EFFECT OF BIOAUGMENTATION PRODUCT BIOWISH® AQUA™ ON NITROGEN REMOVAL IN WASTEWATER

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ABSTRACT

Effect of Bioaugmentation Product BiOWiSH® Aqua™ on Nitrogen Removal in Wastewater

Patrick Cassidy Kalvass

Biological nutrient removal (BNR) from wastewater, and specifically nitrogen removal, is a growing concern to wastewater dischargers such as municipalities. Excess nutrients in effluent can create problems such as eutrophication, toxicity to aquatic life, and dissolved oxygen depletion in receiving waters. BNR systems have been installed in many locations with success, but their operation presents operational and financial demands greater than conventional biological treatment. Nitrogen removal is typically performed in sequential autotrophic nitrification and denitrification, which increases needed energy input, operational complexity, and therefore cost. Simultaneous nitrification-denitrification (SNdN) achieved in a single system has also been successfully implemented, however operational parameters that compromise between ideals for aerobic nitrification and anoxic denitrification result in decreased reaction rates and removal efficiencies. The application of a product that could potentially enhance SNdN reaction rates and removal efficiencies through bioaugmentation could help ease operational and financial strains.

In contrast to common sequential processes, some heterotrophic *Bacillus* bacteria have demonstrated SNdN (Kim et al., 2005), (Zhang et al., 2011). However, their application outside of laboratory setting has yet to be established. Aqua™ is a proprietary bioaugmentation product composed of specific *Bacillus* strains developed by BiOWiSH® Technologies with the intent of improving aerobic, heterotrophic SNdN rates and removal efficiencies. Screening and bench-scale experiments were performed in flasks at 35° C on orbital shakers operated at a range of speeds. Primary wastewater and minimal media were

used for the experiment, and inoculation was performed with both specific *Bacillus* strains and Aqua™.

Rapid total ammonia nitrogen (TAN) removal was observed in initial screening experiments with Aqua™ in sterile wastewater. *Bacillus pumilus* was identified as the fastest growing organism of the Aqua™ assemblage with the greatest TAN removal 1st order rate constant (0.32/ hr.), decreasing TAN 96% within 10 hours from an initial 48.5 ppm.

The orbital shaker speed that maximized TAN removal was 100 rpm, with reduction 47% and 88% more effective than both the upper (150 rpm) and lower (50 rpm) bound tested speeds, respectively. Visible floc growth centered in flasks, along with optical density data indicated cell growth and the possibility the system could support SNdN. Carbon amendments to minimal media were then evaluated, and sodium succinate improved TAN reduction by 53% compared to dextrose amended systems. This was likely because dextrose metabolism requires glycolysis to produce pyruvate for utilization in the TCA cycle for energy production; while succinate avoids glycolysis and thus is more easily utilized. In another experiment, flasks with supplemental trace minerals had a 59% higher TAN removal than the controls. Additions of supplemental vitamin solution or yeast extract improved TAN removal by 18% and 38%, respectively.

Two 10-day experiments assessed Aqua™ performance in municipal primary clarifier effluent. Nitrogen balance and optical density data showed that Aqua™ dosing at 10 ppm had no effect on nitrogen removal. The second 10-day experiment increased Aqua™ dosing to 50 ppm and evaluated product activation through incubation in growth media prior to inoculation. Nitrogen balance analysis showed no effect from Aqua™ on nitrogen removal during the second 10-day experiment as well. Systems amended with dextrose saw an initial

rapid TAN first order removal rate (0.25/ hr.). However, difference between control and inoculated flasks was negligible showing no effect from Aqua™. A lack of total nitrogen losses and a lack of nitrate presence during initial rapid TAN losses confirmed these losses were by assimilation into organic nitrogen.

The above experiments suggest that initial success in TAN removal during screening experiments resulted from lack of competition with other microorganisms, the high 1500 ppm dose of Aqua™, and amended dextrose.

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1. INTRODUCTION

The following thesis work was prompted through research aims of BiOWiSH® Technologies with the goal of validating their proprietary biological product, Aqua™. Aqua ™ is a bioaugmentation product created with the intention of promoting rapid nitrogen removal from wastewater. In the remainder of this thesis BiOWiSH® Technologies will further be referred to as “BiOWiSH,” and any reference to “BiOWiSH” refers to BiOWiSH® Technologies and their representatives. Additionally, reference to Aqua™ implies trademark, and Aqua™ will be referred to simply as “Aqua” from here after.

1.1 Regulatory Drivers

Wastewater treatment in the United States has long focused on removal of conventional pollutants such as Biochemical Oxygen Demand (BOD), Total Suspended Solids (TSS), and oil & grease. Traditionally, proper removal of these substances has also facilitated a reduction in pathogens, coliforms, and turbidity. The removal of conventional pollutants classified effluent wastewater as meeting secondary treatment standards set by the National Pollutant Discharge Elimination System (NPDES) (Secondary, 2015). For decades, meeting secondary treatment standards has been the goal of publicly owned treatment works (POTWs). However, environmental degradation issues due to nutrients in effluent wastewater began to show problematic effects in the 1960’s and 70’s and prompted government action to identify and fix this emerging problem (Michalak, 2013)

NPDES is the regulatory framework established in 1972 which places limits based on acceptable effluent concentrations of all pollutants, more recently including nutrients such as nitrogen and phosphorus (Sewage, 2018). Permitted concentrations are unique to each receiving water and treatment plant but are developed based on technology-based effluent

limitations (TBEL's) and water quality-based effluent limitations (WQBEL's) (NPDES 2015). As some receiving waters may be more sensitive to certain pollutants, this allows for differing final effluent limitations at different locations. For example, in San Luis Obispo, CA their Water Resource Recovery Facility NPDES permit specifies a maximum of 10 part per million (ppm) nitrate as nitrogen for their average monthly discharge (NPDES, 2016). While in contrast, Central Contra Costa Sanitary District (Martinez, CA) has no nitrate limitation, but a limitation of total ammonia as nitrogen of 65 ppm for their average monthly concentration (NPDES, 2016). The increasing frequency with which nutrients, and nitrogen, are being regulated by NPDES has prompted industry wide investigation into systems and processes capable of efficiently removing nitrogen from wastewater.

1.2 Environmental Concerns push Biological Nutrient Removal (BNR)

The nitrogen cycle is the most prominent elemental cycle in our world, yet one which remains not completely defined. Nitrogen exists in all three phases of our environment, elementally dominating our atmosphere, and providing key nutrients for growth in the aqueous and soil phases. The focus of this thesis research was removing nitrogen from wastewater media and understanding the nitrogen transformation pathways taking place. Biological Nutrient Removal (BNR) is the process by which nutrients, predominantly nitrogen and phosphorus are removed from wastewater before discharge (Biological, 2018). The removal of nutrients prior to discharge is key in preventing eutrophication of surface waters. Eutrophication, or an overload of nutrients, manifest itself in algal blooms which deplete water bodies of dissolved oxygen and diminish aquatic life (Biological, 2018). The eutrophication of Lake Erie in the 1960's and 70's is one of the most notorious cases in history which highlighted the need for stricter regulations and the implementation of BNR systems (Figure 1-1) (Eutrophication, 2002). Nutrients from agricultural and urban lands in

the surrounding areas flowed into Lake Erie for decades, earning it the title of a “dead lake” and causing beach shutdowns, extensive aquatic life mortality, and the stench of decaying algae on the beaches. After improved sewage controls over forty years, Lake Erie has once again begun to see growing populations of aquatic life, however algal blooms continue to plague the Lake year after year (Eutrophication, 2002).

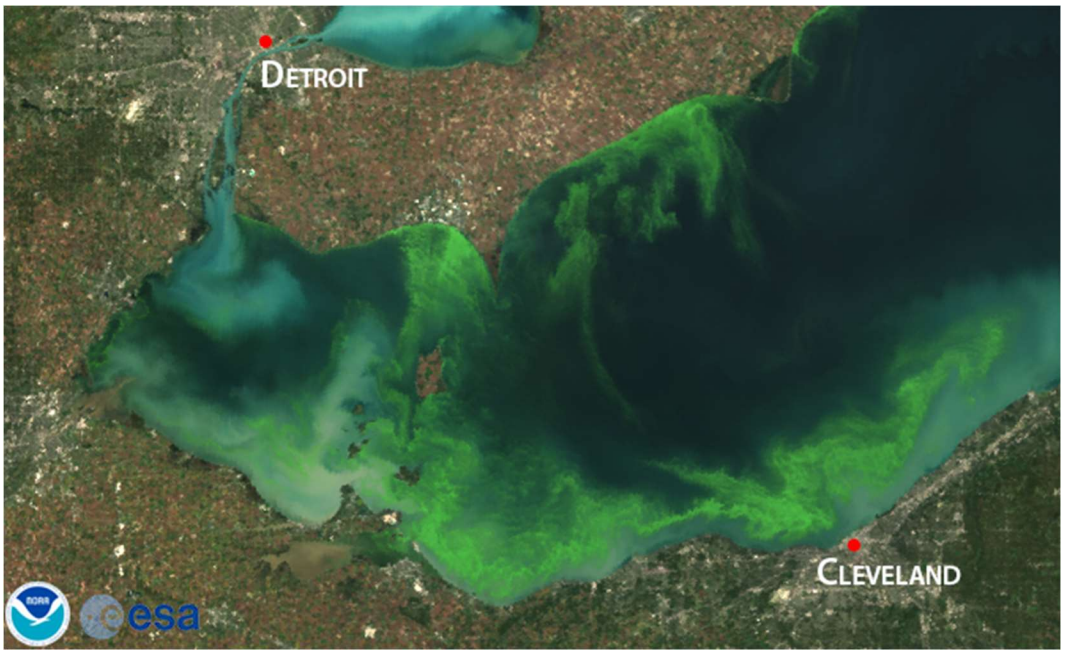

Figure 1-1: Eutrophication of Lake Erie with blue-green algal blooms characteristic of cyanobacteria growth due to excess nutrients. (Lake, 2014)

Prior to 1970’s, BNR systems were not common in the United States, however issues such as Lake Erie and others forced the expansion of regulations to include nutrient limitations in many NPDES permits (Sewage, 2018). Even so, as of 2000 BNR systems treated only 1% of wastewater flow through POTWs in the United States (Wang 2011). With increased awareness of the deleterious effects of high nutrient loadings on surface waters, this number has risen significantly since 2000 and continues upward as permit renewals begin to require greater than secondary effluent standards. The rise in implementation of these plants is predominantly at the requirement of NPDES permit renewals.

While nutrient removal regulations have increasingly required BNR additions and upgrades to POTWs, there remains a variety of different systems through which BNR may be accomplished. Unlike systems such as membrane bioreactors (MBR), package BNR plants are not common today and the means and mechanism by which BNR is accomplished is often left up to the specific municipality of concern. Additionally, as this remains a developing field, there remains room for research and development into more efficient ways with which to remove and even recover these nutrients. While BNR refers to nutrient removal in the general sense, the following *Background* will explore nitrogen removal and the relevant transformation pathways with regard to wastewater treatment. Reference to BNR in the remainder of this thesis will focus solely on nitrogen removal, and not phosphorus or combined nitrogen and phosphorus removal.

2. BACKGROUND

Singular pathways of the nitrogen cycle that are common in BNR systems and relevant to research performed will be covered below beginning with overviews of Nitrification, Denitrification, and Assimilation. The combined cycle of simultaneous nitrification-denitrification (SNdN) will then be covered as it relates to nitrogen removal in wastewater treatment. SNdN will discussed in context of the specific type of bacteria performing the process. Generally referred to as SNdN, this mechanism was the crux of the initial hypothesis and a field in which it is believed there is significant space for discovery of more efficient systems than current industry standard (Bai, 2016).

After nitrogen transformation pathways are discussed below, industry standard treatment systems relevant to these pathways will be covered. Key operational metrics which limit plant efficiencies such as hydraulic retention time (HRT), solids retention time (SRT), and others will be compared against non-nitrogen removal systems. This comparison will demonstrate how nitrogen removal requirements increase treatment times, thereby reducing operational efficiencies of plants. This increased treatment time of typical nitrogen removal systems is ultimately the area of focus for SNdN systems; with the goal of optimizing SNdN to reduce treatment times.

2.1 Nitrification Overview

Nitrification is a process by which ammonia nitrogen in the form of the ammonium ion (NH_4^+) is oxidized to nitrite (Eq. 1) and subsequently oxidized again to nitrate (Eq. 2). The total oxidation reaction is presented in Equation 3, neglecting cell synthesis for simplicity. The transformation begins and ends with each constituent existing in the aqueous phase.

Nitrification is a process needed in wastewater treatment to remove ammonia-nitrogen from effluent. Excess concentrations can have toxic effects on aquatic life, marginalize dissolved oxygen (DO) concentrations in receiving waters, and contribute to eutrophication. Excess ammonia-nitrogen in effluent also limits water reuse applications as regulations limit concentrations that can be injected into groundwater formations (Tchobanoglous, 2003).

Equation 1: $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$

Equation 2: $2NO_2^- + 2O_2 \rightarrow 2NO_3^-$

Equation 3: $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$

While total ammonia-nitrogen (TAN) removal is a common step in BNR systems, it is often the first of two steps in the nitrogen removal process. In addition to limits on TAN concentrations, nitrate limits on effluent discharge also exist for good reason, as nitrate can be toxic to aquatic life and harmful to children causing methemoglobinemia or “blue baby syndrome.” Blue baby syndrome occurs when drinking water containing nitrate is consumed and reduces the oxygen carrying capacity of hemoglobin in babies. This phenomenon results in a “blue baby” face and can result in death (Knobeloch, 2000). For these reasons, the presence of nitrate in considerable quantities and/or out falling to sensitive receiving locations, is necessary to be removed. Nitrate removal processes will begin to be discussed below in *Denitrification Treatment Systems*.

2.1.1 Nitrification Treatment Systems

Nitrification in wastewater treatment facilities can occur in either attached growth (trickling filter) or suspended growth (activated sludge) systems. Both systems are common place in POTWs and each have their operational benefits. Suspended growth systems such as activated sludge or oxidation ditches typically achieve nitrification concurrently with BOD removal in the same tank. These systems often employ return activated sludge (RAS) recycle which provides additional bacteria for the biological treatment to proceed with (Tchobanoglous, 2003). Mechanical aeration and mixing for these systems is necessary to provide adequate oxygen and contact between molecules for the reactions outlined in Equations 1, 2, and 3 to occur. Nitrifying systems and their bacteria are also sensitive to temperature and pH fluctuations. As such, operational care must be taken to ensure that DO, pH and temperature are all maintained for complete nitrification to occur (Tchobanoglous, 2003).

In contrast to suspended growth, attached growth systems must remove the majority of influent BOD before nitrification can occur. BOD removal occurs via heterotrophic bacteria, which are faster growing than typical autotrophic nitrifying bacteria. Thus, if BOD is not reduced before, the heterotrophs will dominate the system and not allow nitrifying organisms to grow. Therefore, this form of nitrification typically takes two unit-processes, one to first remove BOD and the second subsequently for nitrification. Most of attached growth systems exist as trickling filters which employ media such as rock, plastic, or random pack material for microbes to grow on. As wastewater is trickled over the surface of these cylindrical towers, air is brought upwards either passively or actively to provide necessary oxygen for the process. Other attached growth systems such as rotating biological contactors (RBC)

and integrated fixed-film activated sludge are available, but much less common than the trickling filter.

The two-step nitrification process (Eqns. 1 & 2) is typically completed by aerobic autotrophic bacteria; with each step having distinct bacteria that perform the process (Tchobanoglous, 2003). Step one is traditionally observed to be completed by bacteria of the *Nitrosomonas* genera, with step two being performed by bacteria of the *Nitrobacter* genera. Various other autotrophic bacteria are also noted to perform both stages of nitrification (Table 2-1). As mentioned previously, nitrification is a pH sensitive process, with high pH (above 8) resulting in increased ammonia concentrations (unable to nitrify); and depressed pH (below 6.8) significantly reducing the rate at which the nitrifying organisms can perform (Tchobanoglous, 2003). For these reasons, alkalinity is an important parameter in the operation of nitrifying systems.

Table 2-1: Common genera of nitrifying and denitrifying bacteria; however not a comprehensive list of every bacteria observed to perform these transformations (Tchobanoglous, 2003).

	Nitrification Step 1	**Nitrification Step 2**	**Denitrification**
Most Prevalent genera	*Nitrosomonas*	*Nitrobacter*	*Pseudomonas*
Other capable genera	*Nitrosospira*	*Nitrococcus*	*Bacillus*
	Nitrosolobus	*Nitrospira*	*Arthrobacter*
	Nitrosorobrio	*Nitrospina*	*Rhizobium*
	Nitrosococcus	*Nitroeystis*	*Spirillum*

2.2 Denitrification Overview

Nitrate formed during nitrification, is reduced to inert nitrogen gas (N_2) through the process of denitrification (Eq. 4). Equation 4 utilizes a standard composition of wastewater organics to represent the carbon source. Prior to N_2 formation, the intermediaries' nitric oxide and nitrous oxide are formed as nitrogen changes oxidation states from plus five in nitrate to zero in nitrogen gas (Eq. 5). This change in oxidation states is what requires an electron donor (organic carbon) in the denitrification process.

Equation 4: $C_{10}H_{19}O_3N + 10NO_3^- \rightarrow 5N_2 + 10CO_2 + 3H_2O + NH_3 + 10OH^-$

Equation 5: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

Denitrification is integral in BNR systems as it transforms nitrogen from aqueous to gaseous phase. Removing nitrate from wastewater before outfall is key in preventing eutrophication of receiving waters, nitrate toxicity effects in aquatic life, and allowing for beneficial reuse applications (Tchobanoglous, 2003). Additionally, if a receiving water body is used as a water source for drinking water treatment plants (e.g. Mississippi River and San Francisco Bay), any nitrate present in the water must be removed below 10 ppm before being distributed as it can have deleterious effects in humans, as previously discussed (Komor, 2002).

In most applications, denitrification is always a sequential treatment step following nitrification. This is the case in the clear majority of POTW - BNR applications, and as such, systems that perform denitrification along with nitrification are the focus of this background. However, it is worth noting that singular denitrification systems are used for applications that produce nitrate such as: agricultural discharge, aquaculture, contaminated groundwater, explosives wastewater, and flue gas with dissolved oxidized nitrogen (Cyplik, 2011).

2.2.1 Denitrification Treatment Systems

In wastewater treatment, denitrification takes place in either *pre-anoxic denitrification* or *post-anoxic denitrification* systems*,* with reference to the order of the aeration and anoxic tanks (Figure 2-1). Pre-anoxic denitrification indicates that an anoxic tank comes prior to the aerobic tank; while post-anoxic refers to a system with the anoxic tank located after the aerobic tank. Traditionally, nitrification takes places in the aerobic tank, and denitrification in the anoxic tank.

Pre-anoxic is a substrate driven process, meaning BOD present in the influent serves as the electron donor in the reduction of nitrate; while post-anoxic relies on endogenous decay and supplemental carbon for its electron donor (Tchobanoglous, 2003). Pre-anoxic denitrification is often referred to as the Modified Ludzack-Ettinger (MLE) process and relies on an internal recycle of mixed liquor from the aerobic tank to the anoxic tank. This internal recycle provides nitrate produced in the aerobic zone to the anoxic zone, where it utilizes BOD from influent and return activated sludge (RAS) flows as electron donors with which to perform denitrification. Additionally, a benefit of the MLE process is that a portion of influent BOD is reduced by utilizing available nitrate as an electron acceptor in place of oxygen, which helps to reduce organic loading on the aerobic tank (Tchobanoglous, 2003).

Post-anoxic denitrification, also known as the Wurhman process, converts nitrate produced in the aerobic zone to nitrogen gas in the subsequent anoxic tank (Grissop, 2010) This process does not utilize an internal recycle, but does use RAS flow to maintain proper microbe populations in the tanks. To promote denitrification, organic matter is needed in the anoxic tank to provide electron donors. As such, this post-anoxic setup often requires the addition of a carbon source such as methanol or acetic acid to provide sufficient BOD for

nitrate reduction (Tchobanoglous, 2003). In both pre- and post-anoxic denitrification, the anoxic tank is mixed to provide sufficient contact between substrate and microbes, but not aerated as DO levels must be kept below 0.2 ppm to ensure that nitrate is used as the electron acceptor (Skerman and McRae, 1957; Terai and Mori, 1975; Dawson and Murphy, 1972).

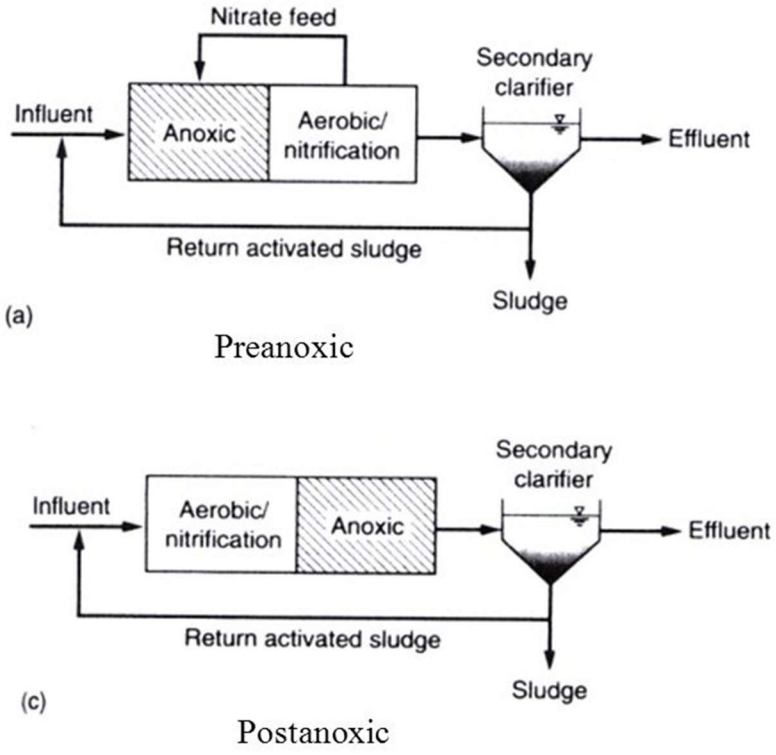

Figure 2-1: Schematic of Pre-anoxic (a) and Post-anoxic (c) system flows including internal return flows between tanks. Aerobic tanks perform nitrification while anoxic tanks perform denitrification (Tchobanoglous, 2003).

Many different bacterial genera have demonstrated the ability to denitrify including both heterotrophic and autotrophic bacteria, with some of the most common ones listed above in Table 2-1. Most denitrifying bacteria are classified as facultative aerobic bacteria with the ability to use either oxygen or nitrate as its electron acceptor (Tchobanoglous, 2003).

2.3 Assimilation

Assimilation is the process by which nitrogen in the form of either ammonia or nitrate is converted to organic nitrogen in the form of cells and biomass. The nitrogen in organic form is often proteins composed of amino acid structures. Assimilation can occur through plants, fungi, and bacteria - and is a vital process in the creation of organic life (Nitrogen, 2018).

While not commonly designed for in wastewater treatment systems, assimilation of ammonia nitrogen could provide a pathway for nitrogen levels to be reduced to levels acceptable for receiving waters. Once assimilated, biomass may be coagulated, flocculated, and gravity settled. These processes may occur naturally or could be chemically and physically enhanced for more efficient settling. As clarifiers and gravity settlers are universally employed in treatment facilities, this process would not require the addition of extra unit processes to complete.

While most abundantly observed in plants and fungi as a means of nutrient uptake, assimilation has been studied in bacteria such as cyanobacteria, Azotobacter, and Bacillus (Lin and Stewart, 1998). The reason that influent ammonia-nitrogen is not assimilated in typical wastewater systems is due to the abundance of ammonia-nitrogen present in wastewater. Additionally, to assimilate nitrogen, enough available carbon is needed to build proteins within cells. As wastewater typically has carbon to nitrogen ratios from 2:1 to 6:1, there is nitrogen that remains after BOD removal that was unable to be assimilated by microorganisms (Nitrogen, 2005).

2.4 Simultaneous Nitrification-Denitrification (SNdN)

Simultaneous nitrification-denitrification (SNdN) is a process that has been observed to transform ammonia-nitrogen in the aqueous phase to nitrogen gas in a single system. Traditionally, this process can occur in a single activated sludge tank that contains both aerobic and anoxic zones configured around mechanical aerators (Tchobanoglous, 2003). As wastewater is mixed and aerated by the aerators in one section of the tank ammonia nitrogen is converted into nitrate. After nitrification takes place and the wastewater flows away from aerators, DO concentrations drop low enough to create anoxic zones. The anoxic zones then provide the denitrification step. This process has been observed to occur in low DO concentrations, with concentrations above 0.2 ppm inhibiting denitrification in activated sludge applications (Skerman and McRae, 1957; Terai and Mori, 1975; Dawson and Murphy, 1972).

In addition to aerobic and anoxic zones within a single tank, SNdN can also occur in a single floc with different zones existing in activated sludge flocs. Within the individual floc, nitrification may occur on the aerobic exterior of the floc, with denitrification occurring in the anoxic, interior of the floc (Tchobanoglous, 2003). Available DO and substrate provide the environment for nitrification to occur on the floc exterior, while produced nitrate and substrate then diffuse into the floc interior. As the nitrification reaction occurring on the floc exterior depletes available DO, this creates favorable DO concentrations in the floc interior to reduce nitrate to nitrogen gas (Tchobanoglous, 2003).

While SNdN treatment options can provide decreased plant footprint for unit processes, they do come with decreased efficiency. Optimal conditions for both individual process are not realized in the combined cycle, resulting in decreased reaction rates. The nitrification rate is

decreased due to less than optimal DO concentrations, and the denitrification rate is decreased due to less available substrate (Tchobanoglous, 2003).

As mentioned previously, this simultaneous process may occur in a single tank with the right conditions. Separate two-tank BNR systems discussed in *Denitrification Treatment Systems* employ one aerobic and one anoxic tank to perform the processes but are not "simultaneous" systems. In addition to these systems, oxidation ditches are a common wastewater treatment system that can maintain environmental and operational parameters to facilitate SNdN reactions. Oxidation ditches typically have large volumes, and thus can afford the long retention times needed for the depressed nitrification and denitrification reactions to occur simultaneously (Tchobanoglous, 2003). Oxidation ditch systems are advantageous in certain situations, such as small communities, that experience low throughput and may prefer financially simple systems.

The microbiology for traditional SNdN systems is like that of the individual processes, with nitrification employing aerobic autotrophic bacteria and denitrification employing either autotrophic or heterotrophic facultative aerobic bacteria. Bacteria common to these individual processes are listed in Table 2-1.

2.5 Comparison of Operational Parameters

After discussion of nitrogen transformations and accompanying wastewater treatment systems, it is useful to discuss key operational parameters of these systems. Analyzing constraints and requirements of discussed systems will provide insight into the efficiency decrease levied on traditional non-BNR plants through nitrogen removal. Thus, these areas are where efficiency improvements can be made. Primarily, hydraulic retention time (HRT)

and solids retention time (SRT) will be compared (Table 2-2), as these parameters control how long flows must be treated and subsequently how much aeration must be provided on a volumetric flow basis.

Table 2-2: Comparison of HRT and SRT between treatment systems with and without nitrogen removal, showing increased residence times needed for nitrogen removal. (Tchobanoglous, 2003).

	HRT (hours)	SRT (days)
Activated Sludge	3-5	3-15
BNR - MLE Activated Sludge	5-15	7-20
BNR Oxidation Ditch	18-30	20-30

As demonstrated in Table 2-2, the need for complete nitrogen removal from wastewater, increases HRT roughly threefold. Traditional autotrophic nitrifying bacteria are slow-growing organisms because their electron donor, ammonia-nitrogen, exists at an oxidation state of -3, which is more oxidized than heterotrophic bacteria that utilize and reduce organics such as BOD. Therefore, less energy is produced for cell growth per mole of ammonia reduction, compared to BOD reduction - thus nitrifying populations grow slower. Due to slower microbial population growth, nitrifying wastewater treatment systems require longer HRT and SRT to allow populations to grow; in addition to the time needed to perform anoxic denitrification (Tchobanoglous, 2003). Given the treatment time increase required for nitrogen-removal systems, it is of financial interest to POTWs and industrial dischargers to find more efficient nitrogen removal systems.

2.6 Heterotrophic Nitrification-Aerobic Denitrification

As discussed, SNdN has typically been performed by bacteria respective to their individual process, meaning autotrophic bacteria perform nitrification aerobically, and heterotrophic bacteria perform denitrification anoxically. However, heterotrophic bacteria demonstrated potential to perform aerobic SNdN using specific bacterial isolates as inoculum. (J.K. Kim et al., 2005), (Mevel and Prieur, 2000), (Sakai et al., 1996).

2.6.1 Success in Bench-Scale Studies

Heterotrophic *Bacillus* strains, like those in Aqua, were shown to perform aerobic nitrification and denitrification within an hour of inoculation, relying on diffuse oxygen via orbital shaking as the sole source of aeration (Kim et al., 2005). Significant TAN reduction corresponding with nitrate presence, and subsequent nitrate reduction and nitrogen gas production indicated occurrence of SNdN. At 30% DO saturation and a molar C: N ratio of 8:1, a maximum of 33% TN reduction in four hours due to heterotrophic *Bacillus* strains was realized. Strain isolate *B. subtilis* was identified as largely involved in nitrification and strains *B. cereus* and *B. licheniformis* involved in denitrification and the formation of nitrogen gas under aerobic conditions (Kim et al., 2005).

More recently, additional *Bacillus* strains such as *Bacillus methylotrophicus* also showed the capability of heterotrophic nitrification-aerobic denitrification and found success in reducing initial ammonia levels by 51.03% within 56 hours, at a maximum removal rate of 51.58 ppm/d (Zhang, et al., 2011). As these studies demonstrated, *Bacillus* strains have the ability to heterotrophically perform SNdN in aerobic conditions, however their application beyond laboratory scale is still in question.

2.6.2 Limitations of Bench-Scale Studies

While the success of studies by Kim, Zhang and others realized the potential of heterotrophic *Bacillus* strains in performing aerobic SNdN, these studies analyzed simple systems that afforded no microbial competition. Sterile, minimal media and pure isolated cultures provided ideal situations for *Bacillus* to grow unimpeded. While potential implications of these studies are exciting and novel, it was previously known that various heterotrophic microorganism perform nitrification on different nitrogen compounds (Focht and Verstraete, 1977). However, the reduced activities of inoculated microbes in complex systems limited interest in their role in nitrification, as their contribution to the process was thought to be limited compared to autotrophic microorganisms.

2.6.3 Microbe Comparison with Aqua

Knowledge of isolate success in aerobic, heterotrophic SNdN was in part the inspiration behind the assemblage of Aqua, produced by BiOWiSH Technologies. Aqua aimed to commercialize the success that these *Bacillus* strains showed in isolated bench scale testing. The key to realizing full potential would rest on replicating results in complex media such as wastewater, which had been observed in sterile, minimal media. As any commercial, municipal, or industrial product application would occur in complex systems, the true efficacy of heterotrophic aerobic SNdN by Aqua must be shown in similar systems.

As can be seen below in Table 2-3, *Bacillus* strains shown to be successful in previously discussed bench scale testing were also present in the Aqua assemblage, along with others of the *Bacillus* genera, deemed to be of use by BiOWiSH. Continued research in the field has continued to identify additional heterotrophic strains capable of nitrification-aerobic

denitrification such as *Alcaligenes faecalis* No. 4 (Joo et al., 2005), *Bacillus* sp*. Strains* (Yang et al., 2011) and *Pseudomonas* sp. (Wan et al., 2011). Research and discovery of novel strains has excited interest into commercialization of these mechanisms, a niche in which Aqua hoped to succeed.

Table 2-3: Common strains of Bacillus shown to perform heterotrophic SNdN (Kim et al., 2005) and (Zhang et al., 2011) in cross-reference with a portion of BiOWiSH Aqua commercial assemblage.

Kim et al., 2005 & Zhang et al., 2011	**Aqua, 2017 "strain shorthand"**
Bacillus licheniformis	*Bacillus licheniformis "276 & 277"*
Bacillus subtilis	*Bacillus subtilis "34"*
Bacillus methylotrophicus	*Bacillus pumilus "275"*
Bacillus cereus	*Bacillus mojavensis*
	Bacillus thuringiensis

2.7 Potential Impact on Wastewater Treatment Operations

The potential application and impact of a commercial product capable of performing heterotrophic, aerobic SNdN would be advantageous to POTWs for multiple reasons. Most overtly, achieving SNdN aerobically would allow for the process to occur in one reactor tank, which would reduce land-use and operational complexity. Additionally, heterotrophic bacteria would utilize organic substrates from influent, helping reduce organic loadings in addition to nitrogen removal. Furthermore, if reaction rates observed in bench scale studies (Kim et al., 2005), (Zhang et al., 2011) could be realized in complex wastewater system, the limiting retention time for nitrogen removal plants would no longer be dependent on the nitrogen removal process but would be dependent upon organic loading removal

requirements. Effectively, this could eliminate any associated operational costs between nitrogen removing and non-nitrogen removing systems.

While nitrogen removal is currently only required by a select portion of NPDES permits, nitrogen removal capability is advantageous for dischargers even without limits. As nutrient loadings increase in receiving waters across the U.S. and globally, permit requirements and regulations will likely only tighten restrictions. Thus, many dischargers currently not required to, will likely need to implement nitrogen removal soon. Additionally, with municipalities and government agencies looking toward waste streams as a revenue source, their ability to reuse and recover wastewater is paramount.

Recovering wastewater for applications such as groundwater recharge, indirect potable reuse (IPR), or direct potable reuse (DPR) in the future would benefit municipalities financially through its sale; and lessen their communities' environmental footprint. For these reasons, achieving a standard of effluent water quality compliant for these applications should be the goal when considering upgrades to wastewater treatment. Currently, both groundwater recharge and IPR applications are regulated under Title 22 of the California Code of Regulations which places a 10 ppm limit on total nitrogen (Regulations, 2014). Thus, any municipality or community looking toward wastewater recycling and reuse to offset water demand or financial costs, complete BNR must first be considered as an addition to their wastewater treatment train.

Current industry standard two-stage BNR systems, such as those covered in the *Denitrification Treatment Systems* section, increase retention times and operational complexity in comparison to non-BNR plants. However, if the promise of studies discussed in the *Heterotrophic Nitrification-Aerobic Denitrification* section can be realized in complex

wastewater application, then significant financial and environmental benefits would be achieved.

2.8 Research Goals & Questions

Research goals were coalesced by myself, undergraduate student assistants, professors invested in the project, and BiOWiSH representatives and were developed to assess the application of Aqua for nitrogen removal capability in wastewater. Goals evolved from overarching questions, and were condensed into the following:

1. Does application of Aqua improve total ammonia-nitrogen (TAN) removal in wastewater system in comparison to rates observed without bioaugmentation?
2. Does application of Aqua promote a decrease in Total Nitrogen (TN) in comparison to systems without bioaugmentation? If so, can TN decrease be attributed to simultaneous nitrification-denitrification (SNdN)?
3. If mechanisms outlined in Questions 1 and 2 above are not observed, are there other mechanisms occurring by which TAN, TN or other nitrogen constituents are being reduced?

3. MATERIALS AND METHODS

Before answering macro-scale questions posed by above stated research goals, the collection of more detailed information was necessary via small-scale screening, or parameter optimization experiments. Parameter Optimization (PO) experiments, consisted of ten different experiments, performed to optimize parameters in which the bacterial assemblage, Aqua, would be analyzed for performance. Experiments manipulated variables such as growth media (simple vs. complex, defined vs. undefined), table shaker speed (controlled DO concentration), growth factor addition (yeast extract, trace mineral supplement, etc.), carbon amendments, individual bacterial strain performance, and dosing method (activated, dry, etc.). These experiments were performed in 250 mL orbital shaken flasks and allowed for operational and environmental optimization prior to experiment scale-up.

In conjunction with PO experiments, additional statistical experiments were performed on TN method analysis to ensure accuracy of results prior to full-scale experimentation. The results of these statistical analysis experiments are discussed below in *Statistical Analysis Experiment Results*. After completion of PO and statistical experiments, two full-scale (FS) experiments were conducted to assess Aqua performance in conditions like those recommended for application. Each experiment aim, environmental conditions, takeaways, and follow-up questions are summarized below in Table 3-1.

Table 3-1: Summary of experimental process flow. This table serves to introduce goals and flow of these experiments. Detailed analysis and data will be discussed in the Analysis, Results, & Process Flow section.

Experiment Name	Experiment Aim	Media & Inoculum	Takeaways	Questions
PO-1 & PO-2	Introduction to product & baseline nitrogen metrics. Individual strain performance.	Wastewater (WW) Inoculum: 275, 275, 277, 34, Aqua	Strain 275: fastest TAN reducer. No NO3- formation	Replicate TAN reduction in Minimal Media (MM)?
PO-3	Replicate TAN reduction rate in MM.	Minimal Media Inoculum: 275, Aqua	Negligible TAN reduction in MM.	Ratio of WW:MM to observe TAN reduction?
PO-4	Determine ratio of WW:MM to replicate TAN reduction rates in WW.	Minimal Media Wastewater Inoculum: 275	Increased WW ratio → increased TAN reduction.	Constituent in WW that catalyzes TAN reduction?
PO-5	Test different Carbon sources in WW and MM media. *	Minimal Media Wastewater Inoculum: 275	Unclear: Succinate = glucose > acetate. *	Optimize Carbon source in MM.
PO-6	rpm optimization Succinate vs. Glucose	Minimal Media Wastewater Inoculum: 275	100 rpm ideal. Succinate preferred Carbon.	Growth factors affecting TAN reduction?
PO-7	Yeast Extract (YE) vs. Vitamin (V) & Mineral (M)**	Minimal Media Inoculum: 275	Vitamin (V) & Mineral (M) >> YE	Vitamin (V) or Mineral (M) vital?
PO-8	Find crucial growth factor.	Minimal Media Inoculum: 275	M = V + M > YE > V	Which specific mineral(s) catalyze TAN reduction?
PO-9 (Aqua Analysis)	Carbon limiting extent of TAN reduction?	Minimal Media Inoculum: Aqua	Succinate = 2x succinate >> dextrose	Effects of V & M hold with Aqua?
PO-10	Test vitamin & mineral performance with	Minimal Media	M = V + M > YE > V	Which specific mineral(s)

	Aqua.	Inoculum: 275, Aqua		catalyze TAN reduction? Begin large-scale experiments.
Statistical Exp-1	TN Analysis Method Accuracy.	Ammonia-nitrogen vs. Organic nitrogen standards	Hach TN > Elementar for TAN accuracy	
Large-scale 1	Dosing: 10 ppm initial versus 1 ppm/ day.	Wastewater Inoculum: Aqua	No observable effect from either dosing.	Too low dose? Carbon limiting microbes? Product need activation?
Large-scale 2	Dosing: 50 ppm "activated" versus stock solution Carbon addition effects.	Wastewater Inoculum: Aqua	No observable effect from either dosing method.	See Analysis, Results, and Conclusion section.

* Carbon sources tested: Sodium Succinate, Dextrose & Sodium Acetate; see appendix E
** Vitamin & Mineral solutions purchased from ATCC; see Appendices B and C

3.1 Microorganism & Inoculum Preparation

Individual microorganism strain isolates and full Aqua product were used in various experiments as outlined above in column three of Table 3-1. Dose inoculation procedures varied depending on analysis of Aqua or individual strain isolates.

3.1.1 Preparation of Aqua Stock Solution

During full-scale product evaluation, 2g of dry Aqua was added to 500 mL sterilized deionized (DI) water to form a 4,000 ppm stock solution. Prepared solution was then stirred via stir bar and stir plate for 20 minutes before appropriately dosing directly from the homogenized stock solution. Media inoculation from stock solution was performed under

sterile conditions in a laminar airflow workstation used for sterile preparation (Airegard Nuaire, Model No. NU-S201-430, Plymouth, MN).

3.1.2 Strain Isolate Preparation

Experiments evaluating individual strain performance required microorganism “activation” or growth prior to inoculation. Strain isolates were initially streaked on agar plates prior to PO-1 and identified based on morphology. Each strain was re-streaked on agar plates to ensure isolation. Isolated strains were then labelled and stored in refrigeration at 4°C for preservation and environmental control (Thermo Scientific, Revco Series, Waltham, MA). Inoculation procedures for individual strains began with preparation of Luria-Bertani (LB) broth. A single colony from the desired agar plate was retrieved by sterile inoculation loop and dispersed in 4 mL of LB in a 15 mL falcon tube. Microorganism growth was accomplished by placing the falcon tube into an orbital table shaker (New Brunswick Scientific Co. Inc., Innova Incubator Shaker Series Model 42 Inc. Shaker, New Edison, N.J.) overnight for 24 hours at 35°C and 200 rpm.

3.1.3 Activated Aqua Preparation

Dosing procedures for “activated Aqua” employed overnight growth in LB broth, like strain isolate activation, for increased microbial populations prior to inoculation. Activated Aqua was prepared by adding 2 g of dry product to 50 mL of TSB broth to form a 40,000 ppm stock solution. Stock solution was placed in a table shaker at 35°C and 200 rpm and allowed to grow overnight for 24 hours. Appropriate volumes were then aliquoted from this solution and used for inoculation.

3.2 Experimental Design: Apparatus & Environmental Conditions

Experiment PO-1 served to provide baseline metrics of Aqua and strain isolate performance. Subsequent experimental design was predicated on prior experimental results and analysis to answer research goals in a logical manner. As no physical apparatus was constructed for experiments, discussion in this section will focus on media selection and inoculum, shaker speed, carbon & growth factor addition, and sample analysis performed. For conciseness, these mentioned parameters will be summarized for each experiment performed in chronological order. Media preparation and composition will be discussed upon initial introduction and stated afterwards.

3.2.1 PO-1 and PO-2 Experimental Design

Primary wastewater shipped from Cincinnati, OH was screened through screening of 1 mm pore size to remove large organic material. Wastewater from Cincinnati POTW was used per BiOWiSH request as it was desired to obtain Aqua performance metrics in wastewater that had been previously characterized. Analysis was performed on TAN, TN, nitrate, nitrite, and OD to track nitrogen transformations (Table 3-2). Sampling was performed at close time intervals to capture the suspected initial rapid TAN drop.

After screening, wastewater was sterilized by autoclave (Steris Amsco Lab 250, LG 250 Laboratory Steam Sterilizer, Mentor, OH) at 121°C and 15 psi for 45 minutes. Volumes of 300 mL of sterilized wastewater were transferred to six 1000 mL volumetric flasks, appropriately inoculated, lightly capped with foil, and placed into the orbital shaker (Figure 3-1).

Table 3-2: *PO-1 and PO-2 Experimental Design*

Media: Screened & Sterilized Primary Wastewater Temperature: 35° C Speed: 100 rpm Carbon Source: PO-1 (none) & PO-2 (dextrose) Growth Factor: None Duration: 96 hr. (PO-1) & 48 hr. (PO-2)	System (Inoculum)* 1. WW (Strain "275") 2. WW ("Strain 276") 3. WW ("Strain 277") 4. WW ("Strain 34") 5. Aqua @ 1500 ppm 6. Control

Sampling Constituents & Frequency:

PO-1: TAN, NO_3^-, NO_2^-, OD, pH, CFU/mL @ 0, 1, 2, 5, 10, 24, 48, 96 hours

TN @ 0 and 96 hours

PO-2: TAN, NO_3^-, NO_2^-, OD, pH, CFU/mL @ 0, 2, 4, 6, 12, 24 hours

TN @ 0 and 24 hours

* see table 2-3 for bacterial isolates related to strain numbers.

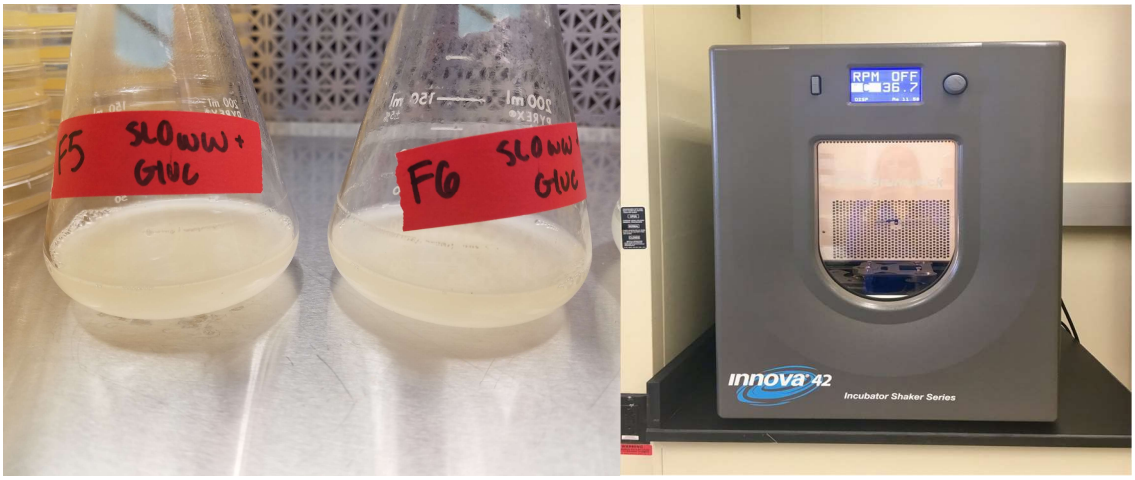

Figure 3-1: Flasks with residual media after completion of PO-2 (left), orbital shaker used for experiment (right).

3.2.2 PO-3 Experimental Design

The aim of experiment PO-3 was to replicate TAN reduction rates observed in sterile wastewater in PO-1 and PO-2, in a defined minimal media (Table 3-3). Defined minimal media recipe was provided by BiOWiSH, composed, amended with ammonium chloride, and sterilized by autoclave. The prescribed recipe contained the following compounds: NH_4Cl, KH_2PO_4, K_2HPO_4, Na_2HPO_4, $CaCl_2$, $MgSO_4$, and $FeCl_3$; the detailed recipe is attached in Appendix A. Each system contained 300 mL of sterile minimal media in 1000 mL volumetric flasks.

Table 3-3: *PO-3 Experimental Design*

Media: Defined Minimal Media	System (Inoculum)
Temperature: 35° C	1. MM (Strain “275”)
Speed: 200 rpm	2. MM (Aqua @ 1500 ppm)
Carbon Source: None	3. Control
Growth Factor: None	
Duration: 48 hr.	

Sampling Constituents & Frequency:

PO-3: TAN, NO_3^-, NO_2^-, OD, pH @ 0, 5, 10, 24, 48 hours

3.2.3 PO-4 Experimental Design

Following the results of PO-3, PO-4 looked to discern whether a specific ratio of wastewater in the system's media would help reproduce TAN reduction rates observed in PO-1 and PO-2 (Table 3-4). Results from this analysis helped determine whether a constituent present in wastewater correlated to TAN reduction in a direct ratio, or if it was a threshold limiting factor.

The scale of PO-4 was reduced from 1000 mL flasks, used in previous PO-1 through PO-3, to 200 mL flasks to analyze more systems at once. Systems contained 50 mL of appropriate media in each flask, which were foil capped, and placed in the orbital shaker.

Table 3-4: *PO-4 Experimental Design*

Media: Screened, Sterilized Primary Wastewater & Minimal Media Temperature: 35° C Speed: 100 rpm Carbon Source: None Growth Factor: None Duration: 24 hr.	System (Inoculum) 1. 100:0 - MM: WW (275) 2. 90:10 - MM: WW (275) 3. 75:25 - MM: WW (275) 4. 50:50 - MM: WW (275) 5. 25:75 - MM: WW (275) 6. 10:90 - MM: WW (275) 7. 0:100 - MM: WW (275)
Sampling Constituents & Frequency: PO-4: TAN @ 0, 10, 24 hours	

3.2.4 PO-5 Experimental Design

As results from experiment PO-3 indicated that TAN reduction in minimal media was lacking some substrate constituent, stock solutions of carbon and yeast extract were prepared for use in analysis in PO-5 (Table 3-5). The following three different carbon source stock solutions were prepared for addition to systems: dextrose, sodium succinate, and sodium acetate. Prepared carbon and yeast extract solutions were sterilized by autoclave, and carbon sources were added at a C: N molar ratio of 12:1. Each system contained a total of 50 mL of media in 200 mL volumetric flasks. As studies on heterotrophic SNdN activity observed maximum TAN reduction rates at C: N ratios of 8:1 or higher, a 12:1 ratio was chosen to provide ample carbon and ensure it did not become the limiting reagent in TAN reduction (Kim et al., 2005) and (Zhang, et al., 2011).

Table 3-5: *PO-5 Experimental Design*

Media: Minimal Media (MM) Temperature: 35° C Speed: 100 rpm Carbon Sources: Dextrose (Dex.), Succinate (Succ.), Acetate Growth Factor: Yeast Extract (YE) Duration: 18 hr.	System (Inoculum) 1. MM & Dex. & YE (275) 2. MM & Succ. & YE (275) 3. MM & Acetate & YE (275) 4. MM & Dex. (275) 5. MM & Succ. (275) 6. MM & Acetate (275) 7. MM (275)
Sampling Constituents & Frequency: PO-5: TAN @ 0, 18 hours	

3.2.5 PO-6 Experimental Design

Optimal dissolved oxygen concentration for TAN reduction was determined indirectly through orbital shaking speed variation. The experimental setup for PO-6 employed three separate orbital shaker tables each set at one of the following speeds: 50, 100, and 150 rpm (Table 3-6). This setup allowed for determination of ideal shaking speed based on maximum TAN reduction rates. Wastewater and minimal media were both utilized to assess impacts in both and observe if variations in shaker speed affected media differently. Results from PO-6 helped determined which shaker speed would be employed in future experiments. All other media and inoculum preparation for PO-6 followed protocols previously outlined.

Table 3-6: *PO-6 Experimental Design*

Media: Screened, Sterilized Primary Wastewater & Minimal Media Temperature: 35° C Speed: 50, 100, 150 rpm Carbon Sources: Dextrose (Dex.), Succinate (Succ.) Growth Factor: Yeast Extract (YE) Duration: 48 hr.	System (Inoculum) 1. WW & Dex. (275) @ 50 rpm 2. MM & Dex. & YE (275) @ 50 rpm 3. MM & Succ. & YE (275) @ 50 rpm 4. WW & Dex. (275) @ 100 rpm 5. MM & Dex. & YE (275) @ 100 rpm 6. MM & Succ. & YE (275) @ 100 rpm 7. WW & Dex. (275) @ 150 rpm 8. MM & Dex. (275) @ 150 rpm 9. MM & Succ. & YE (275) @ 150 rpm
Sampling Constituents & Frequency: PO-6: TAN, OD, pH @ 0, 24, 48 hours	

3.2.6 PO-7 Experimental Design

Media volume was increased from 50 mL to 75 mL during PO-7 to account for increased sampling volumes for total nitrogen analysis. To keep volume to surface area ratios constant from PO-4 - PO-6, volumetric flask size was also increased from 200 mL to 250 mL. In addition to yeast extract, growth factor addition of vitamin (ATCC MD-VS, Vitamin Supplement, Manassas, VA) and trace mineral solution (ATCC MD-TMS, Trace Mineral Supplement, Manassas, VA) was analyzed for impact on TAN reduction (Table 3-7). Autoclave and filter sterilization sterilized mineral and vitamin solutions, respectively. Vitamin and trace mineral supplement solutions were added at 1% by volume, as recommended by the manufacturer. Yeast extract solution was dosed at 1% by volume, based on success at 1.42% addition (Kim et al., 2005).

Table 3-7: *PO-7 Experimental Design*

Media: Defined Minimal Media	System (Inoculum)
Temperature: 35° C	1. MM & YE (275)
Speed: 100 rpm	2. MM & Vit./Min. (275)
Carbon Source: Sodium Succinate	3. Control
Growth Factor: YE, Vitamin & Mineral Supplement	
Duration: 48 hr.	
Sampling Constituents & Frequency:	
PO-7: TAN, NO_3^-, NO_2^-, OD, pH, TN (Elementar) @ 0, 8, 24, 48 hours	

3.2.7 PO-8 Experimental Design

Analysis for nitrate and nitrite was not performed during PO-8 due to the negligible presence in each PO experiment it was previously analyzed (PO-1, 2, 3, and 7). Analysis during PO-7 determined supplemental vitamin and trace mineral additional improved TAN reduction in minimal media better than yeast extract addition. Subsequently, it was desired to know whether both vitamin and mineral solution addition was required, or if only of the two was vital in TAN reduction. Thus, PO-8 was performed under identical conditions as PO-7, with additional systems to separate effects of vitamin and mineral addition (Table 3-8).

Table 3-8: *PO-8 Experimental Design*

Media: Defined Minimal Media	System (Inoculum)
Temperature: 35° C	1. MM & YE (275)
Speed: 100 rpm	2. MM & Vit./Min. (275)
Carbon Source: Sodium Succinate	3. MM & Vit. (275)
Growth Factor: YE, Vitamin, Mineral Supplements	4. MM & Min. (275)
Duration: 48 hr.	5. Control

Sampling Constituents & Frequency:

PO-8: TAN, OD, pH, TN(Elementar) @ 0, 8, 24, 48 hours

3.2.8 PO-9 Experimental Design

Yeast extract was no longer used in experiments after PO-8, due to analysis which showed that supplemental vitamin, and more importantly, mineral solutions improved TAN reduction rates in minimal media much more than yeast extract. Additionally, as PO experiments began to answer questions posed about operational and environmental parameters, inoculum used in experiments switched from *Bacillus pumilus* (Strain 275) to Aqua beginning in PO-9 (Table 3-9). As Aqua analysis during PO-1 and PO-2 showed a plateau of TAN reduction at 50% of initial, carbon was postulated as being the limiting reagent. C: N ratios were not analyzed during PO-1 and 2, thus, this hypothesis was tested by analyzing C: N ratios in PO-9. Stock solutions were prepared for carbon addition at C: N molar ratios of 12:1 and 24:1 respectively.

Table 3-9: *PO-9 Experimental Design*

Media: Defined Minimal Media	System (Inoculum)
Temperature: 35° C	1. MM & Dex. (Aqua @ 1500 ppm)
Speed: 100 rpm	2. MM & Succ. (Aqua @ 1500 ppm)
Carbon Source: Dextrose, Succinate	3. MM & Succ. x2 (Aqua @ 1500 ppm)
Growth Factor: Vitamin & Mineral Supplements	4. Control & Dex.
Duration: 48 hr.	5. Control & Succ.
	6. Control & Succ. x2
Sampling Constituents & Frequency:	
PO-9: TAN, OD, pH, TN (Elementar) @ 0, 8, 24, 48 hours	

3.2.9 PO-10 Experimental Design

Experiments PO-7 and PO-8 determined that trace mineral, and to a lesser extent, vitamin supplement improved TAN reduction in minimal media with *B. pumilus* as inoculum. The focus of PO-10 was to determine if the pattern of growth factor addition in improving TAN reduction held true when Aqua was applied as inoculum (Table 3-10). As previous experiments showed negligible nitrate or nitrite presence, neither was analyzed in PO-10. Additionally, as experiments PO-7, 8, and 9 showed no reduction in total nitrogen, this was not analyzed in PO-10. As with all prior experiments in minimal media, sterilization of all media constituents prior to setup was performed.

Table 3-10: *PO-10 Experimental Design*

Media: Defined Minimal Media	System (Inoculum)
Temperature: 35° C	1. MM & Vit. (Aqua @ 1500 ppm)
Speed: 100 rpm	2. MM & Min. (Aqua @ 1500 ppm)
Carbon Source: Sodium Succinate	3. MM & Vit. /Min. (Aqua @ 1500 ppm)
Growth Factor: Vitamin & Mineral Supplements	4. Control
Duration: 48 hr.	
Sampling Constituents & Frequency:	
PO-10: OD @ 0, 8, 12, 24, 48 hours	

3.3 Full-Scale (FS) Experiment Methods

Upon completion of PO experiments 1 through 10 and statistical evaluation of TN analysis method, experimentation focused on increasing the scale and length of experimentation. The aim of these “full-scale” (FS) experiments was to closer simulate environmental parameters that would be encountered in commercial application of the product. Therefore, they were performed in primary wastewater, that was filtered for homogeneity, but not sterilized. The bacterial competition that native microbes afforded the Aqua assemblage is an environmental hurdle that would be encountered in real-world application and thus a necessary test for product evaluation. Additionally, while previous Aqua experiments were inoculated at 1500 ppm, dosing in full-scale experiments were reduced to levels closer to recommended concentrations (1 to 10 ppm). Carbon and growth factor additions were not planned for full-scale experiment 1 as they would not be present in real-world application.

3.3.1 Full-Scale Experiment 1

Experiment FS-1 was performed in 1000 mL flasks for 11 days and aimed to distinguish effects between dosing frequency and concentration. Each of the three systems was run in triplicate for better statistical accuracy of results - a total of 9 flasks. The 11-day experiment was performed in a large floor table shaker (New Brunswick Scientific Co. Inc., Controlled Environment Incubator Shaker, 15846, New Edison, N.J.) which could accommodate all nine 1000 mL flasks as well as control temperature and orbital shaking speed.

Table 3-11: *FS-1 Experimental Design*

Media: Screen Primary Wastewater (WW)	System (Inoculum)
Temperature: 35° C	1. WW (Aqua @ 10 ppm initial)
Speed: 100 rpm	2. WW (Aqua @ 1 ppm each of 10 days)
Duration: 11 days	3. WW (None)

Sampling Constituents & Frequency:

TAN, NO_3^-, NO_2^- @ 0, 3, 7, 10 days

OD @ 0, 1, 3, 5, 7, 10 days

TN (Hach Kits) @ 0, 5, 10 days

C: N (Elementar) @ 0, 10 days

Eight liters of wastewater was collected from the primary clarifier at the San Luis Obispo Water Resource Reclamation Facility (SLO WRRF). After overnight refrigeration, the wastewater was passed through folded mesh screening to remove particle clumps and increase homogeneity for improved analysis precision (Figure 3-2). Screening used had pore size of 1mm and was folded over twice to obtain smaller pore size. Wastewater volume was then reduced to 3.7 L and analyzed for initial TAN, nitrate, and nitrite levels in quintuplet. Initial TAN levels averaged 35.7 ppm as N, with a standard deviation of 0.86 ppm. The bulk volume of 3.7 L was spiked to 50 ppm TAN-N by adding 202.2 mg of ammonium chloride. Initial nitrate and nitrite levels were negligible (< 1 ppm) as expected in primary wastewater.

Figure 3-2: Primary wastewater in two 4-L containers setup in the laminar flow workstation, next to screening apparatus used to remove oversized organic particles present in the wastewater.

After initial analysis, 400 mL of wastewater was then poured into each of the 9 flasks; each flask having been sterilized by autoclave at 15 psi and 121° C for 15 minutes and hand washed afterward. As outlined above in Table 3-11, analysis for FS-1 examined two different dosing procedures, and one control, with each analyzed in triplicate.

Dosing Concentration and Inoculation Frequency

1. 10 ppm Aqua: Day 0 and none thereafter – System 1
2. 1 ppm Aqua daily: Day 0 - Day 9 – System 2
3. 10 ppm sterilized Aqua: Day 0 and none thereafter – System 3

The purpose of adding sterile Aqua to the control (system 3) was to account for the Carbon being added to systems 1 and 2. Aqua contains 96.9% dextrose, with the remaining makeup specialized bacteria. To sterilize the 4,000 ppm Aqua stock-solution it was heavily autoclaved at 15 psi and 121° C for 45 minutes.

Stock solution preparation is outlined previously in *Microorganism & Inoculum Preparation*. System 1 was inoculated with 1 mL from prepared 4,000 ppm Aqua stock solution on day 0 for a 10-ppm initial dose. System 2 was inoculated daily with 0.1 mL for a 1 ppm dose. After initial dosing and between daily dosing for system 2, stock solution was stored in refrigeration at 4° C. Daily dosing to system 2 were performed with stock solution actively stirred on a stir plate for homogeneity.

3.3.2 Full-Scale Experiment 2

Experiment FS-2 was also performed in 1000 mL flasks for 11 days. Based on results and analysis from FS-1, FS-2 aimed to distinguish effects between dose preparation as well as carbon addition. Five systems were run in duplicate for a total of 10 flask systems, for better statistical accuracy of results. Like FS-1, FS-2 was also ran in a large floor table shaker, which in addition to accommodating all ten 1000 mL flasks, provided ease of sampling and control over temperature, and shaking speed.

Table 3-12: *FS-2 Experimental Design*

Media: Screen Primary Wastewater (WW)	System (Inoculum)
Temperature: 35° C	1. WW (Aqua @ 50 ppm “activated”)
Speed: 100 rpm	2. WW (Aqua @ 50 ppm “stock”)
Duration: 11 days	3. WW & Carbon (Aqua @ 50 ppm “stock”)
Carbon Source: Dextrose	4. WW
	5. WW & Carbon
Sampling Constituents & Frequency:	
TAN, NO_3^-, NO_2^- @ 0, 3, 7, 10 days	
OD @ 0, 1, 3, 5, 7, 10 days	
TN (Hach Kits) @ 0, 3, 7, 10 days	
C: N (Elementar) @ 0, 10 days	

Like FS-1, primary wastewater was collected, screened, and analyzed in quintuplet for initial TAN, NO_3^-, and NO_2^- levels. Initial TAN levels averaged 51.1 ppm TAN as N, with a standard deviation of 1.36 ppm. Due to the elevated initial TAN level, no additional ammonium chloride was added to obtain a 50 ppm TAN as N starting level. Initial nitrate and nitrite levels were negligible (< 1 ppm) as expected in primary wastewater.

Analysis from FS-1 showed no observable effect from Aqua application by either dosing procedure. Subsequently, FS-2 dose concentration was increased to 50 ppm for all inoculated flasks. All FS-1 product dosing was performed from stock solutions; ergo, bacteria in Aqua were afforded no advantage in establishing sizable populations in comparison to native microbes already present in the wastewater. FS-2 analyzed the impact of giving bacteria in Aqua both time and nutrient-rich media (TSB) to grow in before

inoculation into system 1. Procedure for “activated growth” is discussed previously in *Microorganism & Inoculum Preparation.* From the prepared concentrated activated growth solution, 0.5 mL was added to each appropriate system 1 flask of 400 mL of wastewater media for a 50 ppm dose. In addition to activated growth inoculum in system 1, each flask from systems 2, 3, 4, and 5 had 0.5mL of uninoculated growth media (TSB). This addition of TSB was added to normalize for the addition of nutrients present in TSB.

The second variable that FS-2 evaluated was the addition of carbon. Results from FS-1 indicated an average C: N of 2.4 at the commencement of the experiment. This ratio was lower than expected and was considered a possible reason for the lack of rapid TAN reduction in FS-1. Therefore, 977.8 mg of dextrose was added to each flask to bring the molar C: N ratio to 12:1, which had proved successful TAN reduction in previous PO experiments 6, 7, 8, and 9.

PO-5, 6, and 9 confirmed the preferred carbon source of sodium succinate for maximum TAN reduction. However, consultation with the BiOWiSH shifted carbon addition to dextrose for FS-2 as this was the carbon source present in Aqua product. Testing performed confirmed C: N ratios of 14:1 for carbon added flasks, compared to 3:1 for non-amended flasks at the initial time point. While 14:1 is slightly higher than the aim of 12:1, it is likely due to underestimation of ammonia-nitrogen by the Elementar, as discussed in greater detail in *Statistical Analysis Experiment Results.*

3.4 Quality Assurance & Quality Control

Quality Assurance & Quality Control (QA/QC) are important measures to perform during any laboratory or field testing to ensure accuracy and precision of results prior to analysis. Poor

QA/QC can render data statistically inaccurate and unusable. During research performed in support of this thesis QA measures were employed to maintain integrity of experimentation, such as keeping accurate and updated laboratory notebooks, proper training and oversight of research assistants, and experimental design and plan checks with professors and BiOWiSH representatives.

In general, QA/QC methods may be checked through both internal and external measures to check the integrity of sampling, handling, and analysis procedures throughout a specific process (Quality, 2012). However, research performed and analyzed within the same group employed only internal QA/QC methods. Throughout all aspects of experimental planning, sample collection, and sample analysis, QA/QC checks were used to determine the efficacy of results.

Sample collection methods used include running systems in duplicate and triplicate. These replicates ensured precision of laboratory analysis as well as indicating differences between systems. With some PO experiments performed for rapid, baseline analysis, these measures were bypassed in the early stages of screening experimentation. However, latter PO experiments that analyzed Aqua performance (PO-9 and 10) and both full-scale (FS) experiments used robust QA/QC measures to ensure accuracy and precision of results.

In addition to running systems replicates, analysis methods also analyzed split and spiked samples to test precision and accuracy, respectively. Split samples tested precision of laboratory instruments and methods as these lab replicates that should ideally give identical measurements. Spike samples were performed by spiking samples with a known concentration of standard. Spikes tested accuracy of analysis method, as they represent a predictable amount of standard added to a sample.

Calibration standards were used to calibrate respective instruments with standards of known concentration, prior to sample analysis. Calibration curves were developed from calibration standard readings and used to determine accurate sample results. In analysis performed with spectrophotometers (TN), standards were used to convert absorbance into ppm. Calibration blanks were also analyzed to ensure proper “zero” of instruments. Calibration blanks used deionized water to zero the instrument as well as check for instrument drift throughout analysis.

Control verification standards (CVS) were used to ensure instrumentation remained accurate throughout sample analysis. CVS were prepared like calibration standards, but instead of running them prior to analysis, they are dispersed throughout samples to ensure accuracy for the duration of analysis.

3.5 Analytical Methods

The following section will cover all analytical methods that supported this thesis and associated research. During various stages in research samples were tested for the following: TAN, NO_3^-, NO_2^-, C: N, TN, OD, pH and CFU counts. Each discussed method will cover instrumentation, preparation, and operational technique.

3.5.1 Total Ammonia Nitrogen (TAN)

TAN analysis was performed via Ammonia Analyzer (Timberline Instruments, TL-2800 Ammonia Analyzer, Boulder, CO). The sample is mixed with prepared caustic solution, which raises the pH to 11 or higher, converting all ammonium ions into dissolved ammonia

gas. Combined sample and caustic then flow over a tubular membrane where the dissolved ammonia gas diffuses through the membrane and is absorbed by prepared buffer solution. Measurement is made of the change in electrical conductance of the absorbing buffer solution, which is directly proportional to the concentration of ammonium ions present in the sample (TL-2800, 2010).

Prior to sample analysis both the buffer and caustic solution were prepared in accordance with Timberline Instruments manuals. Caustic solution was prepared in 2 L batches by adding 200 mL of 50% KOH solution into 1400 mL of DI water and topping off at 2 L. Boric solution was prepared by adding 50 mL of 10,000 ppm boric acid stock solution to 1800 mL of DI water, and titrating in diluted 1M Ammonium Hydroxide until pH stabilized at 6.9 (Blackwell, 2016).

These boric and caustic solutions provide the mechanism by which the ammonia analyzer reads the amount of TAN present in the sample, and correct preparation is key for consistent, accurate results. Samples were loaded into the Ammonia Analyzer by autosampler for analysis (Cetac, ASX-260 Autosampler, Omaha, NE). Accurate TAN analysis was ensured by running calibration standards prior to each run as well as ammonia standards during sample analysis (Aqua Solutions Inc., Lot # 7060257, Deer Park, TX).

3.5.2 Oxidized Nitrogen (Nitrate & Nitrite)

Nitrate and nitrite analysis was performed by Ion Chromatography (Thermo Scientific, Model DIONEX ICS-1600, Waltham, MA) and accompanying autosampler (Thermo Scientific, Model DIONEX AS-DV, Waltham, MA). Ion chromatography is an industry standard method for anion analysis and works on the mechanism of separating anions based on their affinity

to the present ion exchanger. Eluent was prepared from sodium bicarbonate anhydrous (Fisher Chemical, S495-500, Waltham, MA) for use as the carrier liquid in the ion chromatography process. Once sample is automatically injected into the eluent stream, the system pump pushes the mixture through a separator column which uses polymeric resin to remove contaminants. Sample ions become separated at different rates as they pass through the IC column based on interactions with ion exchange sites. After eluent and sample leave the column, a conductivity cell measures electrical conductance of sample ions and produces a signal which is transmitted to data collection system software (Dionex, 2012).

Similar to TAN analysis, calibrations of sodium nitrate and sodium nitrite solution were run prior to sample analysis which directly related peak height and area to known concentrations. Proper preparation of standards and calibrations is vital for accurate analysis.

3.5.3 Carbon to Nitrogen Ratio (C: N)

The Elementar varioMAX CNS (Elementar, varioMAX CNS 217745, Langenselbold, Germany) was initially utilized for TN analysis in PO experiments, and later for molar C: N ratios. As outlined below in *Sample Collection & Preservation* each sample loaded onto the Elementar was covered with parafilm and frozen at -80° C in specialty fitted Elementar crucibles. The varioMAX CNS instrument is commonly used for soil sample analysis and was fitted with an autosampler. However, due to concerns mentioned in *Sample Collection & Preservation* each frozen sample was manually loaded and individually analyzed, so that on average each sample was exposed to room temperature for a maximum of 10 to 15

minutes. This process prevented samples from thawing, which would have potentially allowed for ammonia volatilization and the resumption of biological activity.

The Elementar varioMAX CNS relies on the Dumas method for nitrogen analysis, which works by initially combusting the sample at 900° C. Carbon dioxide and water are removed through absorption columns, and the nitrogen is quantitatively converted to N_2 gas by subsequent oxidation and reduction tubes. A thermal conductivity detector measures total nitrogen as N_2 and gives a reading in percent nitrogen (Muller, 2017). Samples analyzed by Dumas method were calibrated by analyzing soil standards produced for Elementar nitrogen analysis (Elemental Microanalysis, Medium Organic Content Soil Standard OAS BN 288390, United Kingdom).

3.5.4 Total Nitrogen (TN)

TN analysis was evaluated using Hach TN High-Range Kits (Total Nitrogen Reagent Set, HR, TNT, 2714100, Loveland, CO). Samples were first removed from preservation at -80° C and thawed to room temperature. Standard preparation and analysis setup was performed during sample thawing to begin testing immediately once samples were liquified. Alkaline sodium persulfate and 0.5 mL of sample were added to a provided hydroxide digestion vial, which digested all forms of organic and inorganic nitrogen into nitrate. In concert with addition of persulfate, samples were placed on a heat block (VWR Standard Heat Block, 619 F, Radnor, PA) at 105° C for 30 minutes. After heating, and subsequent sample cooling to room temperature, a second reagent “A,” composed of metabisulfite, was added, and mixed to the hydroxide vial, which eliminated halogen oxide interference. Next, a third reagent “B” was added and mixed which reacted nitrate with chromotropic acid to develop a yellow color in the samples (Blackwell, 2016). After reagent additions and reactions, 2 mL of

digested sample was transferred from the hydroxide vial into “Reagent C” vial, inverted 10 times, and allowed to sit for 5 minutes. After sitting, samples in the “Reagent C” vials were Kim-wiped and measured at 410 nm in the Hach Total Nitrogen Spectrophotometer (Hach, Model DR 3800, Loveland, CO). Absorption readings were compared to calibration curves created from known standards and give concentrations in TN ppm.

3.5.5 Optical Density (OD) Measurements

Optical density (OD) measurements utilized turbidity to estimate microbial cell concentrations in suspension and relate microbial populations among analyzed systems. Measurements taken by spectrophotometer (Molecular Devices, Spectra MAX M2, San Jose, CA) indirectly recorded amount of light absorbed at 600 nm by recording re-radiated or “scattered” light. This scatter was an indication of biomass or active cells in suspension in the sample (Sutton, 2006). For this reason, homogeneity of sample prior to reading was vital for accurate results. See *Sample Collection & Preservation* for methods used to ensure well-mixed samples.

3.5.6 pH Measurements

Periodic measurements of sample pH were recorded to confirm that drops in pH were not inhibiting biological activity. Measurements were recorded with a standard pH Meter (Fisher Scientific, Accumet AB150 pH/mV, Waltham, PA). Additionally, pH measurements were used to determine proper acidification for TAN, NO_3^-, and NO_2^- sample preservation.

3.5.7 CFU plate counts

Experiments PO-1 and 2 utilized plate counting to estimate colony forming unit (CFU) counts per mL. Dilutions were prepared from 10^{-2} to 10^{-8} to observe a plate count in the 30 - 300 CFU range. Each dilution was plated in replicate on laboratory prepared agar plates. Once viable CFU per plate counts were recorded, dilution factor and plated volume were used to calculate CFU per mL. The spread plate method was used, wherein a specific of volume (1 mL) was spread over the agar plate and subsequently incubated before manual CFU counting.

3.6 Sample Collection & Preservation

Sample collection for analysis was performed by pipetting appropriate volumes into 15 and 50 mL volumetric falcon tubes, cuvettes, and crucibles. The following section will detail sample volumes, preparation, and storage & preservation procedures for each of the following analysis performed: TAN, NO_3^-, NO_2^-, C: N, TN, and OD.

3.6.1 Total Ammonia Nitrogen (TAN)

Total Ammonia Nitrogen (TAN) required the collection of 12 mL of sample for each time point and system into a 15 mL falcon tube. Samples were obtained from approximately 2 inches below media surface and away from noticeable flocs. Samples were immediately acidified after collection with 1 drop of 18.4 M sulfuric acid to prevent ammonia volatilization and stop any further metabolic activity. Sample acidification dropped pH below 2 to an average of 1.8 (measured during PO-1 and 2). After collection and acidification, samples were filtered through 0.45 µm nitrocellulose filters using a peristaltic pump and flask system

to remove particles and some colloids before analysis. Samples were stored in refrigeration at 4° C between collection, filtering, and analysis.

3.6.2 Oxidized Nitrogen (Nitrate & Nitrite)

Nitrate and nitrite samples required the collection of 6 mL of sample for each time point and system. Sample collection was performed at the same liquid depth and relative location as TAN sample collection. Like TAN sample preparation, nitrate and nitrite samples were acidified, filtered, and stored prior to analysis.

3.6.3 Carbon to Nitrogen Ratio (C: N)

Samples for analysis by the Elementar required the use of specialty alloy crucibles (Elementar microanalysis, C3064, Langenselbold, Germany) manufactured specifically for use on the varioMAX CNS instrument. Crucibles were prepared by baking them in a furnace for 30 minutes at 900° C. Prior to sample acquisition, crucibles were pre-weighed, and weights recorded. After, 4 mL of appropriate sample was pipetted into crucibles, weighed, covered with parafilm and frozen at -80° C in a deep freezer (Thermo Scientific, TSX Series with V-drive, Waltham, MA) prior to analysis.

3.6.4 Total Nitrogen (TN)

Samples obtained for TN analysis via Hach TN kits were obtained by pipetting 1 mL of appropriate sample from non-flocculated area of each system into 1 mL cryovials (Thermo Scientific, 374130, Waltham, MA) and frozen at -80° C to stop all biological activity before analysis. Sampling method for TN analysis was important to obtain a representative sample

of the system, limitations of utilized TN sampling methods are discussed in *Future Improvements.*

3.6.5 Optical Density (OD) Measurements

Optical density (OD) readings at 600 nm were performed real-time during sampling to evaluate biological growth in systems during experiments. 1 mL of sample was directly pipetted into sterilized cuvettes prior to analysis. Well-mixed samples were vital for accurate readings, and homogeneity was insured by vortexing samples prior to the transfer of 1 mL into a sterile cuvette (Cole-Parmer, EW-83301-10, Vernon Hills, IL). Sample readings above 0.7 were discarded and re-run diluted at a 10 to 1 dilution. These diluted samples were homogenized by drawing up sample and re-injecting three times to prevent settling and concentration gradients.

In general, a maximum of 7 to 10 days from sample to analysis day was used as a benchmark to ensure minimal degradation of sample integrity. However, samples preserved for TN and C: N analysis at -80° C were often kept longer, up to three weeks before analysis.

3.7 Statistical Analysis Experiment Methods

Total nitrogen analysis was necessary to understand if system losses were occurring and thus by which means TAN reduction was taking place. Total Kjeldahl Nitrogen (TKN) was not an option for analysis as it does not account for oxidized nitrogen, as well as being arduous in nature. Therefore, two methods were utilized for TN analysis: 1) Hach High-Range Total Nitrogen Kits and 2) Elementar varioMAX by Dumas Method.

Experiment PO-7 first attempted to analyze TN with the varioMAX, and discovered the instrument recorded increases in TN values at the completion of the experiment. Similar increasing TN patterns continued through PO-8 and PO-9 causing concern that readings were not accurate. Skeptical that microbiology in the systems was fixing nitrogen and increasing TN, other options for TN analysis were considered. Ultimately, Hach TN Kits were chosen for evaluation. Each of the two methods were analyzed for differences in readings based on form of nitrogen being measured: ammonia-nitrogen versus organic nitrogen. The ammonia analyzer served as the calibration instrument for comparisons of ammonia-nitrogen, after confirmation of accurate and precise measurements. Comparison of accurate ammonia-nitrogen readings from the ammonia analyzer to values from the varioMAX and the Hach Kits helped determine which method was more accurate incorporating ammonia-nitrogen into TN readings. Subsequently, TN readings composed of organic nitrogen were compared to ammonia-nitrogen and analyzed for differences in readings.

Standard solutions of ammonium chloride and dl-Aspartate (organic nitrogen) were prepared in sterilized DI water at concentrations of 80, 100, 120, and 500 ppm as N. Each concentration and was prepared in triplicate to afford more accuracy and precision in results.

3.8 Statistical Analysis Experiment Results

Analysis of 80, 100, 120, and 500 ppm ammonia and organic-nitrogen standards by Hach TN revealed an average of 13% error from expected values, compared to 23% by Elementar varioMAX analysis. Average error values,13% and 23%, represented an average of underestimated and overestimated values compared to expected values. However, when

solely ammonia-nitrogen analysis was considered, the Elementar varioMAX showed consistent underestimation of expected values by 19%. In contrast, results from Hach TN Kit Analysis on Ammonia nitrogen showed a 11% error, with error representing both over and underestimates. Normalizing these values to account for cumulative +/- of expected error more starkly shows the pattern of underestimation of ammonia-nitrogen by the Elementar varioMAX (Table 3-13).

The pattern of consistent and magnified underestimation of ammonia-nitrogen during TN analysis performed on the Elementar is the major takeaway from this statistical analysis. Hach TN Kit, Elementar, and even Timberline analysis showed less than ideal error from expected standard concentrations. However, error from Hach TN Kits and the Timberline appeared to be random, while the Elementar showed an inability to properly analyze TN in the form of ammonia-nitrogen.

Table 3-13: Percent Error for each TN analysis method in comparison to expected values. Normalized Ammonia percent error in row 3 showed systematic underestimation of Elementar compared to Hach TN Kits which had more random error associated with it.

	Hach TN Kit	**Elementar**
Combined (Org. & Ammonia)	13%	23%
Ammonia	11%	19%
Normalized Ammonia	-5%	-20%

This systematic underestimation proved to be problematic in assessing TN values during initial time zero sampling of PO-7, 8, and 9. These experiments were performed in minimal media in which 100% of initial nitrogen was in the form of ammonia-nitrogen. Additionally, roughly 85 - 90% of nitrogen in primary wastewater resides in the form of ammonia-nitrogen.

However, as will be discussed further in *Results & Analysis*, with large TAN reductions, but no TN reductions, nitrogen likely was in organic form at the end of experiments. Thus, the Elementar varioMAX analysis underestimated initial readings but returned more accurate final readings, leading to the appearance of an increase in TN.

After statistical analysis shed light on the inaccuracy of TN readings from the Elementar varioMAX, it was no longer used for TN analysis. However, the instrument remained valuable in its ability to evaluate molar C: N ratios. Ratios may have been slightly skewed during initial timepoints, due to discussed issues reading ammonia-nitrogen. However, C: N ratios at the end of experiments were crucial in determining availability of carbon and its possible role as limiting reagent.

4. RESULTS, ANALYSIS & PROCESS FLOW

Parameter Optimization experiments were crucial in determining what conditions Aqua would thrive, and a necessary step prior to assessing its full commercial potential. Biological products can be sensitive to several environmental variables and understanding how those affect the products applicability is of great importance in determining if the product will be effective in each potential unique setting.

After PO experiments, and before full-scale testing, statistical experiments on TN method analysis were performed to ensure accurate and reliable results. Those methods and results were previously discussed in *Statistical Analysis Experiment Methods and Results.* Upon analyzing results from both PO and statistical method experiments, discussion between BiOWiSH, invested professors, and student researchers determined the course for full-scale experimentation. Two 11-day experiments were performed in 400 mL of primary wastewater to assess Aqua performance in realistic conditions. The following subsections will chronologically present and discuss experiment data and results beginning with PO-1 and concluding with two full-scale (FS) experiments. As results from each experiment determined the aim of successive experiments, this process flow from start to finish is the most efficient way to analyze data and results.

4.1 PO-1 & PO-2: Product Introduction & Baseline Performance Metrics

PO-1 & PO-2 took place with a BiOWiSH representative on hand to assist with experiment setup and introduce the research team to the Aqua product. The aim of PO-1, and subsequently PO-2 was to gain a baseline of Aqua's nitrogen removal performance in sterilized wastewater. As initial hypothesis focused on SNdN as the mechanism of TAN

reduction, TN analysis was performed to examine any system losses that could be attributed to nitrogen gas formation.

In addition to performance in nitrogen removal, four individual strains of Aqua's microorganisms were isolated and used as inoculum. Strain isolates were used as inoculum to determine which microorganism(s) were most efficient at reducing TAN relative to each other. CFU counts per mL were also determined from plate counts.

TAN reduction in PO-1 was less complete (41% TAN removal) than initially expected, and as a result dextrose was added to PO-2 media at a molar C: N ratio of 12:1 (Figures 4-1 and 4-2). It was suspected that carbon may have been the limiting substrate for denitrification to occur, and thus its addition would potentially allow for more rapid SNdN. Dextrose addition improved first-order TAN reduction rates by roughly 7-fold (k_1 = 0.044/hr. versus 0.315/hr.) from PO-1 to PO-2, as well as improving the completeness of TAN removal (41% versus 96%). However, analysis from PO-2 showed no statistically reliable or significant drop in TN.

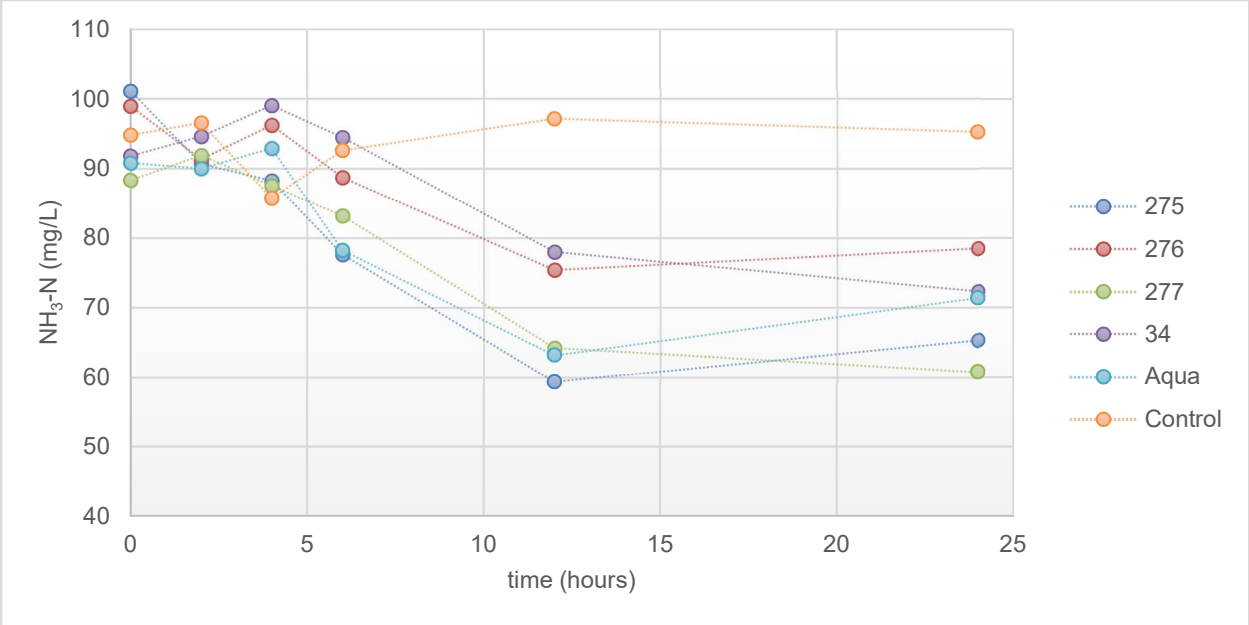

Figure 4-1: TAN analysis results from PO-1 over 24 hours, showing array of isolate and Aqua success in TAN reduction compared to uninoculated control.

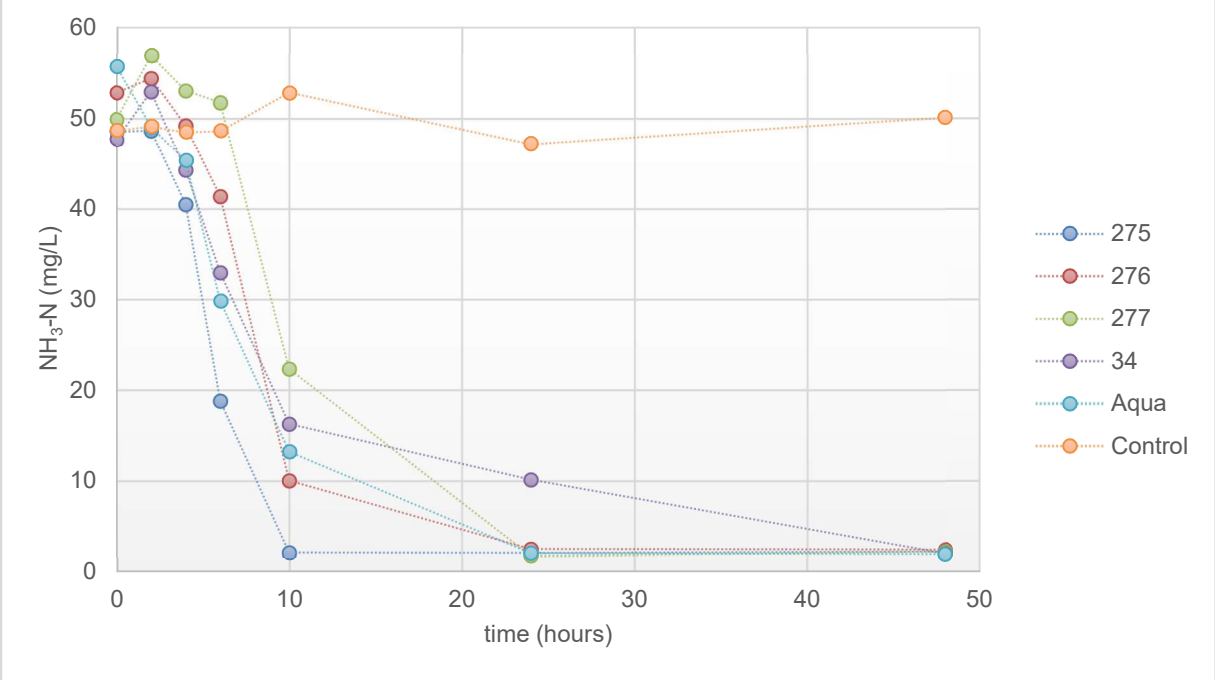

Figure 4-2: TAN over 48 hours from PO-2 showing improved rate and magnitude of TAN reduction in comparison to PO-1 analysis.

TAN data from PO-1 and 2 (Figures 4-1 and 4-2) identified *Bacillus pumilus* “275” as the most rapid TAN removing microorganism of the four tested, with a first-order rate constant of k_1 = 0.315 /hr., compared to k_1 = 0.144/hr. of Aqua. Additionally, OD readings (Figure 4-3) identified 275 as the quickest growing bacterial strain of the four tested. While its population began to crash after 10 hours, this corresponded to a 95.7% substrate reduction (TAN) within 10 hours. Observed OD readings for each system clearly relate to specific isolate growth, as the uninoculated control (orange) remained relatively unturbid (average of 0.32) for the duration of the experiment.

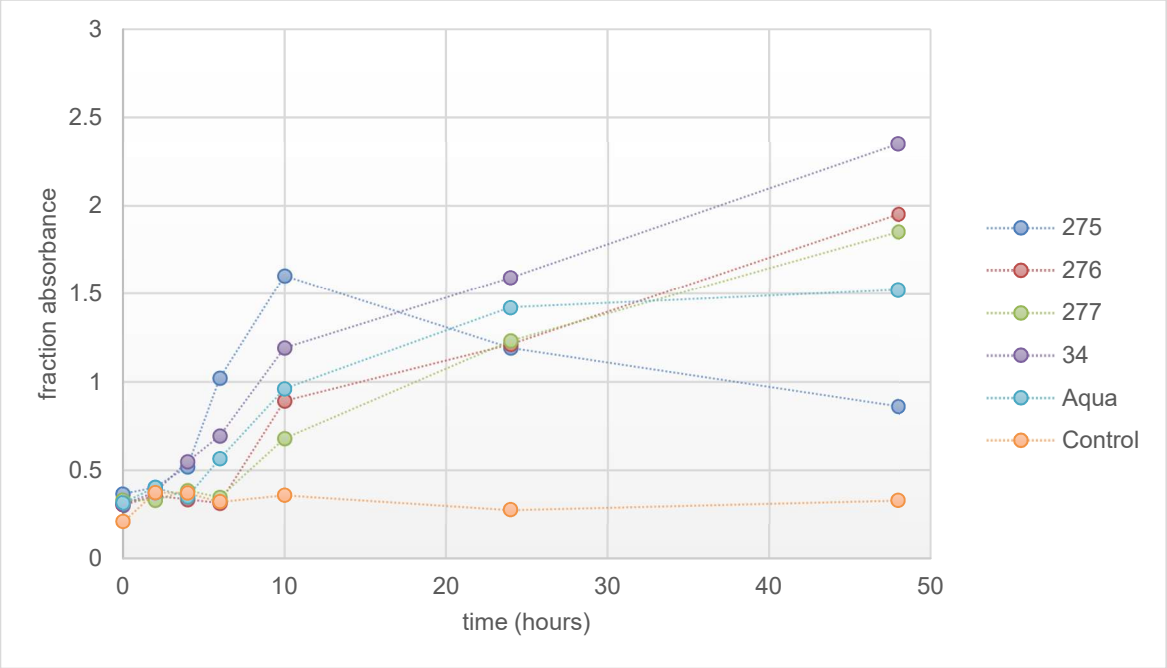

Figure 4-3: OD readings for experiment PO-2 showing strain 275 as the quickest growing microorganism among the 6 tested systems.

One of the initial research goals was to identify the fastest growing organisms in the Aqua assemblage. Identifying the fastest growing microorganism as *Bacillus pumilus* was helpful in determining which bacteria may dominate the system Aqua is applied in. Additionally, determining the truly robust microorganisms in the product was valuable information, so that Aqua could be streamlined and ineffective strains could be removed from the mix. With *Bacillus pumilus* “275” identified as the fastest growing microorganism and one with the

highest TAN removal rate constant, meant it would be the inoculum of focus in subsequent PO experiments.

In addition to observing no drop in TN, PO-1 & PO-2 analysis showed no presence of nitrate or nitrite (<1 ppm as N) throughout the duration of both experiments. This lack of nitrate indicated nitrification was likely not taking place. The question which remained after the introductory experiments was if TAN reduction results could be replicated in a defined minimal media. Replication of rates and magnitude of TAN reduction in minimal media would allow for better understanding of mechanisms by which strain 275 and Aqua performed.

4.2 PO-3, PO-4, and PO-5: TAN Reduction Performance in Minimal Media

Experiment PO-3 aimed to replicate TAN reduction rates observed in PO-2 ($k_{1,\ 275}$ = 0.315/hr.) in a defined minimal media (MM). Replicating results in a simple, yet defined media helped to better understand nitrogen reduction mechanisms of strain 275 and Aqua. Media recipe used was recommended by the BiOWiSH and is defined in *PO-3 Experimental Design*. Data from the control system showed that a lower initial TAN concentration was present compared to inoculated flasks (98 ppm to 122 ppm), likely due to error in experimental setup (Figure 4-4). However, there was no significant drop in TAN across all three flasks, and no difference in system responses to different inoculum.

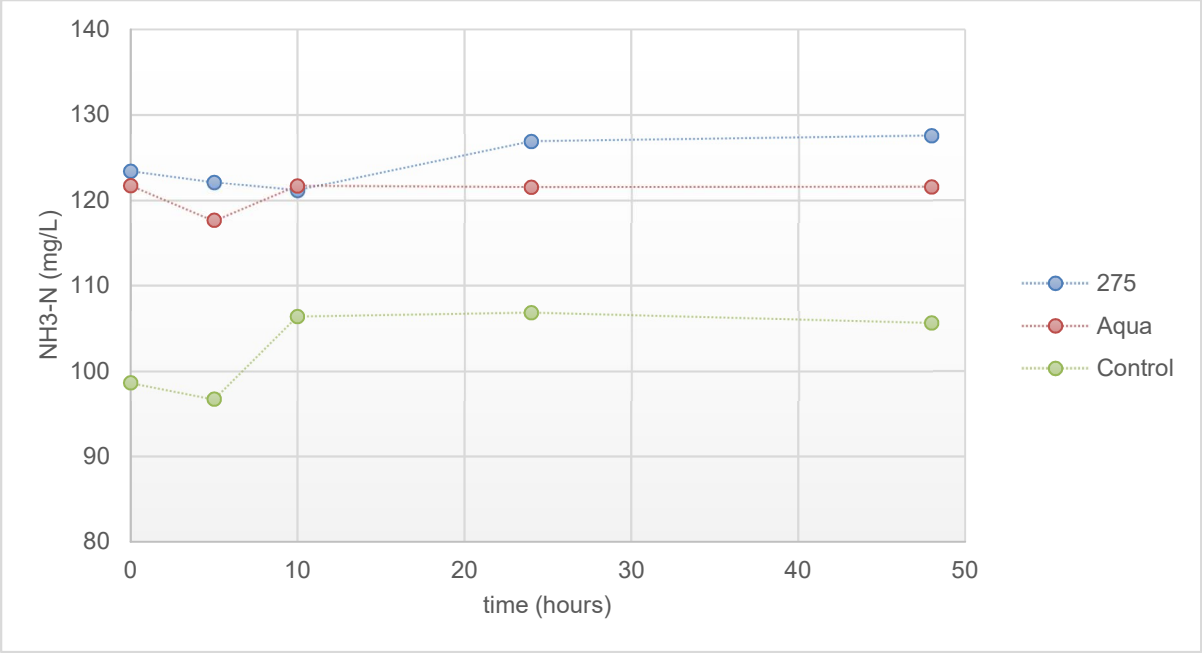

Figure 4-4: Change in TAN over 50 hours during PO-3 showing no effect of inoculum on TAN reduction in minimal media.

After determining that no TAN reduction was realized without the presence of wastewater in media, experiment PO-4 aimed to establish the ratio of wastewater (WW) to minimal media (MM) that would give TAN reduction results like those of PO-2 (Table 4-1). The aim of PO-4 was to determine if a constituent in wastewater was a threshold constituent and any presence of wastewater would induce TAN reduction; or if TAN reduction results directly correlated to amount of wastewater present (Figure 4-5).

Table 4-1: Experiment PO-4 media composition - Wastewater (WW) and Mineral Media (MM).

Flask	1	2	3	4	5	6	7
% WW	0	10	25	50	75	90	100
% MM	100	90	75	50	25	10	0

Experiment PO-4 showed a strong statistical correlation between increased amount of WW present in the media and increased TAN reduction, with an R^2 value of 0.957 (Figure 4-5).

The linear correlation indicated in Figure 4-5 showed that something present in WW was necessary for TAN reduction in a direct ratio, and not a threshold limiting factor. Had each system containing WW saw similar TAN reduction, that would have indicated that a constituent present in WW and not present in the prepared MM was necessary for TAN reduction. However, as this was not the case, PO-4 demonstrated that substrate (carbon) may have been the limiting factor in TAN reduction.

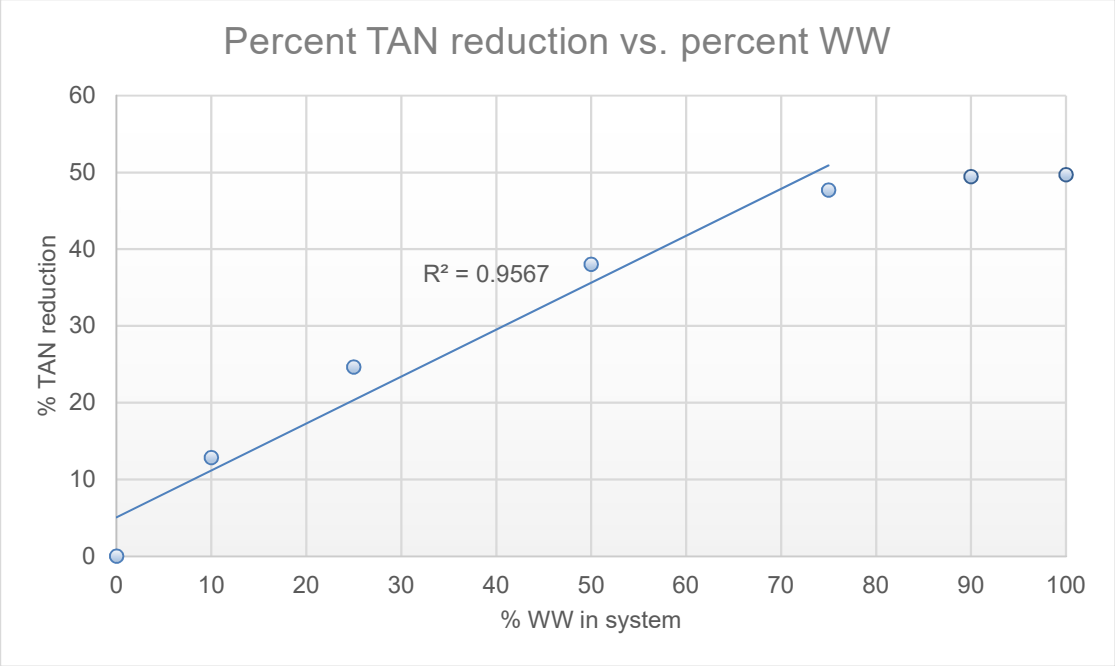

Figure 4-5: Percent wastewater in PO-4 system media and percent TAN reduction from initial values. A strong correlation among the first five flasks is shown, and a plateau in TAN reduction at about 50% thereafter.

While a strong correlation between percent wastewater present in media and percent TAN removal is clear, a plateau like that observed in PO-1 (41% removal) is clear, inhibiting complete TAN removal past roughly 50% from initial levels. For this reason, carbon was suspected as the limiting reagent.

Experiment PO-5 aimed to answer this hypothesis by testing three different carbon source amendments: sodium succinate, dextrose, and sodium acetate in both wastewater and

minimal media. The addition to wastewater media served as a positive control for TAN data analysis. However, data from experiment PO-5 was inconclusive and saw no significant TAN reduction across all systems, with an average percent TAN reduction of 4.4%. The experiment was shifted slightly and re-ran as PO-6.

4.3 PO-6: Optimization of Carbon Source and Table Shaker Speed

As results from PO-5 were inconclusive, experiment PO-6 was performed to properly characterize the effects of carbon amendment to defined minimal media at a 12:1 molar C: N ratio. Table shaker speed was also optimized during PO-6 to determine the speed at which highest TAN reduction rates were observed. Table shaker speed effectively controlled dissolved oxygen (DO) concentration in the systems, as increased speed allowed for increased diffusion from the environment. Optimized table shaker speed was determined by which speed promoted the highest TAN removal.

Maximum dissolved oxygen concentrations depend on both temperature and pressure in water, along with salinity and other media characteristics. With all experiments open to atmospheric pressure and set at a constant 35° C, saturated DO concentrations were thermally capped at 6.93 ppm (Missouri, 2018). In bacteria cultivation systems DO concentration depends on the Oxygen Transfer Rate (OTR) promoted by orbital shaking, which is dependent upon orbital shaft length and increases with shaker speed. Additionally, while transfer rate estimation depends on orbital speed, it is also a function of the bacterial Oxygen Uptake Rate (OUR) (Gomez, 2006). Thus, even a consistent shaking speed does not assure steady DO concentrations within a system. Studies performed with 100 mL of media in 250 mL shaken flasks at 250 rpm have shown maximum DO may remain at 100% saturation depending upon the OUR (Gupta, 2003). However, systems with different growth

rates, exert different OUR and consequently experience different DO concentrations based on growth rates of their microbial populations.

While results from PO-5 were inconclusive at best, it did help determine sodium acetate was not a viable carbon source for TAN reduction and further analysis with carbon addition would focus on sodium succinate and dextrose. TAN reduction in wastewater (WW) systems averaged 92.9% TAN removal during the 48-hour PO-6 experiment and were similar at all three speeds (standard deviation of 1.84 ppm), (Table 4-2). As shaker speed had little effect on TAN reduction in wastewater media, ideal speed was determined from effect on minimal media (MM).

Table 4-2: Shaker speed and carbon source optimization by maximum observed TAN reduction in both Wastewater (WW) and Minimal Media (MM).

Flask	Speed (rpm)	Media	% TAN reduction
F1	50	WW + Dextrose	93.4
F2	50	MM + Dextrose	-8.3
F3	50	MM + Succinate	6.5
F4	100	WW + Dextrose	90.5
F5	100	MM + Dextrose	-0.5
F6	**100**	**MM + Succinate**	**52.0**
F7	150	WW + Dextrose	94.9
F8	150	MM + Dextrose	2.1
F9	150	MM + Succinate	27.5

Analysis from PO-6 showed highest TAN reduction occurred in MM amended with sodium succinate at 100 rpm (Table 4-2). In this system, TAN reduction was 47% and 88% more effective than both the upper (150 rpm) and lower (50 rpm) bound speeds, respectively.

Upon conclusion of PO-6 data analysis, it was determined that no further evaluation of shaker speed was needed.

In addition to determining optimal shaker speed for inoculum effectiveness, experiment PO-6 provided clarity on the most effective carbon addition to minimal media systems. The addition of sodium succinate reduced 31% more TAN, averaged across all three speeds, in comparison to the addition of dextrose (Table 4-2). Flask 6 contained both optimal shaker speed and carbon source, based on TAN removal effectiveness, and is bolded above in Table 4-2.

Results from PO-6 indicated it was the first experiment to show positive TAN reduction in minimal media, as compared to initial observed success in wastewater. While results provided clarity on preferential carbon source and shaker speed; TAN reduction rates in minimal media still did not match those previously observed in wastewater. This is supported in Figure 4-6, by extrapolating growth of bacteria from optical density readings that were measured over the 48 hours of experiment PO-6.

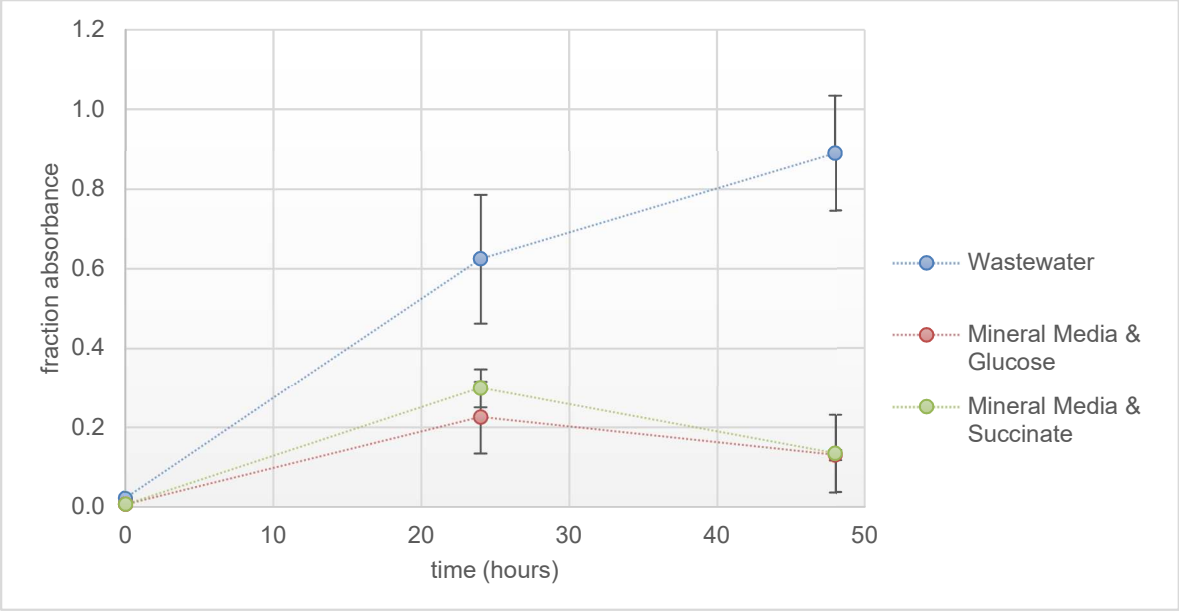

Figure 4-6: Optical density measurements during experiment PO-6 showing greater growth in wastewater compared to minimal media.

Optical density readings for bacterial growth were 6.6 time greater in wastewater than counts in minimal media at the end of the experiment. For this reason, the effect of growth factor addition to minimal media was investigated to determine if this was limiting TAN reduction.

4.4 PO-7: Determining Effect of Growth Factors on TAN Reduction

The investigation of the effect of growth factors on TAN reduction rates was performed by analyzing the effect of yeast extract, and supplemental mineral and vitamin solution additions to defined minimal media. Yeast extract (YE) is a commonly used complex growth factor in biological experiments as it contains nutrients such as peptides, amino acids, purines, and B group vitamins essential for cultivating microorganism growth (Yeast, 2008). Countless experiments have shown the veracity of utilizing yeast extract in facilitating microorganism growth (Li et al., 2011), (Kim et al., 2005).

Trace mineral and vitamin supplement solutions were obtained from ATCC™. Trace Mineral supplement (ATCC MD-TMS, Trace Mineral Supplement, Manassas, VA) solution was based on Wolfe's formulation, a published recipe for trace minerals necessary for stimulation of certain biological activity (Appendix B and C). Both mineral and vitamin supplement (ATCC MD-VS, Vitamin Supplement, Manassas, VA) formulations were manufactured to help speed media preparation and cultivate bacteria growth. The addition of both mineral and vitamin solution (added at 1% by volume) was tested against the addition of yeast extract at 1% by volume (Table 4-3).

Table 4-3: Composition of flask systems in experiment PO-7 with TAN reduction rates and comparison of rates versus control system with no growth factor addition.

Flask	Composition	(TAN reduction rate) k_1 day-1	% rate increase vs. control
C 1&2	MM + YE	0.046	23%
D 1&2	MM + Vitamins/Minerals	0.583	176%
E	Control (no Growth Factor)	0.036	N/A

While the addition of yeast extract proved to increase the TAN reduction rate in comparison to the control, it was markedly less effective than the addition of mineral and vitamin solutions. Table 4-3 shows that replicates of minimal media with mineral and vitamin addition improved TAN removal rates over an order of magnitude in comparison to the control (176% increase), while yeast extract provided a 23% TAN removal rate increase. These TAN removal rates are seen below in Figure 4-7.

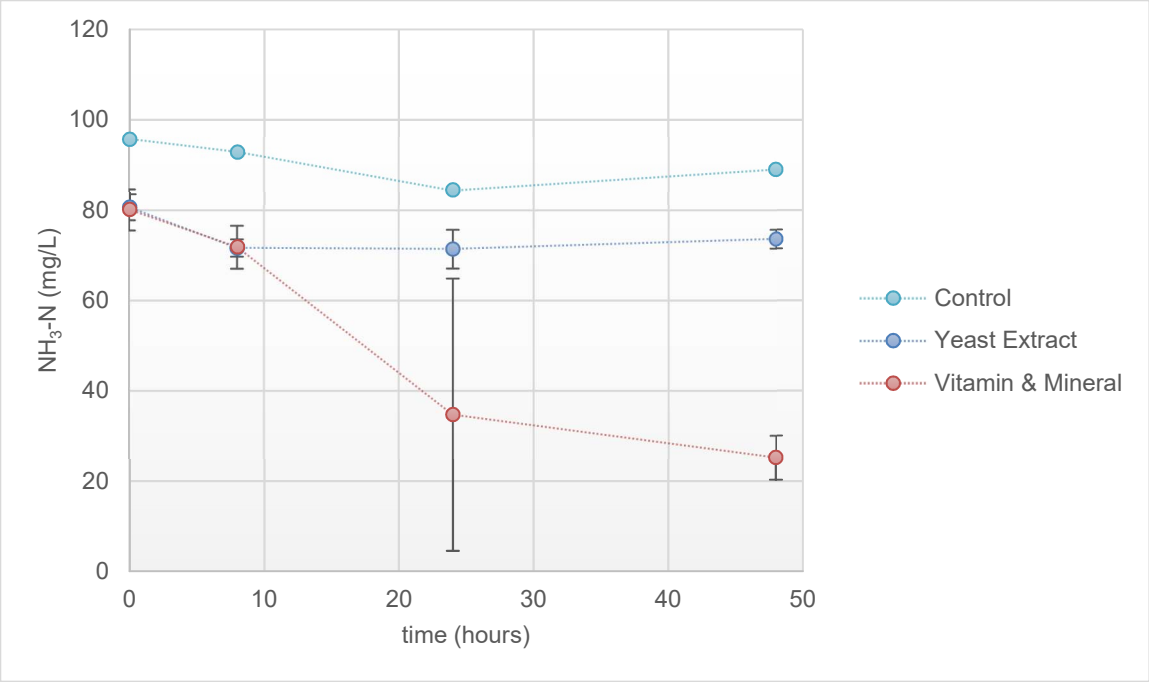

Figure 4-7: TAN versus time during experiment PO-7 showing positive effect of mineral and vitamin addition on TAN reduction.

In addition to TAN reduction rates, system bacterial growth was much more rapid with addition of vitamin and mineral solutions compared to the addition of yeast extract (Figure 4-8).

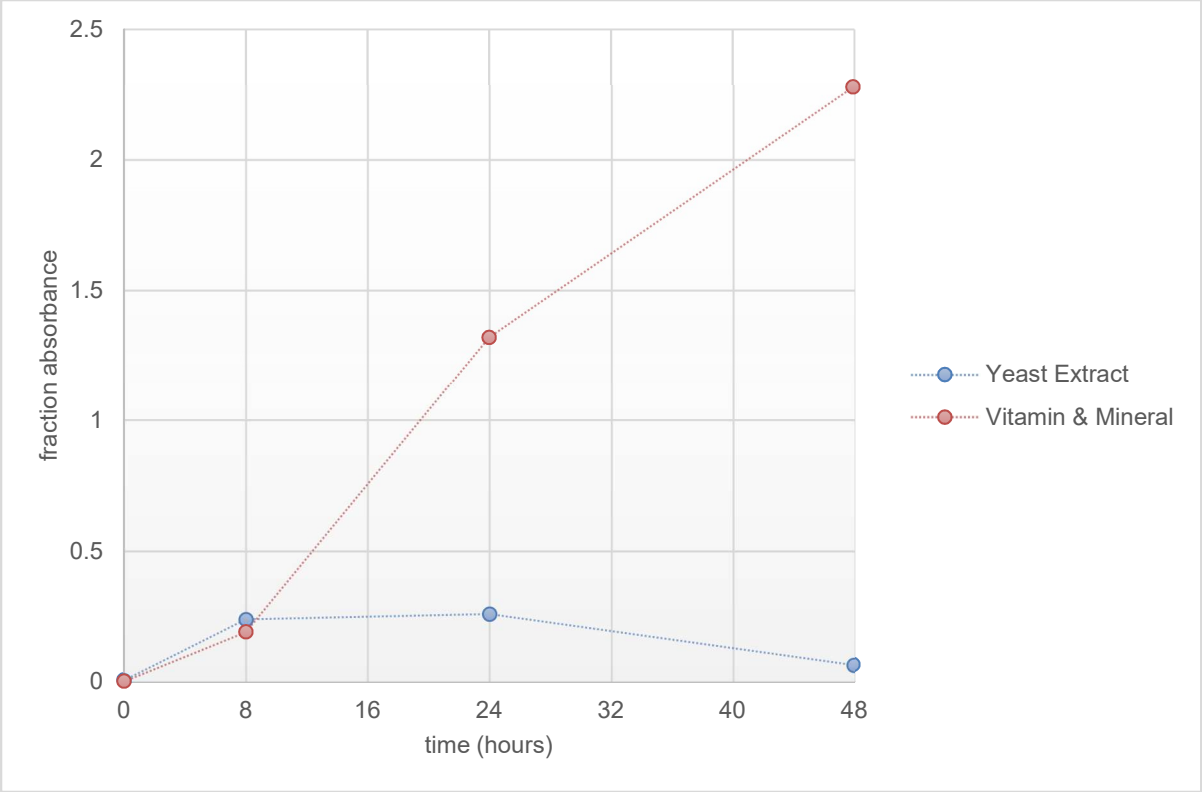

Figure 4-8: Optical density during PO-7 showed increased bacterial growth from 8 to 48 hours with vitamin & mineral supplement addition.

In concert with TAN data, OD data confirmed that vitamin and mineral solutions were a superior growth factor addition to minimal media. Oxidized nitrogen (nitrate and nitrite) was also analyzed during PO-7 and remained under 0.5 ppm for the entirety of the experiment. This lack of nitrate or nitrite presence during TAN reduction, was a pattern observed during PO-1 and PO-2 as well and continued to indicate that nitrification was not occurring while TAN was being reduced.

4.5 PO-8: Continuing Investigation into Growth Factor Effects

Experiment PO-7 served to identify mineral and vitamin solutions as a catalyst source for TAN reduction in minimal media. However, the distinction between which solution, vitamin or mineral, was key in TAN reduction was still unknown and subsequently became the focus of

experiment PO-8. As such, PO-8 analyzed the effect of the following growth factor addition: mineral solution, vitamin solution, mineral & vitamin solutions, and yeast extract. Each growth factor addition was analyzed in minimal media at a molar 12:1 carbon to nitrogen ratio. The primary goal was to identify whether vitamin or mineral solution was the primary growth factor necessary for TAN reduction in minimal media.

Figure 4-9 shows that addition of both mineral and vitamin solutions have the largest TAN reduction (87%), followed closely by mineral addition alone (78%). Data from experiment PO-8 evaluated relative effects of growth factor addition to minimal media with *Bacillus pumilus,* strain 275, as the tested inoculum.

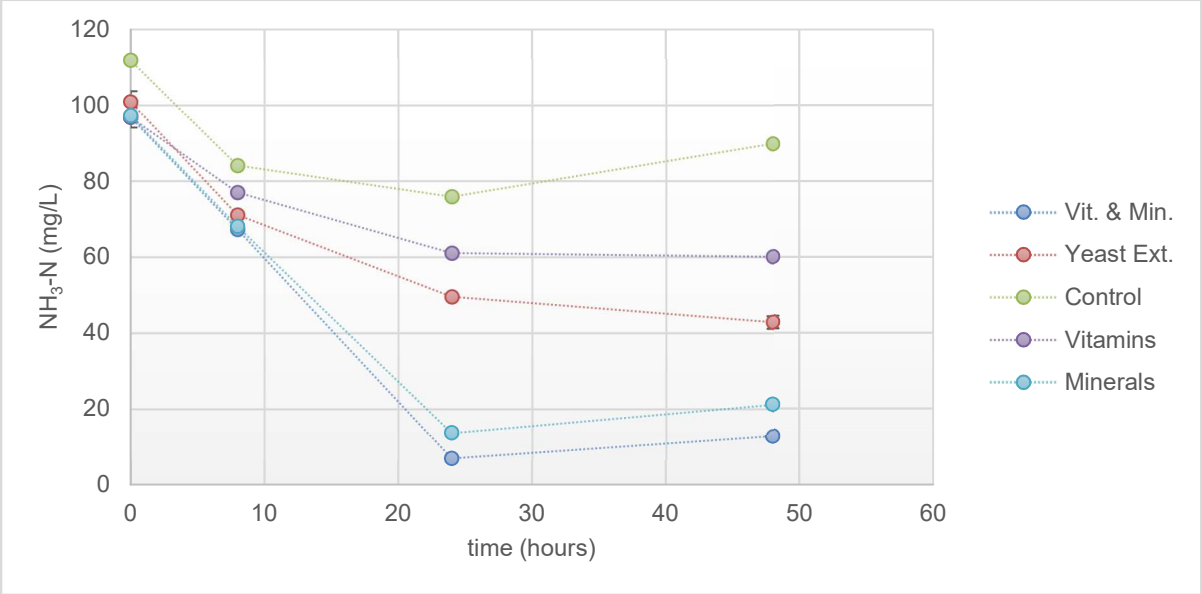

Figure 4-9: TAN concentrations from experiment PO-8 showing most effective TAN removal system with addition of vitamin and mineral solutions, closely followed by mineral addition.

Results also indicated that the addition of vitamin solution was less effective than every other growth factor addition, including yeast extract. The sizable gap of 36 ppm between minerals and yeast extract at 24 hours distinguished between effective and ineffective growth factor addition. Since vitamin addition was the least effective, the 7.6 ppm difference

between mineral and vitamin & mineral addition at 48 hours was determined to likely be due to noise in the data and not significant in determining growth factor effectiveness.

Analysis from PO-7 and PO-8 suggested that yeast extract and vitamin solutions were ineffective growth factors, and thus were no longer considered for individual addition to media systems. Further experiments would likely need to be performed to confirm results from PO-7 and PO-8. These results were tested in experiment PO-10 with Aqua as inoculum. Experiment PO-9 was conducted to test whether the amount of carbon was limiting the extent of TAN reduction in minimal media.

4.6 PO-9: Effect of Carbon Availability on TAN Reduction & Aqua Evaluation

Parameters such as shaker speed, growth factor addition, carbon source, and media composition were analyzed during PO-1 through PO-8. Results and analysis from these experiments were key in understanding product performance under different conditions. After individual strain performance was assessed in PO-1 and PO-2, inoculum was limited to *Bacillus pumilus,* "strain 275," during PO-3 through PO-8 to investigate maximum possible TAN reductions capable. However, after PO-8, it was determined enough screening experiments had taken place and that the complete Aqua product should be evaluated as inoculum. Therefore, PO-9 began Aqua product evaluation that continued through FS-1 & 2.

Data from experiment PO-6 performed in minimal media determined that sodium succinate was the optimal carbon source for TAN reduction. However, the extent of TAN reduction reached a maximum of 52% during the 48-hour experiment. The existence of this TAN reduction plateau helped postulate that bioavailable carbon may have been the limiting reagent in minimal media systems. In addition to plateaus in TAN reduction, the absence of

nitrate or nitrite throughout experiments performed in both minimal and wastewater media indicated that the hypothesized mechanism of SNdN was likely not the method of TAN reduction. As discussed in *Nitrification Overview*, the occurrence of oxidized nitrogen is a marker of nitrification.

As such, TAN removal not due to nitrification, was likely attributable to assimilation into organic nitrogen. Assimilation of ammonia-nitrogen into organic nitrogen requires ample carbon source for the ammonia-nitrogen to bond with. The understanding of the mechanism of assimilation led to the postulation that the availability, or lack thereof, of carbon in minimal media systems was the limiting factor for TAN reduction.

Ergo, experiment PO-9 aimed to evaluate two different molar ratios of carbon to nitrogen (12:1 and 24:1) and their effect on TAN reduction. Dextrose and sodium succinate were both evaluated at 12:1 ratios, and sodium succinate was also tested at a 24:1 ratio. Experiments PO-5 and PO-6 analyzed various carbon sources and their effect on TAN reduction at a 12:1 molar ratio. While those experiments saw rapid TAN reduction, their plateau suggested the initial 12:1 ratio was too low and limited the extent of assimilation. PO-9 analysis of 12:1 and 24:1 C: N ratios is displayed below in Figure 4-10.

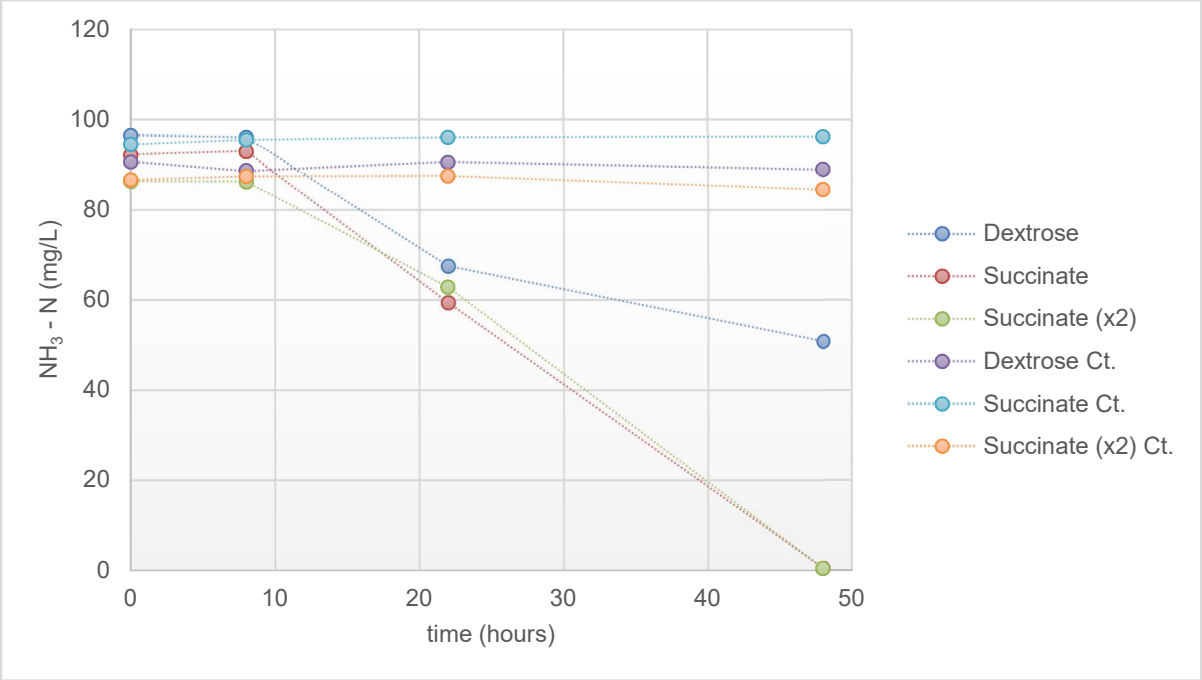

Figure 4-10: Experiment PO-9 showing negligible difference in TAN reduction among C: N ratios of 12:1 and 24:1.

Data displayed above in Figure 4-10 showed complete TAN removal in contrast to PO-6 results; however, no distinction was made between molar ratios of 12:1 and 24:1. In addition, while molar ratios were experimentally planned for 12:1 and 24:1, data analysis revealed actual ratios of 14:1 and 28:1, respectively. This was likely to do the addition of carbon from the Aqua inoculum that was not accounted for during calculations and experiment setup.

While carbon may still act as a limiting reagent in assimilation, results from PO-9 suggested that a 14:1 ratio provided plenty of available carbon. The only distinction that may be made between results from PO-6 and PO-9 is the inoculum used. While PO-9 evaluated the full Aqua product, PO-6 only evaluated strain 275, which may account for the difference in extent of TAN reduction. Aqua was able to provide more complete TAN reduction in comparison to strain 275 alone. This may have occurred because of the additional available

carbon in Aqua or because the entire assemblage of bacteria was available to reduce the TAN in PO-9.

After investigation of carbon as the limiting reagent in TAN reduction, the final parameter optimization experiment returned to the role that growth factor addition played in TAN reduction. Previous experiments PO-3 through PO-8 focused on *Bacillus pumilus,* strain 275 as inoculum; however, PO-10 analyzed the role of growth factor addition with Aqua as inoculum.

4.7 PO-10: Growth Factor Analysis with Aqua

While it was noted that further experimentation was needed to confirm TAN reduction results from PO-8, conversations with BiOWiSH representatives shifted research aims toward focusing on Aqua's effect in wastewater. Thus, the positive correlation that PO-7 and 8 established between TAN reduction and OD readings was used to extrapolate effects observed in PO-10. As such, PO-10 did not analyze TAN, only OD measurements to confirm patterns observed in PO-7 and 8 held true with Aqua inoculum.

Figures 4-11 and 4-12 below demonstrated the same pattern of growth factor effectiveness on bacterial growth as observed in PO-7 and 8. Both figures confirmed the addition of vitamin and mineral solution together promoted the highest rate of bacterial growth, followed by the addition of mineral, and vitamin supplement solution respectively.

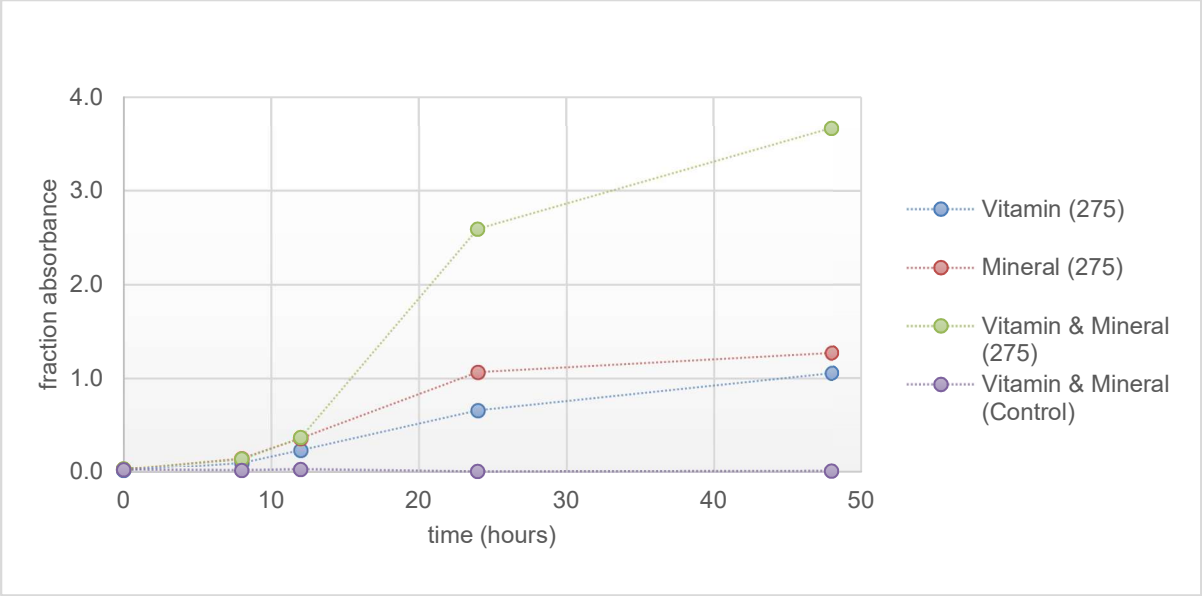

Figure 4-11: Optical density data showing similar pattern of effect on bacterial growth as observed in PO-7 with *Bacillus pumilus* as inoculum.

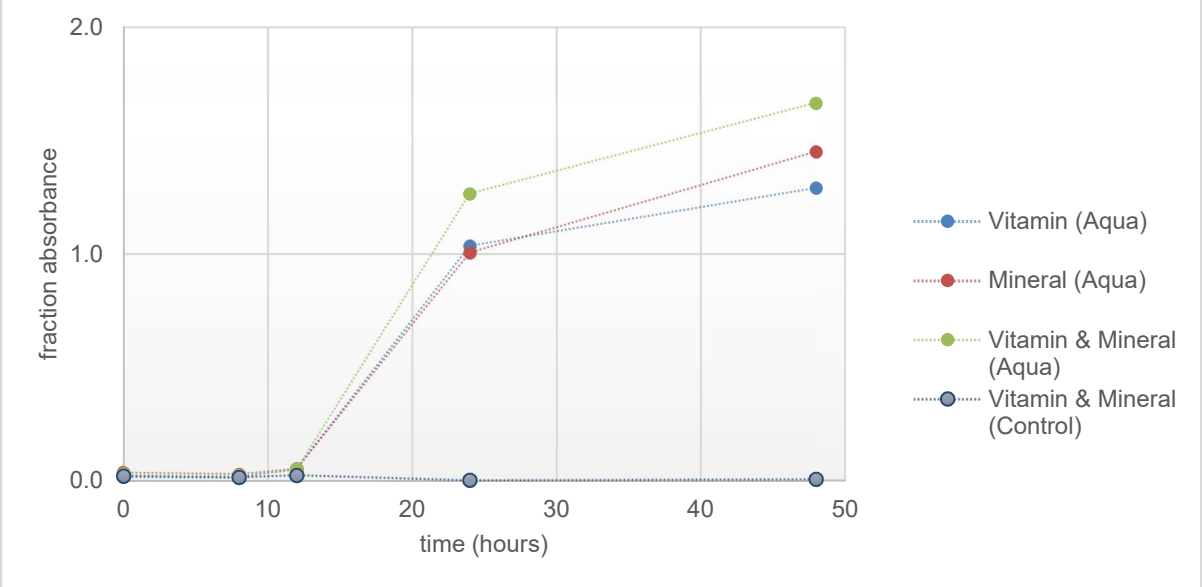

Figure 4-12: Optical density measurements with Aqua as inoculum demonstrating same pattern of effectiveness on biological growth as observed with *Bacillus pumilus* as inoculum.

At the end of parameter optimization experiments, discussion with BiOWiSH representatives and professors shifted experimentation focus. As discussed in *Full-Scale (FS) Experiment Methods,* experimentation would now be evaluated over 11 days on a larger scale.

4.8 Full-Scale (FS) Experiment 1

The aim of full-scale (FS) experimentation was to closer simulate environmental parameters that would be encountered in application of Aqua. Therefore, inoculum levels were reduced closer to BiOWiSH recommended doses (1 to 10 ppm). Additionally, media used was shifted to primary wastewater (unsterilized), as opposed to sterile wastewater or minimal media.

Dose concentration (1 and 10 ppm) and frequency (initial and daily) were analyzed for effect on bacterial cell growth. Results showed there was no discernable difference between un-dosed flasks of primary wastewater and either of the dosing procedures outlined in *Full-Scale (FS) Experiment Methods* (Figure 4-13). This lack of cell growth at both the 10 ppm initial Aqua dose and 1 ppm daily dose suggested the wastewater microbiome was too complex and well-established to afford Aqua bacteria enough substrate to grow to sizable populations. Bacteria native to the system appeared to visibly flocculate and settle out by day 3 of the experiment in each of the three systems. While PO experiments performed in sterile wastewater and minimal media saw notable bacterial growth with time, no such population growth was realized in the primary wastewater media of FS-1. Sample splits indicated precision of laboratory measurements and saw a maximum of 0.011 error for OD measurements performed by spectrophotometer.

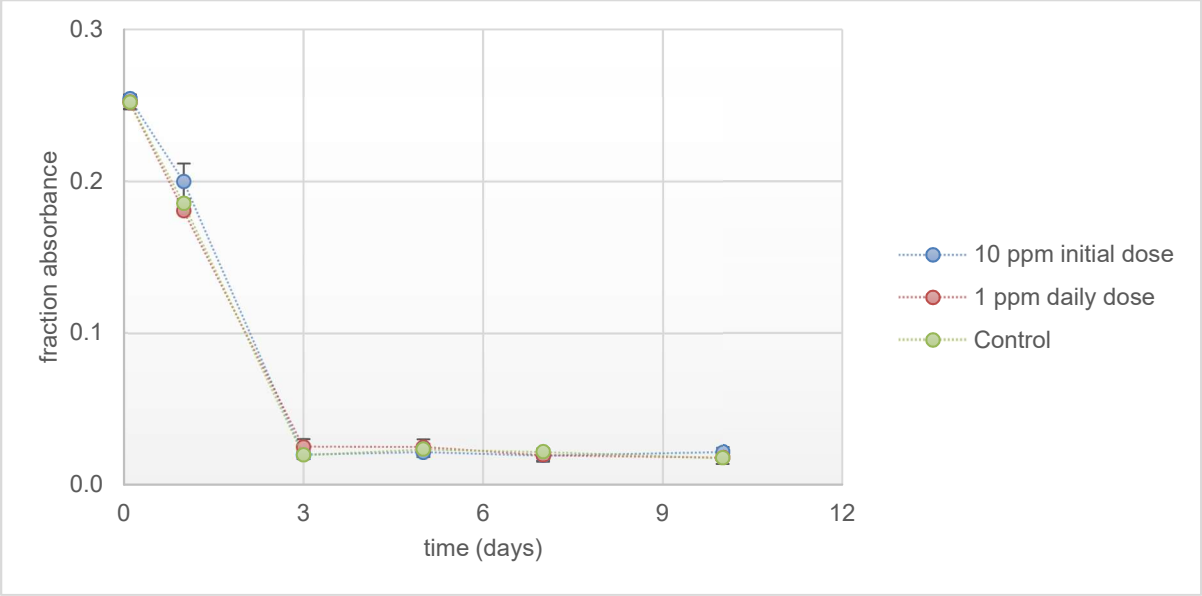

Figure 4-13: Optical density measurements of three FS-1 systems, showing flocculation and settling prior to day 3 measurements. A lack of inoculated bacterial growth was evidenced by an average of 0.02 across all systems from day 3 to 10.

Total ammonia nitrogen (TAN) for all three systems initially started at 45 ppm as N and dropped 84% between days 3 and 7 (Figure 4-14). Throughout the duration of the experiment the standard deviation of the population of nine flasks reached a maximum of 2.09 ppm, which was less than the maximum standard deviation of 3.33 ppm among triplicate flasks (control, day 10). Statistically, this indicated that neither of the two dosing procedures had any effect on observed TAN reduction. Thus, the first-order TAN reduction rate of k_1 = 0.254/ hr. was that of native bacteria present in the systems prior to inoculation.

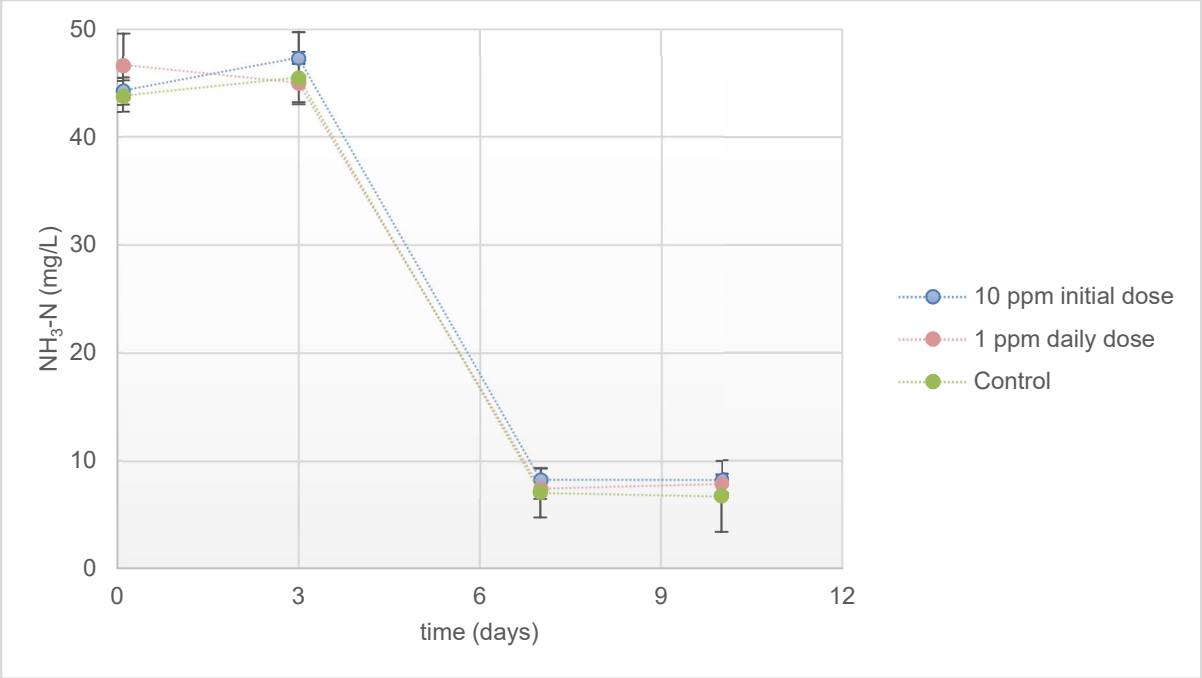

Figure 4-14: TAN showing little variation between three systems tested in FS-1. Error bars on data points represent standard deviation among triplicate for each system and indicated tight precision.

QA/QC measures performed during TAN sample analysis included spikes, splits, control verification standards (CVS), calibration standards, and blanks as discussed in *Quality Assurance & Quality Control*. These metrics are summarized in Table 4-4, below and indicated acceptable levels of both accuracy and precision from TAN analysis.

Table 4-4: QA/QC metrics for TAN analysis by the Timberline Ammonia Analyzer.

QA/QC Metric	Percent difference from expected
CVS 1	4.18
CVS 2	3.64
CVS 3	6.51
CVS 4	3.70
Split 1	0.73
Split 2	4.68
Spike 1	0.52
Spike 2	8.31

Control verification standard (CVS) analysis showed three of four CVS fell within 5% of the expected 50 ppm standard, with CVS number 3 slightly above. This reflected good accuracy of the ammonia analyzer. Split sample analysis confirmed good precision of the ammonia analyzer and experimental method as identical samples were both within 5% of their respective replicate. Likewise, spiked samples determined good accuracy of laboratory methods by returning values within 10% of expected.

Ammonia reduction occurring between days 3 and 7 above in Figure 4-14, corresponded directly with a presence of nitrate (Figure 4-15) which appeared between the same days. Observed nitrate presence with a corresponding reduction in ammonia-nitrogen is typical of nitrification. Additionally, the period between 3 and 7 days is typical of nitrifying bacteria, which traditionally take 5 to 7 days to grow to sizable enough populations to begin nitrifying, as previously discussed in *Comparison of Operational Parameters.*

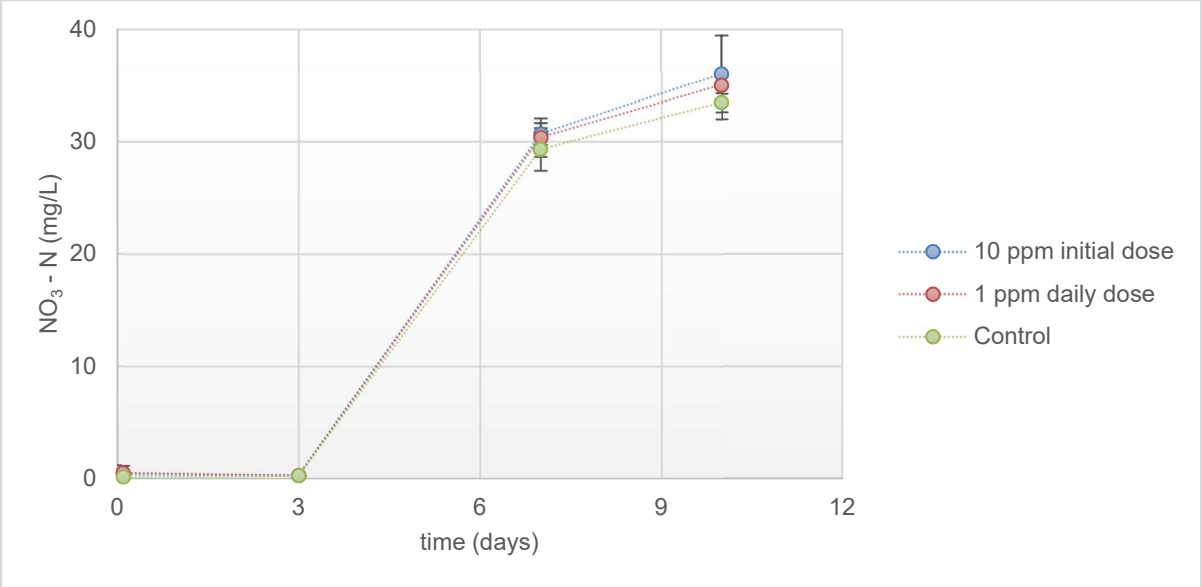

Figure 4-15: Nitrate production beginning after day 3 and corresponding with a drop in TAN (Figure 4-14), with little variation among systems. Results indicated native nitrifying organisms dominated nitrogen transformations within all systems, and not Aqua.

Like statistical analysis of TAN data, nitrate analysis showed less variation among the population of 9 flasks (maximum standard deviation of 2.08 ppm) than within triplicate (maximum standard deviation of 3.42 ppm). This analysis confirmed what was suspected after TAN data analysis; that neither dosing procedure affected nitrogen transformations within the systems. QA/QC analysis for nitrate and nitrite is summarized below in Table 4-5.

Table 4-5: QA/QC metrics for nitrate & nitrite analysis by the IC, with percent difference indicating maximum observed among nitrate and nitrite analysis.

QA/QC Metric	Percent Difference from expected
CVS 1	4.97
CVS 2	1.79
CVS 3	1.99
CVS 4	2.54
Split 1	5.80
Split 2	14.94

Calibration standards ran on the IC returned calibration curves that allowed for the determination of ppm as N readings from integrated peak height. CVS accuracy for the IC instrument was good with all four CVS falling within 5% of expected concentrations based on calibration standards. While this indicated good accuracy of the IC, only one of two split samples was close to the aim of 5% percent difference. This showed less than ideal laboratory and instrument precision but did not invalidate results due to strong precision among triplicate as shown by error bars above in Figure 4-15.

In addition to nitrate production, nitrite was also produced during the latter end of the experiment (Figure 4-16). With more variation among the population (max. standard deviation of 2.26 ppm) than triplicate flasks (maximum standard deviation 1.95 ppm), produced nitrite followed the same trends of TAN and nitrate presence and did not indicate any significant difference between the three systems.

However, with average nitrite concentrations reaching 14.9 ppm as N at day 10, future analysis would be performed to investigate why this value was higher than expected. Nitrite is an intermediary in both nitrification and denitrification processes and is often formed when another necessary reagent limits the extent of the process. In FS-1 oxygen concentrations were held constant by shaker speed, so carbon to nitrogen ratios were examined to determine if they were limiting the extent of natural reactions.

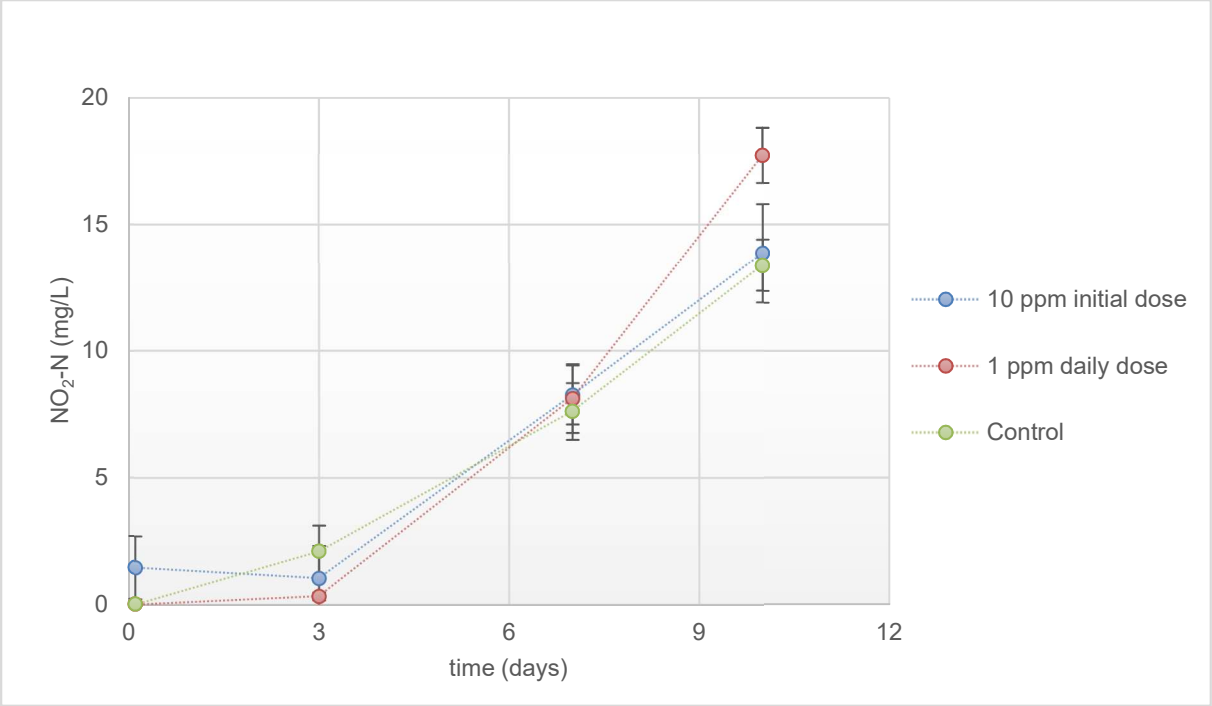

Figure 4-16: Nitrite production during FS-1 beginning after day 3 and progressing until day 10. Error bars indicate standard deviation among triplicate for each system.

Carbon to nitrogen ratios at the commencement of FS-1 averaged 2.4, while at day 10 they dropped to 0.5. For this reason, FS-2 experimented with carbon addition to wastewater. Produced nitrite can be formed during incomplete denitrification. However, since all systems maintained aerobic conditions during the experiment, and not anoxic, this was likely not the reason for nitrite production. Additionally, nitrate was also not observed to reduce at all after its appearance, regardless of nitrite presence (Figure 4-15).

In addition to TAN, NO_3^-, NO_2^-, and C: N ratios, TN analysis was performed to determine if any system losses of nitrogen occurred. While data showed no effect of Aqua dosing during any other constituent analysis, TN (Figure 4-17) was evaluated to confirm suspected conclusions and better understand the nitrogen transformations occurring.

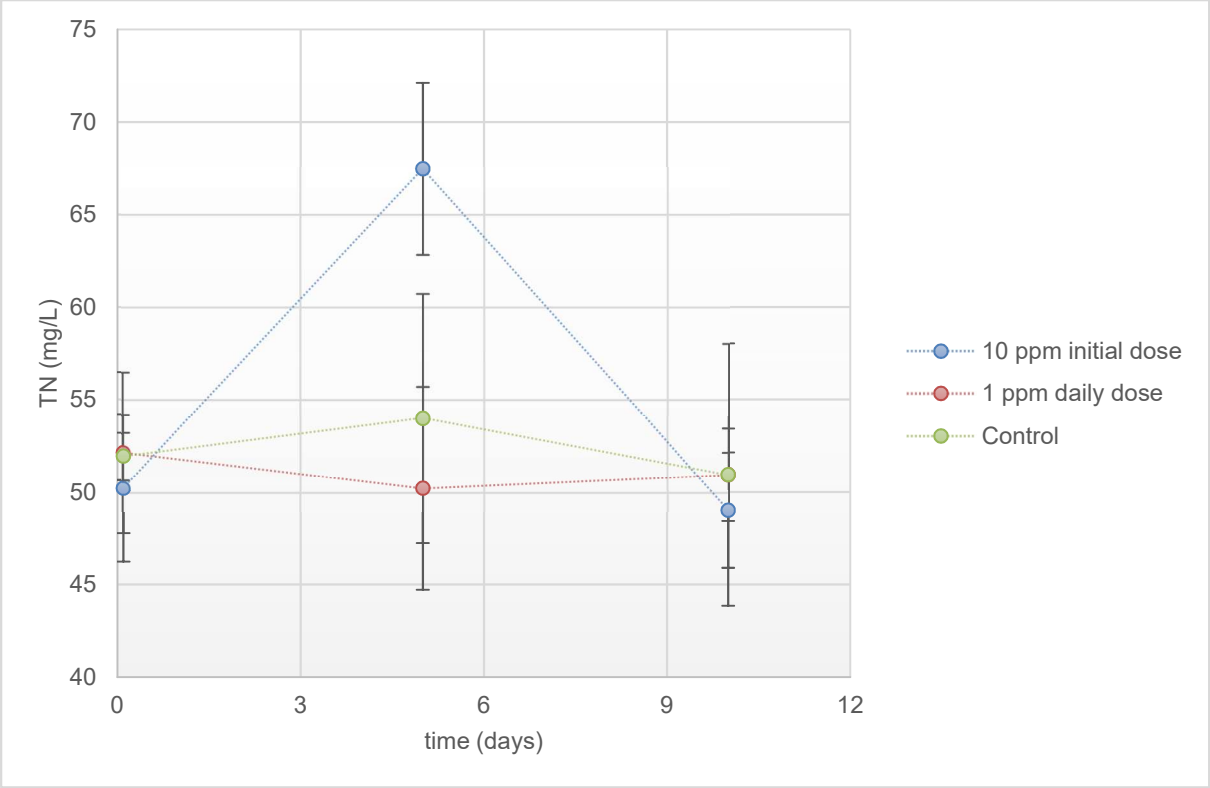

Figure 4-17: Total nitrogen (TN) analysis performed on days 0, 5, and 10 present statistically inaccurate data, but in general show no loss of nitrogen from all systems.

Data from TN analysis was ultimately invalid as standard accuracy was over 18% different from expected values (Table 4-6). TN analysis precision was also poor, with each split lying outside the desired 10% percent difference range. Additionally, one of three spiked samples fell outside 10% percent difference from expected, which indicated a lack of data accuracy. While poor QA/QC metrics limited takeaways, it was also clear that no general reduction in TN was observed.

Day 0 averaged 51.43 ppm across all 9 flasks with a standard deviation of 1.37 ppm, while day 10 averaged 50.31 ppm, with a standard deviation of 2.03 ppm. The apparent drop of 1.12 ppm would not be significant regardless of QA/QC results.

Table 4-6: QA/QC metrics for TN analysis by Hach-HR Kit showing poor accuracy and precision during analysis.

QA/QC Metric	Percent Difference from expected
CVS 1	20.03
CVS 2	18.44
CVS 3	5.96
CVS 4	5.55
Split 1	33.44
Split 2	11.57
Spike 1	3.51
Spike 2	11.38
Spike 3	5.20

Due to the discussed QA/QC issues, TN analysis was solely used for ensuring no drastic system losses. Reasons for such magnitude of inaccuracy and imprecision within TN analysis results are discussed further in *Future Improvements.*

4.9 Full-Scale (FS) Experiment 2

Based on results from *FS-1* that saw no appreciable effect from either dosing procedure, dose concentrations for *FS-2* were increased to 50 ppm Aqua for all inoculated systems. Additionally, dosing method was tested by activating bacteria overnight in tryptic soy broth (TSB) to allow for sizable population growth prior to inoculation. In addition to inoculum activation, the addition of dextrose at a 12:1 carbon to nitrogen ratio in systems 3 and 5, was a manipulated variable of focus for *FS-2* (Systems defined in *Full-Scale Experiment 2, pg. 39).*

Optical density readings from *FS-2* showed appreciable bacterial growth beginning at day 1 in systems 3 and 5 (added carbon) and tapered off thereafter (Figure 4-18). Systems 3 and 5 exhibited a 0.75 increase in OD over systems 1, 2, and 4 at day 1, however by days 3 and 7 this gap had dropped to 0.24 and 0.08, respectively. This initial spike in bacterial concentration was a direct result of added carbon in the form of dextrose, as the heterotrophic bacteria in those systems were afforded ample source of electron donor. Split precision in OD measurements was good during every sample with a maximum split difference of 0.01.

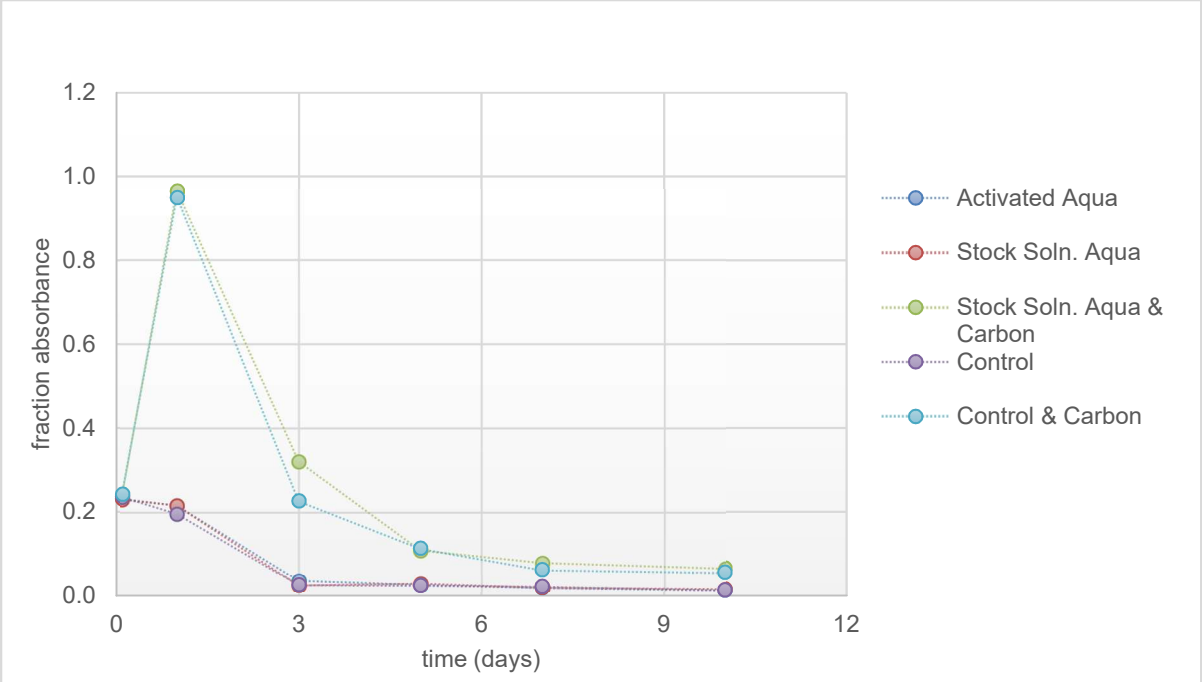

Figure 4-18: Optical density measurements demonstrated a spike in population for systems with added carbon in comparison to systems without.

As bacterial populations spiked in systems 3 and 5, a rapid initial reduction in TAN was observed at a rate of k_1 = 0.25/hr. (Figure 4-19). However, after day 3 these systems saw an increase of 14 ppm at day 7. This phenomenon is discussed in *Conclusions* but was likely due to the ammonification of the organic nitrogen created during the initial TAN reduction. This was because the initial reduction in systems 3 and 5 was accomplished through assimilation and not nitrification; which was confirmed by a lack of nitrate presence in these systems prior to day 3 (Figure 4-20). Between days 7 and 10 a second TAN reduction was observed in systems 3 and 5, this time due to nitrification and positively accompanied by the presence of nitrate.

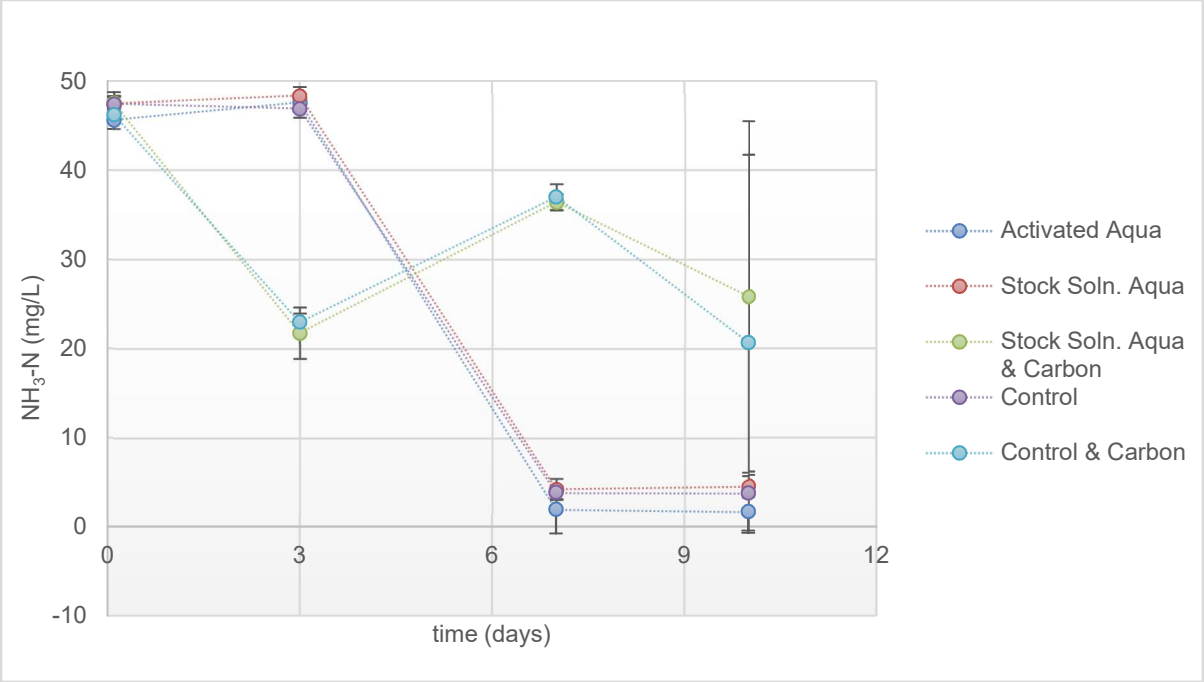

Figure 4-19: TAN data showing initial rapid decline and subsequent rebound in systems with added carbon, and slower nitrification process occurring in systems without. No distinction was observed between control and dosed systems.

Systems 1, 2, and 4 exhibited 93% TAN reduction between days 3 and 7 due to nitrifying organisms at a rate, k_1 = 0.384/hr. No significant distinction was observed among the three systems in either rate or extent of TAN reduction. Thus, dosed Aqua, regardless of procedure (activated or stock solution) had no effect on TAN reduction rates. Nitrification is confirmed by parallel nitrate presence in systems 1, 2, and 4 (Figure 4-20). The lack of distinction between inoculated and uninoculated systems in nitrate production again indicated that Aqua had no observable effect on nitrification processes.

Table 4-7: QA/QC metrics for TAN analysis by the Timberline Ammonia Analyzer.

QA/QC Metric	Percent Difference from expected
CVS 1	9.72
CVS 2	4.67
CVS 3	1.79
CVS 4	1.05
Split 1	3.22
Split 2	3.28
Spike 1	3.25
Spike 2	1.74
Spike 3	1.76
Spike 4	0.21

Control verification standard (CVS) analysis showed three of four CVS fell within 5% of the expected 50 ppm standard, with CVS number 1 at 9.72% difference. This reflected good accuracy of the ammonia analyzer, as had been experienced throughout performed research. Split sample analysis confirmed good precision of the ammonia analyzer as samples were both within 3.5% of their respective replicate. Likewise, spiked samples determined great accuracy of laboratory methods by returning values within 3.5% of expected.

As mentioned previously, no nitrate was detected until appearing between days 3 and 7 (Figure 4-20). Systems 1, 2, and 4 detected nitrate beginning on day 3 - all systems without amended carbon. Each of these systems saw similar nitrate presence, with a day 7 average of 10.14 ppm and standard deviation of 0.363 ppm among the systems. This standard deviation among three systems was less than maximum standard deviation of system 4

(0.48 ppm). This showed more variation between replicate flasks in a system than all three systems combined, again indicating no impact from any Aqua dosing procedures.

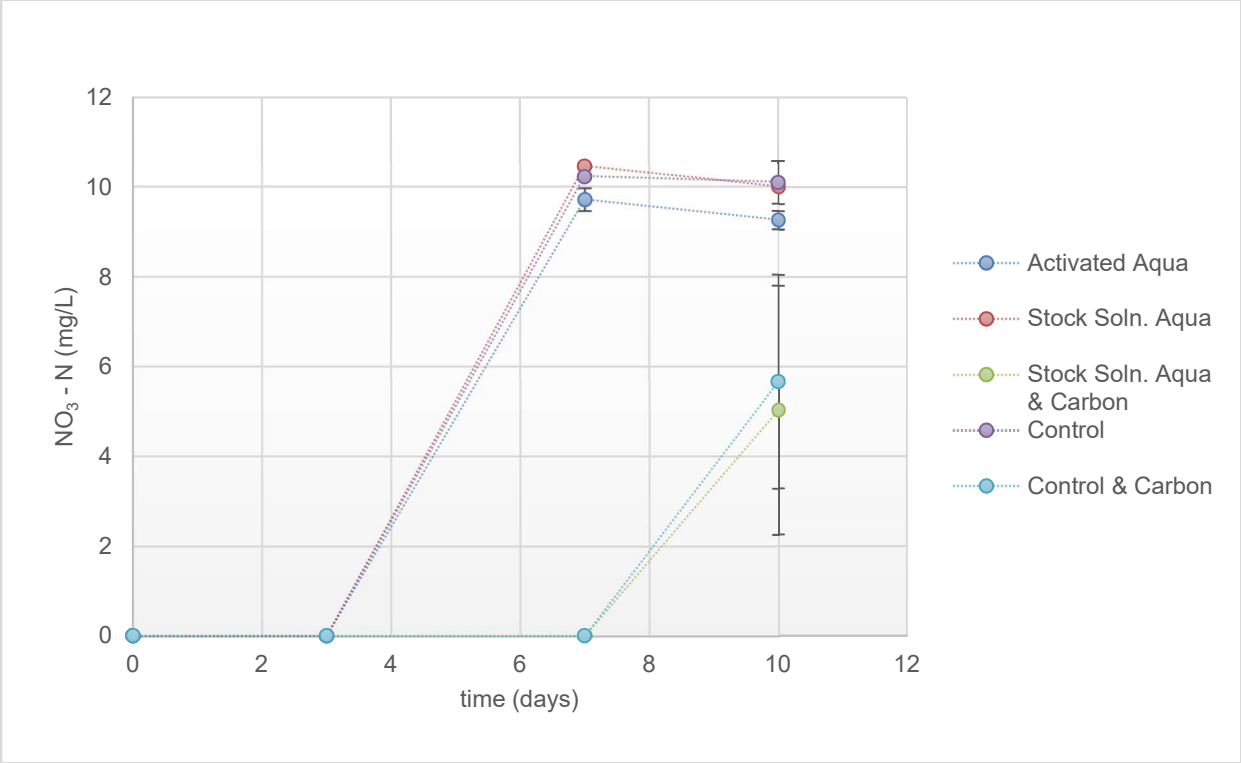

Figure 4-20: Nitrate presence showing natural nitrification processes in systems 1, 2, and 4 between days 3 and 7. Delayed nitrification in carbon dosed systems (3 and 5) due to initial TAN assimilation.

While nitrate presence appeared between days 3 and 7 for non-carbon dosed systems, nitrate presence was delayed until day 7 for systems 3 and 5. These systems with added carbon experienced rapid initial TAN assimilation, until day 3 when available carbon was consumed, and assimilation ceased. After subsequent ammonification of produced organic nitrogen, natural nitrification began. Nitrate in systems 3 and 5 averaged 5.35 ppm as N with a standard deviation of 2.14 ppm at day 10. Delayed nitrification occurred in these systems as heterotrophic assimilating microorganisms outgrew nitrifying bacteria and dominated the system until added carbon was depleted. After which, nitrifying bacteria native to the wastewater grew sizable populations and began reducing TAN into nitrate.

Nitrite presence in FS-2 appeared in all five systems at day 10, with an average of 1.46 ppm as N and standard deviation of 0.10 ppm (Figure 4-21). As mentioned previously, nitrite is an intermediary of denitrification, and often an indicator of incomplete denitrification. It was possible that after reducing TAN, DO levels were low enough to where facultative heterotrophs that reduce nitrate by using it as their electron acceptor, began to transform nitrate to nitrite and begin denitrification. However, this conjecture was not confirmed by any data and would likely need to be corroborated by a drop in TN to show system losses attributable to denitrification. Quality assurance of nitrate and nitrite samples was good and confirmed accuracy and precision during sample analysis (Table 4-8).

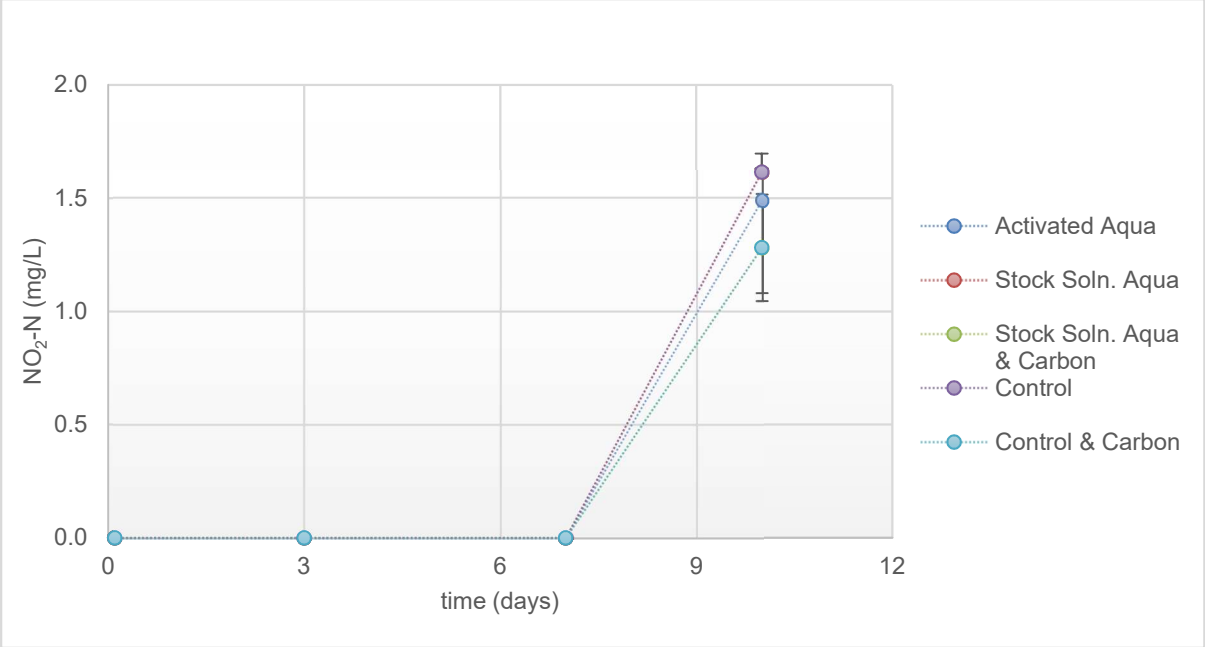

Figure 4-21: Nitrite presence occurring at day 10 in all five systems at an average of 1.46 ppm as N.

Table 4-8: QA/QC summary for nitrate and nitrite analysis on the IC instrument. Percent difference represent the larger error between nitrate and nitrite for each respective QA/QC sample analyzed.

QA/QC Metric	Percent Difference from expected
CVS 1	5.95
CVS 2	6.04
CVS 3	5.33
CVS 4	2.56
CVS 5	2.89
Split 1	0.00
Split 2	2.28
Split 3	0.00
Split 4	3.12

Percent error below 5% for standards indicated the IC instrument returned accurate results. While 3 of 5 CVS analyzed exceeded the ideal 5% error, they were not egregiously errors, as all remained under 6.25%. All four split samples recorded percent error between replicates of less than 3.25 percent, indicating very good instrument and method precision.

Initial day 0 carbon to nitrogen ratios in FS-1 averaged 2.4. Similarly, in FS-2, initial wastewater C: N ratio averaged 3.0. Carbon amended systems (3 & 5) had dextrose added to achieve a molar ratio of 12:1 based on initial ratios from FS-1. Analysis revealed an initial ratio of 14:1, which was slightly higher than designed for, but partially explained by the higher initial ratio in FS-2 wastewater. Carbon to nitrogen ratios of all systems at day 10 averaged 1.0, slightly higher than day 10 of FS-1 (0.5).

However, during FS-2 carbon amended flasks averaged a day 10 molar ratio of 1.4, higher than non-carbon containing flasks ratio of 0.7. This difference could possibly be explained by the delayed natural nitrification processes that occurred in the carbon amended flasks. As nitrification was delayed until day 7 in systems 3 and 5, a shift in the dominant microbe population may have been influenced. Death of nitrifying organisms and growth of carbon consuming heterotrophs at day 10 was delayed compared to systems 1, 2, and 4. Meaning, systems that completed nitrification sooner (between days 3 and 7) saw population growths of heterotrophs on day 10 that began to consume more of the available carbon than the delayed nitrification systems (3 & 5). This 0.7 molar difference, while slight, demonstrated that indeed no impact from Aqua dosing was felt, as microbial population shifts were strictly influenced by available carbon.

Similar to FS-1, TN analysis was performed to determine if any system losses of nitrogen occurred. While data analysis showed no effect of Aqua dosing during any other constituent analysis, TN (Figure 4-22) was evaluated to better understand nitrogen mechanisms occurring. Issues with QA/QC also plagued TN analysis in FS-2, as day 0 systems averaged 75.47 ppm and day 10, 86.74 ppm. Systems were clearly not fixing nitrogen due to the abundance of available ammonia and organic nitrogen in the system, and thus the apparent increase in TN was likely due to method and analysis error.

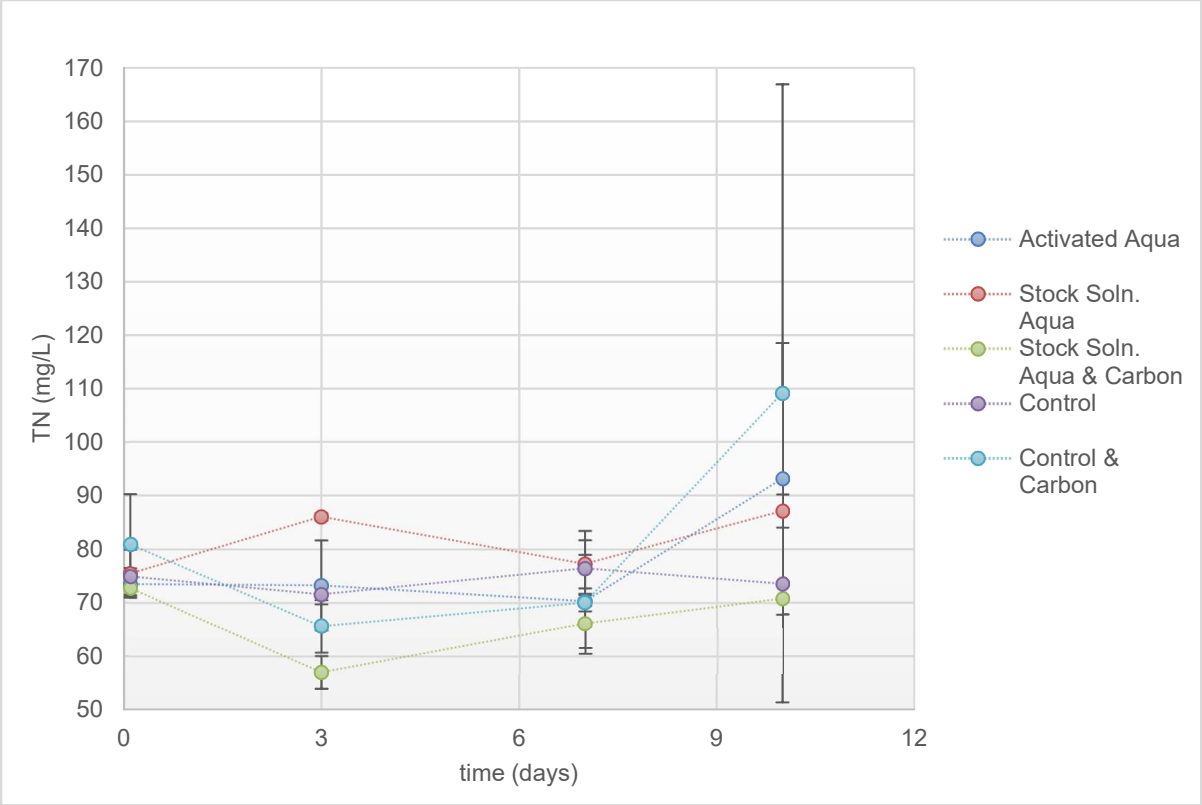

Figure 4-22: TN analysis from FS-2 showing considerable standard deviation, which rendered results invalid.

Spike recovery accuracy and split sample precision averaged 3.5% and 8.9% error, respectively. However, CVS accuracy was very poor with a maximum percent difference of 37.2%, and an average of 17.2%. Issues with TN analysis stemmed both from sampling protocol as well as analysis method and will be discussed in *Future Improvements.* Although QA/QC issues were apparent, TN analysis followed suit with analysis of other nitrogen constituents by not identifying any impact from Aqua dosing and confirming that no system losses of nitrogen were experienced.

5. Discussion, Future Improvements, & Conclusions

Experimental data collection and analysis began with evaluation of Aqua's capability in nitrogen removal. Screening and parameter optimization experiments helped identify conditions and parameters best suited for Aqua performance. Two 11-day experiments evaluated Aqua performance in conditions similar to recommended application setting by BiOWiSH. Data and analysis from performed experiments identified nitrogen transformation mechanisms which occurred and ultimately helped answer the following research questions:

1. Does application of Aqua improve total ammonia-nitrogen (TAN) removal in wastewater system in comparison to rates observed without bioaugmentation?
2. Does application of Aqua promote a decrease in Total Nitrogen (TN) in comparison to systems without bioaugmentation? If so, can TN decrease be attributed to simultaneous nitrification-denitrification (SNdN)?
3. If mechanisms outlined in Questions 1 and 2 above are not observed, are there other mechanisms occurring by which TAN, TN or other nitrogen constituents are being reduced?

5.1 Discussion of Results and Analysis

Analysis from screening experiments PO-1 and PO-2 observed both rapid TAN reduction and rapid bacterial growth. Carbon addition in PO-2 at a 12:1 molar C: N ratio improved TAN reduction rates by 7-fold in comparison to PO-1. The success and rate of TAN reduction in PO-2 set the stage for investigation into the mechanisms of nitrogen transformation occurring. However, while PO-3 through PO-10 helped gain insight into microbial mechanisms and optimized environmental and growth parameters, the success of Aqua would be determined in larger and longer scaled experiments.

The successful TAN reduction observed in PO-2 was due in part to the sterile wastewater media used, the addition of carbon, and a product dose of 1500 ppm. TAN reduction in PO-2 had the maximum first-order TAN reduction rate (k_1 = 0.315/hr.) experienced across the entirety of performed experiments. Experiments performed in minimal media with amended minerals and added carbon saw similar reductions but at lower rates (k_1 = .084/hr.). Ultimately, as detailed above in analysis of FS-1 and 2, when Aqua was dosed at recommended amount of 1 ppm in primary wastewater it did not have any noticeable effect on TAN reduction rates. TAN reduction occurred in these systems, but at first-order rates (k_1 = 0.254/ hr.) and times (3-7 days) typical of nitrifying organisms already present in wastewater, as confirmed by control systems.

Increased dose concentrations and product activation prior to inoculation in FS-2 had no observable effect on TAN reduction in comparison to control systems. Indeed, the only observed mechanism in FS-2 not typical of natural wastewater processes was rapid ammonia-nitrogen assimilation, fueled by the addition of dextrose. However, as control data showed, whether systems with dextrose were inoculated with Aqua or not, had no observable effect.

Analysis of FS-2 data detailed the microbial mechanisms that occurred in systems with dextrose amendment (3 & 5). Rapid TAN reduction occurred in the first three days of the experiment (53% reduction) in dextrose amended systems. However, the lack of a parallel nitrate presence at this stage built the case for the absence of nitrification being the route of TAN reduction. Between 3 and 7 days, TAN levels slowly recovered as available carbon levels reduced and organic nitrogen that has assimilated into biomass cells began to catabolize and release back into the system as ammonia-nitrogen (Hoffman, 1998). As the assimilating microbes in the system became limited by a lack of electron donors (carbon),

their population began to decrease. It was at this juncture that native nitrifying bacteria were observed to shift population dynamics of the system and become the dominant microbial population in the system. This was evidenced by a second decrease in TAN, this time accompanied accordingly with nitrate production. This parallel decrease in TAN and increase of nitrate is characteristic of nitrification. TAN reduction rate in non-carbon stimulated flasks became appreciable between days 3 and 7 of FS-2 - a timeframe like HRT's employed at nitrifying municipal wastewater treatment plants.

In fact, if the end goal of Aqua application was the transformation of ammonia-nitrogen to diatomic nitrogen gas through nitrification and denitrification, the direct conclusion made was that the product may produce opposite of the desired effect. The reason for this being that Aqua is composed of 96.9% dextrose, a constituent that was shown to promote assimilation and delay nitrification. However, the more likely outcome of product application at recommended doses of 1 to 10 ppm was observed in analysis of FS-1 results. These indicated that at such low doses, the product had no effect on applied systems.

Biologically, this lack of impact in a complex system such as wastewater is intuitive. Thousands, if not hundreds of thousands of microbes live in wastewater, growing and existing at various rates and populations. A product dosing at 10 ppm, with active microbes accounting for roughly 3.1% of that, would seem to stand no chance to grow to sizable enough populations to cause an impact or even promote a shift in microbiome populations. Any biology introduced into complex systems needs a marked advantage in growth mechanism or initial introduced population to be able to survive and thrive. Cells counts performed during experiment PO-1 confirmed this logic. Cell counts during PO-1 represented specific isolate bacterial growth in sterile wastewater. Because bacteria strains were first isolated and the inoculated in sterile media, these CFU counts represented the

maximum growth of these bacteria, as they were not challenged with any microbial competition. Maximum CFU counts after 96 hours of uninhibited growth, were on the order of 10^8, which are still on the low end of the range of untreated wastewater total coliform counts (10^7 to 10^9 MPN/100 mL), (Francy et al., 2011). Thus, even unimpeded growth of bacteria would still not create a marked advantage over the numerous microbial populations present in wastewater.

5.2 Future Improvements

Weaknesses that appeared throughout the experimental processes will be discussed and focus on TN sampling procedure, dissolved oxygen concentration limitations, QA/QC improvements, and inherent pitfalls of utilizing orbital shakers to model microbiology systems. As metrics showed throughout PO and FS experiments, TAN, NO_3^-, NO_2^- and OD measurements all routinely passed QA/QC goals for both precision and accuracy. However, TN analysis performed by Hach TN kits saw greater deviation among triplicate as well as greater error in accuracy and precision metrics.

A reason for this, and an area for improvement, was in the sample acquisition process. TN samples were aliquoted from flasks in 1 mL volumes, meaning a sample not well-representative of the system could grossly under or overestimate TN. In FS-1 and 2, systems had formed large flocs in the center of the flasks due to the orbital shaker rotational motion. Thus, when sampling, it became difficult to properly homogenize the flocs with the clearer water around them. Care was taken to avoid flocs as it was thought that in normal wastewater application, flocs would be settled out and removed. However, with such a small sample size of 1 mL, biomass containing organic nitrogen could greatly skew TN

measurements, if not completely homogenized prior to sampling. This issue was reflected in a maximum difference of 81.7 ppm between replicates in FS-2.

To remediate this issue, systems should be completely homogenized with a laboratory blender prior to sampling. Additionally, a larger sampling volume should be taken, and multiple split samples run for each system to determine possible outlying samples which contained abnormally large biomass concentrations.

In addition to issues with TN sampling protocol, no direct dissolved oxygen concentrations were measured. Dissolved oxygen concentration in orbital shaker flasks is difficult to measure as it directly depends on the shaking speed of the flask. To measure DO with a conventional probe, the table shaker must be opened, which stops shaking, and thus diffusion. Ergo, measurements must be made immediately after shaker opening, and still likely have a degree of error associated with them. Novel noninvasive devices such as oxygen-sensitive patches manufactured by Fluorometrix Corp. (Stow, MA) which can be mounted on the interior of the flask and measured with an optrode, have shown success in measuring real time oxygen concentrations (Gupta, Rao, 2003). However, their accessibility at the time of experimentation was not feasible. Additionally, limitations on accuracy at certain percent saturations could limit their reliability as well. For experimentation moving forward, purchasing several of the devices would be a feasible way to directly monitor DO in shaker flasks.

In conjunction with weaknesses that appeared in TN analysis and DO concentration projections, improvements could have been made in QA/QC area. In initial experiments PO-1, 2, 3, 4, 5, 6, and 9, systems were not performed in replicate. This was done intentionally to maximize the number of variables that could be tested, however it did present less

reliable data. Analyzing one experimental variable per experiment and running multiple replicate systems would be ideal to limit the inherent variability that comes with testing multiple variables at once. While this was a weakness, it was taken into consideration, and control flasks that isolated one specific variable were always analyzed as well.

Beyond the weaknesses and proposed solutions discussed above, the experimental apparatus itself had inherent weaknesses. Shaken flasks on an orbital shaker table are ubiquitous with culture experiments and have been used successfully for decades. However, limitations such as oxygen transfer and the predictable shaking pattern are cause for investigation. Flasks are meant to serve as bench-scale simulations of real-world systems, yet the diffusion of oxygen and mixing patterns are not close to these systems. In comparison to bioreactor fermenters, which actively diffuse oxygen through sparging, passive diffusion through orbital shaken systems can severely limit oxygen transfer. Limiting oxygen can impact all other analysis being tested and underestimate differences in microbial activity due to other operating conditions because of the oxygen limitation (Buchs, 2001). When oxygen availability is low it can slow down microorganism's metabolism, essentially negating potential effects from other operational or environmental conditions (Buchs, 2001).

While these limitations did not appear to effect systems tested during experimentation and research in support of this thesis, orbital shaken flasks are not ideal systems with which to model certain wastewater treatment systems. In addition to issues with oxygen transfer, the predictability of the orbital motion separates denser particles and organisms to the center of the flask, while leaving the lighter on the surrounding outside. This created less than ideal mixing conditions and could affect data reliability when analyzing smaller operational and environmental condition changes. Solving these issues could in part be achieved using a bioreactor fermenter, which utilizes active oxygen diffusion and active mixing. However,

these systems have their limitations and drawbacks too, such as cell lysis from active mixing, which prevent them from being a perfect solution to modeled wastewater systems.

5.3 Conclusions

In answering research questions outlined previously in *Background*, it was important to delineate between the two types of media systems in which Aqua was tested: sterile (WW and MM) and unsterile (wastewater). While research questions will be answered with respect to both situations, it was important to note that success in sterile environments had no practical application, only edification purposes of biological processes.

Question 1: Does application of Aqua improve total ammonia-nitrogen (TAN) removal in wastewater system in comparison to rates observed without bioaugmentation?

Conclusion: The application of Aqua at recommended dosing provided no marked advantage in TAN removal in primary wastewater. When applied in sterile media, at elevated dosing (1500 ppm), Aqua was observed to reduce TAN at a rate of -0.044/hr. and decrease initial TAN by 41% in 12 hours.

Question 2: Does application of Aqua promote a decrease in Total Nitrogen (TN) in comparison to systems without bioaugmentation? If so, can TN decrease be attributed to simultaneous nitrification-denitrification (SNdN)?

Conclusion: The application of Aqua at recommended dosing provided no marked TN reduction in primary wastewater or sterile media, and thus SNdN was not observed. However, improved TN sampling method was discussed in *Future Improvements.*

Question 3: If mechanisms outlined in Questions 1 and 2 above are not observed, are there other mechanisms occurring by which TAN, TN or other nitrogen constituents are being reduced?

Conclusion: TAN reductions observed in FS-1 and FS-2, not elucidated by native nitrifying bacteria, occurred due to nitrogen assimilation in systems amended with dextrose.

While interesting to note the extent of natural processes occurring in the wastewater systems, it did not distract from the confirmation that Aqua dosing in any form did not affect the mechanism by which nitrogen was transformed in the systems. Analysis and discussion performed in support of this thesis research, confidently answered the posed research questions. However, while confidence in concluding assertions remained high, weaknesses were present in experimental processes which could have been improved upon and are recommended to be solved in *Future Improvements*.

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Appendix A. Minimal Media Composition as provided by BiOWiSH Technologies.

In addition to the outlined media recipe below, 38.2 g of ammonium chloride was added to solution A, to make a 100x solution, with which media addition would provide media systems 100 ppm NH4-N/L.

QUALITY ASSURANCE STANDARD OPERATING PROCEDURE (SOP)	
Title: Preparation of Mineral Medium	**SOP No.: MLM14** **Revision: 0** **Date of Issuance: 08/15/15** **Effective Date: 08/15/15** **Page 1 of 2**

SOP Owner/Originator: John P. Gorsuch Date: 08/15/15

PURPOSE

To ensure an adequate and sterile supply of mineral medium in the microbiology lab.

DEFINITION OF RESPONSIBILITIES

The research associate or laboratory assistant will prepare mineral medium stock solutions as needed to support microbiological assays.

PROCEDURE

1) Mineral medium is reconstituted from four component stock solutions, labelled A, B, C and D. Prepare solution A as follows:

   KH_2PO_4…… 8.50g
   K_2HPO_4.....21. 75g
   Na_2HPO_4…….33. 40g

   Dissolve in water to make up 1.0L. The final pH of the solution should be 7.4.

2) Prepare Solution B as follows:

$CaCl_2$……27.50g

Dissolve in water to make up 1.0L.

3) Prepare Solution C as follows:

$MgSO_4$……..…22.50g

Dissolve in water to make up 1.0L.

4) Prepare Solution D as follows:

$FeCl_3$……0.25g

Dissolve in water to make up 1.0L. ***If the solution is not for immediate use, add 1 drop of concentrated HCl.***

5) To 800mL DI H_2O, add 10mL of Solution A, 1mL of Solution B, 1mL of Solution C and 1mL of Solution D. Mix on a magnetic stir plate, and add water to achieve a final volume of 1.0L.

6) Autoclave stock solutions and finalized mineral medium at 121°C, 15psi for 15 minutes. Store at 4°C until needed.

Appendix B. Vitamin Supplement Composition

Formulation as provided by ATCC Product Sheet Vitamin Supplement (ATCC® MDVS™)*

*Based on Wolfe's Vitamin solution:

Folic acid 2.0 mg/liter

Pyridoxine hydrochloride 10.0 mg/liter

Riboflavin 5.0 mg/liter

Biotin 2.0 mg/liter

Thiamine 5.0 mg/liter

Nicotinic acid 5.0 mg/liter

Calcium Pantothenate 5.0 mg/liter

Vitamin B12 0.1 mg/liter

pAminobenzoic acid 5.0 mg/liter

Thioctic acid 5.0 mg/liter

Monopotassium phosphate 900.0 mg/liter

Appendix C. Trace Mineral Supplement Composition

Formulation as provided by ATCC Product Sheet Trace Mineral Supplement

(ATCC® MD-TMS™)*

*Based on Wolfe's Mineral solution:

EDTA, 0.5 g/liter

$MgSO_4*7H_2O$, 3.0 g/liter

$MnSO_4*H_2O$, 0.5 g/liter

NaCl, 1.0 g/liter

$FeSO_4*7H2O$, 0.1 g/liter

$Co(NO_3)_2*6H_2O$, 0.1 g/liter

$CaCl_2$ (anhydrous), 0.1 g/liter

$ZnSO_4*7H_2O$, 0.1 g/liter

$CuSO_4*5H_2O$, 0.010 g/liter

$AlK(SO_4)_2$ (anhydrous), 0.010 g/liter

H_3BO_3, 0.010 g/liter

$Na_2MoO_4*2H_2O$, 0.010 g/liter

Na_2SeO_3 (anhydrous), 0.001 g/liter

$Na_2WO_4*2H_2O$, 0.010 g/liter

$NiCl_2*6H_2O$, 0.020 g/liter

Appendix D. Carbon Source Amendments

Sodium Succinate Dibasic for Carbon addition (SIGMA-ALDRICH, S5047, St. Louis, MO)

11.26g into 100mL sterile DI for 20x solution.

Dextrose for Carbon addition (SIGMA-ALDRICH, DX0145-5, St. Louis, MO)

5.004g into 100mL sterile DI for 20x solution.

Sodium Acetate for Carbon Addition (SIGMA-ALDRICH, S2889, St. Louis, MO)

6.84g into 100mL sterile DI for 20x solution.