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Impact of Tetracycline on Microbial Communities in the Secondary Treatment Process of Wastewater Treatment Systems

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IMPACT OF TETRACYCLINE ON MICROBIAL COMMUNITIES IN THE SECONDARY TREATMENT PROCESS OF WASTEWATER TREATMENT SYSTEMS

by

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ABSTRACT

This study examined the impact of the antibiotic tetracycline at environmentally relevant concentrations (1µg/L and 10µg/L) on the composition and function of the microbial community that are responsible for the secondary treatment step in a municipal wastewater treatment plant (MWTP). Specifically, this study examined whether nitrification is inhibited by the presence of tetracycline under high and low nutrient replacement conditions. Aerated semi-batch reactors were set up containing activated sludge samples from a MWTP. Reactors were replenished with a synthetic wastewater media at two constant replacement rates for a period of 4 weeks. Parameters such as ammonia, nitrate/nitrite and total Kjeldahl nitrogen concentrations were monitored to evaluate the nitrogen removal efficiency. Under a low nutrient replacement rate, tetracycline was observed to have a positive impact on ammonia removal and nitrification than at the higher one. However, total Kjeldahl nitrogen concentrations increased in low nutrient replacement reactors under the presence of tetracycline which suggested a potential inhibitory effect on denitrification. At high nutrient replacement rates, tetracycline did not demonstrate an inhibitory effect on both nitrification and denitrification processes. Overall, it appears that both antibiotic presence and nutrient replacement rates can influence the community composition and function of microbial communities found in a MWTP.

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“**If I have seen further, it is by standing on the shoulders of giants**”

-Sir Isaac Newton

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CHAPTER 1: INTRODUCTION

Wastewater is defined as the water after use from residential, industrial and institutional sources. The city of Toronto is home to four MWTPs that process nearly 340 million gallons of wastewater a day. The increased volume of wastewater being produced in Toronto annually is partially due to the expansion and rise of urban communities (City of Toronto, 2008). Ammonia is one of the predominant sources of nitrogen present in wastewater (Kowalchuk and Stephen, 2001). It is important to remove ammonia before the wastewater can be safely discharged into receiving water bodies because the presence of excessive nitrogen can lead to eutrophication of freshwater bodies and can be toxic to aquatic wildlife (Egli et al., 2003). Therefore, the treatment of wastewater has become a crucial process in avoiding the destruction and pollution of Canadian natural water resources. The successful and efficient removal of nitrogenous compounds from wastewater involves microorganisms playing a crucial role. Microorganisms are important in the secondary treatment step of wastewater that is also known as the biological treatment step. Their wide array of abilities to degrade organic and inorganic compounds allows for the removal of nutrients such as carbon, nitrogen and phosphorus. (Wojnowska-Baryla et al., 2010). The process of nitrification achieved by microorganisms is the initial step in the removal of nitrogenous compounds. This process is composed of two steps that are used to convert ammonia to nitrite (ammonia oxidation) and nitrite to nitrate (nitrite oxidation). Denitrification of the nitrate to gaseous nitrogen results in the loss of nitrogen from the wastewater (Burrell et al., 1998).

Pharmaceuticals are a class of emerging contaminants due to their extensive use in human and veterinary medicine (Fent et al., 2006). An increasing number and volume of pharmaceuticals and personal care products (PPCPs) have been found in municipal wastewater

streams, treated effluent and receiving water bodies in Canada (Servos et al., 2007). Pharmaceutical compounds are designed to target specific metabolic pathways in humans and domestic animals in order to provide therapeutic benefits; however their impact on non-target organisms has the possibility of becoming detrimental even at very low concentrations (Daughton and Ternes, 1999; Jones et al., 2005; Dietrich et al., 2002 and Moldovan, 2006). Municipal wastewater is one of the major routes by which these compounds can enter into the environment (Ternes and Joss, 2004). In MWTPs, the nitrification process has been shown to be sensitive to various pharmaceuticals. In one study, wastewater reactors processing wastewater supplemented with a mixture of non steroidal anti-inflammatory drugs (NSAIDS) ibuprofen, naproxen, ketoprofen, diclofenac were found to contain no *Nitrospira* sp. even at low drug concentrations (5ug/L) (Kraigher et al., 2008). Nitrification is often regarded as the "Achilles heel" of wastewater treatment because if nitrifiers are inhibited and removed from a system, reestablishment of nitrification is often a lengthy and expensive mission (Wagner and Loy, 2002). The importance of these nitrifiers and their biotechnological application in wastewater remediation highlights the need to understand the impact of long-term exposure of pharmaceuticals on the ecology and physiology of biological treatment microbial communities.

Unfortunately, there is a lack of understanding on PPCP's and their effects on the environment because of incomplete assessment data (Ternes and Joss, 2004). Since different classes of pharmaceuticals have different modes of action; no one really knows the extent of the effects of these compounds on the environment. For example, tetracyclines (TC) are a group of broad spectrum antibiotics used against the wide variety of gram positive and gram negative bacterial infections in human and veterinary medicine (Wang et al., 2010). They are also used as growth promoters in animal intensive industries. The mode of action of TC is blocking the

binding of aminoacyl tRNA to the ribosome thereby inhibiting protein synthesis (Jury et al., 2011). The widespread use of TC has lead to them being detected in surface water samples at concentrations ranging from 0.07-1.34µg/L (Lindsay et al., 2001).

The objective of this study was to assess the impact of tetracycline on the microbial community involved in the secondary treatment of municipal waste water using a semi-batch wastewater reactor system. In particular, chemical parameters such as ammonia concentration, nitrate/nitrite concentration and total Kjeldahl nitrogen were monitored to study the impact of tetracycline on the community that is specifically important for nitrogen removal. Also flow rates in wastewater treatment plants can vary due to weather conditions and various other factors which can lead to changes in microbial community composition. The effect of high and low nutrient replacement rates under tetracycline pressure was also assessed.

CHAPTER 2: LITERATURE REVIEW

2.1. Wastewater Treatment

The overall goal of wastewater treatment is to remove pollutants from wastewater and to protect and preserve Canadian natural water resources from contamination. Wastewater is the flow of used water that is discharged from homes, businesses, commercial activities and institutions (City of Guelph, 2007). The city of Toronto has a highly concentrated urban population that is responsible for contributing excessive organic, inorganic material and disease causing bacteria into the sewer system. As a result, the treatment of wastewater is an essential process that is used to prevent the sewage from contaminating drinking water and natural water resources (City of Toronto, 2008).

Wastewater treatment begins when wastewater treatment plants (WWTP) receive wastewater from various sources through the sewer system or the collection system. This is a network of pipes specifically designed to transport millions of gallons of wastewater that are generated each day. Wastewater is characterized and identified based upon the source it arrives from. The term "domestic wastewater" is wastewater that is discharged from residential sources. This wastewater is commonly generated by activities such as food preparation, laundry, cleaning and personal hygiene. Industrial/commercial wastewater is flow that is discharged from manufacturing and commercial activities such as printing, food and beverage processing. Institutional wastewater is wastewater that is discharged from institutions such as hospitals and educational facilities (City of Guelph, 2007).

After the wastewater is collected at the WWTP, it is first subjected to a preliminary process designed to mechanically remove large debris from the wastewater. This is achieved by the passing of wastewater through a series of screens or mechanical rakes. Since grit and other

debris present in wastewater can be very abrasive, the removal of these objects protects the plant's piping and makes for easier downstream processing.

Following the preliminary process, conventional wastewater treatment involves 3 main steps that have been maintained as a standard for wastewater treatment with slight modifications over time. Primary treatment begins with the screened wastewater flowing into large settling tanks with a reduced flow velocity of the influent wastewater. The slow rate of flow into the tanks allows the wastewater to be retained in these tanks for about 3-4 hours. These tanks promote the separation of heavy solid organic material from the liquid which tends to settle near the bottom of these tanks forming a raw or primary sludge (City of Guelph, 2007). This sludge is collected and discharged to other processing operations for further treatment. Lighter materials such as fats and oils that float to near the top surface of the tanks are usually skimmed off the surface and also are collected and transported for further treatment. The remaining liquid that contains non-settling, dissolved and suspended material is known as primary effluent. The primary effluent then flows into the secondary treatment which is also called the biological treatment of wastewater. In this step colloidal, dissolved organic and inorganic materials are metabolized and flocculated using microorganisms (City of Toronto, 2008). Biological treatment can involve the activated sludge process, biofiltration, rotating biological contactors or constructed aerated lagoons. Out of all the biological treatment methods, the main advantage of using the activated sludge process is that it produces a high quality effluent at a reasonable operating and maintenance cost (NESC, 2003). Therefore, the activated sludge process is the most commonly biological treatment method of wastewater. In biological treatment, oxygen is often required to provide an optimal condition for the growth of these microorganisms. After mixing the wastewater with microorganisms and allowing flocculation to occur, the wastewater

is directed to a secondary clarifier tank. The solids (usually a mixture of bacteria and organic matter arranged in flocs) are heavier here and settle to the bottom of the tank and are removed. After the secondary clarification step, the wastewater that remains is disinfected via chlorination or other disinfection methods to kill harmful organisms before being released into receiving waters (City of Guelph, 2007).

If necessary the wastewater is subjected to tertiary treatment. Tertiary treatment is usually implemented to further remove nutrients such as phosphorus and nitrogen from the wastewater.

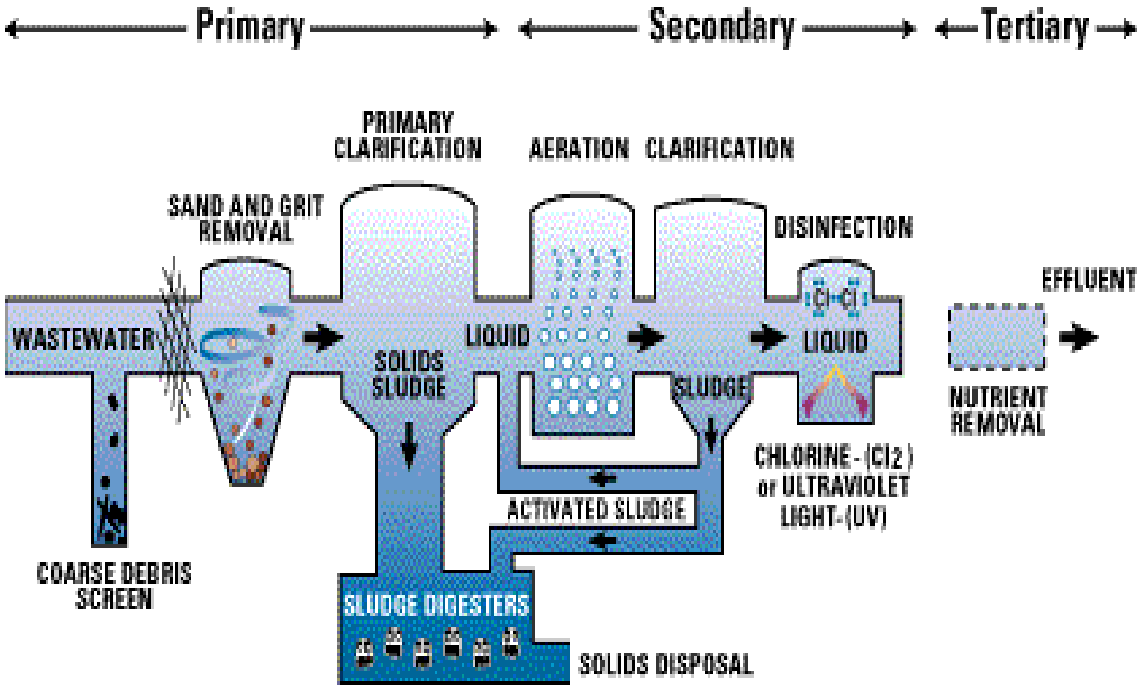


Figure 1. Conventional wastewater treatment process (WATER REUSE, 2010)

2.1.1. North Toronto Wastewater Treatment Plant and Treatment System

North Toronto Wastewater Treatment Plant (NTWTP) is one of the four wastewater treatment facilities owned and operated by the City of Toronto. It is located in the Don Valley at 21 Redway Rd. The plant covers an area of 27 hectares and serves a population of approximately

80,000 residents in the East York community and surrounding area by treating more than 7.5 million gallons per day (City of Toronto, 2008).

North Toronto wastewater treatment plant was one of the first plants in North America to implement the biological activated sludge process as part of their wastewater treatment system. The plant contains four primary, two secondary, and four storage digesters on site (City of Toronto, 2008).

The complete wastewater treatment system at NTWTP involves preliminary treatment, primary treatment and secondary treatment using the activated sludge process followed by disinfection. After the preliminary screening process, primary treatment process begins when the wastewater is directed to a primary settling tank where the velocity of the flow of wastewater is reduced to retain the wastewater in the tank for several hours (City of Guelph, 2007). During this time, heavier solid particles such as soil, sand, gravel and other grit are allowed to settle to the bottom of the tank and are physically removed through the use of pumps. The material that settles at the bottom of the tank during this stage is classified as primary sludge. The primary sludge component is pumped into digestion tanks where the process of solid reduction and gas production occurs (City of Toronto, 2008). Primary treatment removes approximately 60-70% of total solids that is contained in the influent of the plant (City of Guelph, 2007). Digested sludge is pumped from the NTWTP to the Coxwell Sanitary Trunk Sewer and then flows by gravity to the Ashbridges Bay Treatment Plant for collection and final disposal (City of Toronto, 2008). The remaining liquid present in the primary settling tanks known as primary effluent continues to flow into aeration basins for the initiation of the secondary treatment or biological treatment. In the aeration basins, the use of activated sludge process is used to remove organic waste from the wastewater (organic material remains in solution of settled sewage and does not settle during

primary treatment) (City of Guelph, 2007). The organic material that is present is a source of nutrients for microorganisms that exist in activated sludge. Ceramic disks are used to produce air bubbles that promote the growth of these microorganisms by providing oxygen and ensure mixing which allows contact with the reactants. The mixing of wastewater and activated sludge forms what is known as mixed liquor. Mixed liquor is then transferred to the secondary clarifier tank where the microorganisms flocculate and settle to form sludge. The settled sludge is removed from the bottom of the tank. The final product is produced when the remaining liquid from the secondary clarifier tank is disinfected with sodium hypochlorite before being discharged into the Don River (City of Toronto, 2008).

2.1.2. Biological Treatment Step: The Activated Sludge Process

The activated sludge process is the most widely used biological treatment method for treating domestic and industrial wastewater (Pholchan et al., 2010). Its primary function is to remove nutrients. The term nutrient is used to describe carbon, nitrogen and phosphorus compounds present in wastewater. The activated sludge process can be used to reduce nitrogen and phosphorus concentrations in the effluent which will prevent algal and other photosynthetic aquatic organism growth in the receiving water and avoid the eutrophication of receiving water bodies (EPA, 1997). In the activated sludge process, effluent from primary treatment is pumped into an aeration tank and is mixed with bacteria rich slurry known as activated sludge (Maier et al., 1999). The activated sludge consists of sludge particles that arise from the growth of bacteria in the presence of free dissolved oxygen. The term “activated” arrives from the fact that activated sludge contains live aerobic microorganisms such as algae, bacteria, fungi and protozoa (City of Guelph, 2007). As these microorganisms start to grow in the presence of various nutrients present in wastewater, they form solid particles or flocs that clump together (NESC, 2003). Air

or pure oxygen is pumped through the mixture and promotes bacterial growth, flocculation and decomposition of the organic and inorganic material (Maier et al., 1999). After being retained for 4-6 hours in aeration tanks or basins, this mixture called mixed liquor is then transferred to a secondary clarifier tank (City of Guelph, 2007). The floc that settles to the bottom of the tank (usually referred to as sludge) is removed leaving behind a clear liquid that is devoid of organic materials and suspended solids. A portion of the sludge known as return sludge is often returned to the aeration tank to be used as inoculum for the incoming primary effluent (NESC, 2003). The activated sludge process is an economical and an effective method to remove organic compounds from wastewater (Kowalchuk and Stephen, 2001). It is known that the activated sludge process removes certain pollutants with an efficiency of 90% or more (Viessman and Hammer, 1998).

2.1.3. Factors Impacting the Activated Sludge Process

It is common knowledge that the activated sludge process relies on the mixing of microorganisms and wastewater in an aerobic environment to facilitate the decomposition of organic matter. However, there has been only a modest understanding of how activated sludge systems work resulting in limited ability to predict their performance or the causes of malfunction (Pholchan et al., 2010). Since microorganisms are crucial to the efficiency of the activated sludge process, there is a need for increased understanding of the microbial communities and their relationship to treatment performance (Pholchan et al., 2010). That is why information about microbial community diversity and its functions along with parameters that affect their growth and biochemical activities can be very useful (Sanz and Kochling, 2006). If parameters are not controlled then adverse effects on sludge settlement can occur, such as bulking and foaming caused by filamentous organisms (Pholchan et al., 2010). The next section

will discuss the various parameters affecting the activated sludge process in wastewater treatment plants.

2.1.3.1. The Impact of Oxygen, Temperature and pH

The activated sludge process is dependent on microorganisms because their metabolic activity allows for removal of organic and inorganic matter from the wastewater. Due to the sensitivity of microorganisms to environmental factors, specific parameters must be maintained and controlled for desired microbial growth. There is a reason as to why the activated sludge process usually takes place in aeration tanks. The accurate control of oxygen concentrations is vital because oxygen is required for the respiration of the microorganisms present in the activated sludge process. For example, heterotrophic bacteria in the presence of free oxygen can break down fats, carbohydrates and proteins. Too little oxygen can lead to decreased metabolism of the microorganisms and hinder efficiency of the process (EPA, 1997). The biochemical oxygen demand (BOD) can be used to measure the amount of organic matter present in wastewater. The BOD is the amount of molecular oxygen required to oxidize degradable matter present in wastewater by biochemical oxidation processes. The two basic reactions for the oxidation of organic matter can be described as follows:

1) Organic Matter + O_2 $\longrightarrow$ CO_2 + H_2O+ ENERGY
2) Organic Matter + P + NH_3 + O_2 + ENERGY $\longrightarrow$ NEW CELL MATERIAL

(EPA, 1997)

Thus, it is important that the oxygen added must satisfy the influent BOD. For successful biological nutrient removal the dissolved oxygen concentration must be between 1-2 mg/L (EPA, 1997).

The maintenance of pH must also be within the 6.5 and 8.5 range (Grady and Lim, 1980). It is reported that the optimum pH for nitrification which is a crucial process that occurs in this step is 8.2-8.6 range (Viessman and Hammer, 1998). And lastly the temperature of the activated sludge system can also impact the growth of microorganisms and the overall efficiency of nutrient removal. The optimal temperature range for biological treatment systems are from 20°C-40°C. Since the activated sludge process introduces air to provide oxygen and mixing, the temperature is modified to a range of 15-25°C (Viessman and Hammer, 1998).

2.1.3.2. Hydraulic and Organic Loading Rate and Flocculation

Microbial communities in activated sludge systems are likely to be affected by parameters such as influent characteristics, environmental conditions and system design and operation (Pholchan et al., 2010). The hydraulic flow entering a wastewater treatment plant can vary from hour to hour, day to day, week to week and season to season. Also the organic loading rate (OLR) which is determined by the volume and strength of domestic and industrial wastewater entering the plant can also impact the structure of microbial communities and overall efficiency of nutrient removal in activated sludge systems (EPA, 1997). For example, one study demonstrated that reactors operated at low OLR had better carbon removal and nitrification rates than the ones operated at high OLR (Pholchan et al., 2010). Microbial community diversity was also impacted by these parameters as well. A greater number of DGGE bands was present for ammonia oxidizing bacteria (AOB), in reactors operated at low OLR relative to high OLR, indicating higher diversity of AOB in the reactors operated at low OLR (Pholchan et al., 2010).

Microorganisms involved in the activated sludge process can mix with particles in wastewater and form various spatial structures such as activated sludge flocs, biofilm or granules (Wojnowska-Baryla et al., 2010). This is an important characteristic of the microorganisms as

bioflocculation is the key to efficient solid-liquid separation of activated sludge from treated waste (Wilen et al., 2006). The formation of floc is important especially when the wastewater enters the secondary clarifier tank because its characteristics will determine how well it settles and separates from the wastewater.

Only a fraction (5-20%) of the organic matter in the sludge floc is made up of bacteria. The majority of the floc contains mainly extracellular polymeric substances (EPS). The importance of EPS is that it holds the microorganisms together in a matrix onto which organic fibers, organic and inorganic particles as well as various colloids can adsorb. The floc stability can be decreased by environmental stresses such as sudden temperature changes, pH variations and toxic compounds. The process of deflocculation caused by the above factors can cause an increased number of small flocs and free bacteria which will remain suspended and not settle (Wojnowska-Baryla et al., 2010). This will cause problems in the proper treatment of wastewater.

2.2. Microbial Community Composition in an Activated Sludge System

Selection of microorganisms through their ability to produce energy under specific conditions has been used as a basis for biological nutrient removal (Wojnowska-Baryla et al., 2010). The major groups of microorganisms found in activated sludge process include bacteria, protozoa, fungi, algae and filamentous organisms (EPA, 1997). The removal of carbon, nitrogen and phosphorus from wastewater is usually achieved by prokaryotic communities. Due to their ability to mineralize organic and inorganic nutrients, they are observed to dominate in terms of number and thus represent a core component of the activated sludge process (Madoni, 2011). The importance of protozoa and their role in wastewater treatment is often overlooked. Protozoan populations are commonly found in the mixed liquor of activated sludge tanks. The

conditions of low organic load and high sludge retention time in the current systems of wastewater treatment lead to the common occurrence of protozoa such as ciliates, flagellates, amoebae and even small metazoa (Madoni, 2011). It is believed that protozoa contribute to wastewater treatment by clarifying the effluent. The direct effect of protozoa is accomplished by their ability to graze on bacteria (Madoni, 2011). Some protozoa such as crawling ciliates and other forms can eat flocculated bacteria but most protozoa are attached ciliates that can only graze on suspended bacteria and particles resulting in a clearer effluent (Madoni, 2011). Studies have also been performed on the role of protozoa predation in nitrogen cycling in activated sludge (Verhagen and Laanbroek, 1991; Petropoulos and Gilbride, 2005 and Pogue and Gilbride, 2007). It has been determined that the presence of protozoa can increase the per-cell nitrification rate, due to the fact that protozoa have the ability to influence bacterial growth.

2.2.1. Bacteria in Activated Sludge

In the activated sludge process, heterotrophic bacteria can use organic compounds present in wastewater for energy and cell synthesis. These organic compounds are easily biodegradable so the heterotrophic bacteria can grow more efficiently. Their doubling time can be measured in hours and in some cases minutes (EPA, 1997). Most bacteria found in activated sludge systems are chemoheterotrophs. They are responsible for degradation of organic materials present in wastewater because they metabolize these compounds to simpler metabolic end products such as carbon dioxide or form cell biomass. In contrast, autotrophic bacteria derive their cell carbon from CO_2 and obtain energy from inorganic sources. The autotrophic bacteria are slow growers when compared to the heterotrophic bacteria present in wastewater. Autotrophic bacteria have long generation times due to the low energy yield from their oxidation reactions. Most of their energy is devoted to fixing CO_2 and the calvin cycle which leaves very little energy for

reproduction and growth. Due to low number of these bacteria, they are also more susceptible to toxic shocks, temperature and pH fluctutations (EPA, 1997).

Three main groups of bacteria that are most important in the activated sludge process are filamentous bacteria, bacteria that are responsible for nitrogen removal and bacteria that are responsible for phosphorus removal (Wagner and Loy, 2002). Data based on 16s ribosomal ribonucleic acid (rRNA) gene library analysis has led to the identification of major groups of bacteria that are most commonly found in activated sludge. It is found that alpha, beta, gamma *Proteobacteria* along with bacteroidetes and firmicutes were most frequently found in wastewater treatment reactors (Wagner and Loy, 2002). Among these groups the beta-*Proteobacteria* are observed to be the most dominant, followed by alpha-*Proteobacteria* and gamma-*Proteobacteria*. These findings are consistent with results obtained by FISH studies of activated sludge systems (Wagner and Loy, 2002).

A certain number of filamentous bacteria are important in the activated sludge process due to their ability to enhance floc formation. However, the occurrence of large filamentous bacterial populations can hinder the activated sludge process (Wagner and Loy, 2002). Its detrimental problems include foaming or settling problems known as bulking. Among others, the filamentous organism *Microthrix parvicella* is observed to be the most common cause of these problems (Wagner and Loy, 2002).

The process of nitrification and denitrification which results in ammonia (major nitrogen compound in sewage) removal from wastewater is a major biotechnological application of microorganisms. Nitrification is the chemolithoautotrophic oxidation of ammonia to nitrate under strict aerobic conditions. Nitrification is a two step process where two different groups of

bacteria are responsible. The first oxidative step of converting ammonia to nitrite; a process called ammonia oxidation is catalyzed by ammonia oxidizing bacteria (AOB). The AOB are genetically diverse but are related to each other in the beta subdivision of *Proteobacteria* (Ahn, 2006). The second oxidative step of converting nitrite to nitrate; a process called nitrite oxidation is catalyzed by nitrite oxidizing bacteria (NOB). The most famous NOB is known as *Nitrobacter* is related to the alpha subdivision of *Proteobacteria* (Ahn, 2006). The AOB and NOB use ammonia and nitrite respectively as an energy source with oxygen acting as a final electron acceptor and carbon dioxide as the carbon source.

The complete nitrogen removal is achieved by denitrification which occurs by a heterotrophic bioconversion process in an anaerobic environment. The oxidized nitrogen compounds from nitrification (NO_2 and NO_3) are reduced to gaseous N_2 by heterotrophic microorganisms. Common denitrifiers are found usually within the gram-negative alpha and beta classes of *Proteobacteria*.

The remarkable advances in molecular biological techniques have led to the identification of the anaerobic ammonium oxidation process or ANAMMOX process. This process is achieved under anoxic conditions by autotrophic ANAMMOX bacteria that oxidize ammonia to dinitrogen gas using nitrite as their electron source (Strous et al., 1998; Van Hulle et al., 2010). The ANAMMOX bacteria have an unusual physiology because they can consume ammonia in the absence of oxygen (Ahn, 2006). The ANAMMOX process is a promising alternative to traditional nitrification-denitrification technology because it can produce higher nitrogen removal rates, has low operational costs and has smaller space requirements (Jin et al., 2012).

However, the implementation and application of the ANAMMOX process does pose a few challenges. Growth characteristics of the ANAMMOX bacteria are often subjected to inhibition by nitrogen-rich wastewater. In addition, ANAMMOX bacteria have a slow growth rate and are highly sensitive to environmental conditions making them extremely difficult to cultivate (Jin et al., 2012).

2.3. Nitrogen Cycling in Activated Sludge Process

The eutrophication of many ecosystems has led to an increased interest in the ecology of nitrogen transformation (Kowalchuk and Stephen, 2001). In countries such as China, the major surface water resources have been polluted by untreated or insufficiently treated wastewater (Xia et al., 2000). The overall goal of wastewater treatment is to ensure that pollutants are removed from the wastewater before being released into receiving waters. In biological treatment step, this is achieved by the utilizing heterotrophic and autotrophic microorganisms that are able to produce energy from nitrogenous compounds under specific conditions (Wojnowska-Baryla et al., 2010). In conventional treatments, the biological elimination of nitrogen from wastewater is made by possible by two key biochemical processes involving nitrification followed by denitrification (Ahn, 2006). Nitrification is performed by chemolithotrophic oxidation of ammonia to nitrate under strictly aerobic conditions (Halling-Sorensen and Jorgensen, 1993). Denitrification is the second key process and is achieved by heterotrophic bacteria that convert the nitrate to dinitrogen gas that is eventually lost into the atmosphere.

The following sections will provide a detailed explanation of nitrification and denitrification and which nitrifying and denitrifying species are involved in the biological treatment of wastewater.

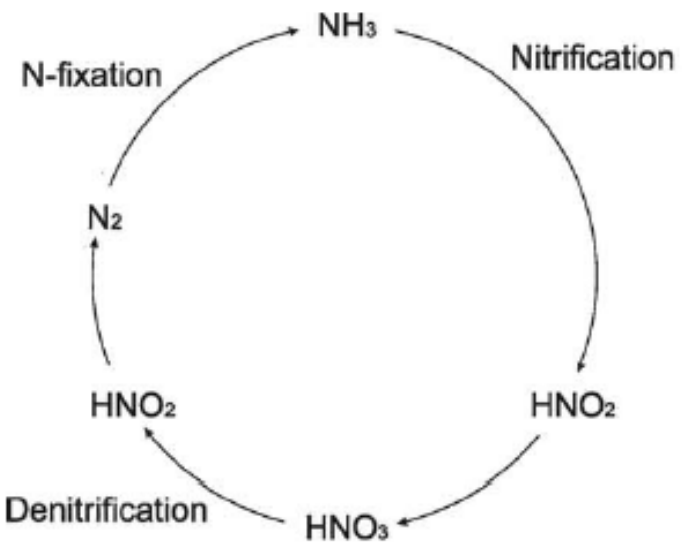


Figure 2. Classical nitrogen cycle (Ahn, 2006. pp. 1710)

2.3.1. Nitrification

Ammonia is one of the pollutants present in high concentrations in domestic wastewater. The primary sources of ammonia in domestic wastewater are excretory materials such as feces, urine and food processing discharges. In wastewater, 40% of the nitrogen present is in the form of ammonia (Viessman and Hammer, 1998). The need to remove ammonia from waste is quite significant because of its presence in the environment can cause concern. An ideal and inexpensive way of ammonia removal is achieved by nitrification in the biological treatment reactors using AOB and NOB (Wang and Liu, 1999). The nitrification process is the conversion of ammonia to nitrate.

2.3.1.1. The Process of Nitrification

Chemolithotropic nitrification is a two-step process. It involves the oxidation of ammonia to nitrite (Step 1); nitrite is then oxidized to nitrate (Step 2). It is found that no known bacteria

can directly catalyze the production of nitrate from ammonia. Thus the two steps in nitrification are carried out by two different groups of slow growing autotrophic bacteria called AOB and NOB (Kowalchuk and Stephen, 2001).

Ammonia oxidation of ammonia (NH_3) to nitrite (NO_2) is also carried out in two steps, involving two enzymatic catalysis reactions. Ammonia is oxidized to hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO), and then NH_2OH is oxidized to NO_2 by hydroxylamine oxidoreductase. Ammonia monooxygenase is an enzyme that has multi-subunits, broad specificity and is common to all AOB (Kowalchuk and Stephen, 2001). In nitrite oxidation, chemolithotropic NOB under oxic conditions extract energy from the oxidation of nitrite (NO_2) to nitrate (NO_3). This nitrite oxidation is the second step in nitrification and represents a major biogeochemical process in aquatic and terrestrial ecosystems (Sorokin et al., 2012).

2.3.1.2 Nitrifying Microorganisms in the Activated Sludge Process

Nitrifying bacteria such as ammonia oxidizers (AOB) and nitrite oxidizers (NOB) are unique in their ability to convert ammonia to nitrite and nitrite to nitrate (Kowalchuk and Stephen, 2001). For AOBs in wastewater treatment systems, different facilities can support different populations and species richness (Rowan et al., 2003). For example, one study demonstrated that the *Nitrosomonas* like populations most dominated activated sludge reactors (Dionisi et al., 2002). While another group suggested that *Nitrospira* like group seem to prevail in some bioreactors (Sofia et al., 2004). These results imply that the species of AOBs found in wastewater treatment may differ based on the type of wastewater, as well as dynamic characteristics of wastewater treatment plants such as aeration intensity, reactor configuration, solid retention time and hydraulic retention time because all these combine to present significantly different environments (Park and Noguera, 2004). However, the bacteria that is

most commonly recognized as an ammonia oxidizer is *Nitrosomonas* (Ahn, 2006). Other genus such as *Nitrosococcus, Nitrosopira, Nitrosovibrio* and *Nitrosolobus* are also able to oxidize ammonia to nitrite.

For nitrite oxidizers, the most famous genus is the *Nitrobacter* which is closely related to the alpha subdivision of *Proteobacteria* (Ahn, 2006). Other nitrite oxidizers such as *Nitrospira, Nitrospina, Nitrococcus, Nitrocystis* are also known to be involved with nitrite oxidation (Ahn, 2006). The cultivation of nitrite oxidizing bacteria is difficult which owes to the restriction of knowledge of the physiological and genomic properties of this group (Sorokin et al., 2012).

2.3.2. Denitrification

Denitrification is essential for complete removal of nitrogen in WWTPs. Biological denitrification enables the conversion of oxidized nitrogen compounds such as nitrite and nitrate to be converted into harmless nitrogen gas by the activity of denitrifying bacteria. Recently developed processes sheds new light on denitrification which was originally thought to be a heterotrophic process and exclusively a facultative anaerobic or a microaerophillic process. New processes such as aerobic denitrification (Robertson and Kuenen, 1984), autotrophic denitrification (Zumft, 1992) and the ANAMMOX process (Schmidt et al., 2003) are novel and cost effective alternatives that can offer advantages such as reduction in energy and low carbon subtrates (Yang et al., 2011).

In conventional denitrification, heterotrophic bacteria under anoxic conditions use nitrite and nitrate instead of oxygen as final electron acceptors and organic matter as a carbon and energy source (Ahn, 2006). As a heterotrophic process, denitrification may appear to be less sensitive to environmental parameters. But it has been demonstrated that variations in dissolved

oxygen, availability of organic carbon and presence or accumulation of NO_2 and NH_4 can affect it (Tiedje, 1988; Islam et al., 2009).

Aerobic denitrification can be advantageous because it occurs directly in aerated bioreactors under the presence of readily biodegradable organics. Most aerobic denitrifiers are heterotrophs. They have the ability to co-respire nitrate and oxygen and are widespread in the environment (Robertson and Kuenen, 1984).

In autotrophic denitrification, the energy can be derived from inorganic oxidation-reduction reactions with elements such as hydrogen and various reduced sulfur compounds (HS^-, H_2S etc.). Autotrophic denitrifiers can use inorganic sulfur compounds, hydrogen, ammonia or nitrite as electron acceptors (Zumft, 1992). It is observed that these autotrophs are also slow growing as their doubling rate requires much more time when compared to the autotrophic bacteria that are involved in nitrification (Jetten et al., 2001; Strous et al., 1999)

Among nitrogen removal processes, ANAMMOX offers a novel, energy saving and cost effective biological nitrogen removal method (Wang et al., 2009). Anaerobic ammonium oxidation process involves autotrophic bacteria that can convert ammonium to N_2 using nitrite as the electron acceptor (Pathak et al., 2006). The ANAMMOX process removes only about 90% of the incoming nitrogen as ammonium/nitrite, leaving about 10% of nitrogen as nitrate in the effluent (Wang et al., 2009). Therefore, it is suggested that a combination of both ANAMMOX and denitrification is needed to provide the complete solution to nitrogen removal (Pathak et al., 2006).

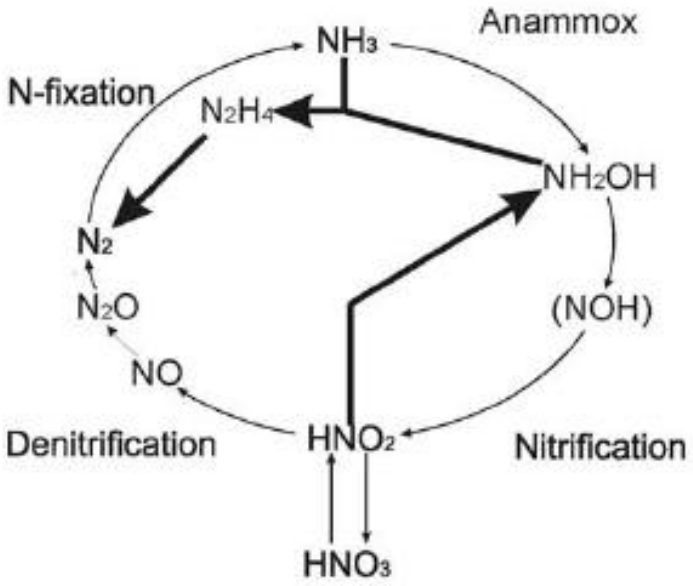


Figure 3. Anaerobic Ammonium Oxidation (ANAMMOX) (Ahn, 2006. pp1710)

2.3.2.1 Denitrifying Microorganisms in the Activated Sludge Process

Denitrifying bacteria are all common to the gram negative alpha and beta classes of the *Proteobacteria* such as *Pseudomonas*, *Alcaligenes*, *Parococcus* and *Thiobacillus* (Ahn, 2006). The first aerobic denitrifier was observed in the *Parococcus* genus named *Parococcus denitrificans* (Ahn, 2006). For autotrophic denitrification, *Thiobacillus denitrificans* is a widely distributed and well known for its ability to couple sulfur compounds (such as hydrogen sulphide and thiosulphate) to denitrification (Ahn, 2006). *Planctomycete* bacteria are one of the groups that are known to be responsible for the ANAMMOX process (Jetten et al., 2001).

Table 1. List of autotrophic and ANAMMOX bacterial species that are important in nitrogen removal in wastewater treatment systems (modified from Ahn, 2006. pp.1714)

Type	Species	References
Aerobic Denitrification	*Paracoccus denitrificans*	Ludwig et al., 1993
	Pseudomonas carboxydoflava	Moir et al., 1996
	Thiosphaera pantotropha	Robertson and Kuenen, 1984
NO_2^- or NH_4^+ oxidation	*Nitrosomonas*	Poth et al., 1985
	N. europea	Bock et al., 1995 and Schmidt
	N. eutropha	and Bock, 1997
	Nitrobacter	Ahlers et al., 1990
	N. vulgaris	
Anaerobic ammonium oxidation	*Candidatus Brocadia anammoxidans*	Jetten et al., 2001
	Candidatus Kuenenia stuttgartiensis	Schmidt et al., 2000
	Candidatus Scalindua brodae	Schmidt et al., 2003
	Candidatus Scalindua wagneri	Schmidt et al., 2003
	Candidatus Scalindua Sorokinii	Schmidt et al., 2003

2.4. Contaminants in Wastewater

2.4.1. Pharmaceuticals in Wastewater

The focus of environmental research has gone beyond studying the impacts of classic pollutants to studies investigating the impact of "emerging" pollutants such as pharmaceuticals and personal care products (PPCPs). Recently, increased attention has been given to the presence of pharmaceuticals in wastewaters, surface waters and ground waters (Verlicchi, 2010). A large proportion of the pharmaceuticals are released into the environment through the wastewater stream. After being consumed, the active substances of these products are metabolized only to some extent. It is observed that 30-90 % of pharmaceutical products ingested by humans are excreted in the urine or feces (Robinson et al, 2007). These compounds can take on a "pseudo-

persistent" quality because of the continuous influx of pharmaceuticals that arrive from various waste streams such as hospital wastewaters and municipal wastewaters (Daughton, 2004). Figure 2.1 demonstrates the routes and occurrences of pharmaceutical wastes.

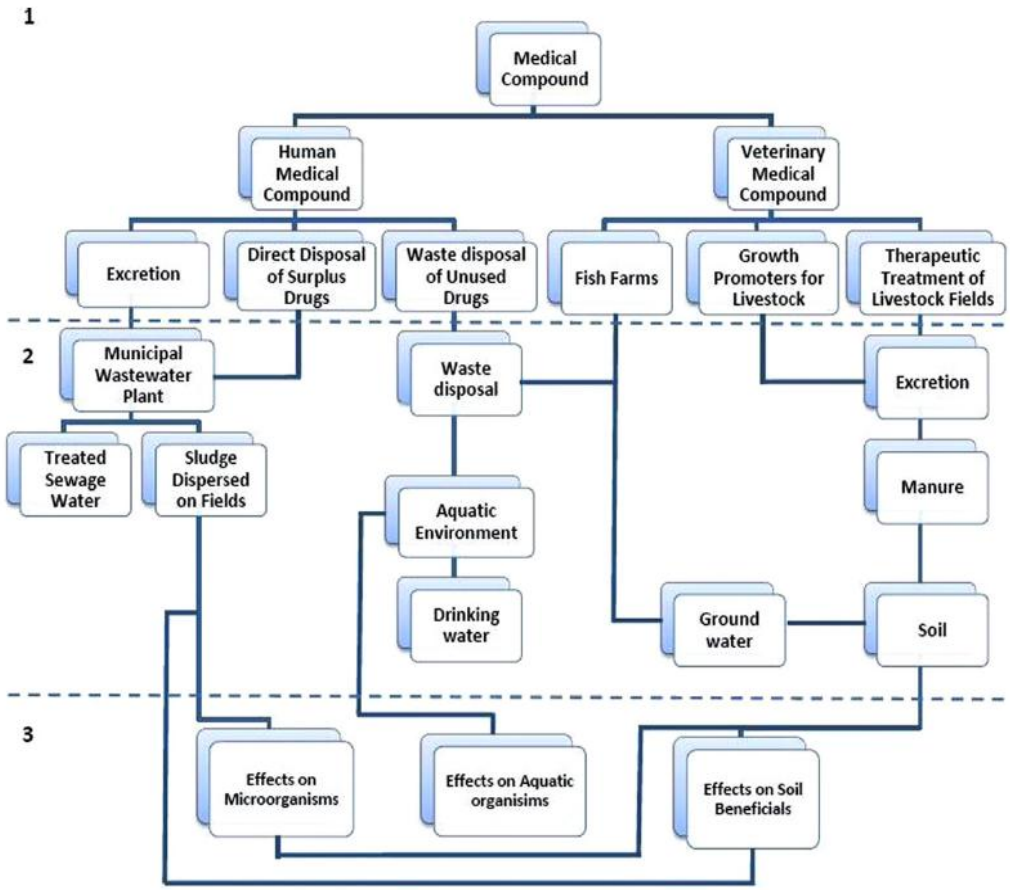


Figure 4. The sources of pharmaceutical waste and their occurrences in the environment (Ziylan and Ince, 2011. pp25)

Although highly efficient, WWTP's are not specifically designed to remove the diverse pharmaceutical products present within the wastewater. This is of concern since the severity of chronic exposure of low concentrations of these drugs on aquatic biota is unknown (Cooper et al., 2007). Specifically, the impact of these compounds on the microorganisms commonly found within the biological treatment system such as the bacteria and protozoa is not known. While

there is variability, the average sewage treatment plant influent concentrations are reported to be in the 0.1-1ug/L range (Verlicchi et al., 2010). Table 2 summarizes a list of pharmaceuticals and their average concentrations that have been detected in hospital and urban wastewater streams. Hospitals are a major source of pharmaceutical wastes and hospital wastewater characteristics can differ from urban waste water. Although analytical methods have allowed for the determination of pharmaceutical concentrations in wastewater, there has not been a complete assessment of the impact of these pharmaceuticals on organisms to those levels. Chemical compounds such as pharmaceuticals can potentially alter the microbial community composition in municipal wastewater treatment. The constant rise in the concentrations of these pharmaceutical products in wastewater streams justifies the need to investigate the effect of these compounds on the wastewater treatment process.

Table 2. Classes of pharmaceuticals and their average concentrations detected in hospital wastewater (HWW) and urban wastewater (UWW) streams (Verlicchi et al., 2010. pp. 422)

Therapeutic class	HWWs, average values	UWWs, average values	$\frac{HWWs_{av}}{UWWs_{av}}$
Analgesics, μg L^{-1}	100	11.9	8–15
Antibiotics, μg L^{-1}	11	1.17	5–10
Cytostatics, μg L^{-1}	24	2.97	4–10
β-blockers, μg L^{-1}	5.9	3.21	1–4
Hormones, μg L^{-1}	0.16	0.10	1–3
ICM, μg L^{-1}	1008	6.99	70–150
AOX, μg L^{-1}	1371	150	7–15
Gadolinium, μg L^{-1}	32	0.7	35–55
Platinum, μg L^{-1}	13	0.155	60–90
Mercury, μg L^{-1}	1.65	0.54	3–5

2.4.2. Presence of Antibiotics in Wastewater Streams

Antibiotics are a class of naturally-occurring, semi synthetic and/or synthesized compounds with antimicrobial activity. These compounds are used to combat bacterial infections and are consumed in large volumes by humans and animals to treat and prevent diseases. They

are also used as growth promoters in animal intensive industries (Jury et al., 2011). In a report for antibiotic consumption in Germany, the total consumption of antibiotics in human medicine was estimated to be approximately 250-300 tons per year (Schmidt et al., 2012). Some of these antibiotics are incompletely metabolized during therapeutic use and are excreted into sewage in their native structure. It is not surprising to find detectable levels of these compounds in wastewater influent. There are several classes of antibiotics found in WWTPs including β-lactams, sulfonamides, trimethoprim, macrolides, fluoroquinolones and tetracyclines (Jury et al., 2011). Antibiotic concentrations in WWTP influents have been detected between 0.1-1.0 µg/L (Jury et al., 2011). Some retention of antibiotics in wastewater could be due to adsorption. For the class of fluoroquinolones, it has been reported that approximately 70% of antibiotic in the aqueous phase remains adsorbed to sludge during wastewater treatment (Lindeberg et al., 2006). Since the mode of action of antibiotics is to inhibit or kill microorganisms, it is understandable that they could affect the composition, structure and function of the bacterial community found in the secondary step of the WWTP. Presence of antibiotics in wastewater treatment plants could influence different degradation processes such as elimination of chemical oxygen demand (COD) and nitrification (Schmidt et al., 2012). Nitrification/ Denitrification are important nitrogen removal processes achieved by microorganisms in the secondary treatment step of wastewater treatment plants.

Nitrification is more susceptible to antibiotics because autotrophic nitrifiers are less numerous and have a slower growth rate. This results in the presence of lower numbers of autotrophic bacteria than heterotrophic bacteria. Although there is a lack of complete understanding, there is evidence of the subtle effects of these compounds on the microbial community responsible for biological nutrient removal. One study demonstrated that a mixture

of antibiotics ciproflaxin (CIP), gentamycin (GM), sulfamethoxaole (SMZ), trimethoprim (TMP) and vancomycin (VA) at a concentration of 30mg/L inhibited nitrification at the nitrite oxidation step resulting in accumulation of nitrite (Schmidt et al., 2012). In another study, Costanzo et al. (2004) found that three antibiotics, erythromycin, clarithromycin, and amoxicillin at 1000 μg/L concentrations significantly decreased denitrification rates by benthic bacteria. There is also convincing evidence that ANAMMOX bacteria and their activity are inhibited by antibiotics. There have been studies published on 3 kinds of antibiotics and their inhibition of ANAMMOX bacteria. These antibiotics belong to the classes of chloramphenicol, β-lactams and tetracycline (Jin et al., 2012).

The exposure of virulent bacteria to antibiotic residues in wastewater could also induce resistance (Cooper et al., 2007). Clinical infections, disease and death caused by resistant bacteria are increasingly common and the resistance to a wide range of antibiotics by microorganisms is a major concern in modern medicine. There is speculation that wastewater treatment plants can provide an environment for the establishment and propagation of antibiotic resistance bacteria (Kim and Aga, 2007). Natural antibiotic producers are already resistant to the antibiotics they produce (Jury et al., 2011). Other bacteria develop resistance by developing or acquiring antibiotic resistance mechanisms. They can achieve resistance by altering permeability barriers across bacterial outer membranes, preventing uptake of the compound by inhibiting its corresponding transport carrier, modifying the target's binding sites so that it no longer can recognize the antibiotic and by chemically or with the use of enzymes degrade the antibiotic (Jury et al., 2011). It is not yet known if the low concentrations found in the environment are able to cause resistance, however high numbers of antibiotic resistant bacteria and genes have been isolated from surface waters close to sewage discharge centers (Al-Bahry, 2009).

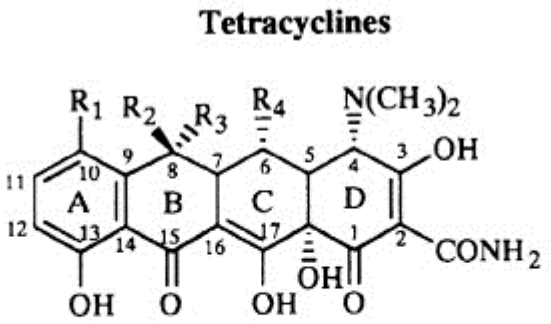
2.4.2.1. Tetracyclines in Wastewater

Tetracyclines are a group of broad-spectrum antibiotics that have been used since the 1940s against a wide range of both Gram-negative and Gram-positive bacteria (Wang et al., 2010). Tetracycline antibiotics act by blocking the binding of aminoacyl tRNA to the ribosome thereby inhibiting protein synthesis (Todar, 2002). Tetracyclines are used frequently in human and veterinary medicine to combat bacterial infections and at sub-therapeutic levels are used to prevent epidemics and to promote growth and weight gain in livestock and aquaculture animals. Tetracycline is the second most used class of antibiotics after penicillin (Sarmah et al., 2006). Due to the widespread use of TC, they have been introduced to the environment through various routes such as hospital and municipal wastewater, manure from the agricultural industry and agricultural runoff. In recent years, TCs have been frequently detected at concentrations of 0.07-1.34 µg/L in surface water samples, 86-199 µg/kg in soils, 4 µg/kg in liquid manure and at 3 µg/L in farm lagoons (Wang et al., 2010). Verlicchi et al., (2010) also report tetracycline has been detected at a concentration of 1 µg/L in urban wastewater and can reach as high as 10µg/L in hospital wastewater.

The increasing number of tetracycline resistance genes also raises concerns. There are 38 known tetracycline and oxytetracycline resistance genes (Roberts, 2005). Out of these genes, 23 encode for efflux pumps, 11 encode for ribosomal proteins, 3 for an inactivation enzyme and 1 for a mechanism not yet known (Jury et al., 2011). Gram negative efflux pump genes are often linked to large conjugative plasmids. Many of these plasmids contain resistance mechanisms for several other antibiotic drugs thus the selection for resistance to tetracycline will generally render recipient bacteria to be multi-resistant (Chopra and Roberts, 2001). The ribosomal proteins are protection proteins for the ribosome from the actions of tetracycline. Other means of resistance

by tet(x), tet(34) and tet(37) genes encode for proteins that can enzymatically alter the tetracycline drug (Diaz-Torres et al., 2003).

Tetracyclines



	R_1	R_2	R_3	R_4
Tetracycline (TC)	H	CH_3	OH	H
Chlortetracycline (CTC)	Cl	CH_3	OH	H
Oxytetracyline (OTC)	H	CH_3	OH	OH

Figure 5. Molecular structure of tetracycline commonly used in human and veterinary medicine (Sarmah et al., 2006)

CHAPTER 3: PURPOSE

The secondary treatment step or the activated sludge process is one of the most important stages of wastewater treatment. In the step, nitrogen removal is a crucial process that must be accomplished in order to avoid contamination and devastating eutrophication of Canadian natural water resources. Nitrification and Denitrification are two key processes achieved by nitrifying and denitrifying bacteria that are present in the secondary treatment step or the activated sludge process of wastewater treatment. Due to their characteristic slow growth rate and generally low population, nitrifiers are more sensitive to toxic shock than other microorganisms. The value of their biotechnological application justifies the need to evaluate the impact of pharmaceuticals on the microbial communities that are responsible for key degradation processes.

Toronto, Canada is an ideal example of a large and growing urban center. Pharmaceuticals and personal care products readily enter the wastewater stream and in high volumes from sources such as the numerous hospitals located in and around the city of Toronto, drug industries, and wastewater that is contributed from every household. This characteristic makes the city of Toronto an ideal location to test effects of pharmaceuticals on the environment. Although analytically the concentrations of various classes of pharmaceuticals have been detected in wastewater streams, there is a lack of research on the chronic effects of these compounds on microorganisms that are crucial to the biological treatment of wastewater. Since tetracyclines are widely used in human and veterinary medicine and are frequently detected in wastewater streams; their impact on the secondary treatment microbial community in the activated sludge process is required.

It is also important to consider that the microbial community in the secondary treatment can be altered by characteristics such as hydraulic retention times, flow rates and organic loading rates. These parameters can change from day to day, week to week and season to season. The introduction of nutrient replacement rates can help simulate these conditions. This study will examine whether the varying nutrient replacement rates will alter the effect of tetracycline on the microbial communities present in the secondary treatment of wastewater.

Therefore the main goal of this study was to monitor the impact of tetracycline on the nitrifying and denitrifying community present in the secondary treatment of wastewater through the assessment of their function (measuring intermediate products in nitrification and denitrification by nutrient analysis) under high and low nutrient replacement rates.

The short term objectives to achieve this goal were:

1. To implement a semi-batch reactor system containing wastewater effluent from the secondary process of a MWTP. The reactors were supplemented with synthetic wastewater at two different replacement rates and contained concentrations of tetracycline of either 1µg/L or 10µg/L to mimic environmental concentrations.
2. To assess nitrogen processing in the reactors by measuring ammonia, nitrate, nitrite and total Kjeldahl nitrogen concentrations.

The lack of data on the impact of pharmaceuticals and personal care products on the biological treatment of wastewater indicates that there is a lack of understanding to how these compounds can affect the wastewater treatment. The expected outcome of this research will examine the effects of low dose antibiotics on the effectiveness of nitrogen removal in wastewater treatment. There is economic interest towards the high value of achieving complete

nitrogen removal and maintaining a stable community that are responsible for this process in wastewater treatment plants.

CHAPTER 4: MATERIALS AND METHODS

4.1. Semi batch reactor system

The reactors were inoculated with an activated sludge sample from the secondary treatment aeration basin of the North Toronto Wastewater Treatment Plant. Approximately 12L of activated sludge was collected and transported from the plant to the lab in two plastic containers, each with a capacity of 6 L. Within 2 hours, the sample was divided into three 4 L flasks and aerated for 7 days to acclimatize the sample to the lab environment. An aliquot of the activated sludge was centrifuged and the pellet was stored at -20°C as a source of DNA from the original sample for later use.

4.1.1. Reactor set up

After 7 days, 18 semi-batch reactors were setup using commercially available 1L plastic containers. Each reactor contained 500mL of activated sludge sample. The reactors did not have lids and were open systems. Aeration of the reactors was important to achieve in the lab to ensure proper simulation of the aeration tank conditions. Commercially available rubber tubing was attached to the lab air supply, then using flow rate limiters or valves air was supplied to the flasks. To minimize the loss of sample through evaporation, tubing from the central air supply nozzle was circulated through a moisture flask which contained autoclaved water before being delivered to the reactors. Fish aquarium bubbling stones were used to achieve aeration and mixing within the flasks.

Figure 6 provides a detailed description of the reactor set up. The 18 reactors were divided into 6 groups of three to mimic various conditions. Nine reactors were treated as high nutrient replacement rate reactors and 9 were low nutrient replacement rate reactors. Overall

there were 6 combinations with triplicate reactors. For high nutrient replacement conditions, there were 3 control reactors that received effluent with no tetracycline added, 3 reactors with 1µg/L tetracycline effluent and 3 reactors with 10µg/L tetracycline effluent. For low nutrient replacement conditions, there were 3 control reactors received effluent with no tetracycline added, 3 reactors with 1µg/L tetracycline and 3 reactors with 10ug/L tetracycline. The 1 µg/L reactors were labeled as the low tetracycline reactors and the 10ug/L were labeled as the high tetracycline reactors. The concentrations that represented low and high concentrations previously reported in environmental water/wastewater samples (Jin et al., 2012). Figure 7 demonstrates the final reactor setup.

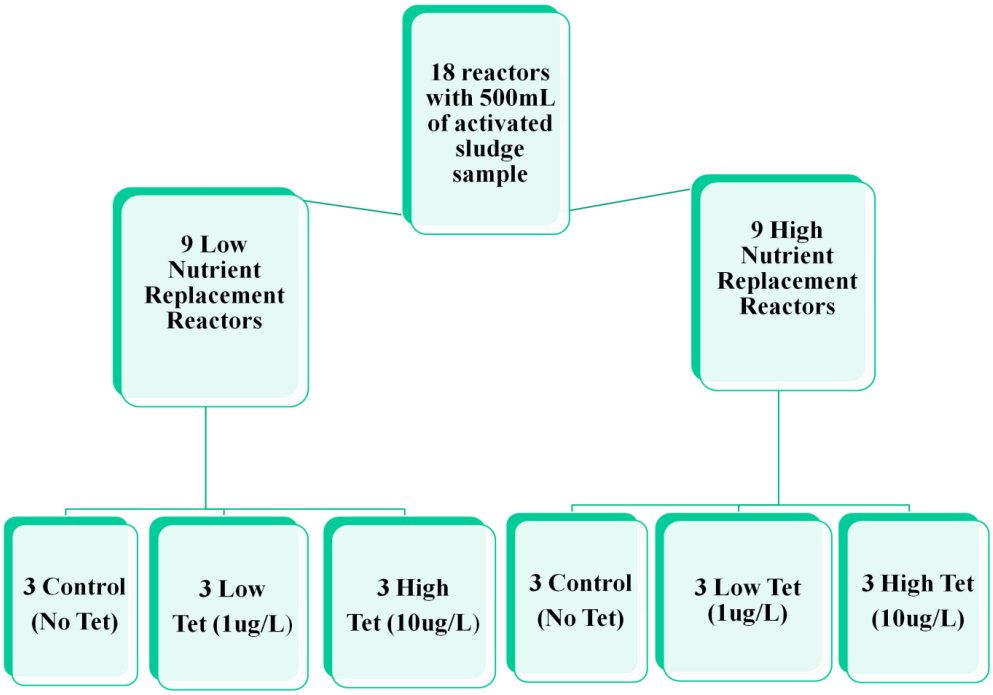


Figure 6. Reactor description and design

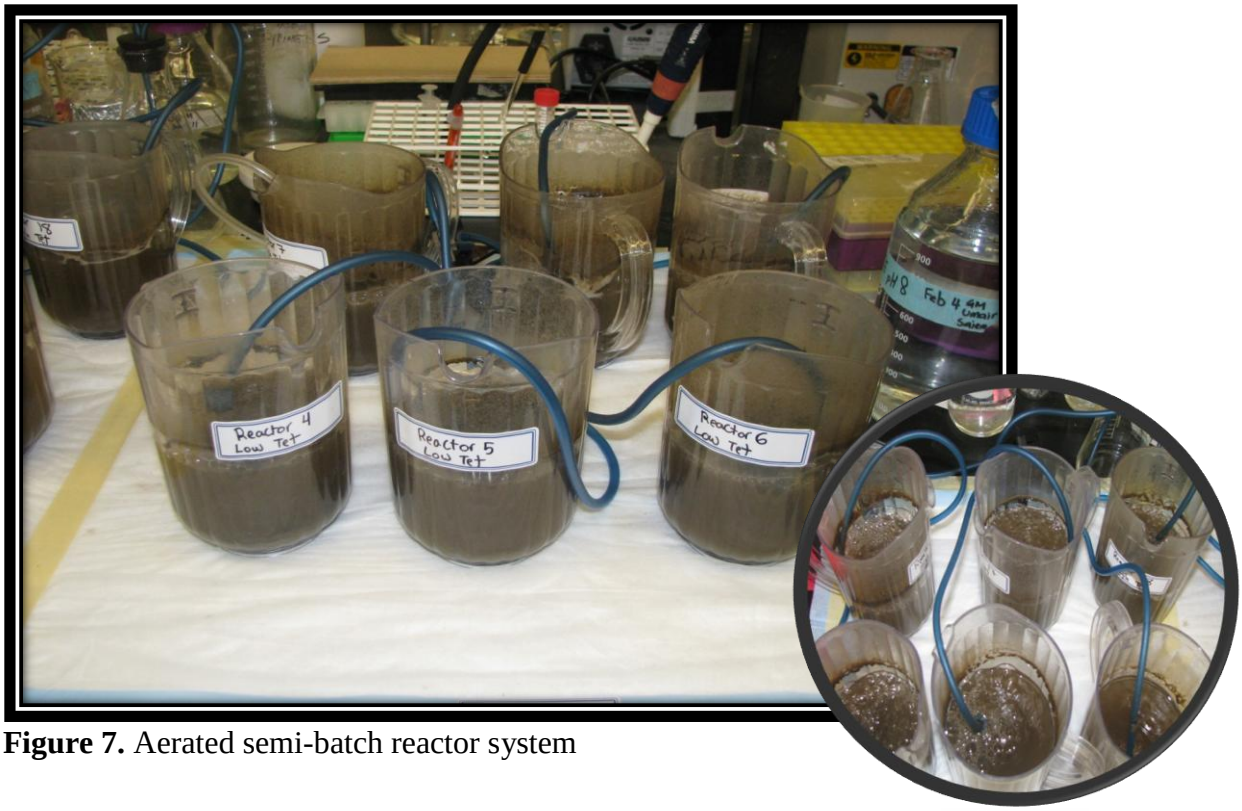


Figure 7. Aerated semi-batch reactor system

4.1.2. Sampling Reactors and Nutrient Replacement

After 7 days of aeration, samples were once again collected just before tetracycline was added and nutrient replacement was implemented in the reactors. For all the high nutrient replacement reactors, 50mL samples were extracted every two days starting on day 9 (November 9th 2011) and until Day 30 (December 7th 2011). For the low nutrient replacement reactors, 10mL samples were extracted every two days starting on day 9 and until Day 30. Samples were stored at -20°C in 50mL and 10mL falcon tubes. Samples were used to extract DNA and for nutrient analysis. Removed volumes were replaced with synthetic wastewater (Liao et al., 2001) supplemented with the appropriate concentration of tetracycline. The synthetic wastewater contained 1506ppm of dissolved organic carbon and 109.2µM of ammonia. Appendix B contains the table that describes the synthetic wastewater composition.

Parameters such as temperature, pH and dissolved oxygen (DO) were not measured because a previous study using a similar reactor setup demonstrated that these conditions were stable in the reactor system and remained in desired levels for microbial growth in activated sludge (Pogue, 2001). The concentrations of total Kjeldahl nitrogen, ammonia, nitrite and nitrate were measured to monitor the nitrogen removal characteristics. Since ammonia, nitrite and nitrate are all intermediary compounds involved in nitrification, monitoring these parameters allowed the assessment of the function of the nitrifying community.

4.2. Chemical Analysis

4.2.1. Ammonium analysis

To measure the amount of ammonia in the samples, a modified version of the phenate method was used (Clesceri et al., 1999; 4500-NH3 F). Dilutions of 1/10 were prepared by combining 0.5mL of each sample and 4.5mL of distilled water. To each of the test tubes, 200µl phenol reagent (refer to APPENDIX B), 200µl of sodium nitroprusside (refer to APPENDIX B) and 400 µl of oxidizing solution (refer to APPENDIX B) were added. The test tubes were then stored away in a dark environment for 30 minutes to allow colour development. Meanwhile, standards were prepared with concentrations of 0, 10, 25 and 50µM from an ammonium chloride (NH_4Cl) standard stock solution. The blank (0µM) was prepared using deionized water. The absorbance of the samples was measured using a spectrophotometer (Perkin Elmer UV/Vis Spectrophotometer, Lambda 20) at 640nm.

4.2.2. Nitrate/Nitrite analysis

To measure the concentrations of nitrate/nitrite; the copperized cadmium reduction method was implemented (Clesceri et al., 1999; 4500-NH3 F). To measure nitrate, 0.5mL of

collected sample from each reactor was added to 2mL of deionized water in test tubes. Additionally 7.5mL of ammonium chloride-EDTA buffer (refer to APPENDIX B) was added. This combined solution was then fed through a stainless steel column (6.4mm ID x 10 cm length). The column was packed with cadmium shavings coated with copper. Samples were pumped through the column at a flow rate of 2mL/min and collected at the end of the column. The first 6mL of the sample was discarded (to remove any residues left from previous samples). The next 5mL of sample was retained in a new test tube. To each of the test tubes containing the collected sample, 400µL of colouring reagent (refer to APPENDIX B) was added. The test tubes were then stored away in a dark environment for 30 minutes to allow colour development. The absorbance of the samples was measured using a spectrophotometer (Perkin Elmer UV/Vis Spectrophotometer, Lambda 20) at 543nm.

To measure nitrite, 0.5mL of collected sample from each reactor was added to 2mL of deionized water in test tubes. Additionally 7.5mL of nitrate buffer was added. To each of the test tubes 400µL of colouring reagent was added. The test tubes were then stored away in a dark environment for 30 minutes to allow colour development. The absorbance of the samples were measured using a spectrophotometer (Perkin Elmer UV/Vis Spectrophotometer, Lambda 20) at 543nm.

4.2.3. Dissolved Organic Carbon (DOC)

Total organic carbon (TOC) is the sum of the organic and inorganic carbon present in the sample. Dissolved organic carbon (DOC) was measured for all reactors. To achieve this, only the liquid portion of the sample was used that contained no pellet or soil component. Samples were diluted to 1/10 by adding 1mL of sample to 9mL of deionized water. A Shimadzu TOC-V Series (Japan) analyzer was used to monitor the DOC. To analyze the total carbon, the instrument used

high temperature (680°C) for combustion of sample and coupling with a carrier gas being passed with a rate 150mL/min in an oxidation-catalyst combustion tube. As the sample passed through the combustion tube, the total carbon in the sample was oxidized to CO_2. The product was then carried to be cooled and dehumidified before passing into the halogen scrubber unit. Next, the sample was transported into the sample cell of the non dispersive infrared detector (NDIR), the site of CO_2 detection. The machine converted the detection signal into a peak. The peak was then compared to the calibration curve (already established using standard total carbon solutions). In order to obtain the dissolved organic carbon concentration, the inorganic carbon concentration value was subtracted from the total carbon concentration. Through an acidification process using hydrochloric acid; the inorganic carbon was converted to CO_2 and then the same procedure for total carbon was implemented in order to obtain inorganic carbon concentration measurements.

There were duplicate measurements obtained for each sample. The average of the two concentrations was calculated.

4.2.4. Kjeldahl Nitrogen Analysis

Kjeldahl nitrogen analysis involved a two step process. First, a digestion of the sample was performed. In a 100mL Kjeldahl digestion flask, 10mL of sample was combined with 5mL of digestion reagent that was a solution composed of potassium sulphate, cupric sulphate and sulphuric acid (Appendix B). The Kjeldahl flasks were heated using a semi-micro Kjeldahl digester. The digester was set to medium high and flasks were heated until the appearance of white smoke was observed. At this point, the digester was set to high and the flasks were heated for 30 minutes. Then the flasks were allowed to cool for 5 minutes. There was appearance of a white residue on the bottom of the flask at which point 10mL of deionized water was added to dilute the residue. The second step involved adding the sample to the BUCHI distillation

apparatus (BUCHI Labortechnik GmbH, Essen, Germany). The distillation process involved the addition of 45% sodium hydroxide (w/w) to produce ammonia gas which was then collected in a specialized BUCHI receiving flask that contained 4% Boric acid. The contents of the receiving flask were then transferred into 85mL capped bottles. The samples were then placed into test tubes and the same protocol used for ammonium analysis (The Phenate method) was implemented.

CHAPTER 5: RESULTS

5.1. Dissolved Organic Carbon (DOC)

The total DOC was monitored on day 0, 7, 16, 21 and 30 (Figure 8). Prior to the implementation of experimental treatments, the concentration of DOC decreased from 972.5ppm on day 0 to an average concentration of 712.1ppm on day 7 for the reactors. After the introduction of high and low nutrient replacement conditions and the addition of tetracycline on day 7, the DOC concentration in all reactors continued to decrease in a similar fashion to nearly 0 on day 21 regardless of the addition of synthetic wastewater every 2 days containing 1500ppm of DOC. By day 30, reactors demonstrated an increase in DOC concentrations to approximately 1/5 of the concentration of the synthetic feed. There were no differences observed between the 6 treatments because all reactors showed nearly identical trends in carbon removal (Figure 8).

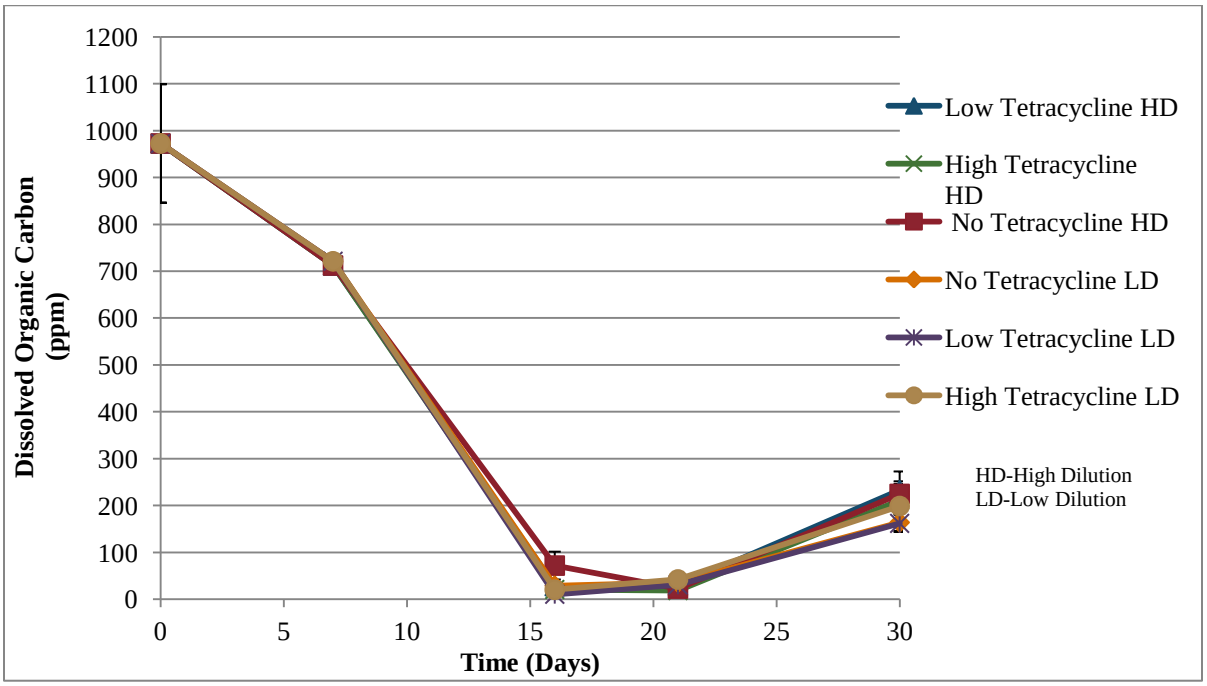


Figure 8. Total dissolved organic carbon (DOC) concentrations in all high nutrient replacement (HD) and low nutrient replacement (LD) reactors containing no tetracycline, low (1µg/L) and high (10µg/L) tetracycline. Mean +/- Standard Deviation (SD) of three replicates.

5.2. Total Nitrogen

The Kjeldahl nitrogen was measured on day 0, 7, 9 and 18. Due to equipment failure, the total measurements for samples after day 18 could not be processed. The Kjeldahl nitrogen decreased in the first seven days in all reactors. The concentrations decreased from 9850µM on day 0 to 8840µM on day 7 (Figure 9 and 10).

The changes in total nitrogen concentrations in high nutrient replacement for control, low and high tetracycline reactors are shown in figure 9. In general, there was a decrease in total nitrogen concentrations in the control reactors over time. From day 7, the total nitrogen continuously decreased to 6500µM on day 9 and finally to 5925µM on day 18. The addition of tetracycline into the high nutrient replacement reactors on day 7 resulted in a slightly greater decrease of total nitrogen when compared to the control reactors. The total nitrogen concentration decreased from 8840µM on day 7 to 2416.67µM and 3850µM on day 9 under low and high tetracycline conditions respectively. This was followed by a gradual increase in nitrogen concentrations to 6350µM and 6750µM on day 18 for the low and high tetracycline levels respectively which was similar to the level in the control reactors (figure 9).

The total Kjeldahl nitrogen profile for low nutrient replacement reactors is shown in figure 10. In general, the control low nutrient replacement reactors demonstrated a gradual decrease in total nitrogen concentration over time. The total nitrogen decreased from 9850µM on day 0 to 4466.67µM on day 18. Interestingly, the presence of tetracycline (high and low) under low nutrient replacement conditions demonstrated a dramatic increase in total nitrogen between day 7 and day 9 before the decrease on day 18 to levels below those seen in the other conditions. The low tetracycline reactors under low nutrient replacement conditions showed an increase of total nitrogen from 8840µM on day 7 to 14466.67µM on day 9. Then the total nitrogen

concentration decreased to 0 by day 18. For the high tetracycline reactors under low nutrient replacement conditions, the total nitrogen concentrations also increased from 8840µM on day 7 to 19533.33µM on day 9 then decreased to below detection levels on day 18.

Figures 11 and 12 demonstrate the impact of nutrient replacement under high and low tetracycline conditions. It can be observed that the introduction of tetracycline regardless of high and low concentrations resulted in an immediate increase of total nitrogen only in low nutrient replacement reactors. These results suggest that the addition of tetracycline under low replacement conditions may influence the nitrogen cycle.

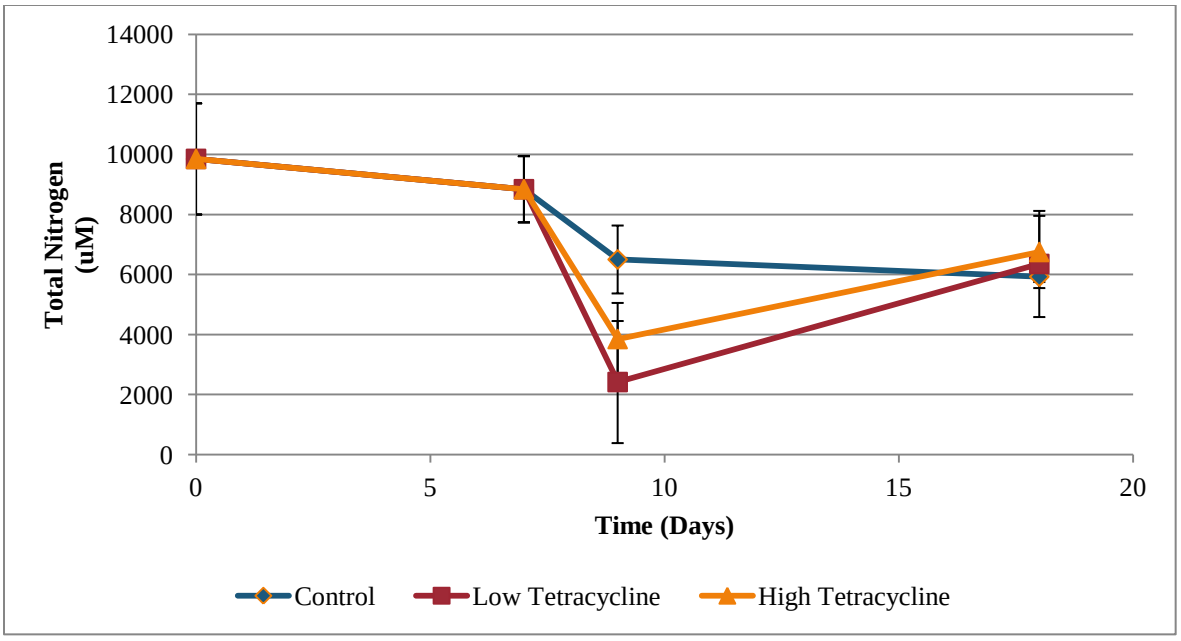


Figure 9. Total Kjeldahl nitrogen profile of high nutrient replacement reactors. Error bars are mean +/- SD for 3 replicates.

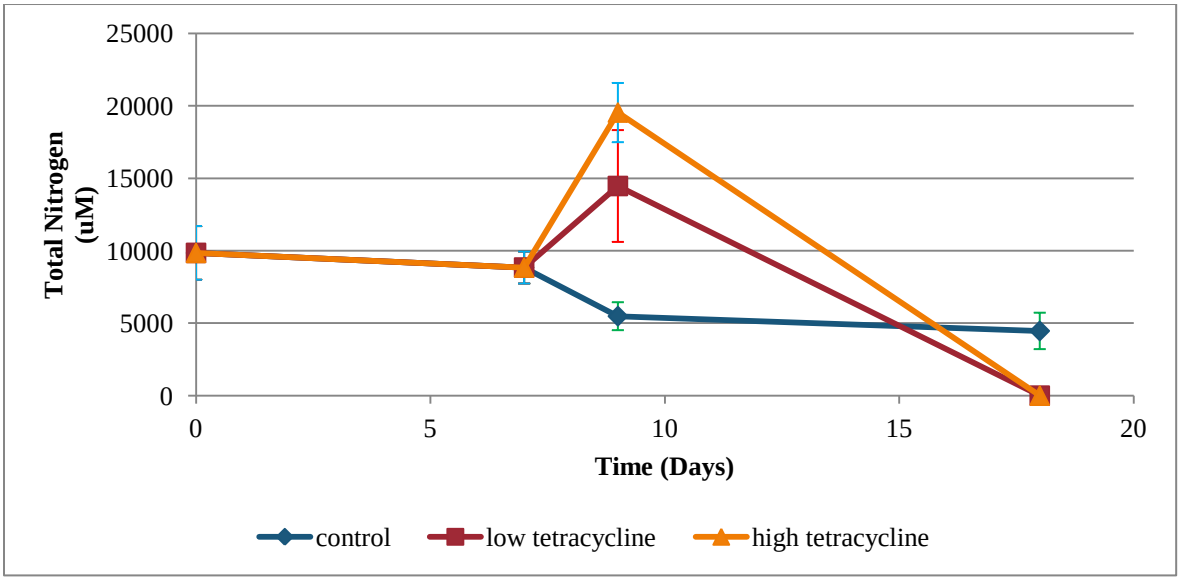


Figure 10. Total Kjeldahl nitrogen profile of low nutrient replacement reactors. Error bars are mean +/- SD for 3 replicates.

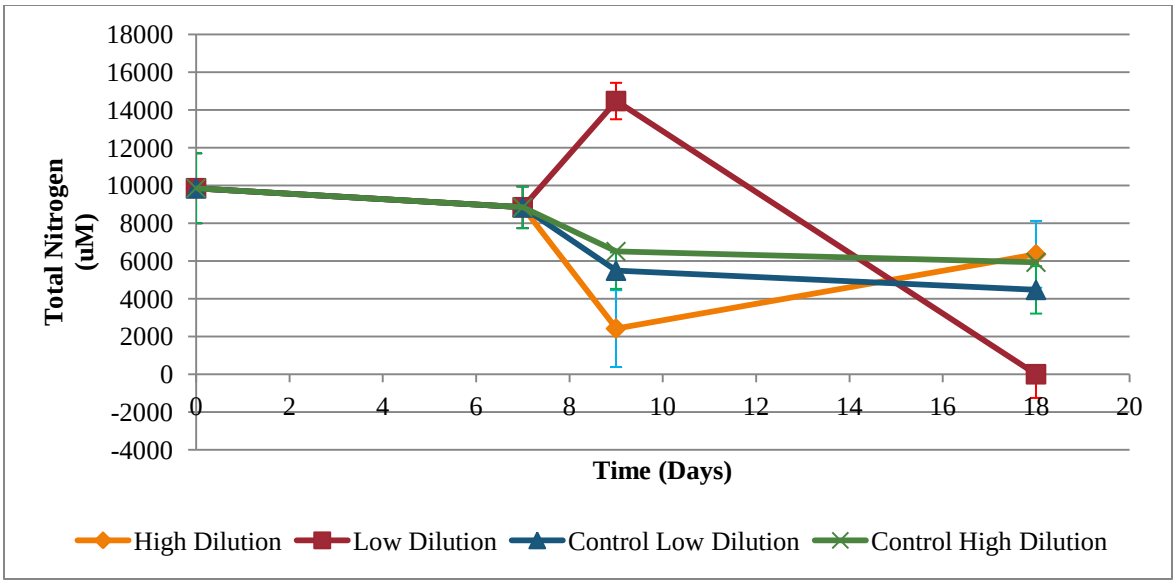


Figure 11. Impact of nutrient replacement on total nitrogen concentrations under low tetracycline conditions. Error bars are mean +/- SD for 3 replicates.

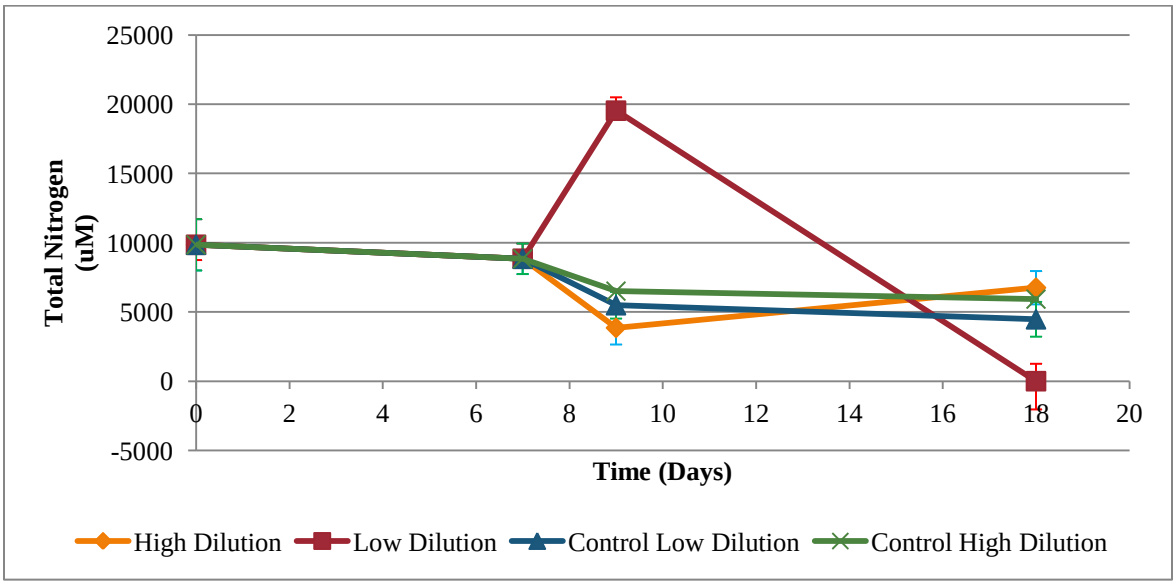


Figure 12. Impact of nutrient replacement on total nitrogen concentrations under high tetracycline conditions. Error bars are mean +/- SD for 3 replicates.

5.3. Ammonia

Ammonia is the first reactant in nitrification and is present in high concentrations in wastewater. In this study, ammonia concentrations were measured on day 0, day 7, day 9, day 21, day 28 and day 30 for all reactors. The change in ammonia concentration in high nutrient replacement control, low tetracycline and high tetracycline reactors is shown in figure 13. In the control reactors the ammonia concentration increased from day 0 to day 9 before gradually decreasing over time. The control reactors demonstrated an increase from 1243.27µM on day 0 to 1297.33µM on day 7 then increased to 1359.82µM on day 9. From day 9 there was a gradual decrease in ammonia concentration to 454.78µM measured on day 30.

For low tetracycline reactors, under high nutrient replacement, the ammonia concentrations followed a similar trend to the control reactors and also gradually declined over time. From 1297.33µM on day 7, the ammonia concentration steadily decreased to 184.67µM on day 28 before increasing to 499.33µM on day 30. For high tetracycline reactors under high nutrient replacement, a slight increase in ammonia concentration was observed from day 7 to day 9. After the addition of the high concentration of tetracycline, the ammonia concentration increased from 1297.33µM on day 7 to 1564.67µM on day 9. After day 9 however, the ammonia concentration decreased similar to the other reactors but only decreased to 754.83µM on day 30. There was little difference in ammonia removal between the low tetracycline, high tetracycline and control treatments. For high nutrient replacement rate reactors by the end of the 30 days, the high tetracycline reactors contained a higher amount of ammonia than the control and the low tetracycline treatments.

For low nutrient replacement conditions, ammonia profiles for control, low and high tetracycline reactors are demonstrated in figure 14. The control reactors demonstrated an overall

increase in ammonia concentrations over time although not significantly. The slight increase was observed from 1243.27µM in day 0 to 1297.33µM on day 7. After slight fluctuations, the ammonia concentration measured on day 30 was 1716µM. However, there was interesting observations made as soon as tetracycline was introduced along with the low nutrient replacement condition on day 7. For low and high tetracycline reactors, there was a drastic decrease in ammonia concentration seen from day 7 to day 9. The ammonia concentration decreased from 1297.33µM on day 7 to 135.18µM on day 9 (Figure 14) in the low tetracycline reactors. In high tetracycline reactors the concentration was 121.53µM on day 9. When comparing to the control reactors, it was observed that there is a greater decrease in ammonia concentration in low nutrient replacement rate reactors in the presence of tetracycline (high and low) between day 7 and day 9. The low tetracycline reactor reached on day 21 before a gradual increase of ammonia concentration to 313.78µM on day 30. The same trend followed in the high tetracycline reactors where the ammonia concentration was 111.13µM on day 21 before gradually increasing to 575.83µM on day 30.

Figure 15 and 16 clearly demonstrates the impact of nutrient replacement rate under low and high tetracycline conditions. There is a more drastic and a larger decrease in ammonia concentrations when tetracycline is introduced in the low nutrient replacement reactors.

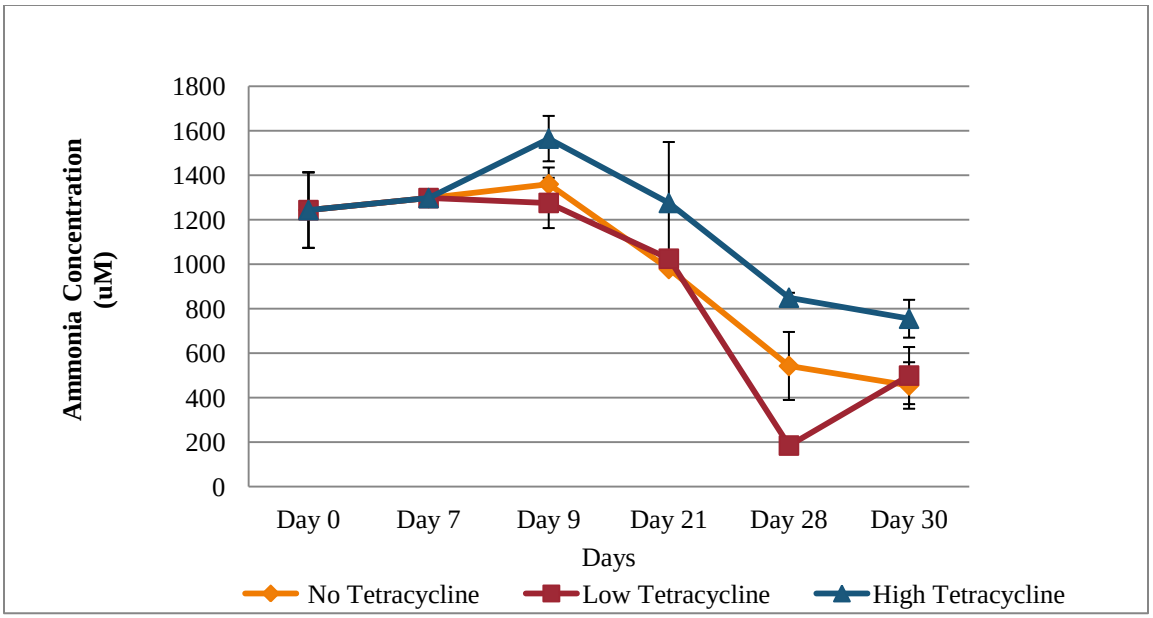


Figure 13. Ammonia concentration profile for high nutrient replacement reactors. Error bars are mean +/- SD for 3 replicates

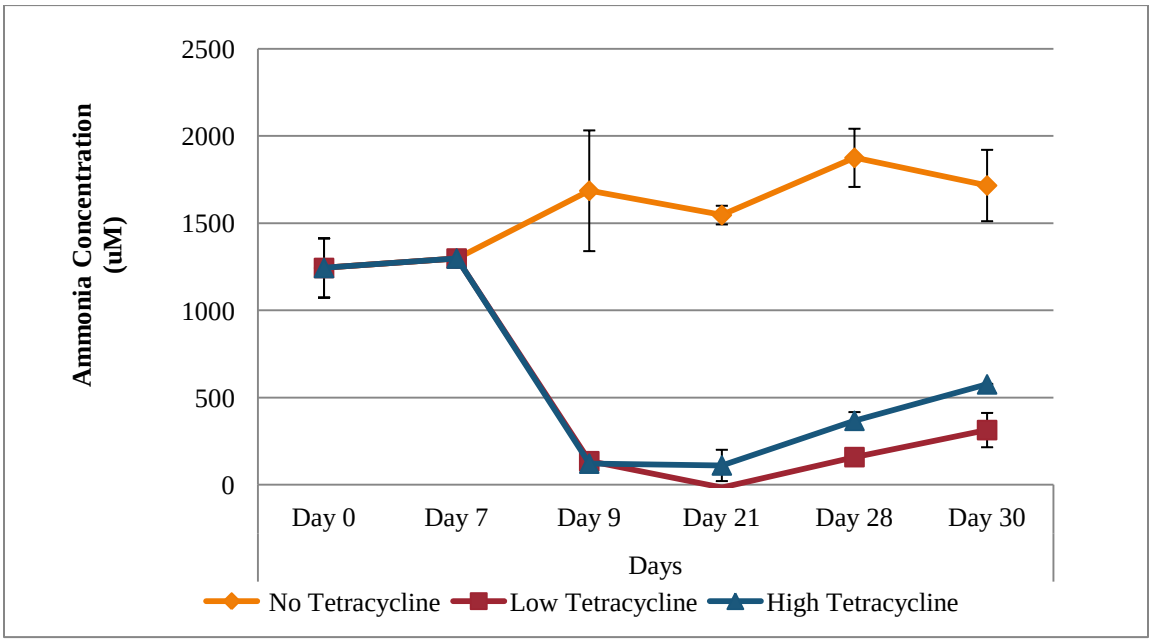


Figure 14. Ammonia concentration profile for low nutrient replacement reactors. Error bars are mean +/- SD for 3 replicates

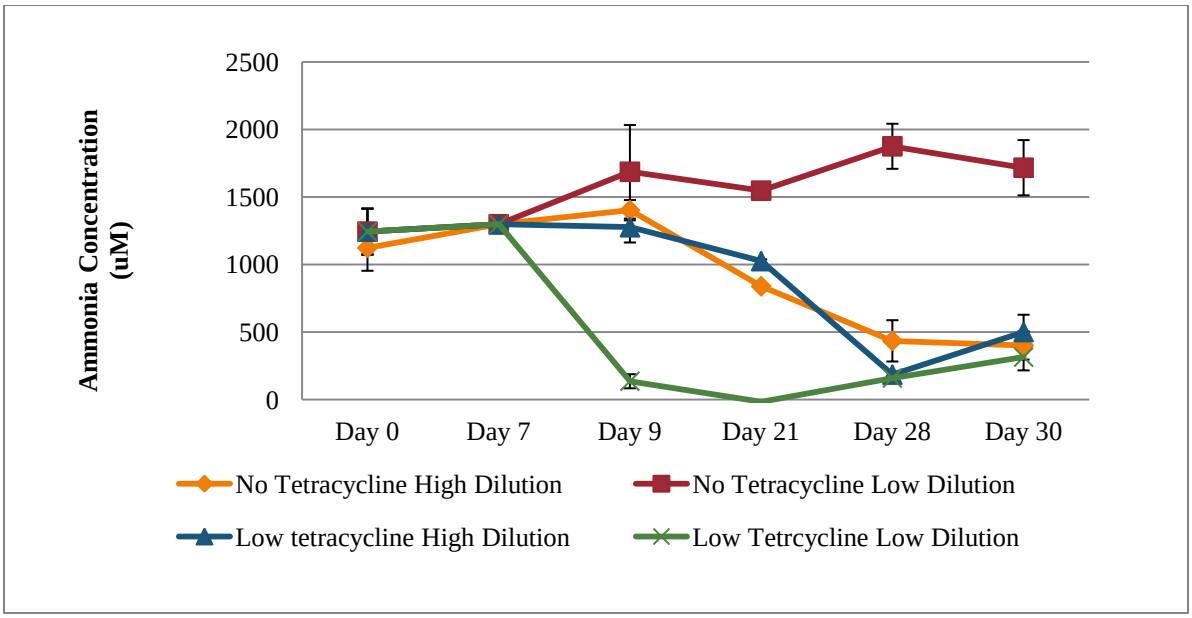


Figure 15. Impact of nutrient replacement on ammonia concentrations under low tetracycline conditions. Error bars are mean +/- SD for 3 replicates

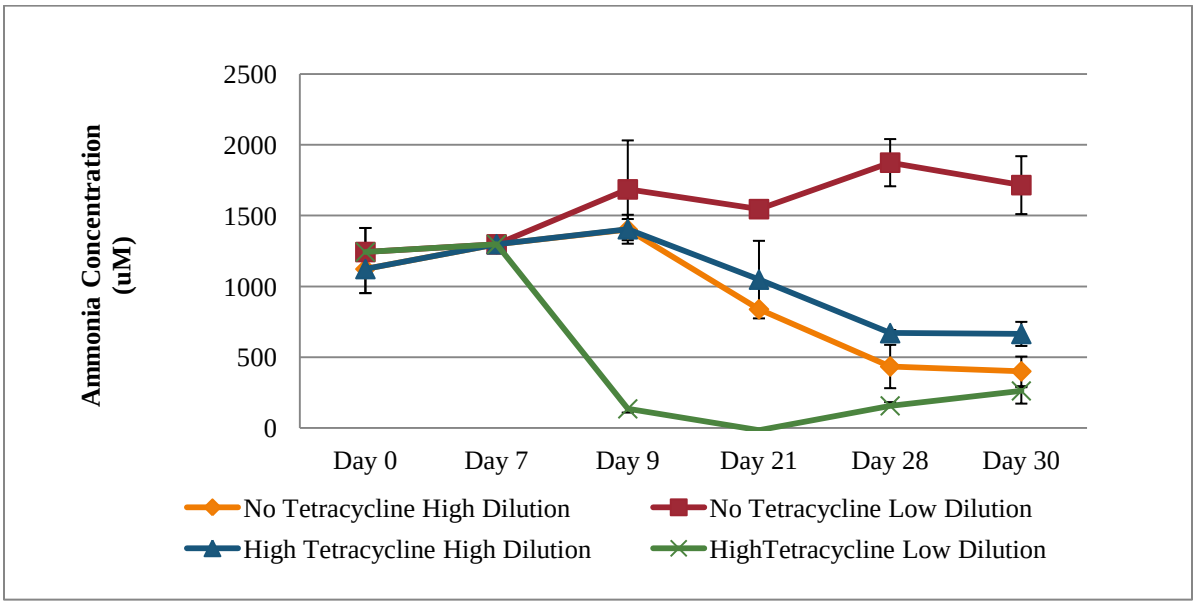


Figure 16. Impact of nutrient replacement on ammonia concentrations under high tetracycline conditions. Error bars are mean +/- SD for 3 replicates

5.4. Nitrate/Nitrite

Nitrite is the intermediary reactant in nitrification and is produced during the ammonia oxidation process. As expected, nitrite concentration was measured but was found to be very low and without significant differences between the treatments. Since nitrite is readily converted to nitrate, it does not accumulate. Please refer to APPEDIX A for Nitrite concentrations.

Nitrate concentrations were measured on day 7, 9, 16, 21, 28 and 30. In low nutrient replacement conditions, all reactors showed a gradual decrease of nitrate concentrations over time. This trend is well demonstrated in figure 17. Control reactors demonstrated a decrease in nitrate concentration from 639.16µM on day 7 to 117.36µM on day 16. There was a slight increase to 300µM observed on day 21 before eventually decreasing to 226.43µM on day 30. The low nutrient replacement reactors that contained tetracycline both high and low demonstrated almost identical trends. In low tetracycline/low nutrient replacement reactors, the nitrate concentration decreased from 639.16µM on day 7 to 86.04µM on day 9. Similar to the reference reactors, an increase in nitrate concentration was observed after day 16 to day 28 before decreasing to 72.02µM on day 30. In high tetracycline/low nutrient replacement reactors, the nitrate concentration decreased from 639.16µM on day 7 to 163.75µM on day 9 after that almost an identical pattern in nitrate concentrations to the low tetracycline reactors was observed.

Figure 18 demonstrates the nitrate profiles for the high nutrient replacement reactors. In the control reactors, there was a steady decrease in nitrate concentration from day 7 to day 16. Nitrate concentration decreased from 639.16µM on day 7 to 368.89µM on day 16. However, there was a significant spike observed in nitrate concentration from day 16 to 638.75µM on day 21. Nitrate concentration again decreased on day 28 and eventually to 139.36µM on day 30. The low and high tetracycline reactors demonstrated very similar trends as well. In the low

tetracycline reactor/high nutrient replacement reactor, the nitrate concentration decreased to 153.95µM on day 16 followed by an increase to 427.71µM on day 21 eventually decreasing to 73.33µM on day 30. High tetracycline/high nutrient replacement reactors demonstrated a decrease in nitrate concentration in a very similar fashion to the low. Nitrate concentration decreased to 54.375µM on day 16 in high tetracycline reactors before drastically increasing to 326.46µM on day 21. Another decrease was shown in nitrate concentration after day 21 with the concentration measured 90.24µM on day 30.

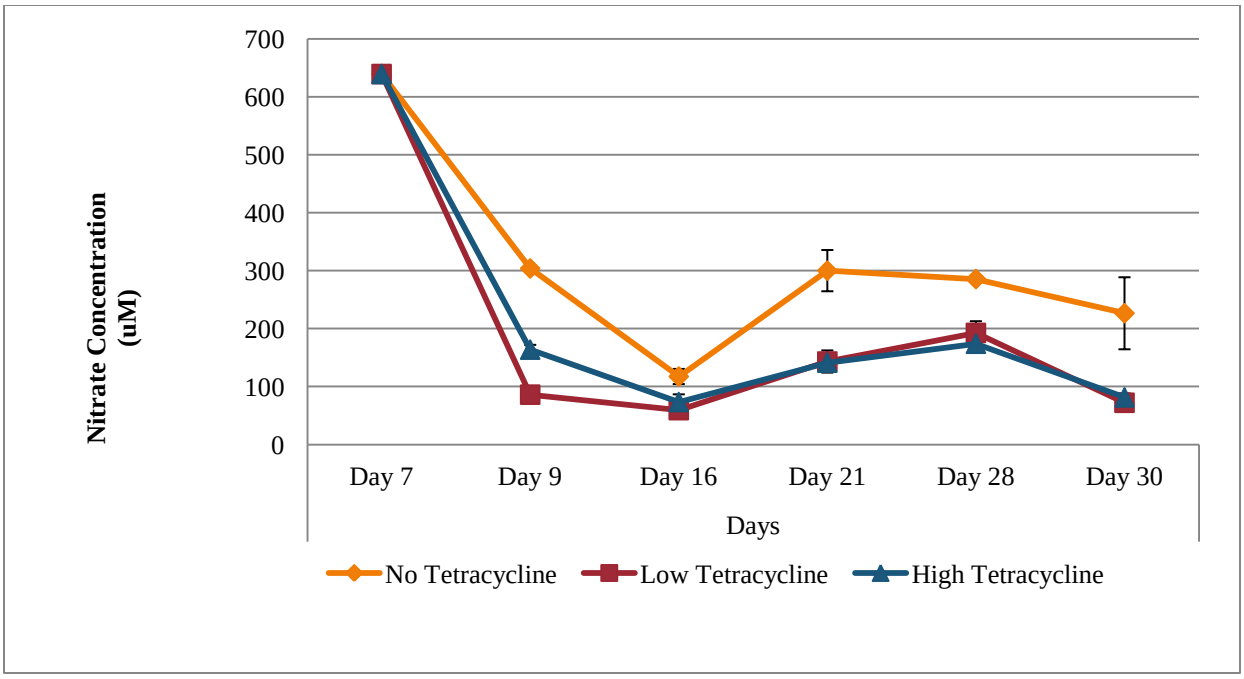


Figure 17. Nitrate concentration profile for low nutrient replacement reactors. Error bars are mean +/- SD for 3 replicates

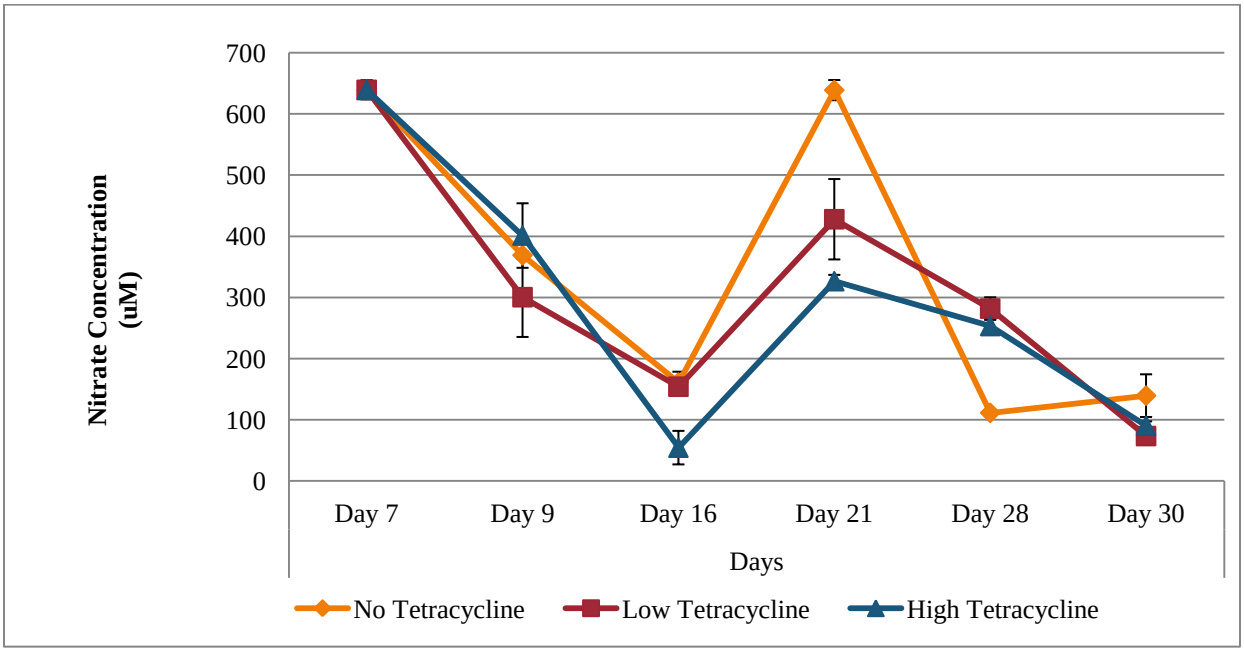


Figure 18. Nitrate concentration profile for high nutrient replacement reactors. Error bars are mean +/- SD for 3 replicates

CHAPTER 6: DISCUSSION

In this present study, the objective was to examine the impact of tetracycline on the microbial community involved in the secondary treatment using a semi-batch reactor system. In particular, chemical parameters such as total Kjeldahl nitrogen, ammonia, and nitrate/nitrite were monitored to study the impact of tetracycline on the community that is specifically important for nitrogen removal in the secondary treatment of wastewater treatment plants. In addition, since the microbial community involved in secondary treatment can also be affected by various other parameters such as organic load, aeration intensity, and hydraulic retention times. The introduction of two nutrient replacement rates for this study allowed for the assessment of the impact of tetracycline on microbial communities in secondary treatment under two different nutrient replacement rates.

6.1. Performance of the Semi-Batch Reactors

It is essential that certain parameters are set to provide the ideal environment to enhance selection of bacteria that are most important in nutrient removal. Nitrifying bacteria are slow growing and are sensitive to toxic shocks, pH and temperature changes that make the establishment of a stable nitrification system in wastewater treatment a challenge. Since nitrification is an aerobic process which requires oxygen, the demand for oxygen must be met in order to achieve nitrification. For successful biological treatment, the dissolved oxygen (DO) concentration must be between 1-2mg/L (EPA, 1997). The pH must also be monitored in order to achieve optimal nitrification rates. The significance of pH depression in the nitrification process is that the reaction rates rapidly decreases as the pH falls below 7 (Ahn, 2006). The temperature is also another parameter that must be monitored. The activated sludge process is observed to be occurring at a range of 15-25°C (Viessman and Hammer, 1998).

The semi-batch reactor system used in this experiment was similar in design but slightly upgraded from a previous study by Pogue, (2001). The aeration technique used aquarium air stones to deliver oxygen instead of a mechanical shaker used previously which produced a more even and robust source of oxygen. The previous study demonstrated this system satisfies the temperature, pH and DO requirements for nitrification and that these parameters did not fall out of the ranges listed above to hinder nitrification rates.

6.2. Dissolved Organic Carbon

The semi-batch reactor system provided an advantage over using a batch system because it allowed the ability to provide fresh synthetic feed to the reactors that would theoretically expand the life cycle of the microbial community. Previously, it was observed that the batch system can sustain a microbial community with a life cycle of approximately one to two weeks and if nutrients were not replaced the system would eventually breakdown (Pogue, 2001). Thus, the addition of fresh synthetic feed was expected to increase the life cycle of the microbial communities in the activated sludge samples. This also provided an advantage in studying nitrifiers and denitrifiers which are considered to be slow growing and require a longer duration to be established. Dissolved organic carbon or DOC is a general description of the organic material dissolved in water. Dissolved organic carbon can be an energy source for the microbial community that is present in the activated sludge system. Like the BOD (Biological oxygen demand) test which can be used to assess the performance of a wastewater treatment plant, monitoring the DOC concentrations can also be used in this regard (EPA, 1997). In this study, the DOC concentrations were monitored to examine the stability and metabolic activity of the microbial community present within the reactors. The DOC was measured in all reactors on day 0, 7, 16, 21 and 30 (Figure 8). The DOC concentration is expected to decrease over time as the

organic matter is metabolized and used as an energy source by microbes in activated sludge. This was observed to be true in all reactors where the concentration of DOC decreased during the first 7 days. After the introduction of high and low nutrient replacement conditions and the addition of tetracycline on day 7, the DOC concentration continued to decrease in all reactors. There were no differences in DOC concentrations between tetracycline treatments in low and high nutrient replacement rates as all reactors demonstrated the same decreasing phenomenon. This suggests that the presence of tetracycline did not have an impact on DOC concentrations in both high and low nutrient replacement rates.

Also, the decreasing trends of DOC concentrations confirmed the presence of a metabolically active microbial community in all the reactors consuming the dissolved organic as an energy source. It was observed that even with the addition of synthetic wastewater every 2 days containing 1500ppm of DOC, the DOC was depleted in the system by day 16 (all reactors nearing 0 on day 16, Figure 8). This suggested that the feeding regime every two days might have not been enough to sustain microbial growth beyond 21 days. Increasing the feeding regime to everyday instead of every two days may provide steady carbon availability to the microbial communities present in the reactors and provide a more stable community for longer term studies.

6.3. Total Nitrogen

The total Kjeldahl nitrogen represents every form of nitrogen including ammonia, nitrate, nitrite and organic nitrogen. In this study, the impact of tetracycline on total nitrogen concentrations was monitored under high and low nutrient replacement rates. The Kjeldahl nitrogen was measured on day 0, 7, 9 and 18. During the first seven days, the wastewater sample was only aerated in the lab without the nutrient replacement and tetracycline conditions. This

was performed to acclimatize the sample to the lab environment. The Kjeldahl nitrogen decreased slightly from 9850µM on day 0 to 8840µM on day 7 in all reactors (Figure 5.2 and 5.3). The control reactors for both high and low nutrient replacement rates demonstrated a general decrease up until day 18 (Figure 5.2 and 5.3). This overall loss of total nitrogen suggested that denitrification may have been occurring in the control reactors. Biological denitrification is the process which enables the transformation of oxidized nitrogen compounds nitrate and nitrite into harmless nitrogen gas that is lost to the atmosphere (Yang et al., 2011). The addition of the tetracycline (high/low) into the high nutrient replacement rate reactors also led to a decrease in total nitrogen concentrations like those seen in the control reactors (2416.67µM and 3850µM on day 9 under low and high tetracycline conditions respectively compared to 5925µM in control reactors). Lower concentrations of total Kjeldahl nitrogen found in the tetracycline reactors indicated a positive effect on denitrification. These results seem to suggest that denitrification was occurring and was not significantly inhibited by the presence of tetracycline under high nutrient replacement rates. The denitrification process provides an important sink for fixed nitrogen and reduces the effects of nitrogen availability in the final effluent when discharged into lakes and streams. These reactors appear to follow the trend seen in WWTP by having an overall decrease in nitrogen over time.

However, the introduction of tetracycline (both high/low) into the low nutrient replacement reactors on day 7 demonstrated a dramatic increase in total nitrogen concentrations. The low tetracycline reactors under low nutrient replacement conditions showed an increase of total nitrogen from 8840µM on day 7 to 14466.67µM on day 9. The high tetracycline reactors showed an increase from 8840µM on day 7 to 19533.33µM. Figures 5.4 and 5.5 which demonstrates the impact of nutrient replacement provide evidence that opposite phenomena was

observed in total nitrogen concentrations for the two different replacement rates under high and low tetracycline concentrations. Both figures demonstrate an increase of total nitrogen soon after the tetracycline was added but only in the low nutrient replacement reactors. This increase in total nitrogen concentration is observed to be an unrealistic assessment. The only source of nitrogen was delivered in the form of ammonia. Since only 109.2μM ammonia was supplemented to the reactors every two days was, it is highly unlikely that the total nitrogen concentrations could increase by a large magnitude as those observed in this study. Subsequent efforts were made to re-assess the total nitrogen concentrations for the day 9 samples. However, the results obtained were inconclusive due to the relative instability of the digested Kjeldahl samples that had been stored. If the increase in total nitrogen concentrations could be confirmed, it could be owed to various factors. The first proposed theory would be that the presence of tetracycline in the low nutrient replacement reactors is inhibiting denitrifying bacteria and the denitrification process. One study demonstrated that a group of antibiotics at 1000μg/L can impact denitrification of benthic bacteria (Costanzo et al., 2004). But this inhibition was tested at concentrations that were much greater than the concentrations used in this study. Since the increase was only observed in low nutrient replacement reactors, the results of this study may indicate the possible notion that the low nutrient replacement rate is altering the microbial community and is inhibiting the denitrification process at much lower tetracycline concentrations (1 and 10μg/L). One possible explanation can be answered by observing the impact of the tetracycline on the ANAMMOX bacteria. These bacteria are unusual in their physiology because of their ability to consume ammonia in the absence of oxygen. The process consists of the oxidation of ammonia using nitrite as an electron acceptor to yield gaseous nitrogen (Ahn, 2006). The ANAMMOX bacteria have a reputation of having an extremely slow doubling rate (Strous

et al., 1999). Due to this, they are present in low numbers which can increase their susceptibility to toxic stresses provided by tetracycline. Although if denitrification was inhibited, it might be indicated by an increase or accumulation in nitrate/nitrite concentrations (assuming the supply of nitrate and nitrite from nitrifying bacteria was not affected). In this study, nitrate concentrations in low nutrient replacement reactors decreased regardless of the addition of tetracycline (Figure 17) suggesting that denitrification is not hindered by the presence of tetracycline under low nutrient replacement conditions. Microbial communities in the activated sludge process can be affected by influent characteristics, design and operation of the reactors and environmental conditions (Pholchan et al., 2010). At a low nutrient replacement rate, it is possible that there is a more sustainable growth of bacteria which can represent a larger biomass in these reactors. It is quite possible that the increase in total nitrogen was observed due to the larger biomass in low nutrient replacement reactors. The next phenomenon which can explain the increase in total nitrogen concentration would be the presence of nitrogen fixating bacteria. The transformation of atmospheric nitrogen (N_2) into a source of nitrogen that is used for bacterial growth is known as nitrogen fixation (Gapes et al., 1999). It is known that the presence of photosynthetic bacteria such as cyanobacteria have the ability to fix nitrogen (Gapes et al., 1999). The semi batch reactor system used in this study did not account for the growth of photosynthetic bacteria. Thus, it is also feasible to assume that the low nutrient replacement rate under the selective pressure of tetracycline is favouring the growth of nitrogen fixing photosynthetic bacteria.

6.4. Ammonia

Ammonia oxidation is the primary step in the biological removal of nitrogen during the treatment of wastewater (Kowalchuk and Stephen, 2001). The high nutrient replacement control reactors demonstrated a general decrease in ammonia concentration over time (from 1243.27µM

on day 0 to 454.78µM measured on day 30 shown in Figure 13). The presence of low tetracycline concentrations demonstrated a similar trend of decreasing ammonia concentrations over time for high nutrient replacement conditions that was comparable to the control reactors. This suggests that the presence of tetracycline at a low concentration did not inhibit ammonia oxidation step of nitrification in high nutrient replacement rate reactors. However, at high tetracycline concentrations, the high nutrient replacement reactors demonstrated lowest rate of ammonia removal (ammonia concentration for high tetracycline on day 30 was 754.83µM compared to 499.33µM and 454.78µM in the low tetracycline and control reactors). This suggests that the presence of high tetracycline may have hindered the ammonia oxidation process under high nutrient replacement rates. Excess ammonium can lead to eutrophication of freshwater bodies and is toxic to aquatic organisms (Egli et al., 2003).

In the low nutrient replacement control reactors, the ammonia concentration remained steady over time with slight fluctuations. Comparing the low nutrient replacement control to the high nutrient replacement control reactors demonstrated that a greater degree of ammonia removal had taken place in the high nutrient replacement conditions. This does not necessarily mean that higher ammonium oxidation has taken place in these reactors. A study performed with high and low OLR rates reported that high OLR reactors demonstrated lower nitrification rates than low OLR reactors. Although the OLR is not exactly the same parameter as nutrient replacement rates, they are comparable. It is observed that high OLR reactors have greater substrate concentrations which lead to higher oxygen consumption and more assimilation of ammonia into the cell by heterotrophs. As a result, more ammonia would be assimilated into the biomass and would decrease the amount of ammonia available for the ammonium oxidizers (Pholchan et al., 2010).

The introduction of tetracycline under low nutrient replacement led to a dramatic decrease in ammonia concentration from day 7 to day 9 (from 1297.33µM on day 7 to 135.18µM in the low tetracycline reactors and to 121.53µM in high tetracycline reactors shown in figure 14). Between days 7-9, the decrease in ammonia concentrations was much greater when tetracycline was present in low nutrient replacement conditions compared to high nutrient replacement (Demonstrated by Figure 15 and 16). This suggests that under low nutrient replacement conditions, the tetracycline is having a positive effect on the ammonia oxidation process. There is a lack of knowledge on the potential effects of antibiotics on microbial nutrient removal processes in wastewater treatment. A study done by Toth et al., (2011) determined whether the presence of veterinary antibiotics chlortetracycline, sulfadimethoxine and monensin at environmentally relevant concentrations would impact soil microbial processes including respiration, nitrification and iron reduction. They found that chlortetracycline did not affect nitrification rates. Schmidt et al., (2012) also observed that ammonia oxidation was not inhibited by increasing concentrations on antibiotic mixture composed of CIP, GM, SMZ/TM and VA at concentrations <40mg/L in a short term nitrification inhibition test. Figure 15 and 16 presented a most compelling observation that the introduction of a low nutrient replacement rate in combination with tetracycline leads to greater decrease of ammonia concentrations over time. This may suggest that a low nutrient replacement rate is providing a microbial environment which selects for ammonia oxidizing bacteria and thus places a positive effect on nitrification. The impact of organic loading rates on nitrification have been reported in several studies that indicate that higher nitrification rates occur in low organic loading rate reactors than in high organic loading rates (Schmidt et al., 2010 and Hanaki et al., 1990). In summary, the present study demonstrated enhanced nitrogen removal in a low nutrient replacement rate only when

tetracycline is present. There is some evidence that could support this finding. It is found that microbial communities in activated sludge can be impacted by organic loading rates. Indicated by the presence of greater number of DGGE bands for AOB, the diversity of ammonia oxidizing bacteria (AOB) in the reactors operated at low OLR is observed to be higher than the reactors operated at high OLR (Pholchan et al., 2010). It is possible to suggest that the low nutrient replacement rate is providing a microbial community that is favouring the AOB bacteria resulting in enhanced ammonium removal rates. It is also important to note that several studies have demonstrated that the ammonia monooxygenase (enzyme which is the membrane bound multi-subunit enzyme that catalyzes ammonia oxidation) is responsible for the biodegradation of several pharmaceutically active compounds that are not easily biodegradable (Kim and Aga, 2006; Yi and Harper, 2007). Among these, one study determined that tetracycline was removed by nitrifying granules by a process of quick adsorption and biodegradation (Yi-Jing et al., 2011). This can explain as to why tetracycline did not have an inhibitory effect on ammonium oxidation and nitrification. However, tetracycline at a low nutrient replacement rate may have influenced other heterotrophic bacteria.

6.5. Nitrate/Nitrite

Nitrate concentrations were measured on day 7, day 9, day 16, day 21, day 28 and day 30. For the high nutrient replacement reactors for all three conditions of no tetracycline, low tetracycline and high tetracycline, the nitrate concentration gradually decreased over time indicating that nitrate reduction was not inhibited and denitrification could be occurring. Denitrification is an anaerobic process and the system implemented for this experiment is taking place under aerobic conditions. However, there are micro-anaerobic zones that are found in flocs and granules that form in biological treatment systems. It has been found that heterotrophic,

nitrifying and denitrifying bacteria can co-exist within the microbial matrix of granules and carry on coupled nitrification-denitrification even when subjected to alternating aerobic and anaerobic conditions (Wojnowska-Baryla et al., 2010). It is quite possible that denitrifiers are surviving within these flocs and carrying out denitrification. The effect of tetracycline in high nutrient replacement reactors on nitrate concentrations were observed to be minimal as all three conditions followed similar trends. There was a faster rate of nitrate removal observed between day 0 and day 16 for all high nutrient replacement reactors (Figure 17). A slight increase in nitrate concentrations was observed in all reactors on day 21 before decreasing again on day 30 (Figure 17).

Figure 18 demonstrates the nitrate profile for low nutrient replacement conditions. All reactors showed a gradual decrease of nitrate concentrations over time suggesting that denitrification was occurring. The presence of tetracycline both high and low when compared to the control reactors did not lead to significant differences in nitrate removal and demonstrated comparable trends. This suggests that tetracycline did not hinder the denitrification process under low nutrient replacement rates.

There was no accumulation of nitrite concentration observed in any conditions. The lack of nitrite accumulation suggested that nitrite oxidation and denitrification were not being uncoupled.

6.6. Future Work and Recommendations

This study has suggested that tetracycline does have an impact on the nitrification and denitrification processes that occur in the secondary treatment of wastewater. By measuring the parameters of key intermediates involved in nitrification and denitrification under the presence of

tetracycline; this study provided insight into the potential impact of tetracycline on the function of nitrifying and denitrifying communities present in the activated sludge system. However, further understanding could be achieved by examining the community composition of the microbial communities present in secondary treatment and monitoring the changes in community composition under the selective pressure of tetracycline. Modern molecular techniques have been applied to identify the microorganisms responsible for nutrient removal in sewage treatment systems (Wagner and Loy, 2002). Numerous tools have been developed in molecular biology such as fluorescence in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes, DGGE or real time PCR to help analyze complex bacterial communities (Wojnowska-Baryla et al., 2010). It is highly recommended that further research is done in order to identify which specific groups of bacteria are being affected by the presence of tetracycline in the activated sludge samples. This study has suggested that nutrient replacement rates may change microbial community composition to allow nitrification rates to be enhanced when tetracycline is present. Analysis of the community composition using molecular methods may be influential in identifying the members responsible for the enhancement of nitrogen removal in the presence of tetracycline.

Due to the slow growing characteristics of nitrifiers and their sensitivity to environmental parameters, it is a challenge to study and analyze them through culture dependent methods. The implementation of the semi-batch reactors can provide some advantages to study the effects of antibiotics and other pharmaceuticals on the biological treatment microbial communities *in situ*. The semi-batch reactor system can sustain the microbial communities for a longer experimental duration. However, an optimal feeding regime must also be found in order to sustain stable microbial growth to test for the toxicity and inhibitory effects of tetracycline. Although the semi-

batch reactors provided the possibility of testing the impacts of nutrient replacement rates in combination with tetracycline, it is however challenging to simulate MWTP flow rates using this particular system. The high and low nutrient replacement rates used in experiment were in actuality both low flow rates when comparing to the flow rates of actual wastewater treatment plants. In the future, a reactor system that takes into consideration similar flow rates to those seen in MWTP could lead to a better simulation.

The accurate monitoring of nitrogen cycling is often challenging because of inadequate measurement techniques. For future studies, it is suggested that nitrification and denitrification should be measured using techniques specific to each process. For example, the stable isotope pairing method can be used in order to measure denitrification processes. Stable isotope pairing process involves enriching the reactors with $^{15}NO_3^-$ and then measuring the mass specific N_2 using a mass spectrometer (Rysgaard et al., 1993; Scott et al., 2008). The ^{15}N isotope is now prominently used in nitrogen cycling studies of various environments (Robinson, 2001). The isotope dilution method can be implemented to measure nitrification rates. In this process, any new nitrate produced in the system will dilute the existing enriched pool of nitrate (Rysgaard et al., 1993).

Finally, the lack of research regarding the impact of pharmaceuticals on microbial communities in secondary treatment of wastewater treatment plants requires further research into other classes of pharmaceuticals like anti-inflammatory drugs, steroidal drugs and other antibiotics.

CHAPTER 7: SUMMARY/ CONCLUSION

Recently, the handling of nitrogenous waste has become a critical factor in environmental management. Nitrogenous waste in wastewater can arrive from various sources including human and animal excreta, industrial wastes and from modern agricultural practices which use nitrogen-rich fertilizers (Kowalchuk and Stephen, 2001). Excess nitrogen discharged into the environment can cause serious problems such as eutrophication of receiving water bodies, deterioration of water resources and pose a risk to human health (Smith, 2003; Wolfe and Patz, 2002). Thus, the proper removal of nitrogenous waste from wastewater is of the utmost importance. The most reliable and economical means of removing nitrogen from the wastewater is achieved by nitrification followed by denitrification (Hanaki et al., 1990). Nitrification is the process that converts ammonia (NH_3) to nitrite ($NO_2^{-)}$ and nitrate ($NO_3^{-)}$. This process is facilitated by two phylogenetically different groups of autotrophic aerobic bacteria which are the AOB and NOB. Wastewater treatment is considered to be the most important biotechnological application of AOB (Kowalchuk and Stephen, 2001). Denitrification plays an essential role in the elimination of nitrogen by converting it into harmless N_2 gas that is eventually lost to the atmosphere. Denitrifying bacteria have the ability to reduce NO_3^- and NO_2^- (both products of nitrification) into N_2O or N_2 in the absence of oxygen. Successful nitrification and denitrification must occur in wastewater treatment to avoid environmental contamination.

Recent studies reveal the findings of increased levels of various pharmaceuticals in effluent of wastewater treatment plants (Lindsay et al., 2001; Verlichhi, 2010). Human and veterinary drugs are being continuously introduced in wastewater treatments plants as a result of metabolic excretion and improper disposal. Approximately 30-90% of an administered pharmaceutical dose ingested by humans is excreted in the urine as the active substance (Rang et

al., 1999). Due to the continuous influx of these pharmaceuticals into the environment, these compounds can become “pseudo-persistent” in a wastewater treatment plant. It is most certain that there is a lack of understanding of the ecological risk that is associated with most of these pharmaceuticals in the environment.

The presence of antibiotics in wastewater treatment systems is of particular interest because the exposure of antibiotic residues to virulent and pathogenic bacteria can induce resistance (Chee-Sanford et al., 2001). However, antibiotics in wastewater treatment systems can also pose a risk to microorganisms that perform beneficial processes such as nitrification and denitrification and organic breakdown. It is feasible that antibiotics that are present in sewage and enter the wastewater treatment system can affect microorganisms that are important for biological treatment of wastewater.

The purpose of this study was to determine if tetracycline can affect the microbial community that are responsible for nitrification and denitrification process by assessing their function. An aerated semi-batch reactor system was set-up for this experiment. The design consisted of eighteen 1L containers containing 500ml of activated sludge from the WWTP. They were aerated using aquarium bubbling stones for a period of 30 days. The reactors were divided into 6 groups with 3 replicate reactors for each experimental condition. The six conditions were low nutrient replacement with no tetracycline, high nutrient replacement reactors with no tetracycline, low nutrient replacement reactors with low (1µg/L) tetracycline, high nutrient replacement reactors with low (1µg/L) tetracycline, low nutrient replacement with high (10µg/L) tetracycline and high nutrient replacement with high (10µg/L) tetracycline. Starting at day 0, every two days 10mL sample was extracted from the low nutrient replacement rate

reactors and 50mL was extracted from the high nutrient replacement rate reactors. The reactors were replenished with the respective volumes using 1X synthetic wastewater.

This study demonstrated that tetracycline in the presence of a low nutrient replacement rate can affect the processes of nitrification and denitrification. In low nutrient replacement reactors, tetracycline was observed to have a positive effect on ammonia and nitrate oxidation. The inhibitory effect of tetracycline at 1 and 10µg/L were not found. There was an observed increase in total nitrogen concentration when tetracycline was present in low nutrient replacement reactors but since no accumulation of nitrate was observed, it is assumed that tetracycline did not have an impact on denitrification rates but may have impacted other microbial species in the reactors that lead to the changes in total nitrogen concentrations.

Overall, this study suggested that antibiotics can have an impact on microbial communities present in the secondary treatment of wastewater treatment plants. However, further studies utilizing molecular techniques for characterizing and identifying the community composition is recommended for a more in depth understanding of this phenomenon.

APPENDIX

APPENDIX A: Nutrient analysis

Table 3. DOC concentrations (ppm) in all reactors. HD-High Dilution, LD-Low Dilution

Treatments	Days									
	0	+/-SD	**7**	+/-SD	**16**	+/-SD	**21**	+/-SD	**30**	+/-SD
No Tetracycline HD	972.5	126.71	712.1	4.26	71.8	29.66	22.7	3.74	224.7	21.36
Low Tetracycline HD	972.5	126.71	712.1	4.26	28.1	5.18	29.2	5.41	233.0	39.67
High Tetracycline HD	972.5	126.71	712.1	4.26	20.9	9.40	18.6	2.89	211.4	40.25
No Tetracycline LD	972.5	126.71	721.1	4.26	28.8	2.32	35.5	7.51	164.0	18.37
Low Tetracycline LD	972.5	126.71	721.1	4.26	10.7	5.49	30.7	12.97	161.9	40.55
High Tetracycline LD	972.5	126.71	721.1	4.26	20.3	9.68	42.2	6.68	199.0	5.73

Table 4. Mean total Kjeldahl nitrogen concentrations (µM) in all reactors +/- SD

Treatments	Days							
	0	**+/-SD**	**7**	**+/-SD**	**9**	**+/-SD**	**18**	**+/-SD**
No Tetracycline HD	9850.0	1852.62	8840.0	1103.09	6500.0	1131.37	5925.0	176.78
LowTetracycline HD	9850.0	1852.62	8840.0	1103.09	2416.7	2033.67	6350.0	1767.77
High Tetracycline HD	9850.0	1852.62	8840.0	1103.09	3850.0	1202.08	6750.0	1202.08
No Tetracycline LD	9850.0	1852.62	8840.0	1103.09	5483.3	964.80	4466.7	1255.32
Low Tetracycline LD	9850.0	1852.62	8840.0	1103.09	14466.7	3858.22	0.0	0.00
High Tetracycline LD	9850.0	1852.62	8840.0	1103.09	19533.3	2041.04	0.0	0.00

Table 5. Mean ammonia concentrations (µM) in all reactors +/- SD

Treatment	Day											
	0	SD	7	SD	9	SD	21	SD	28	SD	30	SD
Control HD	1243.27	169.99	1297.33	1.32	1359.82	74.93	978.43	8.53	542.50	152.97	454.78	104.53
Low Tet HD	1243.27	169.99	1297.33	1.32	1275.44	113.00	1024.60	13.29	184.67	17.91	499.33	128.36
High Tet HD	1243.27	169.99	1297.33	1.32	1564.67	102.11	1275.40	273.98	849.33	22.16	754.83	85.09
Control LD	1243.27	169.99	1297.33	1.32	1685.91	346.13	1546.36	53.51	1874.67	166.76	1716.00	204.59
Low Tet LD	1243.27	169.99	1297.33	1.32	135.18	52.10	-15.93	2.28	158.78	5.74	313.78	98.26
High Tet LD	1243.27	169.99	1297.33	1.32	121.53	26.26	111.13	89.66	367.00	49.97	575.83	3.06

Table 6. Mean nitrate concentrations in all reactors +/- SD

Treatment	Final Conc. (uM)											
	Day 7	SD	Day 9	SD	Day 16	SD	Day 21	SD	Day 28	SD	Day 30	SD
Control HD	639.17	15.17	368.89	6.19	160.83	17.68	638.75	16.50	111.25	2.65	139.37	34.88
Low HD	639.17	15.17	300.28	64.97	153.96	7.37	427.71	65.70	281.67	18.46	73.33	13.13
High HD	639.17	15.17	401.04	52.74	54.38	27.40	326.46	10.31	253.13	11.49	90.24	7.07
Control LD	639.17	15.17	303.75	4.71	117.36	13.06	300.00	35.59	285.42	2.60	226.43	62.14
Low LD	639.17	15.17	86.04	6.19	59.31	1.58	143.19	19.17	192.50	20.24	72.02	0.51
High LD	639.17	15.17	163.75	8.25	73.33	13.24	11.25	8.46	173.89	9.43	81.31	6.90

Table 7. Mean nitrite concentrations (µM) in all reactors +/- SD

Treatments	Final Conc. (uM)											
	Day 0	SD	Day 7	SD	Day 16	SD	Day 21	SD	Day 28	SD	Day 30	SD
Control HD	0.15	0.18	0.13	0.07	0.00	0.00	0.00	0.00	0.00	0.11	0.18	0.14
Low HD	0.15	0.18	0.13	0.07	0.06	0.01	0.00	0.17	0.00	0.05	0.19	0.27
High HD	0.15	0.18	0.13	0.07	0.09	0.04	0.37	1.00	0.00	0.16	0.09	0.31
Control LD	0.15	0.18	0.13	0.07	0.00	0.03	0.00	0.09	0.14	0.34	0.00	0.02
Low LD	0.15	0.18	0.13	0.07	0.00	0.06	0.00	0.09	0.00	0.19	0.21	0.18
High LD	0.15	0.18	0.13	0.07	0.00	0.23	0.00	0.03	0.00	0.11	0.10	0.31

APPENDIX B: Reagent List

Kjeldahl Nitrogen Analysis

Kjeldahl Digestion Reagent: The digestion reagent was prepared by dissolving 134g potassium sulphate and 7.3g of copper sulphate in 800ml of water. To this solution 134ml of concentrated sulphuric acid was added. Due to the heat generated from the acid, the solution was allowed to cool and then was diluted to make 1L. Also the solution must be mixed properly. After solution is made it was stored at room temperature.

Ammonium Nitrogen Analysis

Phenol solution: Must be prepared on a weekly basis by diluting 11.1mg phenol (89%) with 95% (v/v) ethyl alcohol to a final volume of 100mL

Sodium nitroprusside (0.5% (w/v)): 0.5g of sodium nitroprusside was dissolved in 100mL deionized water

Trisodium citrate: 200g trisodium citrate and 10 g sodium hydroxide was added and dissolved in deionized water. After thorough mixing and solution was diluted to make a final volume of 1L

Oxidizing solution: 25 mL of sodium hypochlorite was mixed with 100mL alkaline citrate solution on the day of assay. Commercially available bleach (5%) can be used.

Stock solution for standards: This was prepared by dissolving 3.189g anhydrous NH_4Cl (1mg/mL nitrogen) in 1L water

Nitrate-Nitrite Analysis

Construction of the nitrate reduction column: A cadmium column used for the nitrate/nitrite assay. This was constructed using a copper cylinder with dimensions length 150mm with a 5mm diameter. The column was tightly packed with Cu-Cd granules (must be washed prior to packing, read below). It was ensured that Cu-Cd granules do not leak out because both ends of the column were capped with steel wool and fitted with metal bolts. This also ensured an air tight seal of the column. Commercially available plastic aquarium tube adapter was connected to allow the column to be connected to the external setup.

20g of Cu-Cd granules were washed with 6N HCl followed by deionized water. The granules were then rinsed with 50mL 2% $CuSO_4$ (5g $CuSO_4 \cdot 5H_2O$ diluted to 250mL water) for 5 minutes, until the characteristic copper blue colour diminished. Then using about 50mL 2% $CuSO_4$, washings were repeated until the formation of a brown colloidal precipitate. This was followed by a water wash to remove the precipitate from the washed Cu. These activated granules were then packed into the column using a thin metal rod and a plastic tube filled with the ammonium chloride-EDTA solution. The column was then stored in a plastic case filled with the buffer solution until further use. The washing of the granules allowed running approximately 300 samples before it needed to be packed again.

Colour reagent: A colour reagent was prepared by dissolving 1g of sulphanilamide in 80ml water and 10ml 85% phosphoric acid. After thorough mixing to ensure that everything had dissolved, 0.1g N-(1-

naphthyl)-ethylenediamine dihydrochloride was added to the mixture and the volume of the solution was brought up to 100mL with distilled water. The solution is light sensitive and thus it had to be stored in a brown plastic bottle.

Ammonium chloride-EDTA solution: The buffer solution was made by dissolving 13g NH_4Cl and 1.7g disodium ethylenediaminetetraacetate in 900mL water. Then using a pH meter to monitor the pH, the solution was adjusted to a pH of 8.5 with concentrated NH_4OH. The solution was then diluted to 1L.

Stock solution: A stock solution was prepared by dissolving 7.22g of KNO_3 (previously dried in an oven at 105ºC for 24 hours) in 1L of water. In order to obtain a 10 $mg \bullet L^{-1}$ working standard solution; a 100 fold dilution from the stock solution was made.

Table 8. Synthetic Wastewater Composition (Liao et al., 2001)

Nutrients	Compounds		Constituents	Concentration (mg/L		Sources
C-Source	Glucose		COD	250 (100mg C)		Sigma Chemical, Canada
	Sodium Acetate		COD	283.5 (83.0mg C)		Sigma Chemical, Canada
P-Source	KH2PO4		P	8,78 (2.0mg P)		Sigma Chemical, Canada
	K2HPO4		P	11.24 (2.0mg P)		Sigma Chemical, Canada
N-Source	(NH4)2SO4		NH4-N	89.33 (18.9mg N)		Fisher Scientific, Canada
Others	MgSO4.7H2O		Mg	5.07 (0.5mg Mg)		VWR Scientific, Canada
	CaCl2.2H2O		Ca	2.0 (0.5mg Ca)		VWR Scientific, Canada
	Na2MoO4.2H2O		Mo	0.01 (0.004mg Mo)		VWR Scientific, Canada
	MnCl2.4H2O		Mn	0.036 (0.1mg Mn)		Fisher Scientific, Canada
	FeSO4.7H20		Fe	0.50 (0.1mg fe)		Fisher Scientific, Canada
	CuSO4.5H2O		Cu	0.39 (0.1mg Cu)		Sigma Chemical, Canada
	ZnSO4.7H2O		Zn	0.44 (0.1mg Zn)		VWR Scientific, Canada
	CoCl2.6H2O		Co	0.41 (0.41mg Co)		Fisher Scientific, Canada

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