of the active biomass- (polyphosphate accumulating organism (PAO), ordinary heterotrophic organism (OHO) and ammonia oxidizing bacteria (AOB)) in a BNR activated sludge process, 2) investigate the removal of trimethoprim (TMP) in the BNR process, 3) estimate the biotransformation rate constants of TMP with respect to PAO, OHO and AOB in aerobic BNR activated sludge and 4) assess the contributions of PAO, OHO and AOB towards the removal of TMP in aerobic BNR activated sludge. The results of this study will improve the understanding of the contributions of different biomass groups to TMP biotransformation, developing a comprehensive modeling tool that can facilitate the quantification of TMP removal in advanced wastewater treatment processes.

5.2 Approach

A BNR pilot plant was operated to investigate its performance with respect to the removal of conventional pollutants and TMP. The conventional pollutant data obtained from the BNR process operation were employed to estimate the active biomass fractions in the BNR activated sludge. The mixed liquor in the BNR pilot plant was employed as the source of the inoculum for batch tests that investigated the biotransformation of TMP in aerobic batch tests.

The estimated active biomass fractions and the batch experiment data were then employed to calibrate developed pseudo first order model equations with respect to each of the active biomass fractions. The TMP removal rates obtained from the batch tests were then used to determine the contributions of each of the active biomass fractions towards the overall TMP removal in the aerobic batch test. Figure 5-1 summarizes the approach taken for the study.

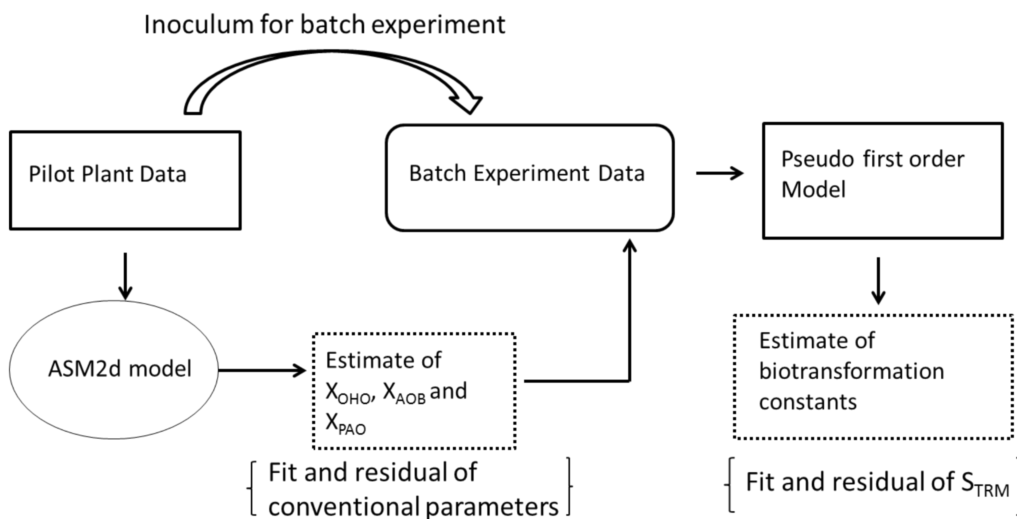

Figure 5-1. Overview of study approach.

5.2.1 BNR Pilot Plant Description

The pilot scale UCT-BNR process consisted of a 0.718 m^3 primary clarifier tank, 0.025 m^3 chemical dosing tank, 0.36 m^3 bioreactor made of stainless steel,

0.286 m^3 final clarifier for solid–liquid separation and associated pumps for fluid transfer. A schematic of the pilot BNR process is shown in Figure 5-2. A UCT-BNR process was selected due to its ability to maintain a PAO population at low influent carbon loading. The pilot BNR was operated on authentic municipal wastewater that was augmented with sodium bicarbonate (22 g/L) to provide alkalinity, di-potassium phosphate (11 g/L) as phosphorus source and sodium acetate (34.36 g/L) to enhance the proliferation of PAOs. The bioreactor was partitioned into six 0.06 m^3 cells to simulate pseudo plug flow. The first cell was operated as an anaerobic zone, the next two cells operated as anoxic zones and the last three cells operated as aerobic zones. The aerobic section of the bioreactor was mixed and aerated using coarse bubble aerators while the non-aerated sections were mixed using mechanical mixers. The solids residence time (SRT) was maintained by wasting mixed liquor from the last aerobic section of the bioreactor. The operating and design conditions of the pilot BNR process are summarized in Table 5-1. The temperature of the bioreactor was maintained at 18 ± 2^oC using a temperature controlled insulated water jacket that was wrapped around the bioreactor while the pH was maintained within the range of 7.2-8.5.

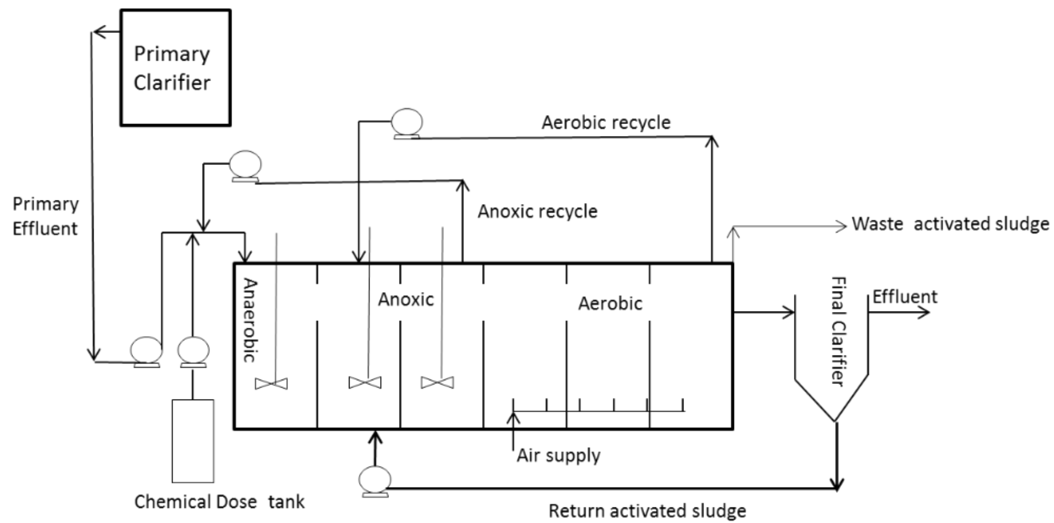

Figure 5-2. Flow schematic of pilot UCT-BNR activated sludge treatment system.

Table 5-1. Pilot BNR operating and design conditions

	Size/description	Unit
Flow rate	1.3	m^3/d
Primary clarifier	Area = 0.46	m^2
	Depth = 1.56	m
Bioreactor	Volume = 0.36	m^3
	Depth = 1.28	m
	DO(aerobic) = 4 - 5	g/m^3
	DO(anoxic) = 1-2.5	g/m^3
	DO(anaerobic) = 0-0.2	g/m^3
Final clarifier	Area = 0.204	m^2
	Depth = 1.4	m
Recycle rate	Aerobic = 2.6	m^3/d
	Anoxic = 1.3	m^3/d
SRT	20	d
Aerobic SRT	10	d
RAS flow rate	0.9	m^3/d
Waste rate	0.018	m^3/d
HRT	7	Hrs.

RAS-return activated sludge,SRT-solid residence time,HRT-hydraulic retension time

5.2.2 Pilot Monitoring and Sampling

The pilot plant was operated for over 12 months with regular monitoring of conventional and operational parameters for 6 months. Stable plant performance was ascertained by monitoring process operating conditions that included, chemical oxygen demand (COD), Total Kjeldahl Nitrogen (TKN), ammonia (NH_3), nitrite (NO_2), nitrate (NO_3), phosphate (PO_4), total suspended solid (TSS) and volatile suspended solid (VSS). Twenty four hour composite influent and effluent samples were collected thrice a week for three weeks (except for the first week with 2 sampling campaigns) for TMP analysis. Eight hour composite samples were also collected for TMP analysis from the anaerobic, anoxic and aerobic zones of the bioreactor. For TMP analysis, the samples were filtered using 1.5 μm glass micro fibre filters (GF/F Whatman), and extracted using solid phase extraction.

5.2.3 Estimation of Active Biomass Fractions

The active biomass fractions in the BNR bioreactor were estimated by simulating the biomass growth in the bioreactor based on growth substrate

uptake as presented in detail elsewhere (Barker and Dold, 1997). The simulation exercise was conducted using the activated sludge model no. 2d (ASM2d) within the wastewater treatment modeling software BioWin 3.0 from Envirosim (Hamilton, Ontario). Average influent wastewater parameters (Table 5-2) and the pilot's design and operating conditions in Table 5-1 were input into the simulator prior to simulating steady state performance of the pilot BNR process. The readily biodegradable fraction of the influent COD (f_{bs}) was adjusted to reflect the sodium acetate that was added to the influent stream. The readily biodegradable fraction of the influent COD has been reported to affect the biological phosphorus removal capability of BNR systems (Comeau et al., 1996; Barnard and Steichen, 2006). Simulated and observed soluble effluent COD and TKN were matched by adjusting the soluble unbiodegradable fractions of the influent COD (f_{su}) and influent TKN (f_{SNI}). Simulated and observed effluent solid concentrations were matched by adjusting the final clarifier solids removal efficiency. The simulated and observed bioreactor mixed liquor volatile suspended solid concentrations were matched by adjusting the particulate unbiodegradable fraction of influent COD (f_{up}). Aside from these calibrations, the

default values were used for the kinetic and stoichiometric parameters (Barker and Dold, 1997).

Table 5-2. Average influent parameter values for BNR pilot plant simulation

Parameter	Value	Unit
Flow rate	1.3 ± 0.4	m^3/d
COD	367 ± 48	gCOD/ m^3
TKN	25 ±11	gN/ m^3
TP	11 ± 6	gP/ m^3
NO_3-N	0.54 ± 0.2	gN/ m^3
NO_2-N	0.06 ± 0.03	gN/ m^3
ISS	16 ± 10	gISS/ m^3
pH	7.2 ± 0.2	
Ca	169 ± 9	g/ m^3
Mg	15 ± 4	g/ m^3
Alkalinity	268 ± 21	g/ m^3 as $CaCO_3$

5.2.4 Batch Experiments

A series of batch tests were performed to investigate the biotransformation of TMP in BNR activated sludge and to assess the contributions of active biomass, PAO, OHO and AOB to the biotransformation of TMP. The batch tests were designed using a combination of experimental conditions to facilitate the growth of different biomass groups in the different tests. Duplicate aerobic batch tests were conducted to asssess the role of PAO, OHO and AOB on the biotransformation of TMP (Aerobic-1) while a third test

(Aerobic-2) was conducted to assess the role of only PAO and OHO in the biotransformation of TMP. A summary of the experimental conditions under which the batch tests were performed is shown in Table 5-3.

The innocula for the batch tests were collected from the aerobic section of the pilot BNR process. The batch reactors (Figure 5-3) had a working volume of 10 L that was filled with 6 L of activated diluted with 4 L of settled raw wastwater to achieve a mixed liquor volatile suspended solid (MLVSS) concentration of approximately 2050 mg/L. An aqueous stock solution of ammonium chloride, di-potassium phosphate and sodium acetate was dosed into the reactors at the same time as the TMP that was dissolved in methanol. The initial TMP concentration in the batch reactors ranged from 0.2 to 1 μg/L. The initial concentrations of the chemicals that were added to facilitate biomass growth in the batch tests are shown in Table 5-3. The mixed liquor in the reactors was mechanically stirred and aerated to maintain dissolved oxygen (DO) concentrations of approximately 2 – 5 g/m^3. The temperature of the reactors were maintained at 18 ± 2°C and the pH was maintained in the ranged of 7.5 - 8.4.

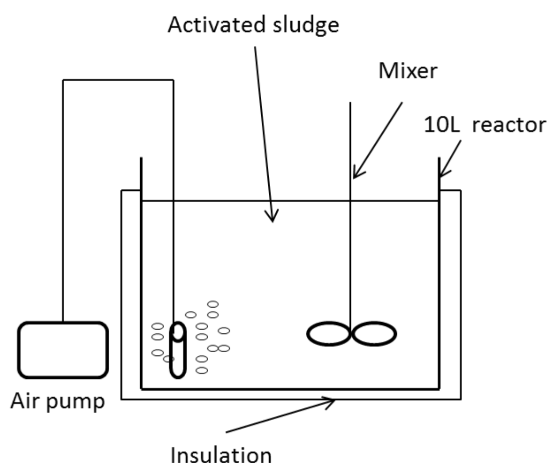

Figure 5-3. Schematic diagram of batch test.

Table 5-3. Initial concentrations of chemicals in batch reactors

Chemical added	Molecular formula		AO-1A	AO-1B	AO-2	Unit
Sodium acetate	$C_2H_3NaO_2$	COD	600	503	535	g COD/m^3
Di-potassium phosphate	K_2HPO_4	PO_4-P	162	193	188	g P/m^3
Ammonium Chloride	NH_4Cl	NH_3-N	114	98	n/a	g N/m^3
Allylthiourea	$C_4H_8N_2S$	ATU	N/A	N/A	13	g/m^3
Target Active Biomass			OHO,PAO,AOB		OHO, PAO	

n/a-not added to reactor; AO-Aerobic, Allylthiourea - Nitrification inhibitor

5.2.5 Batch Experiment Monitoring and Sampling

The time dependent changes in the concentration of TMP and conventional parameters in the effluent samples were characterized to assess the

reactors' performance. From each batch reactor, 500 mL mixed liquor samples were collected in prewashed amber bottles, just before and 15 minutes after the addition of TMP to the reactors and subsequently every four hours for three days. Each sample was centrifuged at 4000 rpm for 5 minutes and the centrate was filtered using 1.5 μm glass fiber filter paper (Whatman 934/AH). A volume of 60 ml of the filtrate was employed for analysis of COD, nitrogen species (NH_4-N, NO_3-N, and NO_2-N), soluble reactive phosphorus (PO_4-P) and the unfiltered samples were analyzed for TSS and VSS concentrations according to *Standard Methods* (Eaton, 2005). A volume of 150 mL of the remaining filtered sample was placed in a prewashed amber bottle and pH adjusted to pH 8 using 0.1M sodium hydroxide. The samples were then spiked with 0.15 mL of a 0.5 ppm isotopically labelled trimethoprim, dissolved in methanol, before solid phase extraction (SPE). All parameters were analyzed in duplicate. The analysis of the conventional parameters and SPE extraction were performed immediately after filtering.

5.2.6 Biotransformation Model

A pseudo first order kinetic expression is commonly used to describe the biodegradation of TrOCs in activated sludge systems (Cowan et al., 1993;

Monteith et al., 1995; Schwarzenbach et al., 2003). Therefore, pseudo first order kinetic expressions were used to estimate the biotransformation rate constants of TMP in the batch experiments. Two different models were employed to investigate the use of MLVSS concentration and estimated active biomass concentration in determining the biotransformation rate constant of TMP in BNR activated sludge. Both models assumed that the MLVSS and the active biomass concentrations were constant throughout the duration of the batch tests. This assumption is typically valid for short duration batch tests. Model 1 did not differentiate between biomass species and hence the rate of biotransformation of TMP was assumed to be a function of the MLVSS concentration in the reactors (equation 5-1)

$$r_i = -k_i X_{mlvss} S_i \qquad 5\text{-}1$$

Where,

r_i = rate of biotransformation of compound i [$\mu g\ L^{-1} hr^{-1}$]

k_i = biotransformation rate constant for compound i [$L\ gCOD^{-1} hr^{-1}$]

X_{mlvss}= mixed liquor volatile suspended solid concentration [$gCOD\ L^{-1}$]

S_i=soluble concentration of compound i [$\mu g.L^{-1}$]

By contrast, model 2 incorporated three biotransformation rates where the rate of biotransformation of TMP was assumed to be dependent on each of the active biomass concentrations (equation 5-2).

$$r_i = -\sum_{j=1}^{n=3} k_{i,j} X_j S_i \qquad 5\text{-}2$$

Where,

$k_{i,j}$ = biotransformation constant for compound i [L $gCOD^{-1}hr^{-1}$] with respect to biomass j

X_j = active biomass concentration [gCOD L^{-1}]

S_i=soluble concentration of compound i [μg.L^{-1}]

$J = PAO, OHO, AOB$

5.2.7 Chemical Analysis

The filtered samples collected from the pilot BNR and the batch experiments were extracted for TMP analysis by solid phase extraction (SPE) (Li et al., 2010) using Oasis HLB 60 mg cartridge from Waters. Prior to extraction, 0.1 mL of a mixture of an internal standard (500 ng/mL) containing the stable isotope labeled surrogates of the analyte was added to the sample. The eluates were evaporated to dryness under a gentle stream of nitrogen and reconstituted in 0.4 ml of methanol. An internal standard (Trimethoprim- $^{13}C_6$) that was prepared by

Prof. Chris Metcalfe's lab in Trent Univerity, was added to the reconstituted samples prior to instrumental analysis to improve the quantitative analysis. The extracted samples were shipped on ice to be quantified at Trent University. The TMP concentrations were measured using high performance liquid chromatography interfaced to a tandem mass spectrometer with an electrospray ionization source using an API 3000 instrument procured from AB SCIEX (Concord Ontario Canada). A detailed description of the sample preparation and LC-MS/MS procedure that was employed to quantify the TMP was described elsewhere (Hoque et al., 2013). The limit of quantification and limit of detection were calculated to be between 0.1 to 2 ng/L.

5.2.8 Statistical Analysis

The conventional and chemical data were analyzed for outliers using the Grubb's test. The significant outliers ($p > 0.05$) were removed before conducting descriptive statistics on the data. The averaged conventional data were introduced into BioWin for the simulation exercise. The fit between the simulated and measured TMP concentrations in the batch experiments was assessed on the basis of r^2 values and the Nash-Sutcliffe Efficiency (NSE) metric (Nash and Sutcliffe, 1970) (equation 5-3). NSE values ranges from $-\infty$ to 1 but are

usually greater than 0. A negative value of NSE indicates that the mean of the measured value is a better predictor than the model. Typically, NSE values greater than 0.7 are considered to indicate a strong predictive characteristic of the model (Moriasi et al., 2007).

$$NSE = \left(1 - \frac{\sigma_e^2}{\sigma_o^2}\right) \qquad 5\text{-}3$$

Where, σ_e = variance of residuals

σ_e = variance of observed values

5.3 Results and Discussions

The pilot BNR process was monitored with respect to the removal of conventional wastewater pollutants. This data was used to ascertain the performance of the BNR treatment plant in terms of conventional contaminants removal and to provide an indication of the metabolisms of the different microbial groups that were active in the bioreactor. The ASM2d model was used to estimate the fractions of active biomass in the bioreactor on the basis of the conventional data. A series of batch experiments was conducted to investigate the biotransformation of TMP in BNR activated sludge. The biotransformation constants were estimated using pseudo first order kinetic expressions. The

estimated biotransformation constants were then used to calculate the contributions of the active biomass fractions towards the biotransformation of TMP.

5.3.1 Conventional Contaminants Removal

The measured responses of the conventional contaminants as presented in Table 5-4 shows that the effluent from the pilot plant was relatively consistent throughout the sampling campaign. This showed the efficient performance of the pilot plant irrespective of the variability in the influent contaminants. Carbonaceous (cBOD5) was consistently removed in the pilot with the effluent concentration ranging from 2 to 9 mg/L. This result suggests a good removal of biodegradable organic matter. In addition, the pilot was expected to have high removal efficiencies for TKN, TAN, NO2-N and TP. These patterns were consistently observed throughout the sampling period as shown in Table 5-4. This suggests that the pilot plant was effectively nitrifying, denitrifying and biologically removing phosphorus. Hence, it was concluded that the BNR pilot plant was achieving levels of treatment that are typical of operations at technical scale (Jeyanayagam, 2005; WEF, 2005).

5.3.2 Pilot Plant Calibration and Active Biomass Estimation

The steady state effluent concentrations of the conventional pollutants and the MLVSS concentration in the bioreactor were simulated using the BioWin modeling software. To simulate the measured effluent and MLVSS concentrations of the pilot BNR, the influent wastewater fractions were adjusted to match the measured data. During the plant operation, the pilot plant influent was augmented with sodium acetate to ensure the proliferation of PAOs for biological phosphorus removal. Hence, the readily biodegradable fraction of the total influent COD (0.55 g COD/ g of total COD) was adjusted in the simulator to reflect the sodium acetate that was added to the influent stream. The simulated and observed soluble effluent COD and TKN were then matched by adjusting the soluble unbiodegradable fractions of the influent COD (0.04 gCOD/ g of total COD) and influent TKN (0.035 gN/g TKN) in the simulator. The solids capture rate in the final clarifier was adjusted to match the observed effluent suspended solid concentration. The simulated and observed mixed liquor volatile suspended solid (MLVSS) concentrations in the aerobic bioreactor were matched by adjusting the particulate unbiodegradable fraction of influent COD (0.043 g COD/ g of total COD). The measured and calibrated MLVSS concentrations in

the aerobic section of the bioreactor were 3600 ± 474 g COD/m^3 and 3600 g COD/m^3 respectively. The simulated concentrations for the active biomass groups- AOB, OHO and PAO in the aerobic section of the pilot BNR bioreactor were 40, 780 and 2710 g COD/m^3. The relative error between the measured and steady state simulated values were below 20% which was deemed to signify good agreement between the measured and simulated data (Table 5-4).

Table 5-4. Measured and predicted effluent concentrations (g/m^3) from the pilot BNR process

Response	Primary Effluent (Influent)		Measured Effluent		Calibrated Effluent	Removal efficiency
	Mean	S.dev	Mean	S.dev		%
COD	367	48	33	12	33	91
TKN	25	12	2.3	0.2	2.3	91
NH_4-N	19	8	0.04	0.03	0.04	100
NO_3-N	0.54	0.2	3	2	3	n/a
NO_2-N	0.06	0.03	0.03	0.01	0.03	50
TP	11	7	3	1.7	3	73
TSS	32	2.5	9	1.6	10	72

n/a-not applicable

5.3.3 TMP Removal in BNR Pilot Plant

The concentrations of TMP in the pilot were measured to investigate the performance of the pilot in terms of TMP removal. The influent TMP concentration (n=8) was 78 ± 39 ng/L while the effluent TMP concentration (n=8)

was 28 ± 8 ng/L, hence the overall TMP removal efficiency was calculated as 59 ± 14%. The TMP removal efficiency obtained in this study was similar to a prior study that investigated the removal of TMP in an MBR system (57 ±10 %) (Radjenovic et al., 2009) but on average, 3-6 times higher than that reported for conventional activated sludge systems (11 ± 31%) (Gobel et al., 2007; Radjenovic et al., 2009; Ogunlaja et al., 2013). Thus, it was concluded that the performance of the pilot BNR process in terms of TMP removal was comparable to the performance of previously investigated advanced wastewater treatment processes.

The bioreactor of the BNR process was divided into three different zones operated at different redox conditions. Therefore, it was expected that the different zones would contribute at different proportions towards the biotransformation of TMP. This expectation was based on the fact that the amount of energy that is usually captured by the microorganisms in aerobic conditions is usually higher than the energy captured in anoxic and anaerobic conditions (Tchobanoglous et al., 2003). This bioenergy could be instrumental to the biotransformation of TMP in the BNR bioreactor. The TMP concentrations in the influent, effluent and the interstage of the BNR bioreactor are presented in

Figure 5-4. It is apparent from Figure 5-4 that the TMP concentration decreased along the interstage of the bioreactor, which suggested that each of the zones contributed to the overall TMP removal.

However, during the operation of the pilot BNR process, mixed liquors were recycled from the aerobic zone to the anoxic zone and from the anoxic zone to the anaerobic zone. The returned activated sludge was also recycled from the final clarifier to the anoxic zone of the bioreactor. These recycle flows can have dilution effects on the pilot plant influent at different points of the treatment system. Therefore, a set of mass balances were employed to characterize the fate of TMP in the pilot BNR process (Figure 5-5).

In the mass balances, the biotransformation efficiency of TMP in each zone was calculated as the difference between the mass flow entering and leaving the zone, divided by the mass flow entering the zone. The difference between the mass inflow and outflow in the aqueous phase across each zone of the bioreactor was assumed to be due to microbial biotransformation within the zone of the bioreactor. This assumption was based on the fact that previous studies had shown negligible TMP removal by sorption in activated sludge systems (Eichhorn et al., 2005; Perez et al., 2005; Batt et al., 2007; Aga, 2008). Therefore, the

TMP biotransformation efficiencies in the anaerobic, anoxic and aerobic sections of the BNR bioreactor were calculated to be 13 ± 12%, 17 ± 10 % and 24 ± 4% respectively. These results show that the biotransformation efficiency in the bioreactor of the BNR pilot increased from the anaerobic to the anoxic and aerobic zone. Hence, it was concluded that TMP was biotransformed in all the redox zones of the BNR bioreactor, albeit at different percentages.

The TMP removals by each of the stages of the BNR process were estimated to compare the contributions of each stage towards the overall TMP removal in the BNR process. The contributions of the stages were calculated to be 22 ± 28%, 29 ± 24% and 41 ±10% for anaerobic, anoxic, aerobic zones respectively. As expected, the results shows that the aerobic zone of the BNR process contributed the highest removal, followed by the anoxic zone and lastly the anaerobic zone. Hence it was concluded that the TMP removal in the BNR process was related to the oxidation-reduction condition in each of the zones.

The energy that is captured by the microorganisms in aerobic conditions is usually higher than the energy captured in anoxic conditions and even higher than the energy captured in anaerobic conditions (Tchobanoglous et al, 2003). This difference in bioenergy could explain why the aerobic zone had the highest

biodegradation efficiency among the three stages for the pilot BNR process. In addition, aerobic heterotrophic organisms have the capability to produce oxygenase enzymes that catalyze the direct incorporation of molecular oxygen into organic compounds. This oxygenase reaction weakens the ring structure in the TMP compounds that can make it accessible for subsequent oxidation steps and more water soluble (Rittman and McCarty, 2001). Thus, the high biotransformation efficiency of TMP in the aerobic zone could have been due to the combination of the aerobic environment and the bacteria activity on the compound. Hence, the aerobic zones as compared to the anoxic and aerobic zones has the potential for significant biotransformation and removal of TMP in a BNR process. However, further study is recommended to investigate methods of improving the removal of TMP in anoxic and anaerobic environments.

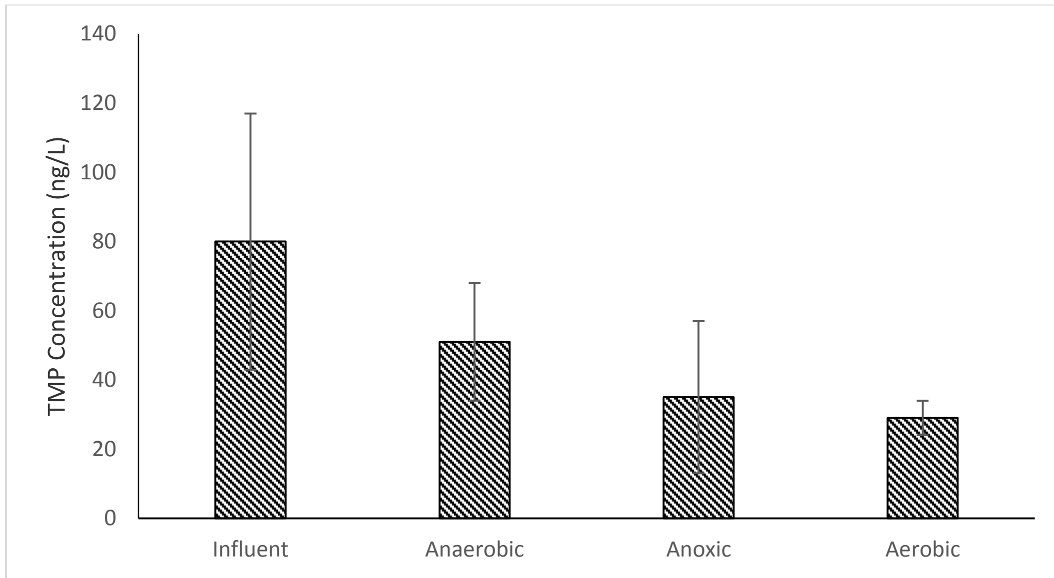

Figure 5-4. Trimethoprim concentrations along BNR pilot plant. (deviation bar represents standard deviation of measurements (n=8)).

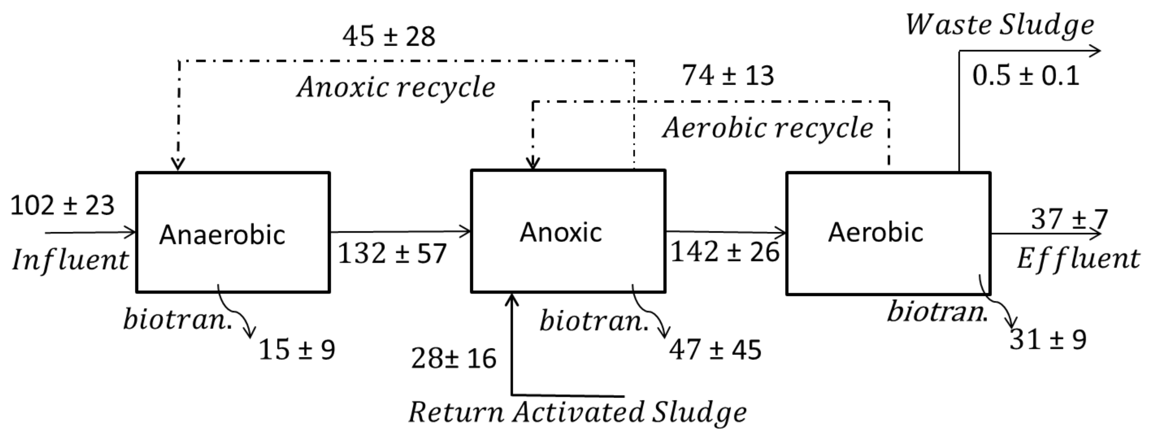

Figure 5-5. Mass balances of Trimethoprim in BNR pilot plant (TMP mass flow rate in μg/d).

5.3.4 Performance of Batch Reactors with respect to Conventional Pollutants

Batch tests were conducted to obtain an improved understanding of the kinetics associated with the removals that were observed in the BNR pilot plant. The performances of the batch tests were initially ascertained by investigating the behavior of the conventional pollutants through the duration of the experiments. Figures 5-6 and 5-7 show the profiles of the conventional pollutants in the batch tests conducted under aerobic conditions without nitrification inhibition (Aerobic-1) and with nitrification inhibition (Aerobic-2) respectively. It is apparent from Figure 5-6 that the AOB activity were active in Aerobic-1 batch test as evidenced by the decrease in ammonia that occurred simultaneously with an increase in nitrate. By contrast, there was no change in ammonia or nitrate concentration throughout the duration of the experiment in Aerobic-2 batch reactor, which indicated the inhibition of AOB activity by the added allylthiourea (Figure 5-7).

Under aerobic conditions, PAOs oxidize intracellular poly-β-hydroxybutyrates (PHBs) to obtain energy for growth and maintenance requirements. The intracellular PHBs support PAO growth and soluble phosphate (PO_4) uptake from the bulk liquid in the reactor. The accumulated

polyphosphates provide the energy required for soluble VFA uptake in the anaerobic zone. Figures 5-6 and 5-7 show a decline in the PO_4 concentrations that ceased after approximately 20-24 hours. The reduction in the PO_4 concentration in the reactors signified that the PAOs were actively taking up PO_4 to form intracellular polyphosphates. The cessation of PO_4 uptake may have been due to the depletion of the intracellular PHBs of the PAO. In the absence of PHBs, the PAOs have no source of energy to carry out their metabolic activities under aerobic conditions. Hence, it was concluded that the PAOs in aerobic 1 and 2 batch tests were active during the 20-24 hours of the tests and became inactive for the rest of the duration of the batch tests.

Under aerobic conditions, ordinary heterotrophic organisms (OHO) oxidize COD for cellular growth and maintenance. Figures 5-6 and 5-7 showed a decline in the soluble COD concentrations, which effectively ceased after approximately 20-24 hours. After the soluble COD concentration in aerobic 1 and 2 reached a minimum, the concentrations tended to gradually increase till the end of the reaction in both aerobic 1 and 2 batch tests. During biomass growth, decay and lysis also occur concurrently. The cell decay and lysis released soluble substrates and particulate substrates into the bulk liquid in the reactor. The

particulate substrates can undergo hydrolysis to produce more soluble substrate for cellular consumption (Grady et al., 1999). These results suggests that the rate at which OHOs oxidized the soluble COD for growth was initially faster than cell death and lysis at the beginning of the batch tests but after approximately 20-24 hours of reaction, the rate of COD consumption became slower than the rate of release of soluble COD into the reactor by biomass death and lysis. This was demonstrated by the slightly increasing COD concentration in both batch tests. Hence, it was concluded that after 20-24 hours of reaction in the batch tests, the biomass in the reactors entered a stationary phase as depicted by the constant soluble COD concentrations.

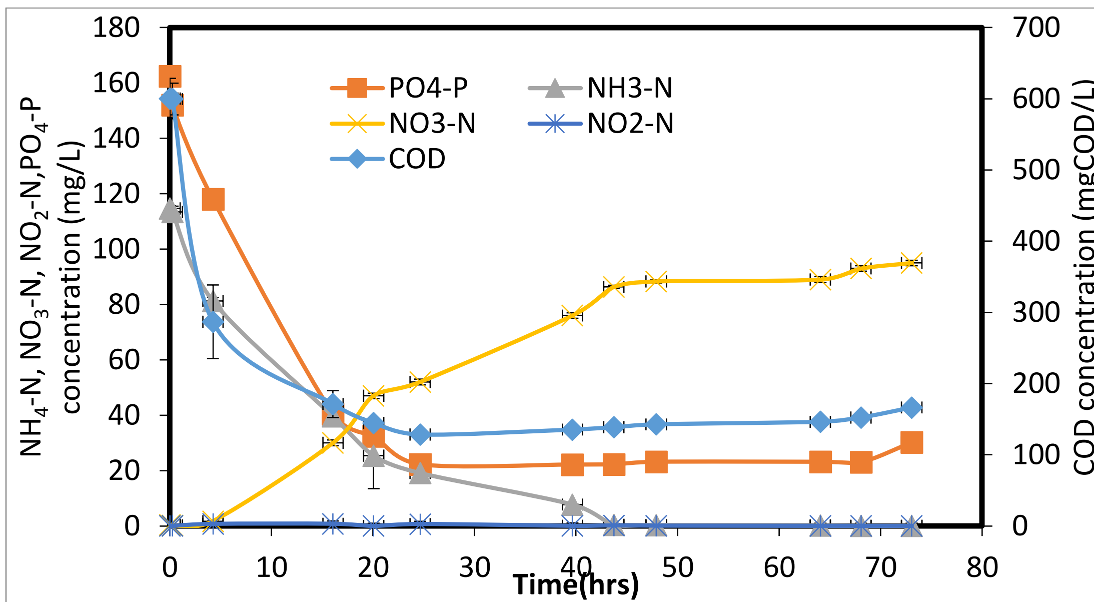

Figure 5-6. Profile of conventional pollutant concentrations in batch experiment without AOB inhibition (Aerobic-1).

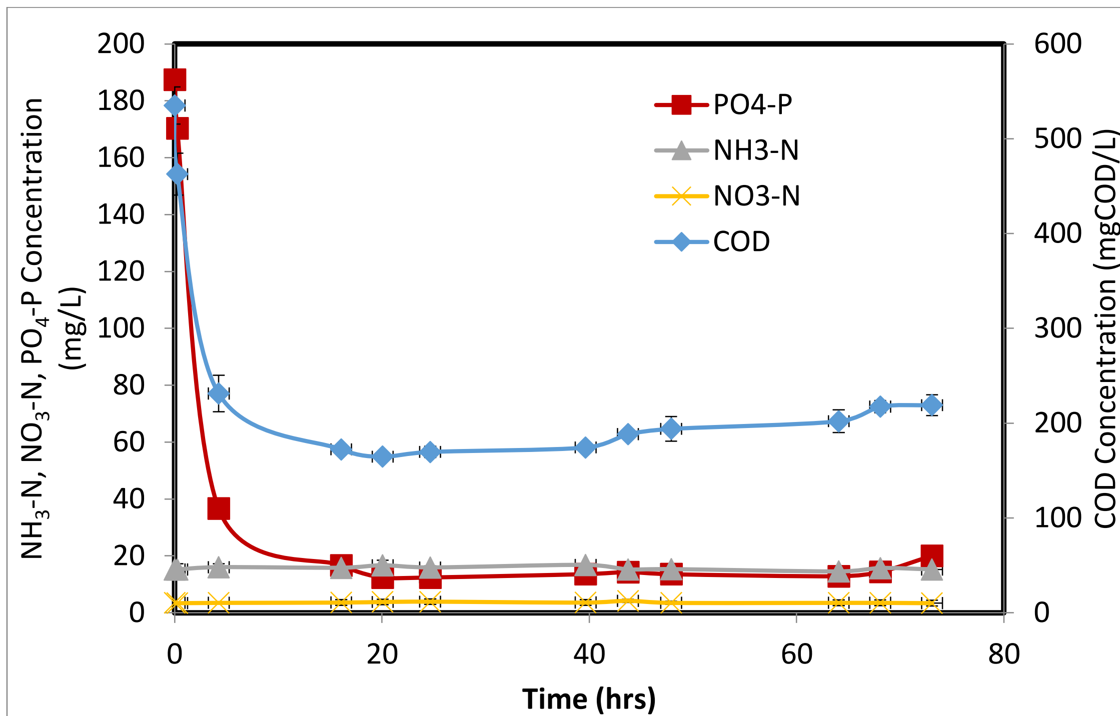

Figure 5-7. Profile of conventional pollutant concentrations in batch experiment with AOB inhibition (Aerobic-2).

5.3.5 Performance of Batch Reactors with respect to Trimethoprim Removal

The TMP concentrations in the water phase were plotted against time (Figure 5-8) so as to investigate the effect of AOB inhibition on TMP removal in the batch reactors. Figure 5-8 shows that the TMP concentrations in the batch experiments with and without AOB inhibition followed similar decreasing trends. It is apparent from the plot that the biotransformation of TMP was faster in aerobic-1 batch tests than in aerobic-2. In Figure 5-8, It can also be observed

that the result of the duplicate aerobic-1 batch tests were consistent with each other, thereby implying good reproducibility of the results.

The previous analysis of the conventional parameters had shown that PAO, OHO and AOB were active in aerobic-1 while PAO and OHO were active in aerobic-2 batch tests. The difference in trends between aerobic-1 and aerobic-2 clearly suggests the impact of the AOB activity on the biotransformation of TMP. The slower decline in TMP remaining in the aerobic reactor with nitrification inhibition as compared to the batch reactor without nitrification inhibition suggest that AOB plays a role in the biotransformation of TMP. Previous studies have suggested the capability of AMO that is produced by AOBs to oxidize a broad range of organic compounds along with the oxidation of ammonia (Batt et al., 2006; Khunjar et al., 2011). The results obtained from the current batch tests suggest that AOBs play a role in the biotransformation of TMP in the BNR activated sludge. Hence, the incorporation of the concentrations of AOB in a biotransformation model could provide a better description of the behavior of micropollutants in activated sludge systems.

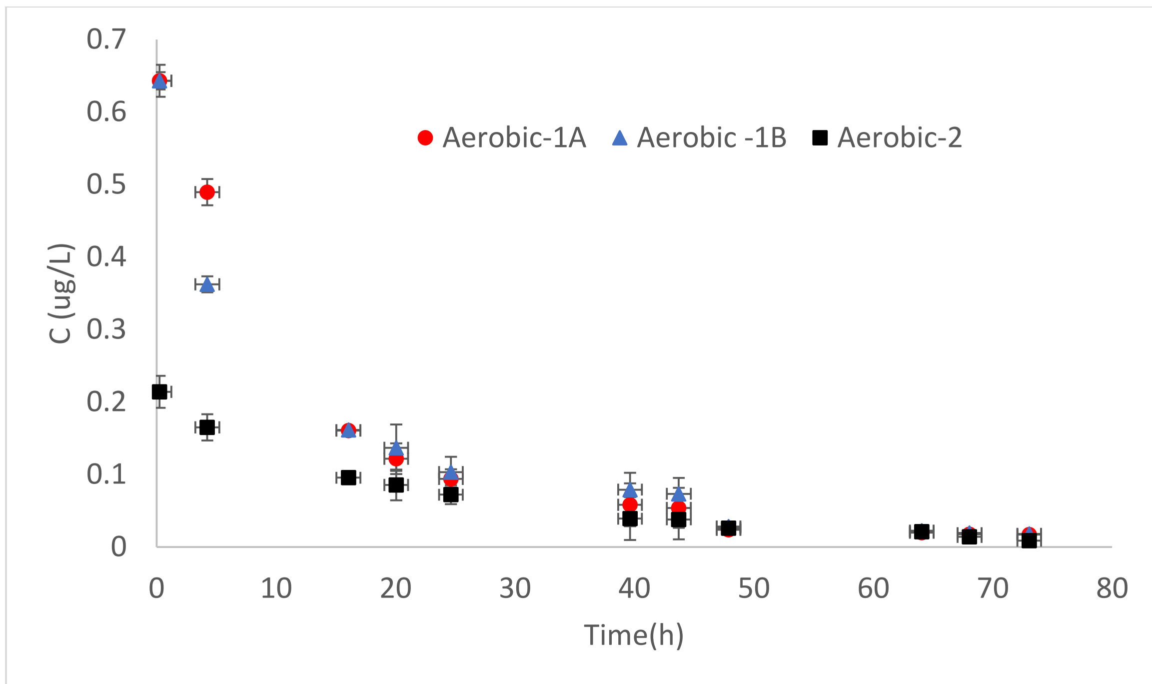

Figure 5-8. TMP Concentrations in the batch reactors: Aerobic 1A&B- without AOB inhibition, Aerobic 2- with AOB inhibition.

5.3.6 Estimation of Biotransformation Rate Constants

The biotransformation of TMP in the batch tests was examined using two pseudo first order kinetic models. In the application of the models, it was assumed that the mixed liquor volatile suspended solids concentration was constant in model 1 and that the estimated active biomass concentrations were constant in model 2. For short duration batch tests, it was considered reasonable to assume that the biomass concentration is constant. Hence, a non-linear integral least square method (Chapra, 1997) that minimized the sum of squares of the

residuals between the predicted and measured TMP data was employed to simultaneously fit the models to the TMP responses from the batch tests with and without nitrification inhibition.

Figures 5-9 and 5-10 show plots of the measured and predicted concentrations of TMP in the aerobic-1 and 2 batch tests respectively. The goodness of fit of the two models were estimated using r^2 and NSE. The results (Table 5-5) show that the r^2 and NSE values for both models 1 and 2 were larger than 0.98 for the tests, indicating that both models could represent the behavior of TMP in the batch tests. However, the higher residuals between the measured TMP concentrations and model 1 predicted TMP concentrations (Figure 5-10) as compared to model 2 predicted TMP concentrations indicated that model 2 was a better fit than model 1 for the aerobic-2 batch test. Hence, it was concluded that model 2 was able to better describe the behavior of TMP in the batch tests. An additional feature of this model is the ability to estimate the contribution of each active biomass group to the overall TMP removal in the BNR activated sludge.

The estimated biotransformation rate constants for TMP in model 1 and model 2 are summarized in Table 5-5. From Table 5-5, it can be seen that the model 2 biotransformation rate constants were inversely proportional to the

active biomass fractions in the BNR mixed liquor employed for the batch test. For example, the AOB had the smallest proportion of the biomass concentration but the highest rate constant while PAO had the highest proportion of the biomass concentration but the lowest rate constant. The ratio of the estimated biotransformation rate constants with respect to PAO, OHO and AOB was 1: 2: 48. The estimated biotransformation rate constant from model 1 was consistent with a previous study that investigated the biotransformation of TMP under aerobic condition using sludge from A^2O-MBR process, two-phase fate model and MLSS as the biomass concentration (Xue et al., 2010). However, the current study was the first to estimate the biotransformation rate constant for TMP with respect to active biomass groups in a BNR activated sludge, hence there was no previous study for comparison.

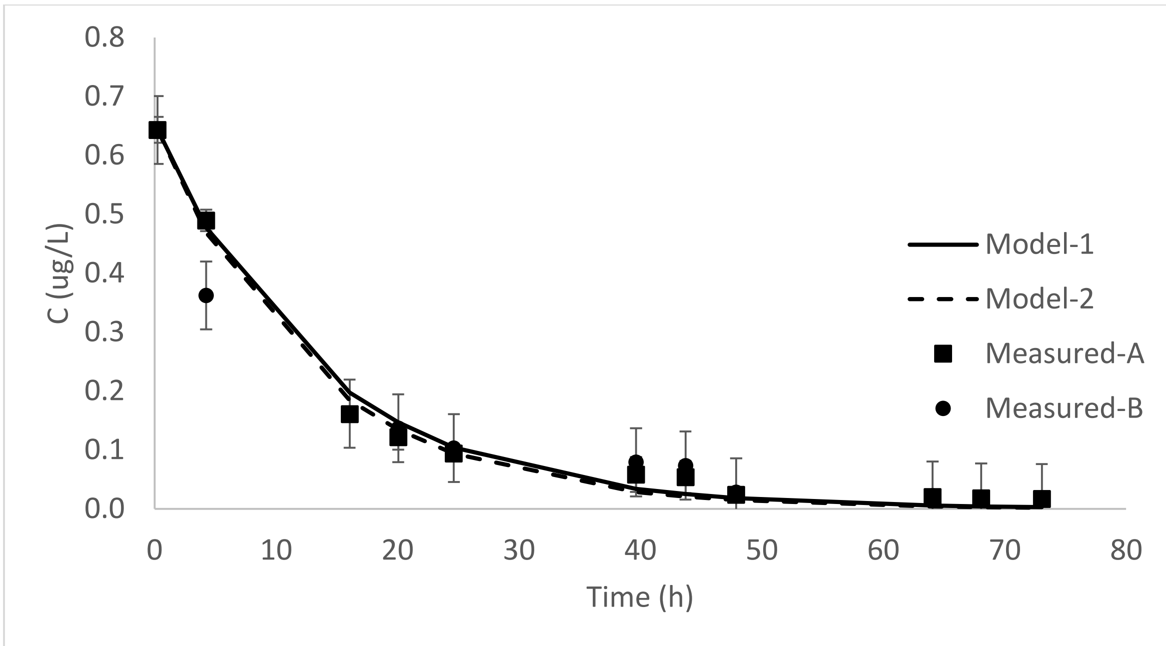

Figure 5-9. Simulated and measured Trimethoprim concentrations in aerobic batch reactors without AOB inhibition.

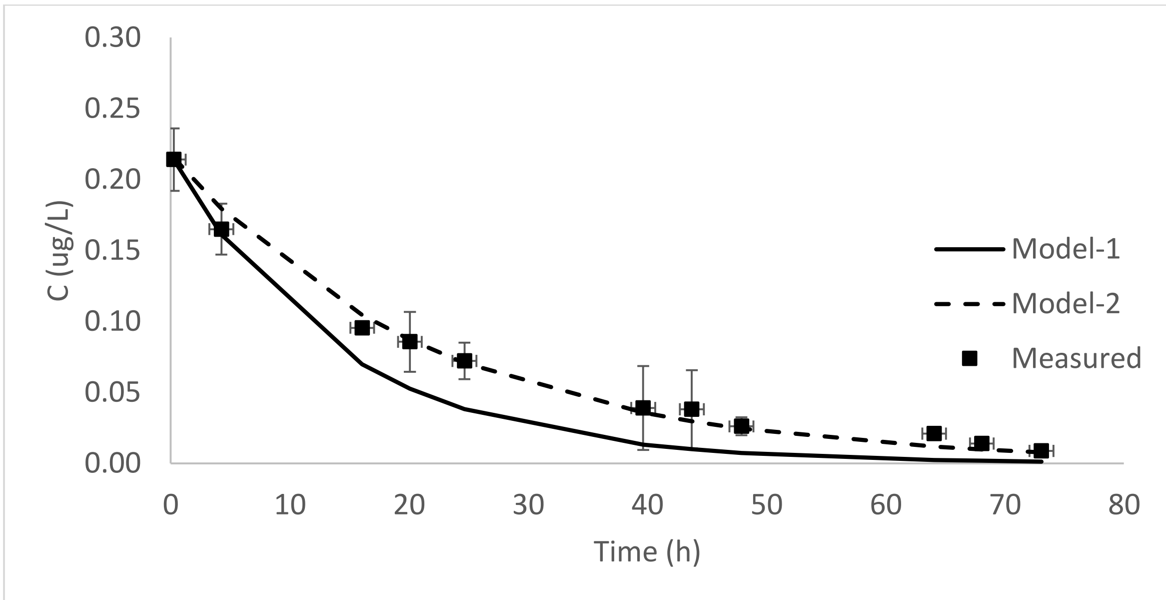

Figure 5-10. Simulated and measured Trimethoprim concentrations in aerobic batch reactors with AOB inhibition.

Table 5-5. Estimated biotransformation rate constant for TMP with respect to PAO, OHO and AOB

	Model-1		Model-2			
	Aerobic-1	Aerobic-2	Aerobic-1	Aerobic-2		
r^2	0.986	0.924	0.986	0.987		
NSE	0.97	0.966	0.971	0.985		
			PAO	OHO	AOB	
k_b	0.58 ± 0.06		0.32 ± 0.06	0.58 ± 0.06	13.7 ± 0.06	L/gCOD/d

5.3.7 Contribution of Active Biomass Groups towards Trimethoprim Biotransformation

To obtain an improved understanding of TMP removal rates by each active biomass, the estimated biotransformation rate constants were employed in model 2 to predict the species-specific rates. Figure 5-11 presents the contributions of PAO, OHO and AOB towards the overall TMP removal rate in the test reactors. It is apparent from Figure 5-11 that the observed TMP removal rates in aerobic-1 were greater than that of aerobic-2. In aerobic-1, the AOB and PAO contributed similarly while OHO contributed the least to the overall TMP removal rate for the first 40 hours of the batch test. A similar trend was also observed in the aerobic 2 batch test where PAO contributed a higher proportion than OHO for the first 40 hours of the biotransformation reaction. Therefore, it was concluded that each of the biomass groups in the BNR mixed liquor worked

collectively to achieve the observed TMP removal but contributed at different proportions in the order AOB = PAO > OHO for aerobic-1 and PAO > OHO for aerobic-2.

In all, these results suggests that both AOB and PAO are instrumental to the significant removal of TMP in the BNR activated sludge. This means that both nitrification and biological phosphorus removal may be beneficial for the biotransformation of TMP in BNR activated sludge. This could explain why the BNR performs better than other conventional activated sludge (CAS) treatment systems for TMP removal. It is recommended that the approach employed in this study for TMP should be extended to other prevalent micropollutants in wastewater.

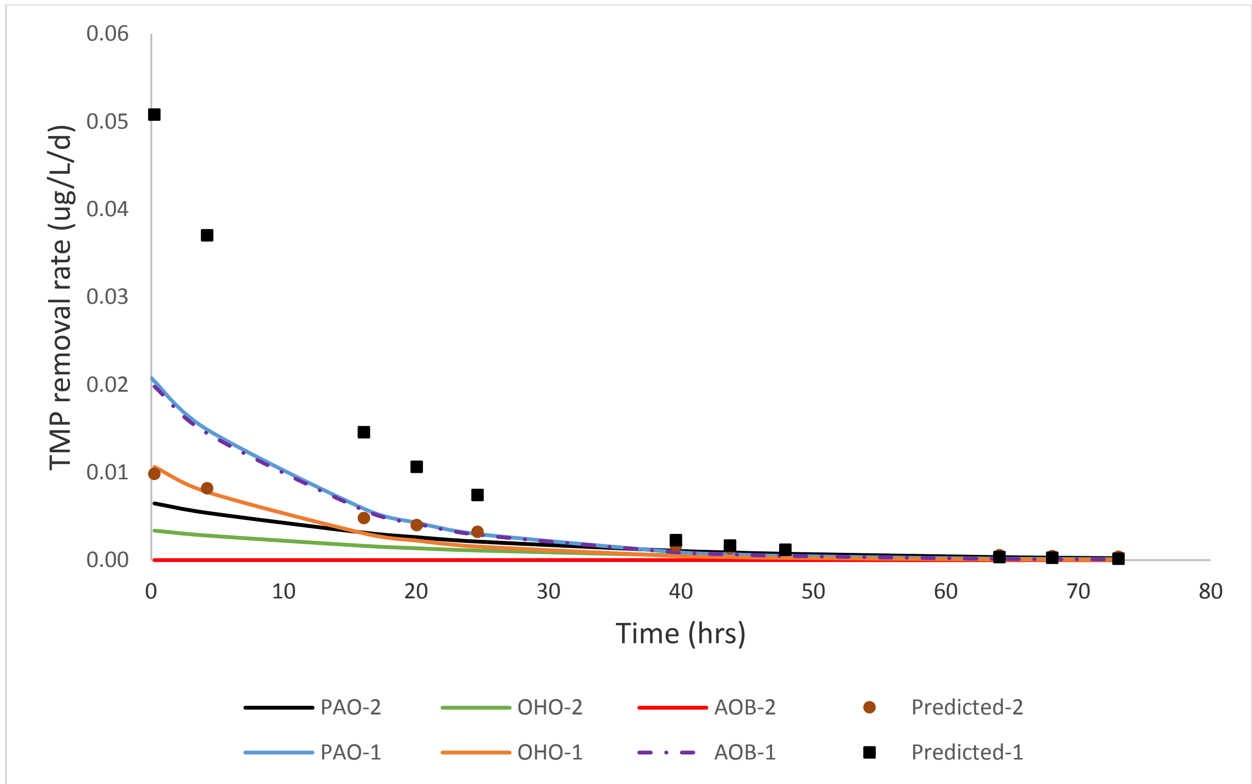

Figure 5-11. Contribution of PAO, OHO, and AOB to the overall TMP removal rate.

Conclusion

A combination of pilot scale BNR activated sludge system, batch experiments and a modeling exercise were employed to investigate the removal and biodegradation kinetics of TMP in a BNR activated sludge. The results showed that TMP can be effectively removed in a BNR activated sludge system with each of the redox zones contributing different proportions. The TMP removal efficiency was calculated as 59 ± 14% while the biotransformation

efficiencies in the anaerobic, anoxic and aerobic sections of the BNR bioreactor were calculated to be 13 ± 12%, 17 ± 10 % and 24 ± 4% respectively. This result indicated that TMP removal in the BNR process was related to the oxidation-reduction condition in each of the zones. A comparison of TMP removal rates in aerobic batch reactors with and without AOB inhibition showed a faster removal rate in the reactor without AOB inhibition, suggesting that AOB plays a role in TMP removal in BNR activated sludge. PAO, OHO and AOB were ascertained to be actively undergoing metabolic activities in the batch tests as monitored by the disappearance of the conventional pollutants associated with their growth and cellular maintenance.

The biotransformation of TMP in BNR activated sludge under aerobic condition obeyed a modified pseudo first order model that incorporated the fractions of the active biomass groups. Biotransformation rate constants for TMP with respect to PAO, OHO and AOB followed a pattern of $k_{AOB} > k_{OHO} > k_{PAO}$. This model showed that PAOs, OHOs and AOBs contributed different proportions towards the biotransformation of TMP in BNR activated sludge with the trend AOBs = PAOs > OHOs. Hence, the developed modeling tool have been shown to provide enhanced quantification of TMP removal in BNR treatment

processes. The results of this study have improved the understanding of the contributions of the active biomass groups in BNR activated sludge towards TMP biotransformation in wastewater treatment. In all, the results of this study indicated one of the possible reasons why BNR treatment processes deliver better TMP removal as compared to CAS.

Chapter 6 Conclusions and Recommendations for Future Research

6.1 Conclusions

A comparison between the performances of three biological wastewater treatment processes (CAS, NAS and BNR) in terms of their capability to remove 10 MPs that spanned a wide range of therapeutic classes indicated that the removal efficiency of TMP improved with the complexity of the three treatment processes configurations and SRTs. IBU, ADR, SMX, NP, E1 and BPA had moderate to high removals (> 65%) while CBZ and MEP remained recalcitrant in the three treatment process configurations. The removal of GEM was better in the NAS than in the BNR and CAS treatment configurations. In order to assess whether the observed chemical removal can be compared to the performance of the processes in terms of estrogenicity removal, a comparison between the performances of the three different process configurations in terms of their estrogenicity removal was investigated. The YES assay analyses showed an improvement in estrogenicity removal in the BNR and NAS treatment configurations as compared to the CAS treatment configuration. Generally, similar trend was observed between the treatment processes in terms of their chemical removal and biological response reduction. The unique contribution of

this study was the side-by-side comparison of the wastewater treatment configurations using both chemical and biological responses (YES assay).

In the comparative study that investigated the performances of the CAS, NAS and BNR configurations in terms of their estrogenicity removal, the BNR had the highest removal efficiency as compared to the other two processes. However, the impact of the different stages of treatment on estrogenicity reduction in the BNR process was not examined in detail. Therefore, the removal of endocrine disrupting compounds in BNR wastewater treatment processes was investigated. The results from the detailed BNR study indicated that the pilot and bench scale BNR processes effectively removed EDCs in the authentic and synthetic wastewater, with removal efficiencies as high as 95% for both systems. The biodegradation of EDCs in the bioreactors of the two BNR processes followed the trend of aerobic >anoxic > anaerobic. In order to understand the fate of E1 and E2 under aerobic, anoxic and anaerobic conditions, a pseudo first order model was calibrated with data generated from batch tests under the target conditions. The biotransformation of E1 was estimated to be the slower step in the two step reaction in the aerobic and anoxic batch tests while under anaerobic conditions there was a balance between the biotransformation of E2 to E1 and the

biodegradation of E1. In order to further understand the transformation dynamics between E1 and E2 under aerobic, anoxic and anaerobic conditions of the BNR process, a mass balance model was used to verify the estimated biotransformation rate constants using the operating conditions of the pilot which was the source of the inoculum for the batch tests. The model effectively predicted the fate of E1, E2, E2 + yE1 and E2-Eq in the different zones of the BNR bioreactor. This study was the first to investigate, model, calibrate and validate the biotransformation of E1 and E2 under different redox conditions in a BNR activated sludge using biological responses.

The role of the active microbial groups on the biotransformation of Trimethoprim in BNR processes was investigated. The results indicated that AOB play a role in TMP removal in BNR activated sludge. TMP removal in the BNR system was related to the oxidation-reduction conditions in each of the zones. A combination of pilot scale BNR activated sludge system, batch tests and modeling exercise were employed to evaluate the contributions of PAO, OHO and AOB to the biotransformation of TMP in BNR activated sludge. Under aerobic conditions, the biotransformation of TMP in BNR activated sludge obeyed a modified pseudo first order model that incorporated the fractions of the

active biomass groups, where the biotransformation rate constants for TMP with respect to PAO, OHO and AOB followed a pattern of $k_{AOB} > k_{OHO} > k_{PAO}$. The contributions of PAOs, OHOs and AOBs to the biotransformation of TMP in BNR activated sludge followed the trend of AOBs = PAOs > OHOs. This study have shown that both nitrification and biological phosphorus removal may be beneficial for the biotransformation of TMP in BNR activated sludge. These results could explain why the BNR performs better than other conventional activated sludge (CAS) treatment systems for TMP removal. This study was the first to investigate the role of PAO, OHO and AOB in the biotransformation of TMP in BNR activated sludge.

6.2 Recommendations for Future Research

It is recommended that the biodegradation models that incorporated the active biomass groups as applied to TMP in this study should be extended to other prevailing MPs in wastewater in order to investigate the role of the active biomass groups in their biotransformation. This approach will refine the current practice of using the lumped parameter of MLSS or MLVSS as the biomass concentration in biodegradation models and replace it with the specific active biomass group directly related to the operating conditions of the treatment

system. This model can be applied in the current structure of activated sludge models to effectively predict the concentrations of MPs in WWTP effluents in order to assist MPs environmental risk analysis for policy formulation.

Additional research is also recommended to generate more data in order to further validate the estimated biotransformation rate constants of TMP with respect to the biomass groups and that of E1 and E2 under aerobic, anoxic and anaerobic conditions. The set of parameters that were calibrated and verified in this study are suitable for the operating conditions of the BNR process investigated in this study and might not be applicable to other treatment processes. Hence, further study is recommended to test the estimated biotransformation rate constants for a wide range of operating conditions and biological treatment processes in order to ascertain the robustness of the models.

It is recommended for future studies to investigate the effect of temperature on the estimated biotransformation kinetics of the MPs in BNR activated sludge. Biologically mediated reactions are usually dependent on temperature because temperature affects microbial activities. Therefore, further research is needed to estimate the temperature correction factor for the estimated rate constants.

Studies should be performed to investigate the effects of varying concentration of dissolved oxygen on the removal of MPs in BNR activated sludge. The results of this study indicated that the degradation efficiencies of the MPs and EDCs improved in the aerobic tests as compared to the anoxic and anaerobic tests. Hence, further research is needed to quantify the effects of different concentrations of dissolved oxygen on the removal of MPs in BNR activated sludge.

Finally, further study of the metabolites formed during the biotransformation of E1 in activated sludge systems is recommended. This research investigated the estrogenic potency of this metabolite. However, it would be beneficial to augment the biological analysis with a chemical identification and quantification of the formed metabolites. This will enable the formulation of a clearer biodegradation pathway for E2 and E1 in activated sludge systems.

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Appendix A

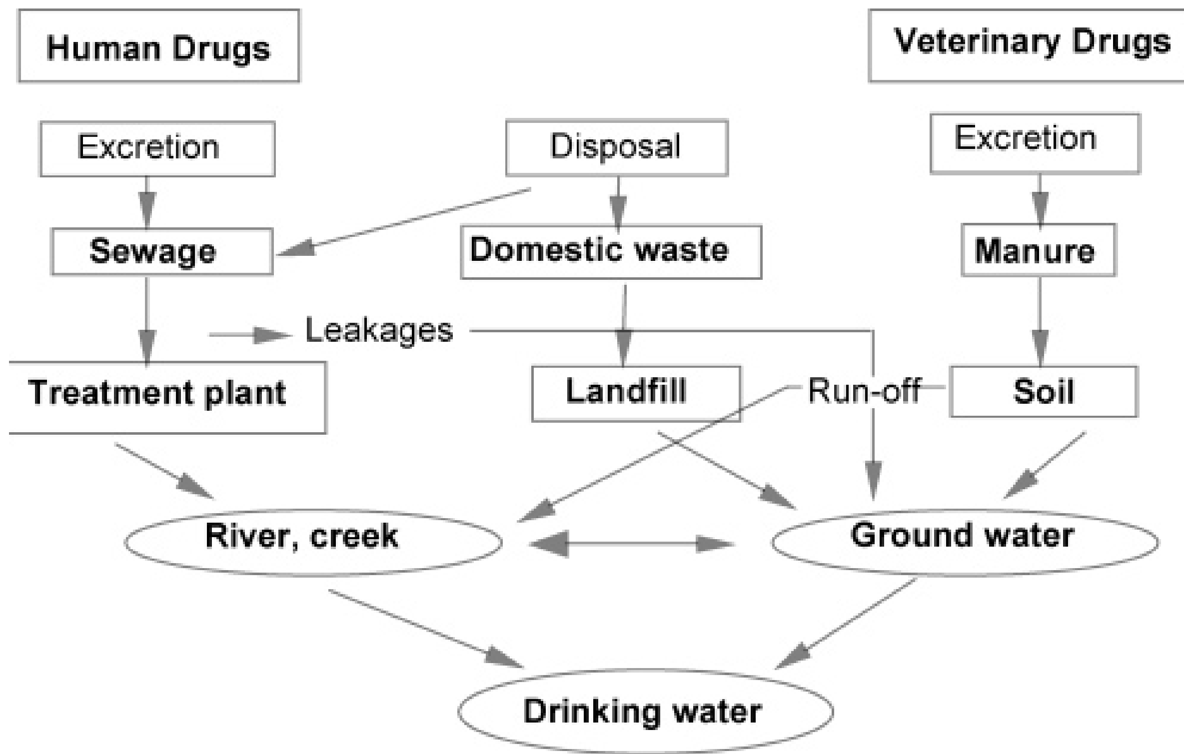


Figure A 1. Sources and distribution of pharmaceuticals in the environment. (Togunde, 2012).

Appendix B

Pilot plant diagrams and operational performance

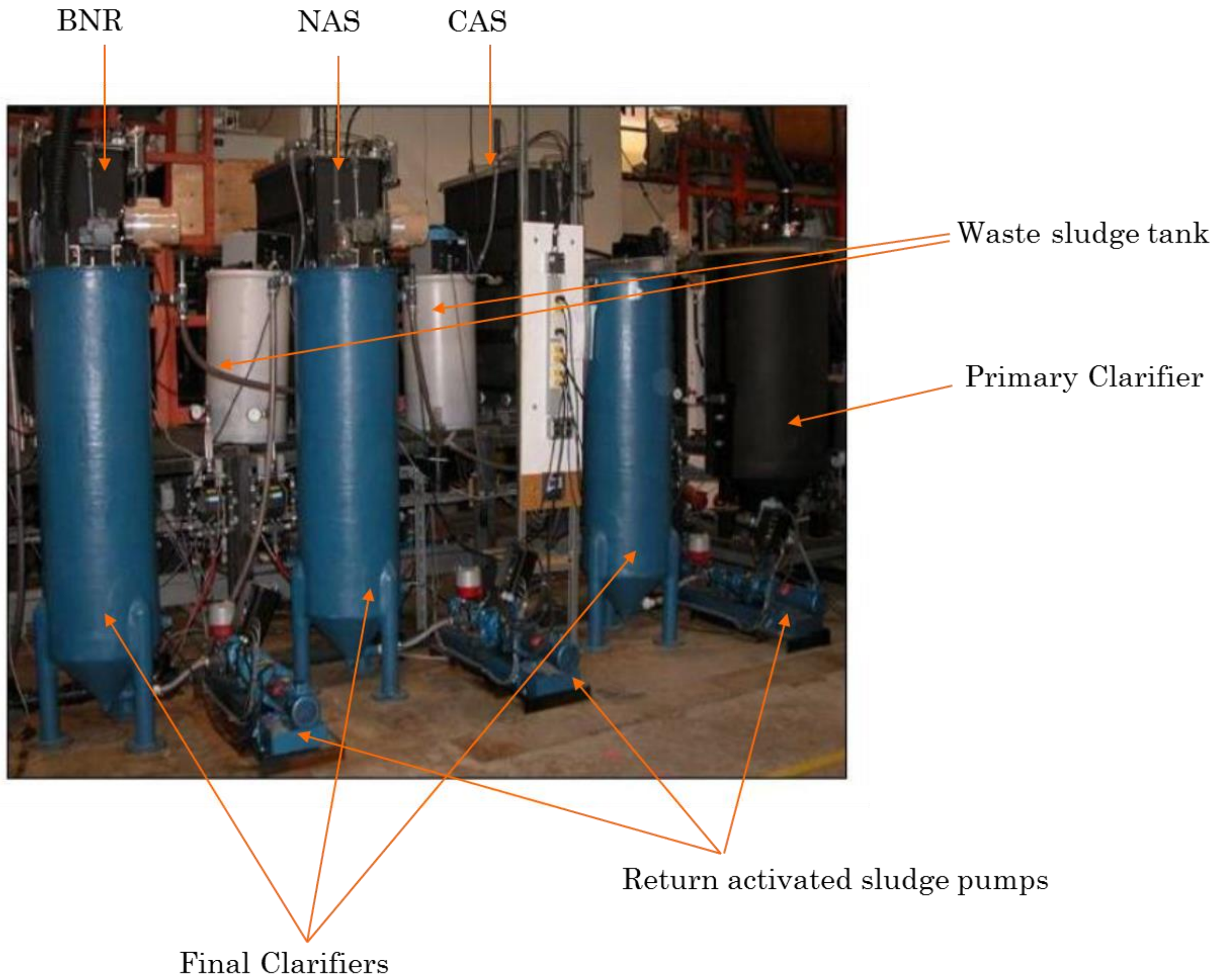


Figure B 1. Pilot Plants (picture taken by Vince Pileggi).

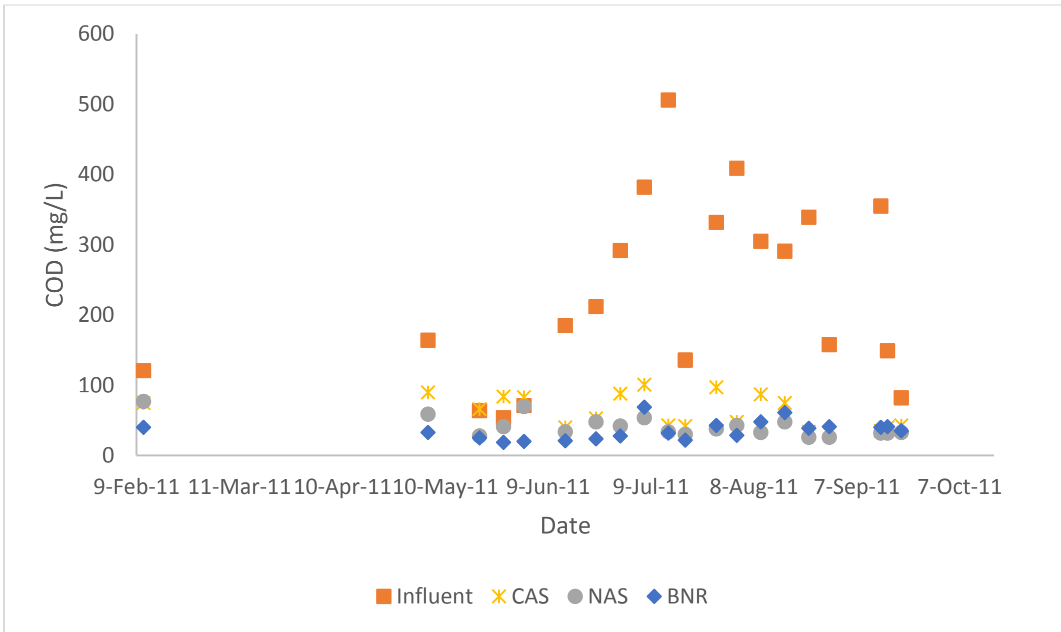


Figure B 2. Daily pilot BNR influent and effluent COD.

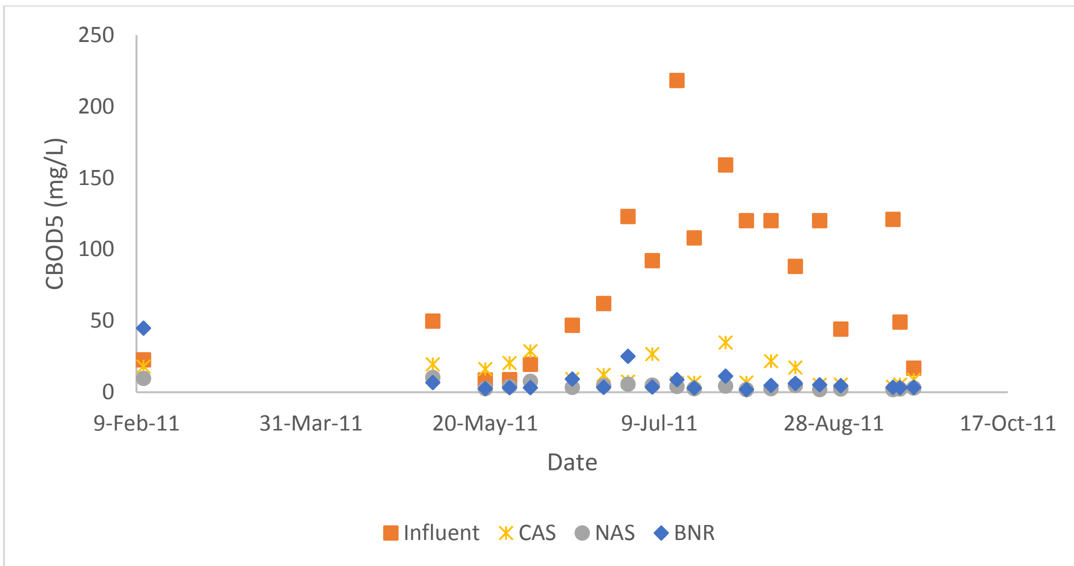


Figure B 3. Daily pilot BNR influent and effluent $CBOD_5$.

Figure B 4. Daily pilot BNR influent and effluent DOC.

Figure B 5. Daily pilot BNR influent and effluent TOC.

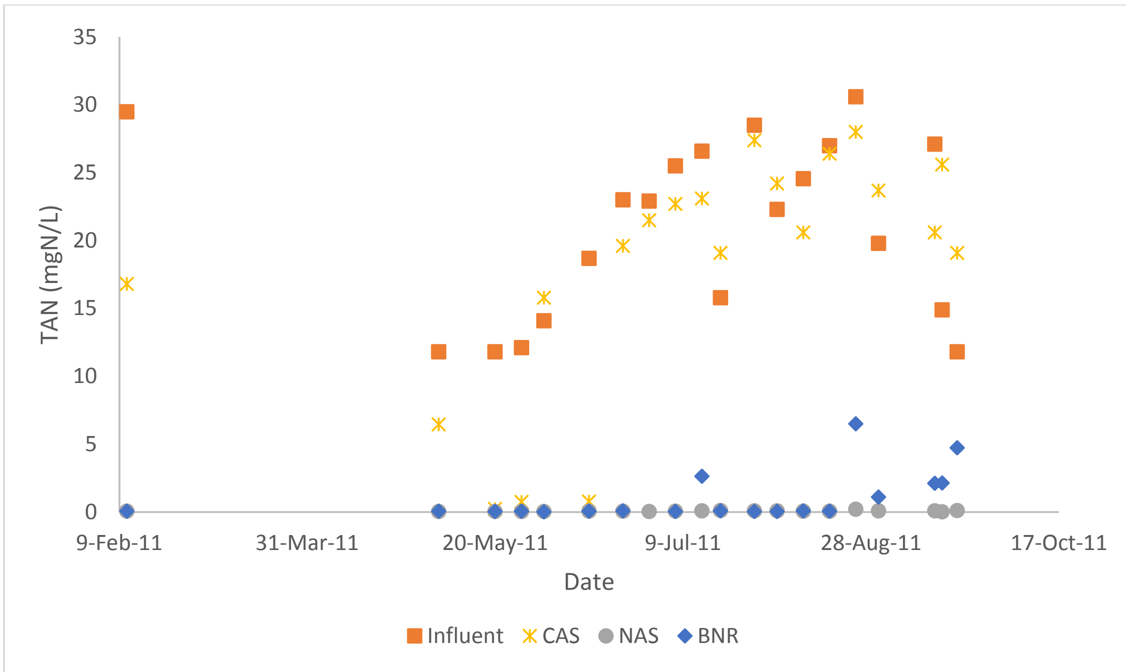


Figure B 6. Daily pilot BNR influent and effluent TAN.

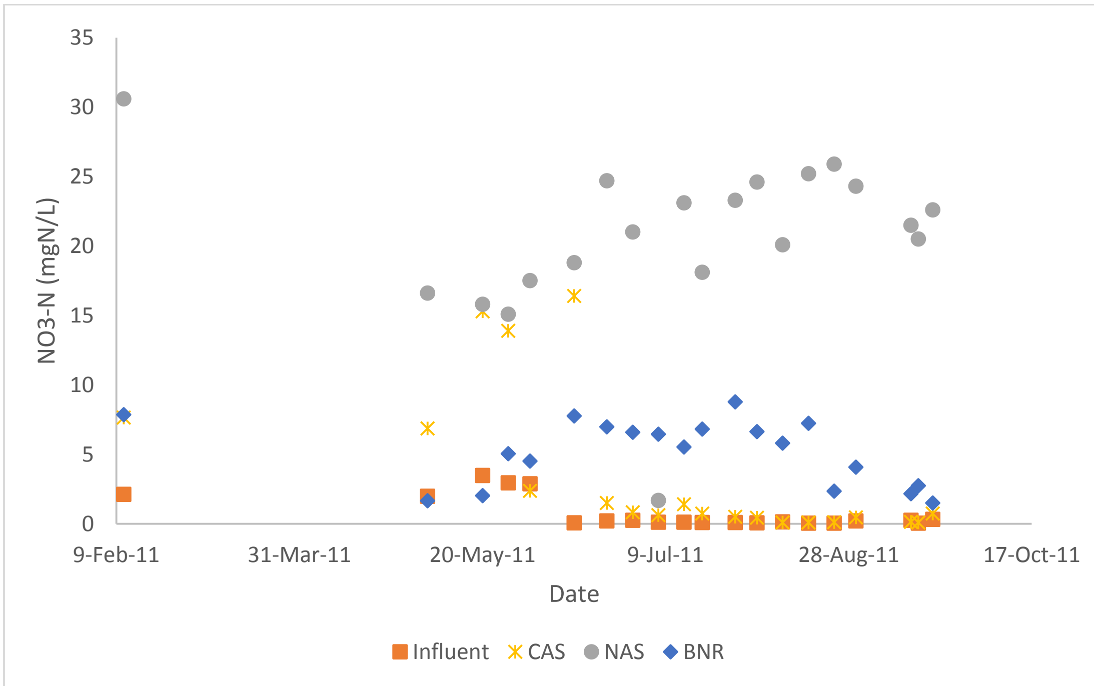


Figure B 7. Daily pilot BNR influent and effluent NO_3-N.

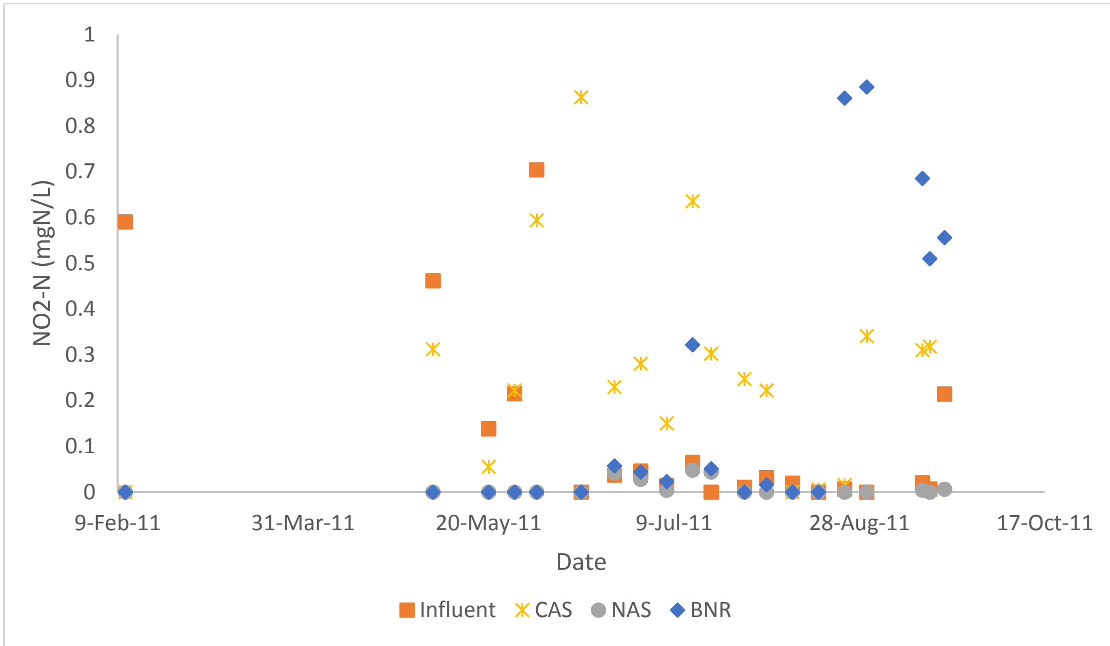


Figure B 8. Daily pilot BNR influent and effluent NO_2-N.

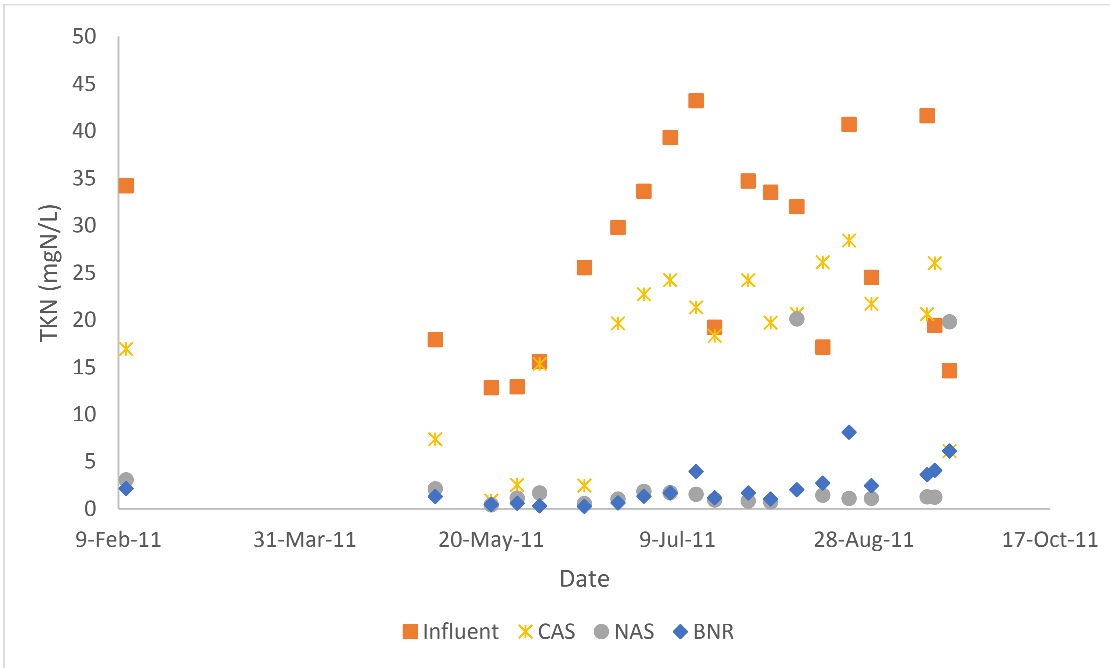


Figure B 9. Daily pilot BNR influent and effluent TKN.

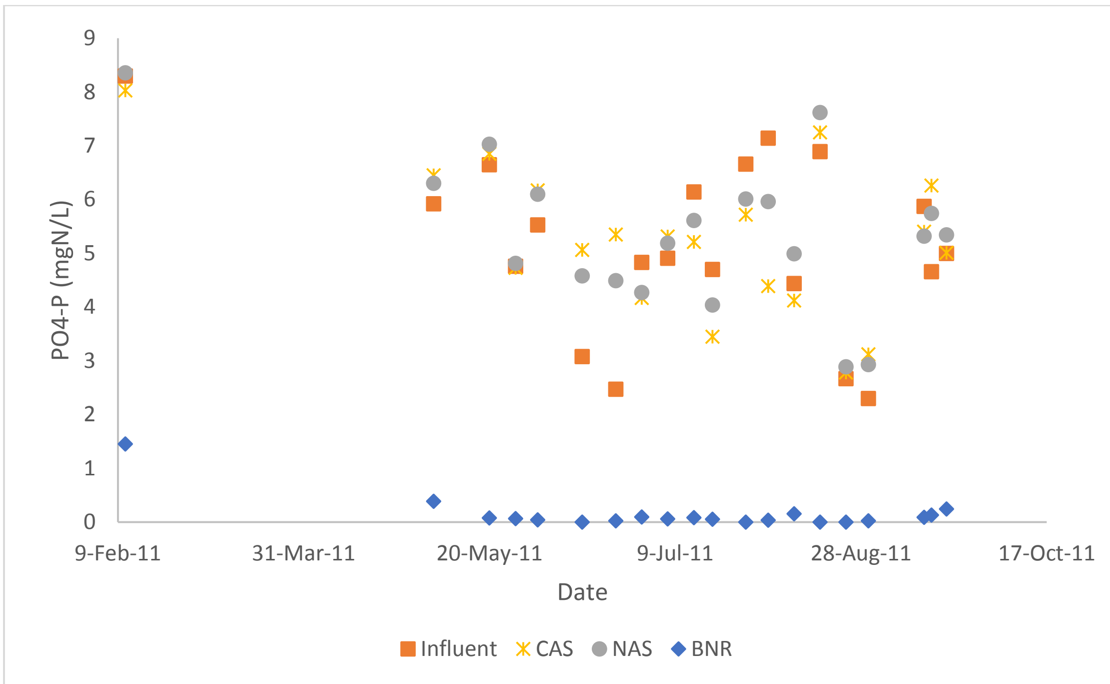


Figure B 10. Daily Pilot BNR influent and effluent PO_4-P.

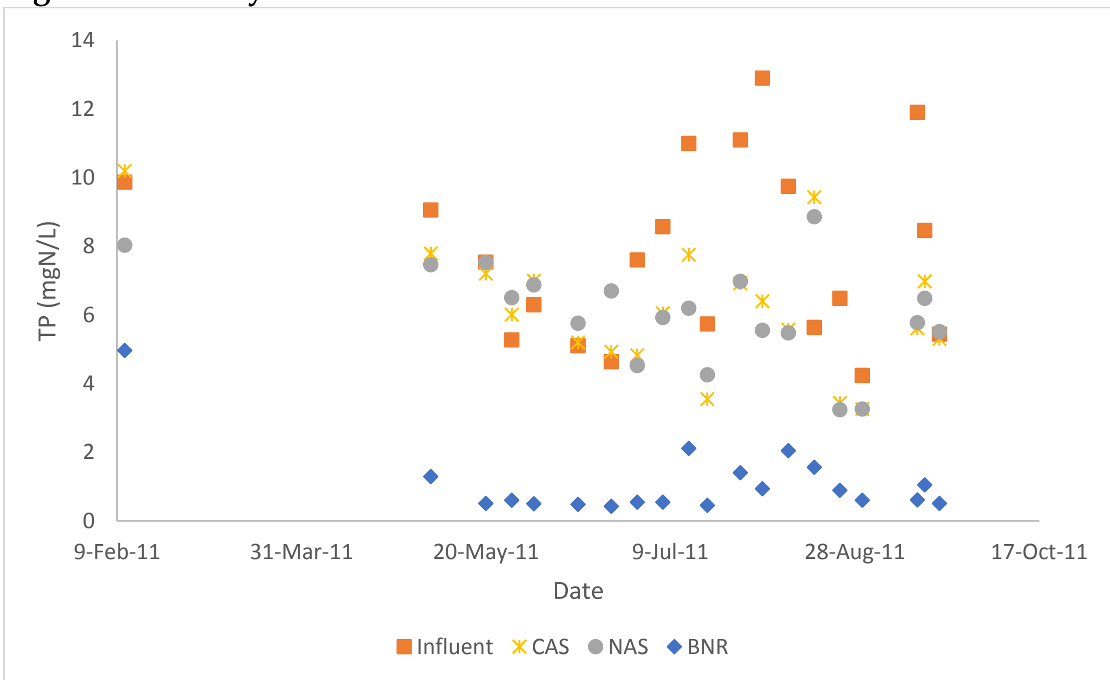


Figure B 11. Daily Pilot BNR influent and effluent TP.

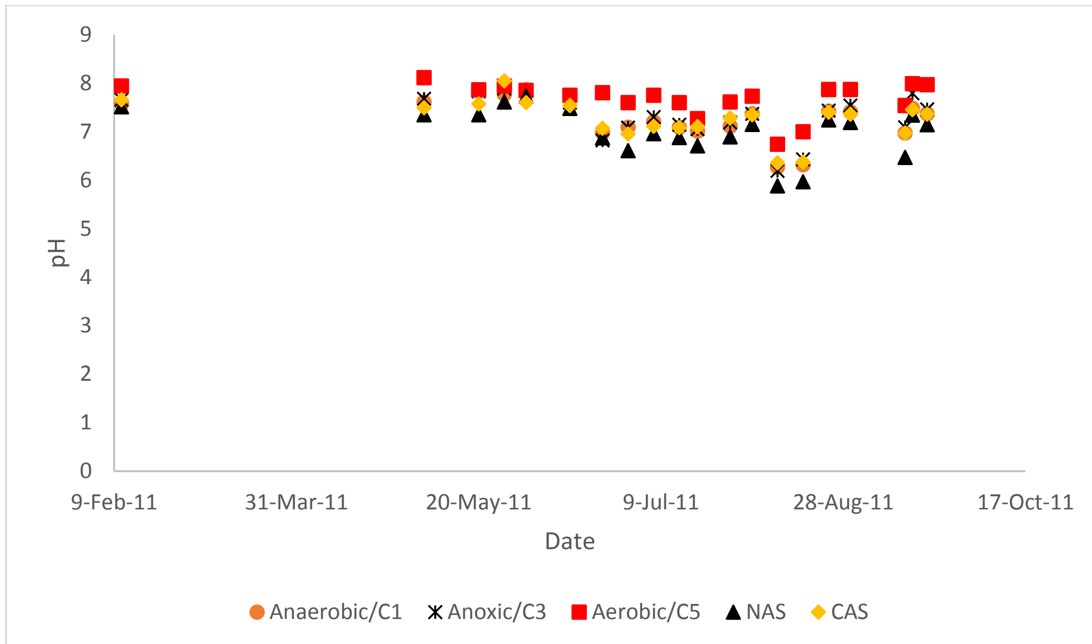


Figure B 12. Daily pilot BNR influent, effluent and BNR stages pH.

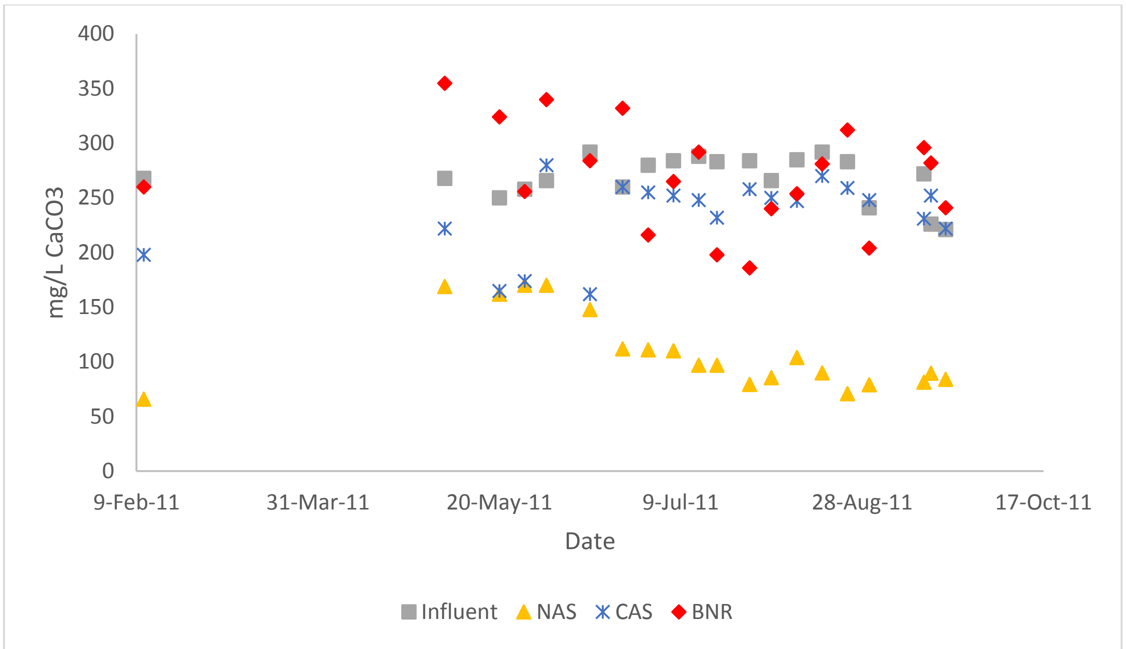


Figure B 13. Daily pilot BNR influent and effluent alkalinity.

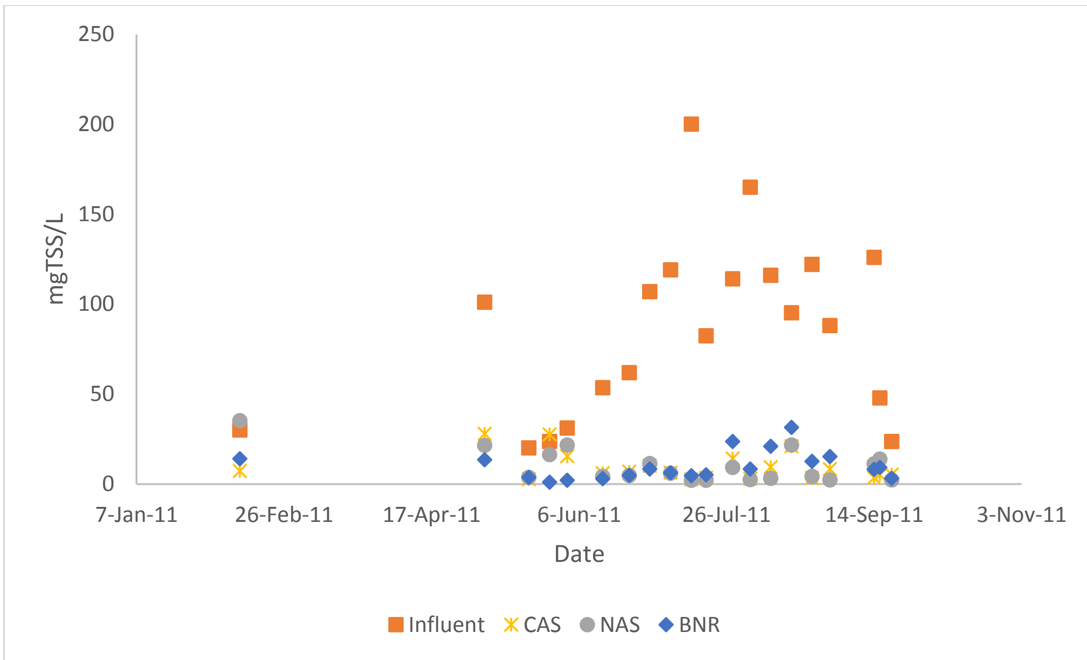


Figure B 14. Daily pilot BNR influent and effluent TSS.

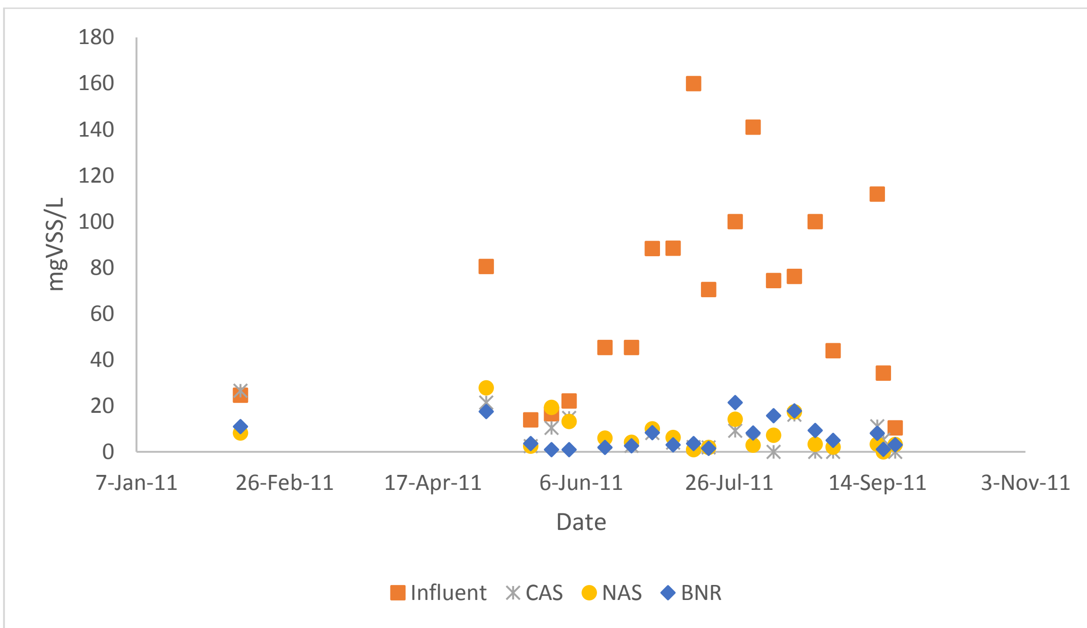


Figure B 15. Daily pilot BNR influent and effluent VSS.

Table A1. QA/QC for Trimethoprim batch test

		O2-2	O2-8	O2-11
Duplicate	A (µg/L)	0.792	0.143	0.031
	B (µg/L)	0.787	0.137	0.037
Relative % difference		**0.68**	**4.77**	**18.04**
in Milli Q Water				
Spike Actual	1	µg/L		
Spike Measured	1.53	µg/L		
% Recovery	153	%		
Blank	ND			
Background (Unspiked Mixed Liquor)		0.049	µg/L	
After Spiking				
Spike Actual		1	µg/L	
Measured	Average (n=3)	0.80	µg/L	
	StDev	0.12		
% Recovery		75	%	

Appendix C

UCT-BNR Simulation Environment

This section describes the modeling environment used to simulate the pilot BNR process. The operation of the pilot plant was simulated using BioWin 3.1® integrated model in the BioWin Platform. BioWin is a widely recognized commercially available wastewater treatment process model and simulation package that was developed by EnviroSim Associates Ltd, Canada. The simulation platform includes activated sludge-anaerobic digestion models which describes various biological processes in wastewater treatment, chemical precipitation reactions, and gas-liquid mass transfer models using fifty state variables and sixty process expressions. BioWin 3.1® modelling tool was chosen because it was suitable for the study objective which requires effective modelling of the different biological and physical processes that impact bacterial growth rate under varying redox and operating conditions. Therefore, the selected modeling environment was well equipped to effectively simulate the pilot UCT-BNR process. Figure C1 shows the BioWin simulator configuration of the pilot UCT- BNR process.

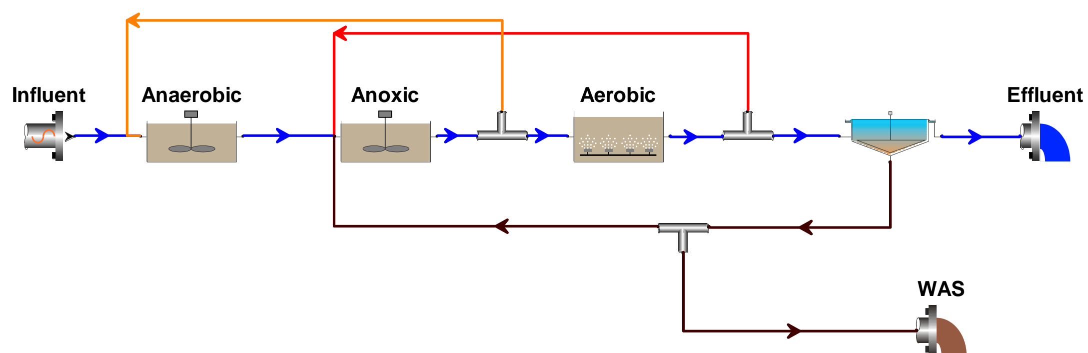


Figure C 1. BioWin Simulator flow diagram of UCT-BNR process.

Appendix D

YES Assay Preparatory Procedures and Solid Phase Extraction

The preparatory procedures in this section were adapted from Routledge and Sumpter (1996) and the operating manual provided by Prof. C. Metcalfe as presented by Citulski (2012).

Preparation of Stock solution

A) Estradiol (E2) Standard

- Weigh 55.6 mg of 98% pure 17-β-estradiol
- Transfer E2 to a 100mL volumetric flask
- Use Pasteur pipette to wash down any remaining E2 from the weighing boat into the volumetric flask using absolute ethanol
- Add ethanol until the volumetric flask is filled; stopper the flask, seal with Teflon tape, and hand-shake for ~30 seconds until the E2 is dissolved
- Charge a disposable 1mL glass pipette x3 with E2 stock solution; transfer 1mL to a second 100mL volumetric flask; reseal the stock solution with stopper and Teflon tape, and place in freezer for storage
- Fill second 100mL volumetric flask with absolute ethanol, stopper, seal with Teflon tape, and hand-shake for ~30 seconds
- Charge a disposable 1mL glass pipette x3 with intermediate E2 solution; transfer 1mL to a third 100mL volumetric flask; dispose of the intermediate solution after use
- Fill third 100mL volumetric flask with absolute ethanol, stopper, seal with Teflon tape, and hand-shake for ~30 seconds, store in the -20°C freezer, but return to room temperature before use (*this is the working E2 standard for the assay, and has a concentration of 54.48μg/L (2 x 10^{-7}M) E2).*

B) Minimal Medium

Add the following to 1000 mL of Milli-Q water and stir with magnetic stirrer on a hot plate set to 75°C until all constituents are dissolved:

- 13.61 g potassium phosphate monobasic (KH_2PO_4)
- 1.98 g ammonium sulphate ($(NH_4)_2SO_4$)
- 4.2 g potassium hydroxide (KOH)
- 0.2 g magnesium sulphate ($MgSO_4$,)
- 1mL of 40 mg/50 mL ferric sulphate ($Fe_2(SO_4)_3$)
- 50 mg L-leucine
- 50 mg L-histidine
- 50 mg adenine
- 30 mg L-tyrosine
- 30 mg L-isoleucine
- 30 mg L-lysine hydrochloride
- 25 mg L-phenylalanine
- 20 mg L-arginine hydrochloride
- 20 mg L-methionine
- 100 mg L-glutamic acid
- 150 mg L-valine
- 375 mg L-serine

- Pipette 45mL aliquots into 100 mL Kimax bottles
- Autoclave at 121°C for 10 minutes
- Store sealed containers at room temperature

Glucose Solution

- Weigh out 20 g of (+)-D-Glucose, anhydrous, add to 250 mL Kimax bottle
- Measure 100 mL of Milli-Q water into the Kimax bottle
- Autoclave at 121°C for 10 minutes
- Store sealed container at room temperature

Vitamin Solution

- Add the following to 180 mL of Milli-Q water in a sterile beaker:
  - 8 mg of pyridoxine
  - 8 mg of thiamine hydrochloride
  - 8 mg (+)-pantothenic acid (D-calcium pantothenate)
  - 40 mg inositol
  - 20 mL of 2 mg/100 mL biotin in Milli-Q water
- Sterilize the solution by syringe-filtering (using a 0.2μm filter tip) into a 250 mL sterilized Kimax bottle
- Store at 4°C

L-Aspartic Acid Solution

- Add 100 mg of L-aspartic acid to 25 mL of Milli-Q water
- Sterilize the solution by autoclaving at 121°C for 10 minutes

- Store at room temperature

L-Threonine Solution

- Add 600 mg of L-threonine to 25 mL of Milli-Q water
- Sterilize the solution by autoclaving at 121°C for 10 minutes
- Store at 4°C

Cupric Sulphate Solution

- Add 320 mg of anhydrous cupric sulphate to 100 mL of Milli-Q water in a sterile beaker
- Sterilize the solution by syringe-filtering (using a 0.2μm filter tip) into a 100mL sterilized Kimax bottle
- Store at room temperature

Chlorophenol Red-β-Galactopyranoside (CPRG) Solution

- Add 100 mg of chlorophenol red-β-D-galactopyranoside to 10mL of absolute ethanol in an amber glass vial
- Store at 4°C

Preparation of HER Yeast Culture

Day One

- Retrieve 10x concentrated yeast stock from the -20°C freezer and thaw in a beaker
- Prepare growth medium in laminar flow hood by pipetting the listed solutions into a 45mL sterile aliquot of minimal medium:
    - 5 mL glucose solution
    - 1.25 mL aspartic acid solution
    - 0.5 mL vitamin solution
    - 0.4 mL threonine solution

- 0.125 mL cupric sulphate solution

- Vortex mix thawed 10x concentrated yeast stock to suspend cells

- Add 250µL of 10x concentrated yeast stock to the growth medium, and place in incubator/shaker set to 28°C and 250 rpm

Day Two (~ 24 hours after initiating incubation)

- Mix a second batch of growth medium, as per Day 1 instructions

- Using a sterile pipette, transfer 2 mL growth medium to a disposable cuvette, and use this as a blank to measure the optical density of the yeast solution; use a second sterile pipette to transfer 2 mL of the cultured yeast solution to a disposable cuvette

- Measure the blank-corrected optical density of the yeast stock at 630nm (Genesys 10 UV-Vis Scanning Spectrophotometer or equivalent)

- If OD_{630nm} ~ 1.0, yeast stock is ready to be used (after a 24-hour incubation period, the OD_{630nm} has typically been 0.90 – 1.25)

- To the remaining growth solution, add cultured yeast in 0.5mL increments, and check the OD_{630nm} after each addition until an absorbance of ~0.1 is achieved (about 2.5mL ± 0.5mL of yeast culture usually added)

- Prepare enough bottles of growth solution to fill the amount of microtiter plates that will be used, and add sufficient yeast culture to each to achieve OD_{630nm} of ~0.1, and then add 0.5mL of CPRG solution to each bottle; this mixture can now be used to fill the microtiter plates

3.6.2 Preparation of the Microplates

- Use 300 µL flat-bottomed microtiter plates (Whatman)

- Prepare a — "dummy row" (sufficient to create a quadruplicate set of standard curves) as follows:

- Pipette 100μL of absolute ethanol into wells C2-C12 of a plate
- Pipette 200μL of E2 working standard into well C1; then withdraw 100μL from this well and add to well C2, fill and discharge the pipette x5 to mix the contents (this will yield a 2x dilution in well C2)
- Withdraw 100μL of solution from well C2, and add to well C3, charging the pipette x5 to mix; repeat this process across the remaining wells, and discard the final excess 100μL from well C12
- Cover the — "dummy row" with lab or autoclave tape, and then – working from the lowest concentration well, C12 – pipette four 10μL aliquots of diluted E2 standard into wells E/F/G/H 12; repeat this process, successively uncovering wells C11 - C1 and adding the solution to the corresponding E/F/G/H wells

➢ Pipette 100μL of absolute ethanol into each of wells A1 - A12 to create a negative blank

➢ Allow ethanol/E2 solution to evaporate by placing the microplate in the laminar flow hood

➢ When all solvent has evaporated from individual wells, add 200μL of the combined growth medium/CPRG/yeast mixture to each well in the sample assay microplate

➢ After addition of yeast culture and incubation, the standard curve/negative control plate should resemble Figure D2.

Environmental Sample Extracts

➢ Add 80μL of cleaned solvent extract from an environmental sample to well 1 of the row; add another 80μL of extract to well 2, and as with the E2 curve procedure, serially-dilute the contents of well 2 across the remaining wells; repeat as desired to obtain replicate curves

➢ Allow solvent to evaporate by placing the microplate in the laminar flow hood

- When all solvent has evaporated from individual wells, add 200µL of the combined growth medium/CPRG/yeast mixture to each well in the sample assay microplate

- Seal the plate(s) with autoclave tape

- Place plates in the incubator/shaker and tape to the shaker plate; incubate at 32°C with the shaker tray set to 150 rpm, for 72 hours

3.6.3 Reading the Microplates

- A microplate reader (TECAN) was used to determine the absorbance of the samples at both 620nm (to evaluate sample turbidity) and 540nm (to evaluate colour development)

- The corrected absorbance values were calculated as follows:
- Corrected Absorbance = AB540 nm – (AB620nm – Control620nm, average)

3.6.4 Preparation and storage of 10X concentrated yeast stock

Day One

- Make growth medium (minimal medium + vitamin solution/etc.) and add 125µL of concentrated yeast stock (stored in the -80°C freezer) thawed in a beaker of ice in the laminar flow hood

- Place in incubator at 28°C with the orbital shaker set to 250 rpm for approximately 24 hours

Day Two

- Make more growth medium and add 1mL of yeast from — ″ Day 1″ culture per ~50mL (several can be made at one time)

- Incubate at 28°C with the orbital shaker set to 250rpm for approximately 24 hours

Day Three

- Transfer each culture made on Day Two to a sterile 50mL centrifuge tube (with closure)

- Centrifuge at 4°C for 10 minutes at 2000*g*

- In laminar flow hood, decant supernatant and re-suspend centrifuged yeast pellet in 5mL of a minimal medium/glycerol mixture (45mL minimal medium + 5.5mL glycerol, previously autoclaved at 121 °C/10 minutes)

- Transfer 0.5 mL aliquots to sterile GC vials (previously autoclaved at 121 °C/10 minutes), cap and crimp, and store at -20 °C

- The prepared yeast stocks are good for 4 months

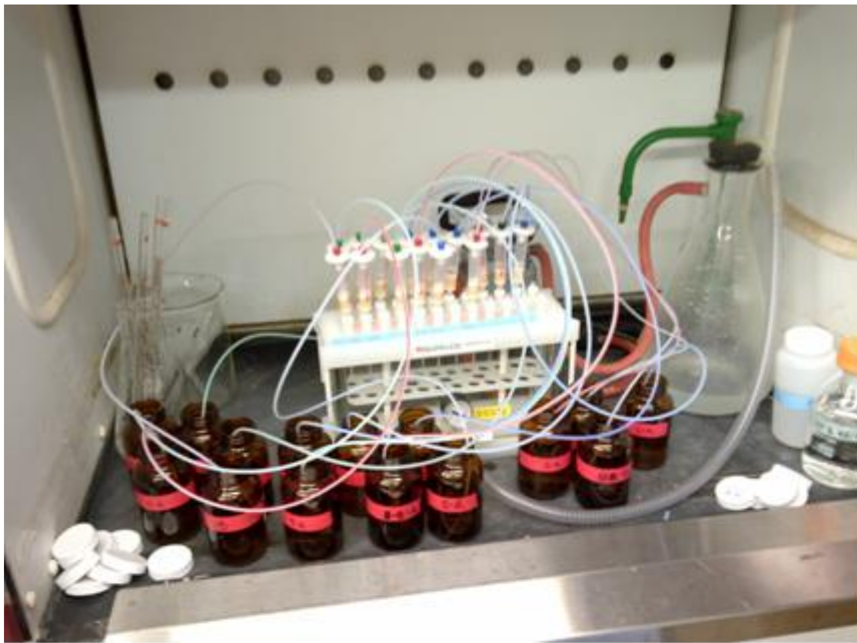


Figure D-1. Sample Extraction Process (Multi-Residue Extraction Technique).

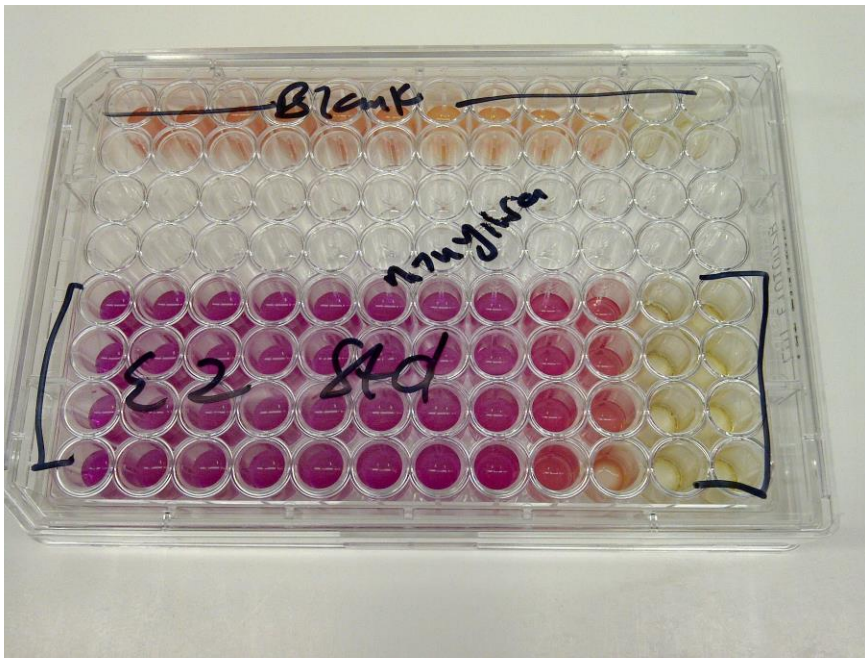


Figure D-2. Example of Ethanol Negative Control Row (blank) and E2 Standard Curve.

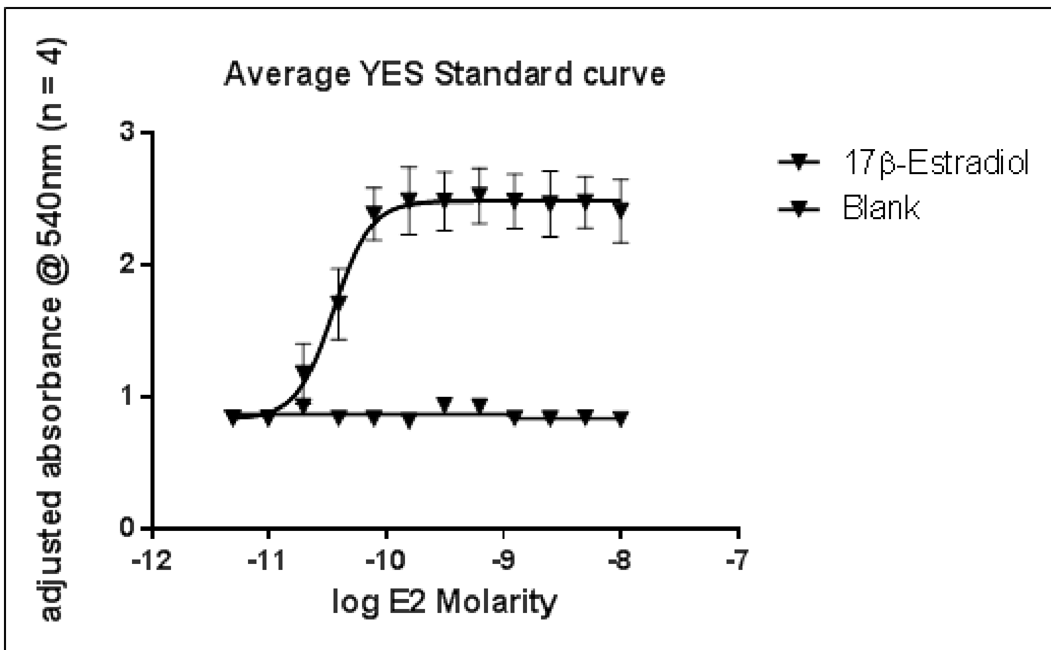
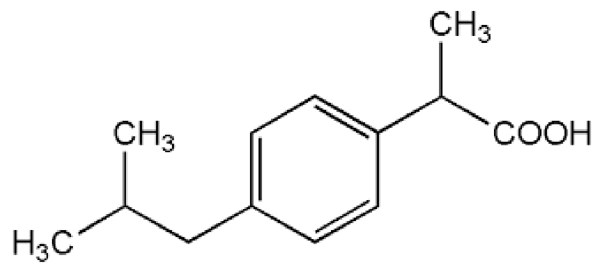


Figure D-3. Example of YES-Assay Dose Response Curve for Blank and E2 Standard Curve.

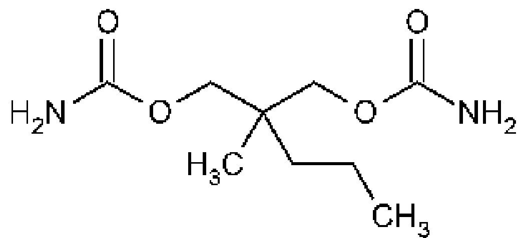
Appendix E

Chemical Structure of Selected Chemical Compounds

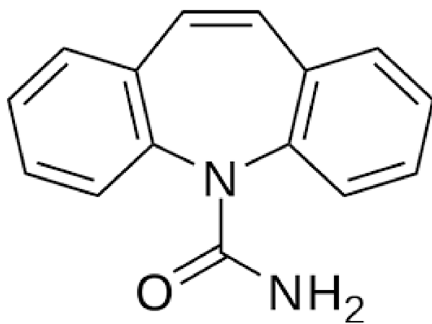
Ibuprofen (C13H18O2)



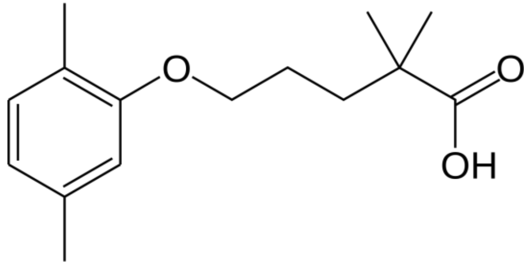
Meprobamate (C9H18N2O4)



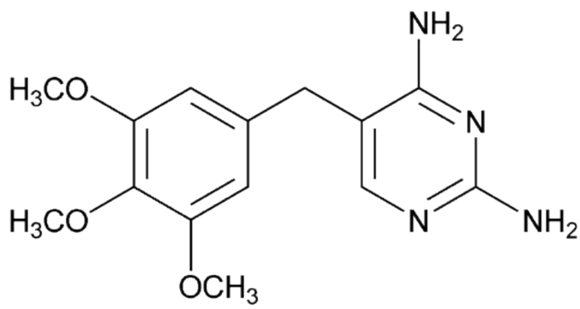
Carbamazepine (C15H12N2O)



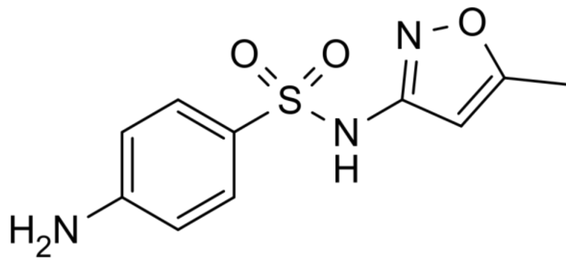
Gemfibrozil ($C_{15}H_{22}O_3$)



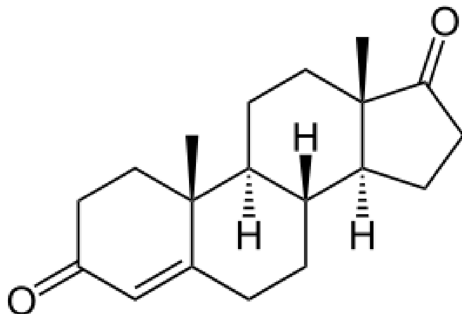
Trimethoprim ($C_{14}H_{18}N_4O_3$)



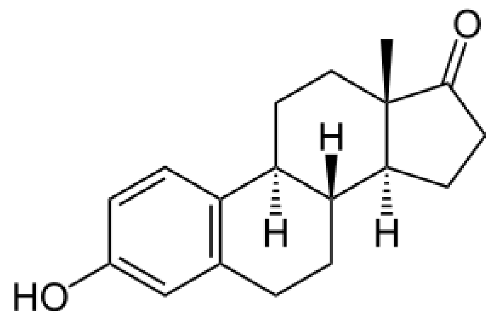
Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$)



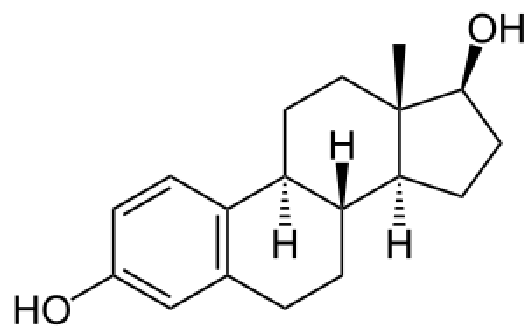
Androstenedione ($C_{19}H_{26}O_2$)



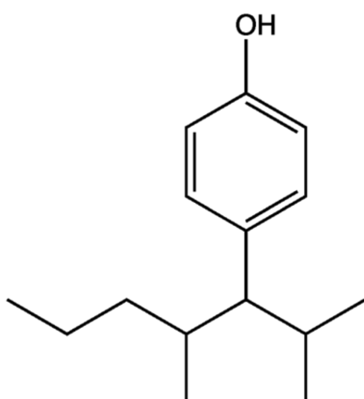
Estrone ($C_{18}H_{22}O_2$)



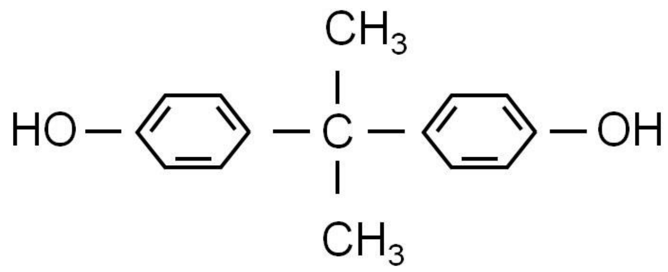
17-β-Estradiol ($C_{18}H_{24}O_2$)



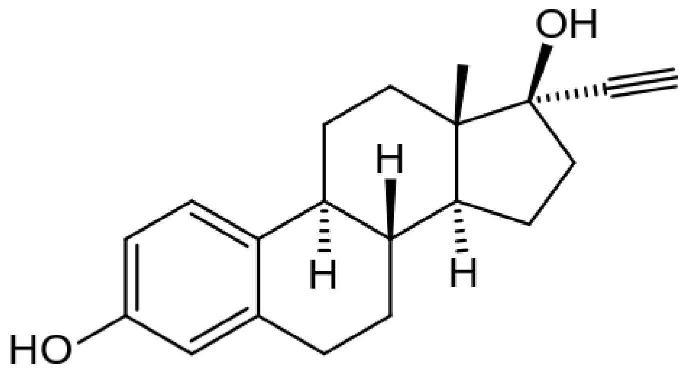
Nonyl-phenol ($C_{15}H_{24}O$)



Bisphenol-A ($C_{15}H_{16}O_2$)



Ethinylestradiol ($C_{20}H_{24}O_2$)



Appendix F

Micropollutant concentrations in treatment trains

ng/L		July	IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Sewage	C1	7/7/2011	1427	16	24	242	134	82	72	40
Influent	C2	7/7/2011	1377	9	21	233	107	80	66	24
CAS - N	C3	7/7/2011	5	8	4	260	71	16	ND	ND
CAS	C4	7/7/2011	141	9	31	280	86	32	7	3
CAS - BNR	C5	7/7/2011	ND	11	9	287	32	42	ND	2
Sewage	D1	7/19/2011	1247	8	22	209	118	107	50	24
Influent	D2	7/19/2011	1267	5	20	194	104	111	36	22
CAS - N	D3	7/19/2011	ND	5	ND	227	92	21	ND	ND
CAS	D4	7/19/2011	440	5	37	223	97	42	8	6
AN	D5	7/19/2011	1327	5	21	198	44	50	24	18
03AX	D6	7/19/2011	657	2	10	146	26	14	ND	7
C5O2	D7	7/19/2011	2	6	9	218	33	22	ND	ND
CAS BNR	D8	7/19/2011	ND	5	9	234	30	16	ND	ND

Average		IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
	Sewage	1337	12	23	226	126	94	61	32
	Influent	1322	7	21	214	105	96	51	23
	CAS - N	5	7	4	244	82	18	ND	ND
	CAS	291	7	34	251	92	37	7	4
	CAS - BNR	ND	8	9	260	31	29	ND	2

Stdev		IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
	Sewage	127	5	2	23	11	18	16	11
	Influent	78	3	1	28	2	22	21	1
	CAS - N	0	1	0	12	7	2	ND	ND
	CAS	106	2	2	20	4	4	1	1
	CAS - BNR	ND	4	0	37	1	19	ND	ND

ng/L		August	IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Sewage	E1	8/3/2011	1257	9	23	175	112	148	59	2
Influent	E2	8/3/2011	1413	9	24	196	116	150	51	16
CAS - N	E3	8/3/2011	nd	14	nd	234	72	34	24	24
CAS	E4	8/3/2011	48	13	30	219	82	94	nd	2
CAS - BNR	E5	8/3/2011	nd	15	8	223	44	121	nd	nd
Sewage	F8	8/24/2011	530	9	33	229	91	196	42	4
Influent	F1	8/24/2011	427	14	26	246	101	20	43	2
CAS	F2	8/24/2011	nd	22	nd	316	46	4	nd	2

CAS - N	F3	8/24/2011	194	11	40	229	32	9	nd	nd
CAS - BNR	F4	8/24/2011	nd	15	nd	274	68	2	54	42
AN	F5	8/24/2011	254	14	17	252	68	17	6	37
AX	F6	8/24/2011	224	11	20	232	116	47	nd	4
O2	F7	8/24/2011	480	11	12	247	94	19	39	3
Sewage	G8	8/30/2011	25	19	9	195	105	36	10	8
Influent	G2	8/30/2011	27	21	11	219	101	34	10	9
CAS	G1	8/30/2011	nd	20	nd	293	97	nd	nd	6
CAS - N	G3	8/30/2011	2	19	nd	258	96	41	nd	2
CAS - BNR	G4	8/30/2011	nd	10	nd	176	18	nd	nd	nd
AN	G5	8/30/2011	nd	22	nd	280	71	nd	3	8
AX	G6	8/30/2011	3	25	nd	308	75	5	nd	1
O2	G7	8/30/2011	4	24	9	297	86	40	nd	2

		IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Average	Sewage	604	12	22	200	103	127	37	5
	Influent	622	14	20	221	106	68	34	9
	CAS - N	nd	15	40	240	67	28	24	13
	CAS	81	18	30	276	75	49	nd	3
	CAS - BNR	nd	13	8	224	44	62	54	42

		IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Stdev	Sewage	619	6	12	27	11	82	25	3
	Influent	661	6	8	25	9	71	22	7
	CAS - N	nd	4	ND	15	32	17	ND	16
	CAS	100	5	ND	51	26	64	ND	3
	CAS - BNR	nd	3	ND	49	25	84	ND	ND
RSD	Sewage	103	47	56	14	11	65	67	71
	Influent	106	41	39	11	8	105	64	75
	CAS - N	ND	29	ND	6	49	60	ND	120
	CAS	123	27	ND	18	35	129	ND	81
	CAS - BNR	ND	22	ND	22	58	136	ND	ND

ng/mL		September	IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Influent	H1	Sept-14-2011	ND	0.017	ND	0.216	0.034	0.009	ND	0.004
CAS	H2	Sept-14-2011	ND	0.024	ND	0.226	0.033	0.004	ND	0.004
CAS-N	H3	Sept-14-2011	ND	0.027	ND	0.284	0.042	0.002	ND	ND
CAS-BNR	H4	Sept-14-2011	ND	0.022	ND	0.254	0.025	ND	ND	0.003
AN	H5	Sept-14-2011	ND	0.021	ND	0.262	0.030	ND	ND	0.009
AX	H6	Sept-14-2011	ND	0.023	ND	0.270	0.025	ND	ND	0.013
O2	H7	Sept-14-2011	ND	0.021	ND	0.258	0.028	ND	ND	0.009
Sewage	H8	Sept-14-2011	ND	0.017	ND	0.208	0.043	0.009	ND	0.005
Influent	I1	Sept-16-2011	1.607	0.008	0.035	0.168	0.031	0.130	ND	ND

CAS	I2	Sept-16-2011	0.034	0.008	0.043	0.193	0.037	0.077	ND	ND
CAS-N	I3	Sept-16-2011	ND	ND	ND	0.002	0.001	ND	ND	ND
CAS-BNR	I4	Sept-16-2011	0.023	0.010	0.036	0.210	0.024	0.086	ND	ND
AN	I5	Sept-16-2011	ND	ND	ND	ND	ND	ND	ND	ND
AX	I6	Sept-16-2011	ND	ND	ND	ND	ND	ND	ND	ND
O2	I7	Sept-16-2011	ND	ND	ND	ND	ND	ND	ND	ND
Sewage	I8	Sept-16-2011	1.529	0.011	0.049	0.225	0.041	0.185	ND	ND
Influent	J1	Sept-20-2011	1.367	0.009	0.026	0.128	0.027	0.103	ND	ND
CAS	J2	Sept-20-2011	0.035	0.008	0.031	0.209	0.031	0.119	ND	ND
CAS-N	J3	Sept-20-2011	0.041	0.009	0.007	0.238	0.033	0.079	ND	ND
CAS-BNR	J4	Sept-20-2011	0.019	0.009	0.031	0.218	0.026	0.116	ND	ND
AN	J5	Sept-20-2011	0.022	ND	ND	0.002	0.001	0.003	ND	ND
AX	J6	Sept-20-2011	0.590	0.009	0.031	0.201	0.022	0.176	ND	ND
O2	J7	Sept-20-2011	0.022	0.009	0.029	0.187	0.022	0.162	ND	ND
Sewage	J8	Sept-20-2011	1.437	0.008	0.024	0.146	0.035	0.164	ND	ND

Average

	IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Sewage	1483	12	36	193	39	119	ND	4
Influent	1487	11	30	171	30	81	ND	4
CAS - N	41	12	7	175	26	41	ND	ND
CAS	35	13	37	209	34	67	ND	ND
CAS - BNR	21	14	33	227	25	101	ND	ND

Stdev

	IBU	MEP	GEM	CBZ	TMP	SMX	ADR	E1
Sewage	65	5	17	42	4	96		
Influent	170	5	6	44	3	64		
CAS - N	0	12	0	151	22	55		
CAS	1	9	8	17	3	58		
CAS - BNR	3	8	4	23	1	21		

Appendix G

Trimethoprim batch test data

Aerobic-1B	ug/L			mg/L	mg/L	mg/L	mg/L	mg/L
hr	TRM-2	std-2	C/Co	COD	PO4-P	NH3-N	NO3-N	NO2-N
0	0.529	0.124	1	503.00	193.76	98.27	4.03	0.42
0.25	0.643	0.012	0.940469	451.00	175.52	94.73	2.83	0.37
4.25	0.362	0.011	0.818182	231.00	148.04	69.54	26.67	0.93
16.07	0.161	0.004	0.174487	166.00	82.54	31.30	65.67	0.06
20.07	0.137	0.032	0.101026	154.00	79.62	18.59	78.33	0.17
24.65	0.103	0.021	0.083284	154.00	76.17	10.78	88.69	0.01
39.65	0.079	0.023	0.017889	168.33	80.44	5.56	91.67	0.02
43.73	0.073	0.022	0.01654	172.33	84.13	0.04	97.33	0.01
47.9	0.028	0.002	0.016129	186.00	85.03	0.01	98.67	0.01
64.07	0.022	0.002	0.016129	208.67	89.01	0.05	100.33	0.02
68.07	0.019	0.003	0.016129	216.00	92.19	0.02	101.00	0.01
73.07	0.018	0.002	0.016129	229.00	95.28	0.01	102.33	0.01

Aerobic-1	(ug/L)												
hr	TRM-1-1	C/Co	Std	COD	SD	PO_4-P	SD	NH_3-N	SD	NO_3-N	SD	NO_2-N	SD
0.00	0.656		0.179	600.33	28.22	162.40	3.81	114.67	0.99	0.40	0.00	0.02	0.00
0.25	0.549	1.000	0.022	599.67	22.14	152.00	4.35	113.40	2.09	0.20	0.00	0.01	0.00
4.25	0.489	0.892	0.018	286.67	51.64	118.00	3.35	81.23	1.24	1.70	0.00	0.73	0.00
16.07	0.160	0.292	0.001	171.30	18.88	40.46	0.65	39.60	1.18	30.00	1.00	0.76	0.01
20.07	0.122	0.222	0.021	144.67	4.04	32.39	0.21	25.40	11.88	47.00	1.00	0.01	0.01
24.65	0.094	0.171	0.013	128.33	4.62	22.30	0.59	19.10	0.50	52.00	1.00	0.69	0.01
39.65	0.058	0.106	0.030	135.33	2.08	22.20	0.21	7.73	0.21	76.00	1.00	0.05	0.02
43.73	0.054	0.098	0.027	138.67	4.51	22.30	0.20	0.40	0.20	86.33	0.58	0.21	0.01
47.90	0.024	0.043	0.006	142.67	1.53	23.20	0.05	0.33	0.06	88.33	0.58	0.10	0.01
64.07	0.020	0.036	0.004	146.30	0.00	23.20	0.00	0.17	0.03	89.00	1.00	0.04	0.00
68.07	0.018	0.032	0.002	152.33	0.58	23.10	0.05	0.06	0.01	93.00	1.00	0.03	0.00
73.07	0.017	0.031	0.002	166.33	1.53	30.20	0.10	0.07	0.01	95.00	1.00	0.02	0.00

Aerobic-2	ug/L										
hr	TRM-2	Std-1	C/Co	COD	SD	NH_3-N	SD	NO_3-N	SD	PO_4-P	SD
0	0.218	0.18		535.00	19.47	15.20	0.99	3.50	0.00	187.52	1.73
0.25	0.214	0.02	1.000	462.67	22.03	15.20	2.09	3.40	0.00	170.32	3.49
4.25	0.165	0.02	0.771	231.33	19.35	16.00	1.24	3.50	0.00	36.68	0.62
16.07	0.095	0.00	0.446	172.33	2.52	15.80	1.18	3.60	1.00	16.68	0.19
20.07	0.086	0.02	0.400	164.67	3.79	16.70	1.80	3.80	1.00	12.42	0.04
24.65	0.072	0.01	0.337	169.67	6.03	15.90	0.50	3.90	1.00	12.44	0.46
39.65	0.039	0.03	0.182	174.33	1.53	16.80	0.21	3.60	1.00	13.61	0.10
43.73	0.038	0.03	0.178	188.33	2.52	15.20	0.20	4.20	0.58	14.35	0.05
47.9	0.026	0.01	0.121	194.00	13.00	15.30	0.06	3.50	0.58	13.58	0.03
64.07	0.021	0.00	0.098	202.00	12.00	14.50	0.03	3.50	1.00	12.87	0.03
68.07	0.014	0.00	0.065	217.50	6.36	15.70	0.01	3.50	1.00	14.39	0.11
73.07	0.009	0.00	0.041	219.00	11.00	15.20	0.01	3.40	1.00	20.07	0.05