

EFFECTS OF TETRACYCLINE AND IBUPROFEN ON THE RELATIVE ABUNDANCE
OF PROTOZOAN AND BACTERIAL POPULATIONS IN WASTEWATER TREATMENT
SEMI - BATCH REACTORS

by

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ABSTRACT

The activated sludge process in Wastewater Treatment Plant (WWTPs) relies on the activities of microbes to reduce the organic and inorganic matter and produce effluent that is safe to discharge into receiving waters. This research examined the effects of non-steroidal anti-inflammatory drug (NSAID) ibuprofen and the antibiotic tetracycline on the microbial population in activated sludge from the Humber WWTP. The current investigation was designated to observe the impact of these contaminants, at low (environmentally relative concentrations) as well as extremely high concentrations of tetracycline and ibuprofen. Using 16S and 18S rRNA gene primer sets, and qPCR the abundance of each population was monitored as well as the relative abundance of two populations under the various conditions. It was found that current environmental concentrations of ibuprofen stimulated protozoan growth but higher concentrations reduced their numbers especially in the presence of tetracycline. Finally using DGGE, the identity for some of the more abundant protozoa were identified and it was noted that high ibuprofen and tetracycline concentrations favored the abundance of some genera.

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CHAPTER 1:

1.1 INTRODUCTION

Wastewater is a compilation of rainwater, agricultural, industrial, and municipal wastewater (Pauli *et al.*, 2015). The city of Toronto houses four wastewater treatment plants (WWTP), Ashbridges Bay, Highland Creek, Humber, and North Toronto. The four plants jointly handle approximately 1,111.6 ML/day of wastewater and serve 2,773,000 residents in 2014 (City of Toronto, 2015). Wastewater can include organic and inorganic matter, nutrients, pathogens, synthetic chemicals, and heavy metals. In order to reduce harm and damages to the environment, and human society, as well as recycle fresh water, wastewater management is critical and their processes are vital to the operation.

The wastewater treatment process includes; i) preliminary, ii) primary, iii) secondary, sometimes a iv) tertiary treatment phase. A vital process of wastewater management is the secondary treatment phase, which operates with the usage of microorganism as clarifiers for waste decomposition. The importance of maintaining a proper environment for these microorganisms is critical for this process in order to maintain efficient biodegradation. Microorganisms are not immune to chemical and physical effects that may cause population reduction, subsequently reducing performance efficiency. The two significant microbial groups in the activated sludge are protozoa and bacteria. While bacteria biodegrade organic matter within the influent (wastewater entering), protozoa graze on free swimming bacteria and help clear the effluent (wastewater existing) (Pauli *et al.*, 2015).

1.2 IBUPROFEN AS A CONTAMINANT OF WASTE WATER

There are lots of contaminants that make their way into wastewater influent. One group are the non-steroidal anti-inflammatory drugs (NSAID) such as ibuprofen. Ibuprofen is an antipyretic and analgesic drug that is commonly used by children and adults (Aslam *et al.*, 2010). Ibuprofen was developed in 1961 by the Boots Company and was approved by the FDA for over-the-counter use by 1984 (Rainsford, 2011). Ibuprofen was first introduced in 1969 in a study conducted by the University of Wollongong with evidence suggesting that some NSAIDs have the capabilities of antibacterial properties (Yin *et al.*, 2014). Health Canada has recently reported that in Canada approximately 780,000 prescriptions of 600 mg of ibuprofen were sold between 2010 and 2014 (Health Canada, 2015). This statistic does not include the over-the-counter ibuprofen sold, such as the regular strength 200 mg and extra strength 400 mg.

1.3 TETRACYCLINE AS A CONTAMINANT

Another contaminant group in wastewater influent are antibiotics such as tetracycline. Tetracyclines are a group of broad-spectrum antibiotics first discovered in the early 1940s and were approved by the FDA in 1948 (Nelson *et al.*, 2011). Tetracyclines also have sub classifications such as short acting (i.e. tetracycline), intermediate-acting (i.e. demeclocycline) and long-acting compounds (i.e. minocycline and doxycycline). Members of the tetracycline family share similar antibacterial effects against pathogens such as, *Chlamydia*, *Mycoplasma* and *Rickettsia* (Do Bugs Need Drugs?, 2014). Tetracyclines function by inhibiting protein synthesis by blocking aminoacyl tRNA and preventing it from binding to the 30S ribosome (Todar, 2002). Tetracyclines have been commonly used for both human and animal care, where the majority of

use today is in veterinary medicine and animal husbandry, such as growth promotion and prevention of bacterial infections (Gujarathi *et al.*, 2005).

According to a report by the BC Centre for Disease Control, from 1996 to 2009, the consumption rate of tetracyclines gradually declined from 3.34 to 2.66 defined daily dose (DDD)/1000 population/day, which was approximately a total percentage drop of 20%. Conversely, an increase of 8% was witnessed from 2.66 in 2009 to 2.88 DDD/1000 population/day in 2013 ("Do Bugs Need Drugs?", 2014). The reason for this change is that although tetracycline has declined, sub group doxycycline has significantly increased in use and is the major contributor for the overall tetracycline increase. The use of doxycycline decreased from 1.27 in 1996 to 1.06 DDD/1000 population/day in 2003, then had a steady increase to 1.78 DDD/1000 population/day up to 2013, which is most likely related to MRSA and treatment of skin and soft tissue infections (Do Bugs Need Drugs?, 2014). Usage of minocycline also increased from 0.43 DDD/1000 population/day in 1996 to 0.82 DDD/1000 population/day in 2013. Figure 1 and Figure 2 illustrate the change of DDD/1000 population/day and daily prescription rates for tetracyclines from 1996 to 2003. The Figures 1 and 2 demonstrate that, tetracycline has decreased while doxycycline and minocycline have steadily increased. Utilization of these antibiotic consumption in British Columbia were comparable in consumption to that of Ontario (Do Bugs Need Drugs?, 2014).

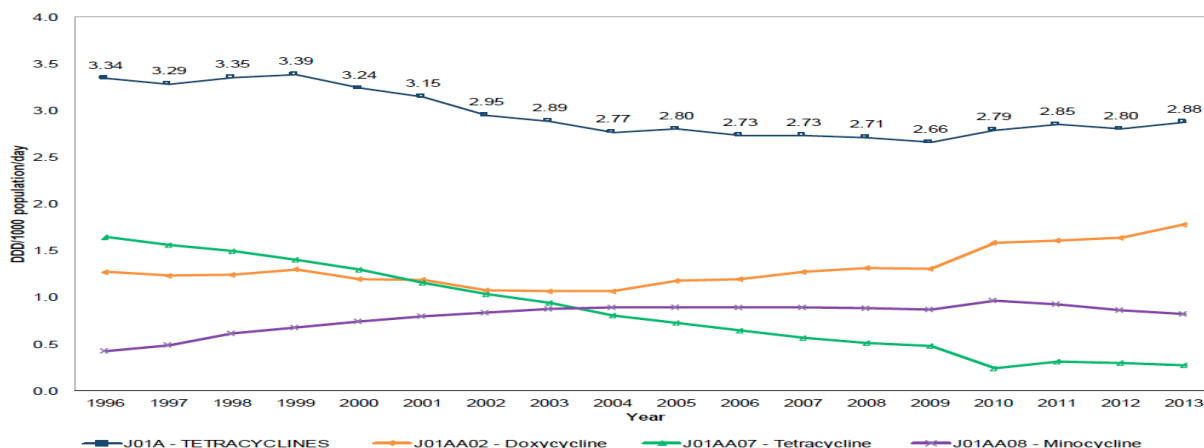


Figure 1 - Daily Consumption of tetracyclines and subclass agents in Canada from 1996 - 2013 (Do Bugs Need Drugs?, 2014)

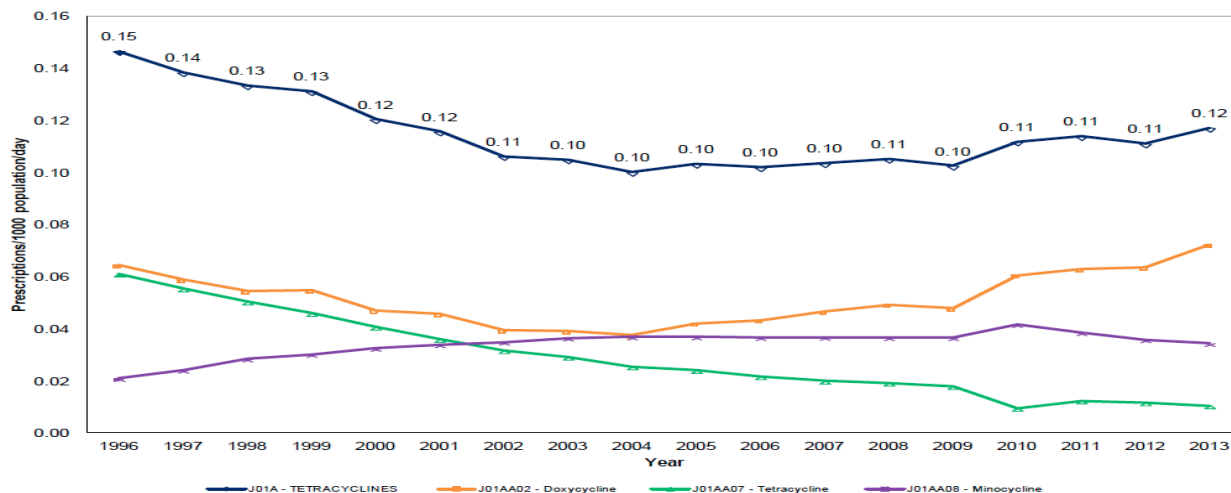


Figure 2 - Daily prescription rates for tetracyclines and subclass agents in Canada from 1996 – 2013 (Do Bugs Need Drugs?, 2014)

There are thousands of contaminants in wastewater, however, for this study tetracycline and ibuprofen were chosen as representatives of antibiotics and NSAIDS respectively because they are currently present in wastewater and it is very difficult to test all contaminants at the same time. In relation to the shown statistics, people are currently using ibuprofen and tetracycline in significant quantities. Although current activated sludge processes are working

very well, microorganisms are being affected albeit on an insignificant scale but nonetheless still affected. This study will examine the effects of tetracycline and ibuprofen on the relative abundance of protozoan and bacterial populations because these two microorganisms play a vital role in the activated sludge process.

1.4 OBJECTIVES

In order to examine the effects of tetracycline and ibuprofen on the relative abundance of protozoan and bacterial populations, this study had the following three objectives.

- I. Determine the abundance of protozoan and bacterial populations in presence of tetracycline and ibuprofen separately and simultaneously.
- II. Comparison of the relative abundance of protozoan and bacterial populations separately and simultaneously through quantitative real-time polymerase chain reaction (qPCR).
- III. Examine the eukaryotic community using 18S rRNA gene primers to determine effects of pharmaceuticals.

CHAPTER 2 LITERATURE REVIEW

2.1 WASTEWATER TREATMENT

The main objective of Toronto's wastewater treatment is to remove contaminants from wastewater before the effluent is released to Lake Ontario. Doing so will preserve Canadian water resources, the environment, and aquatic life. Wastewater is a combination of solids and liquids that derive from matter that has been flushed down toilets, drains and sinks daily (City of Toronto, 2015). The flushed matter travels through the sanitary sewer system throughout the city to one of the four WWTPs (City of Toronto, 2015). Toronto's WWTPs are designed to remove chemicals, solids and any other undesired contaminant in a cost effective, environmentally friendly and reliable approach (City of Toronto, 2015). A conventional wastewater treatment process is used for municipal sewage in Ontario, and its process involves a preliminary, primary, secondary and sometime a tertiary phase.

2.1.1 HUMBER WASTEWATER TREATMENT PLANT

Humber Wastewater Treatment Plant (HWWTP) is one of four WWTPs owned and operated by the City of Toronto. HWWTP is Toronto's second largest WWTP, located along the Queensway in Toronto's west end, near the mouth of the Humber River (City of Toronto, 2015). The HWWTP began operation in 1960 and its current capacity is 473,000 cubic meters. The facility serves a population of approximately 651,999, a population throughout the Etobicoke, a

portion of North York, York and Toronto.

2.1.2 PRELIMINARY PHASE

The preliminary phase is designed to remove large debris from the wastewater before proceeding to the next phase (City of Toronto, 2015). The treatment process starts with raw wastewater entering the HWWTP Head House which functions to provide grit and screening removal processes. The plant has six inlet channels, four channels equipped with front raking mechanical bar screens and two with back raking mechanical bar screens (City of Toronto, 2015). The wastewater then splits into two streams, one stream is within the Head House and the other stream goes to another building, both used for grit removal (City of Toronto, 2015). In the Head House, ferrous chloride is added for phosphorus removal and the grit is removed using six vortex chambers, while in the other building the grit is removed using three Aerated Grit Channels (City of Toronto, 2015). The grit and screenings are then transported to a sanitary landfill location.

2.1.3 PRIMARY TREATMENT

The next step that precedes Preliminary Phase is the Primary Phase, also known as Primary Settling or Clarification. In this phase the flow velocity of the influent is reduced in the Primary Clarification Tanks, allowing for settlement of heavy solids and partially removing non-soluble phosphorus (City of Toronto, 2015). Within the tanks, sludge collectors sweep the settled

sludge into sludge hoppers that are located at the bottom of the tank, the sludge is then pumped to anaerobic digestion tanks. Unsettled, suspended, and dissolved effluent then proceeds to the secondary phase (City of Toronto, 2015).

2.1.4 SECONDARY PHASE

Secondary phase, also known as biological treatment phase, utilizes microorganisms to metabolize and dissolve organic and inorganic materials. These microorganisms can be used as part of processes such as activated sludge, rotating biological contactors, and bio-filtration. Activated sludge is the method of choice for municipal wastewater due to yielding the highest quality effluent at a reasonable maintenance and operating cost (Nesc, 2003). After microorganisms are added, with the addition of oxygen, the microorganisms break down organic solids, followed by flocculation. The effluent is then directed into a final sedimentation tank, where solid matter (a mixture of organic matter and microorganisms) is now heavy enough to settle in the tank and ready for discarding. Within the secondary phase, some of the returned activated sludge from final sedimentation or secondary clarification tanks is recycled back to the primary effluent in the aeration tank and the rest is sent to sludge thickening (City of Toronto, 2015).

Waste activated sludge from the final sedimentation that is sent to sludge thickening is dehydrated in order to reduce volume size and is then sent through the primary anaerobic digestion followed by secondary anaerobic digestion for energy production and other bio-solids use (City of Toronto, 2015).

2.1.5 FINAL EFFLUENT QUALITY & DISINFECTION

The final stage of treatment is chlorination in order to insure eradication of any microorganisms left in the effluent before discarding into bodies of water, in this case Lake Ontario. Figure 3 shows the Humber WWTP process.

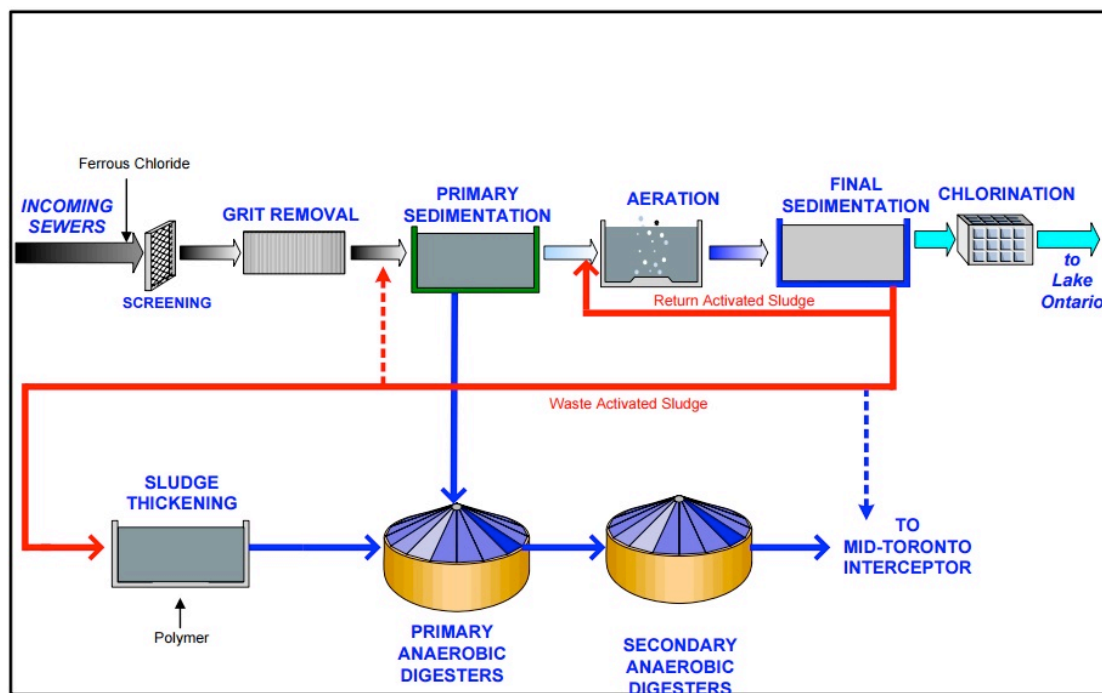


Figure 3 - Humber wastewater treatment process (City of Toronto, 2015)

2.2 MICROBIAL COMMUNITY

Microbial communities have been used for degradation since the discovery of bacteria in the nineteenth century (Pauli *et al.*, 2015). Activated sludge was developed in 1913 in the United

Kingdom by W.T. Lockett and Edward Ardern, utilizes suspended organisms and their self-purification properties to transform the suspended and dissolved constituents into biomass, which then can be removed by sedimentation (Beychok 1967; Pauli *et al.*, 2015). Activated sludge contains bacteria, filamentous bacteria, protozoa, metazoan (which includes rotifers, daphnia, other crustaceans, nematodes), algae, fungi and enteric viruses (Pauli *et al.*, 2015; Prado *et al.*, 2014).

The most important group of microorganisms for wastewater treatment are bacteria, they are responsible for forming floc and degrading organic matter. There are many types of bacteria and their composition usually depends on the nature of the organic material present in wastewater (McKinney, 1962). Activated sludge tanks with suspended flocculate material can contain various amounts of bacteria. Pike, 1975, observed a population size approximately 6×10^9 individual bacteria per ml, while another study by Allen, 1944, examined samples presenting a population size between 9.5 to 48.5 millions per ml, the difference in population size is due to different numeration procedures. The bacterial population represents approximately 90% of the total biomass of the activated sludge.

Although bacteria are the dominating microorganisms in activated sludge, there are other high forms of microorganisms, particularly protozoa, which help in clarifying the effluent. Protozoa are single celled eukaryotes that range from 5 to 1000 μm in magnitude. Activated sludge is added to wastewater and in combination with aeration and agitation, the microorganisms are used for waste decomposition, this process is followed by sedimentation, where the sludge is either discarded or reused (Nesc, 2003). These microorganisms contribute to

wastewater treatment in different forms. Prokaryotic communities are responsible for the removal of carbon, phosphorous and nitrogen from wastewater influent. Due to prokaryotes ability to mineralize inorganic and organic nutrients, it has been found that their population is greater in mass in comparison to other microorganisms and therefore represent an essential component of the activated sludge process (Madoni, 2011).

On the other hand, protozoa graze on free-swimming dispersed bacteria and other suspended particles, therefore contribute to the wastewater treatment processes by clarifying the effluent (Modoni, 2011). The high organic content in wastewater allows for biocoenosis of organisms to exist, with the primary members being decomposers, i.e., saprophytic (obtain nutrients from dead organic matter) bacteria. Bacteria biodegrades organic matter in the presence of oxygen and produces carbon dioxide and water; as well, nitrogen is released in the form of ammonia (NH_3) (Pauli *et al.*, 2015). In wastewater treatment, bacteria are vastly greater in number and smaller in relative size than protozoa are (Pauli *et al.*, 2015). Bacteria and protozoa together form a closely related microbial system, this system allows for the “natural” self-purification of wastewater (Pauli *et al.*, 2015). Filamentous bacteria give awareness to activated sludge conditions; excessive amounts of filamentous bacteria in an activated sludge treatment systems can cause complications with sludge and floc settling (Pauli *et al.*, 2015).

2.2.1 ACTIVATED SLUDGE, PARAMETERS AND EFFICIENCY

Activated sludge is the biomass in aeration tanks that is produced in settled or raw wastewater by the growth of microorganisms in the presence of dissolved oxygen. The sludge is

deemed activated due to the presence of microorganism, particularly bacteria, protozoa and fungi. Wastewater is added to activated sludge in aeration tanks in combination with aeration and agitation, the microorganisms are used for waste decomposition, this process is followed by sedimentation, where the sludge is either discarded or reused (Nesc, 2003). During the activated sludge process, flocculation is induced by agitating wastewater in order to force suspended particles, bacteria, to group or clump together to produce heavier particles; these particles are termed floc. After the floc is formed, it becomes heavy and settles at the bottom of the tank (Wisconsin Department of Natural Resources, 2010).

Numerous parameters are used to describe activated sludge used in wastewater treatment. These parameters are, Sludge Volume Index (SVI), Mixed Liquor Volatile Suspended Solids (MLVSS), Mixed Liquor Suspension Solids (MLSS), and Oxygen Uptake Rate (OUR). These parameters are used to evaluate sludge condition and quality. Biochemical oxygen demand (BOD) can be examined in order to determine the efficiency of the activated sludge process before and after the treatment of wastewater. In aeration tanks, MLSS can be used as a measure of suspended organic and inorganic material (Wisconsin department of natural resources, 2010).

Microorganisms are an essential part of the activated sludge process; microorganisms help by removing nitrifying ammonia and carbonaceous organic material that is present in the secondary influent process. In the activated sludge process, biomass goes through a process of ammonification to form ammonia, which then proceeds to nitrification to form nitrite and then to form nitrate, which then produces nitrogen gas through denitrification. Anammox reaction is another method in which nitrogen is biologically removed; nitrite ions and ammonia ions form

nitrogen gas (Basnyat, 2010). Below, figure 4 shows the nitrogen cycle and the relationship between the processes. Furthermore, some microorganisms, through nitrification, may use energy produced by oxidizing ammonia and nitrogen to nitrate. The major nutrients in wastewater that encourage growth of algae and other organic matter are phosphate and nitrogen. Biological removal of nitrogen and phosphorus is an essential function of the activated sludge process.

The most predominant microbe in activate sludge are heterotrophic bacteria. Heterotrophic bacteria use organic compounds for their carbon supply and energy source. Microbes are able to convert carbon into cell components as well as oxidize the end products, which include water and carbon dioxide (Hanrahan, 2012). Along with bacteria many other microbes are present.

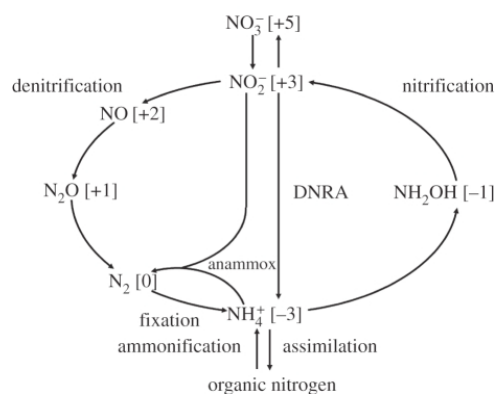


Figure 4 Diagram showing nitrogen cycle and the relationship of ammonification, nitrification, denitrification, and anammox process (Thomson *et al.*, 2012).

In activated sludge, the microbial population exists in a heterogeneous community that is in equilibrium, which supports the treatment plant in adaptability with respect to plant operational changes. Such changes include temperature, flow and other wastewater composition

changes. Perpetrations to the system, such as the presence of pharmaceuticals, may inhibit the performance of the activated sludge, which in turn can result in a loading shock to the system resulting in a decrease in treatment efficiency (Gutierrez *et al.*, 2002).

Carvalho *et al.*, (2013), evaluated the performance of activated sludge in the removal of frequently used veterinary medications, ceftiofur, enrofloxacin (ENR), and tetracycline (TET), from influent. The study was performed using 100 µg/L of the pharmaceutical and resulted in 68% removal for ENR and 77 % for reactor containing TET in the aqueous stage. The research resulted in sorption of sludge having the most significant percent of drug removal.

Puigagut *et al.*, (2007), tested soluble compounds on microfauna and the treatment efficiency of activated sludge. The experiment tested activated sludge containing domestic sewage supplemented with enriched starch or glucose over a period of five months. The study showed that the system with enriched starch presented a lower abundance of filamentous bacteria, which resulted in better sludge sedimentation. Although there was no difference in organic matter removal, a higher rate of nitrification and denitrification was observed which was most likely is a result of more compact flocs. Another observation was ciliated protozoa, specifically attached ciliates, were found to be the dominating microfuna in both tests whereas metazoan, in particular Lecanidae rotifers, were found to be more abundant in the enriched starch system. Furthermore, metazoan feeding caused a reduction in floc leading to a lower nitrogen quality in the effluent.

2.2.2 PHARMACEUTICALS

The definition of pharmaceuticals as defined by the European Union is commonly known as drugs and medicine in real world, is defined as a chemical and biological product which are present in the form of different substances to cure diseases in human life (WHO, 2006). Several uses of pharmaceuticals in humans can be used to treat or correct physiological functions by applying immunological, pharmacological and metabolic action. In Europe, 3000 active components of pharmaceuticals are being used. Pharmaceuticals are classified differently and their structure of classification is usually defined by their chemical formula/structure, receptor interaction, pharmacological activity and physiological classification. An active pharmacological compound has been found in every pharmaceutical (WHO, 2006). Figure 5 shows a schematic figure of potential sources and pathways for the occurrence of pharmaceuticals in the environment. While some contaminants go through the wastewater treatment system, many contaminants go untreated from the source straight to the near by body of water which is often the drinking water source.

The Defined Daily Dose (DDD) and The Anatomical Therapeutic Chemical (ATC) are two main measuring units that are frequently used for drug utilization research on international level. The comparison and exchange of the data is occurred by ATC/DDD system on a major scale that includes local, national and international level around the globe (WHO, 2006). During the last decade, there has been an increase trend in terms of nature, type and presence of pharmaceuticals in wastewater. There are new pharmaceuticals in the market that are rapidly added to the large collection of chemical classes (Daughton *et al.*, 1999; Heberer, 2002).

Pharmaceuticals have been found in the water samples of groundwater, river water, wastewater and drinking water. Most common type of pharmaceuticals found in the water samples are lipid regulator, antibiotics, contraceptives, beta-blockers, anti-inflammatory drugs and tranquilizers (Daughton *et al.*, 1999; Ternes, 2001; Heberer, 2002).

The classifications of pharmaceuticals are divided into various groups such as antibacterial, anti-inflammatory, antiepileptic's, antirheumatics, lipid modifying agents and beta-blocking agents. Antibacterial drugs are further classified into Fluoroquinolones, which are used to treat Urinary Tract Infections (UTI), gonorrhea, respiratory infections, cervicitis, bacterial prostatitis and anthrax. Ciprofloxacin, Ofloxacin and Norfloxacin, Sulamethoxazole and Sulfonamide are Fluoroquinolones. Antiepileptic drugs are used to cure manic-depressive illness and neuropathic pain, Carbamazepine is an example of a broadly researched antiepileptic drug (Rang *et al.*, 2003).

Anti-inflammatory and antirheumatic drugs, also known as non-steroidal anti-inflammatory drugs (NSAIDs), help treat rheumatic musculoskeletal, pain and inflammation amongst other bodily issues. Examples of these drugs are Ibuprofen, Ketoprofen, Diclofenac and Naproxen which are used worldwide (Rang *et al.*, 2003). An example of a lipid modifying agent is Bezafibrate, which is commonly used to treat mixed dyslipidaemia that leads to atherosclerosis (Rang *et al.*, 2003). Beta-blockers, such as Atenolol, Acebutolol and Metoprolol are drugs that help treat hypertension, dysrhythmias and angina (Rang *et al.*, 2003). Therapeutic agents help treat muscle pain, Diclofenac and Ketoprofen in the form of different sprays and gels are examples of such agents (Rang *et al.*, 2003). Osmerod and Efrimsson (1988) conducted

research on the toxicity effects of phenols on heterotrophic microorganisms. Through microscopic examination, the research concluded that with increasing test compounds, ciliates were the first microbes to disappear.

2.2.3 MAJOR PHARMACEUTICALS IN WASTEWATER

Two major pharmaceuticals found in wastewater are Diclofenac and Carbamazepine. It has been found that during the wastewater treatment process (WWTP), pharmaceuticals are abundantly present and are not efficiently removed before effluent is released into water bodies. Pharmaceuticals are found in the waters of America, Asia and Europe (Zhang *et al.*, 2008). The traces of pharmaceuticals are present mainly in wastewater treatment process (WWTP) effluents, ground water, surface water and in drinking water as well. Drugs like Diclofenac and Carbamazepine have different concentration in countries due to pharmaceutical consumption rates, as well as limited resources to conduct an investigation to find high concentration in the wastewater. The classification of Carbamazepine is anthropogenic and its concentration in wastewater effluent varies from nanogram per liter to microgram per liter in different countries (Heberer, 2002; Zhang *et al.*, 2008).

Meige *et al.*, (2009), conducted a study analyze the nature of pharmaceuticals, their occurrence, removal efficiency and their quantities. The list's allows the identification of commonly investigated PPCPs (pharmaceuticals and personal care products) and the most persistent of them present in the wastewater treatment process in order to obtain quantitative and reliable values of their concentrations as well as determine the removal efficiency and frequency of detection in WWTP. Meige and colleagues (2009) has detected and identified hundred's of

pharmaceutical and PPCP'S through prescription drug classes which are measured in WWTPs of countries such as North America, Brazil and European countries. Anti-inflammatory and analgesics drugs, bacteriostatic and antibiotics, beta-blockers, anti-epileptics, contrast media, blood lipid regulators, hormones (including oral contraceptives) cytostatics, musk fragrances, antidepressants and anxiolytic, antiseptics and disinfectants are examples of drugs noted by the review to be in the WWTPs (Miège *et al.*, 2009).

Microorganisms are used for mineralizing contaminants and breaking them down to an acceptable form for nutritional use and discharge. It is done through the activated sludge processes in WWTPs. Moreover, air stripping and then sorption onto sludge are two ways to remove the contaminants present in the wastewater. Overall the removal of pharmaceutical contaminants present in activated sludge process involves four steps i.e. biotransformation, air stripping, sorption and photo transformation (Zhang, 2008). Table 1 shows an array of contaminants and their removal rates, and as shown, the removal rates are not always 100%. Note that tetracycline has a removal rate between 40 and 89 percent with an average of 71 percent removal rate and ibuprofen has a removal rate between 65 and 100 with an average of 91 percent removal rate.

Ibuprofen, an NSAID, is an example of a commonly used pharmaceutical, it has a chemical structure that does not contain chlorine and double aromatic rings, which makes it easier to breakdown. Clofibric acid is a refractory contaminant, meaning difficult to treat, it contains chlorine in its chemical structure, which makes it harder to breakdown. Membrane bioreactors are considered highly effective in the removal of Clofibric acid in WWTPs (Kimura

et al., 2005). Table 2 contains cited ibuprofen concentrations of various influent, effluent and maximal removal rates.

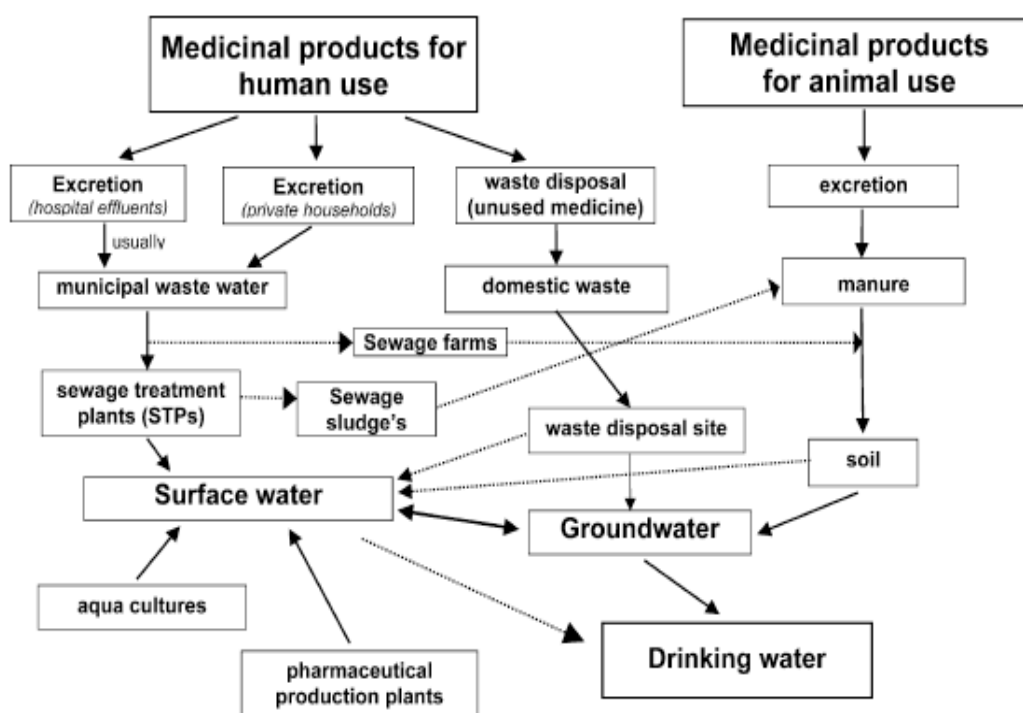


Figure 5: A system showing potential sources and pathways for the occurrence of pharmaceuticals in the aquatic environment (Heberer, 2002)

Table 1: The range of removal rates (%RE) for the most representative compounds of each therapeutic group, in the whole set of WWTP under investigation. (Gros *et al.*, 2010)

Compounds	Range of %RE	Average %RE (\pm RSD)
Sulfadiazine	[43–98]	69 (\pm 32)
Sulfamethoxazole	[30–92]	74 (\pm 22)
Norfloxacin	[30–98]	57 (\pm 54)
Ofloxacin	[20–99]	40 (\pm 64)
Ciprofloxacin	[37–99]	66 (\pm 35)
Tetracycline	[40–89]	71 (\pm 33)
Enalapril	[83–99]	96 (\pm 11)
Salbutamol	[20–99]	60 (\pm 44)
Famotidine	[30–99]	50 (\pm 59)
Ranitidine	[50–98]	66 (\pm 39)
Cimetidine	[30–99]	50 (\pm 64)
Glibenclamide	[22–75]	46 (\pm 39)
Nadolol	[25–99]	60 (\pm 51)
Atenolol	[20–97]	59 (\pm 50)
Bezafibrate	[23–99]	69 (\pm 39)
Gemfibrozil	[30–99]	67 (\pm 48)
Atorvastatin	[40–80]	58 (\pm 44)
Propyphenazone	[30–87]	44 (\pm 68)
Ketoprofen	[40–100]	69 (\pm 40)
Naproxen	[60–100]	86 (\pm 13)
Ibuprofen	[65–100]	91 (\pm 13)
Diclofenac	[30–100]	58 (\pm 53)
Acetaminophen	[96–100]	99 (\pm 1)
Salicylic acid	[82–99]	96 (\pm 8)
Furosemide	[20–96]	50 (\pm 59)

Table 2: Various concentrations of ibuprofen found in influent, effluent, and their removal efficiency.

Influent Conc. µg/L	Effluent Conc. µg/L	Maximal removal %	Reference
3		96	(Buser <i>et al.</i> , 1999)
38.7	4	>90	(Metcalf <i>et al.</i> , (2003)
9.5-14.7	0.01-0.02	99	(Thomas & Foster 2004)
1.5	0.01	12-86	(Strenn <i>et al.</i> , 2005)
2.6-5.7	0.9-2.1	60-70	(Carballa <i>et al.</i> , 2004)
5.7	0.18	97	(Quintana <i>et al.</i> , 2005)
28	3.0	98	(Roberts & Thomas 2005)
2-3	0.6-0.8	53-79	(Tauxe-Wuersch <i>et al.</i> , 2005)
13.1	0-3.8	79-100	(Lindqvista <i>et al.</i> , 2005)

2.3 PROTOZOA

Protozoa are mostly found in biofilms, activated sludge, sedimentation tanks and are associated with aerobic processes, thus they are restricted to certain areas of the wastewater treatment plant. However, there are a few specialists amongst the protozoan species that take part in anaerobic processes (Pauli *et al.*, 2015).

Literature regarding protozoa in wastewater mainly focuses on activated sludge and aerobic processes. Protozoa are a common constituent of the wastewater ecosystem (Madoni, 2011). Studies show that over 200 protozoan species are present in percolating filters, it has been established that the most common groups of protozoa detected in wastewater treatment plants are amoebae, ciliates, and flagellates, whereby ciliates produce the largest fraction with regards to population and biomass, ranging from 500 – 10,000 individuals per ml of liquor (Madoni, 2011; Pauli *et al.*, 2015). Protozoa comprise of about 5% of the dry weight of suspended solids in the mixed liquor (Curds, 1973, 1975, 1982).

Most protozoa such as ciliates often graze on suspended particles and bacteria, subsequently having a significant effect on the quality of effluent and clarification of effluent (Madoni, 2003). It has also been observed that in the presence of ciliates, a decrease in the density of viable *Escherichia coli* occurred (Curds *et al.*, 1969; Mallory *et al.* 1983). In rotating biological contactors (RBC), there is a correlation between the distribution of microorganisms and the organic load. Free-swimming ciliates and heterotrophic flagellates are dominant in early stages due to the higher organic load, whereas attached ciliates and testate amoebae are dominant

in the final stages containing low biochemical oxygen demand (BOD) (Curds & Cockburn, 1970 I). In activated sludge, free-living protozoa have been found to have a population size of $3 - 20 \times 10^6$ cells/l, as well in biomass they can have values of 250 mg/l in dry weight (Madoni, 1994a). Curds (1975), compiled a list of 228 species of protozoa found in activated sludge.

Within wastewater treatment systems, the formation of the protozoan biocoenosis, in addition to that of the total biomass involved in the purification process, is essentially dependent on factors such as wastewater composition, physical conditions, and factors resulting from technologies involved (Pauli *et al.*, 2015). Research has found that depending on the wastewater treatment plant, different protozoa dominated. A study by Sydenham (1971) showed amoebae to be the dominant group with regard to biomass while another study by Curds and Cockburn (1970) demonstrated that sludge with a high organic content showed flagellates to be a higher population density.

Protozoa positively affect bacterial carbon mineralization in activated sludge (Ratsak *et al.*, 1996). Protozoa excrete growth-stimulating compounds and nutritional minerals that result in enhanced bacterial activity and accelerated use of carbon by bacteria (Madoni, 2011). Additionally, it has been discovered that due to influence on bacterial growth, protozoa causes an increase in the per-cell nitrification rates (Madoni, 2011). Research conducted by Curds *et al.*, (1968), found that without the presence of protozoa in the mixed liquor, the effluent parameters such as BOD, organic carbon, and mixed liquor suspended solids (MLSS), were higher. Protozoa are found to be the most important bacterivorous grazers. Various studies have researched and established the relationship between the presences of particular species of ciliates in relation to

the performance of activated sludge process (Madoni *et al.*, 1993; Madoni, 1994). Jenkins and colleagues (1993) noted that stalked ciliates are suggestive of stable activated sludge process.

A side from heavy metals, there are numerous toxic substances entering the WWTP that can damage the protozoan community. The shock load effect of sodium chloride (NaCl) in the protozoan community has been evaluated in (Salvadò *et al.* 2001), the observations have shown that concentrations from 3000 to 10,000 mg/l gradually affected the microbial community with few protozoa surviving (Madoni, 2011).

Concentration of protozoan population are typically larger than 10^6 protozoa/L under normal conditions, 10^7 protozoa/L represents very good pollution reduction. As well, protozoan population that is 10^6 organisms/L is approximately 6 orders of magnitude less than the total number of bacteria in activated sludge. Concentrations that are found lower than 10^5 protozoa/L suggests a low efficiency of the plant (Madoni, 2011).

Ciliates the most often found protozoa in sludge, and they can be classified in four principal groups: crawling, free-swimming, attached and carnivorous (Madoni, 2011). With regards to crawling ciliates, they crawl over the surface of the sludge floc and or attach themselves to the sludge floc by a stalk. As for free-swimming ciliates, they swim through the liquid stage of the mixed liquor (Curds, 1973; Madoni, 1986). It was showed that effluent from activated sludge lacking protozoa was found to be more turbid than those containing protozoa. It is concluded that protozoa play a significant role in the effluent quality of the activate sludge process (Curds 1973; Curds 1975). Table 3 is a listing by Madoni (1994b) of ciliated protozoa

commonly found in activated sludge and grouped by specialization. Table 4, constructed by Madoni (1986, 1988) describes the ciliated group performance and possible causes for the rated performance.

Table 3: Activated sludge ciliates grouped by specialization. Categorized by Madoni 1994b

Carnivorous	Free-swimming	Crawling	Attached
Holotrichs	<i>Colpoda sp.</i>	<i>Aspidisca cicada</i>	<i>Carchesium spp</i>
<i>Acinertia incuroata</i>	<i>Colpidium colpoda</i>	<i>Aspidisca lynceus</i>	<i>Epistylis spp</i>
<i>Amphileptus sp.</i>	<i>Colpidium campylum</i>	<i>Chilodonella uncinata</i>	<i>Opercularia coarctata</i>
<i>Coleps hirtus*</i>	<i>Cinetochilum margaritaceum</i>	<i>Euplotes affinis</i>	<i>Opercularia microdiscus</i>
<i>Litonotus spp</i>	<i>Cyclidium glaucoma</i>	<i>Euplotes moebiusi</i>	<i>Opercularia minima</i>
<i>Spathidium spp</i>	<i>Dextotricha sp.</i>	<i>Euplotes patella</i>	<i>Stentor spp</i>
Suctorians	<i>Glaucoma scinti/lans</i>	<i>Stylonychia spp</i>	<i>Vaginicola crystallina</i>
<i>Acineta spp</i>	<i>Loxocephalus sp.</i>	<i>Trithigmostoma cucul/ulus</i>	<i>Vorticella conrllaria</i>
<i>Metacineta sp.</i>	<i>Paramecium spp</i>	<i>Trochi/ia minuta</i>	<i>Vorticella microstoma</i>
<i>Podophrya spp</i>	<i>Pseudocohnilembus pusillus</i>		<i>Vorticella octam</i>
<i>Tokophrya spp</i>	<i>Sathrophilus sp.</i>		<i>Zoothamnium spp</i>
	<i>Spirostomum teres</i>		
	<i>Tetrahymena pyriformis complex</i>		
	<i>Uronema nigricans</i>		
	<i>Acinertia uncinata</i>		
	<i>Drepanomonas revoluta</i>		
	<i>Trachelophyllum pusillum</i>		

*Omnivorous; Incertae sedis: these species are free-swimming forms but their grazing activity seems to be linked to the Hoc.

Table 4: Performance rating of ciliate groups and possible causes for performance rate (Madoni 1986, 1988)

Dominant group	Performance	Possible causes
Small flagellates	Low	Poorly aerated sludge; overloading; fermenting substances involved
Small swimming ciliates	Mediocre	Too short sewage retention time; poorly aerated sludge
Large swimming ciliates	Mediocre	Overloading; poorly aerated sludge
Crawling ciliates	Good	
Sessile and crawling ciliates	Good	
Sessile ciliates	Decreasing	Transient phenomena (discontinuous load, recent sludge extraction)
Small naked amoebae and flagellates	Poor	Very high load, not easily degradable
Testate amoebae	Good	

2.3.1 PROTOZOA RELATED RESEARCH

Petropoulos *et al.*, (2005), studied effects of protozoan grazing on nitrification process in activated sludge. It was concluded that with the reduction of grazing protozoan, nitrification rates declined. This result suggests that a reduction in either protozoan or bacterial concentrations may be the cause of nitrification decline (Petropoulos & Gilbride, 2005). This research relates to the current study with respect to protozoan and bacterial relationship.

Pogue *et al.*, (2007) reported protozoan grazing on the ammonia and nitrite-oxidizing bacterial communities in activated sludge concluded that without the presence of protozoan grazing, the nitrification rates would be lower. Inhibition in ammonia oxidation also lowered the amount of nitrate and nitrite accumulation. Introducing deflocculation led to high levels of nitrate accumulation, which is an indication of high levels of nitrification. This effect was less pronounced in the absence of protozoan grazing. In addition, nitrite oxidizing bacteria (NOB) and ammonia oxidizing bacteria (AOB) were found clustered in the floc. Inhibiting protozoa and ammonia oxidation, as well inducing deflocculation did not reduce NOB and AOB presence or have influence on their position within the floc. These conclusions suggest that nitrite-oxidizing bacteria and ammonia-oxidizing bacteria are present but less active in the absence of protozoa (Pogue & Gilbride, 2007). This study shows that protozoa play an important role in WWTPs and thus if impacted in a way to reduce their function and abilities then consequential affects may occur.

A third study conducted by Islam 2013, examined the impacts of tetracycline on the composition and function of microbial communities with respect to nitrification and the effect of tetracycline under low and high nutrient replacement conditions. The study concluded that with lower rate of nutrient replacement, tetracycline was observed to have a positive effect on ammonia removal and nitrification than at a higher rate. In addition, under lower nutrient replacement, Kjeldahl nitrogen concentrations increased in the presence of tetracycline, which suggested a potential inhibitory effect on de-nitrification. Finally, at higher nutrient replacement rates, tetracycline had no inhibitory effect on either de-nitrification and nitrification functions (Islam, 2013). In relation to my research, this study shows a causal effect of tetracycline on the microbial system, as well there is a significant protozoan and bacterial relationship with wastewater treatment dependent on their condition.

2.4 TETRACYCLINE

As previously stated, tetracyclines are a group of broad-spectrum antibiotics discovered in the early 1940s and were approved by the FDA in 1948 (Nelson *et al.*, 2011). Tetracyclines affect a wide range or both Gram-positive and Gram-Negative bacteria (Wang *et al.*, 2010). Tetracyclines are used by humans to fight bacterial infections and by veterinarians to treat or fight against bacterial infections. Tetracyclines are also added to food producing animals as well as honeybees as a result of their broad antibiotic spectrum and its advantage of preventing wide spread diseases (Kazemifard, 1997; Oka *et al*, 2000). Furthermore, they are used to promote growth as well as disease prevention in livestock and aquatic agriculture (Boxall *et al*, 2003; Kay *et al*, 2005). Since the discovery of chlortetracycline in 1948, its family has grown with the

addition of eight other tetracyclines that are now commercially accessible. Of those, tetracycline, oxytetracycline, doxycycline, and chlortetracycline are frequently used as veterinary medicines (Chopra & Roberts, 2001). Tetracycline not only encompasses antibacterial properties but also contains antiprotozoal capabilities (Chopra & Roberts, 2001). Tetracycline functions by binding to the 30S ribosomes in the mitochondrial organelle or by thus inhibiting protein syntheses. Examples of protozoan parasites affected by tetracyclines are *Entamoeba histolytica*, *Plasmodium falciparum*, *Trichomonas vaginalis*, *Leishmania major*, *Giardia lamblia*, and *Toxoplasma gondii*, though further research into specific organelles affected by tetracycline still requiring further research (Chopra & Roberts, 2001).

Due to the large quantities of tetracycline used and released into the environment, it can be passed along with wastewater into wastewater treatment facilities. A study in 2005 stated that 0.2 µg/L of tetracycline was measured in WWTP influent in Canada, however the study only tested for tetracycline and not its sub-classes (Yang *et al.*, 2005). Deblonde *et al.* (2011) reported tetracycline levels of 48 µg/L in influent and 2.375 µg/L in effluent. Due to tetracyclines antibacterial capabilities, bacterial biodegradation may play a negligible role in the removal of the antibiotic. To this point, no evidence of microbial degradation of tetracyclines in the environment has been seen (Alexy *et al.*, 2004).

2.5 IBUPROFEN

As previously mentioned in the introduction, ibuprofen was developed by the Boots Company in 1961 and approved by the FDA for over-the-counter use by 1984 (Rainsford, 2011).

Ibuprofen was first introduced in 1969 in a study conducted by the University of Wollongong with evidence suggesting that some NSAIDs have the capabilities of antibacterial properties (Yin *et al.*, 2014). NSAIDs have been shown to have antibacterial capabilities with a mechanism to still be discovered. Yin *et al.* (2014) showed results in regards to testing the NSAIDs bromfenac, carprofen, and vedaprofen and discovered that *Escherichia coli* DNA polymerase III β subunit was inhibited (Yin *et al.*, 2014). The DNA polymerase III subunit is the primary enzyme responsible for DNA replication. By inhibiting this subunit, bacterial reproduction is also inhibited. A study conducted in Spain by Ferrando-Climent and colleagues (2012) showed that ibuprofen concentrations of 13.74 $\mu\text{g/L}$ were found in wastewater influent. A study showed an average of 38.7 $\mu\text{g/L}$ and a maximum of 75.8 $\mu\text{g/L}$ of ibuprofen collected from 14 sewage treatment plants in Canada between 1998 through 1999 (Metcalf *et al.*, 2003). In another study, ibuprofen was detected at levels of 13.482 $\mu\text{g/L}$ in influent and 3.480 $\mu\text{g/L}$ in effluent (Deblonde *et al.*, 2011).

Due to dependence on protozoa and bacteria for wastewater purification processes, dependence of protozoa on bacteria for grazing and other sources, and bacterial benefits from protozoa for nutrients and function enhancement, it is vitally important to keep their ecosystem satisfied. If commonly used compounds such as ibuprofen and tetracycline severely affect either of these organisms, the ecosystem balance will be affected and their purification performance will be compromised.

2.6 16S rRNA gene AND 18S rRNA gene PRIMERS

A primer is a single stranded oligo-nucleotide sequence. The strand length can vary based on its function. A primer consisting of 18 to 24 bp is considered appropriate for a standard PCR (Dieffenbach & Dveksler, 1995). PCR uses a set of two primers, a forward primer and a reverse primer. The forward primer is a sequence that is complementary to a section on the anti-sense strand that follows 3' to 5' in the forward direction whereas the reverse primer is complementary to the section on the sense strand that follows 3' to 5' in the reverse direction of a double stranded DNA fragment. The primers are synthesized in order to target specific DNA fragments to identify a specific species. The 16S rRNA gene is a component of the prokaryotic ribosomes whereas the 18S rRNA gene is a component of the eukaryotic ribosomes. Some primers such as universal primer sets can target a diverse array of species while some primers are used to only target a minor set of species (Yu *et al.*, 2013).

Lin *et al.*, (2014), studied the diversity of microbial communities in each step of treatment plant for potable water generation. The 16S rRNA gene is universal amongst bacteria, and thus their relationships can be measured with a universal 16S rRNA gene primer. By comparing the 16S rRNA gene sequences, differentiation between organisms at the genus level across the phyla of prokaryotes is possible. Furthermore, organism can be identified and classified at multiple strain levels including species and subspecies (Jill, 2004). The ribosomal subunits vary between prokaryotes and eukaryotes, and where 16S rRNA gene is prokaryotic, 18S rRNA gene is the eukaryotic nuclear homologue similar to that of the 16S rRNA gene in prokaryotes.

In order to identify the different bacterial communities, the V1-V3 region of the bacterial 16S rRNA gene was amplified using forward primer F341 (5'- CCT ACG GGA GGC AGC AG-3') and reverse primer V3R (5'- ATT ACC GCG GCT GCT GG -3') (Muyzer *et al.* 1993). For eukaryotes such as protozoa, a qPCR approach was used to amplify the variable V4 region of the 18S rRNA gene. The primers used to examine eukaryotes were 3NDF (5'- GGCAAGTCTGGTGCCAG-3') and reverse primer V4_euk_R2 (5'- ACGGTATCT(AG)ATC(AG)TCTTCG-3') (Lin *et al.*, 2014).

2.7 QUANTITATIVE PCR (qPCR)

Quantitative real-time Polymerase Chain Reaction (qPCR), is a widely used molecular method for microbial detection in real-time (Girones *et al.*, 2010). The qPCR method measures the amplification of a nucleic acid (DNA, RNA) target sequence in the presence of a fluorescent dye. Through the process, fluorescence increases indicating amplification of nucleic acid and is measured in real-time. The crossing point (Cp) is the cycle number at which the targeted sample begins to amplify exponentially, the Cp value is then compared to a standard curve from which a quantitative value is calculated.

An advantage to using qPCR is the quantification of very minuscule samples; an example is the detection of a single microbe such as an amoeba in a water sample (Qvarnstrom *et al.*, 2006). Furthermore, qPCR does not take long and within approximately 3 hours results can be attained. There are a number of studies that have used qPCR for analyzing microbial species, one particular study previously mentioned that stands out in relation to this research is Lin *et al.*,

(2014), which also utilized qPCR to study microbials within potable water generation treatment plant. Other studies have shown the potential of qPCR procedures in monitoring the quality of recreational waters, focusing on bacteria, such as *Enterococcus spp.* and *E. coli* (Haugland *et al.*, 2005; Bartrand *et al.*, 2010; Noble *et al.*, 2010; Wade *et al.*, 2010).

2.8 RESEARCH OBJECTIVE

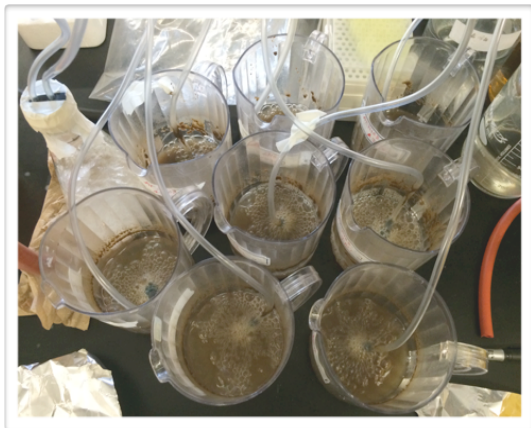
The objective of this research is to examine the affects of tetracycline and ibuprofen on the relative abundance of the bacterial and protozoan community present in activated sludge in a semi-batch reactor system. The study will examine the effects of the pharmaceuticals at different concentrations, tetracycline: 50 ng/ml, 500 ng/ml, ibuprofen: 100 ng/ml, 2000 ng/ml, 10^5 ng/ml. The semi-batch reactors will run for 15 days and samples will be extracted on days, 0, 5, 10 and 15. Synthetic nutrient supplements every 5 days and bubbling stones for aeration in order to simulate wastewater treatment plant conditions. Following the samples extractions from each reactor, DNA extraction is performed on day 0 and day 15 samples. Following DNA extraction, qPCR is performed to determine abundance of bacterial and protozoan populations using 16S rRNA gene and 18S rRNA gene primers. Using C_p values obtained from the qPCR, relative quantification of C_p values will be calculated using $R=2^{(control - sample)}$ formula to normalize the C_p values. In order to examine the effects of the pharmaceuticals on the eukaryotic community, DDGE and 18S rRNA gene primers are used to observe change in community compositions.

CHAPTER 3 MATERIALS AND METHOD

3.1 SEMI BATCH REACTOR SYSTEM

The semi batch reactors were constructed and filled with activated sludge mixture from the secondary-treatment aeration tanks 2, 4, 6, and 8 from the Humber Wastewater Treatment Plant. Approximately 1 L from each aeration tank was collected from the WWTP and transported to the lab in plastic containers. Within approximately 3 hours the containers of activated sludge sample were combined for a collective of 4 L, which was then divided to produce 500 ml reactors (Figure 6). The batch reactors are oxygenated using aquarium bubbler through a flask containing deionized water in order to minimize evaporation. An aliquot of the combined activated sludge was taken and centrifuged at 7000 rcf for 15 minutes to achieve a pellet which was then stored at -20°C. This was considered the time zero sample. At days 5 and 10, 10% of the volume for the reactor was replaced with synthetic wastewater to maintain nutrients but no microbes were added (Liao *et al.* 2001). The reactors were also topped up to 500 ml to account for any evaporation. Aliquots from each reactor were collected after 15 days and used for analyses.

Figure 6. One liter Reactors were set up on the bench at room temperature and aerated with aquarium bubblers. They were maintained for 15 days. a) top view, b) side view.



A.



B.

3.2 EXPERIMENT SETUP

The batch reactors contained 500 ml per reactor of activated sludge from the Humber Wastewater Treatment Plant. Eight batch reactors were set up at a time to simulate aeration tanks from WWTP. Three sets of experiments were carried out. The first two sets contained 8 reactors; two reactors that were set up as a control where no pharmaceutical was added. Two reactors were supplemented with 100 ng/ml of ibuprofen, two were supplemented with 50 ng/ml of tetracycline and two were supplemented with both ibuprofen and tetracycline. The third set of reactors consisted of 10 reactors, each condition in duplicate; control: 100 ng/ml of ibuprofen and 50 ng/ml of tetracycline: 2000 ng/ml of ibuprofen and 50 ng/ml of tetracycline: 100,000 ng/ml ibuprofen and 50 ng/ml tetracycline, and 100,000 ng/ml ibuprofen and 500 ng/ml tetracycline.

Tetracycline:

A stock solution of tetracycline (30 mg/ml) was made using milli q water as a solvent and it was stored at 4°C in foil until needed since daylight can cause breakdown of the chemical structure. Final concentration of 50 ng/ml and 500 ng/ml were added to the appropriate reactors.

Ibuprofen:

A stock solution of Ibuprofen (0.1 mg/ml) was made using milli q water as a solvent and stored at 4°C. Final concentrations of 100 ng/ml, 2000 ng/ml and 100 mg/ml were added to the appropriate reactors.

3.3 DNA EXTRACTION

Reactor samples were processed for community DNA using the *PowerSoil DNA Isolation Kit* (Mo Bio Laboratories, USA). A NanoDrop spectrophotometer (Thermo Scientific, USA) was used to measure the concentrations of each DNA sample in order to determine the concentrations of DNA to adjust volume quantity accordingly for qPCR reactions.

3.4 QUANTITATIVE POLYMERASE CHAIN REACTION

Primers for prokaryotic and eukaryotic rRNA genes were used to quantify the amount of each population that was present in the samples. Primers and protocol for qPCR are followed according to Lin *et al.*, (2014). The 16S rRNA gene bacterial genes were amplified using 200 µM of the forward primer F341 (5'- CCT ACG GGA GGC AGC AG-3') and 200 µM of

the reverse primer V3R (5'- ATT ACC GCG GCT GCT GG -3') (Muyzer *et al.* 1993). The qPCR protocol for 16S rRNA gene prokaryotic amplification was as follows, hot start at 95 °C, followed by 25 cycles at 92 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s with a final extension at 72 °C for 7 min.. For the 18S rRNA protozoan gene, 5 µM forward primer 3NDF (5'- GGCAAGTCTGGTGCCAG-3') and 5 µM reverse primer V4_euk_R2 (5'- ACGGTATCT(AG)ATC(AG)TCTTCG-3') were used (Lin *et al.*, 2014). The qPCR protocol for 18S rRNA gene eukaryotic amplification was as follows 95 °C for 2min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s with a final extension at 72 °C for 5 min. These primers have been chosen because they produce amplicons equal to or less than 500 bp, which is ideal for qPCR. Due to the various concentrations of DNA in each sample, 50 ng/µl of DNA was chosen to be added to each reaction to standardize the procedure therefore different volumes of the DNA solution were used and the total volume of the reaction adjusted with Milli-Q H₂O in order to obtain a final reaction sample of 20 µl. For full procedure and reagents setup up for qPCR refer to Appendix C.

Following the qPCR protocol, the LightCycler software was used to analyze the tested sample and provided information such as amplification curves, melting curves, and Cp values for each sample. The Cp values were then collected and organized on excel, averages of the same samples was calculated, standard deviation was calculated and the normalization equation $R=2^{\Delta C_p}$ ("Real-Time", 2006) where $\Delta C_p = (C_p \text{ control} - C_p \text{ test})$, was used to calculate the relative abundance ratio for each sample. In order to perform relative quantification, an experimental control is required; in this case it's the day 15 control sample. The test samples are expressed as an increase or decrease relative to the experimental control.

Graphs were then produced using the calculated information. The ratio, R, is calculated by using efficiency value 2, which assumes a PCR performance that achieves perfect doubling with each amplification cycle.

3.5 COMMUNITY PROFILES AND PROTOZOA IDENTIFICATION

The Standard Operating Protocol Denaturing Gradient Gel Electrophoresis System (DGGE) was used to analysis the community profiles for protozoa. The gel was performed in a DCode Universal Mutation Detection System (BioRad Laboratories, Mississauga, ON). The PCR products used for DGGE were the Day 15 control: 100 ng/ml of ibuprofen and 50 ng/ml of tetracycline: 2000 ng/ml of ibuprofen and 50 ng/ml of tetracycline: 100,000 ng/ml ibuprofen and 50 ng/ml tetracycline, and 100,000 ng/ml ibuprofen and 500 ng/ml tetracycline. After gel preparation, the gel was run using standard procedures as instructed by the DCode protocol manual (BioRad Laboratories, Mississauga, ON). The denaturing gradient used was 40-70%. The samples were loaded into the gel wells with 30µl of PCR products and 30µl (1:1 ratio) of loading dye (2x). DGGE gel ran with the denaturing gradient parallel to the direction of the electrophoresis for 16 hours at 80 volts in a tank containing 6 liters of 1 X TAE buffer at 60 °C. DGGE gel was stained with Sybr Gold (Thermo Fisher Scientific Invitrogen, USA) for 30 min, the de-stained for 15 min in in 250 ml of 1X TAE. The gel was then placed in a UV trans illuminator for viewing. Each DNA fragment on the gel was then cut out and placed in different eppendorf tubes obtaining a total of 12 samples. Each fragment was submerged in 30µl of deionized water and left to elute for 3 days at 4 °C. Afterwards, 1 µl of the fragment was used for PCR to determine product quality. The PCR products were purified using the PCR purification

kit (Qiagen, Germany). Following a successful PCR purification, the product was sent to ACGT Corp (Toronto, ON) for DNA sequencing. For full procedure and reagents setup refer to Appendix A.

In order to identify the 18S rRNA gene species, Basic Local Alignment Search Tool (BLAST), was utilized. BLAST is a database and its function is to find sections of local similarity between sequences. In order to do so, the program compares proteins or nucleotide sequences to its sequence database and calculates a significance of possible matches. BLAST can also distinguish between sequences in order to identify members of the same gene family (Altschul *et al.*, 1997). The sequences retrieved from ACGT Corp were entered into the BLAST database and a statistical comparison of each species was determined and the ideal identities were collected and charted in Table 6.

CHAPTER 4 RESULTS

Semi- batch reactors were maintained for 15 days at a temperature range between 20 and 22 °C. It was observed that the color and consistency of the sludge did not change over the duration of the experiment except in those that contained the largest amount of Ibuprofen (100,000 ng/ml). It appeared that at this concentration the pharmaceutical caused some foaming in the reactors.

Samples from the reactors were collected on day 0, 5, 10, and 15, (only day 0 and day 15 samples were analyzed for this project) and the microbial community DNA was extracted. To measure the relative amount of the bacterial population compared to the protozoan population, qPCR was performed with primer sets that could distinguish between prokaryotic and eukaryotic DNA. The primers used were specific to rRNA gene sequence for the 16S rRNA gene sequences in bacteria and the 18S rRNA gene sequences in eukaryotes. Although the primers for the 18S rRNA gene were not specific to protozoa, a previous study by Ovez and Orhon (2005), have shown that the majority of the eukaryotic community in wastewater is composed of protists with concentrations ranging from 500 to 1000 cell/ml during optimal conditions to over 5000 cell/ml during bulking conditions. Therefore, it is assumed that the 18S rRNA gene signal will represent the protozoan population.

The PCR experiments were carried out to determine the crossing point (Cp) values that can be used as a measure of the abundance of the target sequences in a DNA sample. The Cp values were measured in triplicate for each reactor sample for both 16S rRNA gene and 18S rRNA gene qPCR reactions.

The averages and standard deviations were calculated for combined reactor sets 1 and 2. Ratio of 16S rRNA gene and 18S rRNA gene sequences were calculated using the relative quantification formula $R=2^{\Delta C_p}$ (“Real-Time”, 2006). The final values are listed in Table 5, for a complete listing of all values refer to Appendix B. For experiment 1 and the 16S rRNA gene set, the average C_p values were between 13.77 and 14.61. With respect to the 18S rRNA gene C_p values, the average was between 17.69 and 19.44. These averages show that there was a higher 18S rRNA gene C_p values than 16S rRNA gene, meaning the need for more cycles for the florescent signal were required to cross the threshold or simply there was less 18S rRNA gene present at the start of the reaction. A variation in the standard deviation was observed, possibly due to numerous qPCR trials for both set 1 and set 2 in experiment 1. For experiment 2, 16S rRNA gene C_p averages are between 13.94 and 15.23, 18S rRNA gene C_p averages are between 17.25 and 20.00. Similar to experiment 1, the standard deviation varied. All control ratios were normalized to a value of 1.00, in accordance to the formula used.

Table 5: Normalization using relative abundance formula of Cp values, normalization of protozoan to bacterial ratio and standard deviation for 16S rRNA gene and 18S rRNA gene for; day 0, no pharmaceuticals (control), tetracycline (50 ng/ml), Ibuprofen (100 ng/ml), and a combination of Ibuprofen (100 ng/ml) and tetracycline (50 ng/ml). Cp values, standard deviation and relative abundance ratios for 16S rRNA gene and 18S rRNA gene; day 0, no pharmaceuticals (control), ibuprofen (100 ng/ml) plus tetracycline (50 ng/ml), Ibuprofen (2000 ng/ml), a combination of Ibuprofen (100,000 ng/ml) and tetracycline (50 ng/ml) and a combination of Ibuprofen (100,000 ng/ml) and tetracycline (500 ng/ml)

	Normalization Bacteria Cp	Normalization Protozoa Cp	Normalization P:B	S.D. Bacteria	S.D. Protozoa
Day 0	0.63	3.09		0.02	1.97
Control	1.00	1.00	1.00		
Tet 50 ng	0.93	0.84	0.91	0.14	0.05
Ibu 100 ng	1.01	1.73	1.71	0.01	0.94
Ibu 100 ng / tet 50 ng	0.89	0.77	0.86	0.04	0.08
Day 0	1.92	7.35		0.22	3.74
Control	1.00	1.00	1.00		
Ibu 100 ng / tet 50 ng	0.81	0.70	0.87	0.05	0.26
Ibu 2000 ng / tet 50 ng	0.99	0.65	0.65	0.09	0.12
Ibu 100000 ng / tet 50 ng	0.98	0.16	0.17	0.13	0.08
Ibu 100000 ng / tet 500 ng	1.28	0.16	0.13	0.18	0.08

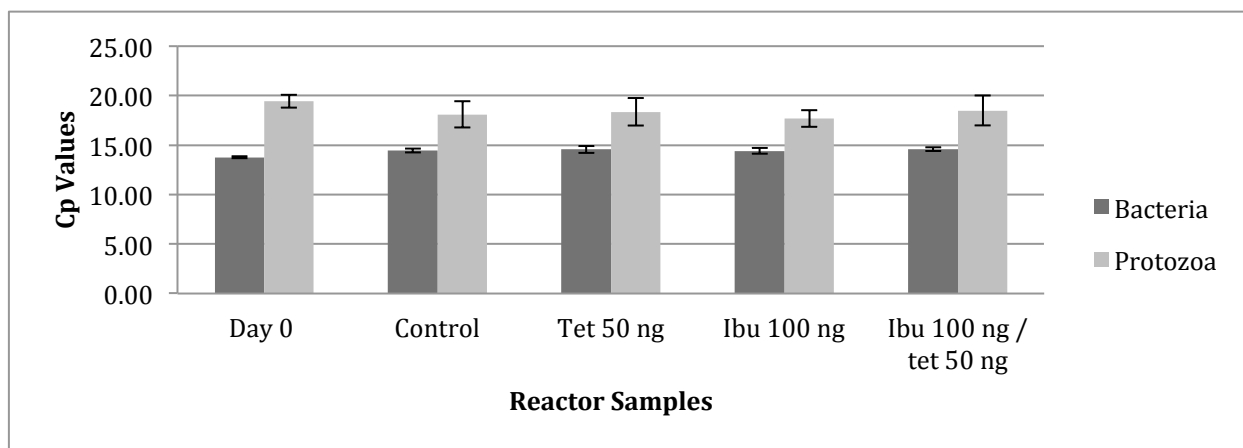


Figure 7: Abundance of bacterial and protozoan populations in each reactors at time zero and then after 15 days after the addition of no pharmaceuticals (control), tetracycline (tet) (50 ng/ml), ibuprofen (ibu) (100 ng/ml), and a combination of ibuprofen (100 ng/ml) and tetracycline (50 ng/ml).

Figure 7, shows a comparison of absolute Cp values for each population on day 0 and day 15. Day 0 and day 15 control did not contain tetracycline or ibuprofen, tetracycline contained 50 ng/ml of tetracycline, ibuprofen contained 100 ng/ml of ibuprofen and tetracycline/ibuprofen contained 50 ng/ml of tetracycline and 100 ng/ml of ibuprofen. A higher Cp value represents a lower detection level while a lower Cp value represents a higher detection level of rRNA gene associated with the primer set. Very little change in the absolute Cp values was observed for the abundance of the day 15 16S rRNA and 18S rRNA genes in comparison to the day 0 sample.

Figures 8A, 8B, 9A and 9B show a control ratio of 1:1 for the bacterial and protozoan population. However, that is not a true ratio, the 1:1 ratio is simply a reference to compare the change of the other reactors, in order to determine if an increase or decrease has occurred in comparison to their own control.

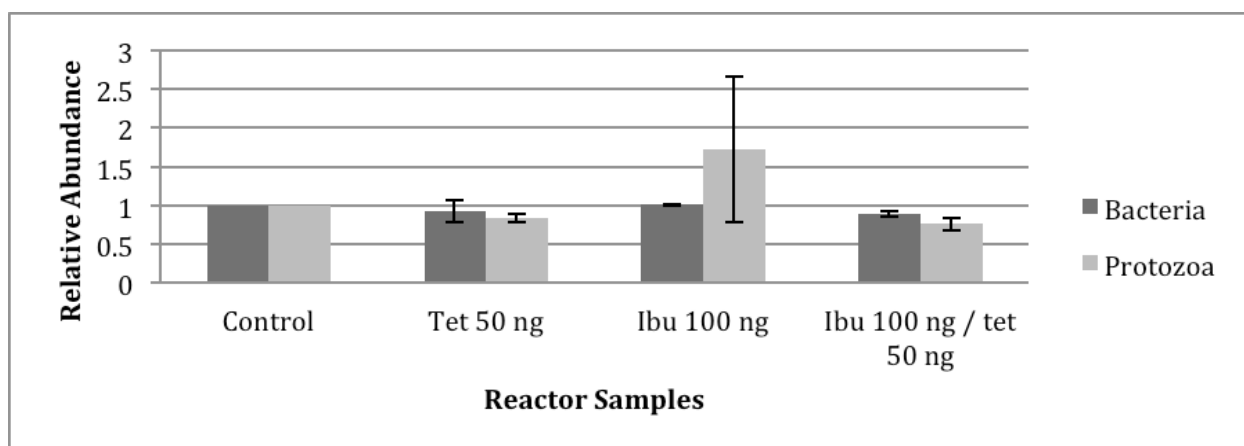


Figure 8A: The relative abundance of the bacterial and protozoan populations in each reactor compared to the abundance seen in the control. No pharmaceuticals (control), tetracycline (50 ng/ml), Ibuprofen (100 ng/ml), and a combination of Ibuprofen (100 ng/ml) and tetracycline (50 ng/ml). Ratios are relative to their own control and the control values are not 1:1 ratio.

Figure 8A shows a comparison of the abundance of each population for control and reactors with pharmaceuticals. In order to calculate the ratios of each sample, a reference sample was used. The day 15 control (no pharmaceuticals) sample was used as a reference in order to determine if a change has occurred. Calculations are not calculated for an absolute quantification but rather a relative expression.

The formula $R=2^{\Delta C_p}$ expressed by “Real-Time” (2006), incorporates an experiment efficiency condition represented by “E”, $R = E^{\Delta C_p \text{ (control - sample)}}$ where E can be rearranged as $R=2^{(\text{control} - \text{sample})}$. Arrangement of the formula assumes a perfect doubling efficiency ($E = 2$) for each cycle. The standard deviations were calculated for the triplicate sets of the C_p values for both 16S rRNA gene and 18S rRNA gene. Same calculation approach was followed for Figure 10A. With regards to the relative abundance of bacteria, observations show a change (decrease)

in the abundance of bacteria in the reactor containing 50 ng/ml of tetracycline and in the reactor containing 100 ng/ml of ibuprofen and 50 ng/ml of tetracycline. In the case of protozoa, observations show a change (decrease) has occurred with 100 ng/ml of ibuprofen and 50 ng/ml of tetracycline.

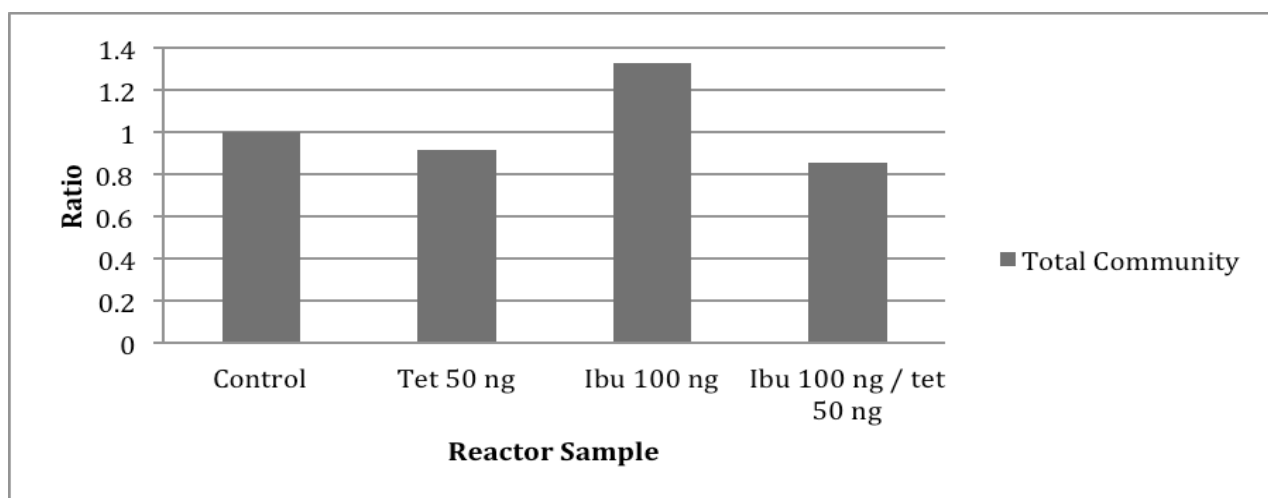


Figure 8B: Using the relative abundance of the protozoan population vs. the bacterial population calculated using the relative expression formula for all day 15 reactors after the addition of no pharmaceuticals (control), tetracycline (50 ng/ml), Ibuprofen (100 ng/ml), and a combination of Ibuprofen (100 ng/ml) and tetracycline (50 ng/ml). Using the protozoa and bacterial ratios to calculate a single community ratio for each reactor.

Figure 8B shows an overall community change. Using the relative abundance values from Figure 8A, a simple ratio of protozoa to bacteria (P:B) was calculated to examine the entire microbial community for each sample. Although the control shows a 1:1 ratio, in reality there are millions of bacteria per protozoa. The 1:1 control ratio is simply a reference point at which the total community is seen as being in a normal state, that is in the absence of pharmaceuticals. The following sample ratios increase and decrease with respect to the pharmaceutical in which the

total population is effected. A lower ratio represents an increase of bacteria relative to protozoa. It does not mean that bacteria has increased relative to the control, instead it represents more bacteria compared to protozoa than there is in the control. Whereas an increased ratio implies a higher protozoa population relative to bacteria, it does not mean that there are more protozoa than bacteria but rather more protozoa compared to bacteria than in the control. Figure 10B reflects a similar understanding of the ratio calculation.

Compared to the control, the reactors containing 50 ng/ml of tetracycline and 100 ng/ml of ibuprofen with the addition of 50 ng/ml of tetracycline have shown an overall decrease of the microbial community due to the decrease of the protozoan population, as seen in Figure 8A. However, the reactor containing 100 ng/ml of ibuprofen has shown an increases in total microbial community, this is due to the increase of the protozoan population seen in Figure 8A.

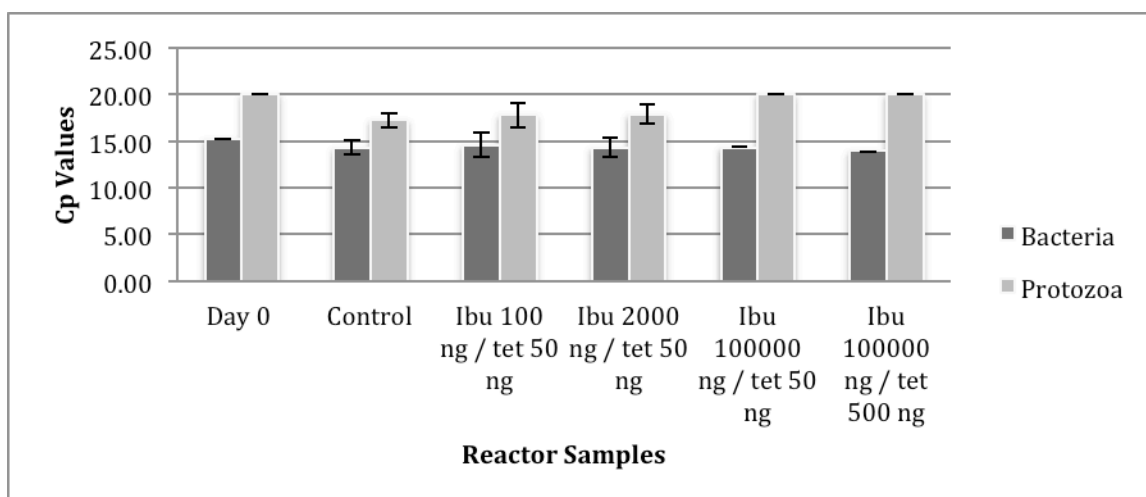


Figure 9: The relative abundance of the bacterial and protozoan populations in each reactors at time zero and then after 15 days after the addition of no pharmaceuticals (control), ibuprofen (100 ng/ml) plus tetracycline (50 ng/ml), Ibuprofen (2000 ng/ml), a combination of Ibuprofen (100,000 ng/ml) and tetracycline (50 ng/ml) and a combination of Ibuprofen (100,000 ng/ml) and tetracycline (500 ng/ml).

Figure 9 shows a similar pattern as Figure 7 when additional reactors were examined with higher concentrations of pharmaceuticals where the 18S rRNA gene had a higher Cp value than the 16S rRNA gene sequence. The 16S rRNA gene Cp values seem to have had a negligible change throughout the 15 days, even with the high concentration of 500 ng/ml of tetracycline, which showed little to no major change. With regards to the 18S rRNA gene, the Cp values increased as the concentrations of ibuprofen increased, representing lower 18S rRNA gene abundance.

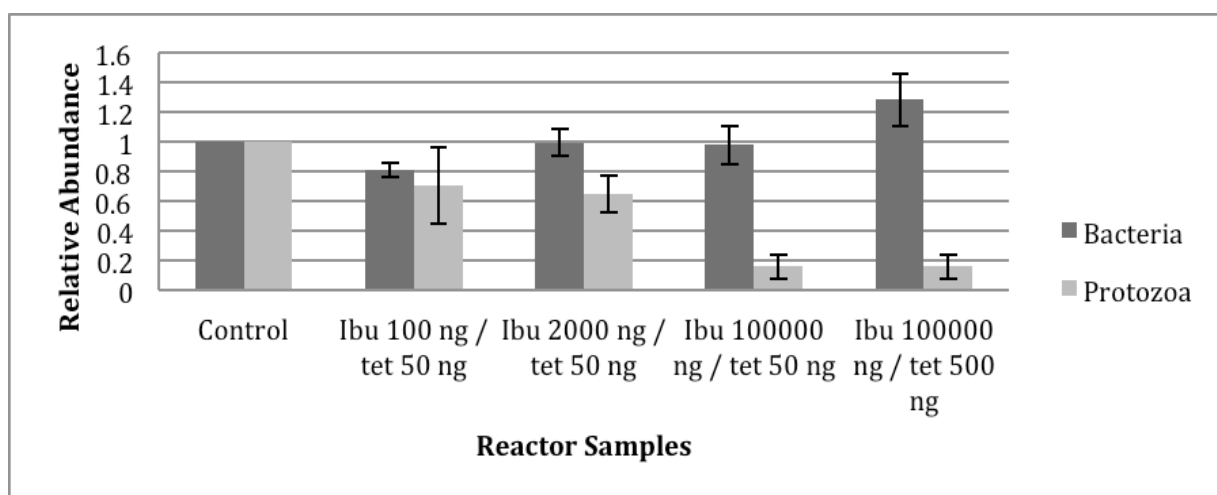


Figure 10A: The relative abundance of the bacterial and protozoan populations calculated using the relative expression formula for all day 15 reactors after the addition of no pharmaceuticals (control), Ibuprofen (100 ng/ml) and tetracycline (50 ng/ml), Ibuprofen 2000 ng/ml and tetracycline (50 ng/ml), Ibuprofen (100,000 ng/ml) and tetracycline (50 ng/ml), and Ibuprofen (100,000 ng/ml) and tetracycline (500 ng/ml). Ratios are relative to their own control and the control values are not 1:1 ratio.

Figure 10A follows the same calculation approach as demonstrated for Figure 8A. Figure 10A shows a comparison of the abundance of each population for control and reactors with pharmaceuticals at extreme concentrations. The values were calculated using the formula $R=2^{\Delta C_p}$. The standard deviations were calculated for the triplicates set of the C_p values for both the 16S rRNA gene and 18S rRNA gene. For bacteria, a decrease was observed for reactors containing 100 ng/ml of Ibuprofen and 50 ng/ml tetracycline, which confirmed the results found in the earlier experiment, both showing a major difference in comparison to their controls. Using Observations showed a decrease in the bacterial population under reactor sample containing 100 ng/ml of Ibuprofen and 50 ng/ml tetracycline and increase for 100,000 ng/ml of Ibuprofen and 50 ng/ml tetracycline. In regards to the protozoan population a major decrease is seen in all reactors with greater decrease with concentrations of 100,000 ng/ml of ibuprofen.

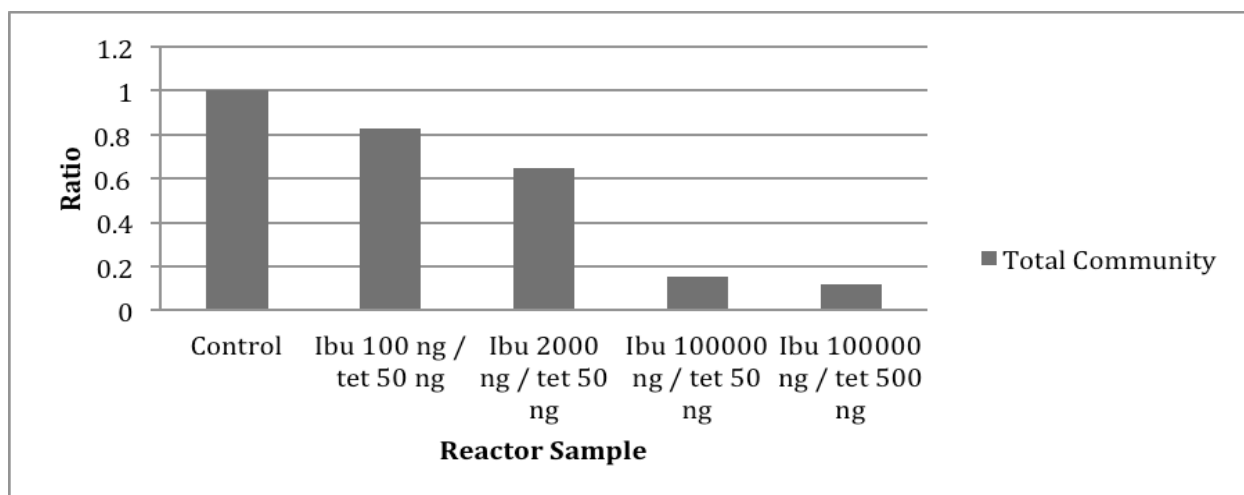


Figure 10B: Using the relative abundance of the protozoan population vs the bacterial population calculated using the relative expression formula for all day 15 reactors after the addition of no pharmaceuticals (control), Ibuprofen (100 ng/ml) and tetracycline (50 ng/ml), Ibuprofen 2000 ng/ml and tetracycline (50 ng/ml), Ibuprofen 100,000 ng/ml and tetracycline (50 ng/ml), and Ibuprofen (100,000 ng/ml) and tetracycline (500 ng/ml). Using the protozoa and bacterial ratios to calculate a single community ratio for each reactor.

Figure 10B shows an overall microbial community change using ratios calculated for Figure 10A. Using the relative abundance values, a single ratio was produced to examine the entire microbial community change. Compared to the control, the reactors demonstrated a continuous decrease in protozoan to bacterial ratio as the concentrations of ibuprofen and tetracycline increased for each reactor. The reactors containing 100,000 ng/ml of ibuprofen showed the largest change in community structure due to the major reduction of protozoa.

The protozoan community profiles were generated by amplifying the 18S rRNA gene and running the amplicons on a DGGE gel. The DGGE gel image is seen in Figure 12. The control lane (lane 3) represents the community on Day zero. Lanes 1 and 2 represent the community on day 15 after being incubated with the pharmaceuticals for that time period. Major differences

between control and the other lanes is the disappearance of bands 2, 3, 4, 5, and 12. There are also other faint bands that did not get tested but can be seen in the control and missing in the lanes with higher ibuprofen concentrations. Bands 6 and 7 appear more prominent in the control and lesser in the other two lanes. Band 8 is present in lanes 1 and 2 but is not observable in the control lane. Band 1 appears faint in the control lanes but prominent in lanes 1 and 2 under band 9 and 10. Disappearance of bands from lanes 1 and 2 can be attributed to the high concentrations of ibuprofen, while the faint bands observed in lane 1 that appear more prominent in lane 2 can be attributed to the high concentration of tetracycline. Bands that were faint or unobservable in the control but can be observed in lanes 1 and 2 can be attributed to the loss of other species allowing the rise of the few to increase.

DNA bands in the 18S rRNA gene lanes were extracted, re-amplified and cleaned, then sent for sequencing to identify the major genera that comprised the protozoan population. Figure 13 shows image of isolated DNA bands extracted from DGGE gel.

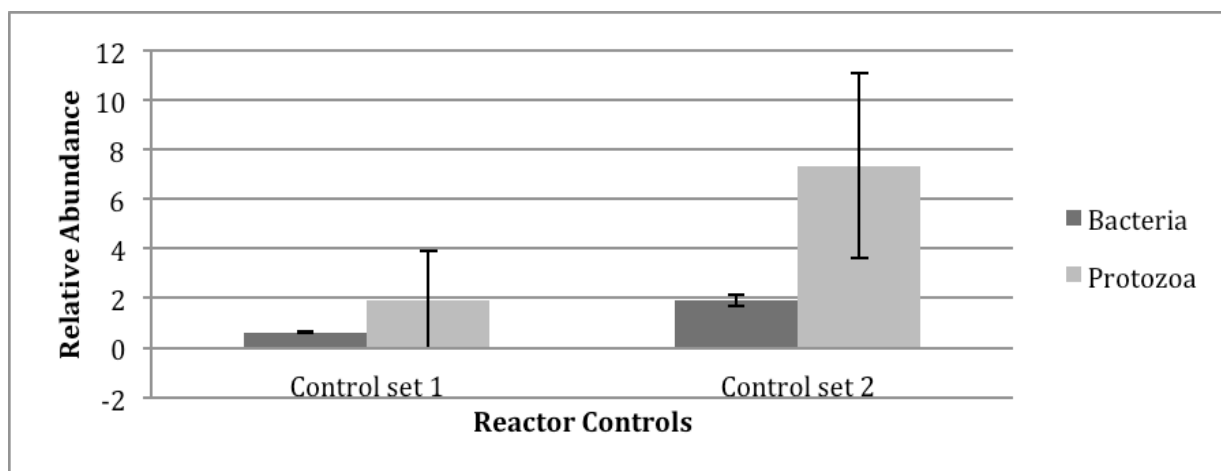


Figure 11: The relative abundance of the bacterial and protozoan populations for two sets of day 0 and day 15 controls. Ratios are relative to their own control and the control values are not 1:1 ratio.

Figure 11 follows the same calculation approach using the relative quantification formula. Figure 11 shows a comparison of the abundance of each population for two sets of day 0 and day 15 controls using $R=2^{\Delta C_p}$. The standard deviations were calculated for the triplicates set of the C_p values for both 16S rRNA gene and 18S rRNA gene. For the low concentration control batch, bacteria observed no change while protozoa doubled. For the high concentration control batch, bacteria doubled while protozoa increased 7x compared to the day 0 sample. The protozoan populations appeared to highly increase relative to the bacterial population. Both sets observed the same trend.

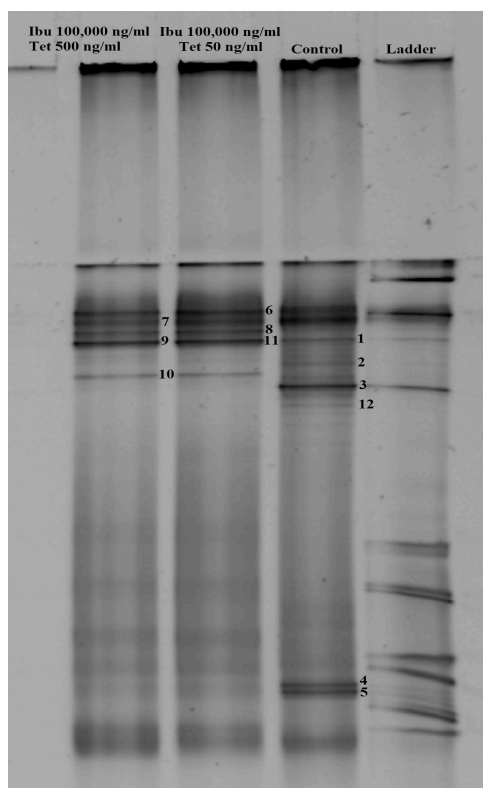


Figure 12: DGGE profile of protozoan population in the reactors with various tetracycline and ibuprofen contractions. From right to left; MW marker ladder, control population, profile from reactors with Ibu 100,000 ng/ml and tet 50 ng/ml, profile from reactors with Ibu 100,000 ng/ml and tet 500 ng/ml. The bands present qPCR fragments generated with the 18S rRNA gene primers.

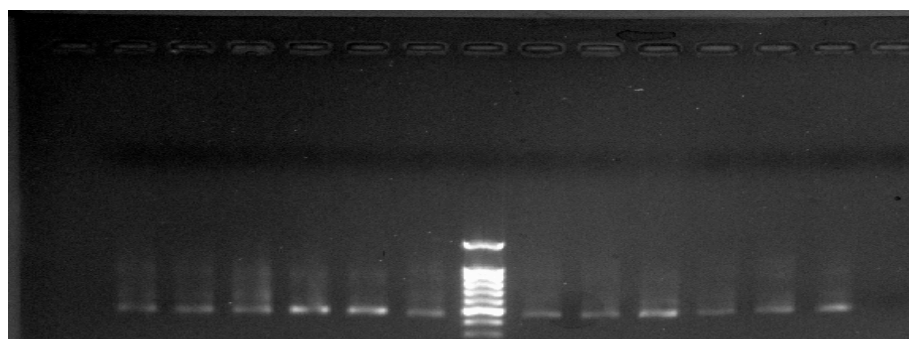


Figure 13: PCR gel image of isolated DNA bands cut from DGGE gel. Image shows DNA bands 1-6, ladder and 7-12. Bands show approximately 500 base pairs.

Table 6: Analyzed nucleotide sequences from DGGE samples 1-12 obtained from ACGT Inc. Using the Basic Local Alignment Search Tool (BLAST) from National Center For Biotechnology Information (NCBI), a compiled list of possible species with their identity percentage is produced. (Altschul *et al.*, 1997)

Number	Species	Identities	Environment
1	<i>Lamproderma sp.</i>	86%	NA
2	<i>Slavina appendiculata</i>	99%	Aquatic
	<i>Paranais litoralis</i>	99%	Aquatic , brackish
	<i>Nais communis</i>	99%	Aquatic
3	<i>Slavina appendiculata</i>	99%	Aquatic
	<i>Paranais litoralis</i>	99%	Aquatic , brackish
	<i>Nais communis</i>	99%	Aquatic
4	<i>Arcella vulgaris</i>	95%	Aquatic
5	<i>Arcella hemisphaerica</i>	98%	Aquatic
	<i>Arcella gibbosa</i>	97%	Aquatic
6	<i>Uncultured eukaryote</i>	95%	Cold desert
	<i>Invertebrate</i>	92%	NA
7	<i>Cercozoa sp.</i>	95%	Soil, freshwater, aquatic
8	<i>Uncultured Cryptomycota</i>	92%	Aquifer, freshwater, aquatic
	<i>Lamproderma sp.</i>	85%	NA
9	<i>Uncultured Cryptomycota</i>	93%	Aquifer, freshwater, aquatic
	<i>Lamproderma sp.</i>	86%	NA
	<i>Eimeriidae environmental sample clone</i>	84%	Soil
10	<i>Uncultured eukaryote</i>	95%	Aquatic
11	<i>Uncultured Cryptomycota</i>	90%	Aquifer, freshwater, aquatic
	<i>Lamproderma sp.</i>	86%	NA
12	<i>Uncultured eukaryote</i>	98%	Karst spring
	<i>Slavina appendiculata</i>	97%	Aquatic
	<i>Paranais litoralis</i>	97%	Aquatic , brackish
	<i>Nais communis</i>	97%	Aquatic

Using the nucleotide sequences obtained from ACGT Inc., a collection of best possible identity match of species was compiled in Table 6. Some of the sequences were identified as uncultured samples but the cultured sample with the highest identity match has been included in Table 6. Identity matching percentage ranged from as low as 84% to as high as 99% match. The samples that ranged in the mid 80's percentile were compiled with an identity match in the 90's,

reason for that is to show a cultured along with an uncultured specie. Nucleotide sequences that were used to identify the species have been compiled and are listed in the appendix. Although all of these species were identified using the 18S rRNA gene and are classified under eukaryote, while most were identified as protozoan others had different classifications. *Lamproderma*, which was identified under samples 1, 8, 9, and 11, is an Amoebozoa, which is under the kingdom protozoa. The genus *Arcella*, identified under samples 4 and 5 is amoebozoa, which is under the kingdom protozoa. *Cercozoa* identified under sample 7 is a group of protist. Lastly, *Slavina appendiculata*, *Paranais litoralis*, and *Nais communis*, identified under samples 2, 3, and 12, are an animalia, a type of worm. The rest of the samples contain uncultured eukaryotic samples. Nearly all of these eukaryotes have been previously found in aqueous environments.

CHAPTER 5 DISCUSSION

5.1 RESEARCH EXAMINATION

The focus of this research is to study the effects of both ibuprofen and tetracycline independently and in combination, on activated sludge microorganisms in relation to sustainability and community composition. Since the focus of this study is the microbial community, it is important to differentiate between natural and toxic variations within the microbial community. A natural variation would be represented by the control where no pharmaceutical is added and only the effects of time and natural uncontrollable events occur, whereas, toxic variations represent the introduction of a contaminant such as pharmaceuticals. Thus, this study was conducted with replicate trials and reactor sets. Throughout the trials, Cp values for reactor samples containing the same pharmaceutical concentrations exhibited the same increasing and decrease trends, indicating consistency.

The microbial communities present in the activated sludge were investigated under the influence of tetracycline and ibuprofen in a semi-batch reactor system containing activated sludge extracted from the secondary wastewater treatment process. Specifically, the study examined the relative abundance of prokaryotes and eukaryotes using 16S rRNA gene and 18S rRNA gene primers to monitor the impact of tetracycline and ibuprofen on the communities that are important in secondary wastewater treatment process. The study was expected to show a decrease in the bacterial population due to the antibiotic, and possible an inhibitory effect of ibuprofen on the eukaryotic population.

The study was designed to examine the effects of these contaminants at two ranges of concentrations of both pharmaceutical compounds in which one was the environmentally relevant concentration and other was an extremely high concentration. Concentrations of 100 ng, 2000 ng, 100,000 ng, per ml of ibuprofen and 50 ng, and 500 ng, per ml of tetracycline were used.

Quantitative PCR and DGGE were reliable in monitoring changes in the systems caused by the different concentrations of tetracycline and ibuprofen. Analysis of the communities was conducted through DNA extraction, qPCR of 16S rRNA gene and 18S rRNA gene sequences, PCR of 18S rRNA gene, and DGGE of 18S rRNA gene amplicons from the eukaryotic community.

The overall change of the relative abundance of microbial communities in the presence of the two pharmaceuticals was measured through qPCR. It is difficult to calculate an exact number of the microorganisms present. A relative calculation was used in order to monitor change in comparison to the control. As to the actual amount of organism per ml, one can use past studies with calculated population numbers. Previous studies showed the bacterial population ranging from as low as 9.5 million to as high as 6 billion cells per ml of effluent, and the protozoan population to be from as low as 3000 to as high as several hundred thousand cells per ml. If one was to use the optimal values or lowest values of both, an ratio can be produced. Example of such ratio can be created using 3000 cells per ml of protozoa compared to 9.5 million cells of bacteria per ml to achieve a ratio of 3×10^{-5} that can be used to represent the relative community

composition.

In the current study, a physical DNA extraction method, bead beating, was used to reduce the possible introduction of biases related to chemical and enzymatic methods because eukaryotes have a diverse cell structures. The choice of primers and their annealing temperatures are essential parameters in obtaining the desired qPCR amplified products. The chosen 16S rRNA gene forward primer F341 and reverse primer V3R, and 18S rRNA gene forward primer 3NDF and reverse primer V4_euk_R2 were selected for this research because of their efficiency, and have been verified by others (Muyzer *et al.*, 1993; Lin *et al.*, 2014). One of the reasons for selecting the primers used is due to their success in identifying 18S rRNA gene species. A limitation of identifying 18S rRNA gene sequences can be attributed to public databases containing a limited quantity of sequences derived from eukaryotes in wastewater treatment plants (Matsunaga *et al.*, 2014). Possibly due to the aforementioned reason, many of the identified species were uncultured eukaryotes. Using different primer sets can increase the identification of the eukaryotic community.

The purpose of DGGE is that DNA fragments of the same length can be separated based on differences in their nucleic acid sequence. Since the target sequence in this study is the 18S rRNA gene fragment containing organism specific strands, the number of DGGE bands ideally represents the number of organisms in the system, in this case eukaryotes, and the intensity of the bands represents the abundance of that organism. Eichner *et al.* (1999), suggested that one species is able to produce more than a single DGGE band and a single band can represent more than a single species, thus the number and intensity of any band may not reflect the actual

number and abundance of a particular species within a given community. Due to the aforementioned reason, the intensity, presence and absence of a band was the target of the DGGE, and was looked upon as a cause and effect of the contaminant, in this case it was the concentration of ibuprofen. As seen in Figure 12, band presence and frequency changes from one reactor to the next as ibuprofen increases. Despite certain limitations of DGGE, the results showed a relationship between high concentrations of toxins, i.e. ibuprofen and tetracycline, and decrease in the eukaryotic community in different activated sludge samples.

5.2 PERFORMANCE OF THE SEMI-BATCH REACTORS

It is important that certain environment conditions are met in order to simulate and environment similar to that in a WWTP in order to achieve a realistic outcome to the tests conducted on the microorganism. To achieve a 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 determine whether greater concentrations would affect pH levels of activated sludge. When tested, the difference in the pH levels for the control reactor and high concentration of ibuprofen and tetracycline reactors were negligible. The activated sludge process is found to occur at temperatures ranging from 15-25°C (Viessman and Hammer, 1998). The temperatures were fairly constant throughout the experiments since reactors were conducted in the same environment and did not differ between reactors. The semi-batch reactor system used in this experiment was similar in design in a previous study by Islam (2013).

Pogue, (2001), showed that in a semi-batch reactor system, the microbial community can be sustained roughly one to two weeks, although without the addition of nutrients the process would eventually fail. The advantage of using a semi-batch reactor system over a batch system allows for fresh synthetic feed or organic load to be added to the reactors, which in theory would sustain and prolong the microbial community long enough to perform the required tests. A limitation of the reactor is the evaporation of water caused by the aeration. The reactors consisting of 100,000 ng/ml of ibuprofen were observed to evaporate at a higher rate. The reason could be attributed to the foaming caused by the high volume of ibuprofen, the foaming can be seen in Figure 6B. The constituents in the ibuprofen capsules could have possibly caused the foaming. Another limitation could be attributed to the aeration component, as it was difficult to ensure all reactors received the same amount of aeration due to the lack of a controlling unit that could monitor airflow rates. Nevertheless, airflow was monitored manually and adjusted as needed. Simulating aeration tank conditions and expecting the microbial community to behave in a similar manner in a laboratory environment can be challenging, because wastewater treatment plants have a continuous inflow of effluent containing high concentrations of nutrients for the microbial community while the tested community is limited to the conditions of a set nutrient schedule.

5.3 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

There is not extensive data in the literature with regards to the effects of NSAIDs particularly on eukaryotic protozoa. Some NSAIDs have been tested on different microbial such

as different bacteria and more often eukaryotic algae than other eukaryotes, and have been found to produce growth inhibiting capabilities in eukaryotic species. Abdul Hussein and Al-Janabi (2011), tested Diclofenac, Naproxen and Aspirin which showed a great ability to inhibit dermatophytes growth. Another study tested other NSAIDs (Diflunisal, Piroxicam, Diclofenac, Mefenamic acid and Ibuprofen), in concentrations of 100,000 ng/ml on different cultures of cyanobacteria and eukaryotic algae. Cyanobacteria were slightly effected but showed more tolerance to the NSAIDs than eukaryotic algae, which had growth reductions caused by all NSAIDs tested (Bácsi *et al.*, 2016).

Ibuprofen is a NSAID that has been exhibited to significantly affect the growth of numerous fungal and bacterial species (Sanyal *et al.*, 1993; Chowdhury *et al.*, 1996). Furthermore, ibuprofen could possess stimulatory effect on bacteria (Pomati *et al.*, 2004). In the study by Pomati (2004) showed that both *Lemna* and *Synechocystis* were inhibited by freshly added ibuprofen but exhibited growth stimulation after the second day, however after further addition exhibited an inhibitory effect by the fifth day (Pomati *et al.*, 2004). This could explain why in Figure 6A, protozoa increased under the 100 ng/ml of ibuprofen, possibly due to its stimulating effect while showing a major decrease under higher concentrations of 2000 and 100,000 ng/ml. As for Figure 10A, the increase in bacteria could be attributed to the stimulating affects of high concentration ibuprofen, but a most likely possibility for the increase is likely due to the decrease in protozoan population or the inability for the protozoa to graze.

Some of the eukaryotes present in the control are absent under higher concentrations of ibuprofen. Figure 12, shows various effects of ibuprofen on eukaryotes. The results show that

while some protists such as amoebzoa are affected by high concentrations of ibuprofen, others are not. *Slavina appendiculata*, is a eukaryote that has a high identity match. It was present in the control but absent due to the high levels of ibuprofen. Cercozoa is a protist that has a brighter band in the control, the brighter band has often been associated with intensity due to higher concentrations of DNA. While Cercozoa was present throughout, the intensity of the band decreases with increased amounts of ibuprofen. It is clear that ibuprofen can either have an extreme effect or minor effect depending on the species present.

5.4 ANTIBIOTIC TETRACYCLINE

Tetracycline is a broad-spectrum antibiotic that is active against prokaryotes through its inhibition of protein synthesis by inhibiting the association of aminoacyl-tRNAs with the bacterial ribosomes (Pomati *et al.*, 2004). Although tetracycline has inhibitory capabilities, in this study, minor affects have been observed. Even in the presence of 10x the relative concentrations, tetracycline appeared to display no effects. This observation could be attributed to resistance gained by the prokaryotic species found in activated sludge. Figures 10A and 10B show a major increase in the reactor that contained 500 ng/ml tetracycline / 100,000 ng/ml ibuprofen in comparison to the reactor that contained 50 ng/ml tetracycline / 100,000 ng/ml ibuprofen. Some studies have shown that antibiotic resistant bacteria have the capability to use antibiotics as a carbon source. Dantas *et al.*, (2008), penicillin resistant soil bacteria were found to contain the capability of utilizing and degrading penicillin as the sole carbon source. Walsh *et al.*, (2013) found that antibiotics were detoxified by bacteria through a resistance mechanisms

and enzyme degradation. This could be the reason for the increase shown for the reactor with 500 ng/ml of tetracycline, where the bacteria could possibly use the high concentration of tetracycline as its carbon source, and that coupled with the inhibited protozoa could cause the change in abundance.

5.5 EQUATIONS AND EXPECTATIONS

The chosen mathematical model for relative quantification for qPCR is $R = E^{(\text{control} - \text{sample})}$ which translates to $R = 2^{\Delta C_p}$ (“Real-Time”, 2006). This model assumes certain conditions. First condition is 100% efficiency of the qPCR process, where $E = 10^{-1/\text{slop}}$ is not calculated but rather assumes a perfect doubling efficiency ($E = 2$) of the target DNA during each performed qPCR cycle (Livak, 1997, 2001; Livak and Schmittgen, 2001). Second condition, requires accurate quantification of starting material which in this case its assumed each sample started with 50 ng/μl of DNA. A limitation of the C_p values obtained from the qPCR is the upper limit of 20, which represents the highest number of cycles needed to reach the crossing point. Possibly, under different qPCR protocol settings, such as the increased number of cycles, a higher upper limit can be achieved in order to observe the 18S C_p values without limitations.

5.6 RESEARCH PROJECT APPLICATION AND FUTURE PROSPECTIVE

The investigation revealed the impact of ibuprofen and tetracycline on the microbial community present in the secondary wastewater treatment activated sludge. Research can be extended to examine the varying effects on microorganisms through different NSAIDs at

varies concentrations, both independently and combination. Furthermore, testing the contaminants during low, medium, and high organic loading may show increased or reduced contaminant effect on the microbial community. Current major antibiotics may also be tested through the same method. Low concentration of tetracycline showed no effect, insight to the extent of varies antibiotic resistance could be beneficial.

Through literature exploration, limited information was available particularly in the last decade in regards to studies examining community populations in wastewater treatment plants in Canada. It could be beneficial to examine the microbial community and study the population sizes of all species, prokaryotic, eukaryotic and archaea. Research can also examine the populations during low, medium, and high organic load in order to establish population ratios. Benefits of such a study can be beneficial when examining effects of toxins and observing community changes in relation to documented population sizes as well as determining domestic levels of antimicrobial resistance.

Improvements to the semi-batch reactors would be beneficial. Evaporation reduction technique can be improved. Saran wrap and aluminum foil top covers were used to reduce evaporation, but failed to meet expectations. Construction of fitted cover top can be fashioned with organic load accessibility. Furthermore, reactors can be constructed to examine open verses closed systems and observe environmental change. Further improvements of sludge settling can be reduced, stir rods can be used to maintain internal effluent flow and reduce settling.

5.7 CONCLUSION

The exploration of effects of tetracycline and ibuprofen separately and in combination, at low and high doses on microbial communities were examined. For this purpose semi-batch reactors containing activated sludge extract from the Humber wastewater secondary treatment plant were used. The relative abundance of prokaryotes and eukaryotes were monitored via 16S rRNA gene and 18S rRNA gene primer sets as well as DGGE. It was concluded that low doses show no inhibitory effect in relation to the prokaryotic community, while high doses of ibuprofen showed an inhibitory effect on the eukaryotic community. Examination of the 18S rRNA genes of the eukaryotic community screened using DGGE showed a reduction of species in reactors containing high dose of ibuprofen while high concentrations of tetracycline showed no effect.

APPENDIX A: STANDARD DGGE PROTOCOL

Standard Operating Protocol

Denaturing Gradient Gel Electrophoresis System

Denaturant Gradient Gel

1. Assemble the glass plates with spacers and clamps. Make sure that the spacers and glass plates are flush against the sides of the clamps. Secure the plates on the casting stand.
2. Clamp (or tape) needle between the glass plates (middle/top) so that it will inject the gel solution between the plates.
3. Set pump at 99. Level the casting stand and the gradient former.
4. Add to the required amount of 0% denaturant and 80% denaturant to two 50 ml Falcon tubes (Low and High).
5. Add 115 μ l of 10% fresh ammonium persulfate (w/v) to each tube. Mix gently by inversion. Be careful not to introduce air in the solution.
6. Add 11.5 μ l TEMED (BioRad Cat#: 161-0800) to each tube. Mix gently by inversion. Be careful not to introduce air in the solution.
7. Add the low denaturant solution gently to the left chamber (Low). Remove air bubble from transfer tube by opening the valve stem quickly until the transfer tube is full of low denaturant solution and you see the solution entering the chamber on the right (High).
8. Transfer the excess low denaturant solution from the right chamber back to the left chamber.
9. Add the high denaturant solution gently to the right chamber (High).
10. Turn on mixer.

11. Turn on pump (setting at 99).
12. Open the valve stem. Let the solution enter the glass plates. DO NOT TILT THE GRADIENT FORMER.
13. Cover the top of the gel with a piece of plastic wrap to prevent it from drying out. See step 16 before you go have a coffee!
14. Let gel polymerize for 1.5 hour at room temperature.

Buffer

1. Add 6 L of 1X TAE to gel tank (*i.e.*, fill to the FILL line).
2. Insert the lid and turn on. Let the buffer warm up until the temperature reaches 60 degrees. This takes more than an hour, so you should do this 30 min after pouring the gel.

Spacer Gel

1. Using filter paper, remove the water on top of the polymerized gel.
2. Insert gel comb fully.
3. Mix 3.75 ml of 8% acrylamide- 0% denaturant with 1.25 ml of 1X TAE and with 45 μ l of 10% (wlv) APS and 4.5 μ l TEMED.
4. Add this to the top of the denaturant gradient with a pipet.
5. Let polymerize for 0.5 hour.

Loading and Running Gel

1. Assemble the plates on the core. Pour approximately 350 ml 1X TAE in the upper chamber to check the integrity of the seal. If buffer is leaking, discard the buffer,

disassemble, lubricate the gasket, reassemble the plates onto the core and test again. Insert into 60°C 1x TAE buffer (tank).

2. Let equilibrate for 15 min.
3. Wash wells with syringe using the 1 X TAE buffer from the tank.
4. Load wells with samples diluted in 2X gel loading buffer (max volume -45µl).
5. Run gel at 80 V for 16 h. at 60°C.

Staining Gel

1. Stain gel for 0.5 hour in 1:10000 dilution of SYBR Gold (Invitrogen Cat# S11494) with gentle shaking (25 µl of SYBR Gold in 250 ml of 1X TAE).
2. De-stain for 10-15 mins in 250 ml of 1X TAE with gentle shaking.

Solutions

1. Ammonium Persulfate-APS (10%) (BioRad cat#: 161-0700)

Add 100 mg of dry APS to 1 ml of ultrapure H₂O, vortex to dissolve. This is used immediately and then discarded.

2. Acrylamide - Denaturant Solutions (BioRad cat#: 161-0148)

The acrylamide solutions are only stable for one month. All glassware should be rinsed with ultrapure water.

8% acrylamide - 0% denaturant: to make 100 ml of solution 20 ml 40%

Acrylamide/Bisacrylamide (37.5:1) (BioRad)

2 ml 50X TAE prepared with ultrapure H₂O and ultrapure reagents 78 ml ultrapure H₂O

Sterilize through a 0.22um filter (150 mL filtration unit). Degas for 5 min (pump) or 10-15 min (lab set up). Store at 4°C in a brown bottle for approx. 1 month.

8% acrylamide - 80% denaturant: to make 100 ml of solution

20 ml 40% Acrylamide/Bisacrylamide (37.5:1) (BioRad)

2 ml 50X TAE prepared with ultrapure H₂O and ultrapure reagents 32 ml deionized Formamide
(see recipe below)

33.6 g Ultrapure Urea (Fluka-Sigma/Aldrich cat#:51456)

Adjust volume to 100 ml

Mix and sterilize through a 0.22um filter (150 mL filtration unit). Degas for 5 min (pump) or 10-15 min (lab set up). Store at 4°C in a brown bottle for approx. 1 month.

APPENDIX B: FULL Cp DATA SET

Table 7: Full experiment 1 Cp values, standard deviation and relative abundance ratios for 16S rRNA gene and 18S rRNA gene for; day 0, no pharmaceuticals (control), tetracycline (50 ng/ml), Ibuprofen (100 ng/ml), and a combination of Ibuprofen (100 ng/ml) and tetracycline (50 ng/ml). Experiment 2 Cp values, standard deviation and relative abundance ratios for 16S rRNA gene and 18S rRNA gene; day 0, no pharmaceuticals (control), ibuprofen (100 ng/ml) plus tetracycline (50 ng/ml), Ibuprofen (2000 ng/ml), a combination of Ibuprofen (100,000 ng/ml) and tetracycline (50 ng/ml) and a combination of Ibuprofen (100,000 ng/ml) and tetracycline (500 ng/ml)

Environmentally relative concentrations	CP 16S- 1	CP 16S- 2	CP 16S- 3	Average s Cp 1	Average s Cp 2	Average s Cp 3	Avg 1 $R=2^{\Delta C_p}$	Avg 2 $R=2^{\Delta C_p}$	Avg $3R=2^{\Delta C_p}$	Average Ratio	Stand Dev.
Bacteria 16S											
Day 0	13.75	13.86	13.83	13.79	13.83	13.71	0.64	0.60	0.64	0.63	0.02
Day 0 (2)	13.92	13.96	13.68								
Day 0 (Experiment 2)	13.76	14.06	13.93								
Day 0 (Experiment 2) (2)	13.72	13.43	13.38								
Control	14.47	14.11	14.01	14.42	14.56	14.36					
Control (2)	13.46	14.03	13.71								
Control (Experiment 2)	15.01	15.15	15.00								
Control (2) (Experiment 2) (2)	14.75	14.96	14.70								
Tet 50 ng	14.49	14.52	14.38	14.41	14.94	14.34	1.01	0.77	1.01	0.93	0.14
Tet 50 ng (2)	13.97	14.40	13.98								

Tet 50 ng (Experiment 2)	14.57	16.12	14.56								
Tet 50 ng (Experiment 2) (2)	14.60	14.71	14.42								
Ibu 100 ng	14.01	14.79	13.85	14.41	14.53	14.34	1.01	1.02	1.01	1.01	0.01
Ibu 100 ng (2)	14.38	13.70	14.39								
Ibu 100 ng (Experiment 2)	14.69	14.65	14.55								
Ibu 100 ng (Experiment 2) (2)	14.57	14.98	14.58								
Ibu 100 ng / tet 50 ng	14.84	14.76	14.50	14.65	14.71	14.47	0.85	0.90	0.92	0.89	0.04
Ibu 100 ng / tet 50 ng (2)	14.49	14.20	14.39								
Ibu 100 ng / tet 50 ng (Experiment 2)	14.67	14.87	14.51								
Ibu 100 ng / tet 50 ng (Experiment 2) (2)	14.60	15.00	14.47								
Protozoa 18S											
Day 0	18.15	20.00	20.00	18.85	20.00	19.46	3.57	4.77	0.92	3.09	1.97
Day 0 (2)	20.00	20.00	20.00								
Day 0 (Experiment 2)	19.85	20.00	20.00								
Day 0 (Experiment 2) (2)	17.39	20.00	17.84								
Control	17.50	17.97	20.00	17.01	17.75	19.58					
Control (2)	16.96	17.72	19.13								
Control (Experiment 2)	17.20	17.90	19.93								
Control (2) (Experiment 2) (2)	16.38	17.40	19.24								
Tet 50 ng	16.58	17.22	19.65	17.25	17.91	19.91	0.85	0.89	0.79	0.84	0.05

Tet 50 ng (2)	17.25	17.54	20.00								
Tet 50 ng (Experiment 2)	17.43	18.34	20.00								
Tet 50 ng (Experiment 2) (2)	17.73	18.55	20.00								
Ibu 100 ng	16.72	17.45	18.11	16.87	17.79	18.42	1.30	1.09	2.81	1.73	0.94
Ibu 100 ng (2)	15.78	16.00	15.58								
Ibu 100 ng (Experiment 2)	17.20	18.47	20.00								
Ibu 100 ng (Experiment 2) (2)	17.77	19.24	20.00								
Ibu 100 ng / tet 50 ng	16.88	18.16	20.00	17.24	18.26	20.00	0.85	0.70	0.74	0.77	0.08
Ibu 100 ng / tet 50 ng (2)	17.06	16.73	20.00								
Ibu 100 ng / tet 50 ng (Experiment 2)	17.85	19.83	20.00								
Ibu 100 ng / tet 50 ng (Experiment 2) (2)	17.17	18.32	20.00								
High Concentration											
Bacteria 16S											
Day 0	15.63	16.03	16.04	14.93	15.40	15.35	1.71	1.93	2.14	1.92	0.22
Day 0 (2)	14.23	14.76	14.66								
Control	14.16	14.43	14.13	14.16	14.45	14.25					
Control (2)	14.16	14.47	14.37								
Ibu 100 ng / tet 50 ng	14.43	14.51	14.43	14.53	14.67	14.60	0.78	0.86	0.79	0.81	0.05
Ibu 100 ng / tet 50 ng (2)	14.62	14.82	14.76								
Ibu 2000 ng / tet 50 ng	14.06	14.13	14.26	14.17	14.34	14.40	0.99	1.08	0.90	0.99	0.09
Ibu 2000 ng / tet 50 ng	14.28	14.54	14.54								

(2)											
Ibu 100000 ng / tet 50 ng	13.89	14.03	14.08	14.30	14.28	14.41	0.91	1.13	0.90	0.98	0.13
Ibu 100000 ng / tet 50 ng (2)	14.70	14.53	14.73								
Ibu 100000 ng / tet 500 ng	14.03	13.91	13.91	14.01	13.90	13.90	1.11	1.46	1.28	1.28	0.18
Ibu 100000 ng / tet 500 ng (2)	13.99	13.89	13.88								
Protozoa 18S											
Day 0	20.00	20.00	20.00	20.00	20.00	20.00	6.66	4.01	11.39	7.35	3.74
Day 0 (2)	20.00	20.00	20.00								
Control	17.36	18.25	16.62	17.27	18.00	16.49					
Control (2)	17.17	17.74	16.36								
Ibu 100 ng / tet 50 ng	17.54	19.36	16.59	17.53	19.29	16.70	0.84	0.41	0.87	0.70	0.26
Ibu 100 ng / tet 50 ng (2)	17.51	19.21	16.80								
Ibu 2000 ng / tet 50 ng	17.22	17.90	16.77	17.85	18.95	16.88	0.67	0.52	0.76	0.65	0.12
Ibu 2000 ng / tet 50 ng (2)	18.48	20.00	16.99								
Ibu 100000 ng / tet 50 ng	20.00	20.00	20.00	20.00	20.00	19.96	0.15	0.25	0.09	0.16	0.08
Ibu 100000 ng / tet 50 ng (2)	20.00	20.00	19.91								
Ibu 100000 ng / tet 500 ng	20.00	20.00	20.00	20.00	20.00	20.00	0.15	0.25	0.09	0.16	0.08
Ibu 100000 ng / tet 500 ng (2)	20.00	20.00	20.00								

APPENDIX C: REAGENTS & NUCLEIC ACID SEQUENCES

Table 8: Reagents and concentrations used for qPCR

Reagents	For 20 µl sample (µl)
Milli-Q H ₂ O	11.81
Bovine Serum Albumin (BSA) (100x, 10 mg/ml)	0.34
Dimethyl Sulfoxide (DMSO)	1.25
Forward/Reverse (5 µM)	0.50
MgCl ₂ (conc?)	1.60
Master Mix (FastStart PCR Master Mix) (co?)	2.00

Table 9: – Reagents and concentrations used for PCR

Reagents	For 25 µl sample (µl)
Milli-Q H ₂ O	8.90
Bovine Serum Albumin (BSA) (100x)	0.34
Dimethyl Sulfoxide (DMSO)	1.25
Forward (10 µM)	0.50
Reverse (10 µM)	0.50
Master Mix (FastStart PCR Master Mix)	12.50

Table 10: 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	KH ₂ PO ₄	P	8.78 (2.0mg P)	Sigma Chemical, Canada
	K ₂ HPO ₄	P	11.24 (2.0mg P)	Sigma Chemical, Canada

N-Source	(NH ₄) ₂ SO ₄	NH ₄ -N	89.33 (18.9mg N)	Fisher Scientific, Canada
Others	MgSO ₄ ·7H ₂ O	Mg	5.07 (0.5mg Mg)	VWR Scientific, Canada
	CaCl ₂ ·2H ₂ O	Ca	2.0 (0.5mg Ca)	VWR Scientific, Canada
	Na ₂ MoO ₄ ·2H ₂ O	Mo	0.01 (0.004mg Mo)	VWR Scientific, Canada
	MnCl ₂ ·4H ₂ O	Mn	0.036 (0.1mg Mn)	Fisher Scientific, Canada
	FeSO ₄ ·7H ₂ O	Fe	0.50 (0.1mg Fe)	Fisher Scientific, Canada
	CuSO ₄ ·5H ₂ O	Cu	0.39 (0.1mg Cu)	Sigma Chemical, Canada
	ZnSO ₄ ·7H ₂ O	Zn	0.44 (0.1mg Zn)	VWR Scientific, Canada
	CoCl ₂ ·6H ₂ O	Co	0.41 (0.41mg Co)	Fisher Scientific, Canada

Nucleotide sequences from DGGE samples 1-12 obtained from ACGT Inc.

TEMPLATE NAME: 1

PRIMER NAME: 3NDF

NNNNNNNNNNNNNNNNNNNTTNAANTTNTTGCAGTTAAAAAGCTCGTAGTTGNATNTCG
 GNNSNNGNCNNNCGGTNNGNCTNCNGGNNATNACTGNNCGNSCNGANYYYWYMYYY
 CKRNKKWMNCNKNGTGCNCTTCNYYGNGTGCKYCGGGNGNCNNGNACGNTTACNT
 TGANAAAANTAGAGTGNTNAAAGCANGCNNACTGNCTGNACNTNNCGCATGGAAN
 TTGNNANNACNNNGNTCTNNNTTNNTTGGTTTTGAGGACCACTGTCATGNNNNTAN
 NNNNANTGNNGGCATTTTCNTCNGGNGTCNNAGGNGNNAATTTGNANTGCNNNAN
 NANNCNANCTNNN

TEMPLATE NAME: 2

PRIMER NAME: 3NDF

NNNNNNNNNNNNNATNNNNNNNNNTNNTGCAGTTAAAAAGCTCGNAGTTGGATCTCG

GGTCCAGGCTNGCNGNTNNNNNTCGCGGCGATTACTGCNCGTCCTGACCTACCTCCCG
GTTTTCCCTTGGTGCTCTTCGTTGAGTGCTTCGGGTGGCCGGAACGTTTACTTTGAAA
AAATTAGAGTGCTCAAAGCAGGCGGACTGCCTGAATAATCGCGCATGGAATAATGG
AATAGGACCTCGGTTCTATTTTGTTGGTTTTTCGGAACCTCGAGGTAATGATTAAGAGG
GACAGACGGGGGCATTTCGTATTACGGTGTTAGAGGTGAAATTCTTGGATCGCCGTAA
GACGAACTACTGCGAAAGCATTTGCCAAGAATGTTTTCNTTAATCAAGAACGAAAG
TNNGAGGTTCTGAAGACGATCNGATACNNNNN

TEMPLATE NAME: 2
PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNNNNNNNNNNNTNTNGGNANTGCTTTCGCAGTAGTTCGTCTTNCG
GCGATCCAAGAATTTACCTCTAACACCGTAATACGAATGCCCCCGTCTGTCCCTCT
TAATCATTACCTCGAGTTCCGAAAACCAACAAAATAGAACCGAGGTCCTATTCCATT
ATTCCATGCGCGATTATTCAGGCAGTCCGCCTGCTTTGAGCACTCTAATTTTTTCAA
GTAAACGTTCCGGCCACCCGAAGCACTCAACGAAGAGCACCAAGGGAAAACCGGG
AGGTAGGTCAGGACGNGCAGTAATCGCCGCGAGGCGAACCGCNAGCCTGGACCCG
AGATCCAACCTACNAGCTTTTTAACTGCAACAACCTTAATATACGCTATTGGAGCTGG
AATTACCGCGGCTGCTGGCANNNACTTGCCAN

TEMPLATE NAME: 3
PRIMER NAME: 3NDF

NNNNNNNNNNNNNNNATATNAANGTTNTTGCAGTTAAAAAGCTCGTAGTTGGATCTCG
GGTCCAGGCTNGCGGTTTCGCCTCGCGGCGATTACTGCYCGTCCTGACCTACCTCCCG
GTTTTCCCTTGGTGCTCTTCGTTGAGTGCTTCGGGTGGCCGGAACGTTTACTTTGAAA
AAATTAGAGTGCTCAAAGCAGGCGGACTGCCTGAATAATCGCGCATGGAATAATGG
AATAGGACCTCGGTTCTATTTTGTTGGTTTTTCGGAACCTCGAGGTAATGATTAAGAGG
GACAGACGGGGGCATTTCGTATTACGGTGTTAGAGGTGAAATTCTTGGATCGCCGTAA
GACGAACTACTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAG
TNAGAGGTTCTGAAGANNATNANATACCGN

TEMPLATE NAME: 3
PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNNNNNNNNNTTCTTGNCNNNGCTTTCGCAGTAGTTCGTCTTACGG
CGATCCAAGAATTTACCTCTAACACCGTAATACGAATGCCCCCGTCTGTCCCTCTT
AATCATTACCTCGAGTTCCGAAAACCAACAAAATAGAACCGAGGTCCTATTCCATTA
TTCCATGCGCGATTATTCAGGCAGTCCGCCTGCTTTGAGCACTCTAATTTTTTCAAAG
TAAACGTTCCGGCCACCCGAAGCACTCAACGAAGAGCACCAAGGGAAAACCGGGA
GGTAGGTCAGGACGRGCAGTAATCGCCGCGAGGCGAACCGCRAGCCTGGACCCGAG
ATCCAACCTACGAGCTTTTTAACTGCAACAACCTTAATATACGCTATTGGAGCTGGAA
TTACCGCGGCTGCTGGCACNNNNNTTGCCNCNGCTGCTGGCACCNAGACTTGC

TEMPLATE NAME: 4

PRIMER NAME: 3NDF

NNNNNNNNNNNNNNNNNGNACGTTNNNTTGCTGCAGTTAAAAAGCTCGTAGTTGAACG
TNCGGGTTCGCGGGACGGGGCCGCCCCGCGAGGGACGGCGCCGCCCYGGGGCYCG
TGNNNTCYSATCTCGCCGCCCGTCGCGAGGCGGACGGAGGGANGGATGAGGTTACC
TGGA AAAAATCGGGGCGATCTAGGCGGGCGAACCTCGCCAACGAACGATMGGACA
TGGGATAACGGAAGAGGGCRTC GGCTCGACTTCGTCGGTTCCTCGNGCYGAGGCAC
GTACGGTGAATAGGGACGGTCGGGGCCGCCCCGTACCGGGTCGYGAGAGGTGAAATT
CTAGGATCGACCCGAGACGAACCGCCGCGAAAGCATTGGCAAGGACGCTTTCATT
AATCAAGAACGAAAGTCGGGGGATCGAAGANGANNANATACCGNNN

TEMPLATE NAME: 4

PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNGANNCGTCCTTGCCGAATGCTTTCGCGGGCGGTTCGTCTCG
GGTCGATCCTAGAAATTTACCTCTCNCGACCCGGTACGGGCGGGCCCNACCGTCCCT
ATTCNCCGTACGTGCCTCNGCNCGAGGAACCNACNAAGTCNAGCCNANGCCCTCNT
CCGTTATCCCATGTCCNATCGTTCGTTGGCGAGGTCNCCCGCCTAGATCGCCCCGA
TTTTTTCCAGGTAACCTCATCCNTCCCTCCGTCCGCCTCGCGACGGGCGGGCGAGATSR
GATNNACGRGCCCCNGGGCGGGCGCCGTCCCTCGCGGGGCGGGCCCGTCCCGCGGA
ACCCGNACGTTCAACTACGAGCTTTTTAACTGCAGCAACTTTAACGTACGCTATTGG
AGCTGGAATTACCGCGGCTGCTGGCANNNACTTGCN

TEMPLATE NAME: 5

PRIMER NAME: 3NDF

NNNNNNNGNGNACGTNNNNNTTGCTGCAGTTAAAAAGCTCGTAGTTGAACGTCCGGG
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CCNATCTCGCCGCCCGTCGCGAGGCGGACGGAGGGACGGATGAGGTTACCTGGA
AAATCGGGGCGATCTAGGCGGGCGAACCTCGCCAACGAACGATMGGACATGGGAT
AACGGAAGAGGGCNTCGGCTCGACTTCGTCGGTTCCTCGNGCYGAGGCACGTACGG
TGAATAGGGACGGTCGGGGCCGCCCCGTACCGGGTCGCGAGAGGTGAAATTCTAGGA
TCGACCCGAGACGAACCGCCGCGAAAGCATTGGCAAGGACGCTTTCATTAATCAA
GAACGAAAGTCGGGGGATCGAAGNNGN

TEMPLATE NAME: 5

PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNNNCCTTGCCGAANGCTTTCGCGGGCGGTTCGTCTCGGGTCGA
TCCTAGAAATTTACCTCTCNCGACCCGGTACGGGCGGGCCCGACCGTCCCTATTAC
CGTACGTGCCTCNGCTCGAGGAACCGACGAAGTCGAGCCGATGCCCTCTTCGTTAT
CCCATGTCCTATCGTTCGTTGGCGAGGTCGCCCCGCTAGATCGCCCCGATTTTTTCC
AGGTAACCTCATCCGTCCCTCCGTCCGCCTCGCGACGGGCGGGCGAGATCNGATCCCA
CGGGCCCCNGGGCGGGCGCCGTCCCTCGCGGGGCGGGCCCGTCCCGCGGAACCCGGA

CGTTCAACTACGAGCTTTTAACTGCAGCAACTTTAACGTACGCTATTGGAGCTGGA
ATTACCGCGGCTGCTGGCNNNNACTTG

TEMPLATE NAME: 6
PRIMER NAME: V4R2

NNNNNNNNNNNNNANNNNTTGNNNANGCTTTCGCATTAGTTAGTCTTcAAaAAATC
CAAGAATTTACCTCTGACTATTGAATACTAATGCCCCCAACTGTCCCTATTAAT
CATTACTTTGGTCCTCAAAMCMAMMAAAATAAAAMCMAAGTCCTATCATGWTAT
TCCMTGSTAACGKATTCAAGCATAAGCCTGCTTTAAACACTCTAATTTCCCTCAAG
GTAARGCTCGGTTCCCTASCGCACCGAASCACGACTAGCTCCCCGAAGGTGAGGC
RCAGCGATCCCAGTACACCCAGAGGGTGACCAGGCCGCCTTGCCTAAAGTCCAA
CTACRAGCTTTTAACTGCAACAACCTTTAATATACKCTATTGGAGCTGGAATTAC
CGCGGCTGCTGGCACCGWACTTGCCNGNNNNNNNNNNNN

TEMPLATE NAME: 7
PRIMER NAME: 3NDF

NNNNNNNNNNNNNNNNNNNTGTTGCAGTTAAAAAGCTCGTAGTTGAATaTCTGG
TATARCGTACCTGGCCCGCAGCCTTTAGGCTTGKGTGTGCCGGCGCACGTTTGCC
ATCTTTCTAGAAAACGGSTCTGCCATTARCTTGGTGGGTTTCGGGATCTAGATCATT
TACTTTGAAAAAATTAKAGTGTTTAAAGCAAGCAATTGCTCTGAATACATTARCA
TGRAATAATAATCTAGGACTTCKGTTCTATTTTGTGTTTCTAAAACCTGAARTAA
TGATTGATAGGGACAGTTGGGGGTGCTAGTATTGRCCGGMCAKAGGTGAAATTC
TTGRATTCGGTCAAGACTAAGTTATGCGAAAGCATTCCRCCAAGGATGTCTTCTTT
AATCAAKAACGAAAGTTGGGRGATCRAARACSATYAGATACCGA

TEMPLATE NAME: 8
PRIMER NAME: 3NDF

NNNNNNNNNNGNANANNANNNTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTTCG
GACGAGGACGTCCGGTCTGGCTTCTGGTCRGCaTGGACGGGCCGAGTCTTACTT
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TTGAGAAAAATARAGTGTTTAAAGCAAGCCGATGCTTGAATACGTTAGCATGGA
ATAATAGAAATAGGACCTTGGTCTTATTTTGTGTTTGTGAGGACCACTGTCATGA
TGAATAGGGACAGTTGGGGGCATTAGTATTCTGGCGTCAGAGGTGAAATTCTTGG
ATTGCCAGAACTAAGTGTGCGAAAGCATTGTTTCCCAAGGATGTTTTTCATTAATC
AAGAACGAAAGTTAGGGGATCGAARACRANNNATNCC

TEMPLATE NAME: 9
PRIMER NAME: 3NDF

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GGACGAGGACGTCCGGTCTGGCTTCTGGTCAGCACTGGACGGGGCCGAGTCTTACTTC
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AGAAAAATAGAGTGTTTAAAGCAAGCCGATGCTTGAATACGTTAGCATGGAATAAT
AGAATAGGACCTTGGTCTTATTTTGTGTTGGTTTTGAGGACCACTGTCATGATGAATAG
GGACAGTTGGGGGCATTAGTATTCTGGCGTCAGAGGTGAAATTCTTGGATTGCCAGA
AGACTAACTAGTGCGAAAGCATTTGCCAAGGATGTTTTTCATTAATCAAGAACGAAA
GTTNNGGGGNTCGAAGNNNNNNNTACCG

TEMPLATE NAME: 9
PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNNCNTTGNNNNNGCTTTCGCACTAGTTAGTCTTCTGGCAATC
CAAGAATTTACCTCTGACGCCAGAATACTAATGCCCCCAACTGTCCCTATTCATCA
TGACAGTGGTCCTCAAAACCAACAAAATAAGACCAAGGTCCTATTCTATTATTCCAT
GCTAACGTATTCAAGCATCGGCTTGCTTTAAACACTCTATTTTTCTCAAGGTAATCGT
CCTAGTTCCCCGGCGCACCCGGTGAAGGGCACGCCGCTTCCCTAGAAGTAAGACTC
GGCCCGTCCAGTGCTGACCAGAAGCCAGACCGGACGTCCTCGTCCGAAATTCAACT
ACGAGCTTTTTAACTGCAACAACCTTTAATATACGCTATTGGAGCTGGAATTACCGCG
GCTGCTGGCNCNNNCTTGCCNNN

TEMPLATE NAME: 10
PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNTGGCGATGCTTTTCGCACTAGTTAGTCTTCCGGCAANCCAA
GAATTTACCTCTGACGCCGGAATACTAATGCCCCCAACTGTCCCTATTCATCATGA
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MWGGYWAWGRATYYMAGCMAGAAGCCTGCTTTGAACACTCTGTTTTCTCAAGGT
AATCGTCTTGTTCCCCGGCGCGCCAGYRAAGGACACGCCGCTTCCCCAGAAGTA
AGACTCGACCCGCGSCGTCGAGGCCGAARCCACACGCCGCGCTCTCGCCCSATGMT
MAMTACSARCTTTTTAACTGCAACAACHTTAATGTGTGCTATTGGARCTGGAATTAC
CGCGGCTGSTGGCACCGAMTTGCCAAGACTTGCNNNCTTTAATATNCNCNNTTGGAG
CTGGAATTANCGCGGCTGNNNNN

TEMPLATE NAME: 11
PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNNCCTTGGcAATGCTTTCGCACTAGTTMGTCTTCTGGMAA
TCCAAGAATTTACCTCTGACKCCRGAACTAATGCCCCCAACTGTCCCTATTCAT
CATGACAKTGGTCCTYAAAACCAACAAAATAARACCAARGTCCTATTYATTATTCC
ATGCTAACGTATTCAAGCAWMRGCTTGCTTTAACTCTATTTTTCTCAAGGTAAT
CGTCCTRGTTCCCCGGCGCACCCGGTGAAGGGCACSCCGCTTCCCTAGAARWAAGA

CTCGRCCCGYCCAGTGCTGACCARAAGCCARACCGGACGTCCTCGTCCGAAATTCAA
CTACGAGCTTTTTAACTGCAACAACCTTTAATATACGCTATTGGAGCTGGAATTACCG
CGGCTGSTGGCACCANACTNNNNNNNN

TEMPLATE NAME: 12

PRIMER NAME: 3NDF

NNNNNNNNNNNNANANNNNNTTNTGTCAGTTAAAAAGCTCGTAGTTGGATCTCGGG
TCCAGGCTNGCGGTTGCCTCGCGGCGATTACTGCYCGTCCTGACCTACCTCCCGGT
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ATTAGAGTGCTCAAAGCAGGCGGACTGCCTGAATAATCGCGCATGGAATAATGGAA
TAGGACCTCGGTTCTATTTTGTGTTGGTTTTTCGGAACCTCGAGGTAATGATTAAGAGGGA
CAGACGGGGGCATTTCGTATTACGGTGTTAGAGGTGAAATTCTTGGATCGCCGNAAG
ACGAACTACTGCGAAAGCATTTGCCAAGAATGTTTTTCATTAATCAAGAACGAAAGT
NNGAGGTTCGAAGACGATCNNATACCNNNNN

TEMPLATE NAME: 12

PRIMER NAME: V4R2

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNGNNNNNNCTTTCGCAGNAGTTCGTCTTNCGG
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NATCATTACCTCGAGTTCCGAAAACCAACAAAATAGAACCGAGGTCCTATTCCATTA
TTCCATGCGCGATTATTCAGGCAGTCCGCCTGCTTTGAGCACTCTAATTTTTTCAAAG
TAAACGTTCCGGCCACCCGAAGCACTCAACGAAGAGCACCAAGGGAAAACCGGGA
GGTAGGTCAGGACGNGCAGTAATCGCCGCGAGGCGAACCGCNAGCCTGGACCCGA
GATCCAACCTACGAGCTTTTTAACTGCAACAACCTTTAATATACGCTATTGGAGCTGGA
ATTACCGCGGCTGCTGGCANNNACTTGCNNNN

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