ABSTRACT

FOGLE, KARA NICOLE KOPF. Quantification of the Denitrification Gene *nosZ* in a Full-Scale Wastewater Treatment Plant Using qPCR. (Under the direction of Dr. Francis L. de los Reyes III.)

Excessive levels of nitrogen in wastewater effluent can result in DO depletion, toxicity, eutrophication, global warming, acid rain, and other undesired effects. Understanding the denitrification process that occurs in wastewater treatment plants is important to allow engineers and plant operators to achieve more efficient nitrogen removal from wastewater. Due to the wide variety of physiological and phylogenetic groups to which denitrifiers belong, quantifying them using ribosomal RNA is not feasible. However, specific denitrification genes can be targeted instead. *nosZ* is the gene that encodes for the enzyme nitrous oxide reductase, the enzyme that catalyzes the transformation of N_2O to N_2, the final step of denitrification.

Most denitrifying bacteria are facultative aerobic chemoorganotrophic organisms, meaning their respiratory networks are designed so that there is preferential electron flow to O_2 under aerobic growth conditions. It is only when O_2 is limiting and NO_3^-/NO_2^- are present that the denitrification enzymes are expressed. Therefore, O_2 plays a major regulatory role in the process of denitrification.

The goal of this research project was to quantify the denitrification gene *nosZ* in a full scale wastewater treatment plant over an entire operational cycle as aeration is turned on and off. In a full-scale plant, the wastewater is cycled through both aerobic and anoxic phases to allow for both nitrification and denitrification, and the expression of denitrification genes therefore likewise cycles. By quantifying this change in gene

expression over time, a better understanding of the regulation of denitrification genes was gained. In this study, the goal was to analyze both DNA and RNA to estimate the concentration of organisms capable of performing denitrification, as well as the concentration of organisms actively performing denitrification. This allowed us to determine the effect of oxygen concentration on the activity and growth of the organisms.

A standard curve of CT value versus log cell concentration was created for a pure culture of *Pseudomonas stutzeri*, a known denitrifier. Samples of water and activated sludge were taken from treatment basins 1 and 2 of the North Cary wastewater treatment plant in Cary, North Carolina over an entire cycle of operation (676 samples over 6 hours). This treatment plant uses a Bio-Denipho process. Dissolved oxygen concentration, pH, and oxidation-reduction potential were measured in the activated sludge basins 1 and 2 over an entire operational cycle. NO_3^- and NO_2^- concentrations in the wastewater were measured. DNA was extracted from selected samples, and qPCR targeting *nosZ* was performed to quantify the microorganisms capable of carrying out denitrification. RNA was also extracted from selected samples, followed by RT-PCR and qPCR targeting *nosZ* in an attempt to quantify the microorganisms actively denitrifying. T-RFLP was then performed on selected samples in order to analyze the microbial community.

From the qPCR performed targeting the nosZ gene present in DNA, it was determined that the concentration of cells with *nosZ* changes significantly over the different phases of operation at a full-scale Bio-Denipho wastewater treatment facility. This change appears to correlate not only with the presence of absence of O_2, but also with availability of organic carbon to utilize as an electron donor. When the air is turned

off, the increase in cells with *nosZ* begins almost immediately. When the air is turned on, there appears to be a slight lag before the concentration of cells with *nosZ* begins to decrease. This may be due to the fact that some residual O_2 remains in the water, or it could be due to carry-over from the previous basin. Also, when there is flow into a basin, whether from the head of the plant of from the previous basin, there is usually at least a slight increase in the concentration of cells with *nosZ*, even when the air is turned on. This is most likely due to an in-flow of organic carbon providing electrons for growth. During some of the anoxic phases, there appears to be a decrease in the concentration of cells with *nosZ* towards the end of the phase before the air is turned on again. This decrease could be due to an insufficient supply of organic carbon.

Attempts to quantify bacteria actively carrying out denitrification by extracting RNA, synthesizing cDNA by RT-PCR, and performing qPCR targeting the *nosZ* gene were unsuccessful and did not result in any usable CT values.

T-RFLP was performed on selected samples to analyze the microbial community. The number of T-RFs was very similar for all samples, meaning that the number of species present was about the same for all of the samples analyzed. The samples taken from anoxic phases were less diverse, while those taken from aerobic phases were more diverse. The relative abundance of the different species present was determined for each sample. Although the same species were present in all of the samples, the abundance of these species changed greatly over the operational cycle of the treatment facility. There did not appear to be species that were present only during anoxic or aerobic phases, and the relative abundances of the species present in the samples did not appear to correlate with whether the sample was taken from an aerobic or anoxic phase.

Quantification of the Denitrification Gene *nosZ* in a Full-Scale Wastewater Treatment Plant Using qPCR

by
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Chair of Advisory Committe

DEDICATION

To my loving family, who will hopefully stop asking me when I am going to graduate, to my husband Rob, for never once cutting me off when I was ranting about my qPCR and gel results, and to Meredith Fotta, without whom I would have quit grad school in the first month.

BIOGRAPHY

Kara Nicole Kopf Fogle, who started out her graduate school experience as Kara Nicole Kopf, was born on Friday, March the 13th, 1987. Upon learning that she entered the world on Friday the 13th, many people insist that this "explains a lot" about her, but she continuously denies this having any impact on her personality and demeanor. The vast majority of her childhood was spent being raised in the South, although this has been disputed mainly because she 1) talks fast, 2) has an extreme dislike of sweet tea and grits, and 3) votes democrat. Kara attended Clemson University where she received her bachelor's of science in Biosystems Engineering in May 2010. Do not ask her to explain what Biosystems Engineering is. She spent the summer after graduation helping care for her newborn twin nieces, ever since which she has lived in constant fear of having twins. She began pursuit of her master's of science in Environmental Engineering at North Carolina State University in the Fall of 2010. Impressively, Kara both planned and executed her own wedding half way through her graduate career (although her mother rightfully insists on taking much of the credit). She and her husband also adopted their canine son, Ramsey, who has successfully fulfilled his goal of being a baby substitute. Upon graduation, she hopes to get a job relevant to her major, although her mother's fears of the republicans gaining power and eradicating all environmental regulatory agencies makes her hesitant to work in the government. Although Kara's peers and colleagues would describe her as being sunny, friendly, and incredibly nice, she would like to clarify that anyone referring to her as "sweetheart" has either a) never met her, or b) is being sarcastic, which would bode well for the two of them being great friends.

ACKNOWLEDGMENTS

First and foremost I would like to thank my advisor, Dr. Francis de los Reyes III, from whom I have learned way more than reactor design and molecular biology. He has been an amazing teacher and mentor whose support, knowledge, and guidance have kept me going, and who has taught me that patience, kindness, and understanding can never be over-used. I would also like to thank Jason So for all of the hours he spent teaching me various molecular biology laboratory techniques, and for never once making me feel stupid for all of the mistakes I made. I would also like to say thanks to all of my other lab team members, Xia He, Bahareh Karami, Tony Sobremisana, Jory Wahlen, Ling Wang, Elvin Hossen, Jim Hurley, and Dominic Libera for all of their support and for just putting up with me, and to David Black, for his assistance in the lab. A special thanks to Meredith Fotta for going through this whole process of grad school with me, and making it all bearable with our weekly Mitch's lunch dates. She has been an amazing friend from the day we met, and I was incredibly lucky to have her. To my mom, dad, sister, brother-in-law, brother, and nieces for their unconditional love, for helping me keep my priorities straight, and never letting me forget that family comes first. And of course, to my amazing husband Rob, for his constant love, support, and understanding, and for never making me feel guilty for all of the times my graduation date was pushed back! Lastly, I would like to thank the National Science Foundation for their generous support of both myself and my project.

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CHAPTER 1: INTRODUCTION

1.1 The Nitrogen Cycle

Nitrogen (N) is a key building block of numerous compounds present in all organisms, accounting for 6.25% of their dry mass on average (Bothe et al., 2007). Nitrogen exists in several oxidation states, and the cycling of nitrogen occurs by oxidation and reduction reactions where nitrogen-containing compounds are used as electron donors or acceptors. The oxidation states range from +5, as it is in nitrate (NO_3^-) to -3, as it is in ammonia (NH_3). These reduction and oxidation (redox) reactions are performed in varying ways by different organisms, and their sum makes up the biological nitrogen cycle, as shown in Figure 1.1 (Bothe et al., 2007).

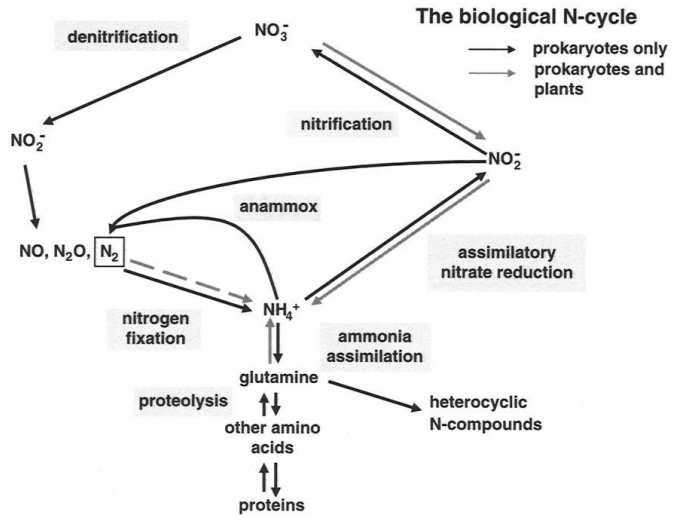


Figure 1.1 The biological nitrogen cycle (Bothe et al., 2007).

All of these reactions are carried out by bacteria, archaea, and some specialized fungi, with the only exception being assimilatory NO_3^- reduction, which occurs in plants (Bothe et al., 2007). The three major processes of microbial nitrogen transformation are nitrification, denitrification, and nitrogen fixation (Madigan and Martinko, 2006).

1.2 Denitrification

Denitrification is the part of the nitrogen cycle whereby nitrate is transformed into dinitrogen gas (N_2). This is a four-step process (Figure 1.2): 1) NO_3^- to nitrite (NO_2^-), 2) NO_2^- to nitric oxide (NO), 3) NO to nitrous oxide (N_2O), and 4) N_2O to N_2 (Ferguson, 1994).

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$$

Figure 1.2 Denitrification

Because denitrification is a reductive process, it is therefore a form of respiration (van Spanning et al., 2007). The N oxyanions, nitrate and nitrite, and the gaseous N oxides, nitric oxide and nitrous oxide, serve in place of dioxygen (O_2) as terminal electron acceptors for electron transport phosphorylation (Zumft, 1997).

1.3 Proteins of Denitrification

The enzymes of denitrification receive electrons by the electron transport chain of the cytoplasmic membrane (Ferguson, 1994). Denitrification is a form of respiration and

therefore shares respiratory chain components with the electron transport system that delivers electrons to O_2 via terminal oxidases (Nicholls and Ferguson, 2002; Baker et al., 1998). The first step of the electron transport process is the electrons transferring from NADH to a flavoprotein (Fp) (Madigan and Martinko, 2006). The two electrons are then transferred from the flavoprotein to an iron-sulfur protein (Madigan and Martinko, 2006). The denitrification-specific components of the chain system start with ubiquinol/ubiquinone (Q) (van Spaning et al., 2007), to which the electrons are next transferred. Following this, the electrons move to cytochrome *b* (cyt *b*) and then to nitrate reductase, the first enzyme in the denitrification process; next they move to cytochrome *cd* (cyt *cd*) and cytochrome bc_1 (cyt bc_1), then to nitrite reductase and nitric oxide reductase, with the final step being nitrous oxide reductase (Madigan and Martinko, 2006). The nitrate and nitric oxide reductases are located in the cytoplasmic membrane while the nitrite and nitrous oxide reductases are periplasmic (van Spanning et al., 2007). This pathway is shown in Figure 1.3 (Madigan and Martinko, 2006).

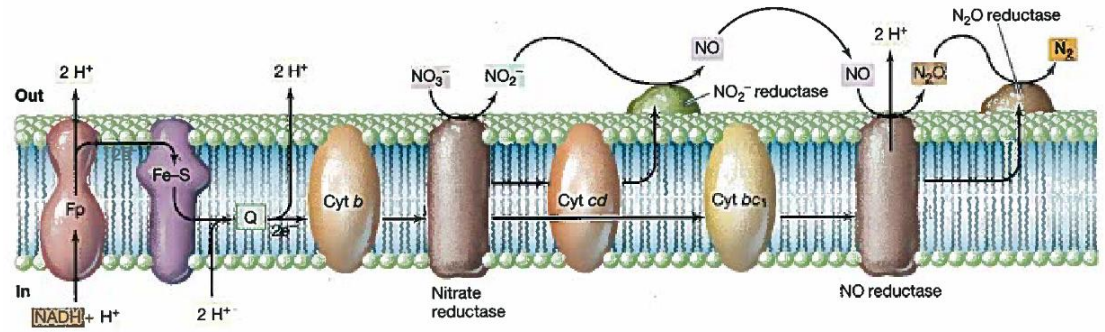


Figure 1.3. Denitrification pathway (Madigan and Martinko, 2006).

1.4 Genes and Enzymes of Denitrification

Each enzyme that catalyzes a different step in the denitrification pathway is encoded for by a different gene. Nitrate respiration, or the reduction of NO_3^- to NO_2^-, is the first step of denitrification. This reaction is carried out by a nitrate reductase that is encoded for by *narG* (Zumft, 2007).

The next step in the denitrification process is the transformation of NO_2^- to NO. This reaction is catalyzed by two different types of nitrite reductases (Nir), either a cytochrome cd_1 enzyme encoded by *nirS* or a copper-containing enzyme encoded by *nirK* (Throbäck et al., 2004). Which gene is present depends on the organism.

Next is the transformation of NO to N_2O. The enzyme that catalyzes this reaction, nitric oxide reductase, is encoded by gene *norB* (van Spanning et al., 2007).

The final step of denitrification is the reduction of N_2O to N_2. The enzyme that catalyzes this reaction is nitrous oxide reductase. *nosZ* is the gene that encoded this enzyme (Throbäck et al., 2004).

1.5 Regulation of the Denitrification Genes

Many of the denitrifying bacteria are facultative aerobic chemo-organotrophic organisms, meaning their respiratory networks are designed so that there is preferential electron flow to O_2 under aerobic conditions (van Spanning et al., 2007). The two dominant signals that induce the synthesis of the denitrification systems are low oxygen tension and the presence of a respirable N oxide (Zumft, 1997). This means that only when there is very

little O_2 present, and NO_3^-/NO_2^- is available, are the denitrification enzymes expressed. Also, because the product of one enzyme is substrate for the next enzyme in the denitrification process, the complete denitrification pathway must be expressed and function at the same time for complete denitrification to take place (Zumft, 1997). Regulation of the enzymes is needed to keep the concentrations of NO_2^- and NO below toxic levels (van Spanning et al., 2007). NO_3^--reductase is the first enzyme that is induced in response to O_2 depletion and NO_3^- availability, and the activity of this enzyme causes an increase in the concentration of NO_2^- (van Spanning et al., 2007). The NO_2^- and NO-reductases are expressed next, and their concentrations and activities are controlled so that the free NO_2^- concentration lowers to the micromolar range while NO is kept in the nanomolar range (van Spanning et al., 2007). Nos is expressed at a later stage in the growth phase than the other reductases (van Spanning et al., 2007). The key molecules that act as signals to the regulation pathways are O_2, NO_3^-, NO_2^-, and NO (Madigan and Martinko, 2006).

1.6 Nitrification

Nitrification is the oxidation of NH_3 to NO_3^- (Madigan and Martinko, 2006). It can be performed by heterotrophic bacteria (Painter, 1977), autotrophic bacteria (Painter, 1977), or Archaea (Park et al., 2006). Many heterotrophic species can form NO_2^- or NO_3^- from NH_3 or reduced organic nitrogen compounds (Schmidt et al., 2003) (Verstraete and Alexander, 1973). However, heterotrophic nitrification is not thought to significantly contribute to nitrogen oxidation in conventional wastewater nitrification (Grady et al., 2011). Instead,

nitrification in wastewater treatment systems is generally considered to be performed by autotrophic bacteria (Grady et al., 2011).

No single chemolithotroph is known to exist that can carry out the complete oxidation of NH_3 to NO_3^- (Madigan and Martinko, 2006). Rather, nitrification in nature results from the sequential action of two separate groups of organisms (Madigan and Martinko, 2006). Aerobic ammonia oxidizing bacteria (AOB) oxidize NH_3 to NO_2^- with hydroxylamine (NH_2OH) as an intermediate product (Grady et al., 2011). The NO_2^- is then oxidized to NO_3^- in a single step by aerobic nitrite oxidizing bacteria (NOB) (Grady et al., 2011). Many species have complex internal membrane systems which are the location of key enzymes in nitrification (Madigan and Martinko, 2006). Ammonia monooxygenase, encoded by the gene *amoA*, catalyzes the oxidation of NH_3 to NH_2OH, and nitrite oxidase, encoded by the gene *hao*, catalyzes the oxidation of NO_2^- to NO_3^- (Madigan and Martinko, 2006). Early studies determined that aerobic AOB were mainly of the genus *Nitrosomonas* and aerobic NOB were mainly of the genus *Nitrobacter* (Grady et al., 2011). However, molecular tools have supplied information in greater detail of the ecology of both groups (Grady et al., 2011). Aerobic AOB that proliferate during the biological treatment of domestic wastewater are primarily of the β-*proteobacteria* and include the *Nitrosomonas* and *Nitrosospira* lineages (Purkhold et al., 2003). The NOB are a diverse group of organisms that contain the α-*proteobacteria*, including *Nitrobacter* and *Nitrospira* (Grady et al., 2011). Aerobic AOB and NOB appear to grow in close physical association (Mobarry et al., 1996), and NOB cluster along NO_2^- gradients generated by AOB (Maixner et al., 2006).

1.7 Nitrogen Fixation

Nitrogen fixation is the process of converting N_2 gas to NH_3 ($N_2 + 8H^+ \longrightarrow 2NH_3 + H_2$) (Madigan and Martinko, 2006). N_2 is the most stable form of nitrogen and is a major reservoir for nitrogen on Earth (Madigan and Martinko, 2006). However, only a small number of organisms are capable of using N_2 as a nitrogen source in the process of nitrogen fixation (Madigan and Martinko, 2006). The microorganisms that carry out biological nitrogen fixation are known collectively as diazotrophs, and they provide about 60% of the total annual input of fixed-N (Newton, 2007). No eukaryote is known that is able to perform nitrogen fixation (Newton, 2007). Nearly all of the diazotrophs use essentially the same enzyme, called nitrogenase, which functions at ambient temperature and pressure in the soil with the sun as the ultimate source of energy (Newton, 2007). Diazotrophs are all prokaryotic and are found in the Bacteria and Archaea (Newton, 2007). They are usually grouped based on their lifestyle; they are either free-living, symbiotic, or in a loose association with plant roots (Newton, 2007). Free-living diazotrophs fix N_2 for their own benefit, can function under aerobic, anaerobic, or micro-anaerobic conditions, and can be either chemotrophs or phototrophs (Newton, 2007). Symbiotic diazotrophs nearly always live and fix N_2 under micro-aerobic conditions inside a specialized structure on plant roots and provide fixed N to their host (Newton, 2007). An example of the free-living obligate aerobes is some species of *Azotobacter*, which fix N_2 in the air (Newton, 2007). *Anabaena* and *Nostoc* are examples of aerobic phototrophs, which fix their own N_2 as well as use photosynthesis to generate their fixed carbon and energy requirements (Newton, 2007). Clostridia represent the anaerobic diazotrophs, which ferment carbon sources mainly to

butyric acid, CO_2, and H_2 (Newton, 2007). *Desulfovibrio* is a diazotroph that is an anaerobic sulfate-reducing bacteria (Newton, 2007). Methanogens are another type of anaerobic diazotroph, which belong to Archaea (Newton, 2007). Anaerobic phototrophs such as *Rhodospirillum rubrum*, a purple nonsulfur bacterium, can also carry out nitrogen fixation (Newton, 2007). Another group of diazotrophs are the facultative anaerobes, which can grow with or without O_2 if provided with fixed N but can only fix N_2 anaerobically (Newton, 2007). The best example of this is a number of species of the genus *Klebsiella* (Newton, 2007). There are also a variety of systems in which bacteria fix N_2 in various symbioses and associations with roots of various plant hosts, the best understood of which are the *Rhizobium*-legume symbioses in which nodules result from the colonization of the legume roots by rhizobia (Newton, 2007). Actinomycetes, such as *Frankia*, also form symbioses, but with the roots of non-legumes such as shrubs and trees (Newton, 2007). Less formalized associations of various grasses with *Acetobacter*, *Azoarcus*, *Azotobacter*, *Azospirillum*, *Gluconacetobacter*, and *Herbaspirillum* also exist (Newton, 2007).

1.8 Nitrate Assimilation

Another part of the nitrogen cycle is assimilatory nitrate reduction (Madigan and Martinko, 2006). It is carried out by higher plants, fungi, algae, and many bacteria, which use NO_3^- as an N source for growth (Moreno-Vivián and Flores, 2007). More than 2×10^{13} kg of N per year is assimilated by this process (Guerrero et al., 1981). NO_3^- is incorporated into the cells by high-affinity transport systems and further reduced to NH_4^+, via NO_2^-, by

two sequential reduction reactions that are catalyzed, respectively, by the enzymes nitrate reductase (NR) and nitrite reductase (NiR) (Moreno-Vivián and Flores, 2007). The resulting NH_4^+ is then incorporated into carbon skeletons mainly by the glutamine synthetase/glutamate synthase pathway (Moreno-Vivián and Flores, 2007). In NO_3^- assimilation, only enough NO_3^- is reduced to satisfy the needs for cell growth (Madigan and Martinko, 2006).

1.9 Effects of the Nitrogen Cycle on the Environment

The presence of nitrogenous compounds can adversely impact or pollute receiving waters (Gerardi, 2002). Principal nitrogenous wastes that pollute these waters are NH_4^+, NO_2^-, and NO_3^- (Gerardi, 2002). Significant concerns include dissolved oxygen depletion, toxicity, eutrophication, methemoglobinemia, global warming, destruction of stratospheric ozone, and acid rain (Gerardi, 2002; Bothe et al., 2007; Noda et al., 2003).

The discharge of nitrogenous wastes to natural waters causes dissolved oxygen depletion. The depletion occurs through the consumption of dissolved oxygen by microbial activity. First, NH_4^+ is oxidized to NO_2^-, and NO_2^- is oxidized to NO_3^- within the receiving water. In other words, nitrification is occurring. The oxidation of each compound occurs as dissolved oxygen is removed from the water by bacteria and added to NH_4^+ and NO_2^-. Second, NH_4^+, NO_2^-, and NO_3^- serve as a nitrogen nutrient for the growth of aquatic plants, especially algae (Gerardi, 2002). When these plants die, dissolved oxygen is removed from the water by bacteria to decompose the dead plants.

All three nitrogenous ions, NH_4^+, NO_2^-, and NO_3^-, can be toxic to aquatic life, especially fish (Gerardi, 2002). NO_2^- causes toxicity by interacting with human molecules, and NO (formed from NO_2^-) is an important signal molecule in many plant and animal reactions (Bothe et al., 2007).

Phosphate (PO_4^{2-}) is the primary source of eutrophication, but nitrogenous wastes also significantly contribute to this water pollution problem (Gerardi, 2002). Eutrophication is the discharge of plant nutrients, mainly phosphorous and nitrogen, in undesired quantities to bodies of freshwater such as lakes and ponds. The presence of these high quantities of plant nutrients causes rapid growth or blooms of aquatic plants, including algae. Eutrophication causes fluctuations in dissolved oxygen concentrations with the death and growth of aquatic plants, the clogging of receiving waters caused by the sudden bloom of aquatic plants, and the production of color, odor, taste, and turbidity problems associated with the growth and death of aquatic plants (Gerardi, 2002).

Another problem associated with the discharge of nitrogenous compounds is methemoglobinemia (Gerardi, 2002; Bothe et al., 2007). Methemoglobinemia is also referred to as blue-baby syndrome. It is a disease experienced mainly by infants when they consume groundwater contaminated with NO_3^- (US EPA, 2007). The most common exposure pathway for children is through consuming infant formula made from drinking water that contains nitrates (US EPA, 1991). Infants of 0 to 3 months are at the highest risk for blue baby syndrome because their normal intestinal flora contribute to the generation of methemoglobin; older children and adults can experience this syndrome, but at higher

concentrations of nitrates (US EPA, 2001; US EPA, 2006). Once the nitrates are in the bloodstream, it binds to hemoglobin (the compound that carries oxygen in blood to tissues in the body) and results in chemically-altered hemoglobin (methemoglobin) that impairs oxygen delivery to tissues, resulting in the blue color of the skin (US EPA, 2001). Reduced oxygenation of the tissues can have numerous adverse implications for the child, the most severe of which are coma and death (US EPA, 2001).

Global warming is yet another problem that is affected by the nitrogen cycle. N_2O is a potent greenhouse gas (Kong el. al., 2010; Noda el. al., 2003). Greenhouse gases are gases such as carbon dioxide, methane, N_2O, and fluorinated gases that absorb heat energy and re-radiate it back to the Earth's surface rather than allowing it to radiate out into space (US EPA, 2010). The increase in concentration of greenhouse gases in the atmosphere has contributed to an increase in the average global temperature (US EPA, 2010). N_2O accounts for 5 to 6% of observed greenhouse warming (Barnes and Owens, 1998). In addition, each molecule of N_2O contributes 200 to 300 times more to the greenhouse effect than carbon dioxide over a 100 year period (Noda et. al., 2003). The concentration of N_2O in the atmosphere has increased significantly since the pre-industrial era; its concentration is approximately 310 ppbv, which is 8% greater than the pre-industrial concentration (Noda et al., 2003), and it also continues to increase at a rate of 0.26% per year (Forster et al., 2007).

Destruction of stratospheric ozone is another concern associated with the nitrogen cycle (Bothe et al., 2007). The reaction of N_2O with atomic oxygen in the stratosphere and the resultant NO induces the destruction of the stratospheric ozone (Upstill-Goddard et. al.,

1999). According to the US EPA (2010), destruction of the ozone leads to less protection from the sun's UVB radiation. This can lead to increased skin cancer, cataracts, damage to plant growth and phytoplankton, and detrimental effects on biogeochemical cycles.

Yet another environmental and human health impact of the nitrogen cycle is acid rain (Bothe et al., 2007). Once in the atmosphere, N_2O can be converted to NO by sunlight, and NO then reacts with ozone in the upper atmosphere to form NO_2^- (Madigan and Martinko, 2006). NO_2^- can then return to the Earth as acid rain, nitrous acid (HNO_2) (Madigan and Martinko, 2006). According to the US EPA (2008), acid rain can lead to many environmental problems, including damage to trees and the acidification of lakes and other water bodies which contributes to reduced biodiversity and fish kills.

1.10 Removal of Nitrogen in Wastewater Treatment

Because of the reasons described above, removal of nitrogen from wastewater before it is discharged to receiving waters is of vital importance. The wastewater nitrogen cycle with the traditional nitrification/denitrification treatment process is given in Figure 1.4 (Gerardi, 2002).

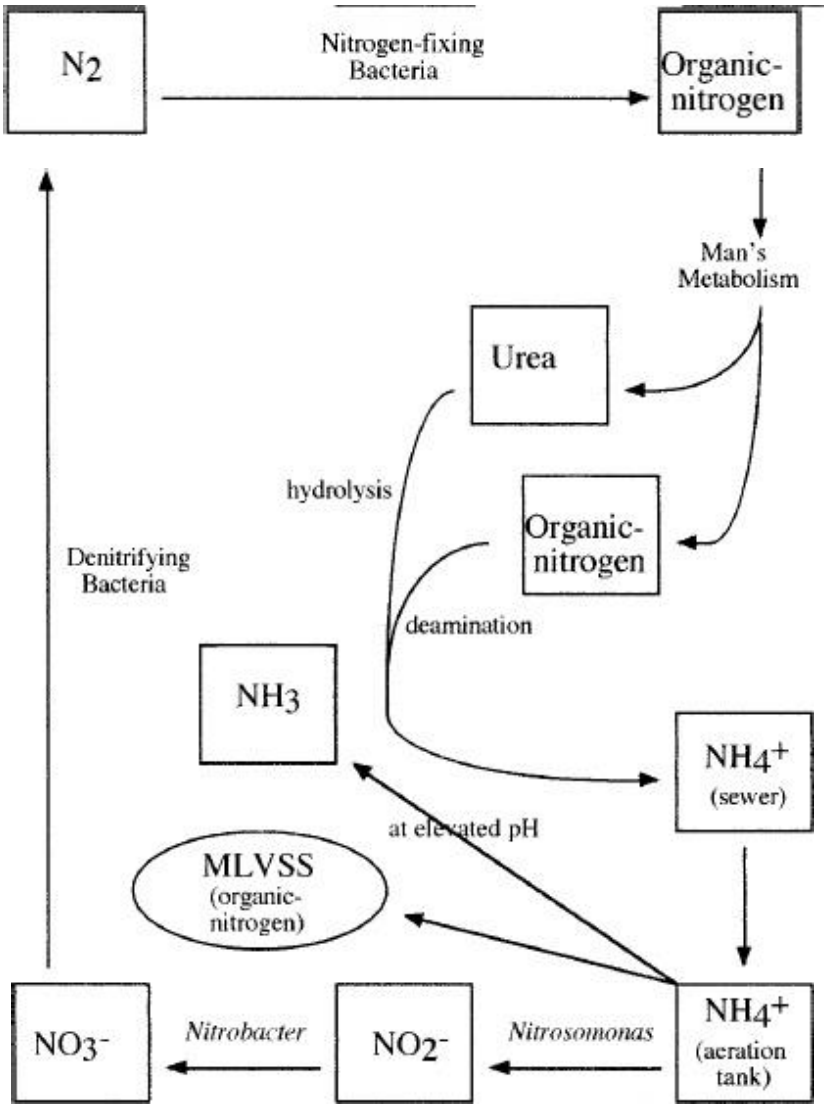


Figure 1.4. The wastewater nitrogen cycle (Gerardi, 2002).

Nitrogen-fixing bacteria convert atmospheric nitrogen, N_2, to organic nitrogen. The organic nitrogen is assimilated by people and excreted as both urea and organic nitrogen. Through hydrolysis and deamination, these are converted to NH_4^+, which is the form of nitrogen

present in the sewer system. The NH_4^+ enters the wastewater treatment facility and is present in the aeration tanks. Nitrifying bacteria convert the NH_4^+ to NO_3^- via NO_2^-. Denitrifying bacteria then convert the NO_3^- back to N_2, and the N_2 is released back into the atmosphere.

1.11 Nitrification/Denitrification in Wastewater Treatment

One of the most common ways to remove nitrogen from wastewater is by nitrification followed by subsequent denitrification. As described previously, the NH_4^+ that enters the treatment facility is converted into NO_3^- by the process of nitrification. The NO_3^- is then converted into N_2 by the process of denitrification, and the N_2 is released into the atmosphere, effectively removing it from the water. There are a number of reactor configurations to achieve nitrogen removal from wastewater. Many of these configurations also achieve phosphorus (P) removal, another goal of wastewater treatment. Together, the removal of these two constituents is known as biological nutrient removal (BNR). Several of these reactor configurations are given below.

The Modified Ladzak-Effinger (MLE) configuration is designed for N removal (Figure 1.5). It contains two basins. The first is anoxic, and the second is aerobic. Nitrate is recycled from the aerobic tank to the anoxic tank. The aerobic tank is placed after the anoxic tank so that there is enough biological oxygen demand (BOD) for denitrification to occur. Sludge is recycled from the clarifier to the anoxic tank.

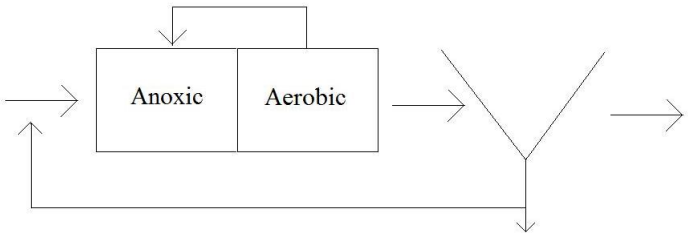


Figure 1.5. Modified Lodzak-Effinger Reactor Configuration.

Another configuration is the Anaerobic-Oxic (A/O) configuration (Figure 1.6). This configuration also has two basins. The first is operated as anaerobic to achieve P removal. The second basin is aerobic. Some N removal occurs through the activity of denitrifying phosphorus accumulating organisms (PAOs). Sludge is recycled from the clarifier to the first basin.

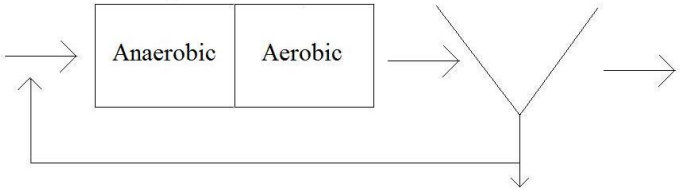


Figure 1.6. Anaerobic-Oxic (A/O) Reactor Configuration.

A modification of the A/O configuration is the Anaerobic-Anoxic-Oxic (A_2/O) configuration (Figure 1.7). This configuration contains three basins. The first is anaerobic to achieve P removal, the second is anoxic, and the third is aerobic. Nitrate is recycled from the

aerobic tank to the anoxic tank to achieve denitrification. This is a more complex configuration than the A/O process, but achieves better denitrification.

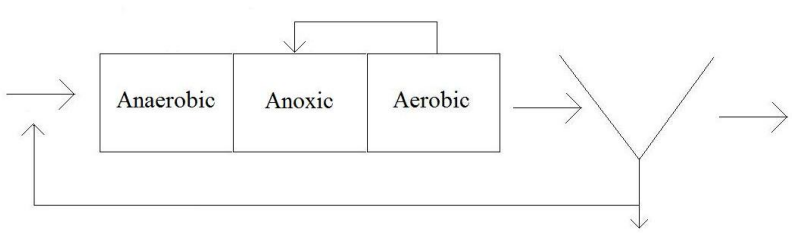


Figure 1.7. Anaerobic-Anoxic-Oxic (A_2/O) Configuration.

The University of Cape Town (UCT) process is another such configuration (Figure 1.8). It contains three basins. The first is anaerobic to achieve P removal, the second is anoxic, and the third is aerobic. Nitrate is recycled from the aerobic basin to the anoxic basin. What makes it different from the A_2/O process is that the sludge is recycled from the clarifier to the anoxic tank rather than the anaerobic tank. This is to ensure that no residual DO is returned to the anaerobic tank to maximize P removal. There is also recycle from the anoxic tank to the anaerobic tank in order to retain biosolids in the anaerobic tank.

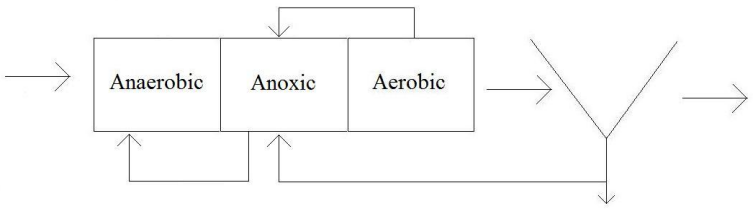


Figure 1.8. University of Cape Town (UCT) Reactor Configuration.

There is also a Modified University of Cape Town (MUCT) reactor configuration (Figure 1.9). This is identical to the UCT configuration except that an additional anoxic tank is present. This further ensures that no DO is recycled to the anaerobic tank.

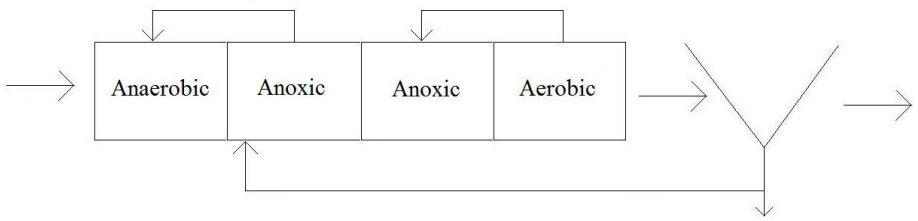


Figure 1.9. Modified University of Cape Town (MUCT) Reactor Configuration.

Another BNR reactor configuration is the 5-Stage Bardenpho (Figure 1.10). This configuration has five basins. The first is anaerobic to achieve P removal. The second is anoxic and the third is aerobic to achieve N removal. The fourth is anoxic to remove any residual NO_3^-, and the fifth is aerobic to strip out gases. Sludge is recycled from the clarifier to the first basin. Nitrate is recycled from the first aerobic basin to the first anoxic basin.

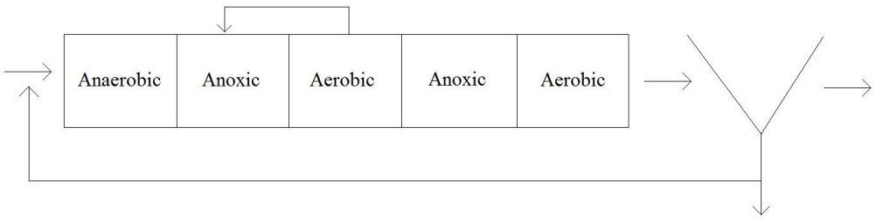


Figure 1.10. 5-Stage Bardenpho Reactor Configuration.

The Biodenipho Process is a set of reactor configurations that also achieves biological N and P removal (Figures 1.11 – 1.1.14). If has five different basins. The first is anaerobic to achieve P removal. There is then a splitter that can direct the flow of wastewater to one of two aeration basins. There is a fourth basin that is anoxic and a fifth basin that is aerobic. There are four different stages to this process. In stage I, the flow is directed to the left basin that is operated under anoxic conditions. The water flows from the anoxic basin to the third basin, which is operated under aerobic conditions. From there, the water flows to the final anoxic tank and then to the final aerobic tank before going to the clarifier. This stage is 90 minutes long. Stage II is identical to stage I except that both aeration basins are operated under aerobic conditions for 30 minutes. In stage III, the flow of water is directed from the anaerobic tank to the right-hand basin, which is operated under anoxic conditions. The water then flows to the third basin, which is operated under aerobic conditions. From there, the water again flows to the final anoxic basin and then to the final aerobic basin before going to the clarifier. This stage is 90 minutes. In the final stage of the process, stage IV, the conditions are the same as in stage III except that both basins are again operated under aerobic conditions for 30 minutes.

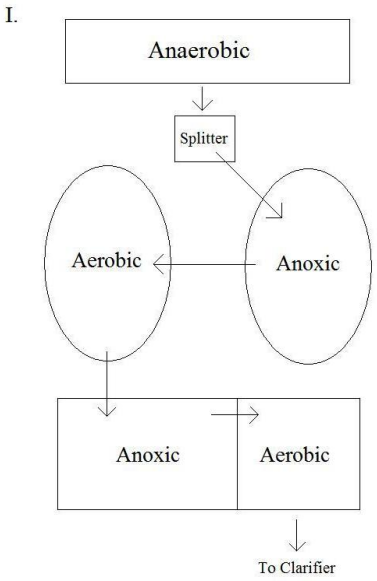


Figure 1.11. Stage I of the Biodenipho Process.

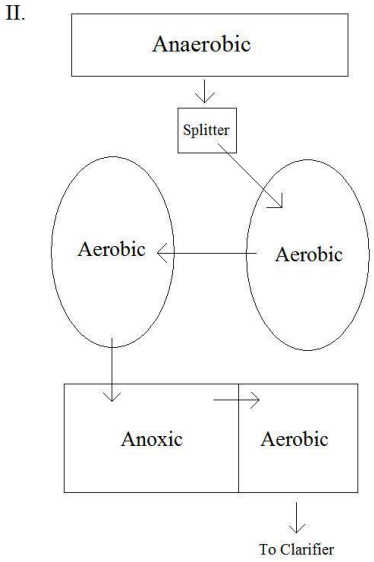


Figure 1.12. Stage II of the Biodenipho Process.

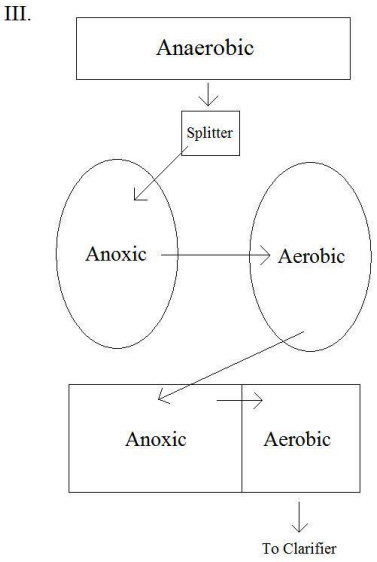


Figure 1.13. Stage III of the Biodenipho Process.

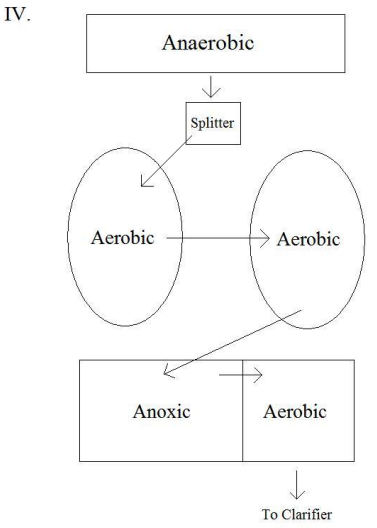


Figure 1.14. Stage IV of the Biodenipho Process.

1.12 Other Nitrogen Removal Processes

In addition to nitrification-denitrification, there are several other systems for removing N from wastewater. Two such systems are Anammox and the Oxygen-Limited Autotrophic Nitrification-Denitrification (OLAND) system.

The anammox process is the oxidation of NH_4^+ to N_2 with NO_2^- as the electron acceptor (Op den Camp et al., 2007). This means that the oxidation of NH_4^+ can take place without any O_2 present. *Brocadia anammoxidans* and *Kuenenia stuttgartiensis* are two bacteria that can carry out the anammox process (Op den Camp et al., 2007). By combining anammox with a preceding partial nitrification step, NH_4^+ can be converted to N_2 directly (Op den Camp et al., 2007). This can result in a 90% reduction of operational costs (Op den Camp et al., 2007). The reasons for this reduction in cost are that an electron donor no longer needs to be supplied for conventional denitrification and that only half of the aeration capacity required for nitrification is required (Op den Camp et al., 2007). The anammox process is particularly desirable for high strength industrial wastewaters that lack a carbon source (Op den Camp et al., 2007). The Sharon process (Figure 1.15) can be used to achieve anammox.

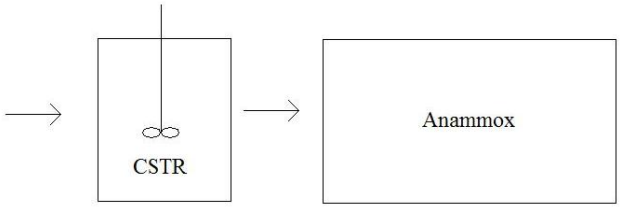


Figure 1.15. Sharon Process.

In the CSTR, a limited supply of O_2 is present so that half of the NH_4^+ is converted to NO_2^-. The retention time is generally around 1 day, and the temperature is between 30 and 40°C. AOB such as *Nitrosomonas* have a higher growth rate than NOB such as *Nitrobacter*, so at this temperature, the NOB are washed out while the AOB are retained. In the anammox reactor, there is a NH_4^+ and NO_2^- mixture and no O_2. Under these anoxic conditions, unique anammox bacteria such as *B. anammoxidans* and *K. stuttgartiensis* convert NH_4^+ and NO_2^- to N_2, using NO_2^- as the electron acceptor as described above. These are autotrophic bacteria, meaning their carbon source is CO_2. The autotrophic bacteria in this process are slow-growing, so they need a longer retention time.

The combination of partial nitrification and anammox in a single, aerated reactor is known as CANON (Completely Autotrophic Nitrogen-Removal Over Nitrite) (Third et al., 2002). In this system, two groups of bacteria perform two reactions simultaneously (Schmidt et al., 2003). Nitrifiers oxidize ammonia to nitrite, consuming oxygen as they do so, and create anoxic conditions that the anammox process needs (Schmidt et al., 2003). The CANON process is completely autotrophic, thereby avoiding the COD addition that is often required in traditional heterotrophic denitrification (Third et al., 2002). The entire nitrogen removal can be achieved in a single reactor with very low aeration, significantly reducing space and energy requirements (Third et al., 2002). The autotrophic system consumes 63% less oxygen and 100% less reducing agent than traditional nitrogen removal systems (Third et al., 2002).

Another alternative nitrogen removal system is known as the NO_x process (Schmidt et al., 2003). By adding NO_x (NO/NO_2), *Nitrosomonas*-like microorganisms nitrify and denitrify simultaneously even under fully oxic conditions with N_2 as the main product (Schmidt et al., 2003). NO_x is the regulatory signal inducing the denitrification activity of the ammonia oxidizers (Schmidt et al., 2003). About 40% of the ammonia load is converted to nitrite, and because nitrite is used as the terminal electron acceptor in the nitrification step, 50% less oxygen is consumed (Schmidt et al., 2003). In the subsequent denitrification step, less COD is consumed (Schmidt et al., 2003).

1.13 Quantification and Identification of Denitrifiers

Despite the importance of N removal, many questions about these transformations remain unanswered. Determining the identity and quantity of microorganisms responsible for these specific biotransformations in complex environments is a major challenge in environmental microbiology and environmental engineering. Approaches based on ribosomal RNA (rRNA) sequence analysis have revealed highly diverse microbial communities in biological wastewater treatement systems. However, rRNA does not provide much information about the metabolic activities of these microorganisms. Thus, the "structure-function" relationship between what organisms are present and what role they are actually playing in the wastewater treatment process has not been determined (de los Reyes III, 2010).

The bacteria that are responsible for carrying out nitrification, the AOB and NOB, come from a phylogenetically similar group of organisms. As described previously, the aerobic AOB that proliferate during the biological treatment of domestic wastewater are primarily of the β-*proteobacteria* and include the *Nitrosomonas* and *Nitrosospira* lineages (Purkhold et al., 2003). The NOB are a diverse group of organisms that contain the α-*proteobacteria*, including *Nitrobacter* and *Nitrospira* (Grady et al., 2011). In other words, these bacteria are close to one another on the evolutionary tree and therefore have common genes and some similarities in their rRNA. It has therefore been possible to study nitrifying bacteria using 16S rRNA-targeted probes. However, denitrification is a different story. Bacteria that are capable of denitrification are a highly diverse and heterogeneous group (Peralta et al., 2010). This means that it is not possible to use 16S rRNA gene sequences to identify denitrifiers in activated sludge; the bacteria are from many different physiological and taxonomic groups and therefore do not share a single denitrifying probe that is based on 16S rRNA. However, it is possible to focus on the gene that encodes for nitrous oxide reductase, *nosZ*, in order to detect microorganisms that are capable of performing complete denitrification (Throback et. al., 2004). Nitrous oxide reductase is the enzyme that catalyzes the final step of denitrification, the transformation of N_2O to N_2.

This gene can be present in the DNA and RNA of the bacteria. If the gene is present in the DNA, it means that the bacteria is capable of expressing the enzyme nitrous oxide reductase, meaning the bacteria is capable of performing the final step of denitrification. If

nosZ mRNA is detected, it means that the bacteria is actively expressing the enzyme, meaning the bacteria is actively performing the final step of denitrification (Figure 1.16).

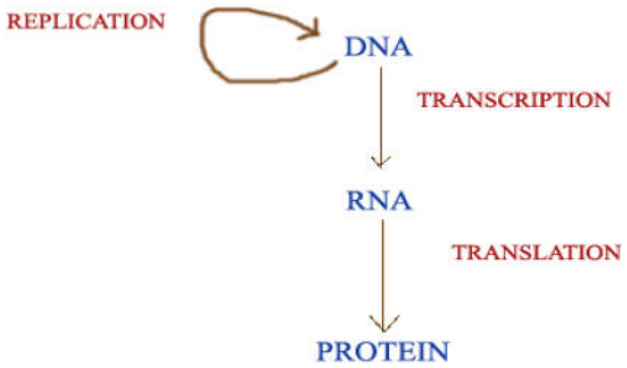


Figure 1.16. Process of protein synthesis.

DNA does not serve as the direct template for protein (enzyme) synthesis. Rather, messenger RNA (mRNA) is transcribed from the DNA. mRNA is the molecule that contains the information for the translation of the DNA into the enzyme. Therefore, the gene *nosZ* being present in the DNA of the bacteria is not evidence of whether or not the nitrous oxide reductase is actually being made by the bacteria. However, if *nosZ* mRNA is present, it means that nitrous oxide reductase is actually being formed in the bacteria.

A number of researchers have taken advantage of this information and have targeted the denitrification genes to quantify, identify, and in general characterize the denitrifying population of bacteria present in various natural and engineered environments. A variety of molecular techniques were used in their studies.

Numerous natural environments and effects of man-made modifications to these environments have been studied. Chon et al. (2009) investigated the abundance of denitrifying genes coding for nitrate (*narG*), nitrite (*nirS*), and nitrous oxide (*nosZ*) reductases in wastewater effluent-fed and estuarine wetlands using quantitative polymerase chain reaction (qPCR). They also looked at the effect of temperature on these abundances. Kong et al. (2010) used qPCR targeting ammonia monooxygenase (*amoA*), *nosZ*, and 16S rRNA genes to investigate the effects of long-term agricultural management practices on potential gross N mineralization and nitrification rates, as well as ammonia-oxidizing bacteria (AOB), denitrifier, and total bacterial community sizes within different soil microenvironments. Levy-Booth and Winder (2010) used qPCR to determine the abundance of *nifH*, *nirS*, and *nirK* genes, which are involved in N fixation and denitrification, in thinned second-growth Douglas-fir forest soil. Dell et al. (2010) used denaturing gradient gel electrophoresis (DGGE) and sequenced clone libraries targeting *nirK* and *nosZ* to study the response of soil-denitrifying bacteria in highly managed turfgrass systems to land-use change and time under management. Peralta et al. (2010) compared the relationship between soil and vegetation factors and microbial community structure and function in restored and reference wetlands. They used terminal restriction fragment length polymorphism (T-RFLP) targeting the 16S rRNA gene (total bacteria) and the *nosZ* gene (denitrifiers). Kennedy and Egger (2010) studied the effects of wildfire and logging on fungal and nitrogen-cycling communities in the rhizosphere of 16 month-old Douglas-fir seedlings as they regenerated in burned and logged soils. They used length-heterogeneity PCR (LH-PCR) of fungal nuclear

rRNA genes and T-RFLP analysis of *nifH* and *nosZ* genes. Zhu et al. (2006) investigated communities of AOB and denitrifying bacteria associated with the leachates from three municipal solid waste disposal sites by examining the diversity of the *amoA* and *nosZ* genes, respectively, using cloning and phylogenetic analysis.

A number of studies have also been made concerning wastewater treatment and the denitrification genes. Bai et al. (2011) investigated the denitrifier community in bioaugmented and general sequencing batch reactors during the treatment of coking wastewater containing pyridine and quinoline. The efficiency and stability of nitrate and nitrite reduction were significantly improved after inoculation with four pyridine- or quinoline-degrading bacterial strains (including three denitrifying strains). T-RFLP based on *nosZ* was used to determine the structure of the denitrifier communities in the bioaugmented and non-bioaugmented reactors. Ducey et al. (2011) used qPCR to quantify the abundance of *amoA*, *nirK*, *nirS*, and *nosZ* in eight different anaerobic swine wastewater lagoons and to see how various measured environmental conditions such as pH, oxidation-reduction potential (ORP), COD, conductivity, and concentrations of nitrogenous compounds affected these abundances. They also evaluated their abundance relative to denitrification enzyme activity. Wan et al. (2010) used DGGE and clone library analysis of the 16S rRNA, *amoA*, and *nosZ* genes to analyze the total, ammonia-oxidizing, and denitrifying bacterial communities monthly in a full-scale membrane bioreactor for over a year. The community fingerprints obtained were compared to those from a conventional activated sludge process running in parallel treating the same domestic wastewater. Geets et al. (2007) used qPCR targeting

nirS, *nirK*, and *nosZ* genes to quantify denitrifiers present in activated sludge from seven different wastewater treatment plants. This study also examined the effect of decreased pH, addition of NaOCl, and addition of allylthiourea, a nitrification inhibitor, on the number of quantified genes present in lab-scale reactors. Gómez-Villalba et al. (2006) used temperature-gradient gel electrophoresis (TGGE) targeting *amoA* and *nosZ* genes to analyze the spatial and temporal diversity of the ammonia-oxidizing and denitrifying bacteria, respectively, in submerged filter biofilms for the treatment of urban wastewater. Sakano et al. (2002) investigated two biological wastewater treatment reactors designed to recover potable water for a spacefaring crew using T-RFLP, sequence, and phylogenetic analyses targeting the 16S rRNA gene for the total bacterial community, the *amoA* gene for the AOB, and the *nosZ* gene for the denitrifying bacteria.

Another area of interest regarding the denitrification process is the effect of dissolved oxygen on the activity of denitrifying bacteria. As described previously, denitrification occurs under anoxic conditions, and the denitrification genes are regulated, in part, by dissolved oxygen concentrations. During most wastewater treatment systems, the wastewater is cycled through aerobic and anoxic conditions to allow for both nitrification and denitrification. Much interest therefore exists about how these transient conditions affect the denitrification process.

Noda et al. (2003) studied the effects of sludge retention time (SRT) and DO on N_2O emission in an anoxic-oxic activated sludge system. They also performed cloning and sequencing based on *nosZ* to better understand the microbial community structure in

the sludge. The results of this study showed that N_2O emission was enhanced under low DO conditions, where the available oxygen was insufficient for nitrification. Bergaust et al. (2010) conducted an investigation of the regulation of denitrification in the model organism *Paracoccus denitrificans* during transition to anoxia both at pH 7 and when challenged with pHs ranging from 6.5 to 7.5. The kinetics of gas transformations (O_2, NO, N_2O, and N_2) were monitored and gene transcription was quantified. *P. denitrificans* demonstrated balanced transitions from O_2 to NO-based respiration at an optimal pH of 7. Transcription of *nosZ* preceded that of *nirS* and *norB* by 5 to 7 hours. Reduction of N_2O was severely inhibited by suboptimal pH while the relative transcription rates of *nosZ* versus *nirS* and *norB* were unaffected by pH. Low pH had a moderate effect on the N_2O reductase activity in cells with a denitrification proteome assembled at pH 7. It was therefore concluded in this study that the inhibition occurred during protein synthesis/assembly rather than transcription. Lu and Chandran (2010) evaluated the impact of organic carbon limitation, nitrite concentrations, and DO concentrations on gaseous N_2O and NO emissions from two sequencing batch reactors operated, respectively, with methanol and ethanol as electron donors. During undisturbed ultimate-state operation, emissions of both N_2O and NO from both reactors were minimal. When the reactors were challenged with transient organic carbon limitation and nitrite pulses, there was little impact on N_2O or NO emissions. However, transient exposure to oxygen led to increased production of N_2O from ethanol grown cultures, owing to their higher kinetics and potentially lower susceptibility to oxygen inhibition. A similar increase in N_2O production was not observed from methanol grown cultures. These results suggest that for DO, but not for carbon limitation or nitrite

exposure, N_2O emission from heterotrophic denitrification reactors can vary as a function of the electron donor used. In addition to these studies, there have been two studies performed at Columbia University (Yu and Chandran, 2010; Yu et al., 2010). These two studies focused on a unique autotrophic AOB that performs denitrification, *Nitrosomonas europaea*, with the end products being NO and N_2O. Yu et al. determined that N_2O production was only observed during recovery to aerobic conditions after a period of anoxia and correlated positively with the degree of ammonia accumulation during anoxia. NO, however, was emitted mainly under anoxia. The production of NO was linked to a major imbalance in the expression of the nitrite reductase gene, which was over-expressed during transient anoxia. In contrast, both genes coding for ammonia and hydroxylamine oxidation and nitric oxide reduction were generally under-expressed during transient anoxia. Yu and Chandran studied the responses of *N. europaea* to DO limitation or sufficiency and exposure to high nitrite concentrations during batch growth. Transcription of genes coding for principal metabolic pathways including ammonia oxidation (*amoA*), hydroxylamine oxidation (*hao*), nitrite reduction (*nirK*), and nitric oxide reduction (*norB*) were quantitatively measured during batch growth at a range of DO concentrations and in the presence of externally added nitrite. They found that exponential phase mRNA concentrations of both *amoA* and *hao* increased with decreasing DO concentrations, suggesting a mechanism to metabolize ammonia and hydroxylamine more effectively under DO limitation. Batch growth in the presence of a high nitrite concentration resulted in elevated exponential phase *nirK* and *norB* mRNA concentrations, potentially to promote utilization of nitrite as an electron acceptor and to detoxify nitrite.

1.14 Description of Project

Although many studies have been conducted to quantify and identify denitrifying bacterial communities using denitrification genes under various conditions and in various natural and engineered environments, there has been no study to date that has quantified denitrification genes at a full-scale wastewater treatment plant as the wastewater is cycled through its aerobic and anoxic phases. This project aimed to do just that. Samples were taken from a full-scale wastewater treatment plant over an entire cycle of operation (676 samples over 6 hours, including both aerobic and anoxic phases). DO, pH, ORP, NO_2^-, and NO_3^- were measured over the same time period. DNA was extracted, and qPCR targeting the *nosZ* gene was performed to quantify microorganisms capable of denitrification and determine how this changes over the operational cycle. Next, RNA was extracted and reverse transcription polymerase chain reaction (RT-PCR) followed by qPCR targeting the *nosZ* gene was performed to quantify microorganisms actively denitrifying and to determine how this changes over the operational cycle. This information can give engineers and wastewater treatment plant operators a better understanding of how denitrification genes are turned on and off during cycling of aeration in the basins, which may lead to better operational control of denitrification. In addition, it will allow us to determine the effect of the absence or presence of oxygen on the activity of the organisms; in other words, we will be able to determine exactly when lower oxygen levels switch on specific denitrification genes at a full scale N-removal plant. T-RFLP was performed and clone libraries constructed based on the *nosZ* gene for selected samples to characterize and identify denitrifiers present.

This information gives us an idea of the number of species present that are carrying out denitrification and identifies what microorganisms are actually present and denitrifying.

1.15 Objectives

- Measure the DO concentration, pH, and ORP of wastewater at a full-scale wastewater treatment plant over an entire cycle of operation and determine if patterns or overall trends exist.
- Measure the NO_2^- and NO_3^- concentrations of wastewater at a full-scale wastewater treatment plant over an entire cycle of operation.
- Extract DNA from sludge samples taken from a full-scale wastewater treatment plant over an entire cycle of operation and perform qPCR targeting the denitrification gene nosZ to observe how the abundance of *nosZ* present in the DNA, and therefore the number of bacteria capable of denitrification, changes over the operational cycle.
- Extract RNA from sludge samples taken from a full-scale wastewater treatment plant over an entire cycle of operation and perform RT-PCR followed by qPCR targeting the denitrification gene *nosZ* to observe how the abundance of *nosZ* present in the RNA, and therefore the number of bacteria actively denitrifying, changes over the operational cycle.
- Perform T-RFLP based on *nosZ* on selected samples to characterize the microbial community of denitrifiers.

CHAPTER 2: METHODOLOGY

2.1 Growth and Cultivation of *Pseudomonas stutzeri*

As described previously, *P. stutzeri* is a well-studied denitrifier. It contains all of the genes that code for the enzymes necessary to convert NO_3^- to N_2. It is a Gram-negative bacterium that belongs to the gamma-group of organisms (van Spanning et al., 2007). *P. stutzeri* was selected to be used as a standard and positive control. Three flasks, each containing 50 mL of Difco Nutrient Broth (containing beef extract and peptone) that was autoclaved at 121°C for 15 minutes, were inoculated with 1 mL of an actively growing culture of *P. sutzeri*. It was incubated at 35°C on a shaker bed for 19 hours. Nine centrifuge tubes, three for each flask, were filled with 1 mL of the cell broth and centrifuged for 5 minutes at 13,700 rpm. The liquid was decanted and another 1 mL of cell broth was added to each centrifuge tube and the process was repeated. Once a sizable pellet was harvested, approximately 0.25 g, the cells were placed in a -20°C freezer for further analysis.

2.2 North Cary Water Reclamation Facility

The North Cary Water Reclamation Facility, located at 1900 Old Reedy Creek Road in Cary, NC, uses a Bio-Denipho process, which was described previously. Briefly, this system comprises an anaerobic tank, two identical activated sludge tanks, and a settling tank. The activated sludge tanks are interconnected and have continuous feed and discharge of wastewater. There are four phases of operation. The phases for the North Cary Water

Reclamation Facility are slightly different than the phases described previously for a general Bio-Denipho process and are as follows. During phase B, the untreated water flows into the first anoxic tank, from which the nitrates accumulated during the previous phase are removed. The mixed liquor then passes into the second tank, which is aerobic, to allow nitrification and removal or organics. Phase B is 90 minutes. During phase E, the wastewater enters into the second tank and flows out of that tank into the third tank. There is no flow into the first tank. Basins 1 and 2 are operated under aerobic conditions for 30 minutes. During phase G, the water flows to the second tank, which is operated under anoxic conditions. The water flows from the second tank into the first tank, which is aerobic. Phase G is 90 minutes. During phase J, the water enters into the first tank and flows out of that tank into the third tank. There is no flow into the second tank. Basins 1 and 2 are operated under aerobic conditions for 30 minutes. These phases are shown in Figures 2.1 to 2.4. The wastewater and return sludge are fed into the first anaerobic tank, and as the biomass is exposed to alternating anaerobic and oxic conditions, phosphorus accumulating organisms take up phosphorus which is then removed with the excess sludge.

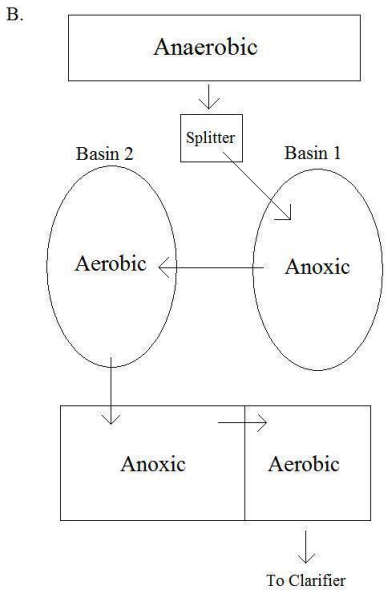


Figure 2.1. Phase B of North Cary facility.

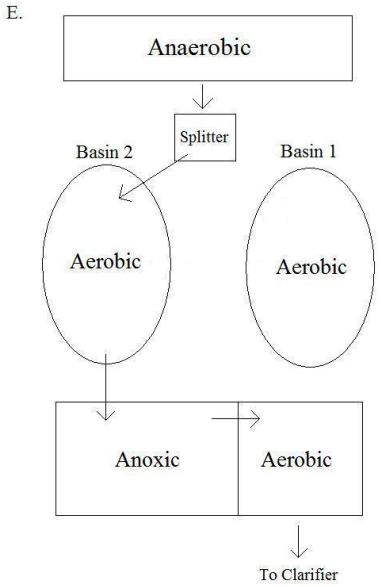


Figure 2.2. Phase E of North Cary facility.

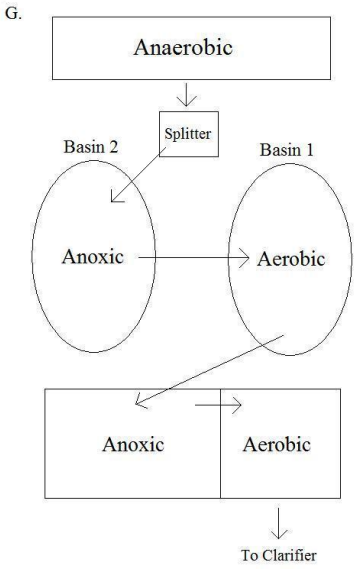


Figure 2.3. Phase G of North Cary facility.

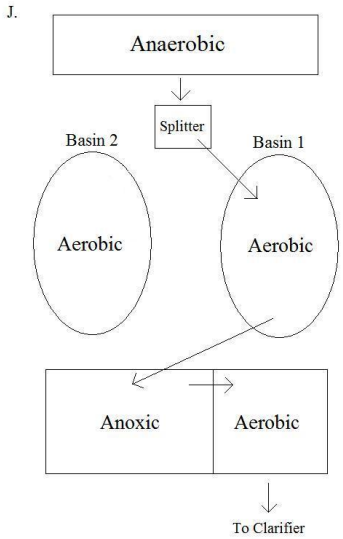


Figure 2.4. Phase J of North Cary facility.

2.3 Sampling and Measurements

Samples and measurements were taken at the North Cary Water Reclamation Facility on May 20, 2011. The sampling scheme for Basins 1 and 2 is given in Figures 2.5 and 2.6, respectively. Samples were taken every 5 to 10 minutes, with a larger number of samples being taken during the transition zones.

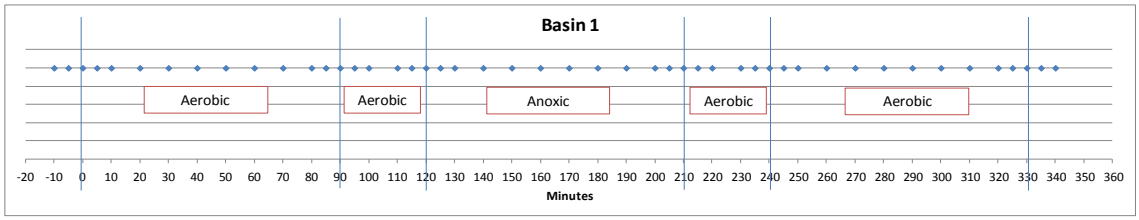


Figure 2.5. Sampling scheme for Basin 1.

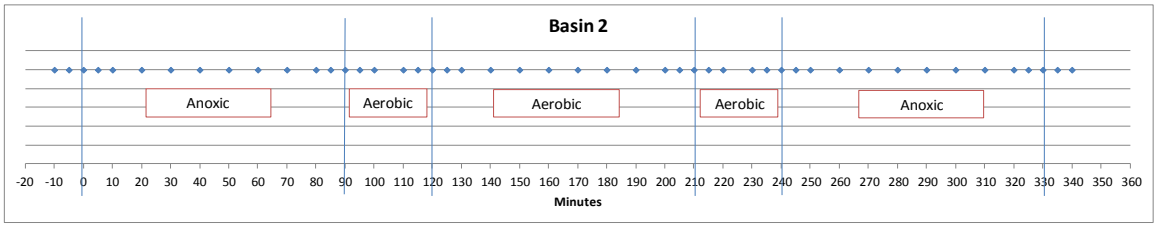


Figure 2.6. Sampling scheme for Basin 2.

Samples of the mixed liquor (15 mL) were taken from basins 1 and 2 at the time intervals shown above. Six samples were taken at each time interval, resulting in six replicates of each sample. The samples were centrifuged on site using an Eppendorf Centrifuge 5810. The sludge was left in the original 15 mL tube while the liquid water sample was poured into a

new 15 mL centrifuge tube. The samples were placed on ice to be transported to the laboratory facility and once there, were placed into a -20°C freezer for further analysis. Dissolved oxygen (DO) concentration, pH, and oxidation-reduction potential (ORP) were measured approximately every 5 minutes in Basins 1 and 2. The DO concentration was measured using a YSI Model 55-12 FT DO probe. The pH and ORP were measured using a HANNA Instruments HI 8424 probe.

2.4 Nitrate and Nitrite Measurements

The NO_3^- and NO_2^- concentrations in the liquid wastewater samples were analyzed using a Dionex ICS-2500 ion chromatograph (IC) with an IonPac AS19 column. The anions were separated using a 20 to 55 mM gradient of hydroxide. The flow rate was 1.1 mL/minute. A 5 µL sample volume was used. All samples analyzed using the IC were first filtered using a 0.45 µm syringe filter.

2.5 DNA Extraction

Total DNA was extracted from selected sludge samples. Two different DNA extraction methods were first tested to see which yielded higher DNA concentrations and better purities. One method was an aluminum sulfate based DNA extraction method developed in our lab (Hicks et. al., in preparation). The other was the MOBIO Laboratories PowerSoil DNA Isolation Kit. The materials and methods for the two DNA extraction procedures are given in Appendix A. The bead beater used was the Biospec mini bead

beater. The vortex used was the Fisher Scientific vortex genie 2. The pH strips used were the Whatman pH indicatior paper Type CF. The zirconium beads used were BioSpec Products 0.1 mm Zirconia/Silica Beads. The aluminum sulfate method was altered slightly due to high protein content in the sludge samples. Optimization of the method was performed to achieve higher purities. This will be discussed further in the Results and Discussion section (Chapter 3).

2.6 qPCR

In order to explain the process of qPCR, a description of PCR is first needed.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a molecular method for the rapid amplification of DNA (Madigan and Martinko, 2006). Amplification of DNA is often necessary when there is not enough DNA present in the original sample to perform techniques such as cloning genes or for sequencing (Madigan and Martinko, 2006). PCR is a very sensitive and specific technique; with correct primers and annealing temperatures, almost no false priming occurs (Madigan and Martinko, 2006). The first step of PCR is the denaturation of the DNA, which occurs at a temperature of around 92°C. This separates the two strands of DNA. Next is the annealing step. This is the step where the primers, which target a specific gene, are attached to the DNA. A primer is a short sequence of DNA, usually around 15 nucleotides, which is complementary to the strand of DNA of interest. This occurs at a temperature of

approximately 55°C. Extension is the final step. DNA polymerase extends the primers using the target strands as templates at a temperature of 72°C. The polymerase that is used is known as *Taq* polymerase because it comes from the microorganism *Thermus aquaticus*. This is a thermophilic organism that was originally isolated from hot springs, meaning that its polymerase is stable up to 95°C. This is important because the denaturation step takes place at 92°C, and if the polymerase enzyme is not able to withstand those high temperatures, it too will be denatured. Once these three steps are completed, the cycle repeats, this time with the products of the first cycle also able to serve as template strands of DNA that can be replicated. Each cycle doubles the amount of the original target DNA. Machines called thermocyclers have been created in which to perform PCR; these machines are capable of heating and cooling the samples to the desired temperatures for each cycle. Generally the limit of cycles that can be run is about 30. Once this number of cycles is reached, the *Taq* polymerase is often no longer active or there are no more nucleotides left to use for extension. A figure depicting the process of PCR is given in Figure 2.7 (Rice, 2012).

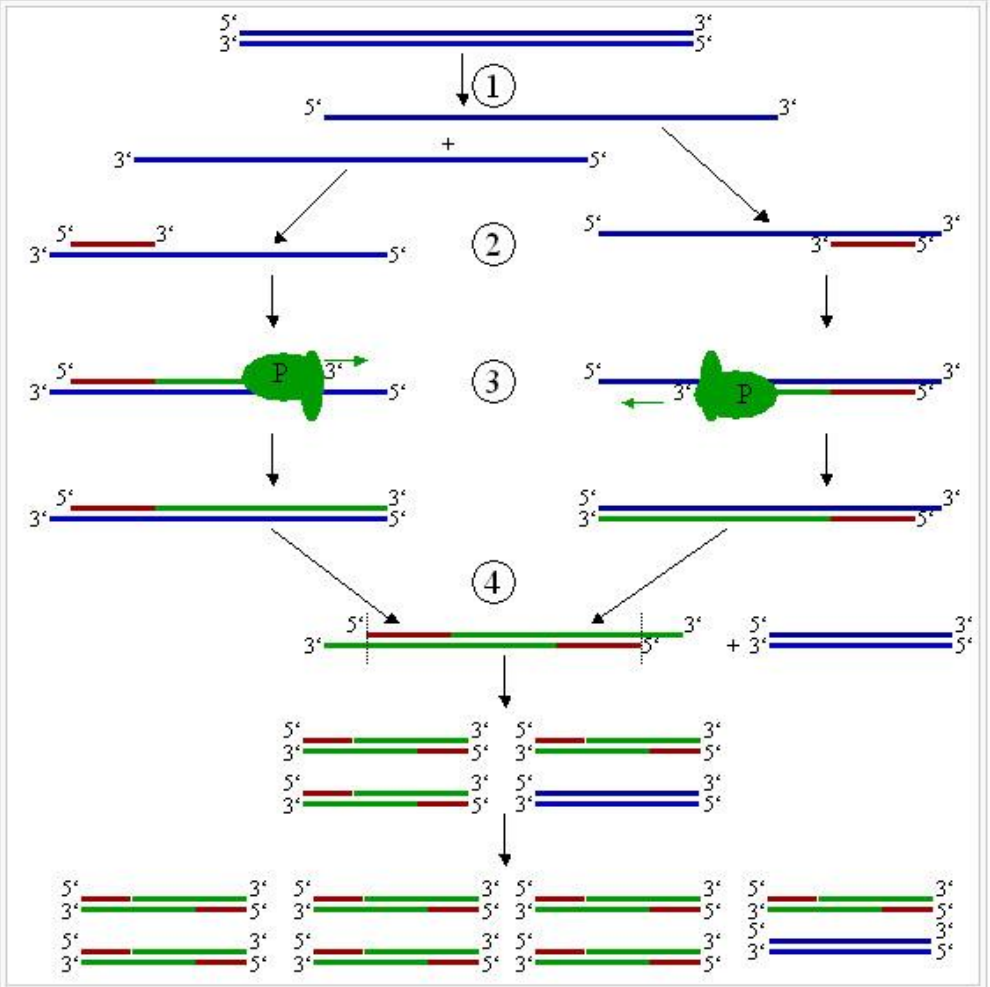


Figure 2: Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at (eg) 68°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle.

Figure 2.7. PCR (Rice, 2012).

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) is a molecular technique used to quantify the amount of DNA present in a sample. PCR is performed on samples of known DNA

concentration. When a higher concentration of DNA is used to begin with, the DNA amplifies at a faster rate, meaning that the point at which rapid exponential increase starts is at a lower cycle number. This point is referred to as the threshold cycle. When a lower concentration of DNA is used to begin with, the threshold cycle is higher because it takes a longer time for the concentration of DNA to begin rapidly increasing. The concentration of DNA is determined using an intercalating dye, a dye that binds to the groove in DNA. The concentration of DNA can be found by observing the intensity of the fluorescence. Once this has been performed on multiple samples of known DNA concentration, a standard curve of threshold cycle versus DNA concentration can be created. A linear trendline can be fit to the data, and the equation of this trendline can then be used to determine an unknown DNA concentration based on the known threshold cycle number. A figure depicting a qPCR curve is given in Figure 2.8 (Langford Veterinary Services, 2009).

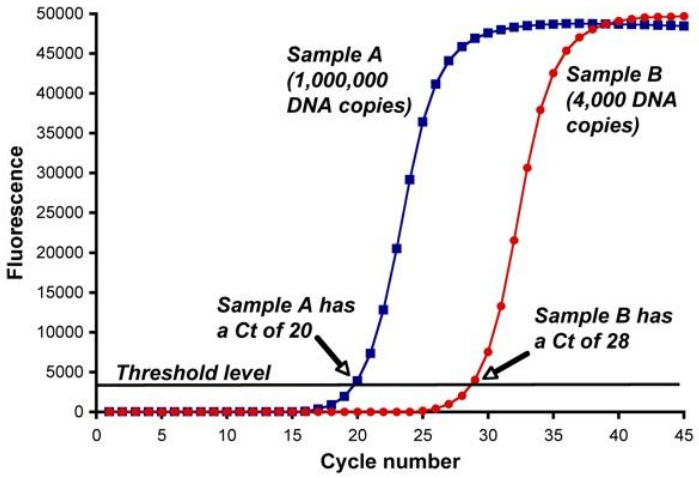


Figure 2.8. qPCR curve (Langford Veterinary Services, 2009).

2.7 Selection of qPCR primers

Primers were selected to perform qPCR targeting the *nosZ* gene. The forward primer chosen was nosZF [5'-CG(C/T) TGT TC(A/C) TCG ACA GCC AG-3'] and the reverse primer chosen was nosZ1622R [5'-CGC (G/A)A(C/G) GGC AA(G/C) AAG GT(G/C) CG-3']. These primers were developed by Throbäck et al (2004) and used for qPCR targeting the *nosZ* gene in several additional studies (Ruyters et al., 2010; Ducey et al., 2011; Geets et al., 2007) with successful results.

2.8 Standard Curve Creation

As described in section 2.1, *Pseudomonas stutzeri* was grown in batch culture, and the cells from three separate reactors were harvested and frozen. Triplicate samples were taken from each of the three reactors. The DNA was extracted from all samples using the Laboratory DNA Extraction Method (section 2.5). The triplicates were then combined, resulting in 1 solution of DNA extract per reactor. The DNA concentration was measured using a NanoDrop Spectrophotometer ND-1000. The DNA concentration of each was then brought to a common concentration of 500 ng/µL. A dilution series containing 0 to -9 dilutions of each of the three DNA extracts was created.

The amount of cells per mL of sample was determined from the DNA concentration. First, the amount of DNA (fg) per cell was determined using the following equation:

$$DNA\ (fg) = (genome\ size\ [bp]) * \left(\frac{600\ Da}{bp}\right) * \left(\frac{1.6*10^{-27}\ kg}{Da}\right) * \left(\frac{10^{18}\ fg}{kg}\right)$$ (Altschul et al., 1990).

The genome size of *P. stutzeri* is 4.6 Mb, or $4.6*10^6$ bp (Ginard et al., 2007). Using the above equation and the known genome size of $4.6*10^6$ bp, it was determined that each cell of *P. stutzeri* contains 4.416 fg of DNA. The number of cells per mL of sample was then determined using the following equation:

$$\frac{cells}{mL} = \left(DNA\ concentration\ \left[\frac{ng}{\mu L}\right]\right) * \left(\frac{100\ \mu L}{extract}\right) * \left(\frac{extract}{15\ mL}\right) * \left(\frac{cell}{4.416\ fg}\right) * \left(\frac{10^6\ fg}{ng}\right)$$

From this, the number of cells per mL of sample for each DNA concentration was determined.

Copy numbers of *nosZ* gene fragments were quantified in triplicate per DNA concentration per extract on a Bio-Rad iQ5 icycler Multicolor Real-Time PCR Detection System. Primers nosZF and nosZ1622R were used (section 2.7). qPCR reactions were performed in a total volume of 25 µL, adding 5 µL DNA as template, 12.5 µL SYBR Green (Bio-Rad iQ SYBR Green Supermix), 7 µL sterile water, and 0.25 µL of both the forward and reverse primers (at a concentration of 0.1 nm/µL). Thermal cycling conditions were as follows: 15 minutes at 95°C; 45 cycles of 15 seconds at 95°C, 15 seconds at 60°C, and 30 seconds at 72°C; 5 minutes at 72°C (Ruyters et al., 2010). Using this procedure, CT values for the samples containing known concentrations of *P. stutzeri* DNA were obtained.

A standard curve of CT value versus log cell count (cells/mL) was then created. This curve was subsequently used to determine the number of cells containing the *nosZ* gene in a mL of activated sludge using the CT value obtained from performing qPCR.

2.9 Quantification of cells containing *nosZ* in activated sludge samples

As described in section 2.3, samples of activated sludge from the North Cary Water Reclamation Facility were collected and frozen. DNA was extracted from selected samples using the Laboratory DNA Extraction Method (section 2.5). The samples selected for DNA extraction from Basin 1 were samples 2, 5, 7, 8, 9, 10, 11, 13, 15, 18, 19, 20, 21, 22, 23, 24, 26, 29, 32, 36, 38, 39, 40, 41, 43, 45, and 47 (three of the six replicates of each sample were analyzed). The samples selected for DNA extraction from Basin 2 were samples 2, 6, 8, 11, 15, 19, 21, 22, 23, 24, 26, 27, 29, 32, 33, 34, 35, 36, 37, 39, 41, 44, 45, 46, 47, and 48 (three of the six replicates of each sample were analyzed). qPCR targeting the *nosZ* gene was then performed on the samples using the same protocol as used for the creation of the standard curve (section 2.8). A positive control of *P. stutzeri* DNA and a negative control of sterile water were included on each qPCR plate. The CT values obtained, along with the standard curve, were then used to determine the number of cells per mL of activated sludge that contained the *nosZ* gene for each sample.

2.10 RNA Extraction

RNA was extracted from selected samples. Two different methods were tested to see which yielded higher RNA concentrations and purities. The first method tested was the RNA Extraction Protocol (Stahl et al., 1988). The materials and method for these procedures are given in Appendix B. The bead beater used was the Biospec mini bead beater. The vortex used was the Fisher Scientific vortex genie 2. The zirconium beads used were BioSpec Products 0.1 mm Zirconia/Silica Beads.

2.11 DNAse Treatment

The RNA extracts were treated with DNAse to remove any carryover DNA. DNaseI from Qiagen was used. The procedure is given in Appendix C.

2.12 RNA Clean-up

Following DNase treatment, impurities were removed from the RNA using the Qiagen RNeasy MinElute Cleanup Kit. The materials and method are given in Appendix D.

2.13 cDNA Synthesis

Following clean-up of the RNA, complementary DNA (cDNA) was synthesized using the RNA as template. The Bio-Rad iScript cDNA Synthesis Kit was used. The materials and method are given in Appendix E.

2.14 cDNA Purification

Following the synthesis of the cDNA from the RNA, the cDNA was purified to remove any impurities. This was achieved using the DNAclear Kit from Applied Biosystems. The materials and method for this procedure are given in Appendix F.

2.15 PCR Targeting 16S rRNA Gene

PCR targeting the 16S rRNA gene was performed on selected cDNA samples as a control. The primers used were BAC-8F [5'-AGA GTT TGA TCC TGG CTC AG-3'] and BAC-1492R [5'-GGT TAC CTT GTT ACG ACT-3'] (Lane, 1991) from Integrated DNA Technologies. The total reaction volume was 15.5 µL, including 7.5 µL of FailSafe PCR 2X PreMix D from Epicentre, 0.3 µL of both the forward and reverse primers (at a concentration of 0.1 nm/µL), 0.18 µL of FailSafe PCR Enzyme Mix, the volume of DNA template required to add 1, 5, and 10 ng of DNA, and the volume of sterile, nuclease-free water required to bring the final reaction volume to 15.5 µL. A Bio-Rad icycler was used to carry out the reactions. Thermal cycling conditions were as follows: 5 minutes at 94°C; 34 cycles of 60 seconds at 95°C, 60 seconds at 56°C, and 90 seconds at 72°C; 10 minutes at 72°C.

2.16 Gel Electrophoresis

Gel Electrophoresis is used to separate DNA fragments based on their size. It is often performed on PCR products to ensure that DNA fragments of the expected size are present and have therefore been successfully amplified using PCR. Electrophoresis uses an electric

current to separate different-sized molecules in a porous, sponge-like matrix. Smaller molecules move more easily through the gel pores than larger molecules. An agarose gel is often used. The gel is submerged in a salt solution, called a running buffer, that conducts electricity. DNA samples are loaded into slots made in the agarose gel. The DNA samples are colorless, but a blue tracking dye is usually added to the samples in order to visually track the DNA migration through the gel. The phosphate groups in the DNA backbone carry negatively-charged oxygens, giving the DNA molecule an overall negative charge. In an electric current, the negatively-charged DNA moves toward the positive pole of the electrophoresis chamber. The DNA molecules move through the gel by snaking through the pores of the agarose matrix. Smaller DNA fragments migrate faster and further over a given time period than do larger fragments. This is how DNA fragments can be separated by size in an agarose gel.

The electrophoresis chamber used was the Classic CSSU911 from E-C Apparatus Corporation. An E-C 1000-90 from E-C Apparatus Corporation was used to apply the current. The running buffer used contained 1X TAE buffer (consisting of EDTA, tris, acetic acid, and dH_2O) and ethidium bromide (EtBr). EtBr is added to stain the DNA so that the bands of DNA can be visualized under UV light. A low DNA mass ladder from Invitrogen was used to supply fragments of known sizes as a standard. The loading dye used was the Qiagen GelPilot Loading Dye, 5X. The gel was made using agarose from Fisher-Scientific (0.7 g) and 1X TAE buffer (70 mL). The agarose was dissolved by heating the solution in a microwave. The solution was then cooled before pouring it into a mold to create the gel.

The gel was allowed to cool and solidify before use. The bands were visualized using the Bio-Rad GelDocXR and Bio-Rad Quantity One 1-D Analysis Software.

2.17 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP targeting the *nosZ* gene was performed on selected samples. T-RFLP is a type of DNA fingerprinting that can be used to examine microbial community structure and community dynamics in response to changes in different environmental parameters or to study bacterial populations in natural habitats. The technique is based on the fact that the *nosZ* gene varies in different organisms. Although the organisms all contain the *nosZ* gene, the exact sequence differs in different organisms. A general description of the technique is as follows. Total DNA is first extracted from the microbial community and the *nosZ* gene is amplified from samples using fluorescently-labeled forward primers. Next, the PCR product is purified and subjected to restriction enzyme digestion with enzymes that have 4 base pair recognition sites. This step generates fluorescently-labeled terminal restriction fragments. The size (length) of one terminal restriction fragment represents one organism. The digested products are then separated and detected on an appropriate electrophoresis platform. For a given sample the terminal fragments will contain a fluorescent label at the 5' end and will therefore be detected. The output will be a series of peaks (fragments) of various sizes and heights that represents the profile of that sample. A figure showing the T-RFLP method is given in Figure 2.9.

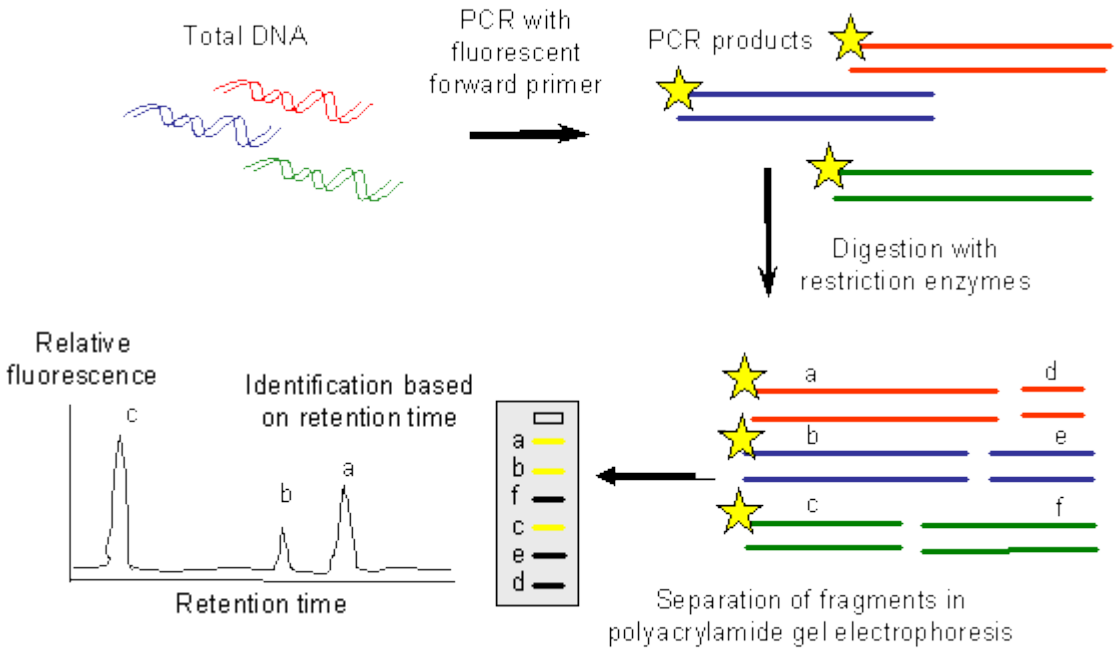


Figure 2.9. T-RFLP (Kaksonen).

2.18 Selection of Primers for T-RFLP

Primers were selected to target the *nosZ* gene during PCR for T-RFLP analysis. Five peer-reviewed research papers in which the researchers performed T-RFLP targeting *nosZ* were examined to determine which set of primers used by these researchers would be the most appropriate. The papers are summarized in Table 2.1.

Table 2.1. Primers used for T-RFLP analysis targeting *nosZ*.

	Primers Used	Number of T-RFs Achieved	Sample Source
Bai et al., 2011	nosZF; nosZR	24	Activated sludge
Kennedy and Egger, 2010	nosZF; nos1773R	43	Forest soils
Zhu et al., 2006	nosZ661F; nosZ1773R	8 to 34	Leachate from MSW landfills
Sakano et al., 2002	nos661F; nos1773R	19 to 57	Biofilm from fixed-film bioreactors
Peralta et al., 2010	nosZF; nosZ1880	47 to 72	Wetland soils

All primer sets used achieved PCR amplification and resulted in an adequate number of terminal restriction fragments (T-RFs). However, Sakano et al. (2002) stated that they were only able to process 10 *nosZ* clones due to poor PCR amplification with the primer set 661F/1772R. Because of this statement, the primer set of nos661F/nos1773R was not chosen. Of the three remaining primer sets used, only one set was used to analyze samples from activated sludge. Because the samples for the current study being conducted were activated sludge samples, this primer set (nosZF/nosZR) was chosen.

2.19 T-RFLP Protocol

The T-RFLP protocol followed was developed by Bryan Staley. It is given in Appendix G.

2.20. PCR Optimization for T-RFLP

For the PCR reaction optimization, the same protocol was used as described in section 2.15. PCR was performed on selected samples in duplicate. The primers used were nosZF [5'- CGY TGT TCM TCG ACA GCC AG-3'] and nosZR [5'-CAT GTG CAG NGC RTG GCA GAA-3'] (section 2.18). DNA template masses of 0.5 ng, 1 ng, 5 ng, and 10 ng were tested. Thermal cycling conditions were as follows: 1 cycle of 96°C for 20 seconds, 65°C for 30 seconds, 72°C for 30 seconds; 2 cycles of 96°C for 20 seconds, 62°C for 30 seconds, 72°C for 35 seconds; 3 cycles of 96°C for 20 seconds, 59°C for 30 seconds, 72°C for 30 seconds; 4 cycles of 96°C for 20 seconds, 56°C for 30 seconds, 72°C for 45 seconds; 5 cycles of 96°C for 20 seconds, 53°C for 30 seconds, 72°C for 50 seconds; 25 cycles of 94°C for 20 seconds, 50°C for 45 seconds, 72°C for 60 seconds; 1 cycle of 72°C for 10 minutes (Rösch et al., 2002). Final PCR reactions were carried out in 50.5 µL reaction volumes. PCR products were visualized using agarose gel electrophoresis as described in section 2.16.

2.21 PCR Product Purification and Quantification

Final PCR products were purified using the Promega Wizard SV Gel and PCR Clean-Up System. The materials and method that were used are given in Appendix H. The purified PCR products were quantified using the NanoDrop Spectotrophotometer ND-1000.

2.22 Digestion of Purified PCR Products

The purified PCR products were digested using three restriction enzymes, HhaI, AluI, and MspI (New England BioLabs). The digestion of the samples with HhaI was carried out in reaction volumes of 20 µL, each containing 0.5 µL enzyme, 2.00 µL NEBuffer4 (New England BioLabs), 0.2 µL bovine serum albumin (BSA) (New England BioLabs), the volume of DNA template required to add 360 ng DNA, and the volume of nuclease-free water required to bring the final reaction volume to 20 µL. The digestions of the samples with AluI and MspI were carried out in reaction volumes of 20 µL, each containing 0.5 µL enzyme, 2.00 µL NEBuffer2 (New England BioLabs), the volume of DNA template required to add 360 ng DNA, and the volume of nuclease-free water required to bring the final reaction volume to 20 µL. The digestion reactions took place in the Bio-Rad icycler at 37°C for 4 hours. The enzymes were then inactivated at 65°C for 20 minutes.

2.23 Purification of Digested PCR Products

The digested PCR products were purified using the Qiagen QIAquick Nucleotide Removal Kit. The materials and method are given in Appendix I.

2.24 Genotyping of Digested, Purified PCR Products

The digested, purified PCR products were placed into a 96-well plate. Each well that contained samples had 4 µL of digested, purified PCR products, 0.5 µL of the standard MapMarker 1000 X-Rhodamine (BioVentures, Inc.), and 5.5 µL of Hi-Di Formamide

(Applied Biosystems). The empty cells were filled with 10 µL of sterile, nuclease-free water. The plate was placed in the Bio-Rad icycler at 95°C for 5 minutes to denature the PCR products. The plate was then submitted to the Genomic Sciences Laboratory at North Carolina State University for genotyping. The analysis was carried out using capillary electrophoresis using the Applied Biosystems 3730xl DNA Analyzer.

2.25 T-RFLP Data Analysis

Analysis of the T-RFLP data was performed using the Applied Biosystems PeakScanner software version 1.0, the R Foundation for Statistical Computing RGui Software version 2.8.1, the Pisces Conservation Community Analysis Package 4 software version 4.1.3, the Pisces Conservation Species Diversity and Richness software version 4.1.2, and the Microbial Community Analysis III (MiCA 3) website from the University of Idaho.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Standard Curve

A standard curve of CT value versus log cell count was created as described in section 2.8. Briefly, DNA was extracted from *Pseudomonas stutzeri*, a known denitrifier. The number of cells was calculated from the DNA concentration using the following equation:

$$\frac{cells}{mL} = \left(DNA\ concentration\ \left[\frac{ng}{\mu L}\right]\right) * \left(\frac{100\ \mu L}{extract}\right) * \left(\frac{extract}{15\ mL}\right) * \left(\frac{cell}{4.416\ fg}\right) * \left(\frac{10^6\ fg}{ng}\right)$$

qPCR targeting the *nosZ* gene was performed on a dilution series (0 to -9 ten-fold dilutions) of the DNA extract, and CT values were obtained. The standard curve is given in Figure 3.1.

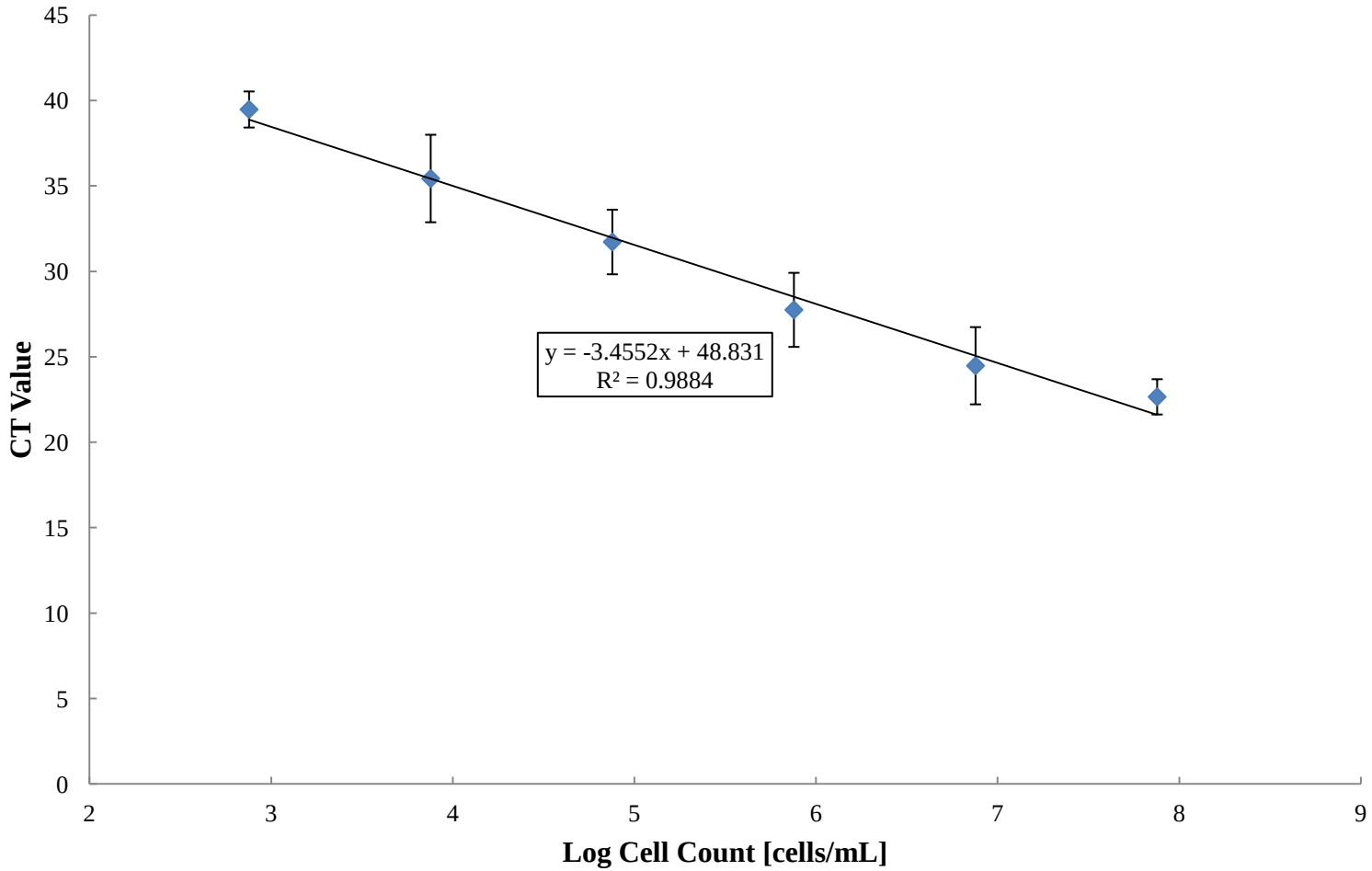


Figure 3.1. qPCR standard curve for *nosZ*.

The undiluted sample (0 dilution) did not yield a CT value, most likely due to a high concentration of inhibitors that did not allow DNA amplification. The -7, -8, and -9 ten-fold dilutions yielded unreliable CT values of 40 or higher and were therefore not used in the standard curve creation. The standard curve therefore was created using the -1 to -6 ten-fold dilutions. A linear trendline was fit to the data, yielding an equation relating the cell count to the CT value. An R^2 value of 0.99 for this trendline indicates that there is a high correlation between the cell count and CT value and that this trendline can therefore be used to accurately calculate the cell count of a sample given that the CT value is known.

3.2 Description of North Cary Water Reclamation Facility

The North Cary Water Reclamation Facility was described in section 2.2. The figures depicting the phases of plant operation are reprinted here in Figures 3.2 through 3.5. In addition, a description of the phases is given in Table 3.1.

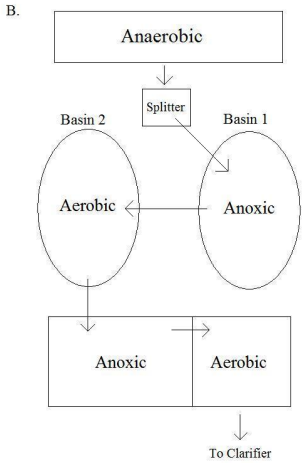


Figure 3.2. Phase B of North Cary facility.

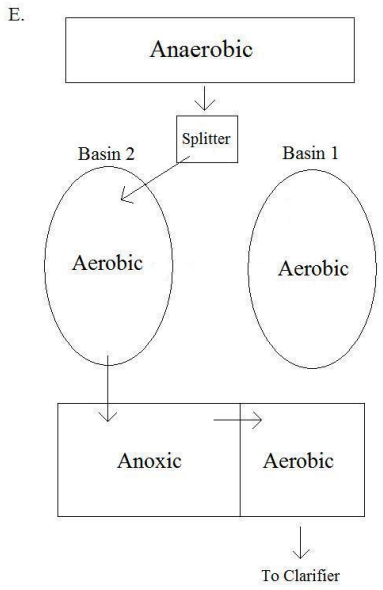


Figure 3.3. Phase E of North Cary facility.

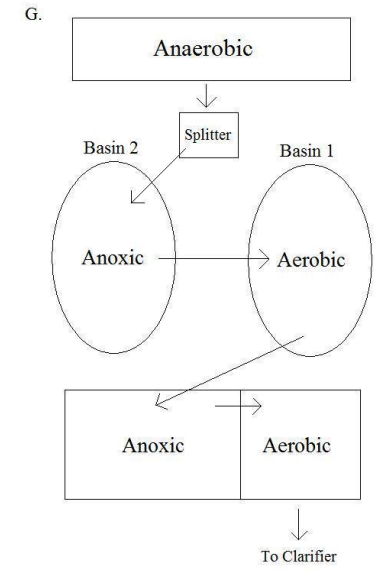


Figure 3.4. Phase G of North Cary facility.

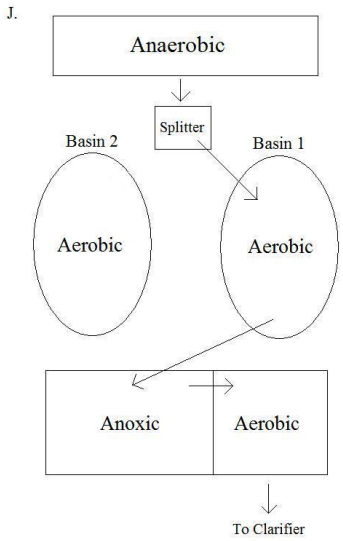


Figure 3.5. Phase J of North Cary facility.

Table 3.1. Description of North Cary Water Reclamation Facility phases.

Description	Phase	Basin 1	Basin 2
Flow into Basin 2; then flows from Basin 2 into Basin 1.	G	Air on	Air off
Flow into Basin 1; then flows out of Basin 1. No flow into Basin 2.	J	Air on	Air on
Flow into Basin 1; then flows from Basin 1 into Basin 2.	B	Air off	Air on
Flow into Basin 2; then flows out of Basin 2. No flow into Basin 1.	E	Air on	Air on

3.3 Dissolved Oxygen (DO) Concentrations

The DO concentrations were measured in Basins 1 and 2 approximately every 5 minutes for 6 hours (Figure 3.6).

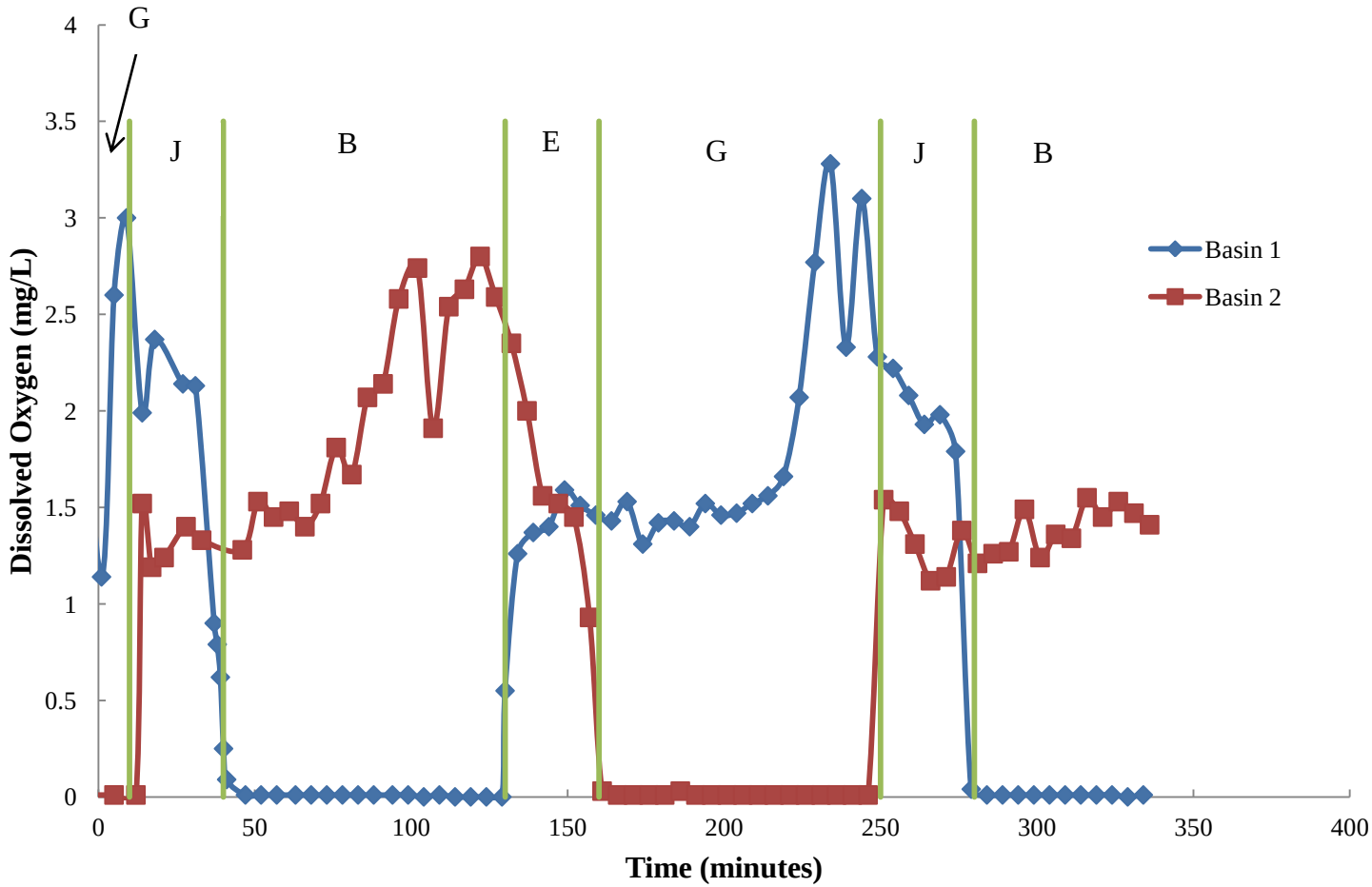


Figure 3.6. DO concentrations over time in Basins 1 and 2.

The green vertical lines represent the transitions from one phase to another. Time "0" on the above graph represents the point at which the plant operation was 80 minutes into phase G. In other words, the first 10 minutes on the graph is the final 10 minutes of phase G. There was then a transition into phase J, which lasted 30 minutes, followed by a transition into phase B, which lasted 90 minutes, followed by a transition into phase E, which lasted 30 minutes. The phases then started over, transitioning back into phase G. The DO concentrations correlate very closely to the expected aerobic and anoxic phases for both basins.

3.4 pH Measurements

The pH was also measured over time in basins 1 and 2 approximately every 5 minutes for 6 hours. The results are shown in Figure 3.7.

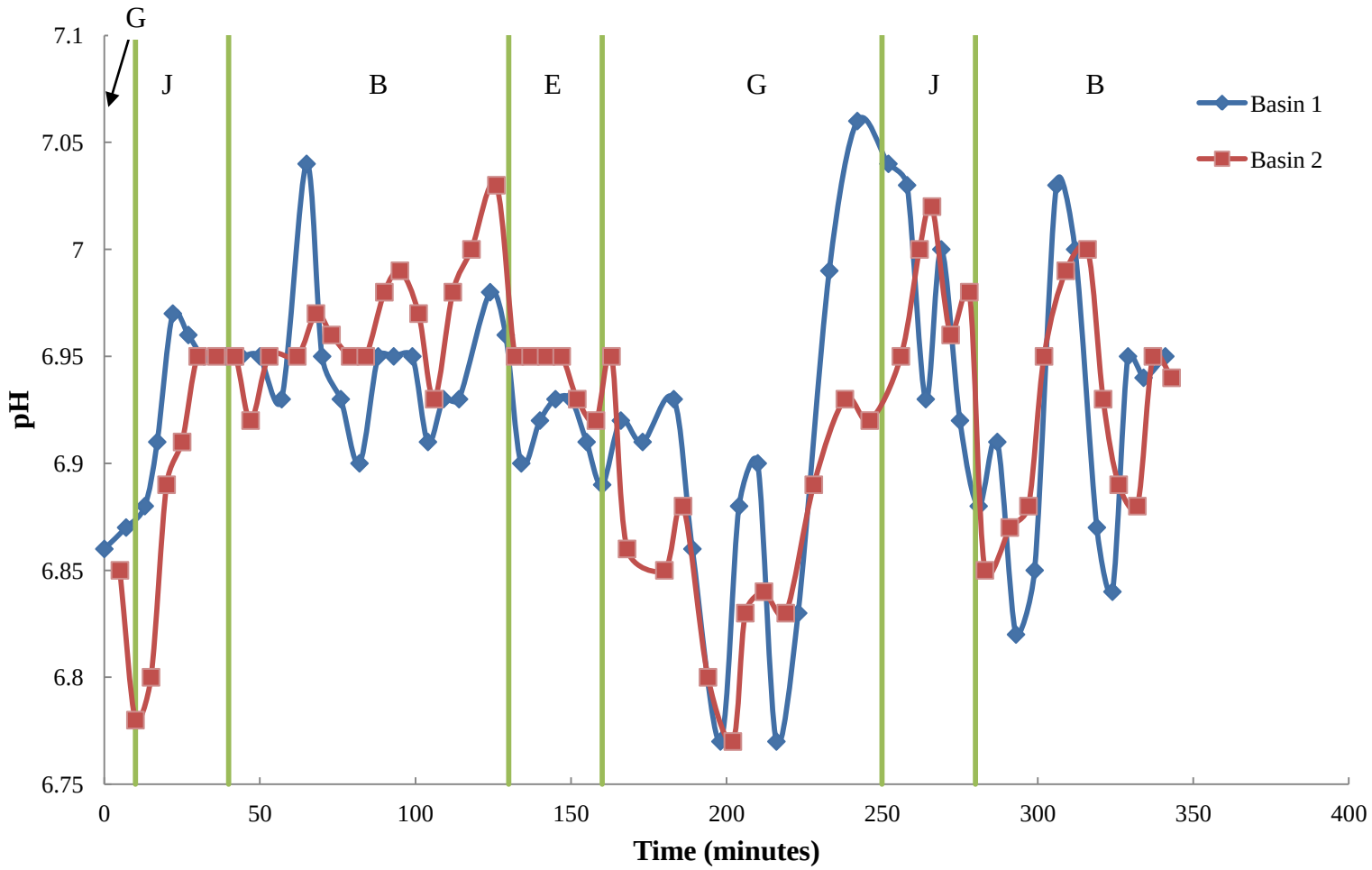


Figure 3.7. pH over time in Basins 1 and 2.

Originally, it was expected that the pH would decrease during periods of nitrification due to the fact that protons are released during this process:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$$

Likewise, it was expected that pH would increase during periods of denitrification due to the fact that protons are taken up during this process:

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

However, as seen in the graph above, this was not observed. Rather, the pH in both basins 1 and 2 seemed to be the same regardless of the fact that when basin 1 was anoxic, basin 2 was aerobic and vice versa. In other words, the pH did not seem to be at all dependent on whether nitrification or denitrification was occurring. Most likely, the pH observed was simply the inherent pH of the incoming wastewater that did not change as it moved through the different basins and phases. Any effects on the pH that nitrification or denitrification may have had were most likely negated by the buffering effects of alkalinity present in the wastewater.

3.5 Oxidation-Reduction Potential (ORP)

The ORP was also measured over time in basins 1 and 2 approximately every 5 minutes for 6 hours. The results are shown in Figure 3.8.

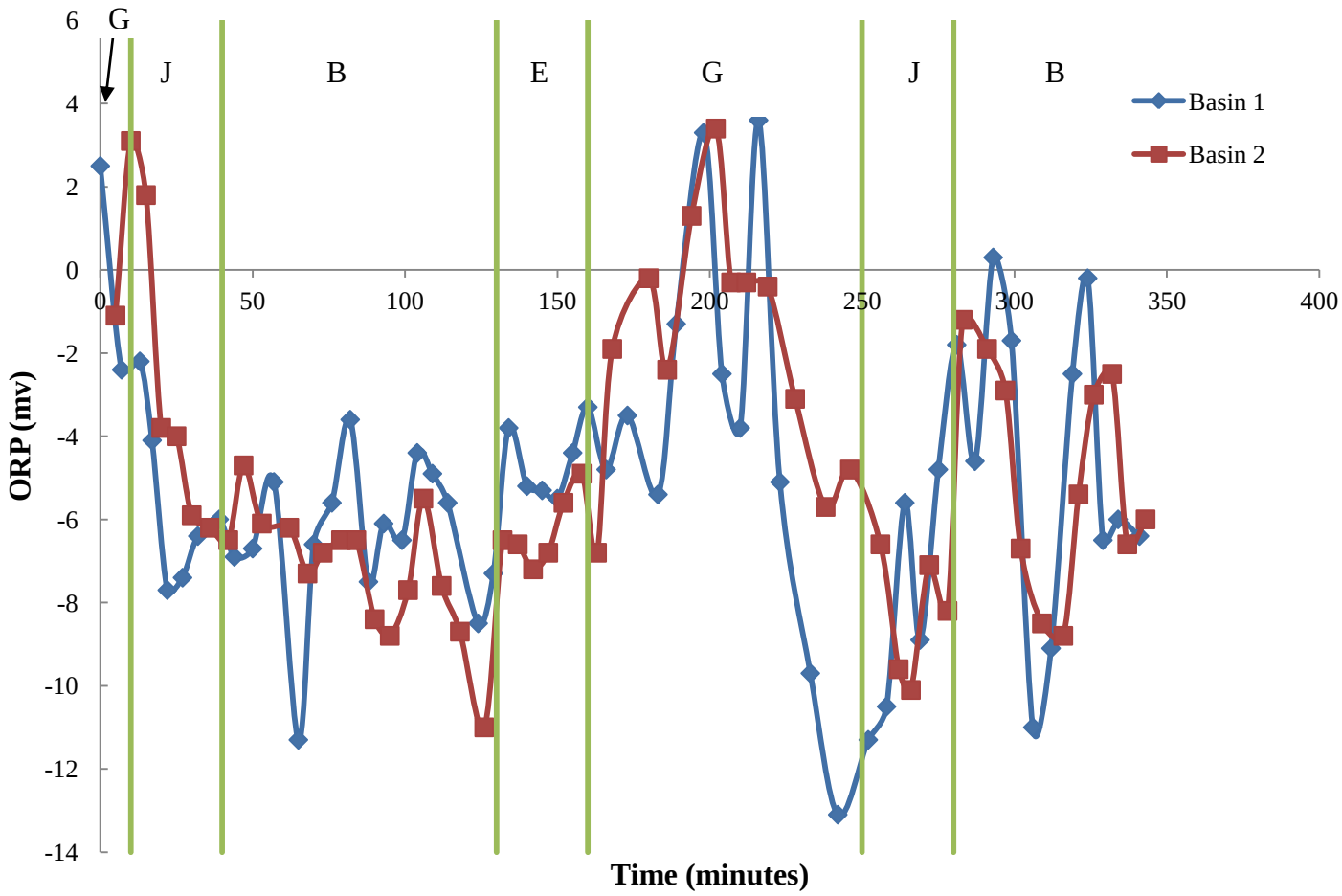


Figure 3.8. ORP over time in Basins 1 and 2.

ORP is a measure of a chemical species' affinity for electrons. Species that have a higher affinity for electrons are more likely to be reduced. A higher ORP indicates that a species has a higher tendency to acquire electrons and therefore a higher tendency to be reduced. Because of this, it was expected that the ORP would be higher during aerobic phases. This is because during aerobic phases, dissolved O_2 is high, and NO_3^- is being produced. The N in NO_3^- has an oxidation state of +5. This means it has the ability to acquire 8 electrons and therefore has a high affinity for electrons and a high tendency to be reduced. Thus, it was expected that the ORP during aerobic phases would be high. Likewise, it was expected that the ORP during anoxic phases would be lower, since denitrification is occurring during anoxic phases and the nitrogenous species present during denitrification have lower oxidation species and a lower tendency to acquire electrons. However, this trend was not observed, as seen in Figure 3.4. Rather, the ORP did not appear to differ significantly between basins 1 and 2, despite the fact that basins 1 and 2 had opposing aerobic/anoxic conditions during several of the phases and should have had notably different ORPs. It is thought that the reason for this unexpected trend is due to improper operation of the ORP probe.

3.6 Nitrate and Nitrite Concentrations

The concentrations of NO_3^- and NO_2^- in the liquid wastewater samples from basins 1 and 2 were measured over time as described in section 2.4. The results are shown in Figures 3.9 and 3.10.

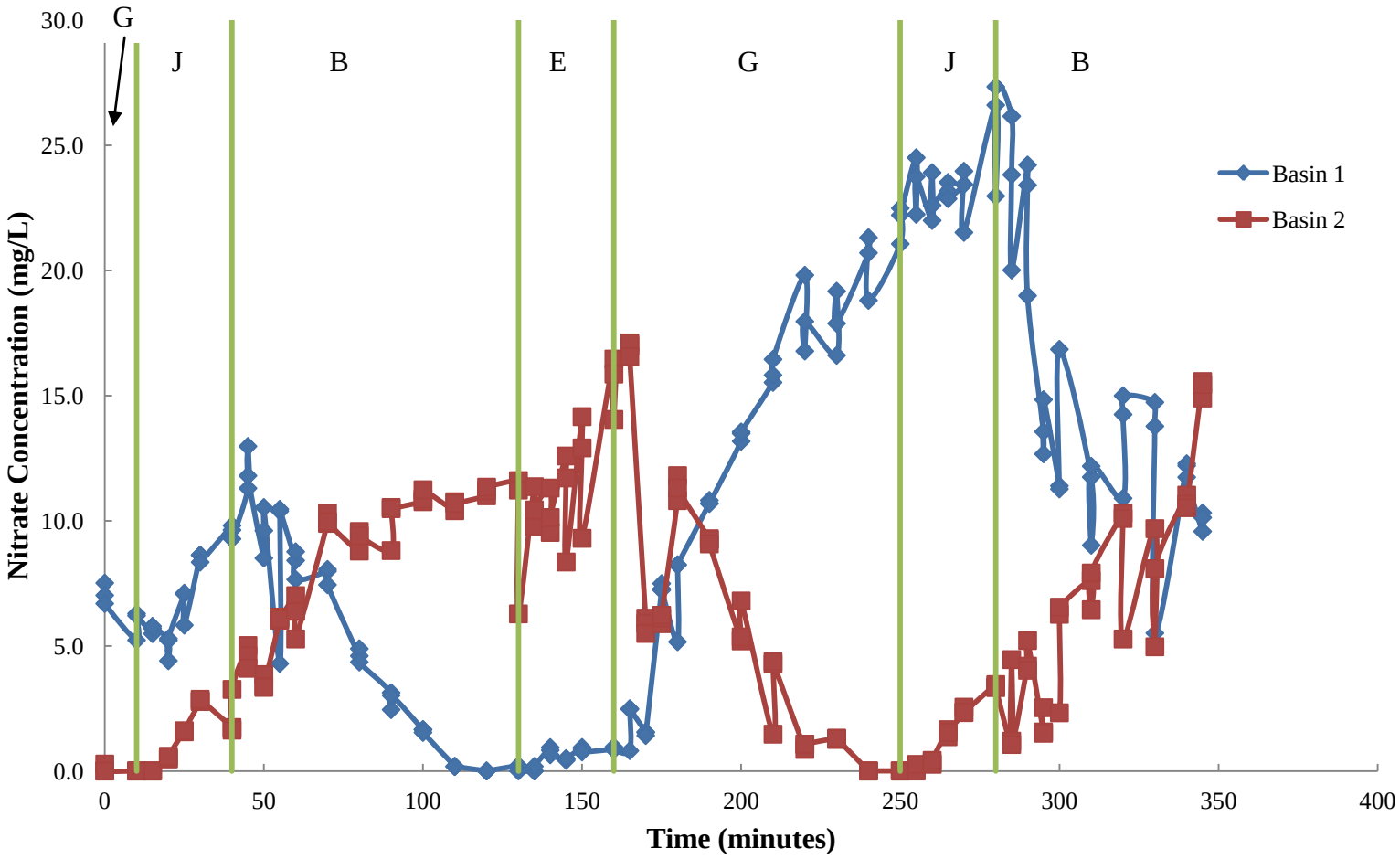


Figure 3.9. NO_3^- concentration over time in Basins 1 and 2.

The NO_3^- concentrations in both basins 1 and 2 were as expected. NO_3^- is produced during nitrification, which takes place during aerobic conditions. NO_3^- is consumed during denitrification, which takes place during anoxic conditions. Therefore, when the air was turned on, the NO_3^- concentration increased until the air was turned off, at which point it began to decrease and decreased until it reached a concentration of 0 mg/L. This occurred in both basins 1 and 2 for all phases.

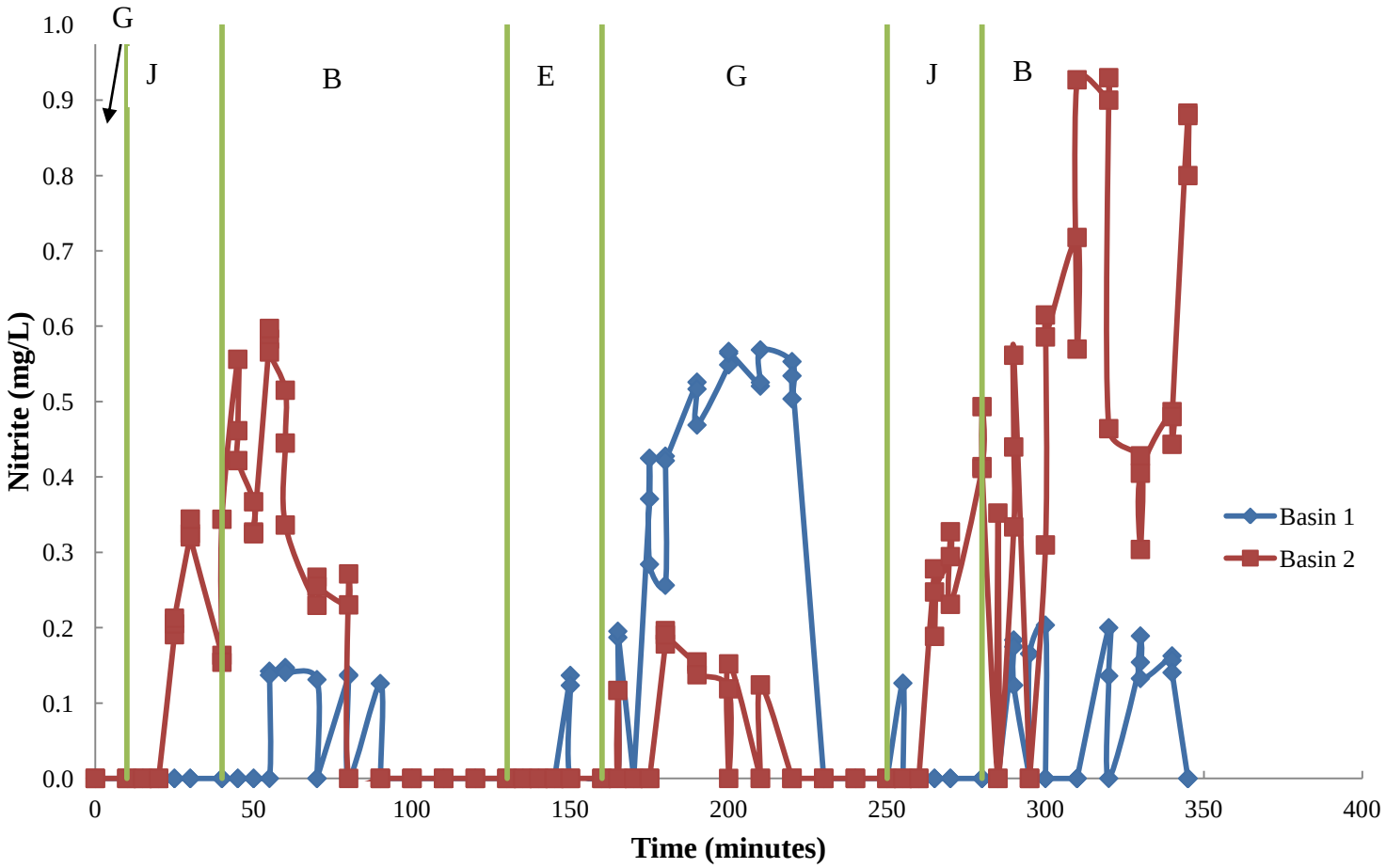


Figure 3.10. NO_2^- concentration over time in Basins 1 and 2.

The NO_2^- concentrations in both basins 1 and 2 were also as expected. NO_2^- is produced during ammonia oxidation, and then subsequently consumed during nitrite oxidation. NO_2^- is also produced and then subsequently consumed during denitrification. The NO_2^- concentration therefore increased at the beginning of aerobic phases but began to decrease before the air was turned off. The same trend was observed during the anoxic phases, with the NO_2^- concentration increasing at the beginning of the anoxic phases and decreasing before the air was turned on.

3.7 Optimizing DNA Extraction Method

Total DNA was extracted from the activated sludge samples as described in section 2.5. Two methods were tested to see which yielded higher DNA concentrations and better purities. Two samples were randomly chosen to test each method. The results are shown in Table 3.2.

Table 3.2. Comparison of DNA Extraction Methods.

Method Used	Sample	DNA Concentration of Extract (ng/µL)	DNA Concentration of Sample (ng/mL)	A_{260}/A_{280}	A_{260}/A_{230}
MOBIO	B1D27-1	29.9	199.3	2.09	0.52
MOBIO	B1D30-1	28.6	190.7	2.10	0.49
Lab Method	B1D2-1	1987.7	13251.3	1.72	1.31
Lab Method	B1D2-3	2370.0	15800.0	1.71	1.30

The Lab Method yielded significantly higher DNA concentrations and A_{260}/A_{230} ratios. Therefore, the Lab Method was selected as the DNA extraction protocol. Total DNA was extracted from the first four selected samples (B1D2, B1D5, B1D7, and B1D1, in triplicate) using this method. However, the A_{260}/A_{280} and A_{260}/A_{230} ratios obtained from this method were not as high as needed. A value close to 2.00 is desirable. These ratios indicate how pure the DNA is. Lower values mean that contaminants, and most likely qPCR inhibitors, are present in higher concentrations. Therefore, the Lab Method was optimized to achieve better purities. Steps 8 through 10 (the protein removal steps) in the Lab Method protocol were performed 1, 2, and 3 times to see if this yielded better purities. Also, incubating the samples in a -4°C refrigerator for Step 9 (instead of incubating on ice for 10 minutes) was tested. The results are shown in Table 3.3.

Table 3.3. DNA Extraction Method Optimization.

	DNA Concentration of Extract (ng/µL)	DNA Concentration of Sample (ng/mL)	A_{260}/A_{280}	A_{260}/A_{230}
protein removal steps once	2224.1	14827.3	1.72	1.28
protein removal steps twice	2087.7	13918.0	1.87	1.57
protein removal steps thrice	1008.1	6720.7	1.98	1.77
removal steps once; incubated overnight	1776.1	11840.7	1.81	1.49
removal steps twice; incubated overnight	1360.9	9027.7	1.84	1.52
removal steps thrice; incubated overnight	969.1	6460.7	1.97	1.79

Repeating the protein removal steps three times significantly improved both the A_{260}/A_{280} and A_{260}/A_{230} ratios. The DNA concentration was lower when the steps were done three times than when they were only done once, but the concentration was still high enough to perform qPCR. Incubating the samples overnight did not seem to make a significant difference in the purities. Therefore, it was determined that performing the protein removal steps (Steps 8 through 10) three times but not incubating the samples overnight yielded the optimal results. The Lab Method with these alterations was then used to extract the total DNA from the remaining samples. The results are given in Figure 3.11.

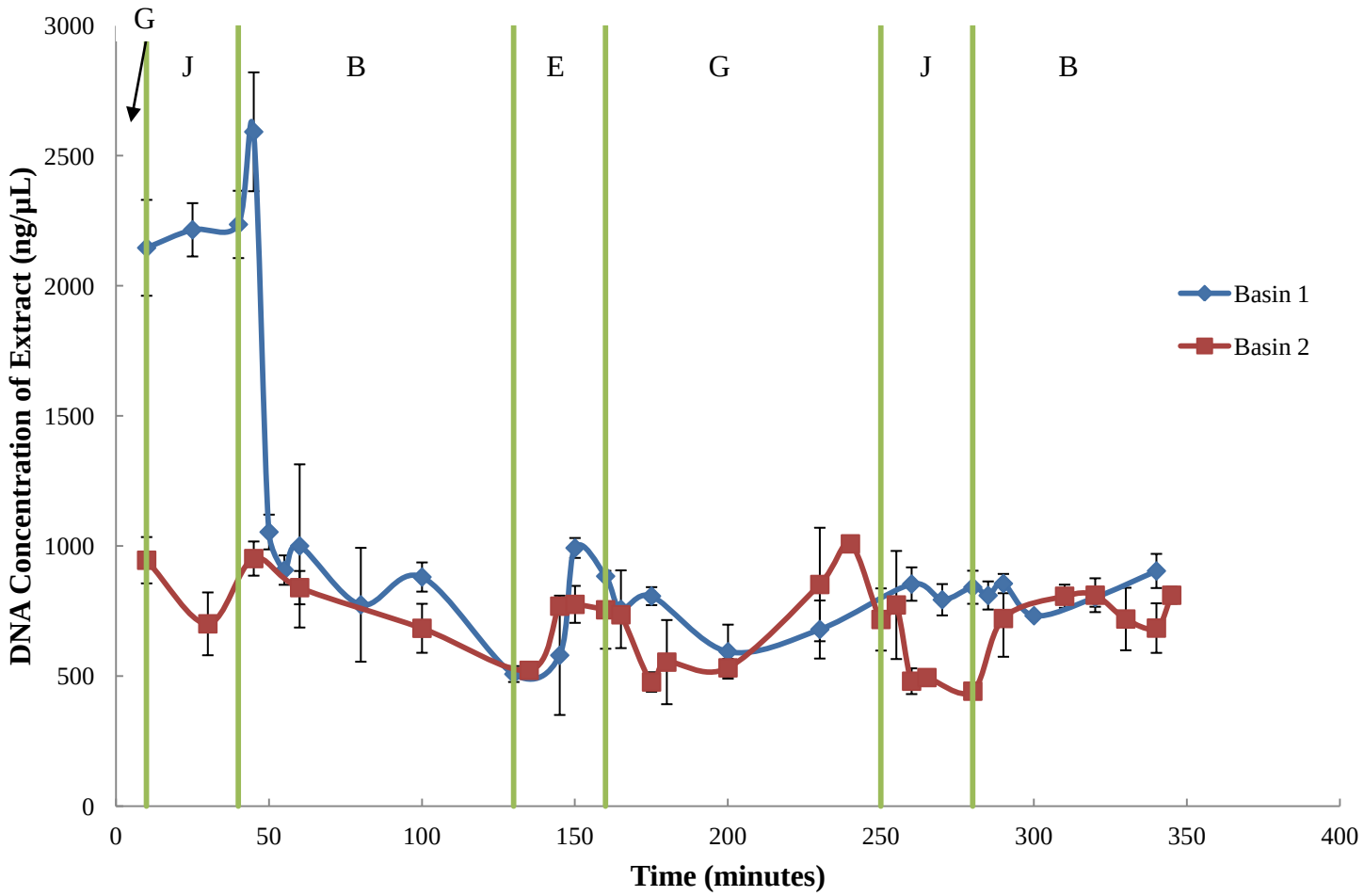


Figure 3.11. DNA Concentration over time in Basins 1 and 2.

The first four samples of basin 1, samples B1D2, B1D5, B1D7, and B1D8, had higher DNA concentrations due to the fact that during extraction, the protein removal steps were only performed once. For the rest of the samples of basin 1 and all of the samples of basin 2, the steps were performed three times, yielding lower DNA concentrations. There did not appear to be any correlation between the total DNA and the air being turned on or off. Rather, the total DNA fluctuated most likely because of the intrinsic characteristics of the incoming wastewater, such as available BOD and the NH_4^+ concentration. The DNA concentrations may also fluctuate slightly due to differing extraction efficiencies.

3.8 qPCR Results

The *nosZ* gene was targeted during qPCR performed on the DNA extracted from the activated sludge samples as described in section 2.9. A positive control consisting of *Pseudomonas stutzeri* DNA was included on every plate, along with a negative control consisting of sterile water in place of the DNA template. It was expected that the CT values for the positive controls would be very similar to the ones used for standard curve creation. It was expected that the CT values for the negative controls would be high, around 40, indicating that no amplification had occurred.

For the first three runs, the CT values for the negative controls were significantly lower than expected, around 29. This meant that there must have been DNA present in the negative controls, indicating contamination. Because of this, the CT values for the samples were not reliable. Through performing qPCR using different batches of reagents, it was

determined that the SYBR Green Supermix being used had been contaminated during production. A new batch of SYBR Green Supermix was obtained. When the new SYBR Green Supermix was used, the CT values for the negative controls were as expected, around 40.

The optimal dilution factor for the DNA extracts to perform qPCR had to be determined. For the samples that only had the protein removal steps of the DNA extraction protocol performed once, samples B1D2, B1D5, B1D7, and B1D8, no successful dilution factor was found. Dilution factors of 0, -1, and -1.5 all returned CT values of NA, meaning there was virtually no amplification and no CT value was able to be calculated. For a dilution factor of -2, the CT values returned were all high, around 39 or 40. These CT values were very close to the CT values for the negative controls, again indicating that very little amplification had occurred. These values were also outside of the range of the standard curve. The most probable reason for these high/NA CT values is that the concentration of contaminants and qPCR inhibitors was too high to allow for successful amplification. This is most likely due to the fact that the protein removal steps were only performed once, meaning a significant amount of contaminants remained in the DNA extracts. For the rest of the samples, the ones in which the protein removal steps were performed three times, a dilution factor of -2 proved to yield CT values solidly in the range of the standard curve and well below the CT values for the negative controls. The 0 and -1 dilution factors still had too high a concentration of contaminants to allow for successful amplification.

Once the appropriate dilution factor was determined, qPCR was carried out for all

samples except samples B1D2, B1D5, B1D7, and B1D8. CT values were obtained, and these values, in conjunction with the standard curve given in section 3.1, were used to determine the *nosZ* DNA concentrations of the activated sludge samples. From the DNA concentration, the number of cells with *nosZ* was determined using the equations given in section 2.8. From this information, it was possible to see how the number of cells with *nosZ* changed over time in basins 1 and 2 as the air was turned on and off. In other words, the number of cells capable of performing denitrification was quantified over the entire operational cycle of the wastewater reclamation facility. The results are given in Figures 3.12 and 3.13.

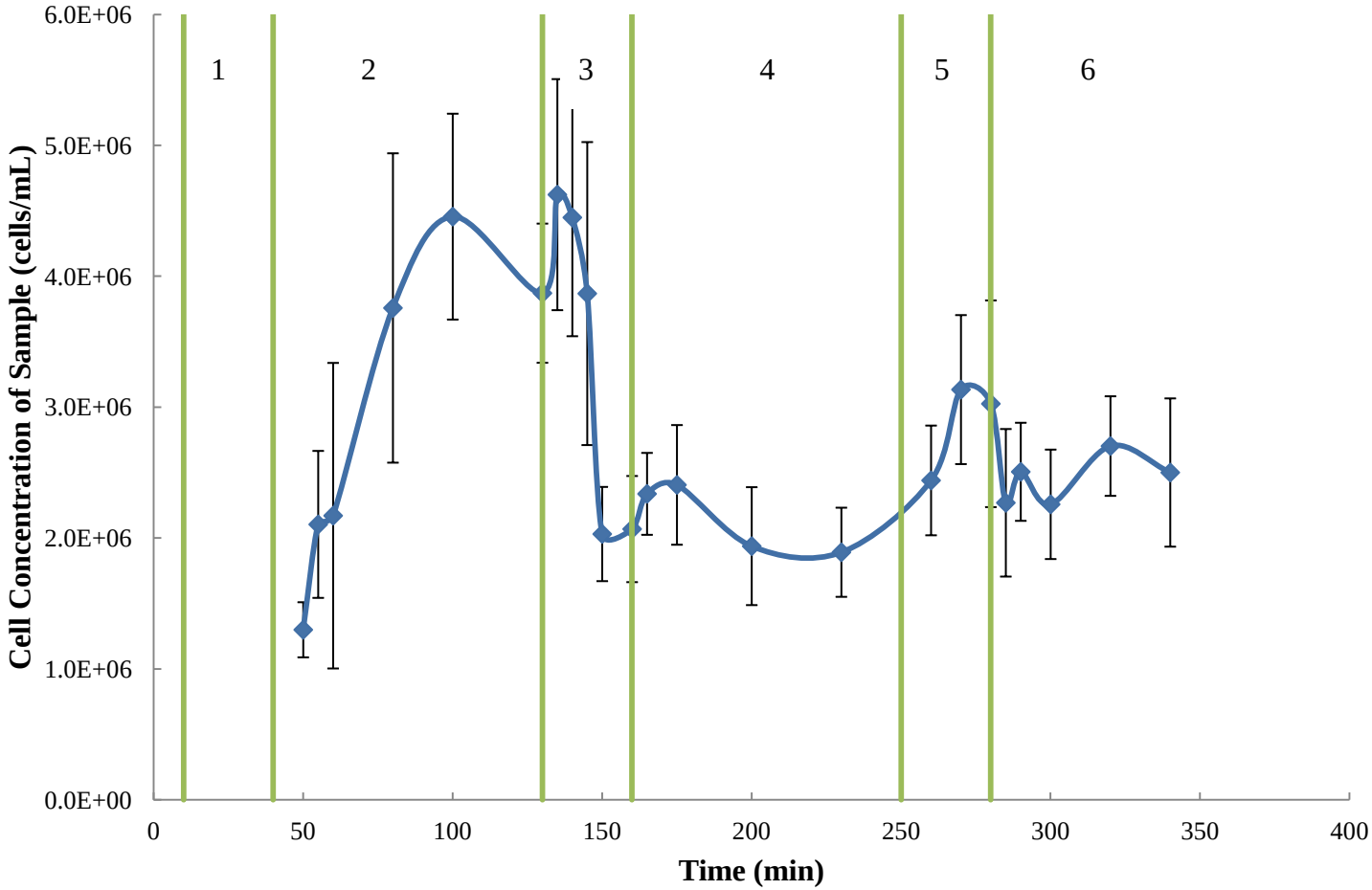


Figure 3.12. Concentration of cells with *nosZ* over time in Basin 1.

The phases have been numbered 1 through 6 to aid discussion. In phase 1, the air was turned on and the wastewater was flowing into basin 1. However, this is the phase in which samples B1D2, B1D5, B1D7, and B1D8 were taken. As described previously, CT values for these samples were unable to be obtained. In phase 2, the air was turned off and the wastewater was flowing into basin 1. The concentration of cells containing *nosZ* begins to increase almost immediately. This increase in cells was expected, as denitrification takes place during anoxic conditions. Because the incoming wastewater was flowing into basin 1 during this phase, there was a sufficient supply of organic carbon to serve as the electron donor. Nitrate was present from the previous aerobic phase. These are the three key factors needed for denitrification to occur and, because all were present, the number of cells with *nosZ*, and therefore the cells capable of performing denitrification, increased. In phase 3, the air was turned on and there was no flow into basin 1. There was an almost immediate decrease in the concentration of cells with *nosZ*. This was again expected, as denitrification does not occur under aerobic conditions and there was no flow of organic carbon into basin 1. During phase 4, the air was turned on and there was flow into basin 1 from basin 2. There does not appear to be significant increase or decrease of cells with *nosZ* as compared to phase 3, which again was expected. There is a very slight increase at the very beginning of the phase which can most likely be explained by carry-over from basin 2. During phase 5, the air was turned on and the wastewater was flowing into basin 1. There is an increase in the concentration of cells with *nosZ*. This is probably due to the in-flow of organic carbon providing a source of electrons. The increase is not as great as the increase observed during phase 2, in which

there were anoxic conditions. During phase 6, the air was turned off and the wastewater was flowing into basin 1. These are the same conditions as phase 2. However, the increase observed during phase 2 was not observed during phase 6. Although the full phase was not captured, it was expected that the concentration of cells with *nosZ* would begin to increase almost immediately. Rather, the cell concentration appeared to remain constant, with maybe a slight increase over the 60 minutes that samples were taken during this phase.

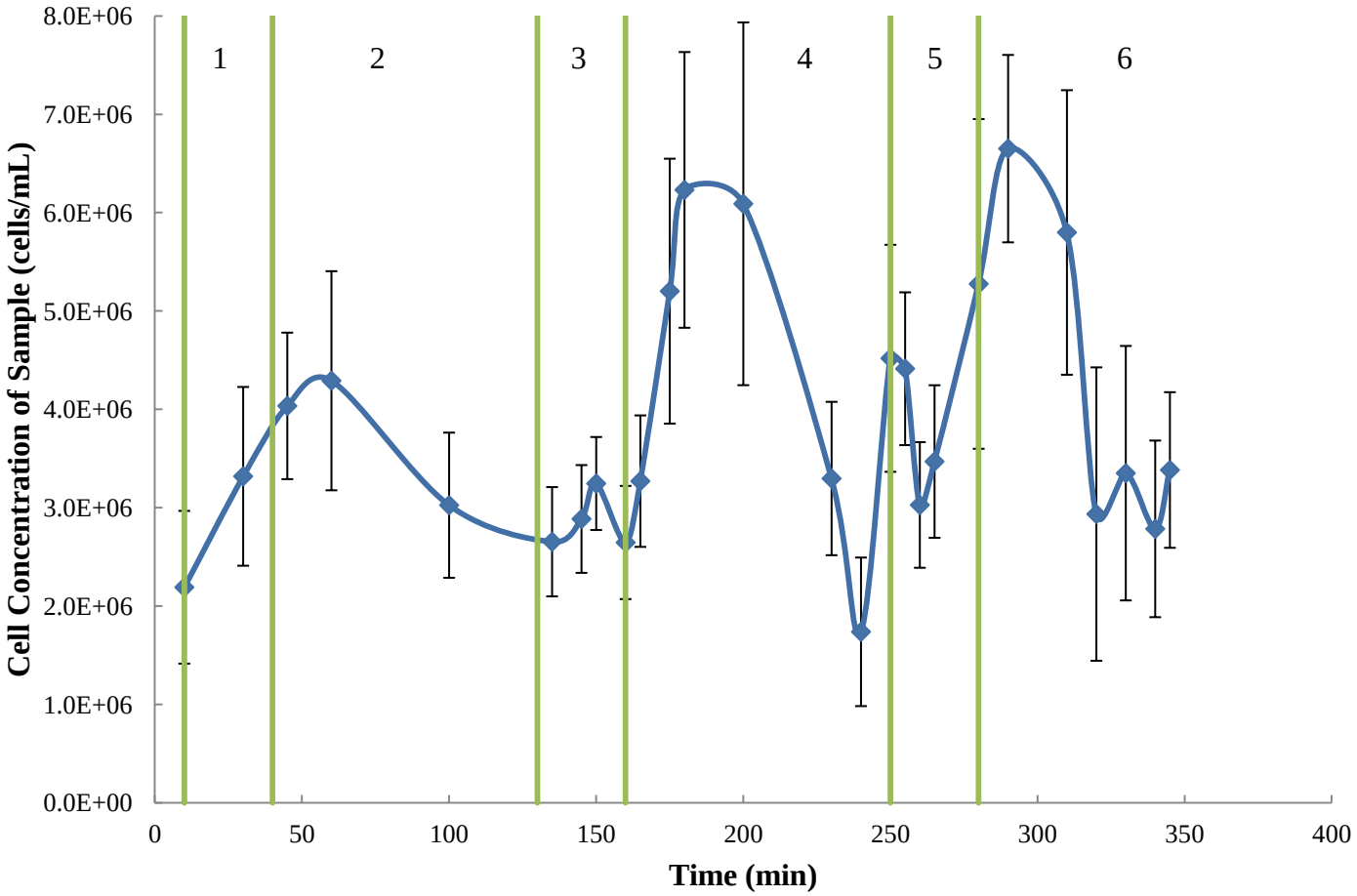


Figure 3.13. Concentration of cells with *nosZ* over time in Basin 2.

During phase 1, the air was turned on and there was no flow into basin 2. However, there was a slight increase in the concentration of cells with *nosZ*. This increase was unexpected. One possible explanation for this increase is that it is due to carry-over from the previous phase. During phase 2, the air was turned on and there was flow into basin 2 from basin 1. There was a slight increase at the beginning of the phase, most likely due to carry-over from basin 2, followed by a decrease in the concentration of cells with *nosZ*. This decrease was expected, as the air was turned on during this phase. During phase 3, the air was turned on and the wastewater was flowing into basin 2. There was a slight increase during this phase that was most likely due to the in-flow of organic carbon. During phase 4, the air was turned off and the wastewater was flowing into basin 2. There was an almost immediate increase in the concentration of cells with *nosZ*. This was expected, as denitrification occurs under these conditions. However, the cell concentration began to decrease before the air was turned on. It is thought that this is due to an insufficient supply of organic carbon, which can be seen in the DO concentration (Figure 3.6) and NO_3^- concentration (Figure 3.9) graphs. Towards the end of phase G, the DO concentration suddenly spikes in basin 1. This spike most likely occurs because there is a decrease in the amount of organic carbon present, meaning not as much as oxygen is being consumed to convert the organic carbon. Likewise, the NO_3^- concentration begins to decrease during phase G in basin 2, showing that there is most likely a sudden decrease in the amount of organic carbon present, meaning nitrification, which produces NO_3^-, is limited. During phase 5, the air was turned on and there was no

flow into basin 2. These are the same conditions as phase 1. A slight increase in the concentration of cells with *nosZ* was observed, just as it was in phase 1. Again, this was somewhat unexpected as these are not the conditions for denitrification. However, as stated previously, this increase could possibly be explained by carry-over from the previous phase. During phase 6, the air was turned on and there was flow into basin 2 from basin 1. These are the same conditions as phase 2. Again, a slight increase in the concentration of cells with *nosZ* was observed at first, followed by a decrease. The increase at the beginning was most likely due to carry-over from basin 2. The subsequent decrease was also expected, as the air was turned on and denitrification was no longer occurring.

As can be seen by these two graphs, the concentration of cells with *nosZ* changes significantly over the different phases of operation at a full-scale Bio-Denipho wastewater treatment facility. This change appears to correlate not only with the presence of absence of O_2, but also with availability of organic carbon to utilize as an electron donor. When the air is turned off, the increase in cells with *nosZ* begins almost immediately. When the air is turned on, there appears to be a slight lag before the concentration of cells with *nosZ* begins to decrease. This may be due to the fact that some residual O_2 remains in the water, or it could be due to carry-over from the previous basin. Also, when there is flow into a basin, whether from the head of the plant or from the previous basin, there is usually at least a slight increase in the concentration of cells with *nosZ*, even when the air is turned on. This is most likely due to an in-flow of

organic carbon providing electrons for growth. During some of the anoxic phases, there appears to be a decrease in the concentration of cells with *nosZ* towards the end of the phase before the air is turned on again. This decrease could be due to an insufficient supply of organic carbon.

3.9 RNA Results

As described previously, using qPCR targeting the *nosZ* gene present in DNA quantifies the bacteria capable of performing denitrification. By targeting the *nosZ* gene present in RNA, the bacteria that are actively performing denitrification can be quantified. Because this information was desired, total RNA was extracted. cDNA was synthesized using RT-PCR with the RNA serving as the template. qPCR was then performed targeting the *nosZ* gene. However, due to complications that arose while performing these steps, no CT values were able to be obtained. The various steps taken are summarized below.

Total RNA was extracted from two samples (samples 2 and 13 from basin 1) using the RNA Extraction Protocol developed by Stahl et al. as described in section 2.10. The RNA concentration was determined using the NanoDrop Spectrophotometer ND-1000. The RNA concentrations for the two samples are given in Table 3.4.

Table 3.4. RNA concentrations.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-1	1775.2	1.97	1.85
B1R13-1	1854.7	1.95	1.78

The RNA concentrations appeared to be sufficiently high with A_{260}/A_{280} and A_{260}/A_{230} ratios that were close to 2.

Because RNA extracted from environmental samples is often contaminated with DNA, the samples are usually treated with DNAse, an enzyme that breaks down DNA. DNaseI from Qiagen was used, as described in section 2.11. The RNA concentration after the DNAse treatment was measured. The results are given in Table 3.5.

Table 3.5. RNA concentrations after DNAse treatment.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-1	410.0	1.81	1.73
B1R13-1	438.2	1.76	1.66

The RNA concentrations were significantly lower than the RNA concentrations obtained previously, before the DNAse treatment. This most likely means that there was a high amount of DNA contamination in the samples. It also could mean that the DNAse had some RNase present, meaning that some of the RNA was destroyed in addition to the DNA.

The RNA was purified using the RNeasy MinElute Cleanup Kit from Qiagen as described in section 2.12. Contaminants as well as the DNAse were removed from the RNA during this process. The RNA concentrations were measured after the RNA had been purified. The results are given in Table 3.6.

Table 3.6. RNA concentrations after DNAse treatment and purification.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-1	452.6	1.87	1.75
B1R13-1	750.5	2.10	1.80

The RNA concentration and A_{260}/A_{280} and A_{260}/A_{230} ratios did not significantly change for sample 2 after purification. For sample 13, all three values increased significantly. The RNA was more concentrated after purification for sample 13, and both the A_{260}/A_{280} ratio and the A_{260}/A_{230} ratio increased.

cDNA was synthesized using the RNA as template with the Bio-Rad iScript cDNA Synthesis Kit as described in section 2.13. The results are given in Table 3.7.

Table 3.7. cDNA concentrations.

Sample	DNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-1	1045.9	1.86	2.21
B1R13-1	969.1	1.86	2.21

The DNA concentrations obtained were sufficiently high. The A_{260}/A_{280} and A_{260}/A_{230} ratios were also close to 2, indicating a high level of purity.

qPCR targeting the *nosZ* gene was performed on the cDNA in triplicate per sample. Dilution factors of -1 to -8 were used. *Pseudomonas stutzeri* DNA was used as the positive control, and sterile water was used as the negative control. The CT values for the positive controls were very similar to the ones obtained for the standard curve. The average CT value for the negative controls was 39.6. The CT values for the samples are given in Table 3.8.

Table 3.8. CT values obtained from qPCR targeting the *nosZ* gene performed on cDNA.

Sample	Dilution	CT Value
B1R2-1	-1	42.44
B1R2-1	-2	40.47
B1R2-1	-3	42.61
B1R2-1	-4	38.90
B1R2-1	-5	40.08
B1R2-1	-6	40.65
B1R2-1	-7	38.61
B1R2-1	-8	42.08
B1R2-1	-1	NA
B1R2-1	-2	41.55
B1R2-1	-3	39.24
B1R2-1	-4	NA
B1R2-1	-5	39.46
B1R2-1	-6	38.67
B1R2-1	-7	39.68
B1R2-1	-8	38.38
B1R2-1	-1	38.56
B1R2-1	-2	41.20
B1R2-1	-3	41.88

Table 3.8 Continued.

B1R2-1	-4	40.27
B1R2-1	-5	41.59
B1R2-1	-6	40.95
B1R2-1	-7	40.81
B1R2-1	-8	40.97
B1R13-1	-1	39.67
B1R13-1	-2	40.77
B1R13-1	-3	40.83
B1R13-1	-4	40.86
B1R13-1	-5	39.66
B1R13-1	-6	40.71
B1R13-1	-7	41.07
B1R13-1	-8	39.07
B1R13-1	-1	38.92
B1R13-1	-2	39.98
B1R13-1	-3	36.47

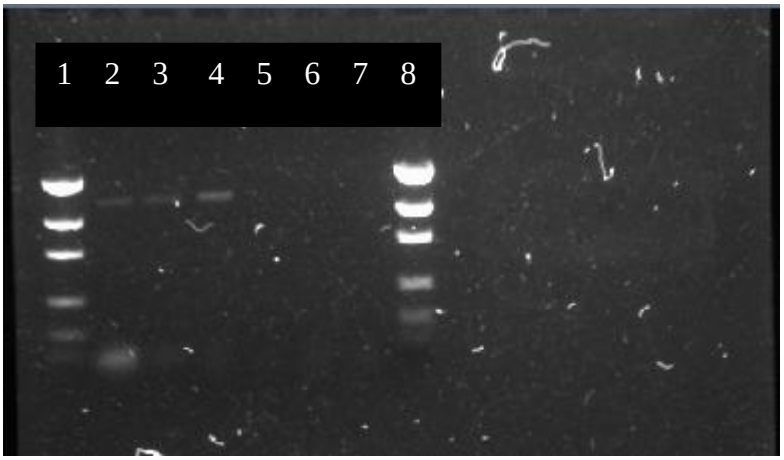
Table 3.8 Continued.

B1R13-1	-4	41.90
B1R13-1	-5	38.83
B1R13-1	-6	42.39
B1R13-1	-7	38.89
B1R13-1	-8	36.63
B1R13-1	-1	39.73
B1R13-1	-2	39.01
B1R13-1	-3	40.92
B1R13-1	-4	40.93
B1R13-1	-5	40.32
B1R13-1	-6	41.66
B1R13-1	-7	38.38
B1R13-1	-8	39.92

The CT values for these samples were too high to be reliable. They were very similar to the average CT value for the negative controls, meaning very little or no *nosZ* amplification occurred. Nearly all of the values were outside the range of the standard

curve or very close to the upper limit. Because of this, the CT values that were obtained were not able to be used to determine the concentration of cells with *nosZ*.

As a control, PCR targeting the 16S rRNA gene was performed on the cDNA. By performing PCR targeting this gene, it could be determined if the cDNA was properly synthesized. PCR was carried out as described in section 2.15. Gel electrophoresis was then performed on the PCR products as described in section 2.16. The resulting gel is shown in Figure 3.14.



Lanes 1 and 8: Low mass DNA ladder

Lanes 2-3: B1R2-1 with 1 ng DNA as template

Lanes 4-5: B1R2-1 with 5 ng DNA as template

Lanes 6-7: B1R2-1 with 10 ng DNA as template

Figure 3.14. Gel of PCR products targeting the 16S rRNA gene.

The first and last wells contained the low mass DNA ladder for use as a standard. The wells in between the ladders contained the PCR products with 1, 5, and 10 ng DNA as template in duplicate. Bands can be seen in the second, third, and fourth wells. The PCR was not optimized since this was only to be used to test whether or not cDNA was present. Since bands can be seen, this means that the cDNA was properly synthesized.

Therefore, the most likely cause of the qPCR targeting the *nosZ* gene resulting in little or no amplification is a high concentration of inhibitors.

Because of this, the cDNA was purified as described in section 2.14 in an attempt to remove any impurities and inhibitors. The cDNA concentration was measured after the purification was performed. The results are given in Table 3.9.

Table 3.9. cDNA concentrations after purification.

Sample	DNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-1	8.9	1.58	0.60
B1R13-1	7.4	1.69	0.64

The resulting cDNA concentrations were very low, as were the A_{260}/A_{280} and A_{260}/A_{230} ratios. This purification step does not appear to have been successful.

qPCR targeting the *nosZ* gene was performed on the purified cDNA in triplicate per sample. Dilution factors of -1 to -6 were used. *Pseudomonas stutzeri* DNA was used as the positive control, and sterile water was used as the negative control. The CT values for the positive controls were very similar to the ones obtained for the standard curve. The average CT value for the negative controls was 39.9. The CT values for the samples are given in Table 3.10.

Table 3.10. CT values obtained from qPCR targeting the *nosZ* gene performed on purified cDNA.

Sample	Dilution	CT Value
B1R2-1	-1	40.50
B1R2-1	-2	39.78
B1R2-1	-3	39.06
B1R2-1	-4	37.58
B1R2-1	-5	40.24
B1R2-1	-6	39.94
B1R2-1	-1	37.44
B1R2-1	-2	38.66
B1R2-1	-3	37.67
B1R2-1	-4	38.70
B1R2-1	-5	41.09
B1R2-1	-6	41.74
B1R2-1	-1	40.72
B1R2-1	-2	39.73
B1R2-1	-3	38.80
B1R2-1	-4	40.75
B1R2-1	-5	40.35
B1R2-1	-6	38.28

Table 3.10 Continued.

B1R13-1	-1	36.19
B1R13-1	-2	38.25
B1R13-1	-3	38.83
B1R13-1	-4	40.76
B1R13-1	-5	37.52
B1R13-1	-6	42.79
B1R13-1	-1	36.51
B1R13-1	-2	40.57
B1R13-1	-3	39.69
B1R13-1	-4	39.30
B1R13-1	-5	37.93
B1R13-1	-6	40.07
B1R13-1	-1	34.63
B1R13-1	-2	39.43
B1R13-1	-3	40.58
B1R13-1	-4	38.00
B1R13-1	-5	41.19
B1R13-1	-6	40.16

The CT values for these samples were again too high to be reliable. They were very similar to the average CT value for the negative controls, meaning very little or no *nosZ* amplification occurred. Nearly all of the values were outside the range of the standard

curve or very close to the upper limit. Because of this, the CT values that were obtained were not able to be used to determine the concentration of cells with *nosZ*. It is thought that the low cDNA concentrations and low ratios that resulted from the cDNA purification is the cause of this lack of amplification.

Because this method of RNA extraction did not yield usable CT values from the qPCR, a different RNA extraction method was used. The MOBIO RNA PowerSoil Total RNA Isolation Kit was used to extract total RNA from two samples (samples B1R2-2 and B1R13-2) as described in section 2.10. The RNA concentrations were measured and are given in Table 3.11.

Table 3.11. RNA concentrations obtained from MOBIO RNA extraction protocol.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-2	41.4	1.78	2.38
B1R13-2	37.5	1.62	2.56

This extraction protocol yielded significantly lower RNA concentrations.

The RNA extracts were treated with DNAse as before to remove any DNA present in the samples. The RNA concentration after the DNAse treatment was measured. The results are given in Table 3.12.

Table 3.12. RNA concentrations from MOBIO kit after DNAse treatment.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-2	12.8	2.03	1.70
B1R13-2	10.6	1.88	1.61

Again, the RNA concentrations were significantly lower than the RNA concentrations obtained previously, before the DNAse treatment. This most likely means that there was a high amount of DNA contamination in the samples. It also could mean that the DNAse had some RNase present, meaning that some of the RNA was destroyed in addition to the DNA.

The RNA was purified using the RNeasy MinElute Cleanup Kit from Qiagen as described in section 2.12. Contaminants as well as the DNAse were removed from the RNA during this process. The RNA concentrations were measured after the RNA had been purified. The results are given in Table 3.13.

Table 3.13. RNA concentrations from MOBIO kit after DNAse treatment and purification.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-2	8.9	1.90	0.73
B1R13-2	6.5	1.50	0.29

The RNA concentrations and purity ratios obtained after purification were very low. This purification does not appear to have been successful. Because the concentrations were so low, there was not enough RNA to perform the cDNA synthesis.

The next step performed was to extract RNA from two additional samples (samples B1R2-3 and B1R13-3), again using the MOBIO kit. The resulting RNA concentrations are given in Table 3.14.

Table 3.14. RNA concentrations obtained from MOBIO RNA extraction protocol.

Sample	RNA Concentration of Extract (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1R2-3	431.0	2.00	2.16
B1R13-3	399.4	2.01	2.17

Because it was previously determined that the DNAse treatment destroys a significant portion of the RNA, resulting in inadequate amounts of RNA after purification to perform cDNA synthesis when this extraction protocol is used, the DNAse treatment was not performed. It was thought that there may not be significant DNA contamination. If there is very little DNA contamination, then the DNAse treatment does not need to be performed and cDNA can be synthesized directly from the original RNA extracts. In order to determine if there was significant DNA contamination, qPCR targeting the *nosZ* gene was performed on the RNA samples. In theory, qPCR does not result in amplification of RNA. It only results in amplification of DNA. Therefore, if there was very little or no DNA present in the RNA extracts, no amplification would occur during qPCR. qPCR targeting the *nosZ* gene was performed on the RNA in triplicate per sample. Dilution factors of 0 to -6 were used. *Pseudomonas stutzeri* DNA was used as the positive control, and sterile water was used as the negative control. The CT values for the positive controls were very similar to the ones obtained for the standard curve. The average CT value for the negative controls was 37.6. The CT values for the samples are given in Table 3.15.

Table 3.15. CT values obtained from qPCR targeting the *nosZ* gene in RNA.

Sample	Dilution	CT Value
B1R2-3	0	NA
B1R2-3	-1	25.33
B1R2-3	-2	26.34
B1R2-3	-3	30.21
B1R2-3	-4	34.75
B1R2-3	-5	35.44
B1R2-3	-6	34.87
B1R2-3	0	NA
B1R2-3	-1	25.28
B1R2-3	-2	26.42
B1R2-3	-3	30.50
B1R2-3	-4	34.30
B1R2-3	-5	33.88
B1R2-3	-6	34.31
B1R2-3	0	NA
B1R2-3	-1	25.30
B1R2-3	-2	26.80
B1R2-3	-3	30.67

Table 3.15 Continued.

B1R2-3	-4	34.45
B1R2-3	-5	33.64
B1R2-3	-6	36.12
B1R13-3	0	25.70
B1R13-3	-1	NA
B1R13-3	-2	26.82
B1R13-3	-3	30.61
B1R13-3	-4	34.14
B1R13-3	-5	34.68
B1R13-3	-6	35.84
B1R13-3	0	25.11
B1R13-3	-1	NA
B1R13-3	-2	26.54
B1R13-3	-3	29.85
B1R13-3	-4	33.23
B1R13-3	-5	33.98
B1R13-3	-6	34.77
B1R13-3	0	24.97
B1R13-3	-1	NA
B1R13-3	-2	26.34

Table 3.15 Continued.

B1R13-3	-3	29.73
B1R13-3	-4	33.46
B1R13-3	-5	34.28
B1R13-3	-6	34.35

The 0 and/or -1 dilutions had CT values that were in the mid-20's. This is significantly lower than the average CT value for the negative controls, indicating that amplification occurred. This means that a significant amount of DNA was present in the samples, since qPCR only amplifies DNA. However, DNAse treatment could not be performed on the samples because it had been determined previously that the DNAse destroyed a significant portion of the RNA as well as the DNA. Therefore the DNAse treatment, along with the purification to remove the DNAse, resulted in RNA concentrations too low to perform cDNA synthesis with this extraction method.

No CT values were able to be obtained from the qPCR using cDNA, synthesized using RNA as template, for any of the wastewater samples. Therefore, no information about how the quantity of bacteria actively performing denitrification changes over time in a full scale wastewater treatment plant was able to be obtained.

3.10 T-RFLP Results

T-RFLP was performed on selected samples following the protocol described in section 2.19. The samples selected were B1D9, B1D15, B1D38, B2D22, B2D27, B2D36, and B2D41. These samples are pointed out in the graphs of cell concentration versus time, shown in Figures 3.15 and 3.16.

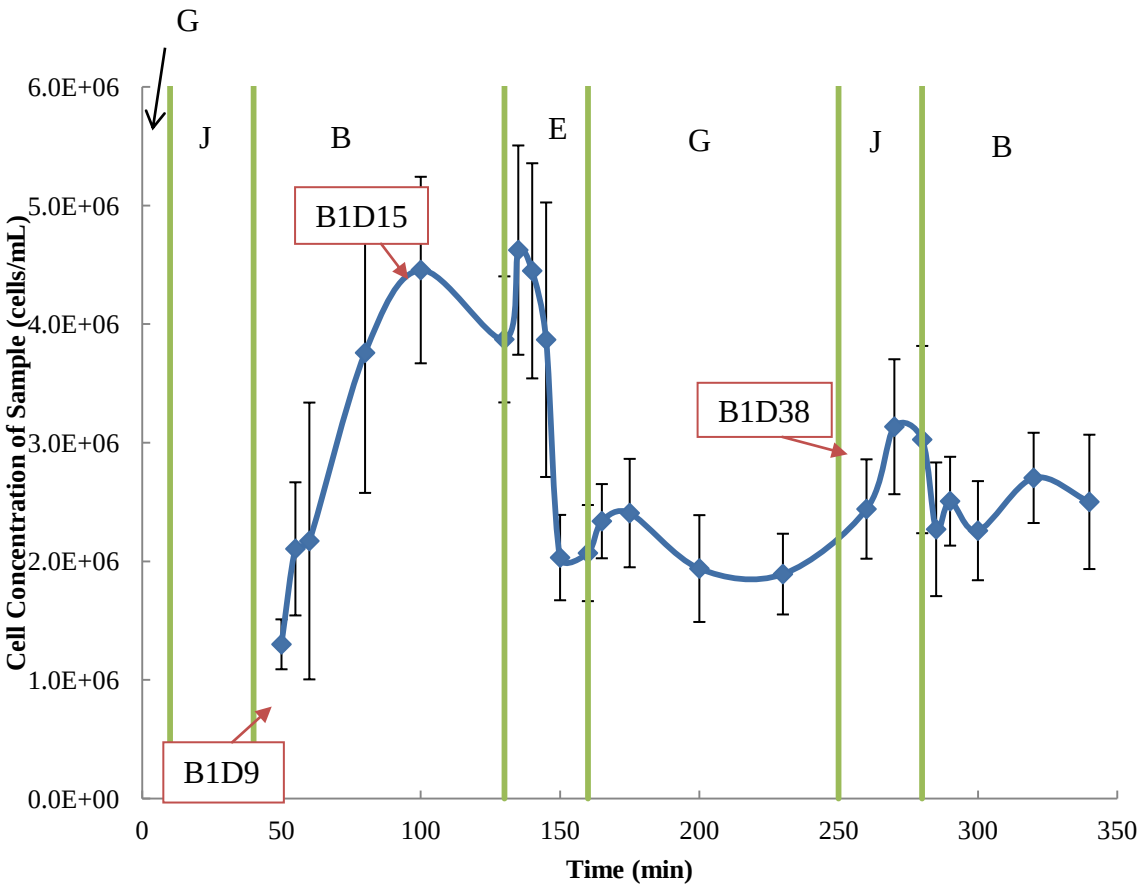


Figure 3.15. Concentration of cells with *nosZ* over time in Basin 1.

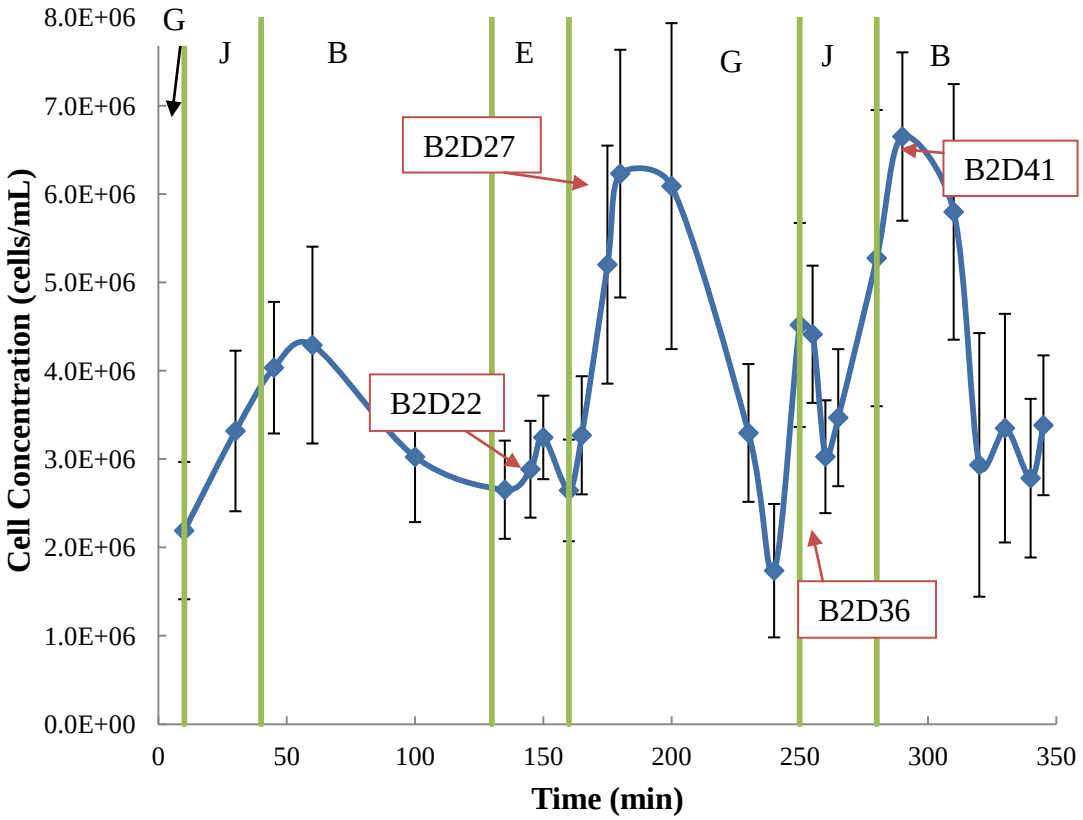
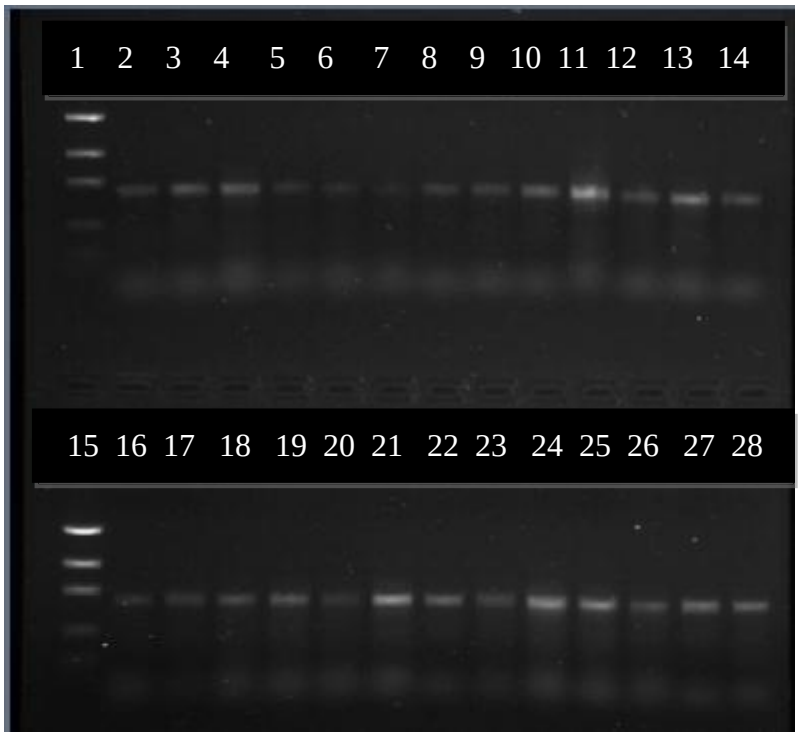


Figure 3.16. Concentration of cells with *nosZ* over time in Basin 2.

These samples were chosen because they represented both the highest and lowest points in both anoxic and aerobic phases in both basins.

The first step of T-RFLP was PCR optimization. First, two samples (samples B1D9 and B1D15) in triplicate were selected to test DNA template masses of 1 ng, 5 ng, and 10 ng. The PCR was performed with *nosZ* primers as described in section 2.20. The gels of the PCR products are shown in Figures 3.17a and 3.17b.



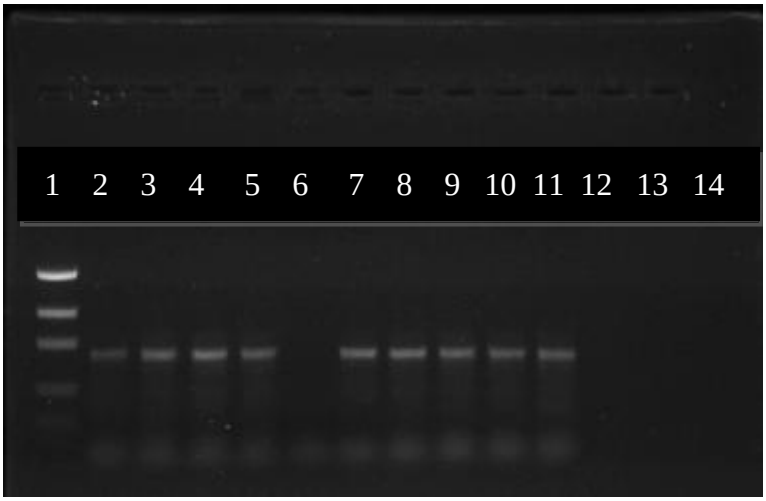
Lanes 1 and 15: low DNA mass ladder

Lanes 2,3,8,9,14,16,21,22, 27, 28: 1 ng of DNA as template

Lanes 4,5,10,11,17,18,23, 24: 5 ng of DNA as template

Lanes 6,7,12,13,19,20,25, 26: 10 ng of DNA as template

Figure 3.17a. Gel 1 testing 1, 5, and 10 ng of DNA on samples B1D9 and B1D15.



Lane 1: low DNA mass ladder

Lanes 6 and 7: 1 ng of DNA as template

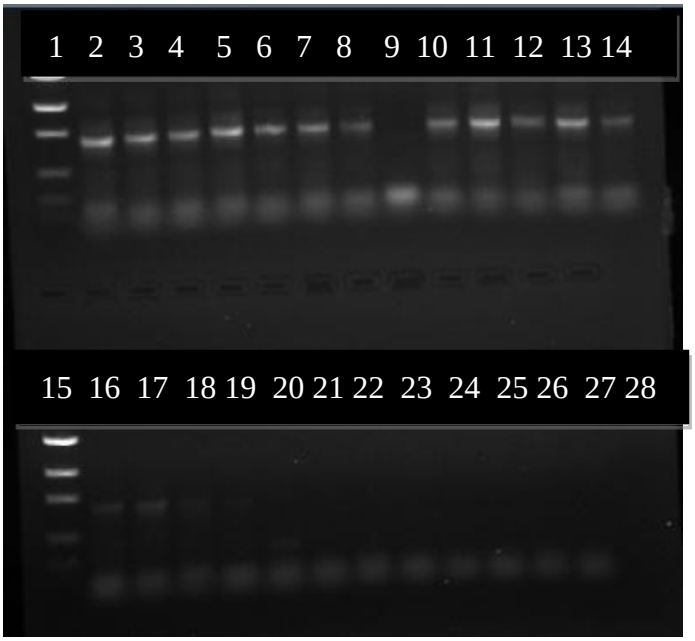
Lanes 2,3,8,9: 5 ng of DNA as template

Lanes 4,5,10,11: 10 ng of DNA as template

Figure 3.17b. Gel 2 testing 1, 5, and 10 ng of DNA on samples B1D9 and B1D15.

Wells 1 and 15 on Gel 1 and well 1 on Gel 2 contained the low DNA mass ladder. Wells 2, 3, 8, 9, 14, 16, 21, 22, 27, and 28 on Gel 1 and wells 6 and 7 on Gel 2 contained 1 ng of DNA as template. Wells 4, 5, 10, 11, 17, 18, 23, and 24 on Gel 1 and wells 2, 3, 8, and 9 on Gel 2 contained 5 ng of DNA as template. Wells 6, 7, 12, 13, 19, 20, 25, and 26 on Gel 1 and wells 4, 5, 10, and 11 on Gel 2 contained 10 ng of DNA as template. After observing the gels, it was determined that the samples that contained 5 ng of DNA template had the greatest intensity bands for the majority of the samples.

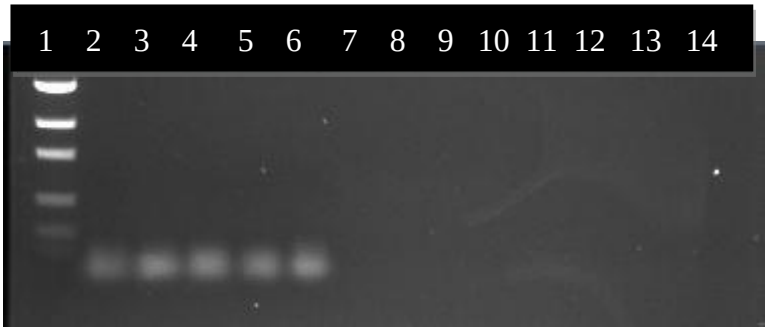
Next, PCR was performed using 5 ng of DNA as template for the remainder of the selected samples (samples B1D38, B2D22, B2D27, B2D36, and B2D41 in triplicate). The resulting gels are shown in Figures 3.18a and 3.18b.



Lanes 1 and 15: low DNA mass ladder

All other lanes: 5 ng of DNA as template

Figure 3.18a. Gel 1 testing 5 ng of DNA on samples B1D38, B2D22, B2D27, B2D36, and B2D41.



Lane 1:
low DNA mass ladder

Lanes 2-6:
5 ng of DNA as template

Figure 3.18b. Gel 2 testing 5 ng of DNA on samples B1D38, B2D22, B2D27, B2D36, and B2D41.

Samples B1D38 and B2D22 appeared to have good, intense bands. The other samples, however, had either very faint bands or no bands at all. So, PCR was performed again on these samples (samples B2D27, B2D36, and B2D41) using 1 ng of DNA as template. The resulting gel is shown in Figure 3.19.

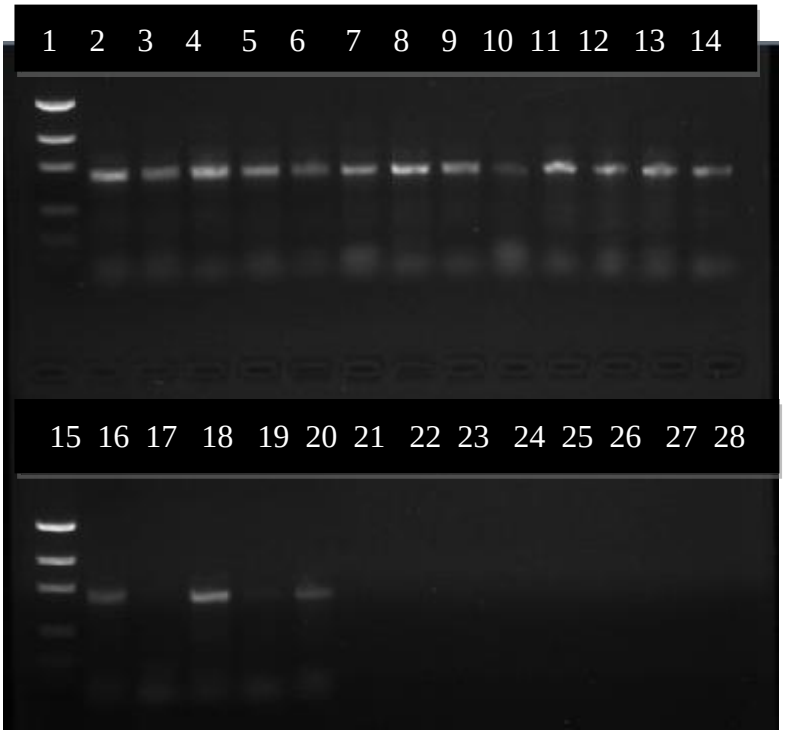
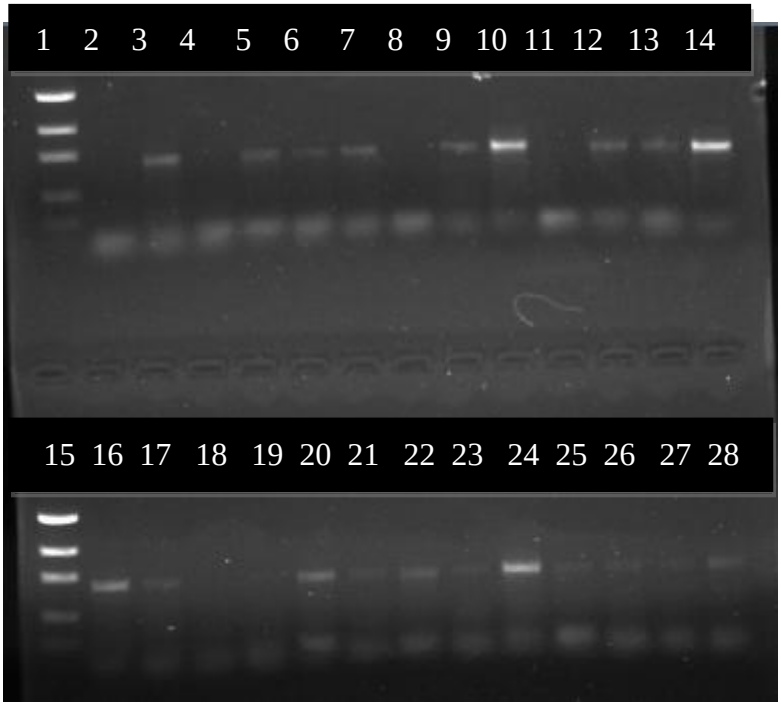


Figure 3.19. Gel testing 1 ng of DNA on samples B2D27, B2D36, and B2D41.

Using 1 ng of DNA as template for these samples yielded strong bands for nearly all wells. So, it appeared at this point that 5 ng of DNA as template was optimal for samples B1D9, B1D15, B1D38, and B2D22 while 1 ng of DNA as template was optimal for samples B2D27, B2D36, and B2D41. Another PCR run was performed using these DNA masses for these specific samples to confirm that these were optimal. The results are shown in Figures 3.20a and 3.20b.

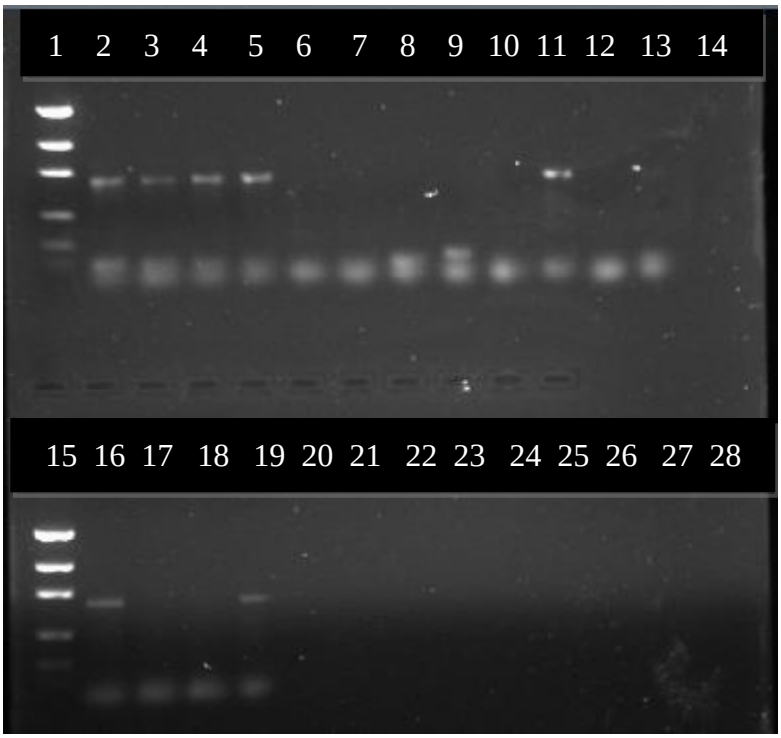


Lanes 1 and 15:
low DNA mass ladder

Lanes 2-14 and 16-26:
5 ng of DNA as template

Lanes 27-28:
1 ng of DNA as template

Figure 3.20a. Gel 1 testing 5 ng of DNA on samples B1D9, B1D15, B1D38, and B2D22 and 1 ng of DNA on B2D27, B2D36, and B2D41.

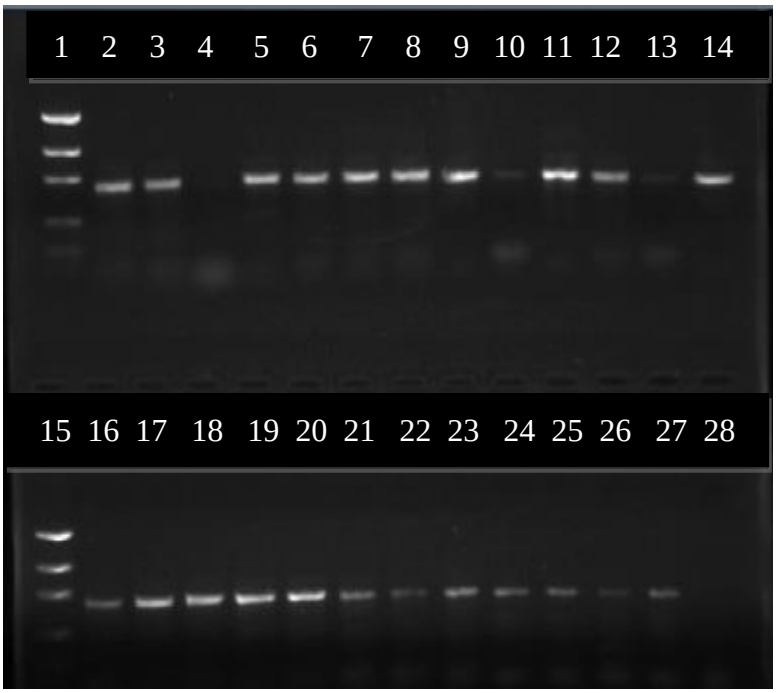


Lanes 1 and 15: low DNA mass ladder

Lanes 2-13 and 16-19 1 ng of DNA as template

Figure 3.20b. Gel 2 testing 5 ng of DNA on samples B1D9, B1D15, B1D38, and B2D22 and 1 ng of DNA on B2D27, B2D36, and B2D41.

As can be seen, the goal of bands of equal intensity for all samples was not achieved. A DNA template mass of 1 ng was then used to perform PCR on all samples. The results are shown in Figures 3.21a and 3.21b.



Lanes 1 and 15: low DNA mass ladder

Lanes 2-14 and 16-28: 1 ng of DNA as template

Figure 3.21a. Gel 1 testing 1 ng of DNA on B1D9, B1D15, B1D38, B2D22, B2D27, B2D36, and B2D41.

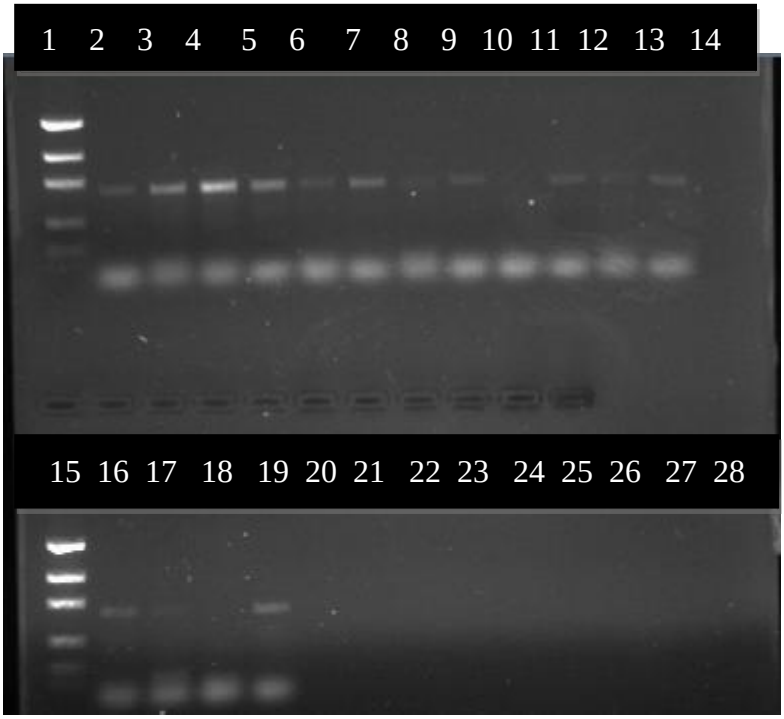


Figure 3.21b. Gel 2 testing 1 ng of DNA on samples B1D9, B1D15, B1D38, B2D22, B2D27, B2D36, and B2D41.

1 ng of DNA template appeared to yield strong intensity bands for samples B1D9, B1D15, and B1D38. However, samples B2D22, B2D27, B2D36, and B2D41 had fainter bands. Therefore, PCR was repeated on these samples using 0.5 ng of DNA as template. The resulting gel is shown in Figure 3.22.

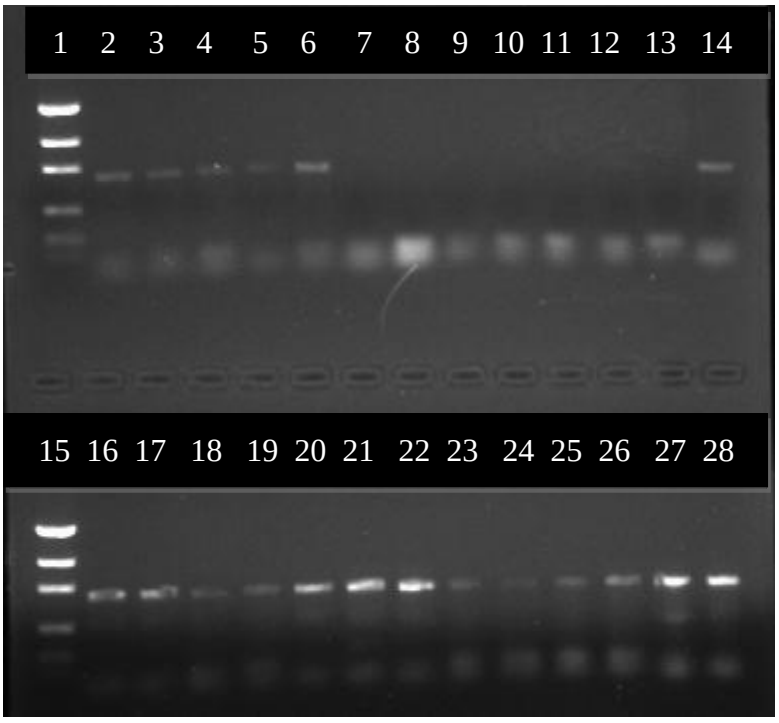
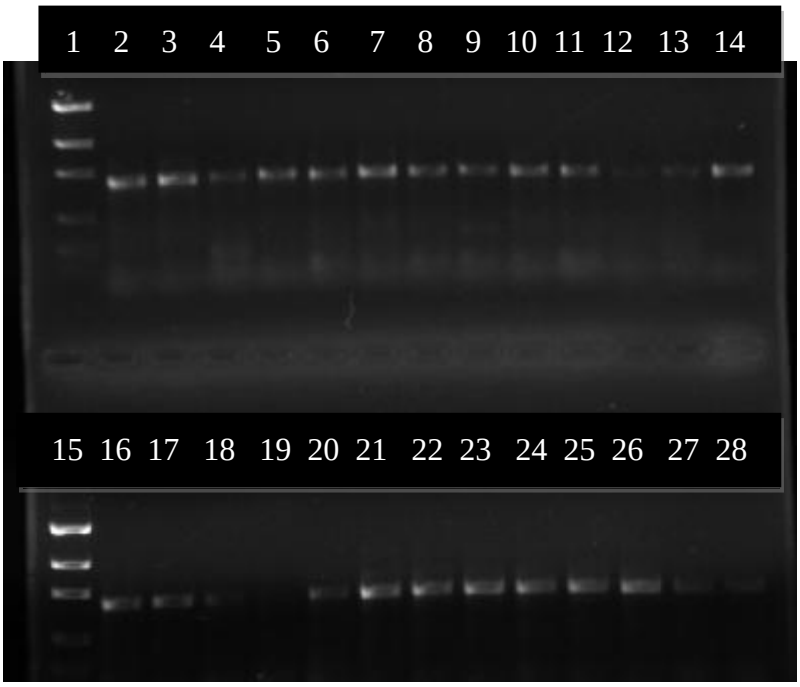


Figure 3.22. Gel testing 0.5 ng of DNA on samples B2D22, B2D27, B2D36, and B2D41.

After comparing all of the gels, it was determined that using 1 ng of DNA as template yielded the brightest bands for samples B1D9, B1D15, B1D38, B1D22, and the first two replicates of B2D27 while using 0.5 ng of DNA as template yielded the brightest bands for the last replicate of sample B2D27 as well as samples B2D36 and B2D41. PCR using these masses of DNA as template was carried out, and the resulting gels are shown in Figures 3.23a and 3.23b.



Lanes 1 and 15: low DNA mass ladder

Lanes 2-14 and 16-28: 1 ng of DNA as template

Figure 3.23a. Gel 1 testing 1 ng of DNA on samples B1D9, B1D15, B1D38, B2D22, and B2D27 (first 2 reps) and 0.5 ng of DNA on B2D27 (last rep), B2D36, and B2D41.

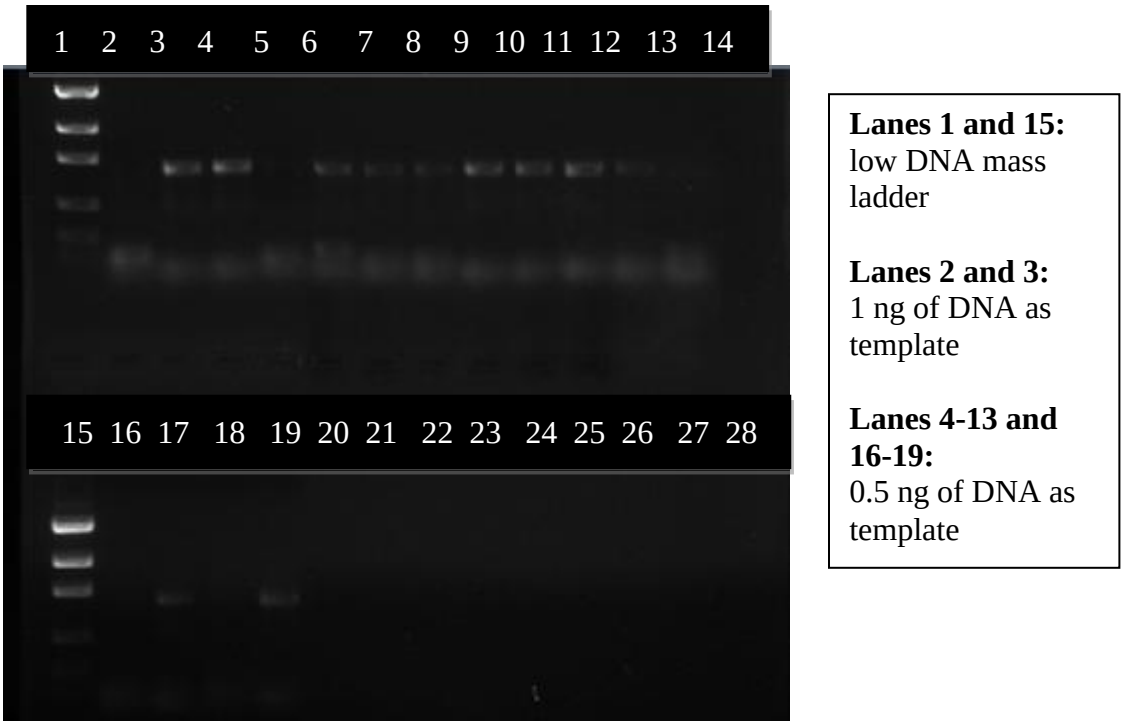


Figure 3.23b. Gel 2 testing 1 ng of DNA on samples B1D9, B1D15, B1D38, B2D22, and B2D27 (first 2 reps) and 0.5 ng of DNA on B2D27 (last rep), B2D36, and B2D41.

The bands for all samples were again not of uniform intensity. It appeared that every gel had some variation in band intensity, and that masses of DNA template that produced bright bands for one gel might produce very faint bands for the following gel when repeated. Although all of the reagents used for PCR were the same for all runs, there appeared to be variations in the results regardless of how many times it was repeated. Because of this, all gels were closely observed and it was determined that the DNA template mass of 1 ng gave bands for nearly all samples, even though some bands were of varying intensity. The replicate for each sample that had the brightest bands was

selected, and final PCR using these replicates with 1 ng of DNA as template was performed. As a control, two replicates were chosen for two of the samples, with one replicate having bright bands and one replicate having dim bands to see if these produced differences in the T-RFLP results. The final samples chosen were B1D9-1, B1D9-3, B1D15-1, B1D38-2, B2D22-2, B2D27-3, B2D36-1, B2D41-1, and B2D41-2.

The final PCR products for the above samples were purified using the Promega Wizard SV Gel and PCR Clean-Up System as described in section 2.21. The purified PCR products were then quantified, and the results are given in Table 3.16.

Table 3.16. DNA concentrations of purified PCR products for T-RFLP.

Sample	DNA Concentration of Purified PCR Products (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
B1D9-1	54.1	1.76	1.86
B1D9-3	65.6	1.87	1.67
B1D15-1	75.4	1.83	1.83
B1D38-2	63.0	1.92	1.69
B2D22-2	64.7	1.85	1.81
B2D27-3	55.2	1.96	1.67
B2D36-1	63.9	1.85	1.81
B2D41-1	59.9	1.92	1.95
B2D41-2	58.2	1.85	1.77

The DNA concentrations were in the expected range and were all very similar, despite

some differences in band intensity.

The purified PCR products were then digested using the restriction enzymes HhaI, AluI, and MspI as described in section 2.22. The digested PCR products were then purified using the Qiagen QIAquick Nucleotide Removal Kit as described in section 2.23. The digests were then placed in a 96-well plate and sent to the Genomic Sciences Laboratory at North Carolina State University for genotyping as described in section 2.24. The results were analyzed using the software listed in section 2.25.

The number of terminal restriction fragments (T-RFs), or the species number, is given in Table 3.17.

Table 3.17. Species number of selected samples.

Sample	Species Number
B1D9-1	22
B1D9-3	25
B1D15-1	24
B1D38-2	25
B2D22-2	32
B2D27-3	23
B2D36-1	24
B2D41-1	25
B2D41-2	24

Sample B2D22-2 had the highest number of T-RFs with 32. The other samples had a very similar number of T-RFS, ranging from 22 to 25. These similar values indicate that although the concentration of bacteria changed significantly over the operational cycle, the number of species present did not vary significantly.

The Shannon Diversity Index was determined for all of the samples. The results are given in Table 3.18.

Table 3.18. Shannon diversity index of selected samples.

Sample	Shannon Diversity Index (H)
B1D9-1	2.566
B1D9-3	2.540
B1D15-1	2.477
B1D38-2	2.641
B2D22-2	2.776
B2D27-3	2.516
B2D36-1	2.647
B2D41-1	2.764
B2D41-2	2.459

The Shannon Diversity Index is a measure of the diversity of the sample, both in terms of the abundance and the number of species present, with a higher Shannon Diversity Index indicating a more diverse sample. Sample B2D22-2 again had the highest value at 2.776.

Sample B2D41-1 was right behind it at 2.764. The other samples had values ranging from 2.459 to 2.647. Four of the samples with the lowest values, samples B1D15-1, B2D27-3, B1D9-3, and B1D9-1, were all taken from anoxic phases with wastewater flowing into that specific basin, either from the head of the plant or from the previous basin. The samples taken from aerobic phases, samples B1D38-2, B2D36-1, B2D41-1, and B2D22-2, all had higher values. Therefore, it appears that the samples taken from anoxic phases are less diverse, while those taken from aerobic phases are more diverse. The only sample that does not fit this trend is sample B2D41-2, which had the lowest value despite being taken from an aerobic phase.

The relative abundance of different fragment lenghts in each sample is given in Figure 3.24.

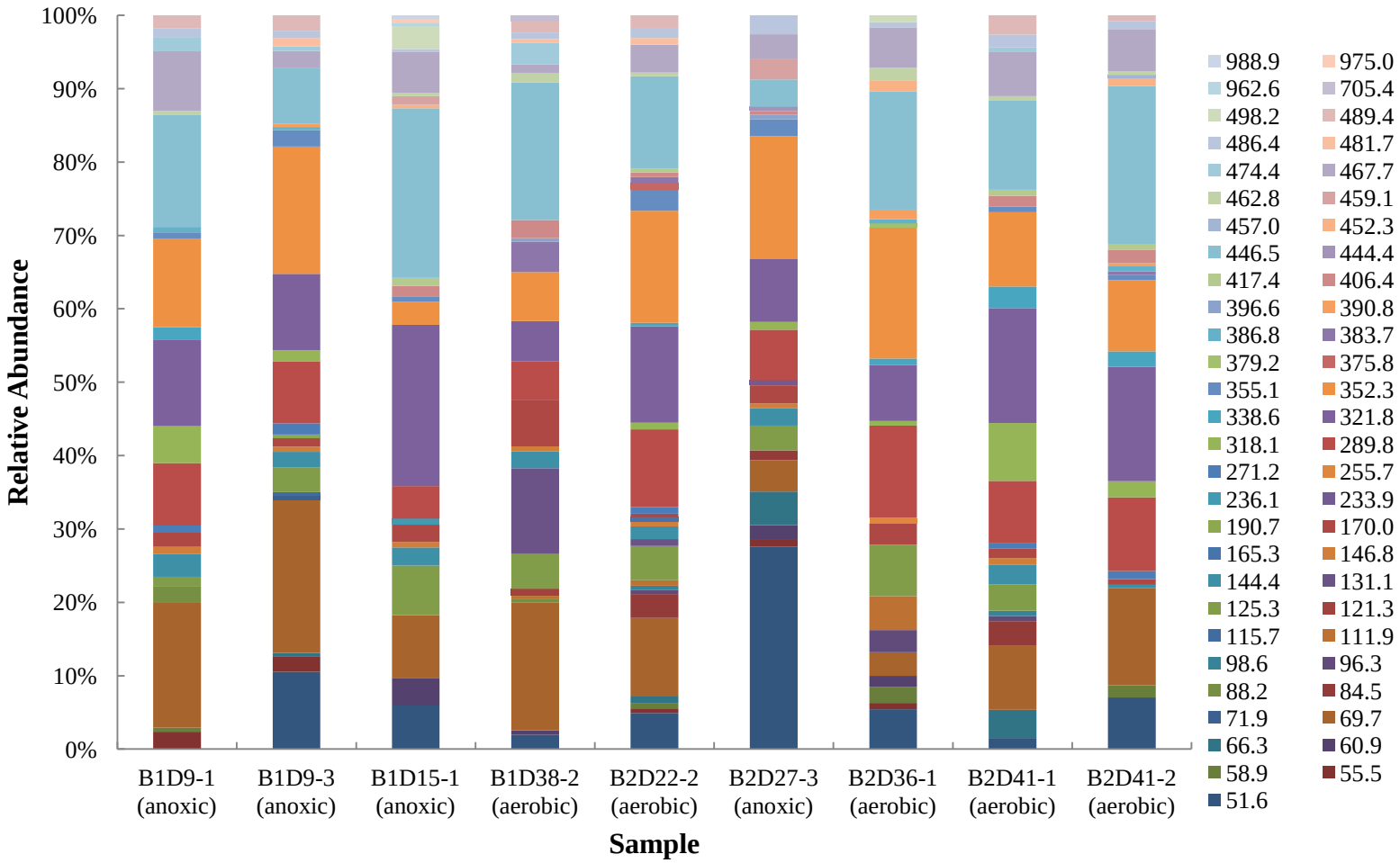


Figure 3.24. Relative abundance of fragment lengths.

The fragment lengths that are present and have the highest relative abundance in all samples are 51.6, 69.7, 289.8, 321.8, 352.3, 446.5, and 467.7. The relative abundance of these fragment lengths differs between the samples, but these fragment lengths are present in nearly all of the samples in significant quantities. This plot shows that although the same species are present in all of the samples, the abundance of these species changes greatly over the operational cycle of the treatment facility. There do not appear to be species that are present only during anoxic or aerobic phases, and the relative abundances of the species present in the samples do not appear to correlate with whether the sample was taken from an aerobic or anoxic phase. For example, fragment length 69.7 is present in all samples. For the anoxic samples, samples B1D9-1, B1D9-3, B1D15-1, and B2D27-3, the relative abundances are 17%, 21%, 9%, and 4%, respectively. For the aerobic samples, samples B1D38-2, B2D22-2, B2D36-1, B2D41-1, and B2D41-2, the relative abundances are 17%, 11%, 3%, 9%, and 13%, respectively. So, the relative abundance is high in both anoxic and aerobic samples and is also low in both anoxic and aerobic samples. This pattern is observed for all of the significant fragment lengths.

A phylogenetic analysis was performed on the T-RFLP results. Out of the 32 fragment lengths, 7 were able to be identified using an established *nosZ* database. The results are given in Figure 3.25.

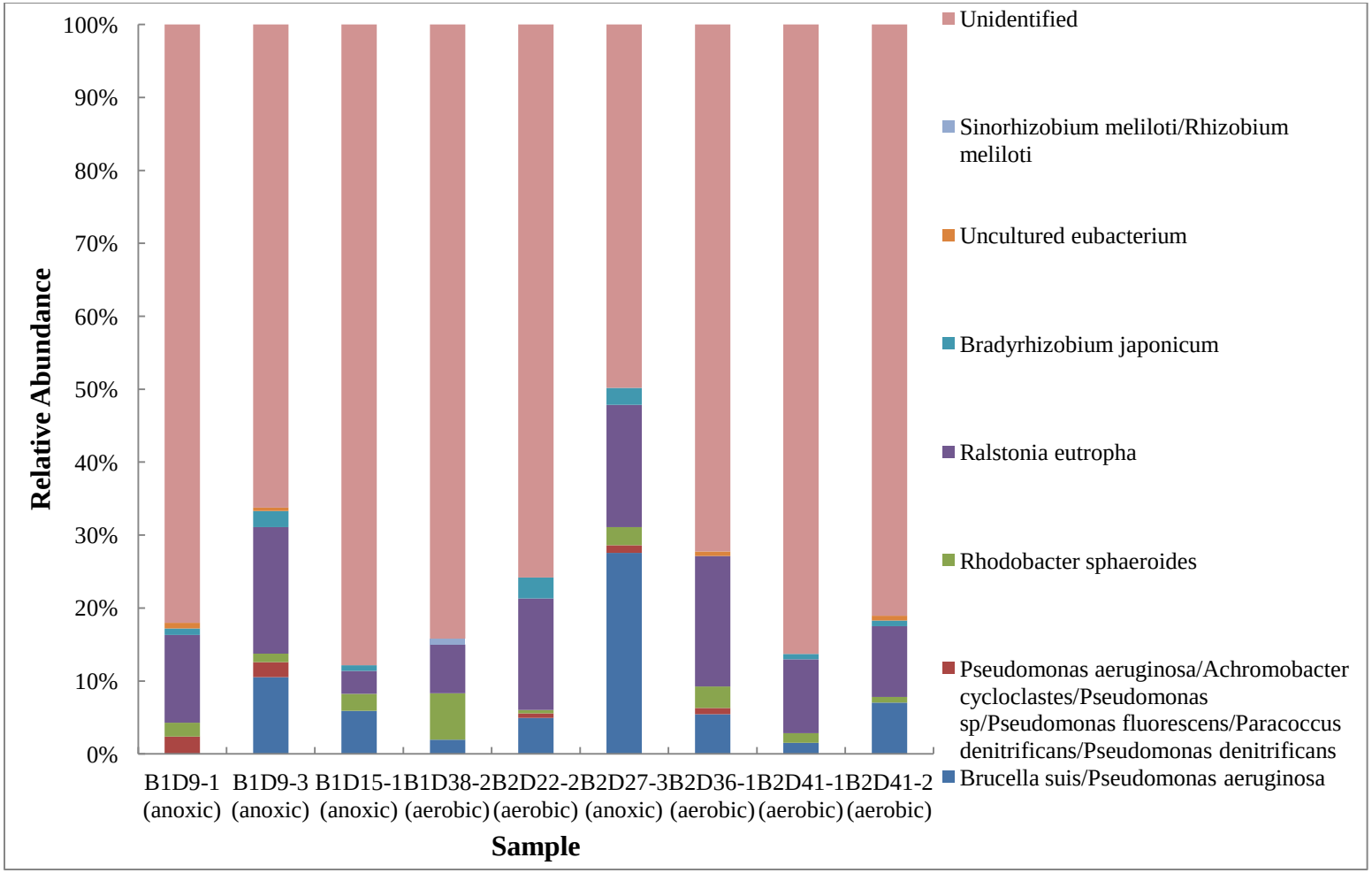


Figure 3.25. Relative abundance of microorganisms for selected samples.

Fragment length 51.6 was identified as most likely being *Brucella suis* or *Pseudomonas aeruginosa*, 55.5 as *Pseudomonas aeruginosa*, *Achromobacter cycloclastes*, *Pseudomonas sp*, *Pseudomonas fluorescens*, *Paracoccus denitrificans*, or *Pseudomonas denitrificans*, 170.0 as *Rhodobacter sphaeroides*, 352.3 as *Ralstonia eutropha*, 355.1 as *Bradyrhizobium japonicum*, 386.8 as uncultured *eubacterium*, and 705.4 as *Sinorhizobium meliloti* or *Rhizobium meliloti*. All of these organisms are denitrifiers (Pickering et al., 2012; Costanzo et al., 2012; Hirayama et al., 2011; Cramm et al., 1999; Mackenzie et al., 2007; Rinaldo et al., 2011; Fujita et al., 2007; Vacková et al, 2011; Parvanova-Mancheva and Beschkov, 2009; Dahouk et al., 2009; Hartsock and Shapleigh, 2010). The majority of the species present were unidentified. This is most likely because targeting *nosZ* during T-RFLP to characterize denitrifiers is a relatively new procedure, and an extensive database based on *nosZ* has not yet been constructed. Over time, as T-RFLP targeting *nosZ* becomes more common, a more complete database will most likely be constructed, giving a more complete picture of which denitrifiers are present.

CHAPTER 4: SUMMARY AND CONCLUSIONS

Nitrogen removal is an important part of wastewater treatment. Failure to do so can result in DO depletion, toxicity, eutrophication, global warming, acid rain, and other undesired effects. Understanding the denitrification process that occurs in wastewater treatment plants is important to allow engineers and plant operators to achieve more efficient nitrogen removal from wastewater. Due to the wide variety of physiological and taxonomic groups to which denitrifiers belong, quantifying them using ribosomal RNA is not possible. However, specific denitrification genes can be targeted instead.

Denitrification is a complex and highly regulated process. It is the conversion of NO_3^- to N_2 gas via stepwise reductions to NO_2^-, NO, and N_2O. Each step of the denitrification process is catalyzed by specific enzymes, and each enzyme is encoded for by a specific gene. *nosZ* is the gene that encodes for the enzyme nitrous oxide reductase. Nitrous oxide reductase is the enzyme that catalyzes the transformation of N_2O to N_2, the final step of denitrification.

Most, possibly all, of denitrifying bacteria are facultative aerobic chemoorganotrophic organisms, meaning their respiratory networks are designed so that there is preferential electron flow to O_2 under aerobic growth conditions. It is only when O_2 is limiting and NO_3^-/NO_2^- are present that the denitrification enzymes are expressed. Therefore, O_2 plays a major regulatory role in the process of denitrification. NO_3^-, NO_2^-, NO, and N_2O also have regulatory roles because of the fact that denitrification is a stepwise process where each product is a substrate for the subsequent reaction.

The goal of this research project was to quantify the denitrification gene *nosZ* in a full scale wastewater treatment plant over an entire operational cycle as aeration is turned on and off. Although nitrogen removal gene expression has been studied before in lab-scale reactors, a full-scale study has not been conducted. In a full-scale plant, the wastewater is cycled through both aerobic and anoxic phases to allow for both nitrification and denitrification, and the expression of denitrification genes therefore likewise cycles. By quantifying this change in gene expression over time, a better understanding of the regulation of denitrification genes was gained. In this study, the goal was to analyze both DNA and RNA to estimate the concentration of organisms capable of performing denitrification, as well as the concentration of organisms actively performing denitrification. This allowed us to determine the effect of oxygen concentration on the activity and growth of the organisms.

A standard curve of CT value versus log(cell concentration) was created for a pure culture of *Pseudomonas stutzeri*, a known denitrifier. Samples of water and activated sludge were taken from treatment basins 1 and 2 of the North Cary wastewater treatment plant in Cary, North Carolina over an entire cycle of operation (676 samples over 6 hours). This treatment plant uses a Bio-Denipho process. Dissolved oxygen concentration, pH, and oxidation-reduction potential were measured in the activated sludge basins 1 and 2 over an entire operational cycle. NO_3^- and NO_2^- concentrations in the wastewater were measured. DNA was extracted from selected samples, and qPCR targeting *nosZ* was performed to quantify the microorganisms capable of carrying out denitrification. RNA was also extracted from selected samples, followed by RT-PCR and qPCR targeting *nosZ* in an attempt to

quantify the microorganisms actively denitrifying. T-RFLP was then performed on selected samples in order to analyze the microbial community.

For the standard curve, an R^2 value of 0.99 for the linear trendline indicated that there was a high correlation between the cell count and CT value. This meant that the trendline could therefore be used to accurately calculate the cell count of a sample given that the CT value is known.

The DO concentrations that were measured in basins 1 and 2 correlated very closely to the expected aerobic and anoxic phases for both basins. The pH was also measured in basins 1 and 2. From the results, the pH did not seem to be at all dependent on whether nitrification or denitrification was occurring. Most likely, the pH observed was simply the inherent pH of the incoming wastewater that did not change as it moved through the different basins and phases. Any effects on the pH that nitrification or denitrification may have had were most likely negated by the buffering effects of alkalinity present in the wastewater. The ORP was also measured in basins 1 and 2. However, the ORP did not appear to differ significantly between basins 1 and 2, despite the fact that basins 1 and 2 had opposing aerobic/anoxic conditions during several of the phases and should have had notably different ORPs. It is thought that the reason for this unexpected trend was due to improper operation of the ORP probe.

The NO_3^- concentrations in both basins 1 and 2 were as expected. NO_3^- is produced during nitrification, which takes place during aerobic conditions. NO_3^- is consumed during denitrification, which takes place during anoxic conditions. Therefore, when the air was

turned on, the NO_3^- concentration began to increase and increased until the air was turned off, at which point it began to decrease and decreased until it reached a concentration of 0 mg/L. This occurred in both basins 1 and 2 for all phases. The NO_2^- concentrations in both basins 1 and 2 were also as expected. NO_2^- is produced during ammonia oxidation, and then subsequently consumed during nitrite oxidation. NO_2^- is also produced and then subsequently consumed during denitrification. The NO_2^- concentration therefore increased at the beginning of aerobic phases but began to decrease before the air was turned off. The same trend was observed during the anoxic phases, with the NO_2^- concentration increasing at the beginning of the anoxic phases and decreasing before the air was turned on.

From the qPCR performed targeting the nosZ gene present in DNA, it was determined that the concentration of cells with *nosZ* changes significantly over the different phases of operation at a full-scale Bio-Denipho wastewater treatment facility. This change appears to correlate not only with the presence of absence of O_2, but also with availability of organic carbon to utilize as an electron donor. When the air is turned off, the increase in cells with *nosZ* begins almost immediately. When the air is turned on, there appears to be a slight lag before the concentration of cells with *nosZ* begins to decrease. This may be due to the fact that some residual O_2 remains in the water, or it could be due to carry-over from the previous basin. Also, when there is flow into a basin, whether from the head of the plant of from the previous basin, there is usually at least a slight increase in the concentration of cells with *nosZ*, even when the air is turned on. This is most likely due to an in-flow of organic carbon providing electrons for growth. During some of the anoxic phases, there appears to

be a decrease in the concentration of cells with *nosZ* towards the end of the phase before the air is turned on again. This decrease could be due to an insufficient supply of organic carbon.

Attempts to quantify bacteria actively carrying out denitrification by extracting RNA, synthesizing cDNA by RT-PCR, and performing qPCR targeting the *nosZ* gene were unsuccessful and did not result in any usable CT values.

T-RFLP was performed on selected samples to analyze the microbial community. The number of T-RFs was very similar for all samples, meaning that the number of species present was about the same for all of the samples analyzed. The Shannon Diversity Index, which is a measure of the diversity of the sample in terms of the abundance and the number of species present, was calculated for all samples. From this, it appeared that the samples taken from anoxic phases were less diverse, while those taken from aerobic phases were more diverse. The relative abundance of the different species present was determined for each sample. Although the same species were present in all of the samples, the abundance of these species changed greatly over the operational cycle of the treatment facility. There did not appear to be species that were present only during anoxic or aerobic phases, and the relative abundances of the species present in the samples did not appear to correlate with whether the sample was taken from an aerobic or anoxic phase. Lastly, PAT was used to identify the fragment lengths. Through this analysis, 7 of the 32 fragment lengths were identified as most likely belong to specific bacteria. The rest of the fragment lengths remained unidentified.

CHAPTER 5: FUTURE WORK

Important information was gathered about nitrogen removal in a full-scale wastewater treatment plant over an entire cycle of operation. However, in order to fully understand the regulation and expression of the denitrification genes and how this changes over cycling aerobic and anoxic phases, the following needs to be performed:

a. Optimize the RNA extraction method so that the concentration of inhibitors is low enough for qPCR to successfully be performed. This will provide information about the quantity of bacteria actively performing denitrification and how the expression of denitrification genes changes over a full operational cycle.
b. Perform qPCR targeting other denitrification genes to see if the trends observed for *nosZ* hold for all genes.
c. Operate lab-scale bioreactors under conditions conducive to nitrification/denitrification with cycling aerobic and anoxic phases. Change various parameters, such as DO concentration, pH, NO_3^- concentration, temperature, organic carbon concentration, in order to see how these factors impact denitrification and the expression and regulation of denitrification genes.

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APPENDICES

APPENDIX A

Laboratory DNA Extraction Method (Aluminum Sulfate Method)

Materials:

- Humic acid removal solution: 100 mM NaH_2PO_4; 100 mM aluminum sulfate (pH 6.0)
- Lysis solution: 100 mM NaCl; 500 mM Tris; 10% w/v sodium dodecyl sulfate; 1% sodium pyrophosphate (pH 9.0)
- 100% isopropanol
- 70% ethanol
- 7.5 M ammonium acetate
- TE Buffer (pH 8.0)
- Zirconium beads (100 µm, previously baked at 200°C for 2 hours)
- pH strips

Method:

1.) Humic substances are precipitated prior to cell lysis by addition of a low pH aluminum sulfate solution (400 µL of Humic Acid Removal Solution) to 0.25 g sample.

2.) Vortex and measure pH. If needed, adjust to 6.5 by addition of HCl. Record pH.

3.) Raise pH to 9.0 to 9.5 with sodium hydroxide (NaOH) and vortex briefly. Be careful not to go above pH 10.0.

4.) Add 0.25 g acid washed zirconium beads (100 µm diameter).

5.) Add 400 µL of Lysis Solution.

6.) Bead beat at max speed 1 minute.

7.) Centrifuge tubes for 5 minutes at 13,200 rpm and transfer clean supernatant. Centrifuge again if necessary to remove debris.

8.) Remove protein by adding 0.5 volume of 7.5 M Ammonium Acetate.

9.) Invert to mix. Incubate on ice for 10 minutes.

10.) Centrifuge tubes for 5 minutes at 13,200 rpm and transfer supernatant. Centrifuge again if necessary to remove white precipitate.

11.) Add 1 volume of 100% isopropanol to supernatant and invert to mix.

12.) Incubate at room temperature for 5 minutes.

13.) Centrifuge tubes for 5 minutes at 13,200 rpm and carefully decant supernatant.

14.) Add 1 mL of 70% ethanol to DNA pellet. Vortex briefly.

15.) Centrifuge tubes for 5 minutes at 13,200 rpm and carefully remove all ethanol using a pipette.

16.) Allow pellet to air dry. Do not over dry.

17.) Resuspend pellet in 100 µL of TE Buffer (pH 8.0).

MOBIO Laboratories PowerSoil DNA Isolation Kit

Materials**:**

- PowerBead Tubes
- PowerSoil Solution C1: aqueous solution of sodium dodocyl sulfate and other proprietary salts

- PowerSoil Solution C2: aqueous solution of acetate and other proprietary salts
- PowerSoil Solution C3: proprietary
- PowerSoil Solution C4: Aqueous solution of guanidine HCl, isopropanol, and other proprietary salts
- PowerSoil Solution C5: Aqueous solution of ethyl alcohol and proprietary salts
- PowerSoil Solution C6: Aqueous solution of Tris (hydroxymethyl) aminomethane
- PowerSoil Spin Filters
- PowerSoil 2 mL collection tubes

Method:

1.) To the PowerBead Tubes provided, add 0.25 g of soil sample.

2.) Gently vortex to mix.

3.) Check Solution C1. If Solution C1 is precipitated, heat solution to 60□C until dissolved before use.

4.) Add 60 µl of Solution C1 and invert several times or vortex briefly.

5.) Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

6.) Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature.

7.) Transfer the supernatant to a clean 2 ml Collection Tube (provided).

8.) Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

9.) Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

10.) Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube (provided).

11.) Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.

12.) Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

13.) Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean 2 mL Collection Tube (provided).

14.) Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.

15.) Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature.

16.) Add 500 µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

17.) Discard the flow through.

18.) Centrifuge again at room temperature for 1 minute at 10,000 x *g*.

19.) Carefully place spin filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

20.) Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

21.) Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

APPENDIX B

RNA Extraction Protocol

Materials:

- 5X pH 5.1 buffer (50 mM sodium acetate; 10 mM EDTA)
- 20% SDS (pH 7.2)
- Phenol (pH 5.1)
- 4:1 Phenol:Chloroform (pH 5.1)
- Baked Zirconium beads
- 1X Chloroform
- 10 M ammonium acetate
- Absolute ethanol
- 80% ethanol
- RNAse-free water

Method:

1.) Into 2 mL screw capped centrifuge tubes place:
    a. 0.5 g baked zirconium beads
    b. 0.5 mL sample and 5X pH 5.1 buffer
    c. 50 µL 20% SDS
    d. Fill remaining volume with phenol (pH 5.1) to around 1 mL
2.) Beat 2 minutes on bead beater
3.) If using phenol, incubate 10 minutes at 60°C

4.) Beat another 2 minutes

5.) Centrifuge at 5,000 rpm for 5 minutes to pellet beads

6.) Remove aqueous and organic phase to clean tube

7.) Wash beads and recover residual lysate by doing the following:

    a. Rinse beads with 200 µL pH 5.1 buffer

    b. Beat 1 minute

    c. Centrifuge at 5,000 rpm for 5 minutes

    d. Pool the aqueous portion with the previous aqueous/organic phases from step 6

8.) Centrifuge aqueous and organic phases at 10,000 rpm for 10 minutes to separate the two phases

9.) Remove the aqueous phase (containing the nucleic acid) to a clean vial

10.) Repeat phenol extraction by adding an equal volume (compared to the recovered lysate) of 4:1 phenol:chloroform (pH 5.1) to the lysate. Vortex for 5 to 10 seconds then centrifuge at 10,000 rpm for 10 minutes. Transfer the aqueous phase to a new 2 mL tube.

11.) Repeat step 10

12.) Repeat step 10 using 1X chloroform (instead of 4:1 phenol:chloroform). In this step, be careful that you do not transfer the organic phase into the new tube.

13.) Precipitate nucleic acid from the final aqueous phase by adding ½ volume of 10 M ammonium acetate and 2 volumes of absolute ethanol. Vortex well.

14.) Keep at -20°C for at least an hour (can also leave overnight if desired).

15.) Centrifuge at 14,000 rpm for 30 minutes. Pour off the supernatant, taking care to retain the RNA pellet (if one is visible).

16.) Wash by soaking the RNA pellet with 2 mL of 80% ethanol and gently inverting the tube 3 to 4 times.

17.) Pour off the ethanol and invert the tube onto a clean Kimwipe to dry for 5 to 10 minutes.

18.) Resuspend the RNA in 100 µL of sterile, RNAse-free water and vortex briefly to dissolve the pellet.

MOBIO Laboratories RNA PowerSoil Total RNA Isolation Kit

Materials:

- RNA PowerSoil Bead Tubes (with 1.5 g beads)
- RNA PowerSoil Bead Solution: Aqueous solution of Guanidine Thiocyanate and proprietary salts
- RNA PowerSoil Solution SR1: Aqueous solution of Sodium Dodocyl Sulfate and proprietary salts
- RNA PowerSoil Solution SR2: Proprietary
- RNA PowerSoil Solution SR3: Proprietary
- RNA PowerSoil Solution SR4: Proprietary
- RNA PowerSoil Solution SR5: Proprietary
- RNA PowerSoil Solution SR6: Proprietary

- RNA PowerSoil Solution SR7: DEPC treated water
- RNA PowerSoil RNA Capture Columns
- RNA PowerSoil 15 mL collection tubes
- RNA PowerSoil 2.2 mL collection tubes
- Phenol:chloroform:isoamyl alcohol (25:24:1)

Method:

1.) Add up to 2 g of soil to the 15 ml **Bead Tube** (provided).
2.) Add 2.5 mL of Bead Solution to the Bead Tube and vortex to mix.
3.) Add 0.25 mL of Solution SR1 to the Bead Tube and vortex to mix.
4.) Add 0.8 mL of Solution SR2 and place the Bead Tube on the vortex adapter and vortex at maximum speed for 5 minutes.
5.) Remove the Bead Tube from the vortex adapter and add 3.5 mL of phenol:chloroform:isoamyl alcohol (pH 6.5 to 8.0), and vortex to mix until the biphasic layer disappears.
6.) Place the Bead Tube on the vortex adapter and vortex at maximum speed for 10 minutes.
7.) Remove the Bead Tube from the vortex adapter and centrifuge at 2,500 x g for 10 minutes at room temperature.
8.) Remove the Bead Tube from the centrifuge and carefully transfer the upper aqueous phase (avoiding the interphase and lower phenol layer) to a clean 15 mL collection

tube (provided). The thickness of the interphase will vary depending on the type of

9.) soil used. Discard the phenol:chloroform:isoamyl alcohol in an approved waste receptacle. NOTE: The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.

10.) Add 1.5 mL of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 4°C for 10 minutes.

11.) Centrifuge at 2,500 x g for 10 minutes at room temperature.

12.) Transfer the supernatant, without disturbing the pellet, to a new 15 mL collection tube (provided).

13.) Add 5 mL of Solution SR4 to the collection tube containing the supernatant, invert or vortex to mix, and incubate at -20°C for 30 minutes.

14.) Centrifuge at 2,500 x g for 30 minutes at room temperature.

15.) Decant the supernatant and invert the 15 mL collection tube and resuspend the pellet completely. (NOTE: Depending on the soil type, the pellet may be large and/or dark in color.)

16.) Add 1 mL of Solution SR5 to the 15 mL collection tube and resuspend the pellet completely. (NOTE: Depending on the soil type, the pellet may be difficult to resuspend. Resuspension may be aided by placing the tubes in a heat block or water bath at 45°C for 10 minutes, followed by vortexing. Repeat until the pellet is resuspended.)

17.) Prepare one RNA capture column (provided) for each RNA isolation sample:

a. Remove the cap of a 15 mL collection tube (provided) and place the RNA capture column inside the 15 mL collection tube. The column will hang in the 15 mL tube.

b. Add 2 mL of Solution SR5 to the RNA capture column and allow it to gravity flow through the column and collect in the 15 mL collection tube. Allow Solution SR5 to completely flow through the column (OPTIONAL: the collection tube may be emptied after Solution SR5 has completely flowed through the column. NOTE: do not allow the column to dry out prior to loading the RNA isolation sample.)

18.) Add the RNA isolation sample from step 15 onto the RNA capture column and allow it to gravity flow through the column. Collect the flow through in the 15 mL collection tube.

19.) Wash the column with 1 mL of Solution SR5. Allow it to gravity flow and collect the flow through in the 15 mL collection tube.

20.) Transfer the RNA capture column to a new 15 mL collection tube (provided) and add 1 mL of Solution SR6 to the RNA capture column to elute the bound RNA into the 15 mL collection tube. Allow Solution SR6 to gravity flow into the 15 mL collection tube.

21.) Transfer the eluted RNA to a 2.2 mL collection tube (provided) and add 1 mL of Solution SR4. Invert at least once to mix and incubate at -20°C for 10 minutes.

22.) Centrifuge the 2.2 mL collection tube at 13,000 x g for 15 minutes at room temperature to pellet the RNA.

23.) Decant the supernatant and invert the 2.2 mL collection tube onto a paper towel for 10 minutes to air dry the pellet.

24.) Resuspend the RNA pellet in 100 µL of Solution SR7. (NOTE: Although DNA carryover does not occur with the majority of soil types, certain soils high in organic matter may present unique carryover situation. In situations where the absence of DNA contamination is critical, the purified RNA should be tested for potential DNA carryover by performing PCR with qualified primers on the isolated RNA without performing prior reverse transcription amplification. The absence of a detectable amplification fragment by agarose electrophoresis indicates the absence of detectable carryover DNA. In the event DNA is detected, DNAse treatment of the isolated RNA is recommended.)

APPENDIX C

DNase Treatment Procedure

1.) Prepare 1 mL of 10X DNase buffer (500 mM Tris-Cl, pH of 8; 120 mM $MgCl_2$; 10 mM $CaCl_2$ as follows:
    a. 500 µL of 1 M Tris-Cl
    b. 120 µL of 1 M $MgCl_2$
    c. 10 µL of 1 M $CaCl_2$
    d. 370 µL of RNase/DNase free water
2.) Add the following to the extracted RNA (50 µL):
    a. 20 µL of 10X DNase buffer
    b. 5 µL of DNaseI from Qiagen
    c. Bring the volume to 200 µL by adding 125 µL of RNase/DNase free water
    d. DNaseI is a very sensitive enzyme. Mix solution by carefully inverting the tube.
3.) Incubate at 37°C for 30 minutes.
4.) Following the DNase digestion, the DNaseI can be inactivated at 70°C for 10 minutes.

APPENDIX D

Qiagen RNeasy MinElute Cleanup Procedure

Materials:

- RNeasy MinElute spin columns
- Collection tubes (1.5 mL)
- Collection tubes (2.0 mL)
- Buffer RLT: guanidine thiocyanate and other proprietary additions
- Buffer RPE (concentrate): proprietary
- RNase free water
- Absolute ethanol

Method:

1.) Add 4 volumes of ethanol to buffer RPE for a working solution.

2.) Adjust the sample to a volume of 100 µL with RNase-free water. Add 350 µL Buffer RLT and mix well.

3.) Add 250 µL ethanol to the diluted RNA and mix well by pipetting. Do not centrifuge. Proceed immediately to step 4.

4.) Transfer the sample (700 µL) to an Rneasy MinElute spin column placed in a 2 mL collection tube (supplied). Close the lid gently, and centrifuge for 15 seconds at ≥ 10,000 rpm. Discard the flow-through.

5.) Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied).

Add 500 µL Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 seconds at ≥ 10,000 rpm to wash the spin column membrane. Discard the flow-through.

6.) Add 500 µL of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 minutes at ≥ 10,000 rpm to wash the spin column membrane. Discard the flow-through and the collection tube.

7.) Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 minutes to dry the membrane. Discard the flow-through and collection tube.

Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14 µL RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 minute at full speed to elute the RNA.

APPENDIX E

Bio-Rad iScript cDNA Synthesis Procedure

Materials:

- 5X iScript Reaction Mix
- Nuclease-free water
- iScript Reverse Transcriptase

Method:

1.) Add 4 µL of 5X iScript reaction mix, 1 µL of iScript reverse transcriptase, the volume of RNA template necessary to achieve 100 fg to 1 µg total RNA, and the necessary volume of nuclease-free water to bring the total reaction volume to 20 µL to a collection tube.

2.) Incubate complete reaction mix for:

   a. 5 minutes at 25°C
   b. 30 minutes at 42°C
   c. 5 minutes at 85°C
   d. Hold at 4°C (optional)

APPENDIX F

cDNA Purification Procedure

Materials:

- cDNA Binding Buffer
- cDNA Wash Buffer
- Micro Filter Cartridges + Collection Tubes
- Micro Elution Tubes
- Nuclease-free Water
- 100% Ethanol

Method:

1.) Add 9.6 mL ACS grade 100% ethanol to the cDNA Wash Buffer. Mix well.

2.) Preheat the 5 mL bottle of Nuclease-free Water to 55°C for at least 10 minutes.

3.) Immediately before starting the cDNA purification, equilibrate one Micro Filter Cartridge for each sample:

   a. Add 30 µL cDNA Binding Buffer to a Micro Filter Cartridge assembled in a Collection Tube.

   b. Incubate for 5 minutes at room temperature (the cDNA Binding Buffer does not need to be spun through).

4.) If the DNA sample volume is less than 100 µL, bring it to 100 µL using Nuclease-free Water.

5.) Add 250 µL of cDNA Binding Buffer to each sample, and mix thoroughly by repeated pipetting or gentle vortexing.

6.) Apply the mixture to an equilibrated Micro Filter Cartridge

   a. Pipet the mixture from step 5 into an equilibrated Micro Filter Cartridge (from step 3).

   b. Centrifuge for 1 minute at 10,000 x g or until the mixture is through the filter.

   c. Discard the flow-through and replace the Micro Filter Cartridge in the Collection Tube.

7.) Wash the Micro Filter Cartridge with 500 µL cDNA Wash Buffer:

   a. Apply 500 µL of cDNA Wash Buffer to each Micro Filter Cartridge.

   b. Centrifuge for 1 minute at 10,000 x g or until all the cDNA Wash Buffer is through the filter.

   c. Discard the flow-through and spin the Micro Filter Cartridge for an additional minute to remove trace amounts of ethanol.

   d. Transfer the Micro Filter Cartridge to a Micro Elution Tube, and discard the Collection Tube.

8.) Elute the cDNA with 2 aliquots of preheated Nuclease-free Water:

   a. Apply 8 to 50 µL Nuclease-free Water (preheated to 55°C) to the filter in the Micro Filter Cartridge.

   b. Leave at room temperature for 2 minutes and then centrifuge for 1 minute at 10,000 x g or until all the Nuclease-free Water is through the filter.

c. Repeat the elution with a second 8 to 50 µL of preheated Nuclease-free Water. The double-stranded cDNA will now be in the Nuclease-free Water in the Micro Elution Tube.

d. Discard the Micro Filter Cartridge.

APPENDIX G

T-RFLP Procedure

1.) Optimize the PCR product.

a) Select 3-5 representative samples from your study.

b) Identify 2-3 template masses or volumes as starting point and run PCR using FAM (or other fluorophore) forward labeled primer.

If masses are used, typically 1 ng, 5 ng and 10 ng template masses are good starting points using 25 uL PCR reaction volumes.

c) View the PCR product on an agarose gel and identify the template mass used that gave the most consistent and highest amplification (band intensity) between samples.

d) Visually inspect the band intensity between samples. If band intensities between samples have reasonably similar band intensities then optimization has likely been achieved.

NOTE: This is a subjective evaluation and may require some practice. A more definitive determination will be done later, or, one can quantify the bands using Gel-Doc or equivalent software.

e) If some samples do not have product or the band intensity is low, analyze any trends observed and re-run PCR with a new range of template masses.

f) Iterate steps 1c and 1d until all samples have been optimized.

2) Perform a 'final' PCR on all samples using the optimum template mass determined. Duplicate reactions should be run for each sample.

3) Combine duplicate reactions into a single tube and purify using the Qiagen or Promega PCR purification kit.

4) Quantify the purified PCR product using the NanoDrop. All concentrations should be within ~20-30%, however; a larger range of variation may be warranted for hard to optimize samples.

   Another exception is for low PCR concentrations <10 ng/uL. Experience has shown the NanoDrop can fluctuate at lower concentrations nearing the devices detection threshold (2 ng/uL). Thus, if samples are within 1-2 ng/uL when their concentration is <10 ng/uL then this is likely satisfactory.

5) Select 4-bp cutter restriction enzymes (REs) that have optimal or acceptable resolving power for the primer set(s) being used. Resolving power refers to enzymes that yield the greatest number of terminal restriction fragments for a particular primer set. RE optimization and selection can be performed at the MICA website (http://mica.ibest.uidaho.edu).

   For example, if you are using the ARC 109f/915r primer set for *Archaea*, the enzymes AluI, BfaI, and TaqI offer the best resolving power.

   If you are using the 8f/1492r primer set for *Bacteria* then the most widely used REs are HhaI, RsaI and MspI. However, these enzymes do not necessarily have the highest resolving power. Other primer sets will typically have different REs that perform optimally.

6) Set up a restriction digest such that 360 ng of purified PCR product are placed into a digest vol. of 20 uL. This needs to be quantitative so you will want to Nanodrop after

purification of the 'final' PCR. After purification (Step 7), this starting mass will yield ~300 ng in a volume of 25 uL (roughly 10-15 ng/uL).

For the digest reaction, typically this is:

0.5 uL restriction enzyme (correlates to 5-10 units of activity),

2 uL 10X buffer (comes w/ the enzyme),

X uL PCR product, and the remainder is water

(If the instructions say add BSA then add BSA as this increases digest efficiency.)

IMPORTANT NOTE: Each digest is separate so for 1 sample you have 1 purified PCR product then you set up 3 tubes. So each tube will contain 1 enzyme. In this way you will have, for example: Sample1-AluI, Sample1-Bfa1 and Sample1-TaqI.

7) Run the digest according to the stated digest temp for 4 hours then inactivate per the instructions. You do not need to run them overnight. Programs are set up on both the Bio-Rad and Eppendorf thermocyclers entitled "REDIGEST" that you can use.

IMPORTANT NOTE: Not all digest and inactivation temperatures are the same for each restriction enzyme. Thus, you WILL need to edit the thermocycler program AND perhaps digest each enzyme separately from one another.

8) After digesting, purify each digest using the Qiagen Nucelotide Removal kit. DO NOT use a PCR purification kit. So, if you started w/ 10 samples you will have 30 digests and 30 purifications to do. Elute the purified digest using 25 uL of elution buffer, not the 30-50 uL they say in the protocol.

9) Transfer the entire volume to a 96-well plate and you are ready to prepare your plate for submission to GRL.

a) Submit a plate record on-line to GRL with your sample listing.

b) Prepare a new plate to run on the sequencer. Each well should contain 10 uL total of the following:

2 - 5 uL sample digest, typical (~20-40 ng digested PCR product)

0.5 uL Size Standard Marker (MapMarker1000 or similar)

X uL Hi-Di formamide

10 uL Total

10) Submit plate for sequencing.

APPENDIX H

PCR Product Purification Procedure

Materials:

- Membrane binding solution: guanidinium thiocyanate and other proprietary additions
- Membrane wash solution: ethanol and other proprietary additions
- Nuclease-free water
- Wizard SV minicolumns
- Collection tubes

Method:

1.) Add an equal volume of Membrane Binding Solution to the PCR amplification.
2.) Insert SV Minicolumn into Collection Tube.
3.) Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
4.) Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5.) Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
6.) Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
7.) Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
8.) Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.

9.) Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.

10.) Discard Minicolumn and store DNA at 4°C or –20°C.

An elution volume of 30 µL was used.

APPENDIX I

Digested PCR Product Purification Procedure

Materials:

- QIAquick spin columns
- Buffer PN (binding buffer)
- Buffer PE (wash buffer)
- Buffer EB (elution buffer)
- 2 mL collection tubes

Method:

1.) Add 10 volumes of Buffer PN to 1 volume of the reaction sample and mix.

2.) Place a QIAquick spin column in a provided 2 ml collection tube.

3.) To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min at 6000 rpm.

4.) Discard the flow-through and place QIAquick column back into the same tube.

5.) To wash QIAquick column, add 750 µl of Buffer PE and centrifuge for 1 min at 6000 rpm.

6.) Discard the flow-through and place the QIAquick column back in the same tube, which should be empty. Centrifuge for an additional 1 min at 13,000 rpm (17,900 x g).

7.) Place the QIAquick column in a clean 1.5 ml microcentrifuge tube.

8.) To elute DNA, add 100–200 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH

7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min at 13,000 rpm (17,900 x g). Alternatively, for increased DNA concentration, add 30–50 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

An elution volume of 25 µL was used.