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Investigation of the Optimal Dissolved CO₂ Concentration and pH Combination for the Growth of Nitrifying Bacteria

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Investigation of the Optimal Dissolved CO₂ Concentration
and pH Combination for the Growth of Nitrifying Bacteria

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Dedication

This dissertation is dedicated to my wife, Sandra Kay Morris, who without her support and understanding, this research and document would not have become a reality.

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Abstract

Ammonium (NH_4^+) is a biological nutrient that is transformed in a wastewater treatment plant (WWTP) in a process called activated sludge. This is accomplished in an aerobic environment using microorganisms and inorganic carbon that convert the ammonium to nitrate (NO_3^-). This process is termed nitrification. Removal of ammonium is necessary due to its oxygen demand and toxicity to the environment.

Nitrification is considered a slow process due to the slow growth rate of the nitrifying bacteria. Ammonia oxidizing bacteria (AOB) first convert the ammonium (NH_4^+) to nitrite (NO_2^-) followed by conversion to nitrate (NO_3^-) by nitrite oxidizing bacteria (NOB). These slow rates limit the treatment capacity of the WWTP.

The initial hypothesis suggested that these slow rates were due to limited carbon in the aeration basin of a WWTP. A series of designed experiments and observational studies revealed substantial dissolved CO_2 exists throughout a WWTP. Based on these findings, the central research focused on determining if an optimum dissolved CO_2 concentration/ pH combination exists that maximizes nitrification.

Experimentation conducted at a pH of 7.0 and varying concentrations of dissolved CO₂ concentration revealed inhibition at low (<5 mg/l) and high (>30 mg/l) dissolved CO₂ concentration levels. Further research found that optimum nitrification can be attained in a dissolved CO₂ concentration range of 10 - 15 mg/l and a pH range of 7.5 – 8.0. A maximum specific growth rate of 1.05 – 1.15 days⁻¹ was achieved. A partitioning of the sums of squares from these designed experiments found that pH accounts for approximately 83 percent of the sums of squares due to treatment with the dissolved CO₂ concentration accounting for 17 percent. This suggests that pH is the dominant factor affecting nitrification when dissolved CO₂ concentration is optimized.

Analysis of the growth kinetics for two of the designed experiments was conducted. However, a set of parameters could not be found that described growth conditions for all operating conditions. Evaluating the results from these two experiments may suggest that a microbial population shift occurred between 16 and 19 mg/l of dissolved CO₂ concentration. These dissolved CO₂ concentrations represent pH values of 7.1 and 7.0, respectively, and were compared to experimentation conducted at a pH of 7.0. Though the pH difference is minor, in combination with the elevated dissolved CO₂ concentration, a microbial shift was hypothesized.

Microbial samples were collected from the designed experiment that optimized dissolved CO₂ concentration (5, 10 and 15 mg/l) and pH (6.5, 7.0, 7.5 and 8.0).

These samples were evaluated using Fluorescence *in situ* hybridizations (FISH) to determine the population density of common ammonium oxidizing bacteria (AOB) (*Nitrosomonas* and *Nitrospira*) and nitrite oxidizing bacteria (NOB) (*Nitrobacter* and *Nitrospirae*). The dominant AOB and NOB microbes were found to be *Nitrosomonas* and *Nitrospirae*.

These results suggest that increased nitrification rates can be achieved by incorporating appropriate controls in a wastewater treatment plant (WWTP). With higher nitrification rates, lower nitrogen values can be obtained which will reduce the WWTP effluent nitrogen concentration. Conversely, these increased nitrification rates can also reduce the volume of an aeration basin given similar effluent nitrogen concentrations.

Chapter 1

Research Objective

1.1 Main Objective

The main objective of this research is to determine if an optimum pH/ dissolved CO₂ concentration exists that will minimize the time required for nitrification in an activated sludge wastewater treatment facility.

1.2 Research Goals

This research will focus on answering the following questions:

- Do ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) exhibit reduced growth due to carbon limitation?

- Is there a preferred dissolved CO₂ concentration that provides for optimum nitrifier growth?

- Is there a preferred pH value in combination with dissolved CO₂ concentration that provides for optimum nitrifier growth?
- Can the microbes most abundant in the nitrification process be quantified at varying pH/dissolved CO₂ concentrations that bracket this optimum combination?

1.3 Hypothesis and Approach

It is hypothesized that the autotrophic nitrifying bacteria in activated sludge systems grow slowly due to CO₂ limitation. Elevated levels of dissolved CO₂ concentrations above atmospheric concentrations will improve the nitrifier growth rate and thus reduce the nitrification time. In order to answer this research question, a series of designed experiments were conducted.. Testing protocol is outlined as follows:

- Conduct a series of preliminary experiments to determine if elevated dissolved CO₂ concentration at specified pH levels using synthetic feed as well as influent from a wastewater treatment facility exhibit increased nitrifier growth as compared to air systems.

- Determine operating conditions, dissolved CO₂ concentrations and pH at several wastewater treatment facilities. Evaluate these conditions as compared to preliminary experiments discussed above.
- Based on results from previous experimentation and assessment of field studies, determine a range of dissolved CO₂ concentration/ pH combinations that encompass the optimum combination of these two variables to achieve maximum nitrification growth.
- Quantify the microbial percent abundance of the most common nitrifiers at the optimum dissolved CO₂ concentration/ pH combination.

Chapter 2

Wastewater Treatment Industry, Literature Review and Preliminary Research

2.1 Wastewater Treatment in the United States

There are 16,024 publicly-owned wastewater treatment processes (WWTP) currently in operation in the United States, serving a population of approximately 190 million people (approximately 72 percent of the U.S. population). Their treatment capacity represents a wastewater flow of approximately 32,175 million gallons per day. Of these plants, 9,388 facilities provide secondary treatment, 4,428 facilities provide advanced treatment, and 2,032 facilities do not discharge to surface waters. In addition, there are 176 facilities that provide a treatment level that is less than secondary (these include facilities with ocean discharge waivers and treatment facilities discharging to other facilities meeting secondary treatment or better [1]).

There are several types of wastewater treatment facilities currently in operation in the U.S. The most prevalent type utilizes an aeration basin to treat and remove biological matter (secondary treatment). The removal of nitrogen and phosphorus are considered advanced treatment methods and in many facilities

are dealt with separately from the secondary treatment. In recent years, several waste treatment designs have been developed that incorporate these advanced removal processes into the aeration basins [2, 3].

The energy impact of the water industry is considerable. Most wastewater treatment systems require a high level of energy to operate, especially advanced treatment systems [4]. It is estimated that more than 5 percent of all global electricity is used to treat wastewater [5] and approximately 3 percent of electrical usage in the United States [6]. In addition, energy costs can account for 30 percent of the total operational and maintenance costs of a wastewater facility [6] with 50 percent of the energy costs for the aeration system [7].

2.2 Biological Nutrient Removal (BNR) Systems

Biological nutrient removal (BNR) is defined as the removal of total nitrogen (TN) and total phosphorus (TP) from wastewater through the use of microorganisms under different environmental conditions in the treatment process [2]. This activated sludge process dates back to the 1880's but was not officially described until 1914 by Arden and Lockett. During experimentation, they discovered that aerating a mass of microorganisms provided for stable organic material in wastewater. The aeration process was termed activated sludge and gave rise to the modern wastewater treatment processes (WWTP) we have today [2].

2.2.1 BNR Wastewater Treatment Processes

There are a number of BNR process configurations available. Some BNR systems are designed to remove only total nitrogen (TN), or both (TN) and total phosphorus (TP). The configuration most appropriate for any particular system depends on the target effluent quality, operator experience, influent quality, and existing treatment processes. BNR configurations vary based on the sequencing of environmental conditions (i.e., aerobic, anaerobic, and anoxic) and timing [3]. Some common BNR system configurations based on their biological nutrient removal focus are discussed below [2, 8].

2.2.1.1 Total Nitrogen Removal Only

- Modified Ludzack-Ettinger (MLE) Process – continuous-flow suspended-growth process with an initial anoxic stage followed by an aerobic stage
- Step Feed Process – alternating anoxic and aerobic stages; however, influent flow is split to several feed locations and the recycle sludge stream is sent to the beginning of the process
- Bardenpho Process (Four-Stage) – continuous-flow suspended-growth process with alternating anoxic/aerobic/anoxic/aerobic stages

- Sequencing Batch Reactor (SBR) Process – suspended-growth batch process sequenced to simulate the four-stage process; used to remove TN (TP removal is inconsistent)
- Extended Aeration Process (EAAS or usually called EA) [2] - a process used on wastewaters that have not been treated in a physical operation to remove suspended organic matter (primary clarifier). In this case, the insoluble organic matter becomes trapped in the biofloc and undergoes some oxidation and stabilization. Most other activated sludge systems are used on wastewaters from which settleable solids have been removed [9]. EA processes utilize long solid retention times (SRT) to stabilize the biosolids resulting from the removal of biodegradable organic matter. SRTs of 20 to 30 days are typical, which means hydraulic retention times (HRT) around 24 hours are required to maintain reasonable mix liquor suspended solids (MLSS) concentrations. Long SRT's offer two benefits: reduced quantities of solids to be disposed of and greater process stability. These benefits are obtained at the expense of the large bioreactors required to achieve the long SRT's, but for many small installations the benefits outweigh the drawbacks [9]. It has good capacity for nitrogen removal; less than 10 mg/l effluent TN is possible. However, nitrogen removal capability is related to skills of operating staff and control methods. (The Extended Aeration process identified in this research was

originally built as an Oxidation Ditch. Due to its operation, it is classified as an Extended Aeration process.)

2.2.1.2 Total Nitrogen and Total Phosphorus Removal

- A²/O Process – MLE process preceded by an initial anaerobic stage
- Modified Bardenpho Process (Five Stage) – Bardenpho process with addition of an initial anaerobic zone
- Modified University of Cape Town (UCT) Process – A²/O Process with a second anoxic stage where the internal nitrate recycle is returned
- Oxidation Ditch – continuous-flow process using looped channels to create time sequenced anoxic, aerobic, and anaerobic zones

A comparison of the TN and TP removal capabilities of common BNR configurations is provided (Table 2-1). This table provides only a general comparison of treatment performance among the various BNR configurations; site-specific conditions dictate the performance of each process [3].

Table 2-1: Comparison of Common BNR Process Configurations

Process	Nitrogen Removal	Phosphorus Removal
MLE	Good	None
Four-Stage Bardenpho	Excellent	None
Step Feed	Moderate	None
SBR	Moderate	Inconsistent
A2/O	Good	Good
Modified UCT	Good	Excellent
Five Stage Bardenpho	Excellent	Good
Oxidation Ditch	Excellent	Good

Although the exact configurations of each system differ, BNR systems designed to remove TN must have an aerobic zone for nitrification and generally incorporate an anoxic zone for denitrification. BNR systems designed to remove TP must have an anaerobic zone free of dissolved oxygen and nitrate. Often, sand or other media filtration is used as a polishing step to remove particulate matter when low TN and TP effluent concentrations are required. Sand filtration can also be combined with attached growth denitrification filters to further reduce soluble nitrates and effluent TN levels [10].

Choosing which system is most appropriate for a particular facility primarily depends on the target effluent concentrations (usually permit driven), and whether the facility will be constructed as new or retrofit with BNR to achieve more stringent effluent limits. New plants have more flexibility and options when deciding which BNR configuration to implement because they are not constrained by existing treatment units and sludge handling procedures [8].

2.2.1.3 Wastewater Treatment Plant Configurations

The four WWTP's used in this research included the Extended Aeration (EA), a Modified Ludzack-Ettinger (MLE), a Bardenpho 4 stage and a Bardenpho 5 stage facility. A schematic of each plant configuration is provided on the following pages [2]:

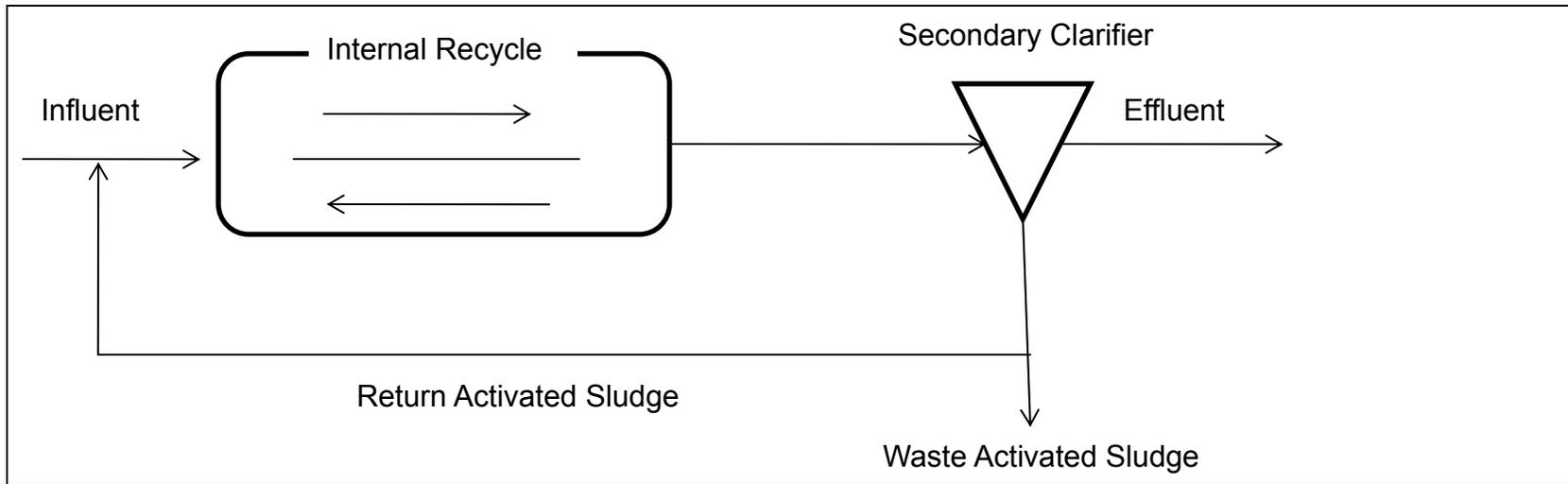


Figure 2-1: Extended Aeration (shown in Oxidation Ditch Configuration)

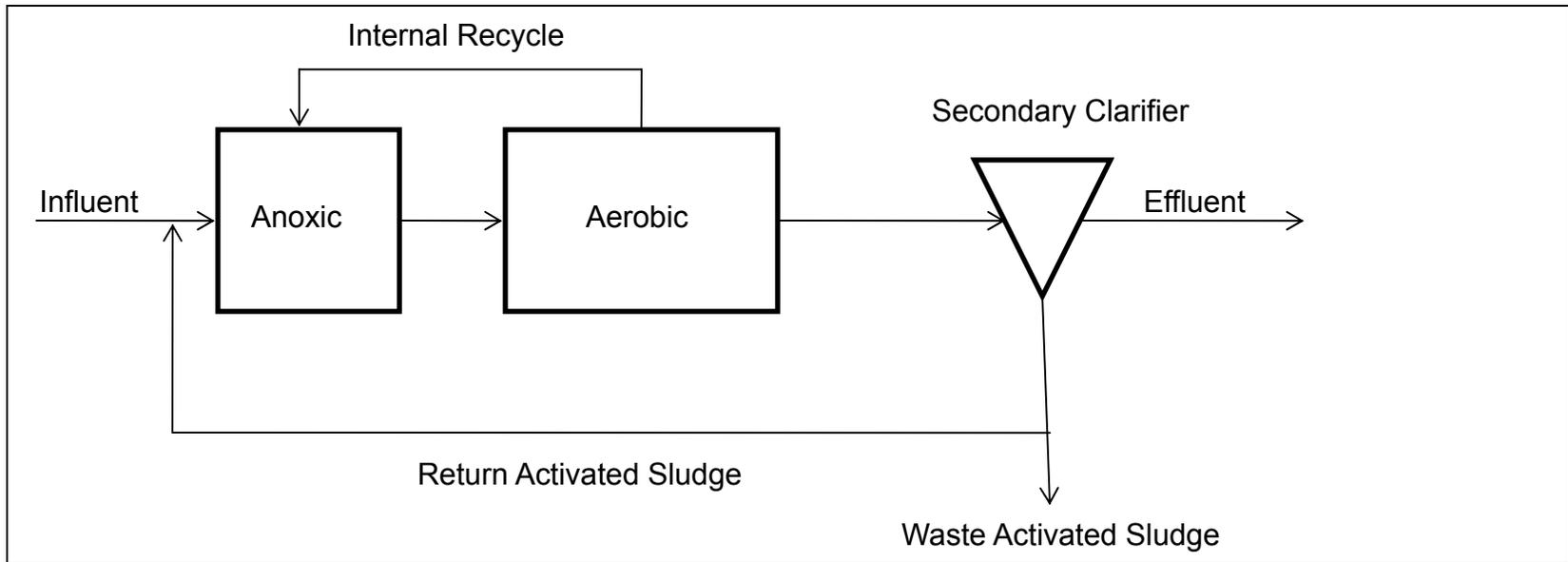


Figure 2-2: Modified Ludzack-Ettinger (MLE)

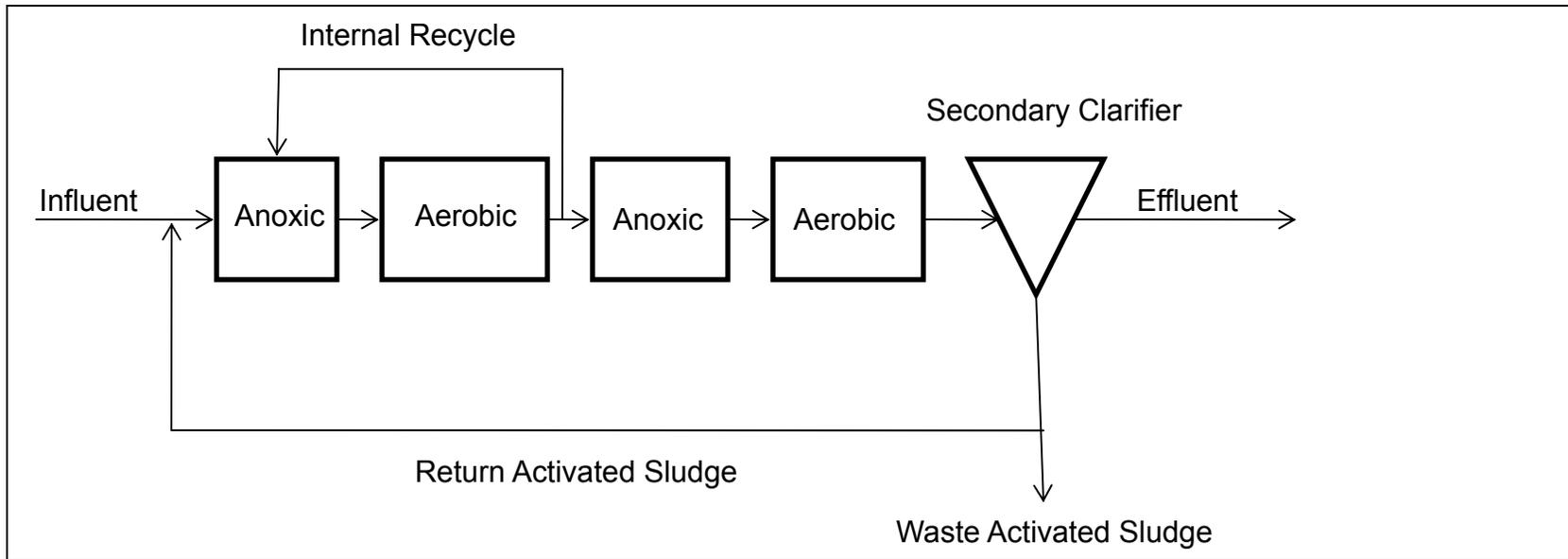


Figure 2-3: Bardenpho 4 stage

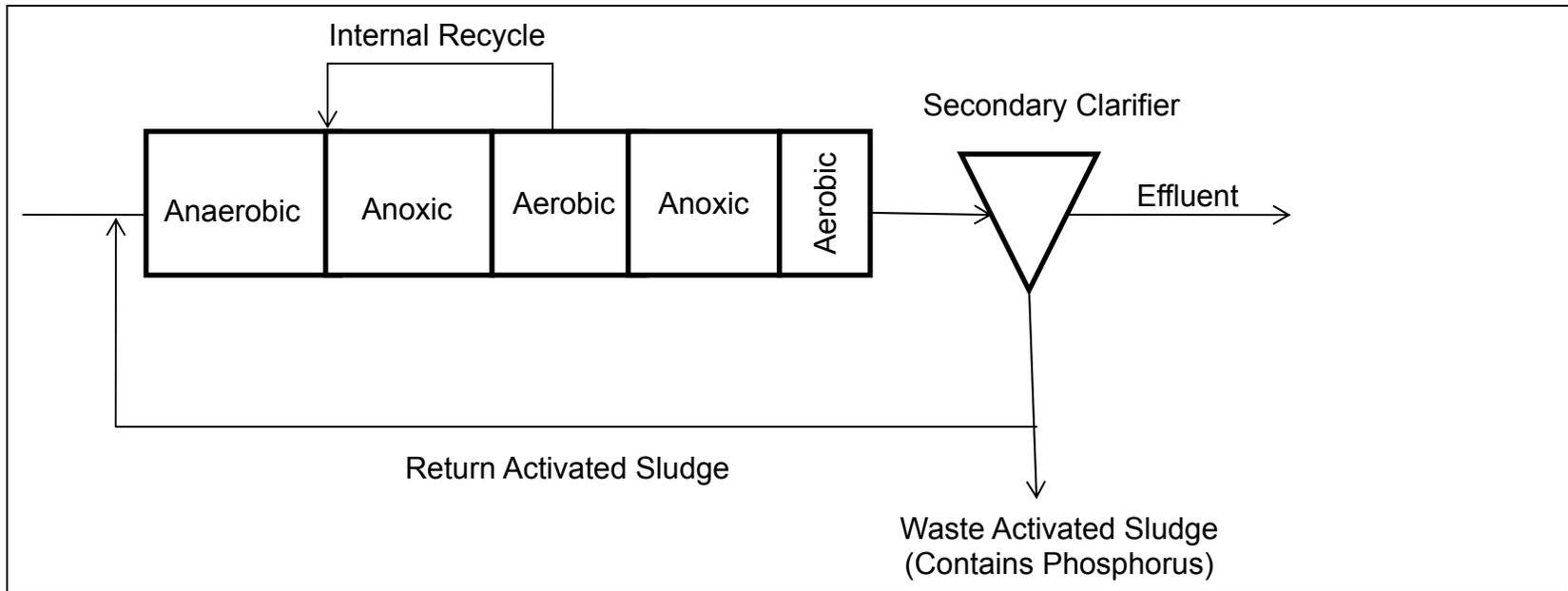


Figure 2-4: Bardenpho 5 stage

2.3 Nitrifying Bacteria and Nitrification

Bacteria found in the aeration basin of a wastewater treatment system are defined as either heterotrophs or autotrophs. Heterotrophs use organic carbon for formation of biomass and are primarily responsible for the reduction of organic matter (BOD). Autotrophic bacteria derive cell carbon from carbon dioxide and are responsible for converting ammonium (NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-) [2].

Concentrations of the types of bacteria found in wastewater vary depending on operating conditions (SRT, influent qualities, domestic/ industrial percentages, activated sludge operating temperature, etc.) and results vary widely. One study that evaluated the waste activated sludge (WAS) from a membrane bioreactor found bacteria percentages in the following ranges [11]:

Heterotrophs 15 - 50 percent with an average percentage of 35 percent.

Autotrophs 2 - 8 percent with an average of 3 percent.

Another study evaluated the effect of the mixed liquor suspended solids (MLSS) and heterotrophic and autotrophic biomass as a function of solids retention time (SRT). At a 12 day SRT, the MLSS concentration was 3000 mg/l with the heterotrophic and autotrophic concentrations at 1300 and 85 mg/l, respectively. (All values reported as mg/l as COD (carbonaceous oxygen demand)). Thus, heterotrophs represent approximately 43 percent of the biomass with autotrophs

representing approximately 3 percent. Additionally, this represents approximately a 15:1 ratio of heterotrophs to autotrophs [9].

Protozoa are also found in wastewater and may contribute as much as 5 percent of the biomass [9]. They are the main predators in suspended growth bioreactors that feed on bacteria. Ciliates are usually the dominant protozoa, both numerically and on a mass basis. Almost all are known to feed on bacteria and the most important are either attached to or crawl over the surface of biomass flocs. Viruses and polyphosphate accumulating organisms comprise other microbes found in wastewater [9].

Total effluent nitrogen comprises ammonia, nitrate, particulate organic nitrogen, and soluble organic nitrogen. The biological processes that primarily remove nitrogen are nitrification and denitrification [3]. In BNR systems, nitrification is the controlling reaction because ammonia oxidizing bacteria lack functional diversity, have stringent growth requirements, and are sensitive to environmental conditions [3]. Nitrification by itself does not actually remove nitrogen from wastewater. Rather, denitrification is needed to convert the oxidized form of nitrogen (nitrate) to nitrogen gas. Nitrification occurs in the presence of oxygen under aerobic conditions, and denitrification occurs in the absence of oxygen under anoxic conditions.

Microorganisms use an electron donor substrate to meet their growth needs, cell synthesis (f_s), and their cell maintenance needs (f_e) [12]. These two values, f_s and f_e , add to one and are expressed in terms of electron equivalents (e^-eq). The fraction f_s can be converted into mass units such as g cell produced/ g COD consumed. When expressed in mass units, it is termed the true yield and given the symbol Y . The conversion from f_s to Y is given as:

$$Y = f_s (M_c \text{ g cells/ mol cells}) / [n_e e^-eq/ \text{mol cells}] (8 \text{ g COD/ } e^-eq \text{ donor})$$

Where:

- M_c = the empirical formula weight of cells
- n_e = the number of electron equivalents in an empirical mole of cells

When cells are represented by $C_5H_7O_2N$ and ammonium is the nitrogen source, $M_c = 113 \text{ g cells/ mol cells}$, $n_e = 20 e^-eq/ \text{mol cells}$. This conversion gives $Y = 0.706 f_s$ and Y is in g cells/ g COD. The numbers used in the conversion change if the cell formula differs or if the cells use oxidized nitrogen sources, such as NO_3^- [12]. From a practical viewpoint, low f_s values translate into slow cell growth as they have high maintenance needs. As a comparison, ammonium oxidizers have a f_s value of 0.14, nitrite oxidizers have a f_s value of 0.10, and aerobic heterotrophs have typical f_s values of 0.6 - 0.7. These low f_s values for the ammonium and nitrite oxidizers translate into low autotrophic biomass growth. In characterizing a biochemical process, investigators can use substrate removal or

biomass growth to describe this activity [9]. This relationship is given by the formula:

$$\hat{\mu} = Y \cdot \hat{q}$$

Where:

- $\hat{\mu}$ = maximum specific growth rate
- \hat{q} = maximum specific rate of substrate utilization
- Y = yield for cell synthesis

Each of these parameters is related but describes different aspects of the biochemical process. \hat{q} is influenced by variation in Y as well as variation in $\hat{\mu}$. Like $\hat{\mu}$, Y is influenced by the substrate being consumed and the microorganisms performing the consumption. However, Y is a reflection of the energy available in a substrate whereas $\hat{\mu}$ is a reflection of how rapidly a microorganism can process that energy and grow. Because they represent different characteristics, there is no correlation between the two parameters. For example, some substrates that are consumed very slowly (low $\hat{\mu}$) provide more energy to the degrading organism (higher Y) than do substrates that are degraded rapidly [13]. This suggests that inferences about the variability of \hat{q} cannot be made on $\hat{\mu}$ alone, and vice versa. Knowledge of the true growth yield is also important in assessing these relationships [9].

Nitrification is a two-step process utilizing aerobic, autotrophic, nitrifying bacteria to complete the conversion process. Ammonium (NH_4^+) is first converted to nitrite (NO_2^-) according to the energy yielding equation [12]:

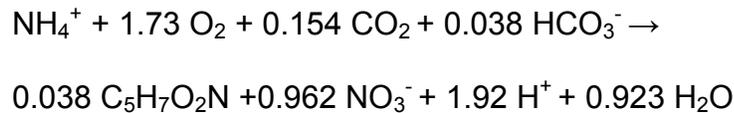


Nitrosomonas, an ammonia oxidizing bacteria (AOB), is considered the predominant bacteria species for this conversion [2]. The nitrite is further oxidized to nitrate (NO_3^-) according to the energy yielding equation [12]:



Of the nitrite oxidizing bacteria (NOB), *Nitrobacter* has been considered the predominant microbe, but in recent years *Nitrospirae* bacteria has been found to play a more significant role. Both AOB and NOB are thought to have slow growth rates and are sensitive to pH and temperature swings, making nitrification difficult to maintain in activated sludge systems [14, 15]. Although autotrophic bacteria are the dominant microbe in nitrification, ammonium oxidation can be performed by archaea [16, 17]. Ammonium-oxidizing archaea were found to occur in WWTP's that were operated at low dissolved oxygen levels and long solid retention times [18].

A complete reaction for the conversion of NH_4^+ to NO_3^- ($f_s = 0.1$) is written as follows [12]:



Denitrification involves the biological reduction of nitrate to nitric oxide, nitrous oxide, and nitrogen gas [2]. Both heterotrophic and autotrophic bacteria are capable of denitrification. The most common and widely distributed denitrifying bacteria are *Pseudomonas* species, which can use hydrogen, methanol, carbohydrates, organic acids, alcohols, benzoates, and other aromatic compounds for denitrification [2]. Table 2-2 provides a review of the different forms of nitrogen and removal capability from wastewater [3].

Table 2-2: Effluent TN Components and Achievable Limits

Form of Nitrogen	Common Removal Mechanism	Technology Limit (mg/l)
Ammonia-N	Nitrification	<0.5
Nitrate-N	Denitrification	1 – 2
Particulate organic-N	Solids separation	<1.0
Soluble organic-N	None	0.5 – 1.5

(Organic nitrogen is not removed biologically. Only the particulate fraction can be removed through solids separation via sedimentation or filtration [8].)

Nitrosomonas and *Nitrobacter* have been considered the predominant AOB and NOB bacteria involved in nitrification and have been investigated extensively [19-22]. In recent years, *Nitrosospira* and *Nitrospirae* have been identified as important microbes involved in nitrification (Table 2-3 and [12, 20, 23, 24]). And

in one study, *Nitrospirae* was found to be the most abundant nitrite oxidizer in wastewater treatment systems [24].

The properties of these predominant AOB and NOB bacteria are provided in Table 2-3. Some properties are similar among the bacteria types but differences do exist. *Nitrosomonas* and *Nitrospirae* have similar optimum pH ranges but differ from the optimum pH for *Nitrobacter*. A WWTP optimized for pH may not obtain optimum nitrification if *Nitrosomonas* and *Nitrobacter* are the predominant AOB and NOB bacteria due to their optimum pH ranges. Additionally, *Nitrosospira* growth may be enhanced at low temperature and *Nitrospirae* may dominate under low concentrations of NH_4^+ and NO_2^- [24, 25]. Information is limited as evidenced by several missing cells in the table. This may be due to the limited availability of pure cultures of nitrifying bacteria to study.

Table 2-3: Properties of Predominant Nitrifying Bacteria

	Genus	Morphology	pH	Optimum pH	Oxygen (mg/l)	Temp Range (°C)	Optimum Temp (°C)	Nutrients	Maximum Specific Growth Rate, days ⁻¹ (Optimum @ 20°C)	Comments
AOB	<i>Nitrosomonas</i>	gram negative, rod shaped or pear shaped	6-9	7.9 - 8.2 [26, 27]	>2.0		25-30		0.76	Inhibited at pH of 6.5, Nitrification ceases at pH 6.0. Can grow in low salinity environments [28]
	<i>Nitrospira</i>	spiral		7.5 -8.0						Enhanced at low temp
NOB	<i>Nitrobacter</i>	gram negative; rod shaped, pear-shaped or pleomorphic		7.2-7.6 [26, 27, 29]	>2.0, More strongly affected by low DO than Nitrosomonas	0-49	25-30	Needs phosphates	0.81	More likely to dominate nitrite oxidation under conditions with low ammonium and nitrite concentrations
	<i>Nitrospira</i>	Long, Slender rods		8.0-8.3 [22]						

Although all of the nitrifiers were once included in the same family because of their activities, it is now recognized that they are phylogenetically diverse [23]. With improvements in genetic techniques, other species have been identified that are not necessarily the most common or most active in the environment. Hence, nitrifying activity should not be assigned to these genera unless they are actually identified [30].

The AOB include genera within the Proteobacteria: *Nitrosomonas* (β), *Nitrospira* (β), and *Nitrosococcus* (γ). *Nitrosococcus* bacteria is considered to dominate in marine environments [30]. *Nitrosococcus mobilis* was originally isolated from brackish water [31] but has been identified as a major contributor in the nitrification process of sewage treatment [32]. The NOB also includes genera within the Proteobacteria: *Nitrobacter* (α), *Nitrococcus* (γ), and *Nitrospina* (δ) [33, 34]. In addition, *Nitrospirae*, a member of the Xenobacteria has been identified as a NOB. Recently, *Nitrosomonas* has been found in low salinity environments

In recent years it has been found that wastewater treatment plants are highly diverse microbial systems and are usually not represented by one nitrifying bacteria [19, 20, 23, 24, 32, 35-37]. The coexistence of different nitrifiers implies functional redundancy which may allow communities to maintain physiological capabilities when conditions change. Thus, a high level of nitrifier diversity is thought to confer performance stability [23].

Juretschko [20] evaluated waste from a WWTP in Germany that received high ammonia concentrations (5,000 mg/l) from a high protein-rich animal waste processing facility. Analysis revealed the predominate AOB to be *Nitrosococcus mobilis*-like bacteria [20]. It should be noted that *Nitrosococcus mobilis* is now considered to be a member of the genus *Nitrosomonas* [20]. This animal waste could have influenced the selection of this AOB. The major NOB genus was *Nitrospirae*.

Dionisi [37] investigated two WWTP's. The first was a 40 million gallon/day WWTP (6 hour HRT) treating primarily municipal waste with some industrial and hospital discharges. *Nitrosomonas* (AOB) and *Nitrospirae* (NOB) were identified as the predominant microbes. The second was a 27 million gallon/day industrial WWTP treating fibers, plastics and chemicals. Its waste consisted mainly of acetic acid, propionic acid, n-butyric acid, ethylene glycol, ethanol, methanol, isopropanol, and acetone and no municipal waste. *Nitrosomonas* (AOB) and *Nitrospirae* (NOB) were identified as the predominant nitrifying bacteria, but the AOB were different species of *Nitrosomonas* between the WWTP's.

Using a fluidized bed reactor, Schramm (1998) used low concentrations of NH_4^+ (40 μM) and identified the predominate AOB as *Nitrosospira* and the NOB as *Nitrospirae* [19]. No members of the genus *Nitrosomonas* (AOB) or *Nitrobacter* (NOB) could be detected. This is agreement with other studies conducted in natural systems in which the ammonium concentration was low [38-40].

Green et. al [41] conducted a similar study using a fluidized bed reactor with chalk (solid calcium carbonate). In this study, the pH established in the reactor varied between 4.5 and 5.5 with higher nitrification rates obtained at the lower pH. In spite of the low pH, a high nitrification rate was observed and found similar to nitrification rates observed in a biological reactor operated at a pH>7.0 [41]. *Nitrosomonas* (AOB) and *Nitrospirae* (NOB) were identified as the predominant microbes. Over time these microbes may have become acclimated to these environmental conditions or may represent new species.

The pH of a WWTP does have an effect on the nitrification rate. A pH of 7.5 - 8.0 is considered optimum with rates declining below a pH of 6.8 [2]. Studies conducted by researchers confirm these pH ranges [22, 42, 43]. However, a study conducted by Tarre and Green found that nitrification could be achieved at a low pH [44]. When using a biofilm reactor, a specific nitrification rate of 0.55 days⁻¹ were achieved at a pH of 4.3±0.1. This is similar to values reported for nitrifying reactors at optimum pH. When conducted using a suspended-biomass reactor, a specific nitrification rate of 0.24 days⁻¹ was achieved at a pH of 3.8±0.3. *Nitrosomonas* (AOB) and *Nitrospirae* (NOB) were identified as the predominant microbes in both systems. (Note: The suspended-biomass study was repeated in the USF - Stroot lab using equipment to conduct the elevated dissolved CO₂ concentration study. Nitrification could not be achieved below a pH of 6.0.)

Temperature impacts nitrifier growth rates with lower temperatures producing lower nitrification rates [2]. In one study, a temperature difference of 10°C (30°C versus 20°C) showed a three-fold increase in maximum growth rates [45]. Studies conducted by Siripong and Rittman [23] showed that *Nitrosomonas* has the potential to grow twice as fast as *Nitrosospira* in the optimum temperature range. This growth advantage favors detection of *Nitrosomonas* rather than *Nitrosospira* with culture based methods. When investigating WWTP's during summer and winter conditions, which had 6.7-13.4°C lower temperatures and 13-49% higher solids retention time (SRT), higher levels of *Nitrosospira* were detected during the winter [23].

Other research has suggested that AOB and NOB are quite versatile in their ability to adapt [46]. Under anaerobic conditions, *Nitrosomonas* (AOB) was found to be capable of nitrite denitrification with molecular hydrogen, hydroxylamine or organic matter (pyruvate, formate) as electron donors resulting in production of N₂O and N₂ [47-50]. It has been suggested that this is a protection mechanism against the negative effects of high nitrite concentration [51, 52]. Alternatively, it has been recognized as a process of high importance for anaerobic growth [51, 52] as well as for the supply of NO necessary for ammonium oxidation [53, 54]. Under oxygen-limited or anoxic conditions, ammonium could act as an electron donor that is oxidized with nitrite instead of oxygen as the electron acceptor [50, 55].

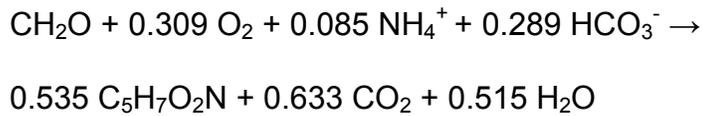
Several strains of *Nitrobacter* are capable of heterotrophic growth under oxic as well as anoxic condition [33, 56, 57]. Some strains of *Nitrobacter* were shown to be denitrifying organisms as well. Under anoxic conditions, nitrite can be used as an acceptor for electrons derived from organic compounds to promote anoxic growth [58]. Since the oxidation of nitrite is a reversible process, the nitrite oxidase-reductase can reduce nitrate to nitrite in the absence of oxygen [59].

The aeration basin of a WWTP is a complex microbial community probably containing several different genera of microbes capable of nitrification [23]. Their food source and operating conditions (temperature, pH, DO) undoubtedly have a significant effect as to which species dominates.

2.4 Heterotrophic Bacteria, Chemical Oxygen Demand (COD) and Ammonium Removal

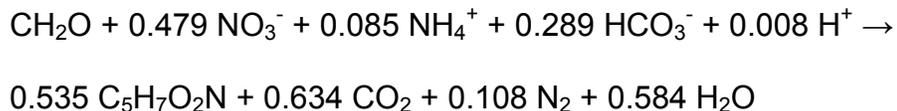
Heterotrophic bacteria consume COD and nitrogen in order to produce biomass. Ammonium (NH_4^+) and organic nitrogen compounds are the preferred nitrogen sources but nitrate will also be utilized in the absence of ammonia [9].

A complete mass based stoichiometric equation for the consumption of carbohydrate COD removed using ammonia as the nitrogen source ($f_s = 0.71$) is written as follows [9]:



Based on this stoichiometric equation, one mg of NH_4^+ is required to convert approximately 19.6 grams of the carbohydrate COD to the COD biomass. For 300 mg/l of influent COD, approximately 15.3 mg/l of ammonia is necessary to convert the COD into biomass. The ammonium not consumed will be converted to nitrate (NO_3^-) through nitrification utilizing autotrophic bacteria. Though uncommon, processes with limited influent nitrogen sources (high COD:N ratio) will experience difficulties in converting all of the COD.

During anoxic conditions, heterotrophic bacteria will use nitrate as an electron acceptor (instead of oxygen) and ammonium as a nitrogen source. A complete mass based stoichiometric equation ($f_s = 0.71$) is provided [9]:



The above reaction, known as denitrification, occurs in wastewater treatment plants that incorporate an anoxic zone to convert nitrate (NO_3^-) to nitrogen gas (N_2). The denitrification rate ($\text{g NO}_3^- \text{-N reduced/g MLVSS d}$), which determines the amount of nitrate denitrified, is primarily a function of availability of rapidly biodegradable organic matter (RBOM) and temperature [3].

Denitrifiers, typically heterotrophs but certain autotrophs are capable of denitrification[60], use organic matter as the energy and carbon source. As a first approximation, a minimum BOD:TKN ratio of approximately 3:1 is required in the bioreactor influent for reliable denitrification. The actual ratio will depend on operating conditions and substrate biodegradability. Within limits, higher F/M ratios in the anoxic zone achieve higher denitrification rates due to the presence of increased RBOM. Likewise, the type of substrate also impacts the denitrification rate. Significantly higher denitrification rates are possible with methanol and fermentation end-products, such as volatile fatty acids (VFAs) present in the influent wastewater. Denitrification supported by endogenous decay is associated with slow denitrification rates [3].

2.5 Carbon Dioxide and Wastewater Treatment Plants

Aquatic systems can be modeled with dissolved CO_2 in open or closed systems. With rare exceptions, wastewater treatment facilities are open systems as they are exposed to the atmosphere and liquid is entering and existing continuously.

Additionally, depending on the pH, the CO₂ will dissociate into three species within the aquatic systems, H₂CO₃^{*}, HCO₃⁻, CO₃²⁻ (Figure 2-5). As most wastewater treatment facilities operate in the pH range of 6.8 – 7.3, HCO₃⁻ is the predominant carbon dioxide species. This is true for both open and closed CO₂ systems. At a pH of 7.0 in the closed system, approximately 81% of the carbon dioxide exists as bicarbonate (HCO₃⁻) with the remainder as H₂CO₃^{*}. It should also be noted that 99% of carbon dioxide in solution exists in the form of dissolved carbon dioxide [61].

Speciation is governed by the following equations:



Where:

- g = gas
- aq = aqueous
- K_H = Henry's constant
- pK_a = acid dissociation constant

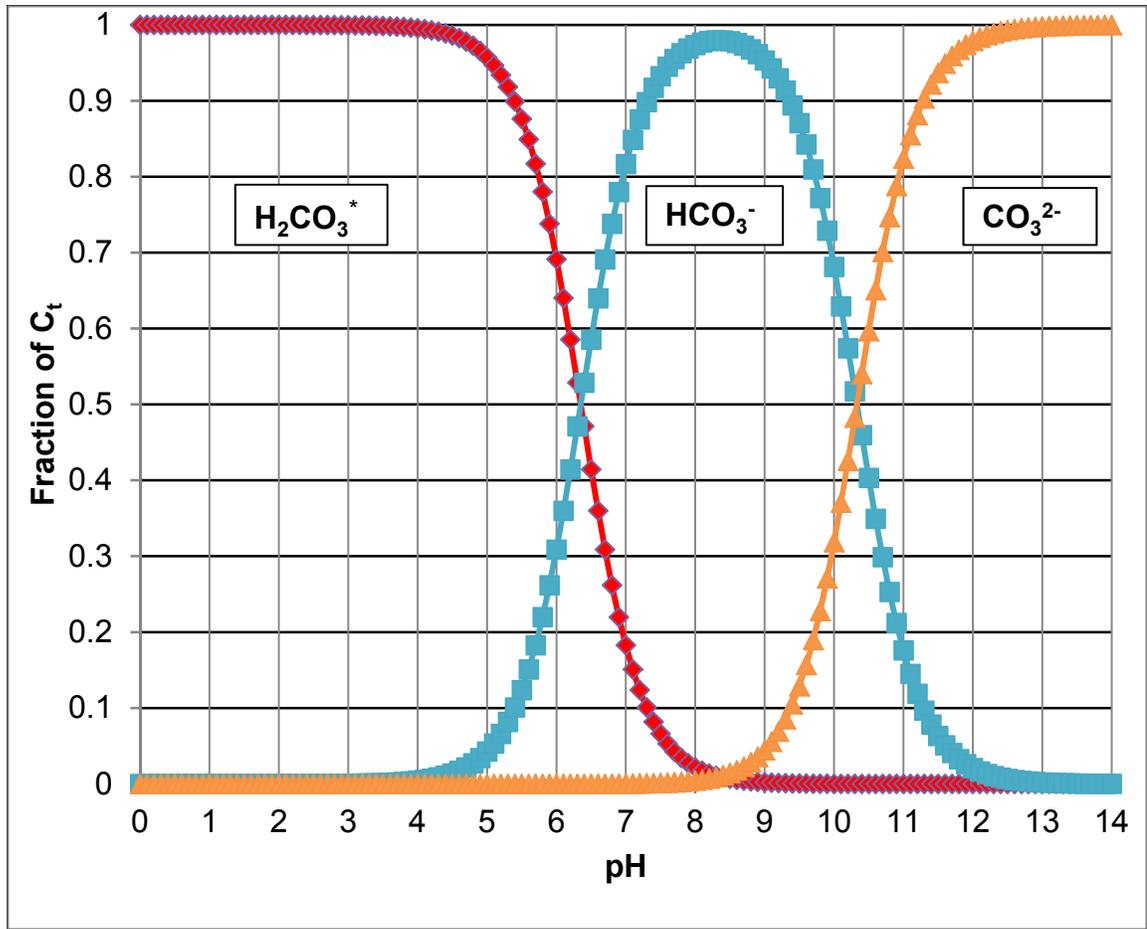


Figure 2-5: Fraction of Dissolved Carbon Dioxide in Species Form as Function of pH in a Closed System

Closed and open systems do have some differences [62]:

- In open systems, $H_2CO_3^*$ remains constant.
- The total carbonate concentration, $[H_2CO_3^*] + [HCO_3^-] + [CO_3^{2-}]$, is constant in a closed system but varies with pH in the open system (Figure 2.6).

Nitrification results in the destruction of 7.1 mg of alkalinity ($CaCO_3$) per mg of NH_4^+ -N oxidized. As ammonium is oxidized, it produces two strong acid

equivalents per mole of NH_4^+ removed [12]. If the influent contains inadequate alkalinity, nitrification would be compromised. As alkalinity is destroyed, pH is decreased and this could potentially reduce the nitrification rate as the alkalinity is needed to buffer the system. Most WWTPs operate in a pH range of 6.8 to 7.3.

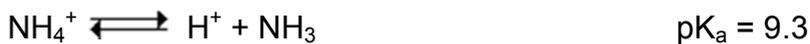
Denitrification results in the recovery of 3.6 mg of alkalinity as CaCO_3 and 2.9 mg of oxygen per mg of NO_3^- -N reduced. This oxygen equivalent is a useful factor when calculating the total oxygen required for nitrification-denitrification biological treatment systems [2]. Therefore, by combining nitrification (aerobic) and denitrification (anoxic), partial alkalinity recovery and oxygen credit can be attained. An additional benefit of incorporating an anoxic selector is improved sludge settleability [3].

Carbon dioxide concentrations in a WWTP will vary depending on the unit operation. Dissolved CO_2 in the influent is usually low (10 mg/l or less) but can be high if anaerobic conditions exist in the sewer system. In the aeration basin, carbon dioxide is produced in the consumption of carbohydrate COD and during denitrification. (See the section 2.4, Heterotrophic Bacteria and COD and Ammonia Removal, for a review of the stoichiometric equations.) However, during nitrification CO_2 is consumed at the rate of 0.085 moles of CO_2 for every mole of NH_4^+ consumed. From secondary clarification to discharge, the CO_2 concentrations will decrease. Typical discharge concentrations (effluent) of 12

mg/l or less are common. (See chapter 4, "Evaluation of Nitrifying Bacteria Specific Growth Rate Sensitivity to Carbon Dioxide for Full-Scale Activated Sludge and Municipal Wastewater," for a complete review of measured CO₂ concentrations at several WWTP's by unit operation.)

A pC-pH diagram showing concentrations for a 1 percent (17 mg/l dissolved CO₂ concentration) CO₂ air mixture in an open carbonate system with a 60 mg/l NH₄⁺-N concentration is provided (Figure 2-6). This ammonium concentration is shown as it was selected in subsequent experimentation conducted in this research. The varying species concentration of the carbon dioxide as a function of pH is shown. At a neutral pH, which most wastewater treatment plants operate, bicarbonate (HCO₃⁻) is the predominate species, H₂CO₃^{*} remains constant across pH levels and CO₃²⁻ is low. The bicarbonate remains dominant to a pH of approximately 10 where the concentration of the CO₃²⁻ attains equality.

Ammonium speciation is governed by the following equations:



$$[\text{H}^+] = [\text{NH}_{3(\text{aq})}] + [\text{OH}^-] \quad \text{Proton Condition}$$

The governing equations for CO₂ were listed previously.

NH_4^+ remains at a constant concentration until it reaches its pK_a value. Upon reaching this value, it transitions to NH_3 . The decrease in concentration as pH increases is due to NH_3 being a base.

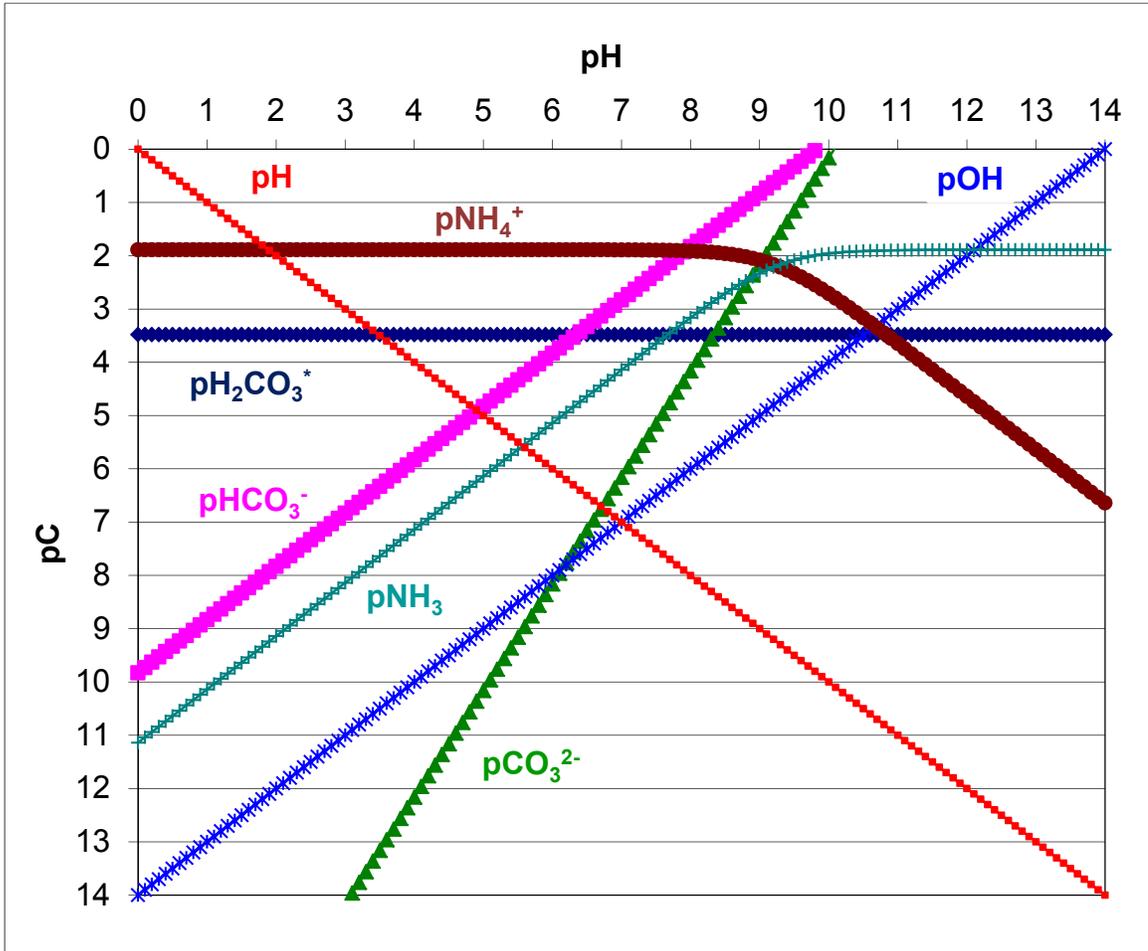


Figure 2-6: 1% CO_2 - Air Mixture and 60 mg/l of NH_4Cl in an Open System

2.6 Substrate Utilization in Wastewater Treatment Plants

At steady state, the mass balance equation for substrate in an activated sludge system may be written as:

Substrate in influent - Substrate consumed =

Substrate in effluent - Substrate in WAS

The change in substrate concentration with time can be determined by starting with the substrate mass balance for a completely stirred tank reactor (CSTR) [2, 63]:

$$dS/dtV = Q \cdot S_o - Q \cdot S + r_{su} \cdot V \quad 2-1$$

where:

- r_{su} = substrate utilization rate = $-(\mu_{max} \cdot S \cdot X) / [Y \cdot (K_s + S)]$
- V = volume of wastewater in the aeration tank
- Q = flow rate of wastewater
- S_o = substrate concentration in influent, $t = 0$, mg/l
- [substrate for growth of heterotrophs (aerobic) and nitrifiers]
- S = substrate concentration in effluent at time t , mg/l
- Y = fraction of substrate mass converted to biomass
- K_s = half saturation constant, mg/l = concentration of limiting substrate when $\mu = 0.5 \mu_{max}$
- μ_{max} = maximum specific growth rate, days^{-1}
- X = concentration of biomass, mg/l

Experimentation conducted in this research used a batch reactor. Since Q is equal to zero for a batch reactor and volume is constant, equation 2-1 can be simplified to:

$$dS/dt = - (\mu_{\max} \cdot S \cdot X) / [Y \cdot (K_s + S)] \quad 2-2$$

Integration of equation 2-2 with respect to time yields:

$$K_s \cdot \ln(S_o/S_t) + (S_o - S_t) = X \cdot (\mu_{\max} / Y) \cdot t \quad 2-3$$

Where:

- S_o = substrate concentration in influent, time = 0, mg/l
- S = substrate concentration at time t, mg/l
- t = time, days

For nitrification, the Monod kinetic coefficients are substituted in equation 2-3 to yield:

$$K_s \cdot \ln(N_o/N_t) + (N_o - N_t) = X_n \cdot (\mu_{\max}/Y) \cdot [DO / (K_o + DO)] \cdot t \quad 2-4$$

Where:

- X_n = concentration of nitrifier biomass, mg/l
- DO = dissolved oxygen concentration, mg/l
- K_o = half saturation constant for oxygen, mg/l
- N_o = substrate concentration (ammonium) in influent, time = 0, mg/l
- N_t = substrate concentration (ammonium) at time t, mg/l

2.7 Estimation of the Maximum Specific Growth Rate, μ_{\max} , from NO_x Generation Rate in Batch Reactor

The rate of generation of the NO_x concentration (nitrite + nitrate) is equal to the disappearance of ammonium utilized for nitrification. Its relationship is given by:

$$dS_{\text{NO}_x}/dt = - dS_{\text{NH}_4^+}/dt \quad 2-5$$

Initial reactor conditions must provide a high ammonium concentration, relative to the half velocity constant from Monod kinetics, K_s to ensure that the nitrification rate is at a maximum [21]. From Monod kinetics, $\mu = \mu_{\max} (S_{\text{NH}_4^+}/(K_s + S_{\text{NH}_4^+}))$. With high concentrations of ammonium, the specific growth rate, μ , will essentially equal the maximum specific growth rate, μ_{\max} . Its relationship is given by:

$$dS_{\text{NO}_x}/dt = \mu_{\max} \cdot (X_{\text{AUT}}/Y_{\text{AUT}}) \quad 2-6$$

And, rearranging the right side of equation 2.6 results in,

$$dS_{\text{NO}_x}/dt = (\mu_{\max}/Y_{\text{AUT}}) X_{\text{AUT}} \quad 2-7$$

The change in nitrifier biomass concentration, X_{AUT} , is determined by growth and decay.

$$\begin{aligned} dX_{AUT}/dt &= (\mu_{max} \cdot X_{AUT}) - (b_{AUT} \cdot X_{AUT}) \\ &= (\mu_{max} - b_{AUT}) \cdot X_{AUT} \end{aligned} \quad 2-8$$

Integrating this equation from time zero to time t yields:

$$X_{AUT,t} = X_{AUT,0} \cdot e^{(\mu_{AUT} - b_{AUT}) t} \quad 2-9$$

where:

- S_{NOx} = oxidized nitrogen concentration
- μ_{AUT} = maximum specific nitrifier growth rate
- $X_{AUT,t}$ = nitrifier concentration at time t
- $X_{AUT,0}$ = nitrifier concentration at time zero
- b_{AUT} = nitrifier decay rate
- Y_{AUT} = nitrifier yield coefficient

Substituting equation 2-9 into equation 2-7 and integrating from time zero to time t yields:

$$S_{NOx,t} = S_{NOx,0} + [(\mu_{AUT} \cdot X_{AUT,0}) / (Y_{AUT} \cdot (\mu_{AUT} - b_{AUT}))] \cdot [(e^{(\mu_{AUT} - b_{AUT}) \cdot t} - 1)] \quad 2-10$$

where:

- $S_{NO_x,t}$ = oxidized nitrogen concentration at time t
- $S_{NO_x,0}$ = oxidized nitrogen concentration at time zero
- Y_{AUT} = nitrifier yield coefficient

For estimating $(\mu_{AUT} - b_{AUT})$, non-linear regression is used to fit equation 2-10 using the measured NO_x data versus time [21]. In high F/M (food to microorganisms) experimentation, which was used in this research, b_{AUT} values from 0.14 – 0.17 were recommended. These range of decay rates were selected based on a series of experiments conducted using various methods, testing and temperature conditions as communicated by various authors [9, 21, 64-68]. Based on these conditions, a value of 0.15 days^{-1} was selected for the nitrifier decay rate, b_{AUT} , used in this research.

2.8 Carbon Dioxide and Nitrification

The slow growth rate and associated nitrification rate requires a lengthy solids retention time (SRT), as much as 20 days. Previous work has demonstrated that the growth of some autotrophic bacteria is carbon limited [69-71]. Inorganic carbon was found to be a limiting factor in biological nutrient removal (BNR) systems due to the low partial pressure of carbon dioxide (pCO_2) of the atmospheric air introduced, and the loss of CO_2 by stripping [72]. These factors were reported to limit the bulk concentration of CO_2 in wastewater and

consequently affect nitrification. Wett and Rauch suggest that pH is not a limiting factor *per se*, but instead the limiting factor is the low bicarbonate concentration resulting from the low pH [72]. Additional evidence of the influence of CO₂ on the specific growth rate of nitrifying bacteria has been demonstrated in a lab-scale, ideal mixed aerated reactor with CO₂ concentrations of up to 17% in air [71]. These preliminary results suggested a strong influence of dissolved CO₂ concentration on nitrification rates. Green et al. found a correlation between the concentration of CO₂ and the ammonium oxidation rate on a nitrifying chalk reactor [70]. In this experiment, the oxidation rate of ammonium increased as the pCO₂ increased. The authors reported that increasing pCO₂ improved the rate of nitrification up to 1% CO₂. Beyond wastewater treatment, elevated pCO₂ was also reported to stimulate nitrification in the soil and is usually measured at a pCO₂ of 10⁻² (1% CO₂). Kinsbursky and Saltzman reported that CO₂ was a possible limiting substrate for nitrifying bacteria in the soil [73].

Research conducted by these authors suggests that providing elevated pCO₂ to the activated sludge system should increase nitrification rates. Based on published literature, a one percent CO₂ mixture in air (17 mg/l dissolved CO₂ concentration) was chosen as an initial condition for this study.

2.9 Preliminary Research

A series of experiments were conducted utilizing two 3-liter beakers set up as sequential batch reactors. One reactor received air while the other received a one percent CO₂ mixture in air (17 mg/l dissolved CO₂ concentration). All other parameters were consistent between reactors. Results indicate that a significant increase in NH₄⁺ conversion (three to five fold) occurred in the reactor supplied with a one percent CO₂ mixture. These reactors were not pH controlled so some loss of NH₄⁺ probably occurred as the air supplied reactor reached pH values as high as 8.57, thus affecting the conversion rate. However, the loss of NH₄⁺ could not fully account for the differences observed. (See chapter 3, "Stimulation of Nitrification by Carbon Dioxide in Lab-Scale Activated Sludge Reactors," for a complete review of this study.)

Based on results from the previous research and the fact that most aeration basins are open systems with minimal pCO₂ available, it was hypothesized that these organisms maybe carbon limited. Optimization of the nitrification process could be achieved by understanding the relationship of dissolved CO₂ concentration on nitrifier growth rates.

Based on this initial research, a series of experiments were conducted to determine nitrifier growth rates at controlled pH comparing varying level of pCO₂ versus an air system. Synthetic feed and influent were incorporated into the

study and a phosphate buffer was used for pH control. (See chapter 3, “Stimulation of Nitrification by Carbon Dioxide in Lab-Scale Activated Sludge Reactors,” for a complete review of methods used to conduct this study.) A partial list of the experiments is provided (Table 2-4).

Table 2-4: Specific Growth Rate at Selected pCO₂ and pH

WWTP	Feed	pH	Source	μ (days ⁻¹)	% Improvement
MLE 1	Synthetic	Not Controlled	Air	0.41	56
			1%	0.64	
MLE 1	Synthetic	7	Air	0.29	107
			1%	0.6	
MLE 2	Influent	7	Air	0.56	50
			1%	0.84	
EA	Synthetic	7	Air	0.45	33
			1%	0.6	
EA	Influent	7	Air	0.22	91
			1%	0.42	
EA	Influent	7	Air	0.5	78
			2%	0.89	
EA	Influent	7.5	Air	0.74	37
			0.1%	1.013	

As can be observed from the study, in all cases the elevated levels of pCO₂ provided enhanced nitrification rates. The varying pCO₂ concentrations above atmospheric levels provided enhanced nitrification, and thus may not be limited to specific dissolved CO₂ concentrations.

Chapter 3

Stimulation of Nitrification by Carbon Dioxide in Lab-Scale Activated Sludge Reactors

3.1 Abstract

It is hypothesized that the autotrophic, nitrifying bacteria in activated sludge systems grow slowly due to CO₂ limitation. To test this hypothesis, four experiments were conducted with two lab-scale reactors fed synthetic wastewater or influent from a wastewater treatment facility. The control reactor was supplied with air (0.03% CO₂), while the experimental reactor was supplied with air containing elevated pCO₂ (1%). The first experiment was conducted with a small inoculum, no carbon source, and phosphate buffer used to maintain pH 7. A 6.9 fold increase in the rate of nitrate formation was observed in the reactor with elevated pCO₂. The last three experiments operated both reactors as sequencing batch reactors fed with synthetic wastewater with acetate as a carbon source. The second experiment demonstrated that providing elevated pCO₂ for the entire react cycle improved the nitrate formation rate, but severely degraded the solids settling performance. The last two experiments demonstrated a five-fold increase by providing elevated levels of pCO₂ for the

final five hours of the 7-hour react cycle without affecting solids settling or COD removal performance.

3.2 Keywords

Activated Sludge, Autotrophic, Carbon Dioxide, Nitrification, Nitrifying Bacteria

3.3 Introduction

Nitrification is the first step for the removal of nitrogen from wastewater, where ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) by aerobic, autotrophic, nitrifying bacteria. These bacteria are thought to have slow growth rates and are sensitive to pH and temperature swings, making nitrification difficult to maintain in activated sludge systems [14, 15]. The slow growth rate and associated nitrification rate requires a lengthy solids retention time (SRT), as much as 20 days. Previous work has demonstrated that the growth of some autotrophic bacteria is carbon limited [69-71]. Inorganic carbon was found to be a limiting factor in biological nutrient removal (BNR) systems due to the low partial pressure of carbon dioxide (pCO_2) of the atmospheric air introduced, and the loss of CO_2 by stripping [72]. These factors were reported to limit the bulk concentration of CO_2 in wastewater and consequently affect nitrification. Moreover, Wett and Rauch [72] suggest that pH is not a limiting factor *per se*. Instead, the limiting factor is the low bicarbonate concentration resulting from the low pH. Additional evidence of the influence of CO_2 on the specific growth rate of nitrifying bacteria has been demonstrated in a lab-scale, ideal mixed aerated

reactor with CO₂ concentrations of up to 17% [71]. These preliminary results suggested a strong influence of pCO₂ on nitrification rates. Green et al. [70] found a correlation between the concentration of CO₂ and the ammonium oxidation rate on a nitrifying chalk reactor. In this experiment, the oxidation rate of ammonium increased as the pCO₂ increased. They reported that increasing pCO₂ improved the rate of nitrification up to 1% CO₂. Beyond wastewater treatment, elevated pCO₂ was also reported to stimulate nitrification in the soil. Carbon dioxide is usually measured in the soil at a pCO₂ of 10⁻² (1% CO₂). Kinsbursky and Saltzman [73] reported that CO₂ was a possible limiting substrate for nitrifying bacteria in the soil.

These results suggest that providing elevated pCO₂ to the activated sludge system should increase nitrification rates, however, additional research is needed to answer three fundamental questions:

- Does elevated pCO₂ or pH depression increase the nitrification rate in activated sludge systems?
- When an activated sludge system is challenged with a lower target SRT, does nitrification persist with elevated pCO₂?

- Does elevated $p\text{CO}_2$ negatively impact the general performance (i.e., chemical oxygen demand removal and adequate solids settling) of the activated sludge system?

Experimentation was conducted using lab-scale reactors to investigate these three research questions.

3.4 Materials and Methods

3.4.1 Experiment 1

This experiment was conducted to determine whether elevated $p\text{CO}_2$ or pH depression caused by elevated $p\text{CO}_2$ was the principal cause of higher nitrification rates in bench-scale activated sludge systems. In addition, the conversion rate of $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$ and a complete nitrogen mass balance was determined. The experiment was conducted based upon previously published guidelines [21]. Two 3 liter beakers were used for the reactors. The control reactor was fed air, while the experimental reactor was fed a mixture of air and 1% CO_2 .

Both reactors were fed a synthetic wastewater with the following composition (per L): 3.33 mL of nutrient solution consisting of (per L): , 22.65g $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$, 27.00 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 10.80 g KCl, 4.20 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.90 g

EDTA, 0.30 g Yeast Extract, and 90 mL of trace metal solution. The trace metal solution consisted of (per L): 5.00 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g H_3BO_3 , 1.60 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01g KI, 5.00 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.10 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 2.20 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 50.0g EDTA. The synthetic wastewater and stock solutions were prepared with deionized water from a reverse osmosis system. A series of preliminary experiments were conducted to establish appropriate operating conditions. Based on these results, 58 mg/l of NH_4^+ -N was used as the sole nitrogen source. The dissolved oxygen was relatively constant at 7.3 mg/l as O_2 , which ensured that oxygen was not limiting. Each reactor had an initial addition of 0.5 grams of sodium bicarbonate with 0.5 gram additions at 49 and 94 hours for a total of 1.5 grams. This approach prevented interference with the nitrite probe, while providing adequate bicarbonate for nitrification.

The pH was maintained between 6.95 and 7.05 through the addition of a phosphate buffer. Three phosphate buffers with pH values of 9.1, 7.0, and 4.4 were prepared with $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (pH = 9.1) and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (pH = 4.4). The pH 7.0 buffer was prepared by mixing 57.7 ml of the $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ solution and 42.3 ml of the $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ solution. Each reactor received identical phosphate buffer additions. The pH 7 buffer was used to equilibrate the total addition. For example, if the control reactor required 8 ml of the pH 4.4 buffer to reach pH 7.0 and the experimental reactor only required 5 ml of the same phosphate buffer, then an additional 3 ml of the pH 7.0 buffer was added to the experimental reactor to maintain the phosphate concentration. A total of 0.042

moles of phosphate buffer was added to each reactor during the course of the experiment.

Each reactor was inoculated with 35 ml of mixed liquor suspended solids (MLSS), that was collected from the nitrification basin of a full-scale activated sludge system (Glendale Wastewater Reclamation Plant of the City of Lakeland, FL) on the same day that the experiment was initiated. Throughout the experiment, NH_4^+ , NO_2^- , NO_3^- , pH, and dissolved oxygen (DO) were periodically measured. Experiments were discontinued when ammonium was less than 20 mg/l NH_4^+ -N in either the control or experimental reactor.

3.4.2 Experiments 2-4

The reactors were operated with a working volume of 3 liters and were seeded with 1 liter of MLSS from the nitrification basin of a full-scale activated sludge system (Northside Wastewater Reclamation Plant of the City of Lakeland, FL), which was operated at an SRT of 22 days (Figure 3). For three cycles per day, both reactors were fed every cycle with 2 liters of synthetic wastewater as described for experiment 1 with the following modifications (per liter): 0.168 g of NaHCO_3 and 0.850 g of $\text{C}_2\text{H}_3\text{O}_2\text{Na}\cdot 3\text{H}_2\text{O}$ were added directly to the solution; and 32.10 g of NH_4Cl was added to the nutrient solution. For Experiments 2 and 3, the synthetic wastewater and stock solutions were prepared with deionized water provided by Culligan Water (Lakeland, FL). For Experiment 4, the

synthetic wastewater and stock solutions were prepared with deionized water from a reverse osmosis system. Synthetic wastewater for experiments 2-4 had the following characteristics: Alkalinity of 100 mg/l as CaCO₃, chemical oxygen demand (COD) of 400 mg/l as O₂, ammonium concentration of 28 mg/l NH₄⁺-N, and pH of 7.6.

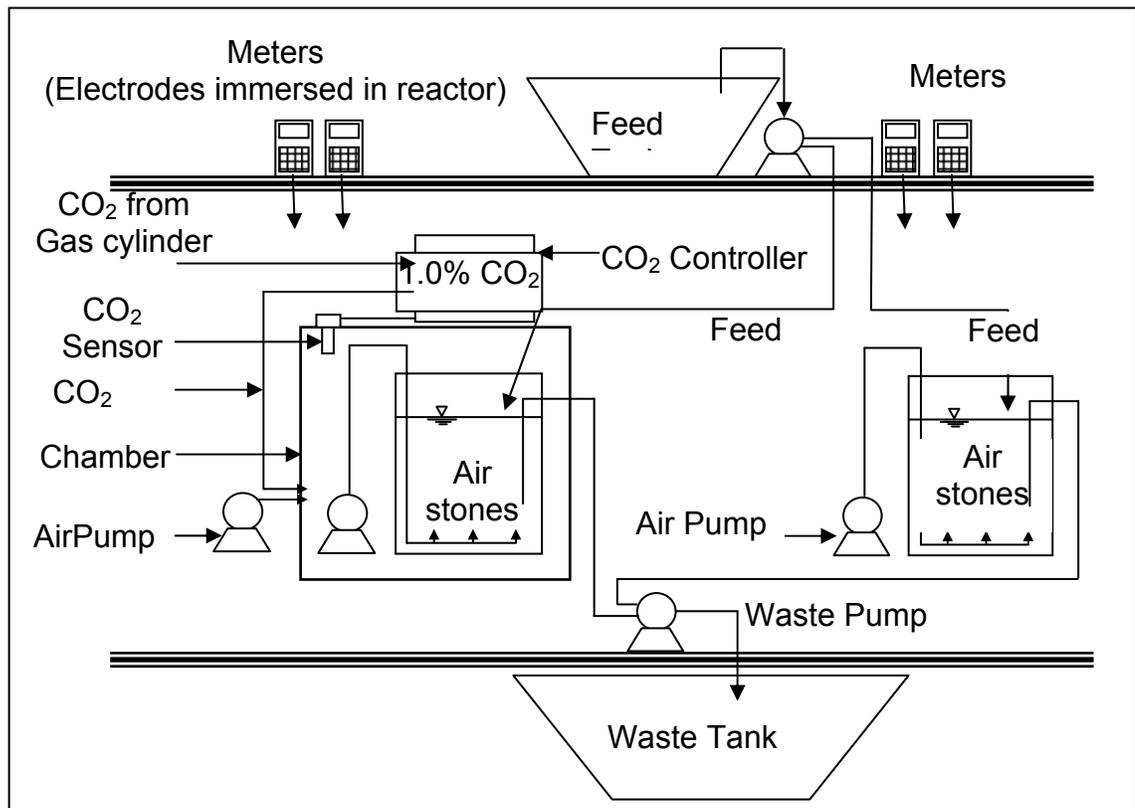


Figure 3-1: The Experimental SBR System that Features pCO₂ Control in the Experimental Reactor (left) and the Control Reactor (right)

The target hydraulic retention time (HRT) for both reactors was 0.5 days, which is similar to common values for municipal activated sludge systems [2]. The cycles were automatically operated with a Chronrol XT-4 (ChronTrol Corporation, San Diego, CA), that controlled the feed pump (Masterflex® L/S Pump Drive, Model

7518-10, Cole-Parmer Instrument Company, Vernon Hills, IL), waste pump (Masterflex® L/S Fixed Flow Drive, Model 7531-01, Cole-Parmer Instrument Company), and air supply system. Each sequence of cycles was 8 hours with three distinct cycles: Fill for 10 minutes at the beginning of the React cycle; React cycle for 7 hours; and Settling and Decanting for 45 and 15 minutes, respectively. The reactors were operated at room temperature (20-22°C).

Information regarding target SRT, length of experiment, and CO₂ addition for experiments 2-4 is provided (Table 3-1). For Experiment 2, CO₂ was supplied during the entire React cycle, whereas for Experiments 3 and 4, CO₂ was added during the last 5 hours of the React cycle. For these experiments, the activated sludge biomass was challenged by decreasing the SRT from 8 days sequentially to 6, 4, and 2 days. Experiment 4 was designed to operate the reactors for a period equal to three times each target SRT, in order to evaluate the impact of pCO₂ on nitrification for extended operation and performance.

Table 3-1: Description of Experiments 2 through 4 Conducted in a SBR

Experiment	SRT (days)	Days Tested per SRT	Total Days Tested	Hours 1% CO ₂ was supplied during React cycle
2	8	8	11	Entire 7 hours
	6	3		
3	8	8	20	Last 5 hours
	6	6		
	4	4		
4	2	2	60	Last 5 hours
	8	24		
	6	18		
	4	12		
	2	6		

3.4.3 Data Collection and Sample Analyses

For experiment 1, measurements were taken at least 4 times per day with a 4 hour time interval between measurements. Instruments used for chemical measurements included: ion selective electrodes (Ammonium combination glass body electrode, Cole-Parmer® 27502-03 and Nitrate combination glass body electrode, Cole-Parmer® 27502-31, Cole-Parmer Instrument Company), (Nitrite glass body electrode (Orion 9700BNWP, Thermo-Electron Corporation), Dissolved Oxygen Meter (Traceable* Portable Dissolved Oxygen Meter, Fisher Scientific), pH meter (pHTestr3+, Oakton Instruments) and ion meters (Oakton® Benchtop Ion 510 Meter and Oakton® Ion 6 Meters, Cole-Parmer Instrument Company). All instruments were calibrated daily before use. The ammonium electrode used a 0.1M NaCl filling solution (Cole Parmer® 27503-78 reference filling solution, Cole-Parmer Instrument Company) and was calibrated with a 1,000 mg/l NH_4^+ -N standard solution (prepared in the laboratory with reagent-grade NH_4Cl) and a 5M NaCl Ionic Strength Adjuster (ISA).

The nitrate electrode used a 0.1M $(\text{NH}_4)_2\text{SO}_4$ filling solution (Cole Parmer® 27503-79 reference filling solution, Cole-Parmer Instrument Company) and was calibrated with a 1,000 mg/l NO_3^- -N standard solution (prepared in the laboratory with reagent-grade NaNO_3) and a 1M NaSO_4 ISA prepared in the laboratory.

The nitrite electrode used an Optimum Results Type F filling solution and was calibrated with a 1,000 mg/l NO_2^- -N standard solution (prepared in the laboratory with reagent-grade NaNO_2). A nitrite interference suppressor solution (NISS) was used for the nitrite probe measurements to negate any bicarbonate or nitrate interference.

In experiments 2-4, samples were collected daily during the entire React cycle to determine NO_3^- formation rates, pH, and DO. Samples of MLSS were collected daily at the end of the React cycle for settling evaluation and biomass analysis. Nitrate concentration, expressed as NO_3^- -N, was measured every 30 minutes during the React cycle to determine nitrification rates.

Samples for total suspended solids (TSS), volatile suspended solids (VSS), and COD analysis were collected once per day from the mixed liquor during the last 15 minutes of the React cycle. For the solids samples, 45 mL of MLSS was collected and transferred to 50 mL conical tubes and stored at 4°C. The sludge settling performance was evaluated by allowing 100 mL of MLSS collected at the end of the React cycle to settle in a graduated cylinder for 30 minutes and recording the sludge blanket volume. The TSS and settled sludge blanket volume measurements were then used to calculate the sludge volume index (SVI). The TSS and VSS were measured in triplicate according to Standard Methods for the Examination of Water and Wastewater Analysis [74] sections 2540D and 2540E respectively. Samples for COD analysis were withdrawn from

both reactors (10 mL of MLSS) at the end of the React cycle and settled for 30 minutes. Next, the supernatant was filtered by a syringe filter with a 25 mm diameter and 0.2 μm pore size (Fisher Scientific). Filtered samples were stored in 15 mL conical tubes at -20°C . Later, determination of COD was performed using the Reactor Digestion Method 8000 [75] for the COD range of 3 - 150 mg/l as O_2 . The vials used for this procedure (Digestion solution for COD 0-150 mg/l as O_2 range, HACH Company, Loveland, CO) were mixed with 2 ml of sample as indicated in the Method 8000 and digested for 2 hours at 150°C in a digital reactor block DRB 200 (HACH Company). Vials were placed in a rack for cooling to room temperature ($\sim 21^{\circ}\text{C}$). A portable spectrophotometer DR/2400 (HACH Company, Loveland, CO) adjusted to a wavelength of 420 nm (program 430 COD LR) as indicated by the Method 8000 was used to read the COD concentrations of the samples. A vial mixed with 2 mL of deionized water was used as a blank. Additional vials each mixed with 300 mg/l as O_2 standard solution at different dilutions were digested to check the calibration curve of the spectrophotometer with defined COD concentrations. The effluent COD concentration was compared to the initial COD concentration of 267 mg/l as O_2 corresponding to two thirds of the COD in the synthetic wastewater (400 mg/l as O_2) to obtain the COD removal efficiency.

3.5 Results

3.5.1 Experiment 1

The results from the experiment that compared the effect of elevated $p\text{CO}_2$ on nitrification rates at constant pH 7.0 are presented (Figures 3-2 and 3-3). The nitrate formation rate for the control reactor was 1.50×10^{-6} mg NO_3^- -N/l-min, which remained relatively constant throughout the experiment. By contrast, the experimental reactor showed an overall conversion rate of 10.3×10^{-6} mg NO_3^- -N/l-min, which represents a 6.9 fold increase. The conversion rate in the experimental reactor increased throughout the experiment. During the first 42 hours, the conversion rate was 5.90×10^{-6} mg NO_3^- -N/l-min, while the conversion rate for the remaining 101 hours more than doubled to 12.2×10^{-6} mg NO_3^- -N/l-min. A loss of ammonium was observed in both reactors, but was pronounced in the control. The experimental reactor lost 6.9 mg/l of NH_4^+ -N or 12 % of the initial ammonium, while the control reactor lost 23 mg/l of NH_4^+ -N or 40% of the initial ammonium. Nitrite was not detected in the control reactor, while nitrite was present in the experimental reactor at low concentrations with a maximum concentration of 1.3 mg/l NO_2^- -N.

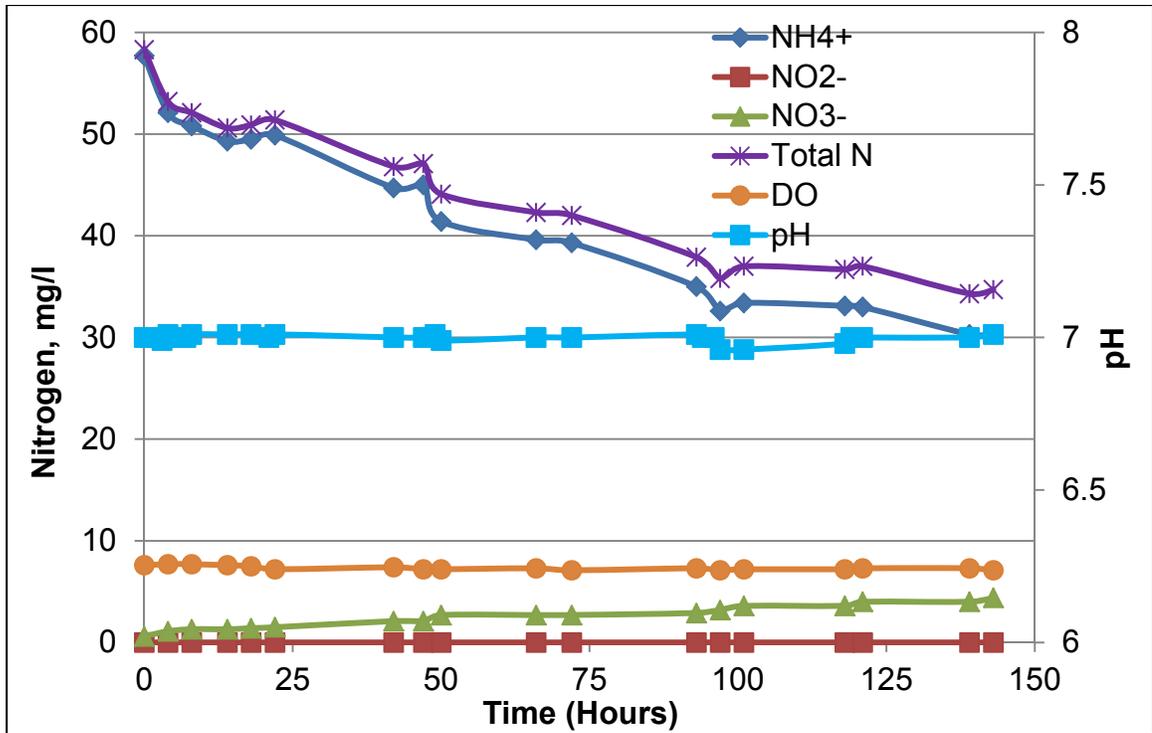


Figure 3-2: Ammonium, Nitrite, Nitrate, Total Nitrogen, pH, and DO for the Control Reactor in Experiment 1

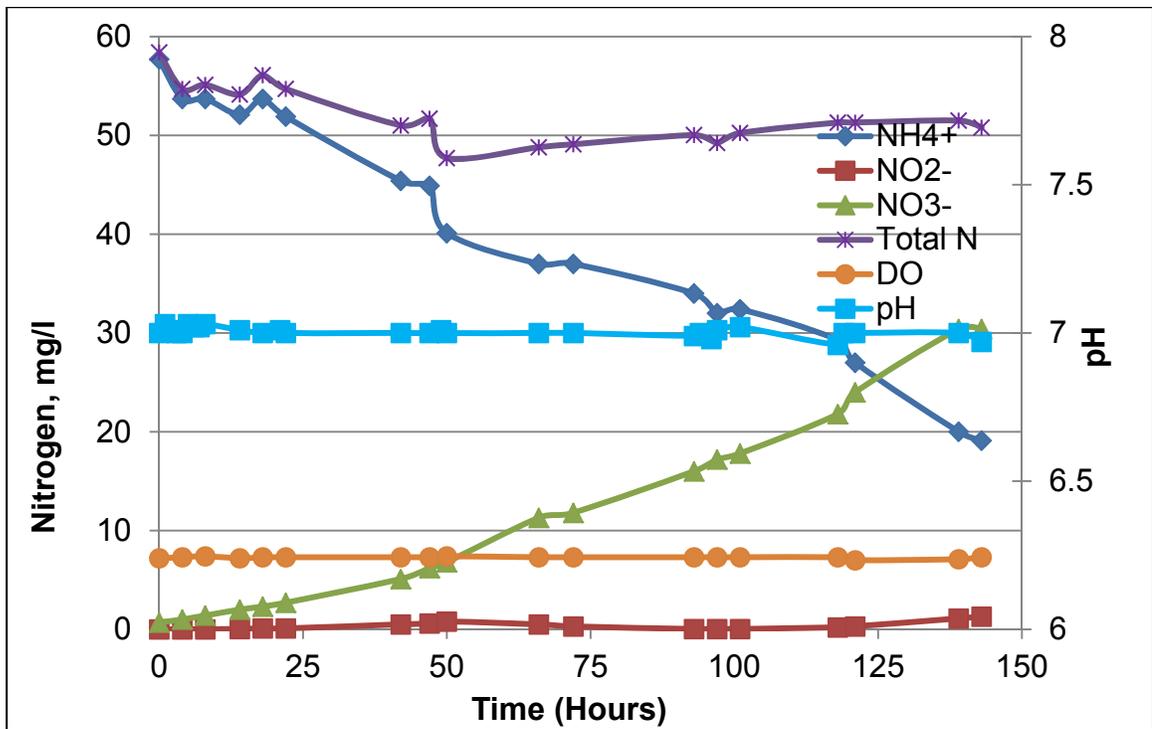


Figure 3-3: Ammonium, Nitrite, Nitrate, Total Nitrogen, pH, and DO for the Experimental Reactor in Experiment 1

3.5.2 Experiment 2

An experiment was performed to determine the effect of providing elevated $p\text{CO}_2$ with aeration throughout the React cycle in bench-scale activated sludge reactors operated as sequencing batch reactors. The positive impact of adding 1% CO_2 during aeration was evident, where nitrate formation rates in the experimental reactor were more than five times greater than the control (data not shown). Maximum nitrate formation rates were 0.0140 and 0.0040 $\text{mg NO}_3^- \text{-N/l-min}$ for the experimental and control reactors, respectively, while the average nitrate formation rates were 0.0080 and 0.0020 $\text{mg NO}_3^- \text{-N/l-min}$ for the experimental and control reactors, respectively. Sludge blanket volumes were greater than 40 ml/100mL and washout of biomass was only observed in the experimental reactor, whereas the control reactor demonstrated adequate solids settling performance. For both reactors, the COD removal efficiencies were greater than 90%. The pH in both reactors was consistent with an average pH of 7.59 and 8.45 in the experimental and control reactors, respectively, which constituted a difference in the average pH of 0.86. Upon completion of the React cycle, a difference in the pH of 0.77 was observed between the reactors with an average pH of 7.91 in the experimental reactor and pH of 8.68 in the control reactor. The significant reduction in the pH of the experimental reactor was due to the elevated $p\text{CO}_2$. In summary, when CO_2 was supplied throughout the 7-hour React cycle, the nitrate formation rates were significantly greater and the COD

removal efficiency was unaffected, but the solids settling performance was impacted severely.

3.5.3 Experiment 3

Based on the results of Experiment 2, the operational conditions were altered to reduce the impact on solids settling by supplying elevated $p\text{CO}_2$ to the experimental reactor after the first two hours of every 7-hour React cycle. With this change in strategy, it was assumed that 2 hours would be ample time for the heterotrophic bacteria to consume the bulk of the COD (i.e. acetate) without being impacted by elevated CO_2 levels. The remaining five hours of the React cycle would provide sufficient time for nitrification. In order to challenge the biomass in both reactors with washout pressure, the target SRT was decreased consecutively from 8 days to 6, 4, and 2 days.

The nitrate formation rates in both reactors during Experiment 3 are provided (Figure 3-4). As can be seen from the graphic, the daily nitrate formation rate was always greater in the experimental reactor compared to the control reactor. Nitrate formation rates were much higher in the experimental reactor (maximum: $0.0160 \text{ mg NO}_3^- \text{-N/l-min}$; average: $0.0070 \text{ mg NO}_3^- \text{-N/l-min}$) compared to the control reactor (maximum: $0.0040 \text{ mg NO}_3^- \text{-N/l-min}$; average: $0.0020 \text{ mg NO}_3^- \text{-N/l-min}$). For operation at lower SRT, the nitrate formation rates were lower in both reactors, which may indicate washout of the nitrifying biomass. Due to

equipment failure, the rates of NH_4^+ oxidation were not measured. Peak sludge blanket volumes greater than 40 ml/100 mL were observed twice in the control reactor whereas the experimental reactor showed adequate settling performance (≤ 33 ml/100 ml). This significant improvement in solids settling in the experimental reactor contrasts sharply with the results from Experiment 2. The COD removal efficiencies were greater than 90% throughout the experiment in both reactors. Similar to Experiment 2, the average pH at the beginning of the React cycle were 7.32 and 8.40 in the experimental and control reactors respectively. By the end of the React cycle, the average pH values were 8.07 and 8.78 in the experimental and control reactors, respectively, which were consistent with the results from Experiment 2. In summary, the results for Experiment 3 suggest that the nitrifying bacteria grew faster when provided 1% CO_2 and were able to maintain nitrification at a lower SRT without affecting the general performance of the system (i.e. solids settling and COD removal efficiency). At a very low SRT of 2 days, nitrification rates were much lower compared to operation at an SRT of 4 days, which may be due to washout of nitrifying bacteria.

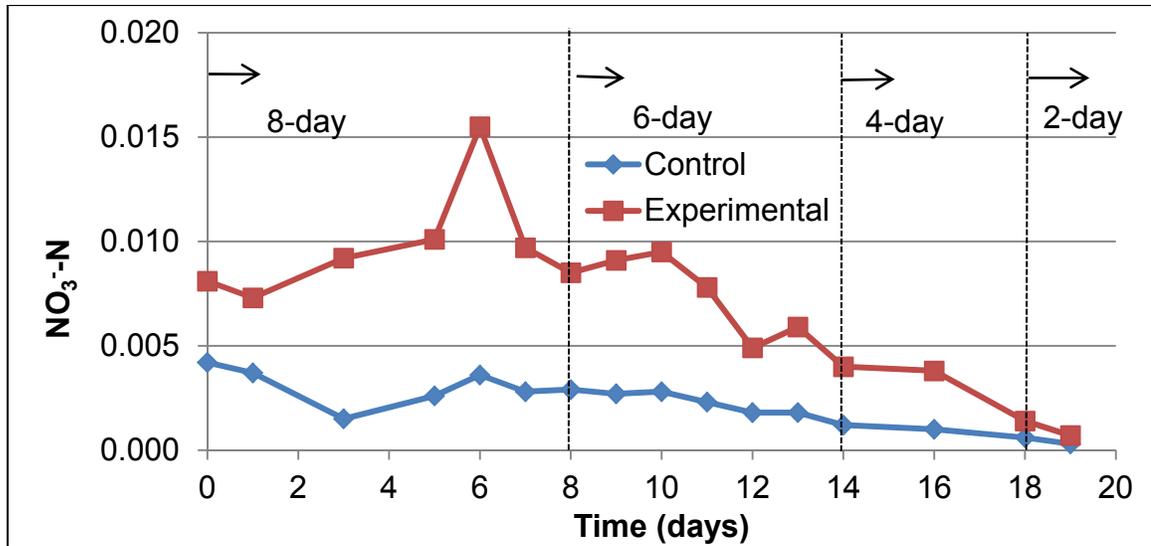


Figure 3-4: Nitrate Formation Rates for Experiment 3. The Start of Each SRT Period is Indicated by an Arrow

3.5.4 Experiment 4

To confirm the results from Experiment 3, a final experiment was designed with the same operational parameters, but the operational period for a target SRT was extended for a period equal to three times each target SRT value. This experimental approach provided sufficient time for the biomass to acclimate to the conditions for each target SRT. Similar to Experiments 2 and 3, the average pH was 8.45 for both reactors at the beginning of the React cycle. By the end of the React cycle, the pH values were 7.85 and 8.66 in the experimental and control reactors, respectively, which were consistent with the results from Experiments 2 and 3.

The nitrate formation rates for both reactors are presented in Figure 3-5. Similar to Experiment 3, the daily nitrate formation rate in the experimental reactor was

greater than the control reactor. Maximum nitrate formation rates were 0.0120 and 0.0050 mg NO₃⁻-N/l-min for the experimental and control reactors, respectively, which were slightly lower than Experiment 3. For both experiments, the maximum nitrate formation rates were observed during operation at an 8-day SRT, which can be attributed to high levels of nitrifying bacteria in the inoculum. The average nitrate formation rates over the course of the entire experiment were 0.0050 and 0.0010 mg NO₃⁻-N/l-min for the experimental and control reactors, respectively. This five-fold increase in the average nitrification rate is greater than the three and a half-fold increase from Experiment 3. In addition, the results provide evidence of high rate nitrification at a lower SRT when elevated pCO₂ is provided during aeration and the biomass is allowed to acclimate to the lower SRT operation.

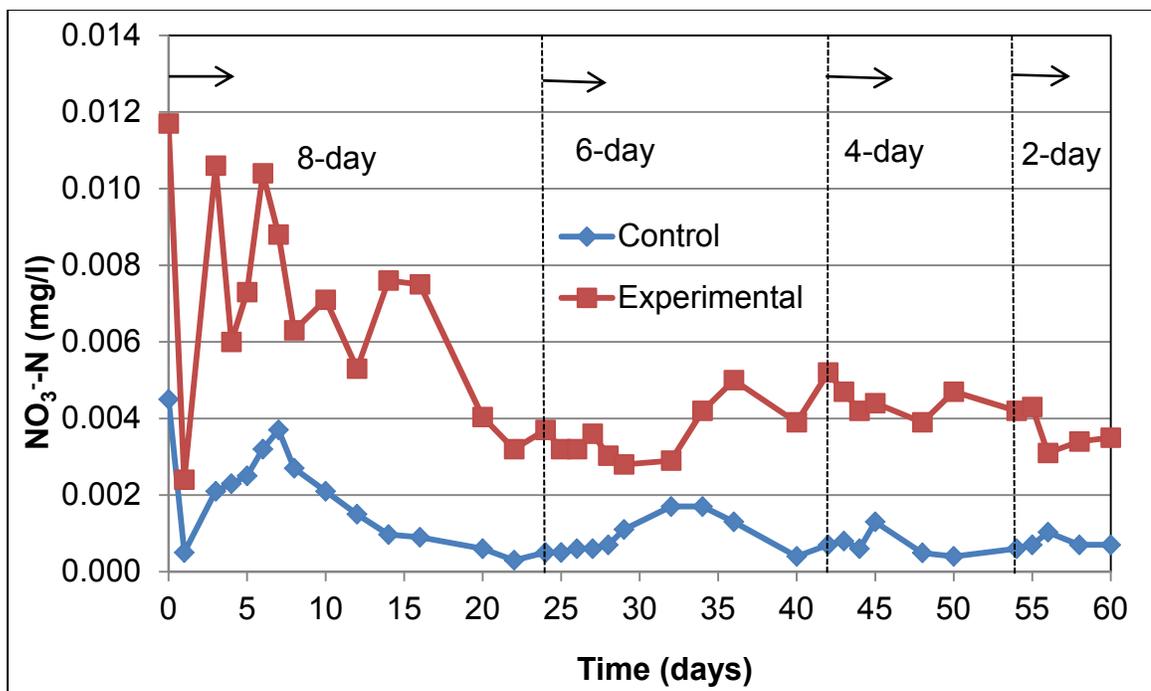


Figure 3-5: Nitrate Formation Rates for Experiment 4. The Start of Each SRT Period is Indicated by an Arrow

Nitrate concentrations in the samples collected at the end of the React cycle for both reactors were low throughout the experiment. These low levels of nitrate and the high SVI values (presented below) may indicate that denitrification occurred during the settling period. However, the average concentration in the experimental reactor was twice the average concentration in the control reactor (data not shown). Nitrate concentrations in the supernatant did not exceed 10 mg NO₃⁻-N/l in the experimental reactor.

In both reactors, no significant impact of elevated pCO₂ and low SRT operation was observed in COD removal efficiencies. Both reactors showed the same trends and had comparable values meeting the required removal efficiency of COD for secondary treatment (90%), and the supernatant concentrations were always below 30 mg/l as O₂, indicating adequate performance of the system. Even though the experimental reactor exhibited slightly higher COD removal efficiencies, they were not significant.

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured during Experiment 4 (Table 3-2). Both reactors had similar solids values and operating performance during the 8-day-SRT period. During the 6-day-SRT period, a significant difference was observed between the two reactors. The experimental reactor showed significantly lower, but stable solids concentrations. The control reactor was significantly impacted by poor solids settling performance and unintentional wasting of biomass was observed during

the Decant cycle. However, the solids concentration in the control reactor was much higher relative to the experimental reactor, which is difficult to explain. During the 4-day SRT period, no discernable differences were observed in the solids concentration or operating conditions for both reactors. During the 2-day SRT period, a reduction in the solids concentration and poor settling performance was observed in both reactors.

Table 3-2: Average Solids Concentration – Experiment 4

SRT-Reactor	TSS, mg/l	%VSS	VSS, mg/l
8-Experimental	1,803	92	1,659
8-Control	1,696	92	1,560
6-Experimental	963	95	915
6-Control	1,456	87	1,267
4-Experimental	1,170	92	1,076
4-Control	1,350	87	1,175
2-Experimental	931	92	857
2-Control	864	92	795

The solids settling performance during Experiment 4 was evaluated by the use of the sludge volume index (SVI). Although the SVI measurement is associated with the evaluation of clarifier performance in full-scale activated sludge systems, it was utilized in this study to provide some guidance on the impact of elevated pCO₂ on solids settling [2]. A comparison of the SVI for both reactors during Experiment 4 revealed better overall settling performance in the experimental reactor, as well as better ability to recover from the reduction of the SRT (data not shown). An SVI value greater than 150 ml/g TSS indicates poor settling and the possible proliferation of filamentous bacteria in full-scale systems. For the experimental reactor, the maximum SVI was 446 ml/g TSS which was less than

the control reactor maximum SVI of 636 ml/g TSS. Similarly, the daily average SVI for the experimental reactor was 210 ml/g TSS whereas the control reactor maintained an average daily value of 254 ml/g TSS.

The settling performance of the experimental reactor was acceptable throughout the experiment except for the 6-day-SRT period (days 25 to 42), when poor settling and bulking problems were observed in both reactors. Foaming was only observed during the poor settling period (days 30 to 40). The reduction of the SRT from 6 days to 4 days on day 42 and the subsequent absence of foaming may indicate that the foaming was due to the slow growth of foam-causing microorganisms such as *Nocardia* and *Microthrix* [76]. Poor settling was observed in the control reactor from day 6 - 50. Approximately 100 ml of MLSS per 8-hour cycle was unintentionally wasted on days 27 through 29 and days 32 through 38 with corresponding SVI values greater than 300 ml/g TSS. This value is twice the value reported for biomass washout, which highlights the limitations of using an SBR system to fully represent full-scale systems [2]. During these periods of poor settling, bubbles were observed in the rising sludge blanket and may indicate denitrification. Additionally, viscous bulking, as suggested by the jelly-like appearance of the MLSS, was associated with the high SVI values and washout of biomass. Overall, the experimental reactor exhibited improved solids settling performance compared to the control reactor. These results are consistent with Experiment 3 and suggest that providing elevated pCO₂ for the latter portion of the React cycle reduces the negative impact on solids settling.

3.6 Discussion

3.6.1 Effect of pH on Nitrification

Results from Experiment 1 clearly demonstrate increased nitrification as shown by the generation of NO_x ($\text{NO}_2^- + \text{NO}_3^-$) as a result of elevated pCO_2 while pH is held constant. However, significant ammonium loss was observed in the control reactor. Some of the loss may have resulted from stripping; however, the ammonia concentration at pH 7 only constitutes 0.8% or 0.46 mg/l $\text{NH}_3\text{-N}$ of the initial ammonium concentration. An alternative and perhaps better explanation of the ammonium loss may be attributed to uptake of ammonium by ammonia oxidizing bacteria without subsequent nitrite formation. Schmidt et al. reported that starving *Nitrosomonas* cells rapidly take up and accumulate ammonium/ammonia without simultaneous nitrite formation [77]. Based on the results of Experiment 1, it appears that the autotrophic nitrifying bacteria in the experimental reactor were converting the ammonium due to the elevated pCO_2 . In the control reactor, the autotrophic nitrifying bacteria were able to accumulate ammonium in the cell, but were unable to convert the ammonium to nitrite because of carbon limitation.

3.6.2 Nitrification in Activated Sludge Systems

These experimental results are consistent with the findings of other researchers, which have found a positive effect of elevated pCO_2 on nitrification rates and in

the specific growth rate of nitrifiers [71, 72, 78-80]. Although nitrate formation rates were not reported by these researchers, observed growth rates based on the increase of $\text{NO}_x\text{-N}$ concentration were reported to be approximately three times higher (1.5% CO_2 vs. 0% CO_2) after two hours of operation, which is similar to results from Experiments 3 and 4 [71]. Additionally, Denecke and Liebig [71] reported that the specific growth rate (μ_{obs}) of mixed autotrophic and heterotrophic sludge increased by 20% when the pCO_2 was elevated to approximately 1%. Other authors also suggested a positive impact of elevated pCO_2 on the specific growth rates of nitrifying bacteria [78, 81].

The role of pH was not evaluated on the nitrate formation rate in Experiments 2-4, however, it is important to consider. The average pH for the experimental and control reactors were 8.03 (s.d. 0.24) and 8.57 (s.d. 0.02), respectively, which are slightly higher than the optimal range of 7.5 – 8.0 [2]. Although the specific growth rate of microorganisms is sensitive to pH, it is difficult to attribute the substantial increase in nitrate formation rates to a half-unit difference in pH especially when considering the results from Experiment 1.

The results of all four experiments demonstrate a positive effect of elevated pCO_2 on nitrate formation rates. The results from Experiments 3 and 4 suggest that CO_2 -sensitive nitrifying bacteria require adequate acclimation periods for low, target SRT operation, which will result in consistently higher rates of nitrification. Furthermore, the rapid improvement in the nitrate formation rates at the

beginning of Experiments 2 - 4 suggest that the CO₂-sensitive nitrifying bacteria are not exotic, but are commonly found in full-scale activated sludge systems. Molecular biology based methods, such as fluorescence *in situ* hybridizations (FISH), may be useful in identifying these CO₂-sensitive nitrifying bacteria.

Finally, it is unknown whether elevated pCO₂ may increase the specific growth rates of other autotrophic bacteria that are of importance in wastewater treatment, such as the ANAMMOX bacteria [82, 83]. These results suggest that the investigation of the effect of elevated pCO₂ on these autotrophic bacteria may prove to be beneficial.

3.7 Conclusions

The experimental results suggest that supplying elevated pCO₂ in the aeration basin of an activated sludge system may significantly increase the nitrification rate. The primary cause of the higher nitrification rates was determined to be the elevated pCO₂ and not the pH depression caused by increasing the pCO₂. These findings also challenge the notion that nitrification is a slow process and the recommendations of a lengthy SRT for adequate nitrification in activated sludge systems. This is significant, since it suggests that nitrification in full-scale activated sludge systems may be improved by providing elevated pCO₂ to a portion of the aeration basin. In addition, this strategy may provide additional flexibility for operation with respect to the SRT.

Chapter 4

Evaluation of Nitrifying Bacteria Specific Growth Rate Sensitivity to Carbon Dioxide for Full-Scale Activated Sludge and Municipal Wastewater

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4.1 Abstract

Biological ammonia removal in wastewater treatment plants is a slow process. It has been theorized that the dissolved CO₂ concentration and pH are important parameters in optimizing the specific growth rate of nitrifying bacteria. Five wastewater treatment plants (WWTP) representing the three major plant configurations, extended aeration (EA), Modified Ludzack-Ettinger (MLE), and

Bardenpho, were evaluated based upon their operating conditions and activated sludge properties. The specific growth rates of the nitrifying bacteria were calculated for field and optimal conditions for pH and dissolved CO₂ concentrations and suggest potential for improvement. Evaluation of nitrification in activated sludge at defined dissolved CO₂ concentrations and constant pH 7 verified these findings. Fluorescence *in situ* hybridizations (FISH) were used to determine the abundance of nitrifying bacteria populations in the activated sludge from each WWTP and lab-scale reactors. Changes in the community structure of the nitrifying bacteria suggest sensitivity to dissolved CO₂.

4.2 Keywords

Nitrification, CO₂, pH, Wastewater, FISH

4.3 Introduction

Nitrification is the first step for the removal of nitrogen from wastewater, where ammonium (NH₄⁺) is oxidized to nitrate (NO₃⁻) by aerobic, autotrophic, nitrifying bacteria. These bacteria are thought to have slow growth rates and are sensitive to pH and temperature swings, making nitrification difficult to maintain in activated sludge systems [14, 15]. The slow growth rate and associated nitrification rate requires a lengthy solids retention time (SRT), as much as 20 days. Previous work has demonstrated that the growth of some autotrophic bacteria is carbon limited [69-71]. Inorganic carbon was found to be a limiting

factor in biological nutrient removal (BNR) systems due to the low partial pressure of carbon dioxide ($p\text{CO}_2$) of the atmospheric air introduced, and the loss of CO_2 by stripping [72]. These factors were reported to limit the bulk concentration of CO_2 in wastewater and consequently affect nitrification. This paper evaluates the effect of elevated $p\text{CO}_2$ on the specific growth rate of nitrifying bacteria using activated sludge from three different types of BNR processes: extended-aeration, Modified Ludzack-Ettinger (MLE), and Bardenpho [3].

4.4 Methodology

4.4.1 Field Evaluation of Nitrification in Three BNR Systems

Five wastewater treatment plants (WWTP) representing the three major biological nutrients removal (BNR) configurations, were evaluated in this study that include an Extended Aeration, two MLE, 4-stage Bardenpho, and 5-stage Bardenpho. Dissolved CO_2 and pH were measured in each unit operation where dissolved CO_2 would be present. Dissolved CO_2 measurements were collected with the OxyGuard CO_2 meter. All pH values in the field were measured with an Oakton pH Tester 10. Field measurements were collected during June and July 2009. All pH values in the laboratory were measured with an Oakton model 510 pH meter.

4.4.2 pH vs. Dissolved CO₂

An activated sludge sample was collected from the aeration basin of each WWTP evaluated. Within one hour of collection, the sample was evaluated in the laboratory to determine the pH at varying dissolved CO₂ concentrations. The sample was placed in a one liter beaker in a sealed desiccant cabinet and air or an air/CO₂ mixture was introduced into the cabinet. An air pump inside the cabinet subsequently introduced the atmosphere into the beaker. The atmosphere was maintained for a minimum of 15 minutes at which time dissolved CO₂ and pH were measured.

4.4.3 Specific Growth Rate Measurement in Lab-Scale Bioreactors

The experiments were conducted based upon previously published guidelines [21]. Two 3 liter beakers were used for the reactors. The control reactor utilized air, while the experimental reactor was aerated with a mixture of air and pure CO₂ to produce dissolved CO₂ concentrations of 12 and 103 mg/l. The pH was maintained between 7.0±0.05 through the addition of a phosphate buffer. Each reactor received identical phosphate buffer additions.

Both reactors were fed influent from the MLE #1 WWTP. A series of preliminary experiments were conducted to establish appropriate operating conditions. Based on these results, 60mg/l of NH₄⁺-N was added to the influent wastewater

which contained, on average, 25 mg/l of NH_4^+ -N. The dissolved oxygen was held constant at 8.3 mg/l as O_2 , which ensured that oxygen was not limiting. Each reactor had an initial addition of 0.5 grams of sodium bicarbonate with 0.5 gram additions during the reaction sequence based on NH_4^+ -N conversion.

Each reactor was inoculated with activated sludge that was collected from the aeration basin of the MLE #1's activated sludge system on the same day that the experiment was initiated. A MLVSS target value of 35 mg/l was specified in these experiments. Throughout the experiment, NH_4^+ , NO_2^- , NO_3^- , pH, and dissolved oxygen (DO) were routinely measured. A non-linear regression model was used to regresses the NO_x^- concentration levels ($\text{NO}_2^- + \text{NO}_3^-$) versus time. An estimate the maximum specific growth rate, μ , of the nitrifying bacteria was calculated using a non-linear regression software package (Oakdale Engineering, Oakdale, PA.).

4.4.4 Estimation of Specific Growth Rate of Nitrifying Bacteria

Growth rate optimization was based on Monod kinetics. An Andrew's equation was used to determine the effect of the dissolved CO_2 concentration on the specific growth rate [30]. The pH sensitivity of the specific growth rate was calculated by using an optimal pH of 8 as reported optimum values range from 7.5 to 8.5 [2]. Specific growth rate optimization was based on results previously reported [71]. The parameters and coefficients are provided in Table 4-1.

Table 4-1: Constants Used to Calculate the Optimal Specific Growth Rate for Nitrifying Bacteria

Constant	Value
K _{CO2} , mg/l	0.5
K _i , mg/l	42
K ₁ for pH	1.58E-07
K ₂ for pH	6.31E-10
μ _{max}	0.75
b	0.1
pH Term Max	0.88
CO ₂ Term Max	0.82

The formula to determine the field and optimum specific growth rate of the nitrifying bacteria is provided:

$$\mu_{obs} = \mu_{max} \cdot \frac{\frac{[CO_2]}{[CO_2] + K_s + \frac{[CO_2]^2}{K_i}}}{CO_2 \text{ Term Max}} \cdot \frac{1}{\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right)} - b$$

The CO₂ term max is the value obtained at a dissolved CO₂ value of 5 mg/l. The pH term max is the value obtained at a pH of 8. These values are used to normalize the formula by using the maximum specific growth rate for ideal dissolved CO₂ concentration and pH. Denecke reported that a 5 mg/l dissolved CO₂ concentration is equivalent to 0.4% CO₂. When calculated using Henry's constant, 0.4% equates to 6.89 mg/l. For purposes of this study, 5 mg/l was used as the optimum CO₂ concentration. Field pH measurements used in this study were calculated from activated sludge evaluated at varying levels of CO₂ concentrations in the laboratory. Although actual field measurements are

reported later in this paper, there was concern as to how well they represented actual pH values at the specified dissolved CO₂ concentrations.

4.4.5 Evaluation of Nitrifying Bacteria Abundance by Fluorescence *in situ* Hybridization

Four fluorescently-labeled oligonucleotide hybridization probes, that target two ammonia oxidizing bacteria (AOB) and nitrifying oxidizing bacteria (NOB) groups were used in this study (Table 4-2) and were synthesized and conjugated with the cyanine dye, Cy3, before purification with oligonucleotide probe purification cartridges. Fluorescently labeled probes were diluted to 50 ng/μl with RNase-free water and stored at -20°C in the dark. Samples (1 ml) were collected from the aeration basin from each WWTP and fixed with 1 ml of 4% PFA for 12-24 hours. The samples were centrifuged and supernatant decanted, and suspended in 2 mL of ethanol PBS (EtOH-PBS). The samples were stored at -20°C until further analysis. Fixed samples were applied to a sample well on a 10 well Heavy Teflon Coated microscope slide (Cel-Line Associates, New Field, NJ) and air-dried. After dehydration with an increasing ethanol series (50, 80, 95% [vol/vol] ethanol, 1 min each), each sample well was covered with a mixture of 18 μl of hybridization buffer (20 % [vol/vol] formamide, 0.9 M NaCl, 100 mM TrisHCl [pH 7.0], 0.1% SDS) [84] and 2 μl of the stock fluorescently labeled oligonucleotide probe. The hybridizations were conducted in a moisture chamber containing excess hybridization buffer (to prevent dehydration of buffer on

sample wells) for 1.5 h, in the dark, at 46°C. The slides were washed for 30 min at 48°C with 50 ml of pre-warmed washing buffer solution (215 mM NaCl, 20 mM TrisHCl [pH 7.0], 0.1% SDS, and 5 mM EDTA) [84]. Fixed, hybridized cells were mounted with Type FF immersion oil (Cargille, Cedar Grove, NJ) and a cover slip. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 1 µg/ml for 1 minute and rinsed with DI water.

Table 4-2: FISH Probe Information

	Probe	Targeted bacteria	Reference
AOB	NSM156	<i>Nitrosomonas</i> spp., <i>Nitrosococcus mobilis</i>	[14]
	Nsv433	<i>Nitrospira</i> spp.	[14]
NOB	NIT3	<i>Nitrobacter</i> spp.	[15]
	Ntspa0712	most members of the phylum <i>Nitrospirae</i>	[85]

Whole cell fluorescence was visualized with an upright epifluorescence microscope (Leitz DiaPlan, Heerbrugg, Switzerland), and digital images were captured using a Spot-FLEX charge coupled device (CCD) camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were collected using a 100X oil objective and constant exposure time of 1.2 sec and gain of 2. For each FISH probe, ten images were collected for each sample and analyzed based on the relative abundance of Cy3 fluorescent cells. Direct measurement of abundance was difficult due to the background fluorescence of the samples, thus a simple scale (Figure 4-1) was used to estimate the abundance. The value of each set of images was totaled and averaged.

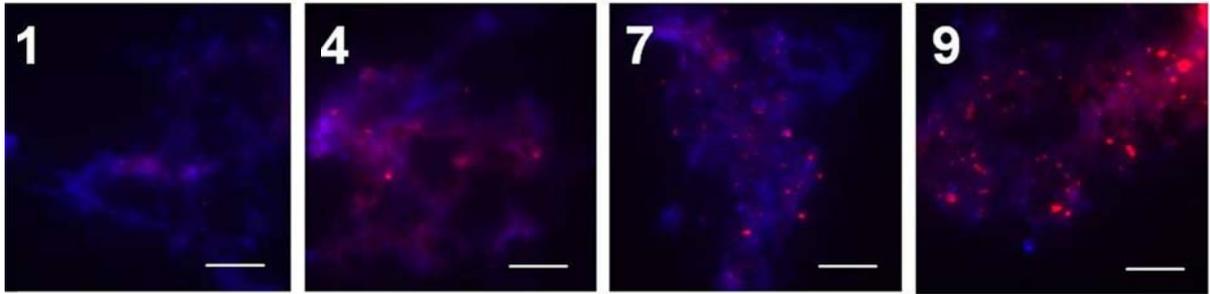


Figure 4-1: FISH Analysis Scale Representation. Scale bars equal 10 μm

4.5 Results

4.5.1 Field Evaluation of Three BNR Systems

An analysis of the three major types of wastewater treatment plant (WWTP) configurations was evaluated based on the dissolved CO_2 and pH of the influent, unit processes, and effluent (Table 4-3) and influent properties and operating conditions (Table 4-4). Dissolved CO_2 concentration and pH were the parameters of primary interest. The dissolved CO_2 values are representative of the measurements for the different unit processes. The pH values were determined by obtaining surface samples, which may not be representative for the particular unit process. As an example, the anoxic zone for the MLE #1 facility provided results ranging from 26 to 58 mg/l of dissolved CO_2 in its basin, where the probe was inserted 8-10 feet below the surface. Although the pH was reported as 7.35, we expected a pH of 6.7-7.0. The 4-stage Bardenpho process uses magnesium hydroxide at their lift stations to negate the effects of hydrogen sulfide which causes odor problems. This would account for the elevated influent

pH at this facility. Only the MLE #1 WWTP received anaerobic sludge brought in from other sources.

Table 4-3: Dissolved CO₂ Concentration and pH of Influent, Unit Processes, and Effluent of Five Wastewater Treatment Plants

	Extended Aeration		MLE #1		MLE #2		4-Stage Bardenpho		5-Stage Bardenpho	
	CO ₂ mg/l	pH	CO ₂ mg/l	pH	CO ₂ mg/l	pH	CO ₂ mg/l	pH	CO ₂ mg/l	pH
Influent	31	6.5	17	7.4	12	7.4	6	7.9	20	7.1
% Domestic	100		95		81		100		95	
1 ^o Clarifier	N/A	N/A	9	7.6	29	6.9	N/A	N/A	N/A	N/A
Zone 1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	31	7.0
Zone 2 (ANX)	24	6.7	26-58	7.35 ^a	23-24	7.2	11	7.3	20	7.1
Aeration	13.5 ^a	6.8	34	6.9	15-24	7.3 ^a	12	7.3	16	7.1
Zone 4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	22	7.0
2 nd Aeration	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	23	7.0
2 ^o Clarifier	12	6.9	23	7.1	23	7.3	11	7.4	19	7.2
Post-Filtration	N/A	N/A	16	7.3	N/A	N/A	6	7.5	12	7.4
Effluent	9	7.0	16	7.3	12	7.4	6	7.7	10	6.9

^a the average of several measurements

N/A: unit processes are not part of the configuration or were not in use.

Large differences in the influent dissolved CO₂ concentrations were observed among the WWTP. The influent of the extended aeration plant had a high dissolved CO₂ level but receives its influent through a large collection system where anaerobic conditions are quite probable and lead to these high readings. The 4-stage Bardenpho process, which has a low dissolved CO₂ concentration, is located in a residential community with a limited collection system. Little time is afforded for the influent to reach anaerobic conditions.

The MLE #2 exhibited a lower influent dissolved CO₂ concentration than observed in the primary clarifier. This WWTP is fed by a large underground piping system which suggests that anaerobic conditions are possible. On the day of the plant visit, a thunderstorm was in-progress and had increased the influent rate by 30 percent during the last hour. A diluted CO₂ influent concentration was recorded, while the primary clarifier had probably not seen the full effect of this dilution. In addition, the primary clarifier is a covered and sealed tank, which may promote anaerobic activity.

The influence of the WWTP configuration is readily seen in the dissolved CO₂ concentration of the aeration basins. The dissolved CO₂ concentration in the anoxic basin is influenced by the mixture of the influent, internal recycled wastewater, and RAS combined with generation of dissolved CO₂ by denitrification. The 5-stage Bardenpho system has the additional contribution of dissolved CO₂ from the anaerobic treatment basin. This treated wastewater enters the aeration basin with an elevated dissolved CO₂ concentration that ranges from 11 to 58 mg/l. In the aeration basin, dissolved CO₂ is produced through the metabolism of the carbonaceous BOD by the heterotrophic bacteria, but dissolved CO₂ is also removed by stripping due to the intensive aeration.

The dissolved CO₂ concentration and pH were measured in unit processes beyond the activated sludge system. All WWTP are discharging final effluent with elevated dissolved CO₂ concentrations when compared to the dissolved CO₂

concentration of water in equilibrium with the atmosphere (0.6 mg/l). The elevated level of dissolved CO₂ is not surprising since the terminal unit processes do not provide adequate stripping.

Table 4-4: Influent Properties and Activated Sludge Operating Conditions for Five Wastewater Treatment Plants

Property	units	Extended Aeration	MLE #1	MLE #2	4-Stage Bardenpho	5-Stage Bardenpho
BOD	mg/l	300	200	550	207	200
NH ₄ ⁺ -N	mg/l	25	28	25	35	31
COD	mg/l	587	N/A	1,250	N/A	N/A
MLSS	mg/l	3,190	2,900	4,092	2,815	3,200
MLVSS	mg/l	2,490	2,320	3,384	2,252	2,240
SRT	days	17	12	9	25.9	15
Aeration DO	mg/l	1-3	2-5	1.5-3	0.8-1.2	0.4

N/A: Not available.

MLE #2 has the lowest domestic wastewater percentage of all the plants evaluated. It services major food processing industries as indicated by its high influent BOD and COD, which requires an elevated solids concentration (MLSS) to ensure proper treatment.

The dissolved oxygen (DO) concentrations are markedly different among the WWTPs. The extended aeration and the MLE plants show expected DO levels typically encountered at wastewater facilities. The Bardenpho processes utilize reduced DO levels to achieve their BOD and ammonia conversions as higher DO concentrations interfere with conversion in their anoxic and anaerobic zones.

4.5.2 Estimation of Specific Growth Rate of Nitrifying Bacteria

A sample of activated sludge from the aeration basin of each process was obtained and evaluated at different dissolved CO₂ concentrations (Figure 4-2). The numbers in the figure represent the dissolved CO₂ concentrations in the aeration basin for the WWTP.

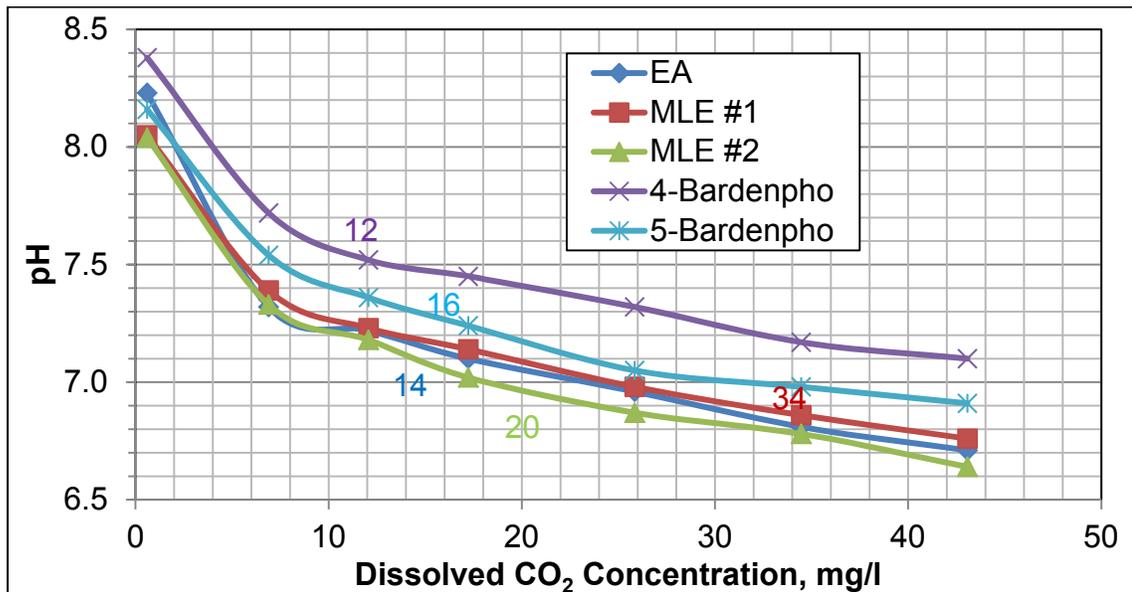


Figure 4-2: Effect of pH at Varying Dissolved CO₂ Concentrations

Results show a general downward trend (lower pH) with increasing levels of CO₂. Although different configuration types appear to segregate, this difference maybe more related to their MLVSS concentrations.

Each WWTP was further evaluated to determine the potential for increasing the specific growth rate of the nitrifying bacteria by optimizing the dissolved CO₂ concentration and allowing for pH adjustment. Our results suggest that improvements are possible for each WWTP evaluated in this study with the MLE

facilities offering the greatest potential (Table 4-5). The Bardenpho processes offer less potential for improvement due to the low dissolved CO₂ concentrations and higher operating pH values, which are near the optimum levels.

Table 4-5: Optimum Specific Growth Rate of Nitrifying Bacteria for Optimal Dissolved CO₂ Concentration of 5 mg/l and Corresponding pH

Properties	Extended Aeration	MLE #1	MLE #2	4-Stage Bardenpho	5-Stage Bardenpho
CO ₂ , field	14	34	20	12	16
pH, field	7.17	6.92	7.01	7.57	7.26
pH, corresponding to optimal CO ₂	7.54	7.56	7.51	7.89	7.7
μ, observed	0.4238	0.22	0.3226	0.5501	0.4368
μ, optimum	0.6016	0.6058	0.595	0.6473	0.6297
% Improvement	42%	175%	84%	18%	44%

4.5.3 Evaluation of the Specific Growth Rate of Nitrifying Bacteria Sensitivity to Dissolved CO₂ Concentration using Lab-Scale Bioreactors

An initial study of the effect of dissolved CO₂ concentration on the specific growth rate of nitrifying bacteria was conducted using activated sludge from the extended aeration facility. The results of an analysis with pH 7.0 and CO₂ concentration at 7 mg/l versus air are provided (Figure 4-3). The selection of the 7 mg/l dissolved CO₂ (0.4%) concentration was based on previous research [71].

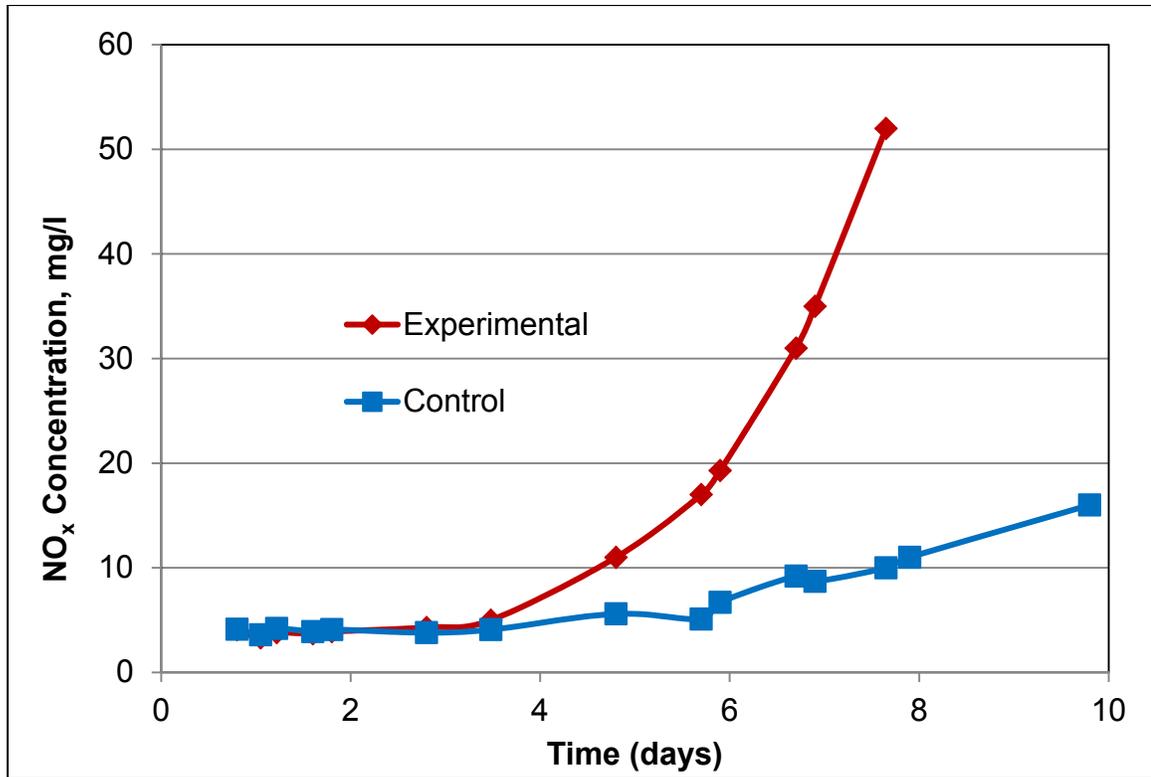


Figure 4-3: Evaluation of Specific Growth Rate of Nitrifying Bacteria Using Air (Control) or 7 mg/l (Experimental) Dissolved CO₂ Concentration

Both reactors display a buildup of NO_x⁻ concentration (NO₂⁻ and NO₃⁻) over a 10 day period. However, it is evident that the rate of NO_x⁻ concentration buildup is significantly higher in the experimental reactor. The specific growth rate of the nitrifying bacteria was estimated by fitting the non-linear response. The maximum specific growth rate, μ_{max} , for both conditions and the associated 95% confidence interval are provided (Table 4-6). The regression analysis was conducted to NO_x values of approximately 20 mg/l. Inhibition effects were observed at values greater than this concentration (data not shown).

Table 4-6: Estimated Specific Growth Rate of Nitrifying Bacteria and 95% Confidence Interval of the Activated Sludge from the WWTP with Extended Aeration for Two Defined Dissolved CO₂ Concentrations

Reactor	Dissolved CO ₂ (mg/l)	μ (days ⁻¹)	Lower Limit	Upper Limit
Control	0.6	0.578	0.479	0.677
Experimental	7	1.011	0.802	1.219

Further research was conducted using activated sludge from the WWTP with MLE #1. The sludge was evaluated at varying levels of pCO₂ from 7 to 17 mg/l at a constant pH of 7. An optimum specific growth rate of 0.84 days⁻¹ was achieved at a dissolved CO₂ of 12 mg/l.

4.5.4 Evaluation of Nitrifying Bacteria by Fluorescence *in situ* Hybridization

Representative FISH images for the samples collected from the MLE #1 and the 4-stage Bardenpho are provided in Figures 4-4 and 4-5. Individual cells and small clusters of cells are present in the flocs for each of the major ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Frequent background fluorescence made enumeration difficult, which required a more qualitative approach that utilized a relative abundance scale (Figure 4-1).

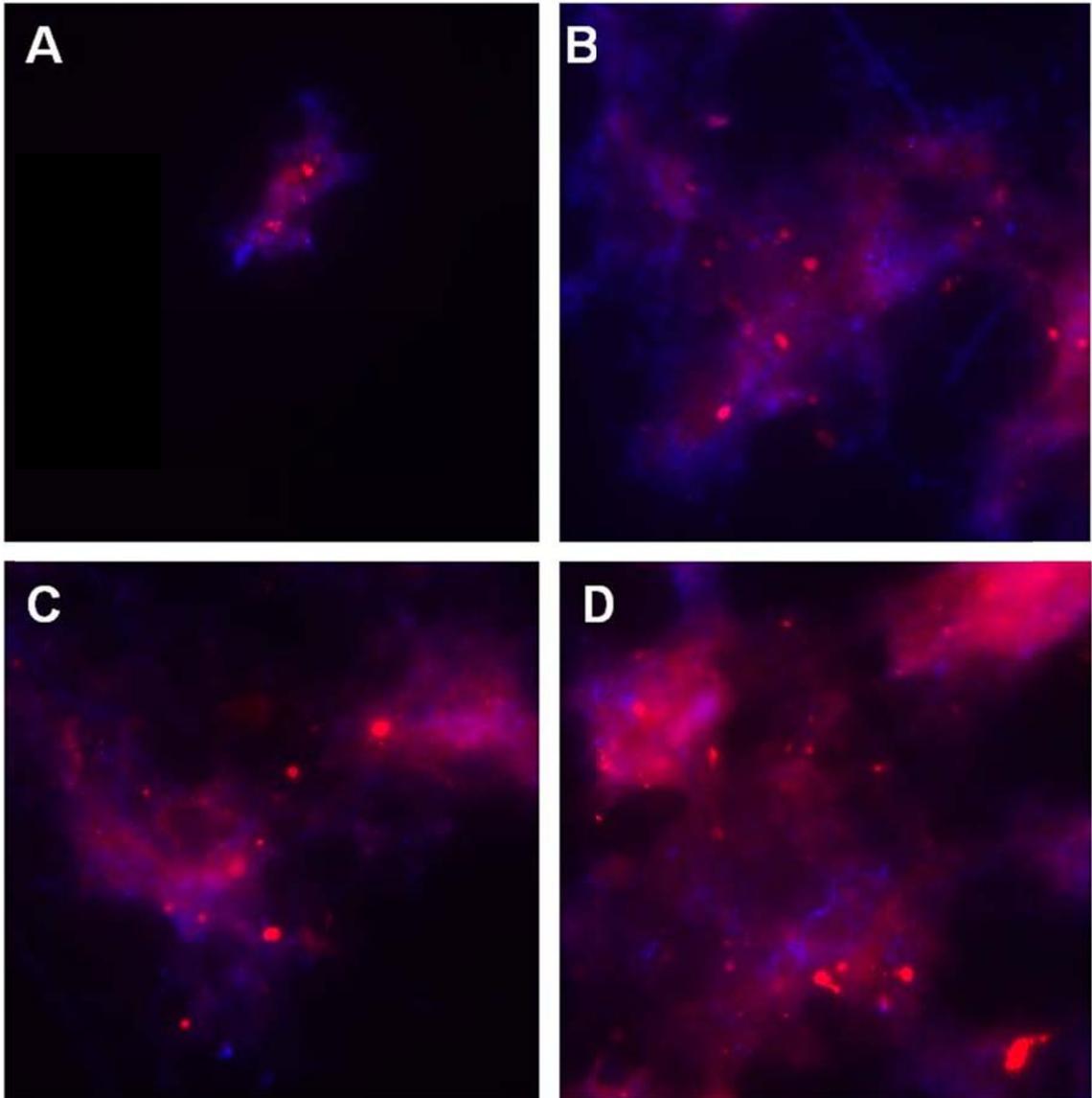


Figure 4-4: Representative FISH Images for Nitrifying Bacteria in MLE #1 including (A) *Nitrosomonas* spp., *Nitrosococcus mobilis*; (B) *Nitrosospira* spp.; (C) *Nitrobacter* spp. and (D) most members of the phylum *Nitrospirae*

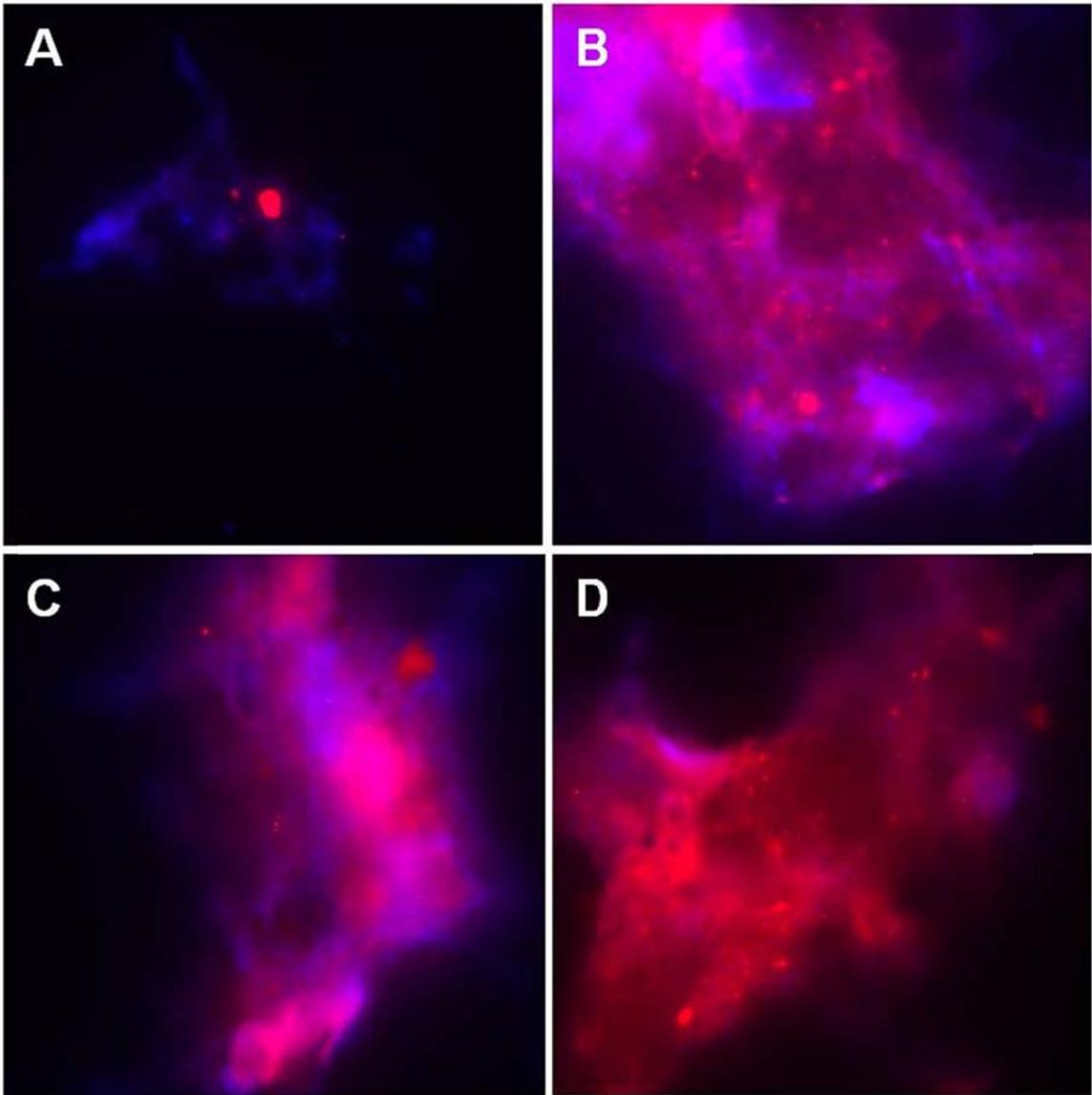


Figure 4-5: Representative FISH Images for 4-Stage Bardenpho including (A) *Nitrosomonas* spp., *Nitrosococcus mobilis*; (B) *Nitrospira* spp.; (C) *Nitrobacter* spp. and (D) most members of the phylum *Nitrospirae*

Analysis of the digital FISH images using the relative abundance scale is provided (Table 4-7). These values show a strong presence of each major AOB and NOB in each WWTP. The extended aeration system appears to have the lowest abundance of AOB and NOB compared to the other WWTPs, although it appears to have a similar community structure to the 5-stage Bardenpho. The

two MLE samples have similar NOB community structure; however the AOB appear to have some differences.

Our attempts to alter the specific growth rate of the nitrifying bacteria by operation at extreme dissolved CO₂ concentrations of 12 and 103 mg/l produced interesting results. For optimal dissolved CO₂ concentration (12 mg/l), the AOB populations appear to be even, while the *Nitrospirae* spp. appears to dominate the *Nitrobacter* spp. amongst the NOB. For the extreme suboptimal dissolved CO₂ concentration (103 mg/l), the *Nitrosomonas* spp. dominate the *Nitrosospira* spp. for the AOB and the NOB populations are higher but more even compared to the field sample. When compared to each other, the abundance of the *Nitrosomonas* spp. and *Nitrospirae* spp. appear to be similar, while *Nitrosospira* spp. are much higher for the reactor operating under optimal CO₂ concentration and the *Nitrobacter* spp. are much higher for the reactor operating under suboptimal CO₂ concentration.

A careful review of the dissolved CO₂ and pH values suggest that the 4-stage Bardenpho system should be operating at near optimal conditions for nitrification. In this system, the dominant AOB appears to be the *Nitrosospira* spp. and the dominant NOB appears to be the *Nitrospirae* phylum. In contrast, the 5-stage Bardenpho system has a higher abundance of *Nitrosomonas* spp., but the *Nitrosospira* spp. is still dominant amongst the NOB. The members of the

phylum *Nitrospirae* are much lower relative to the 4-stage Bardenpho, while the *Nitrobacter* spp. is similar.

Table 4-7: FISH Analysis of Five WWTP and Lab-Scale Reactors Operated at Extreme Dissolved CO₂ Concentrations

		EA	MLE #1			MLE #2	4-Stage BP	5-Stage BP
			Field	12 mg/l CO ₂ *	103 mg/l CO ₂ *			
AOB	NSM156 <i>Nitrosomonas</i> spp., <i>Nitrosococcus mobilis</i>	2.40	3.30	4.90	5.00	5.60	1.00	3.50
	Nsv433 <i>Nitrosospira</i> spp.	4.50	5.60	4.80	3.30	4.50	5.00	5.40
NOB	NIT3 <i>Nitrobacter</i> spp.	2.73	7.20	4.00	6.20	6.73	2.80	2.44
	Ntspa717 most members of the phylum <i>Nitrospirae</i>	1.90	5.80	6.00	6.80	5.50	5.00	1.70

* pH 7

4.6 Discussion

One important finding in this study is the high concentration of dissolved CO₂ in the aeration basins and other unit processes. Significant differences are evident and upon investigation are quite plausible. As an example, the aeration system on an MLE process uses three anoxic and four aerobic zones in a carousel arrangement to convert BOD and ammonia. A mixture of influent, RAS, and internal recycle from the aeration basin enter the anoxic basin, where

denitrification generates additional dissolved CO₂ as a by-product. This treated wastewater with a high level of dissolved CO₂ then flows into the aeration basin where additional dissolved CO₂ is generated with minimal stripping. Evidence of the impact of anoxic treatment and minimal CO₂ stripping are observed in the MLE and Bardenpho systems. Plant influent also impacts the dissolved CO₂ concentration in the aeration basin and appears to be a function of the influent quality and collection system. Finally, the dissolved CO₂ concentration in the effluent is much higher than expected, when you consider that water in equilibrium with the atmosphere has a CO₂ concentration of 0.6 mg/l. It is unknown whether this elevated dissolved CO₂ concentration negatively impacts receiving water by providing a carbon source for the growth of algae and cyanobacteria.

Evaluation of the activated sludge from the WWTPs with Extended Aeration and MLE #1 showed differences in the specific growth rates of the nitrifying bacteria when the dissolved CO₂ concentration was optimized. The EA facility achieved a maximum growth rate at 7 mg/l CO₂ while the MLE #1 facility achieved a maximum growth rate at 12 mg/l, which are both near the optimal dissolved CO₂ concentration reported previously [71]. The community structure of the nitrifying bacteria in the activated sludge is expected to have a significant influence on the optimal dissolved CO₂ concentration. It should be noted that pH was held constant at 7 and optimization of the dissolved CO₂ concentration will increase the pH (Figure 4-2).

The FISH results indicate differences in the community structure of the nitrifying bacteria amongst the WWTPs. Each facility appears to have its own established community of nitrifying bacteria. These results show that several AOB and NOB bacteria coexist in the same system, which is similar to a previous study [23]. The four stage Bardenpho process, which operates near the ideal dissolved CO₂ concentration, shows a dominance of one AOB (*Nitrosospira* spp.) and NOB (phylum *Nitrospirae*). Due to its long SRT of nearly 26 days, the presence of other microbes is not unexpected. This suggests that as a process approaches the ideal dissolved CO₂ concentration for the growth of nitrifying bacteria, the community structure may become less diverse.

The differences in the observed presence of microbes among the WWTPs as seen in the FISH analysis have one distinct possible cause (Table 4-7). The community structure of the nitrifying bacteria may simply be different due to the influent variability. This is evident in observing the differences in the contribution of domestic wastewater in the influent between the plants. MLE #1 and MLE #2 have distinct variability in their AOB and NOB concentrations despite having essentially the same configuration and operational parameters. MLE #1 has a very low contribution of industrial wastewater, but is more diverse in the type of industrial wastewater it receives. MLE #2 has a large contribution of industrial wastewater, but consists mainly of wastewater from food processors as indicated by the high average BOD concentration.

FISH was used to investigate the nitrifying bacteria in lab-scale bioreactor experiments, which were conducted at dissolved CO₂ concentrations of 12 and 103 mg/l at a pH of 7.0. Compared to the seed material (MLE #1), the community structure of the nitrifying bacteria changed dramatically in unanticipated ways. Surprisingly, similar levels of *Nitrosomonas* spp. and *Nitrospirae* members were observed for both extreme dissolved CO₂ concentrations. However, levels of *Nitrosospira* spp. were much greater for the optimal dissolved CO₂ concentration and levels of *Nitrobacter* spp. were much greater for the suboptimal dissolved CO₂ concentration. In our attempts to provide optimal conditions for nitrification for the MLE #1 sludge, we were unable to produce a community structure of the nitrifying bacteria that was similar to the 4-stage Bardenpho. There may be several explanations for this failure. First, failure may be attributed to vastly different nitrifying bacteria in both samples, which would make it impossible to achieve this dominance of AOB and NOB populations present in the 4-stage Bardenpho. Second, it may be due to a lack of a wasting operation, which would remove slow-growing nitrifying bacteria. Third, we may be underestimating the difference in the effect of the influent wastewater properties. Fourth, we may be experiencing a pH effect, since the ideal dissolved CO₂ concentration increases the pH of the activated sludge to 7.56, which is more than half a pH unit above the lab-scale bioreactor experiment.

4.7 Conclusions

The dissolved CO₂ concentration in the influent, unit processes, and effluent of the five WWTPs evaluated in this study proved to be quite different. The dissolved CO₂ concentration in the aeration basin was a function of the influent dissolved CO₂ concentration, generation of dissolved CO₂ through denitrification in the anoxic basin and fermentation in the anaerobic basin, dissolved CO₂ concentration of both internal recycled wastewater and RAS, heterotrophic conversion of carbonaceous BOD to CO₂ in the aeration basin, and limited CO₂ stripping in the aeration basin. The microbial ecology of the nitrifying bacteria of the plants appears to be plant specific, but commonalities are evident. Further research is planned to optimize the conditions for nitrification for each type of process and to evaluate the microbial ecology of the nitrifying bacteria for those conditions.

Chapter 5

Determination of the Relationship of Dissolved CO₂ Concentration and pH and a Design Space for Optimum Nitrification

Based on the field study results as reported in chapter 4, a series of designed experiments were conducted to ascertain if an optimum dissolved CO₂ concentration/pH condition exists that maximizes specific growth rate. Experiment one was conducted to determine the effect of varying concentrations of dissolved CO₂ at a constant pH of 7.0. Experiment 2 was conducted at varying concentrations of dissolved CO₂ at specific pH levels that coincide with sludge from a WWTP. Experiment three was conducted at varying concentrations of dissolved CO₂ and pH to determine a design space for optimum nitrification.

5.1 Methodology and Materials

Three experiments were conducted to determine the maximum specific growth rate of the microbes at varying levels of dissolved CO₂ concentrations. The experiments were conducted based upon previously published guidelines [21]. In experiments 1 and 2, six one liter beakers were used for the batch reactors and were filled to 800 ml using influent from a commercial wastewater facility.

The dissolved CO₂ concentrations fed to the batch reactors in the first experiment were 134, 61, 29, 12, 7 and 2 mg/l, respectively. In the second experiment, the dissolved CO₂ concentrations were 34, 25, 19, 16, 12 and 8, respectively. In the third experiment, 12 one liter beakers were used for the batch reactors and were filled to 800 ml using influent from a commercial wastewater facility with dissolved CO₂ concentrations maintained at 5, 10 and 15 mg/l, respectively. Deionized water from a reverse osmosis system was used to replenish water in the reactors during the experiment.

Establishment of the CO₂ percentages was conducted using a dissolved CO₂ meter (OxyGuard CO₂ Portable Analyzer). The measured dissolved CO₂ was compared to the theoretical dissolved value based on Henry's constant. An R² of 0.9978 was achieved.

A series of preliminary experiments were conducted to establish appropriate operating conditions. Based on these results, 60 mg/l of NH₄⁺-N was used as the sole nitrogen source and added to influent waste water from a commercial waste water treatment facility. A MLE process was selected. The dissolved oxygen concentration was constant at 8.2 mg/l as O₂, which ensured that oxygen was not limiting. Alkalinity was maintained in all experiments at approximately 250 mg/l as CaCO₃.

In experiment 1, each reactor had an initial addition of 0.2 grams of sodium bicarbonate with 0.1 gram additions at 94 hours for a total of 0.3 grams. The pH was maintained between 6.95 and 7.05 through the addition of a phosphate buffer. Three phosphate buffers with pH values of 9.1, 7.0, and 4.4 were prepared with $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. Each reactor received identical phosphate buffer additions. The pH 7 buffer was used to equilibrate the total addition. For example, if the control reactor required 8 ml of the pH 4.4 buffer to reach pH 7.0 and the experimental reactor only required 5 ml of the same phosphate buffer, then an additional 3 ml of the pH 7.0 buffer was added to the experimental reactor to maintain the phosphate concentration. A total of 0.019 moles of phosphate buffer was added to each reactor during the course of the experiment.

In experiment 2, each reactor had an initial addition of 0.3 grams of sodium bicarbonate with no further additions. The pH was maintained at values appropriate for the dissolved CO_2 concentration. These values were determined by aerating a sample of activated sludge from the treatment facility used in this study and recording the pH value at varying levels of dissolved CO_2 concentration. A total of 0.011 moles of phosphate buffer was added to each reactor during the course of the experiment. Additional measurements were taken to minimize variation in the growth rate parameter.

In experiment 3, each reactor had an initial addition of 0.3 grams of sodium bicarbonate with further additions to maintain pH. The pH was maintained at 8.0, 7.5, 7.0 and 6.5. A total of 0.02 moles of phosphate buffer was added to each reactor during the course of the experiment. Additional measurements were taken to minimize variation in the growth rate parameter.

Six sealed desiccant cabinets were used to maintain the appropriate atmospheres. PVC tubing was used to connect the cabinets in series. Rena air pumps (Air 50, 2.0 watts) were used to introduce air into the cabinets. An Optima air pump (4.5 watts) was used in the first cabinet to ensure an adequate system air flow. A carbon dioxide sensor (COY laboratory products) was installed in the first cabinet to establish the initial pCO₂ atmosphere.

Each reactor was inoculated with an appropriate volume of mixed liquor suspended solids (MLSS), that was collected from the nitrification basin of a full-scale activated sludge system on the same day that the experiment was initiated (South Cross Bayou Water Reclamation Facility of the City of St. Petersburg, FL). These volumes were 14, 11 and 12 ml for experiment 1, 2 and 3, respectively. (The volumes were based upon the MLSS concentration on the day the sample was obtained.) Throughout the experiment, NH₄⁺, NO₂⁻, NO₃⁻, pH, and dissolved oxygen (DO) were periodically measured. Experiments were discontinued when the combined NO₂⁻ and NO₃⁻ concentrations totaled 30 mg/l or greater. This was done to negate inhibition effects.

Experimentation was conducted with an ammonia concentration in the reactor high enough (relative to the half velocity constant from Monod kinetics, K_s) to ensure that the nitrification rate is at a maximum [21]. Sixty mg/l of NH_4^+ was significantly greater than the K_s values reported for the ammonia oxidizers, 1.0, and the nitrite oxidizers, 1.3, at 20°C [12]. The growth rates were modeled using a non-linear regression equation as described in *Methods for Wastewater Characterization in Activated Sludge Modeling* [21]. The growth rate expression is provided:

$$S_{\text{NO}_x,t} = S_{\text{NO}_x,0} + \left(\frac{\mu_{\text{max}} \cdot X_{\text{AUT},0}}{Y_{\text{AUT}} \cdot (\mu_{\text{AUT}} - b_{\text{AUT}})} \right) \cdot (e^{(\mu_{\text{max}} - b_{\text{AUT}}) \cdot t} - 1)$$

The parameters $S_{\text{NO}_x,0}$, (oxidized nitrogen concentration at time zero), μ_{max} (maximum specific nitrifier growth rate) and $X_{\text{AUT},0}$ (initial nitrifier concentration) were calculated using this equation. Y_{AUT} (nitrifier yield coefficient) and b_{AUT} (nitrifier decay rate) were given values of 0.15 mg VSS/mg NH_4^+ and 0.15 days⁻¹, respectively. Software from Oakdale Engineering (Oakdale, PA) was used to conduct the non-linear regression modeling. Software from Minitab, Inc. (State College, PA) was used to analyze the experimental design and generate other statistics.

Microsoft Excel was used to estimate growth kinetics for both experiments and compared to findings published by Denecke. The following equation, which is based upon an Andrews's model [30], was used to estimate the kinetics:

$$\mu_{\text{obs}} = \mu_{\text{max}} \cdot \left(\frac{[\text{CO}_2]}{[\text{CO}_2] + K_s + \frac{[\text{CO}_2]^2}{K_i}} \right) \cdot \left(\frac{1}{\left(1 + \frac{[\text{H}^+]}{K_1} + \frac{K_2}{[\text{H}^+]} \right)} \right) - b$$

The parameters μ_{max} , K_s (saturation constant for substrate), K_i (inhibition constant), K_1 and K_2 are calculated using this equation. Specific growth rate (μ_{obs}), dissolved CO_2 concentration ($[\text{CO}_2]$), and proton concentration ($[\text{H}^+]$) were measured or specified during experimentation.

5.1.1 Data Collection and Sample Analyses

Measurements for experiment 1 were taken at least 3 times per day with a 4 hour time interval between measurements. Measurements for experiment 2 were taken approximately every one and one-half hour over 10-12 hours per day. Measurements for experiment 3 were taken every 1.5 hours over 18-20 hours per day. Holes were drilled into the top of the cabinets where rubber stoppers were installed. During measurement taking, electrodes were lowered through the holes and placed into the reactors. This was done to minimize atmospheric loss. The electrode wires were encapsulated in a rubber stopper cut to facilitate the wire. Stoppers were replaced after taking measurements. Instruments used for chemical measurements included: ion selective electrodes (Ammonium combination glass body electrode, Cole-Parmer® 27502-03 and Nitrate combination glass body electrode, Cole-Parmer® 27502-31, Cole-Parmer

Instrument Company), (Nitrite combination electrode (4230-A94, Thomas Scientific), Dissolved Oxygen Meter (Traceable* Portable Dissolved Oxygen Meter, Fisher Scientific), pH meter (pHTestr3+, Oakton Instruments) and ion meters (Oakton® Benchtop Ion 510 Meter and Oakton® Ion 6 Meters, Cole-Parmer Instrument Company). All instruments were calibrated daily before use. The ammonium electrode used a 0.1M NaCl filling solution (Cole Parmer® 27503-78 reference filling solution, Cole-Parmer Instrument Company) and was calibrated with a 1,000 mg/l NH_4^+ -N standard solution (prepared in the laboratory with reagent-grade NH_4Cl) and a 5M NaCl Ionic Strength Adjuster (ISA).

The nitrate electrode used a 0.1M $(\text{NH}_4)_2\text{SO}_4$ filling solution (Cole Parmer® 27503-79 reference filling solution, Cole-Parmer Instrument Company) and was calibrated with a 1,000 mg/l NO_3^- -N standard solution (prepared in the laboratory with reagent-grade NaNO_3) and a 1M NaSO_4 ISA prepared in the laboratory. The nitrite electrode was calibrated with a 1,000 mg/l NO_2^- -N standard solution (prepared in the laboratory with reagent-grade NaNO_2).

The nitrite combination electrode was found to be pH sensitive. Using a pH of 7.0 as the reference pH, a pH of 6.5 exhibited a 10 percent higher nitrite reading. A pH of 7.5 and 8.0 exhibited lower readings of 91 percent and 77 percent, respectively. Adjustments were made to the maximum specific growth rates results as these were based on NO_x ($\text{NO}_2^- + \text{NO}_3^-$) concentration. However, these adjustments had minor to no effect if the results were not corrected.

5.2 Results

5.2.1 Experiment 1

The results from experiment 1 compared the effect of dissolved CO₂ concentrations on nitrification rates at constant pH 7.0. In analyzing the results, the nitrite (NO₂⁻) and nitrate (NO₃⁻) concentrations were added together and then regressed against time in order to estimate the specific growth rate. The growth curve for a dissolved CO₂ concentration of 12 mg/l and at a pH of 7.0 using the Oakdale Engineering software is provided (Figure 5-1).

Concentrations of the NO₂⁻ ranged from 0.5 - 0.7 mg/l in the 7 - 103 mg/l dissolved CO₂ concentrations. The NO₂⁻ in the 2 mg/l dissolved CO₂ concentration ranged from 0.8 - 1.2 mg/l. These represented small concentrations compared to the nitrate, remained relatively constant, and did not accumulate over time. The concentrations of the NO₂⁻ and NO₃⁻ were regressed separately to ascertain differences from their combination (data not shown). No differences were noted.

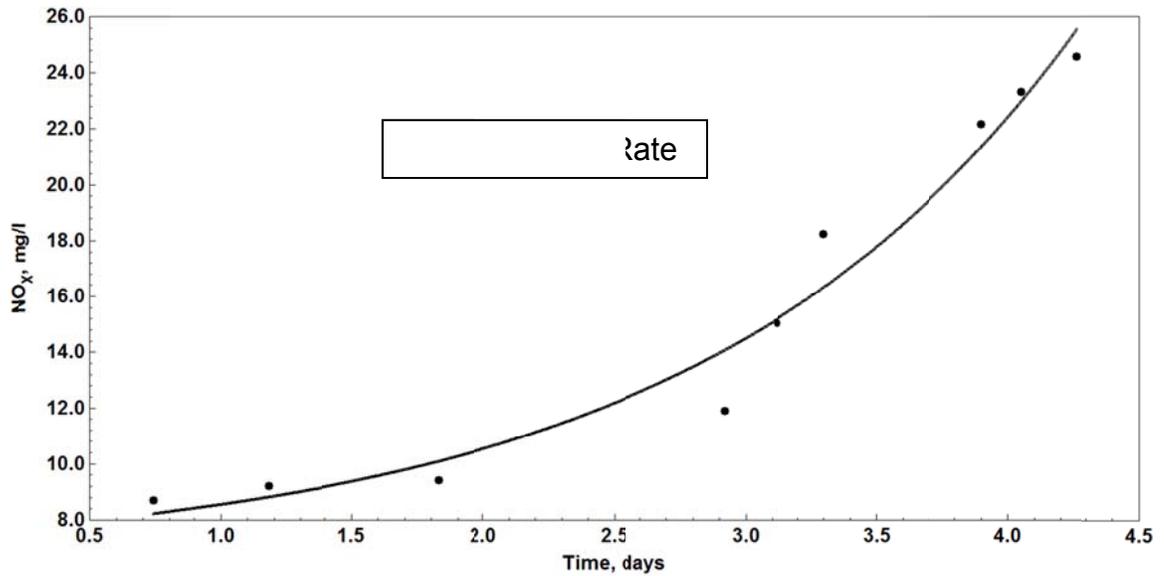


Figure 5-1: Estimated μ_{max} at 12 mg/l of Dissolved CO_2 Concentration and a pH of 7.0

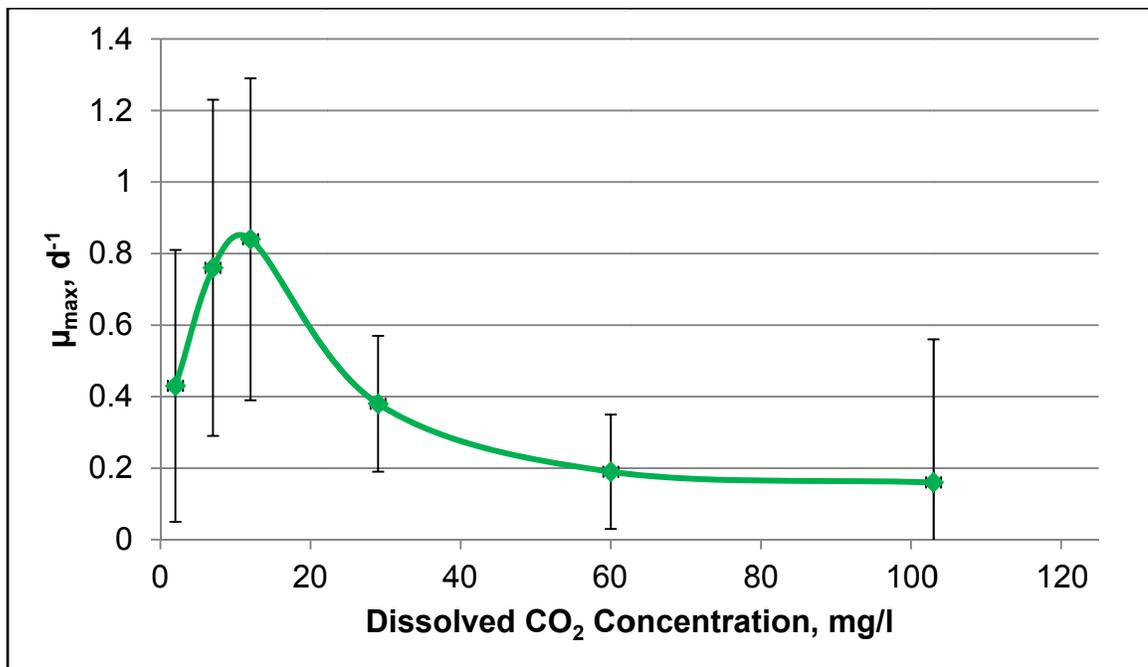


Figure 5-2: Experiment 1 Specific Growth Rate of Nitrifying Bacteria at Varying Levels of Dissolved CO_2 Concentration at pH 7.0

A maximum specific growth rate of 0.84 day^{-1} was achieved at a dissolved CO_2 concentration of 12 mg/l (Figure 5-1). This is in agreement with typical published results of 0.8 day^{-1} for ammonium-oxidizing and nitrate-oxidizing bacteria [30].

The shape of the curve in figure 5-2, which appears similar to a log normal distribution, is indicative of inhibition and can be described by means of an Andrews model [30]. The Andrews model is based on a modification of the Monod equation and incorporates an inhibitory coefficient. Extremely low and elevated dissolved CO₂ concentrations produce unfavorable growth conditions. Good model fits were achieved for each dissolved CO₂ concentration with regression model R² values ranging from 0.92 - 0.98.

5.2.2 Experiment 2

Based on the results from experiment 1, a second experiment (replicated) was conducted to determine an optimum specific growth rate based upon a dissolved CO₂ concentration and its associated pH value (Table 5-1). The pH values were determined using activated sludge from a WWTP (Figure 4-2). (See chapter 4 for a complete review of this study.)

Table 5-1: Experiment 2 pH vs. Dissolved CO₂ Concentration

Dissolved CO ₂ Concentration, mg/l	pH
8	7.32
12	7.23
16	7.16
19	7.1
25	6.98
34	6.86

An optimum specific growth rate of 1.05 days⁻¹ was achieved at a dissolved CO₂ concentration of 8 mg/l (Figure 5-3).

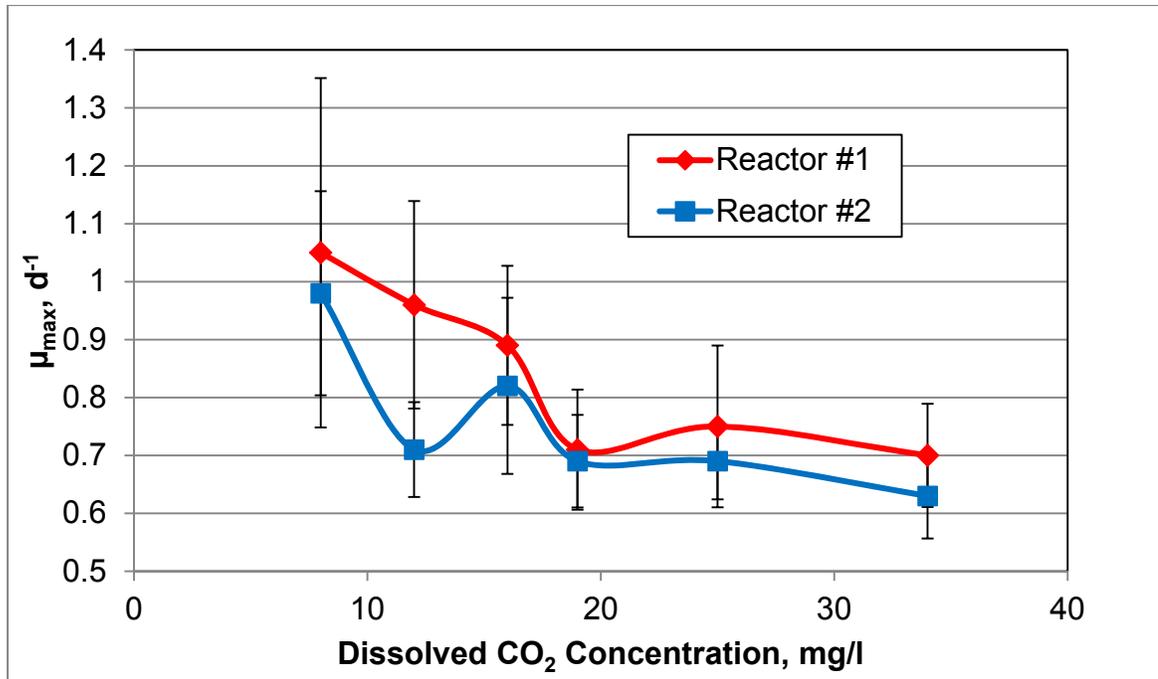


Figure 5-3: Experiment 2 Growth Curve at Selected Dissolved CO₂ Concentrations with 95% Confidence Levels

Except for the 8 mg/l dissolved CO₂ concentration in reactor 1 ($R^2 = 0.81$), good model fits were achieved for each dissolved CO₂ concentration with regression model R^2 values ranging from 0.92 - 0.98. (The nitrate electrode used for the 8 mg/l dissolved CO₂ concentration in reactor 1 failed six days into the experiment and was replaced. This was the cause of the increased variation. The experiment was conducted for ten days ensuring adequate observations were taken.) The decrease in specific growth rate appears linear but both reactors due show a marked decrease when the dissolved CO₂ concentration increases from 16 - 19 mg/l. From 19 - 34 mg/l, the specific growth rate remained relatively constant.

5.2.3 Growth Kinetics

Experiments 1 and 2 were evaluated and their growth parameters were compared to those reported by Denecke (Tables 5-2 & 5-3).

Table 5-2: Combined Growth Parameters for Experiment 1

Parameter	Units	Biomass 1	Biomass 2	Denecke Values [71]
K_s	mg CO ₂ /l	1.5	0.45	0.5
K_i	mg CO ₂ /l	50	25	42
μ_{max}	days ⁻¹	2.5	0.9	0.75
K_1		2E-7	9E-6	6.99E-7
K_2		1E-9	5E-8	1.25E-10

Table 5-3: Combined Growth Parameters for Experiment 2

Parameter	Units	Biomass 1	Biomass 2	Denecke Values [71]
K_s	mg CO ₂ /l	1.1	1.1	0.5
K_i	mg CO ₂ /l	44	70	42
μ_{max}	days ⁻¹	2.2	1.7	0.75
K_1		2E-7	9E-6	6.99E-7
K_2		1E-9	5E-8	1.25E-10

(The values reported by Denecke were for mixed sludge at 0.99 percent CO₂.) A set of parameters could not be found that described both sets of operating conditions describing experiments 1 and 2. Evaluating the curves generated by experiment 2 (Figure 5-3), it was hypothesized that a microbial population shift occurred between 16 and 19 mg/l dissolved CO₂ concentration. These dissolved CO₂ concentrations represent pH values of 7.1 and 6.98, respectively, and experiment 1 was conducted at a pH of 7. Though the pH difference is minor, in combination with the elevated dissolved CO₂ concentration, a microbial shift is

possible. Therefore, a proportion of the microbial populations was hypothesized and given values of 0.3, 0.35, 0.45, 0.75, 0.80, and 0.83. These values represent the 2 - 103 mg/l dissolved CO₂ concentrations in experiment 1. Their complement, 1 - proportion, represents the 8 - 34 mg/l dissolved CO₂ concentration in experiment 2. These percentages were developed by first evaluating experiment 2 so an appropriate set of parameters could be developed that adequately described the inflection from 16 to 19 mg/l dissolved CO₂ concentration (Figure 5-3). Parameters developed produced a good fit for the specific growth rate at its dissolved CO₂ concentration (Tables 5-2 and 5-3 and Figures 5-4 and 5-5). The values reported by Denecke were used as starting point values and fits were calculated by minimizing the difference sums of squares for the model.

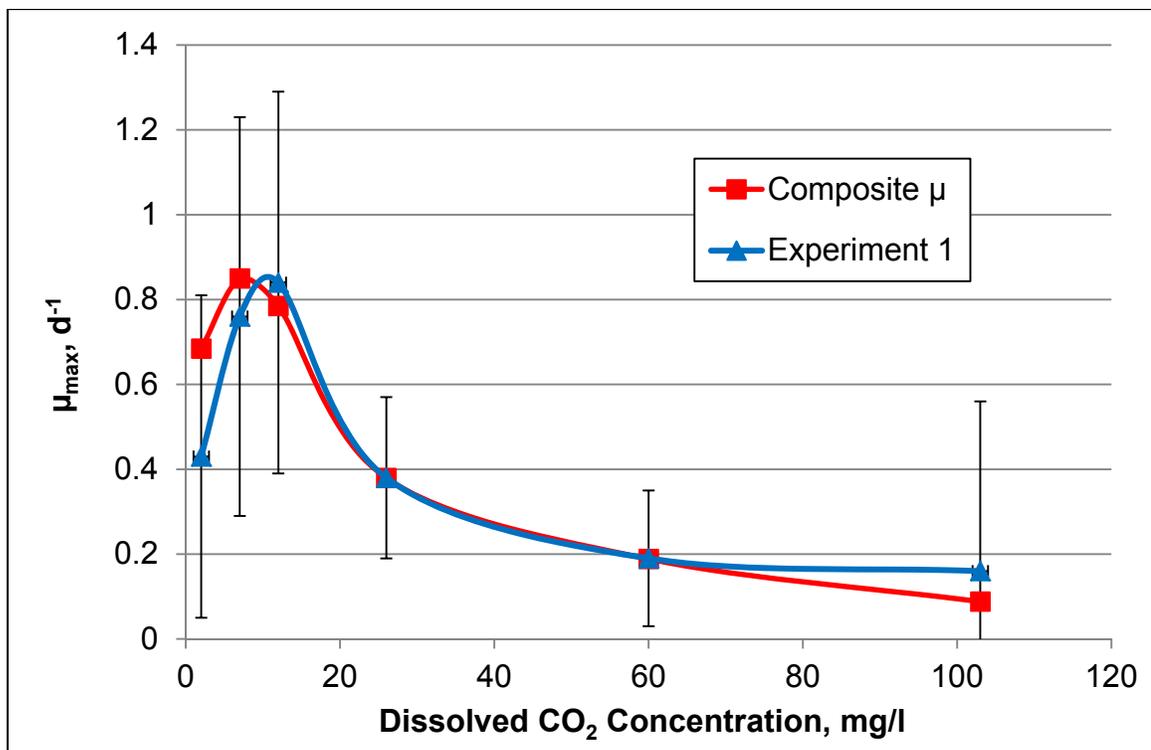


Figure 5-4: Composite Biomass Describing μ_{max} from Experiment 1 with 95% Confidence Levels

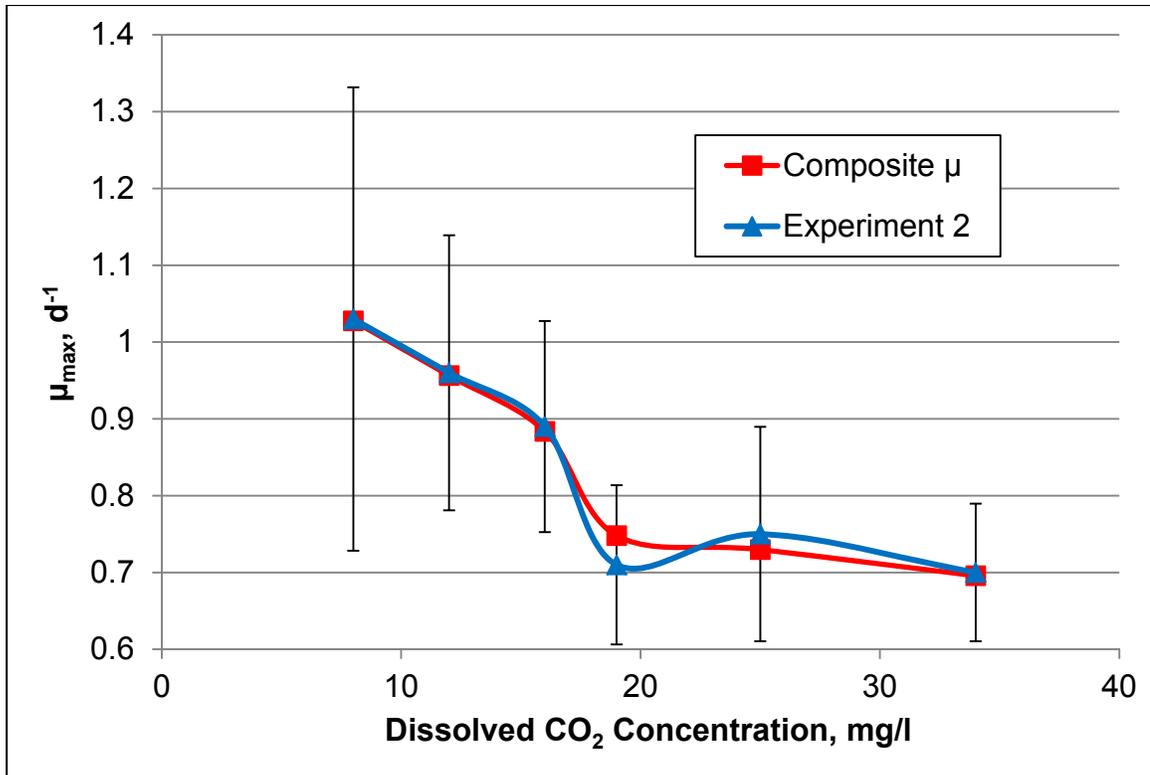


Figure 5-5: Composite Biomass Describing μ_{max} from Experiment 2 with 95% Confidence Levels

5.2.4 Experiment 3

Based on the results from experiment 2, a third experiment was conducted to determine an optimum specific growth rate based upon an observed optimum dissolved CO_2 concentration (8-12 mg/l) from previous experimentation. Results indicate that a combination of dissolved CO_2 concentration and pH produce significant growth rate differences (Figures 5-6 to 5-9).

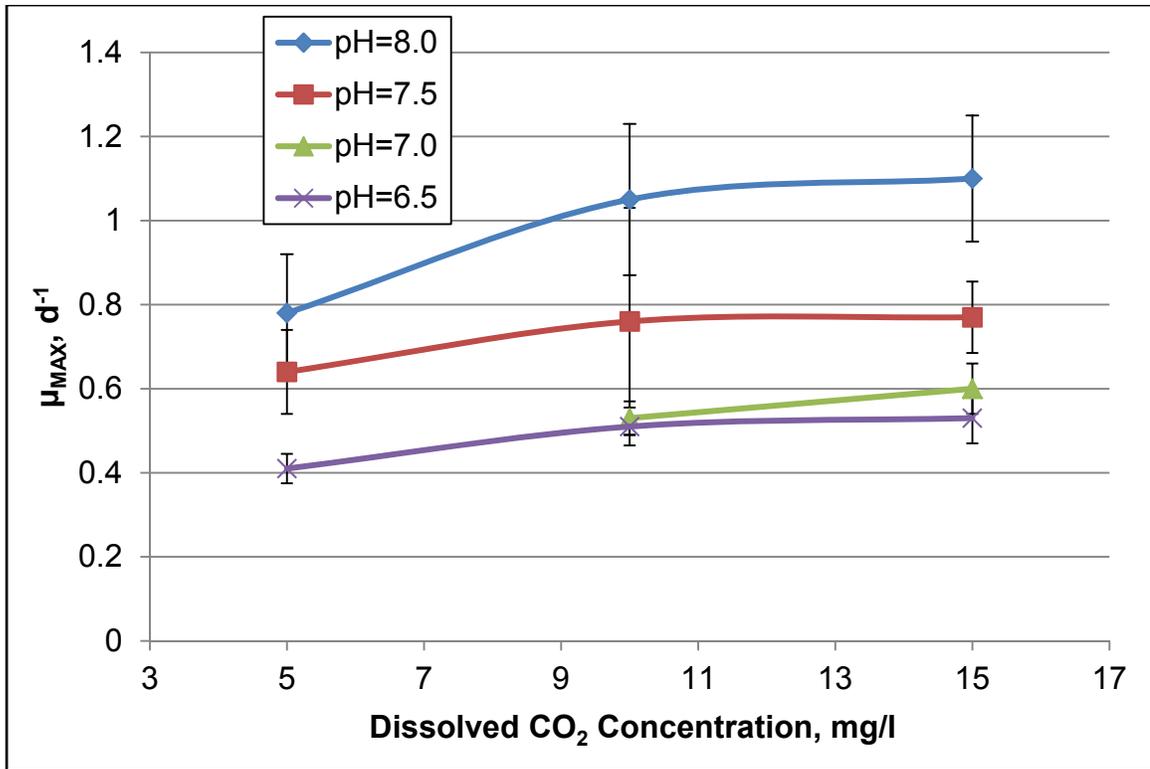


Figure 5-6: Experiment 3 Results of μ_{max} at Selected pH and Dissolved CO_2 Concentration with 95% Confidence Levels

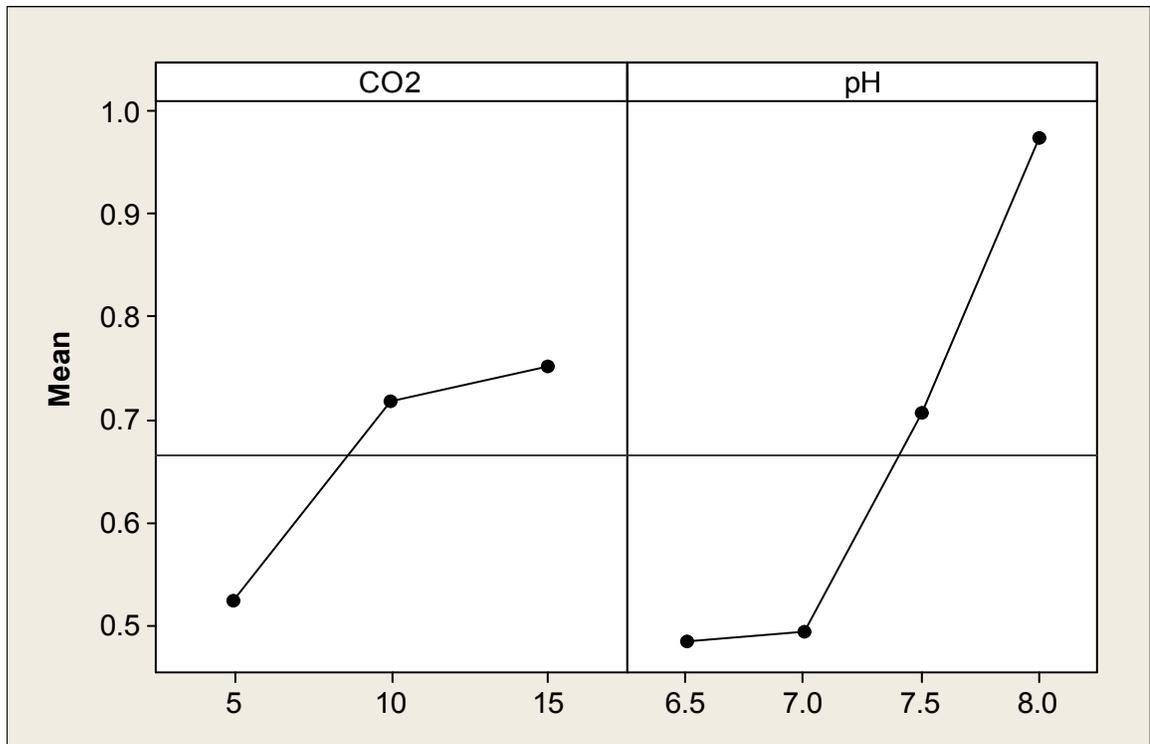


Figure 5-7: Experiment 3 Results of Main Effects Plot for μ_{max}

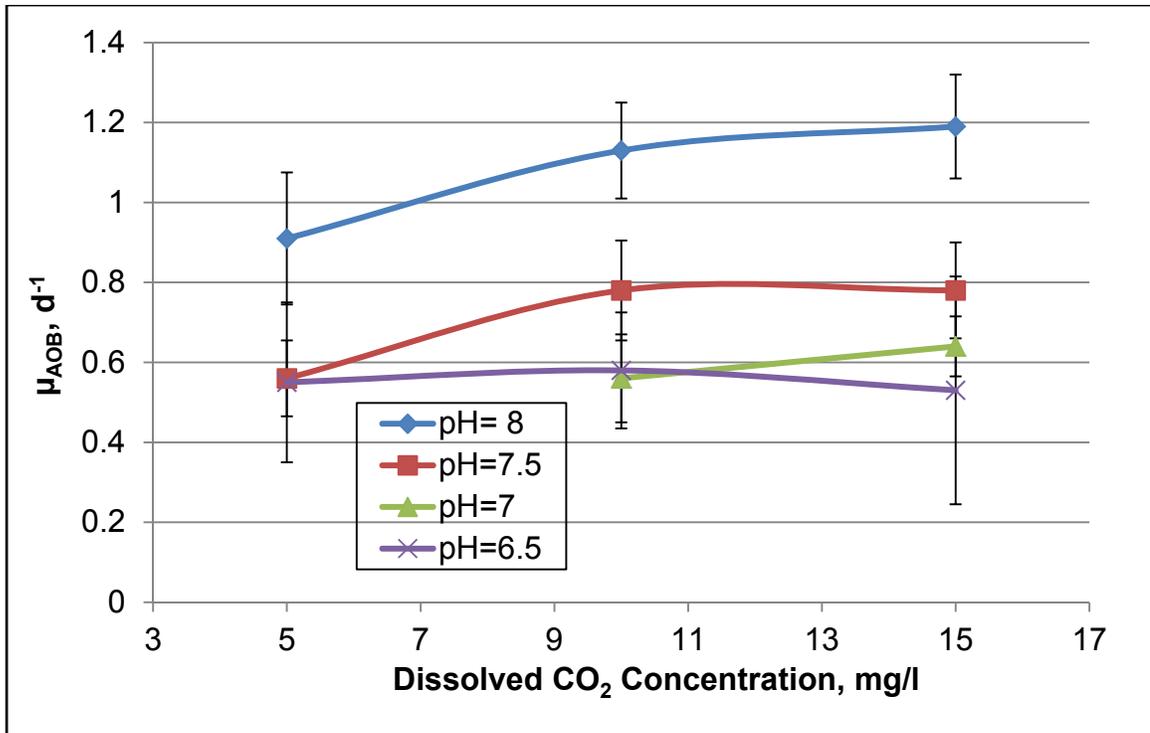


Figure 5-8: Experiment 3 Results of μ_{AOB} at Selected pH and Dissolved CO_2 Concentration with 95% Confidence Levels

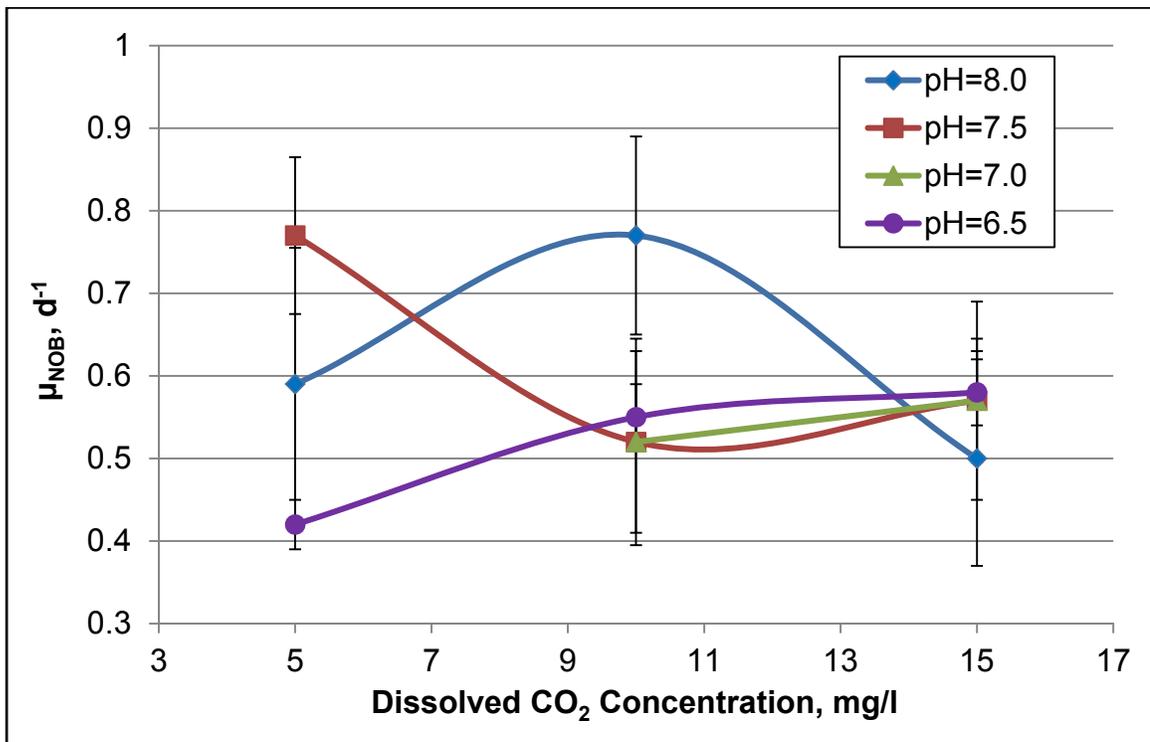


Figure 5-9: Experiment 3 Results of μ_{NOB} at Selected pH and Dissolved CO_2 Concentration with 95% Confidence Levels

Growth rates at a pH of 8 are approximately twice the values of those at pH of 6.5 and 7. It is also evident that lower growth rates are observed at low dissolved CO₂ concentrations. This relationship was observed in previous experimentation (Figure 5-2). The data from the dissolved CO₂ concentration of 5 mg/l at a pH of 7.0 is not displayed as this reactor received twice the activated sludge aliquot, thereby skewing the results. A main effects plot for μ_{\max} clearly shows the effect of dissolved CO₂ concentration and pH on specific growth rate (Figure 5-7).

The growth rates for the AOB and NOB microbes were evaluated at each CO₂/pH combination using non-linear regression as previously reported. It is clearly evident that at dissolved CO₂ concentrations of 10 and 15 mg/l at a pH of 8, significant AOB growth rates occur. This relationship is not observed at lower pH. Also included are the 95% CI for each microbe at the specific dissolved CO₂ concentration/pH combination. With additional measurements, low standard errors were achieved.

5.3 Discussion

5.3.1 Experiment 1

Experiment 1 was conducted as an un-replicated completely randomized design (CRD). The NO₂⁻ and NO₃⁻ concentrations were summed and regressed against

time to determine the maximum specific growth rate (μ_{\max}). As this experiment was non-replicated, degrees of freedom are not available to calculate an error term and thus generate an appropriate analysis of variance (ANOVA).

Evaluation and comparison of the specific growth rate curves was possible by conducting a two-sample Kolmogorov-Smirnov (KS) test. As a non-parametric test, no assumptions about the parameters of a distribution nor is its underlying distribution are made. The null hypothesis for this test is that the two samples have the same distribution. Evaluation of the 7 and 12 mg/l dissolved CO₂ curves ($\mu = 0.76 \text{ days}^{-1}$ and 0.84 days^{-1} , respectively) provided a p-value of 0.829 indicating the underlying distributions are very similar. Evaluating the 103 and 12 mg/l dissolved CO₂ curves, which had the largest differences in μ values (0.16 days^{-1} and 0.84 days^{-1} respectively), provided a p-value of 0.147. Although not statistically significant, results indicate a marked departure in their underlying distributions. This is not unexpected given the large differences in μ_{\max} values. Evaluation of the 103 mg/l dissolved CO₂ curve showed the results to be linear ($R^2 = 0.951$) indicative of a normal distribution instead of an expected exponential distribution. Indicating growth inhibition is evident.

5.3.2 Experiment 2

Experiment 2 was conducted as a replicated CRD. Analysis indicated significant differences among the varying dissolved CO₂ concentration (Figure 5-3). An

average optimum specific growth rate of 1.0 days⁻¹ was achieved at a dissolved CO₂ concentration of 8 mg/l. The dissolved CO₂ concentrations from 8 - 16 mg/l show a downward trend to a constant growth rate from 19 - 34 mg/l. Growth rates in reactor 2 were always lower than reactor 1. The significantly lower growth rate in reactor 2 at a dissolved CO₂ concentration of 12 mg/l cannot be explained. Based on the results, a predicted specific growth rate of 0.91 days⁻¹ should have been observed. It is believed that the reactor was inadvertently contaminated during the experiment. The ANOVA conducted for this experiment shows significant differences at the specified dissolved CO₂ concentrations (Table 5-4).

Table 5-4: Experiment 2 Results of Completely Randomized Design of μ_{max} at Selected Dissolved CO₂ Concentrations

Source	DF	SS	MS	F	P-Value
Dissolved CO ₂ , mg/l	5	0.16167	0.03233	4.92	0.039
Error	6	0.03940	0.00657		
Total	11	0.20107			

S = 0.08103 R-Sq = 80.40% R-Sq (ad) = 64.07%
Pooled Standard Deviation = 0.0810

Individual 95% CIs For Mean Based on Pooled Standard Deviation

Level	N	Mean	Std Dev	
8	2	1.0050	0.0354	-----+-----+-----+-----+-- (-----*-----)
12	2	0.8350	0.1768	(-----*-----)
16	2	0.8550	0.0495	(-----*-----)
19	2	0.7000	0.0141	(-----*-----)
25	2	0.7200	0.0424	(-----*-----)
34	2	0.6650	0.0495	(-----*-----)

-----+-----+-----+-----+--
0.64 0.80 0.96 1.12

As this is a balanced experimental design, a Tukey multiple comparison test was selected and shows only the 34 and 8 mg/l dissolved CO₂ concentrations to be statistically different (p-value = 0.039). It is evident that major differences do exist but are masked by the large variation occurring at the 12 mg/l dissolved CO₂ concentration (Figure 5-10).

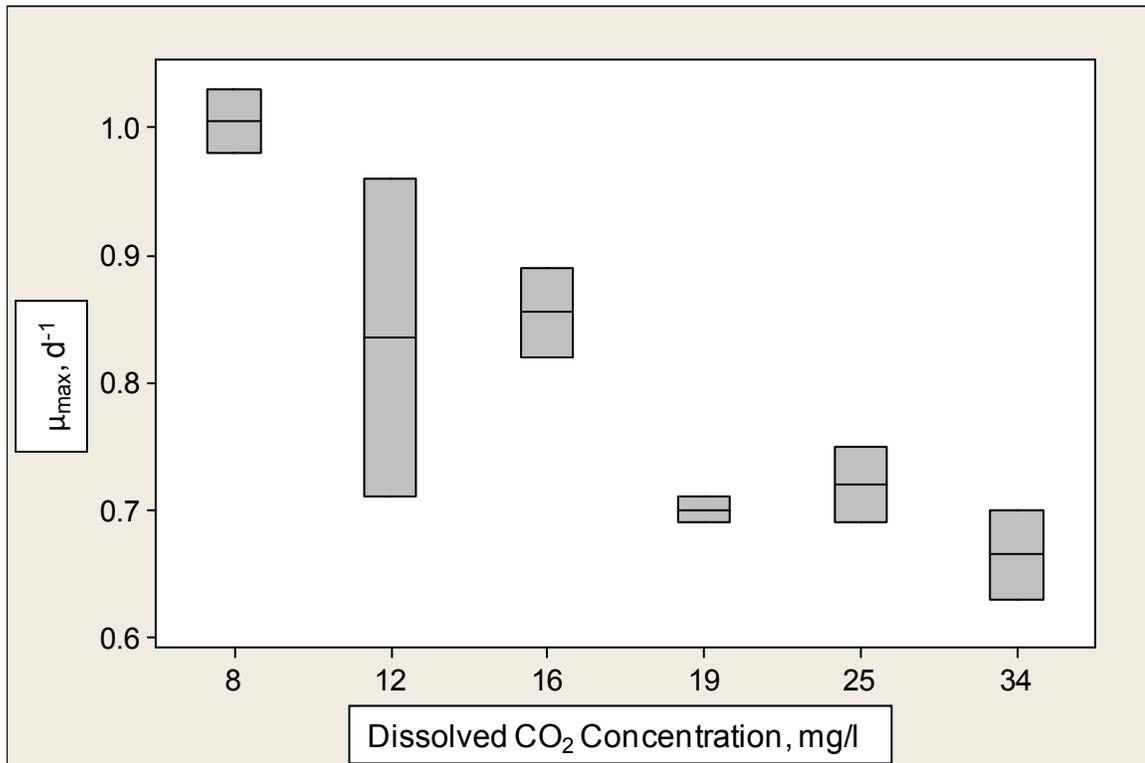


Figure 5-10: Experiment 2 Results of Boxplot of Completely Randomized Design Showing μ_{max} at Selected Dissolved CO₂ Concentrations

5.3.2.1 Effect of pH on Nitrification

A study was conducted to determine the pH of an activated sludge sample at varying levels of dissolved CO₂ concentration (Table 5-1). Measurement taken in the aeration basin of two Modified Ludzack-Ettinger (MLE) wastewater plants had

dissolved CO₂ concentrations of 35 and 26 mg/l. This would equate to pH values of 6.86 and 6.98, respectively. Achieving a pH of 7.5 would require that the dissolved CO₂ concentration be reduced to a value less than 6 mg/l. However, this “optimum pH value” is moderately higher than the pH value of 7.32 which is achieved at the optimum growth rate value of 8 mg/l found during experiment 2.

Although experimental error does exist, the effect of pH cannot be understated. This is evidenced in the different specific growth rates at similar dissolved CO₂ concentrations for the two experiments. The 7 mg/l dissolved CO₂ in the first experiment had a growth rate of 0.76 days⁻¹ while the 8 mg/l dissolved CO₂ concentration in the second experiment had a growth rate of 1.0 days⁻¹. The increased growth rate was achieved with a pH difference of only +0.3 units (pH 7.0 versus pH 7.32).

5.3.2.2 Growth Kinetics

Good fits of the model parameters were achieved. However, these are not optimum as other conditions may satisfy and achieve good model fits. Values were selected that had reasonable agreement with those reported by Denecke. These results indicate that determining specific growth rates may be more complicated than previously reported. It appears that different microbial populations can exist at different pH and experimental conditions will dictate which set of AOB/ NOB organisms are most in abundance. In evaluating these

models, different maximum specific growth rates were obtained when evaluating these two hypothesized sludges. Thus, suggesting that maximum specific growth rates of some AOB/ NOB organisms may be much higher than previously reported.

5.3.2.3 Nitrification in Activated Sludge Systems

These experimental results are consistent with the findings of other researchers, which have found a positive effect of elevated $p\text{CO}_2$ on nitrification rates and in the specific growth rate of nitrifiers [71, 72, 78-80]. Although nitrate formation rates were not reported by these researchers, observed growth rates based on the increase of $\text{NO}_x\text{-N}$ concentration were reported to be approximately three times higher (1.5% CO_2 vs. 0% CO_2) after two hours of operation. Evaluation of nitrification using air was not conducted in this study as observations at various treatment plants show much higher dissolved CO_2 concentrations in their processes.

Additionally, Denecke and Liebig [71] reported that the specific growth rate (μ_{obs}) of mixed autotrophic and heterotrophic sludge increased by 20% when the $p\text{CO}_2$ was elevated to approximately 1% (17 mg/l dissolved CO_2). Other authors also suggested a positive impact of elevated $p\text{CO}_2$ on the specific growth rates of nitrifying bacteria [78, 81].

5.3.3 Experiment 3

Experiment 3 was conducted as a non-replicated CRD due to limited laboratory equipment. The ANOVA conducted for this experiment shows significant differences for pH levels but dissolved CO₂ concentrations were not significant at the $\alpha = 0.05$ level (Figure 5-6 to 5-9 and Table 5-5). However, a p-value of 0.058 does indicate that dissolved CO₂ concentration is influencing the growth rate of the nitrifiers. A partitioning of the sums of squares of the treatment effects shows that dissolved CO₂ contributes 17.4% to model understanding while pH contributes 82.6%. This is shown graphically in Figure 5-7. These results indicate that pH is the dominant factor that affects the nitrification rate at the specified dissolved CO₂ concentrations and pH levels used in this experiment.

Table 5-5: Experiment 3 ANOVA of Main Effects of μ_{\max}

Factor	Type	Levels	Values
CO ₂	fixed	3	5, 10, 15
pH	fixed	4	6.5, 7.0, 7.5, 8.0

Analysis of Variance for μ_{\max}					
Source	DF	SS	MS	F	P-Value
CO ₂	2	0.092056	0.046028	5.33	0.058
pH	3	0.438156	0.146052	16.91	0.005
Error	5	0.043194	0.008639		
Total	10	0.573406			

S = 0.0929456 R-Sq = 92.47% R-Sq (adj) = 84.42%

A multiple comparison test for the main effects indicates differences (Table 5-6). Dissolved CO₂ concentration does not indicate differences at the $\alpha = 0.05$ level. However, pH does show differences where a pH of 8.0 is different from pH values of 7.0 and 6.5. At a pH of 7.5, results show that this level exists in both groups.

Table 5-6: Multiple Comparisons of Factor Effects Using Tukey Method with a 95.0% Confidence Level

Factor	Level	N	Mean (μ_{\max})	Grouping*
Dissolved CO ₂ Concentration	15	4	0.8	A
	10	4	0.7	A
	5	4	0.5	A
pH	8.0	3	1.0	A
	7.5	3	0.7	A & B
	7.0	2	0.5	B
	6.5	3	0.5	B

*Means that do not share a letter are significantly different.

An interaction plot was generated to graphically assess the relationship between the main effects (Figure 5-11). These results indicate that as pH increases the maximum specific growth increases. Some effect of the dissolved CO₂ concentration on this increased growth rate can be observed. The effect of dissolved CO₂ concentration shows similar growth effects at different pH values indicating that a significant interaction effect is probably not evident. It must be emphasized that these results are not accompanied by a statistical analysis and therefore a p-value cannot be generated to confirm the presence of an interaction effect.

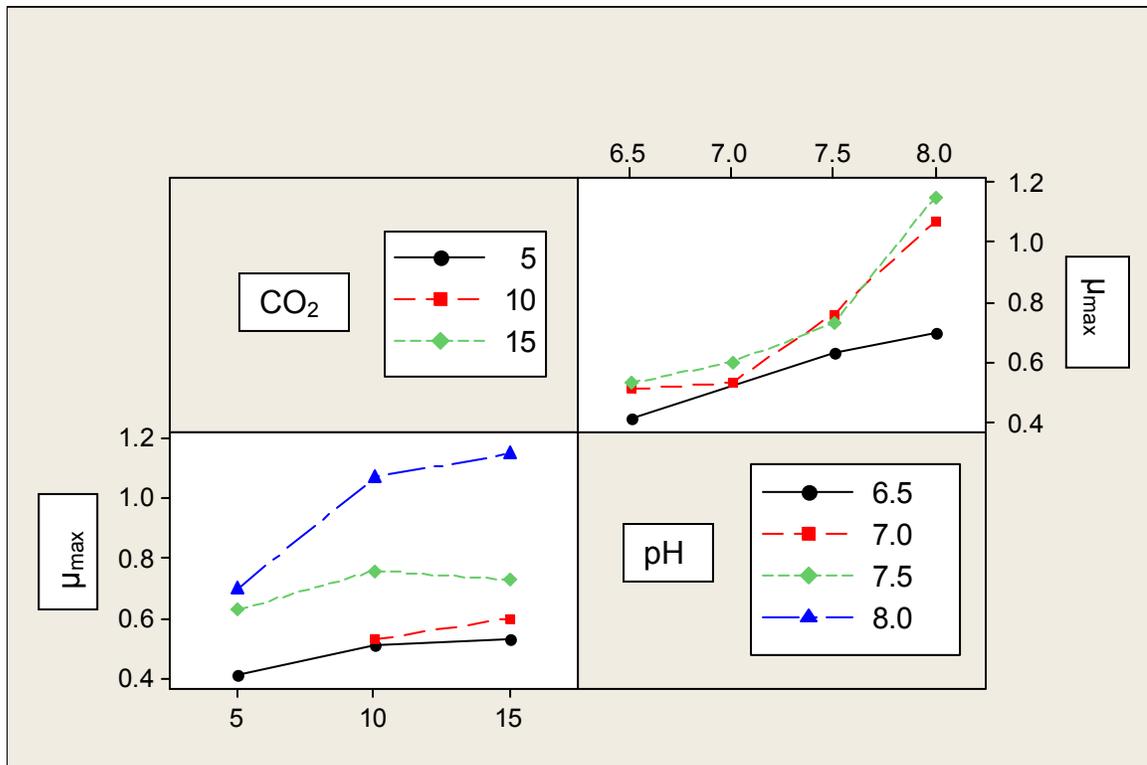


Figure 5-11: Interaction Plot of μ_{max} as a Function of Dissolved CO₂ Concentration and pH

Increased maximum specific growth rates were observed at each dissolved CO₂ concentration as pH increased from 6.5-8.0. At a dissolved CO₂ concentration of 10 and 15 mg/l, the maximum specific growth more than doubled when the pH increased from 6.5 to 8.0 (Figures 5-6 and 5-7). The results from the dissolved CO₂ concentration at 10 and 15 mg/l and at a pH of 8.0 were surprising but not unexpected. *Nitrosomonas*, identified as the major AOB bacteria found in wastewater, has an ideal growth condition at pH 7.8-8.0. As conditions were selected that promote the growth of this microbe, high growth rates were observed. AOB bacteria are considered limiting in the conversion of ammonia to nitrate, but this combination of dissolved CO₂ concentration and pH favored elevated AOB concentrations (Figure 5-8).

Nitrobacter, identified as the major NOB bacteria found in wastewater, has an ideal growth condition at pH 7.3-7.5. Many WWTP's operate at these pH conditions favoring the growth of this microbe. Unfortunately, at this pH the NOB does not display the elevated growth rate observed at pH 8.0. Growth rates for the NOB bacteria were mixed depending on the dissolved CO₂ concentration and pH (Figure 5-9).

Observations from experiments 1 and 3 do suggest that there is a lower dissolved CO₂ concentration limit. Experiment 1 had a lower dissolved CO₂ concentration of 2 mg/l and a growth rate of 0.43 days⁻¹. Experiment 3 had a lower dissolved CO₂ concentration of 5 mg/l and a growth rate of 0.41 – 0.53 days⁻¹ depending on the pH. The experimental results suggest a minimum dissolved CO₂ concentration between 5-10 mg/l is needed to obtain satisfactory nitrification rates.

5.4 Conclusions

Low and high levels of dissolved CO₂ concentration result in inhibition and reduce the nitrification rate. Though experimentation was only conducted at one pH level (7.0), similar results are expected at other pH levels. Further experimentation with adjusted pH levels based on activated sludge from a WWTP (Table 5-1), showed reduced nitrification rates at these higher dissolved CO₂ concentrations. It was hypothesized that these were due to shifts in

microbial ecology that were less conducive to nitrification. Even so, whether the effect was due to a lower pH level or increased dissolved CO₂ concentration is unknown requiring further experimentation.

Incorporating results from previous experimentation, an optimization experiment found an optimum dissolved CO₂ concentration range of 10 -15 mg/l and a pH range of 7.5 – 8.0. A partition of the sums of squares treatment show that pH contributes to 83 percent of model understanding. Dissolved CO₂ concentration does contribute to nitrification (17 percent) but is minor when pH is optimized.

Chapter 6

FISH Analysis of Microbial Samples Collected from Batch Reactors Operated at Different Dissolved CO₂ Concentrations and pH

6.1 Introduction

The optimization studies conducted in this research (Chapter 5) showed that maximum specific growth rates vary depending on the dissolved CO₂ concentration and pH. Dissolved CO₂ concentration was established at 5, 10 and 15 mg/l with pH values maintained at 6.5, 7.0, 7.5 and 8.0. These values were selected based on previous research and literature recommendations. Upon completion of each CO₂/pH combination reactor experiment, a biomass sample was obtained for microbial assessment. It was theorized that certain ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) would predominate at these reactor conditions. The AOB bacteria *Nitrosomonas* and *Nitrosospira* and the NOB bacteria *Nitrobacter* and *Nitrospirae* were selected as they are the most frequently mentioned bacteria species found in literature as related to wastewater.

6.2 Methods and Materials

A total of 52 samples (10 digital images each) were prepared for this study. These samples were obtained from experiment three reviewed in chapter 5. The samples were the combination of dissolved CO₂ concentration and pH by bacteria microbe (AOB and NOB) as well as the seed activated sludge by microbe. The large sample size was obtained to minimize variation.

Fluorescence *in situ* hybridizations (FISH) was used to evaluate the population abundance of the common AOB and NOB bacteria listed above. For a complete review of this molecular biological technique, see the section entitled, “Evaluation of nitrifying bacteria abundance by fluorescence *in situ* hybridization”, from Chapter 4. FISH probe and additional information on the AOB and NOB bacteria studied in this research can be found in Table 4-2.

From Chapter 4, see the section entitled, “Evaluation of nitrifying bacteria abundance by fluorescence *in situ* hybridization”, for a complete review of this molecular biological technique. Also see Table 4-2 for FISH probe and additional information on the AOB and NOB bacteria studied in this research.

The digital images from the FISH analysis were analyzed using the software daime (digital image analysis in microbial ecology) [86]. Each biomass was initially stained with DAPI, a blue-fluorescent nucleic acid stain that preferentially

stains dsDNA but will also bind to RNA, though it is not as strongly fluorescent [87]. Next, the sample was hybridized with the specific Cy3 probe which targets a specific sequence DNA presence associated with the microbe of interest. The Cy3 probe is a reactive water-soluble fluorescent dye of the cyanine dye family. The Cy3 will appear as a red fluorescent color when bonded with the appropriate sequence. After hybridization, its abundance was compared to the total biomass contained within the microbial image. Digital images of DAPI, Cy3 and their merged images were conducted for each sample (Figure 6-1). Blue fluorescence can be seen in the merged image (image C) indicating areas where the bacteria of interest is not present. (The length measure shown in each image represents 10 μ m). A two dimensional automatic segmentation with custom thresholding was used to determine these concentrations. Items appearing smaller than 10 pixels were ignored. A total bio-volume fraction was calculated based on these image concentrations. These are reported as percent (percent of total biomass). Ten observations were measured for each combination and are reported as percent (percent of total biomass). Additional statistics were also generated. Statistical and graphic assessment was conducted using Minitab statistical software (State College, PA.).

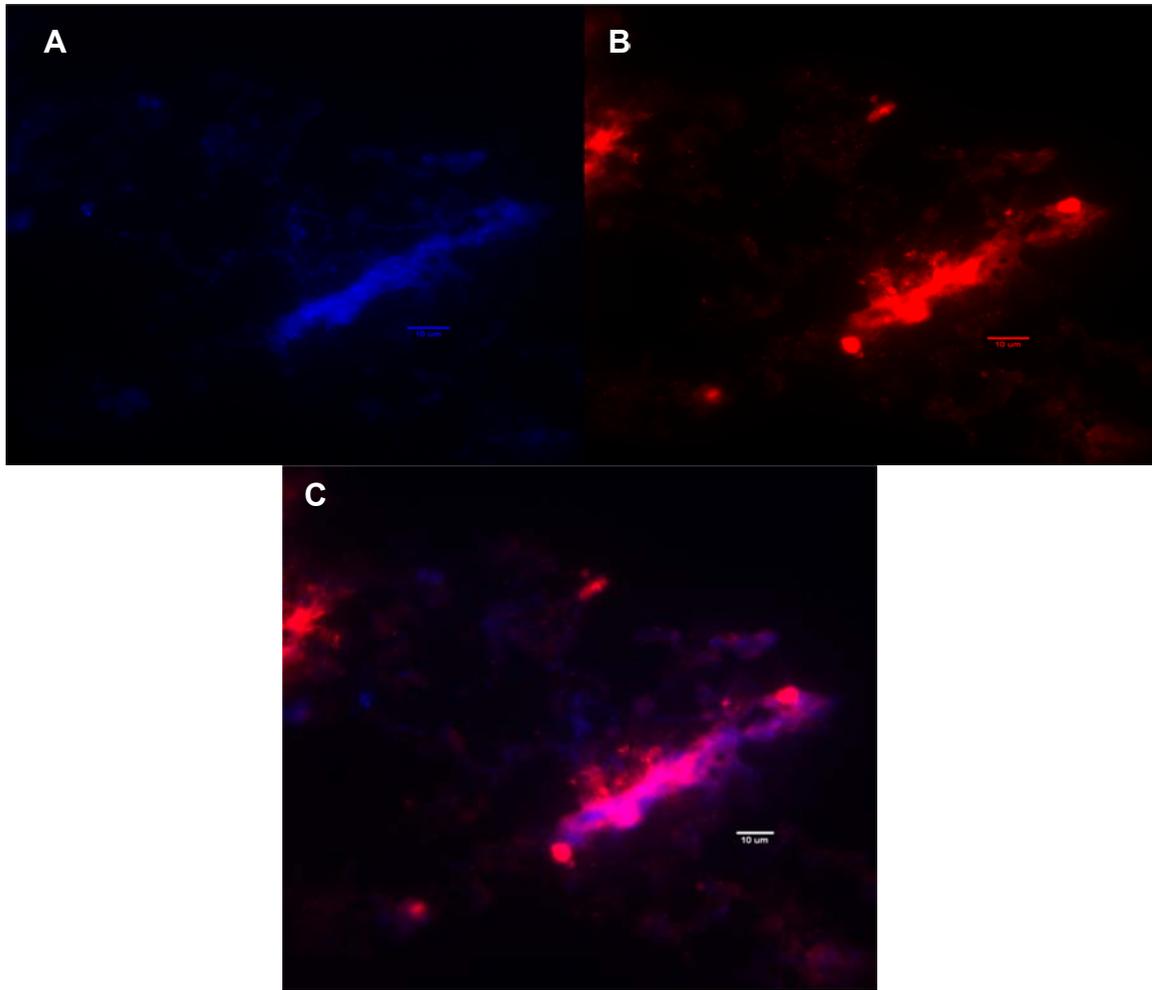


Figure 6-1: Typical Digital Images of (A) DAPI Stain, (B) Cy3 Stain, and (C) Merged Image of DAPI and Cy3 Stain

6.3 Results

Digital merged images for each microbe were generated from the activated sludge sample (Figure 6-2 and 6-3). Figure 6-2 represents the AOB and depicts the initial abundance of *Nitrosomonas* and *Nitrosospira* and then compares them to samples obtained after experimentation. The abundance of the microbes after growth at near optimum conditions is clearly evident. Figure 6-3 represents the NOB and depicts the initial abundance of *Nitrobacter* and *Nitrospirae* and then compares them to samples obtained after experimentation. As with the AOB, the

abundance of the microbes after growth at near optimum conditions is clearly evident. The measurement bar in each digital image represents 10 μm .

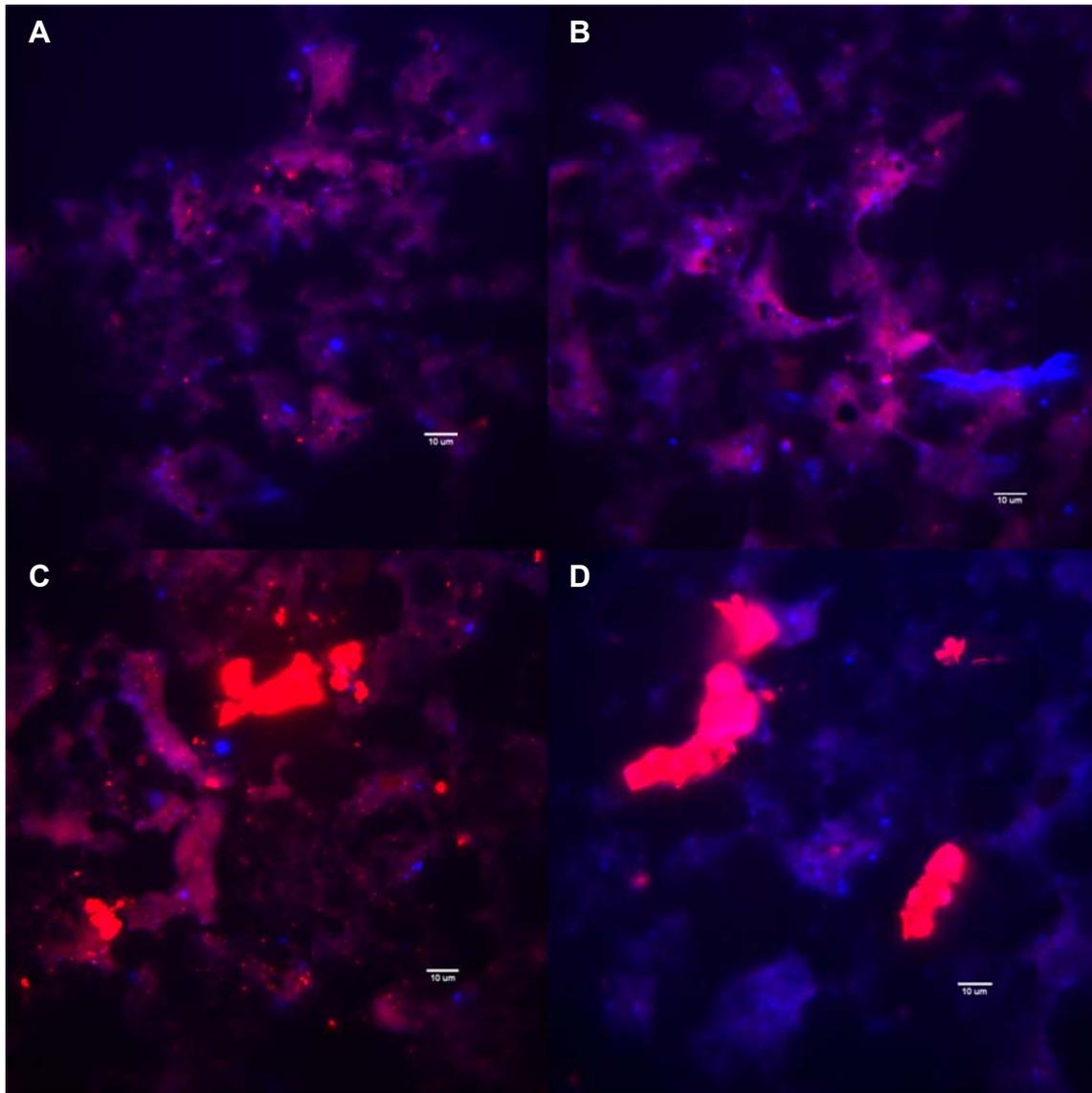


Figure 6-2: AOB Bacteria, Representative FISH Results Showing Percent Abundance (A) *Nitrosomonas* – WWTP Sample, (B) *Nitrospira* – WWTP Sample, (C) *Nitrosomonas* at Dissolved CO₂ Concentration = 5.0 mg/l and pH = 6.5, and (D) *Nitrospira* at Dissolved CO₂ Concentration = 5.0 mg/l and pH = 7.5

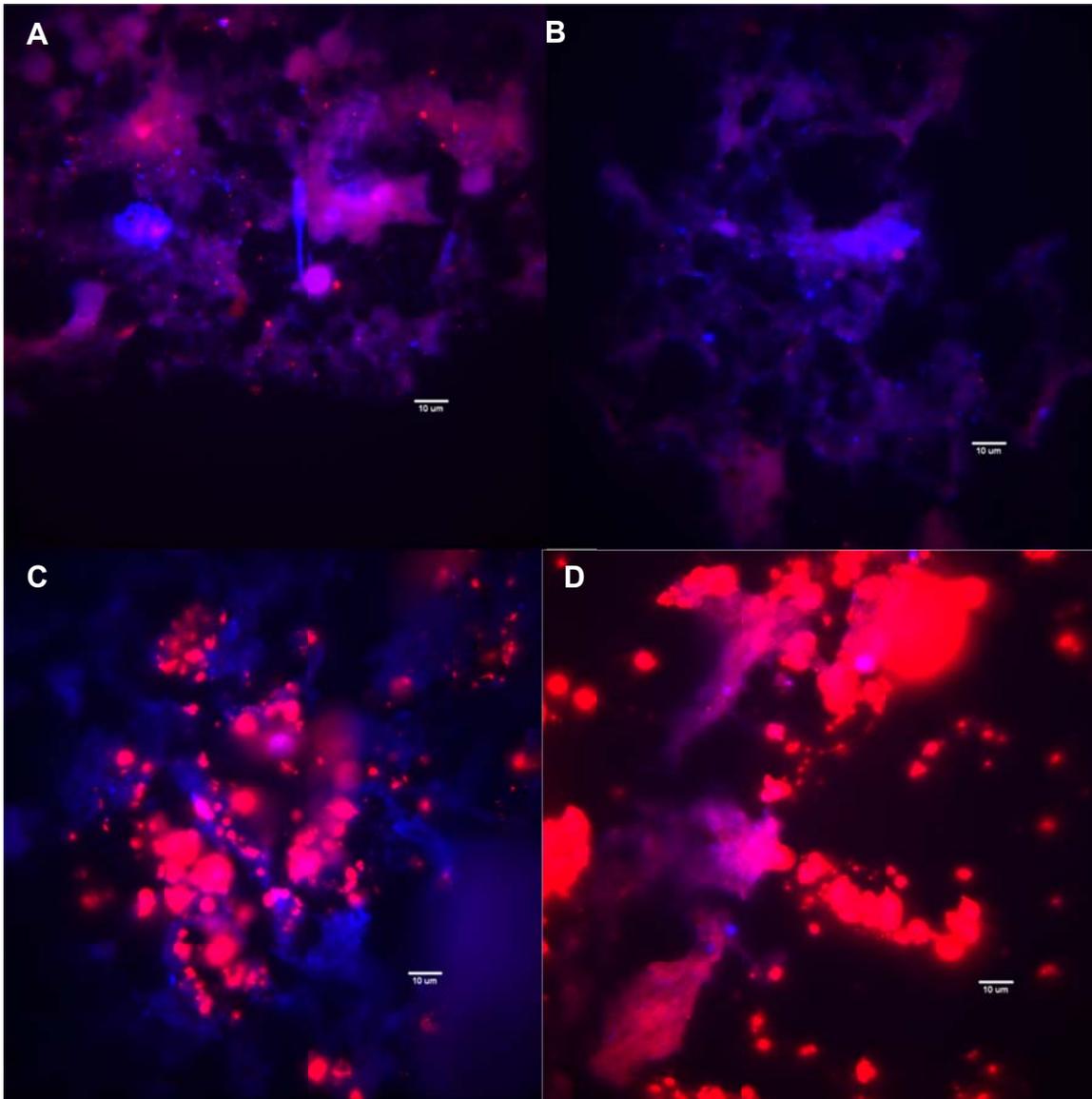


Figure 6-3: NOB Bacteria, Representative FISH Results Showing Percent Abundance (A) *Nitrobacter* – WWTP Sample, (B) *Nitrospirae* – WWTP Sample, (C) *Nitrobacter* at Dissolved CO₂ Concentration = 10.0 mg/l and pH = 7.0, and (D) *Nitrospirae* at Dissolved CO₂ Concentration = 5.0 mg/l and pH = 7.0

Descriptive statistics for each microbe are provided: AOB (Tables 6-1 and 6-2) and NOB (Tables 6-6 and 6-7). The average microbe concentration, its standard deviation, range and the number of digital images depicting a high accumulation of the specified bacteria are presented in these tables. The results for the

activated sludge sample obtained from the WWTP are presented in these tables as well and are listed first.

The high accumulation ratio represents the number of slides out of a total of 10 that exhibit this phenomenon. Microbe colonies exhibiting a size greater than 10um are considered high accumulation. Digital images depicting this phenomenon are presented with digital images of the seed material obtained from the wastewater treatment facility (Figures 6-2 and 6-3). These results are not unexpected as ideal growth conditions, based upon previous designed experiments, could account for these high microbe accumulations.

Analysis of the dissolved CO₂ concentration/ pH combinations were evaluated by bacteria type (AOB and NOB) as a randomized block design with a factorial arrangement. Each bacteria type will be presented separately.

6.3.1 AOB Results

An initial analysis was conducted to determine if differences in the AOB abundance exist between the bacteria obtained for use as the seed material. Operating conditions at the wastewater treatment plant from which this sample was received exhibited a dissolved CO₂ concentration of 34 mg/l and pH of 6.86. A two sample t-test indicated a statistically significant difference exists for the

AOB bacteria (p -value = 0.036) and shows *Nitrosospira* to predominate (Figure 6-4).

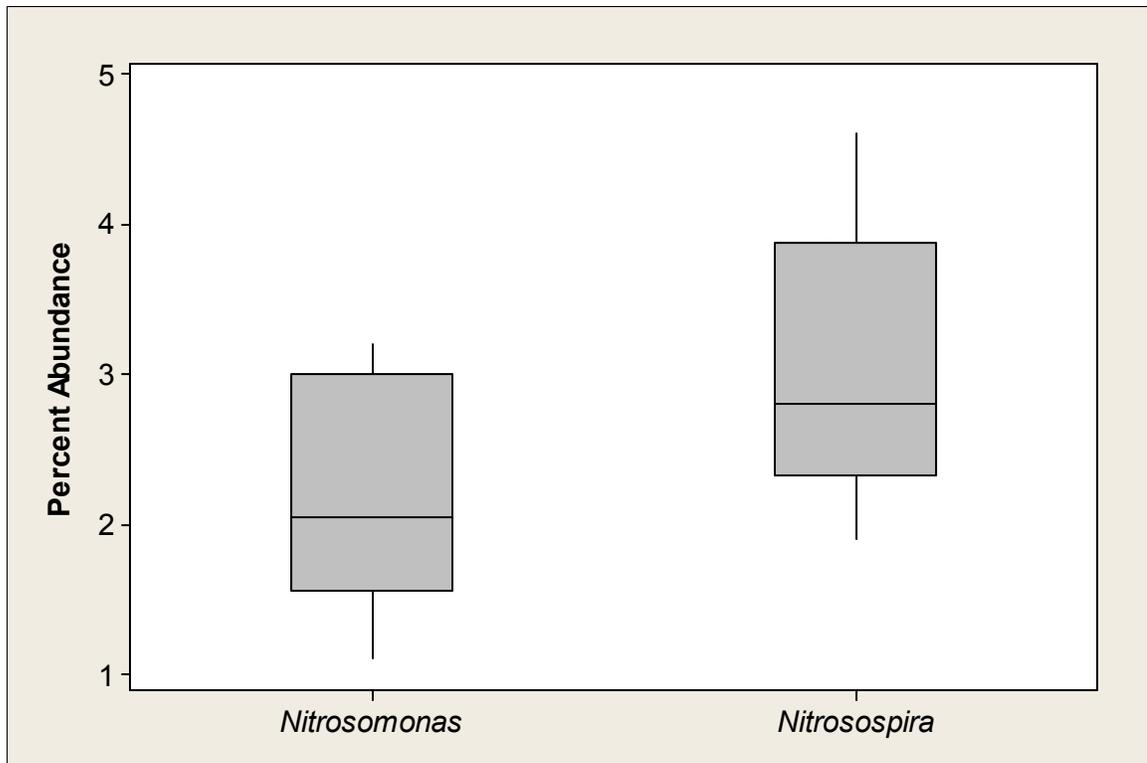


Figure 6-4: Percent Abundance of AOB Bacteria from Activated Sludge

Descriptive statistics for the experimental results of the AOB bacteria are presented in Tables 6-1 and 6-2.

Table 6-1: *Nitrosomonas* Percent Abundance Results

Dissolved CO ₂ Conc	pH	μ_{\max}	Average Microbe Conc	Standard Deviation	Range	Coefficient of Variation	Images w/ High Accum
34	6.86		2.2	0.74	2.1	0.34	0/10
5	6.5	0.55	15.6	5.8	20.5	0.37	7/10
5	7.0		19.7	15.72	38.9	0.80	7/10
5	7.5	0.56	17.8	9.7	32.1	0.54	10/10
5	8.0	0.78	54.1	31.2	84.1	0.58	8/10
10	6.5	0.58	18.3	6.3	18.3	0.34	10/10
10	7.0	0.56	22.4	10.2	31	0.46	9/10
10	7.5	0.62	15.5	4.7	16.5	0.30	10/10
10	8.0	1.15	38.9	23.4	57.5	0.60	10/10
15	6.5	0.53	15.1	6.5	15.8	0.43	4/10
15	7.0	0.63	17.5	4.6	16.6	0.26	7/10
15	7.5	0.7	14.4	7.1	20	0.49	2/10
15	8.0	1.04	18.1	17.4	59.9	0.96	7/10

Table 6-2: *Nitrospira* Percent Abundance Results

Dissolved CO ₂ Conc	pH	μ_{\max}	Average Microbe Conc	Standard Deviation	Range	Coefficient of Variation	Images w/ High Accum
34	6.86		3.0	0.91	2.7	0.30	0/10
5	6.5	0.55	11.7	5.5	14.6	0.47	1/10
5	7.0		16.7	11.8	41.5	0.71	3/10
5	7.5	0.56	21.1	11.2	36.3	0.53	6/10
5	8.0	0.78	4.8	2.1	5.5	0.44	1/10
10	6.5	0.58	16.4	5.0	15.7	0.30	1/10
10	7.0	0.56	13.9	3.3	9.5	0.24	2/10
10	7.5	0.62	23.2	13.5	37.7	0.58	7/10
10	8.0	1.15	7.4	4.1	10.4	0.55	3/10
15	6.5	0.53	20.8	6.5	20	0.31	4/10
15	7.0	0.63	29.5	14.1	43.2	0.48	9/10
15	7.5	0.7	18.5	6.1	18	0.33	3/10
15	8.0	1.04	6.9	2.6	8.3	0.38	0/10

Analysis of the AOB bacteria type showed the blocking variable, AOB Type, to be significant with *Nitrosomonas* being predominant (Table 6-3). The *Nitrosomonas* had a percent abundance across all treatment combinations of 22.3 percent compared to 15.9 percent for the *Nitrosospira*. The main effects, dissolved CO₂ concentration and pH, were not significant but their interaction effect is significant. As a significant interaction is present, analysis of the main effects is not appropriate. Due to the complexity of the interaction, treatment effects will be evaluated for each AOB type microbe separately.

Table 6-3: ANOVA of Percent Abundance of AOB Bacteria

Source	DF	SS	MS	F	P-Value
AOB Type	1	2453.8	2453.8	11.9	0.001
CO ₂	2	285.2	142.6	0.69	0.502
pH	3	944.8	314.9	1.53	0.208
CO ₂ *pH	6	3368.1	561.3	2.72	0.014
Error	227	46820.1	206.3		
Total	239	53871.9			

S = 14.3616 R-Sq = 13.09% R-Sq (adj) = 8.5%

6.3.1.1 *Nitrosomonas*

Analysis of the *Nitrosomonas* bacteria shows all treatment effects to be statistically significant (Table 6-4). As the interaction is significant, analysis of the main effects is not appropriate. A graphical display and review of the interaction is provided (Figure 6-5).

Table 6-4: ANOVA of Percent Abundance of *Nitrosomonas* Bacteria

Source	DF	SS	MS	F	P-Value
CO ₂	2	2343.6	1171.8	5.68	0.005
pH	3	9021.2	3007.1	14.57	0.000
CO ₂ *pH	6	4429.8	738.3	3.58	0.003
Error	108	22286.2	206.4		
Total	119	38080.8			

S = 14.3650 R-Sq = 41.48% R-Sq (adj) = 35.52%

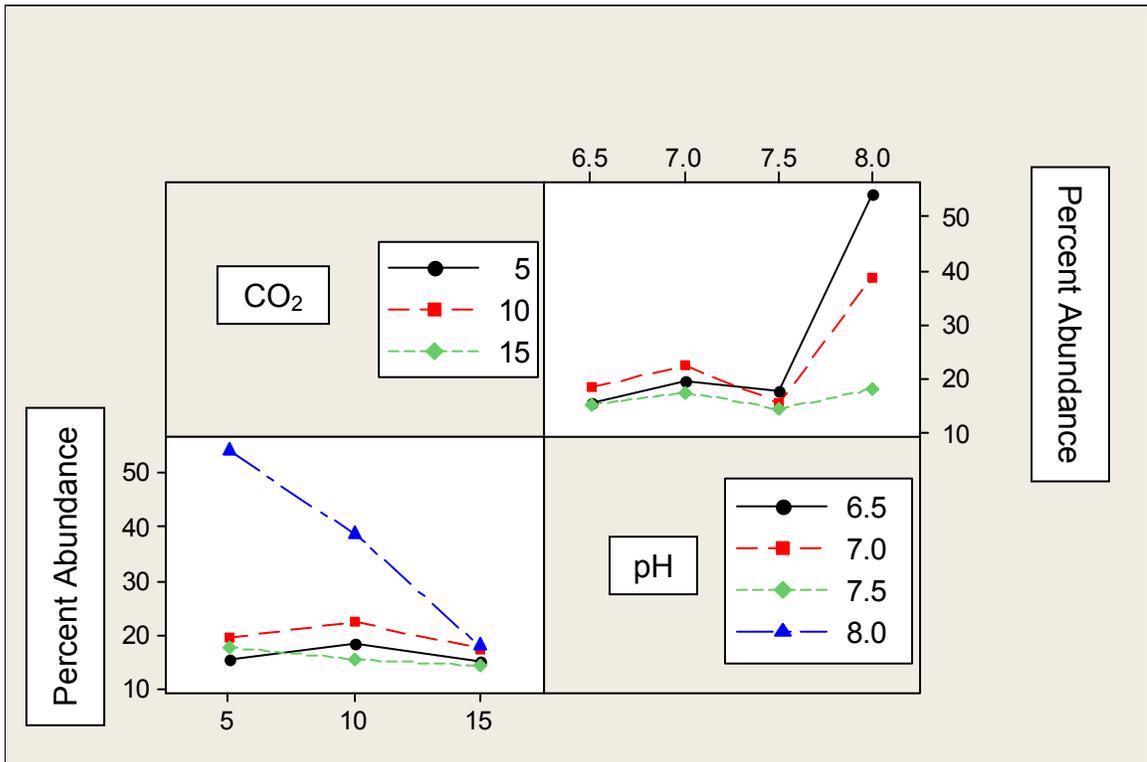


Figure 6-5: Interaction Effect of *Nitrosomonas* Bacteria

Results indicate that a higher percent abundance exists at a pH of 8.0 at the 5 and 10 mg/l dissolved CO₂ concentrations. This is not unexpected as *Nitrosomonas* has a preferred optimum pH range of 7.9 to 8.2. However, all appear to converge to a lower percent abundance as dissolved CO₂ concentrations increase for all pH levels.

6.3.1.2 Nitrosospira

Analysis of the *Nitrosospira* bacteria shows all treatment effects to be significant (Table 6-5). As the interaction is significant, analysis of the main effects is not appropriate. A graphical display and review of the interaction is provided (Figure 6-6).

Table 6-5: ANOVA of Percent Abundance of *Nitrosospira* Bacteria

Source	DF	SS	MS	F	P-Value
CO ₂	2	602.42	301.21	4.42	0.014
pH	3	4012.61	1337.54	19.62	0.000
CO ₂ *pH	6	1360.44	226.74	3.33	0.005
Error	108	7361.93	68.17		
Total	119	13337.41			

S = 8.25627 R-Sq = 44.80% R-Sq (adj) = 39.18%

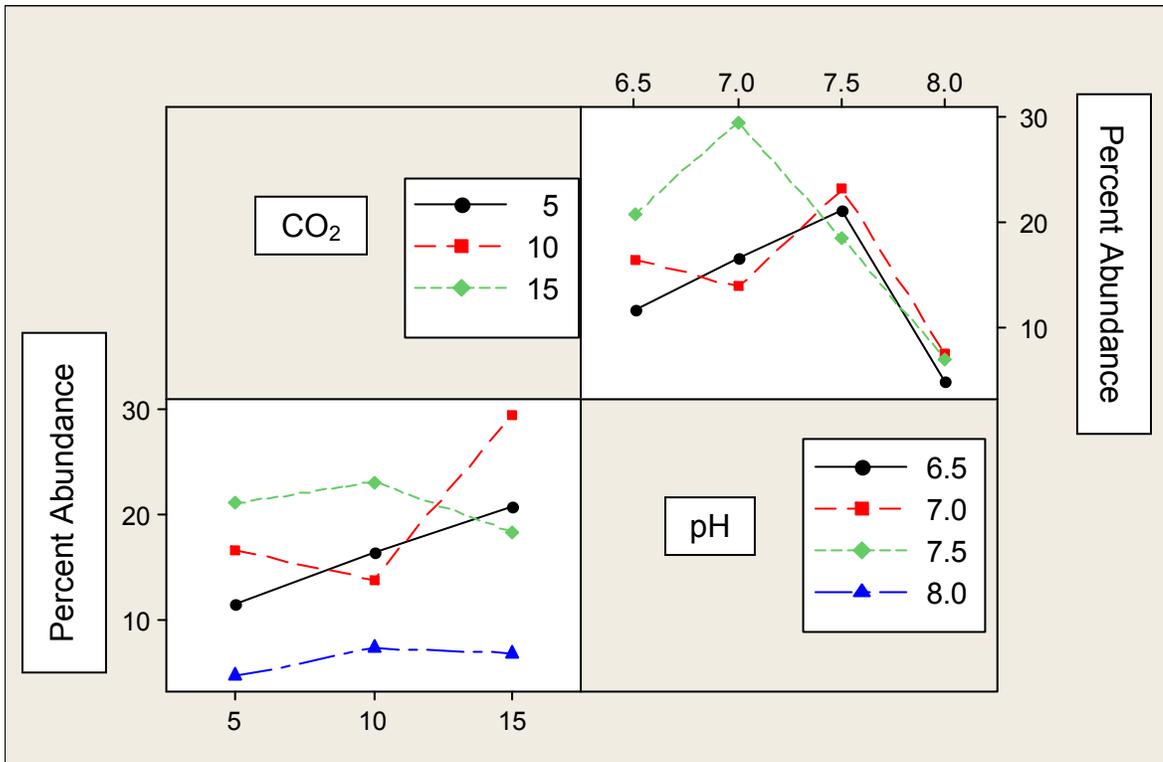


Figure 6-6: Interaction Effect of *Nitrosospira* Bacteria

Percent abundance results for the *Nitrosospira* are mixed. For two of the pH levels, 7.5 and 8.0, the percent abundances are relatively equal with the other two pH levels showing trends that increase with dissolved CO₂ concentration. However, all three dissolved CO₂ concentrations converge at a pH of 7.5 which may not be unexpected as the optimum pH for *Nitrosospira* is 7.5-8.0. At a pH of 8.0, they again converge to low percent abundances. *Nitrosomonas* also showed similar low percent abundances at a pH of 8.0 although its percent abundance was still greater than the *Nitrosospira* (18.1 versus 6.9). This may suggest another AOB may be present.

6.3.2 NOB Results

An initial analysis was conducted to determine if differences exist between the NOB bacteria obtained for use as the seed material. Operating conditions at the wastewater treatment plant from which this sample was received showed a dissolved CO₂ concentration of 34 mg/l and a pH of 6.86. A statistical difference of the abundance of the NOB was not evident (p-value = 0.152, Figure 6-7).

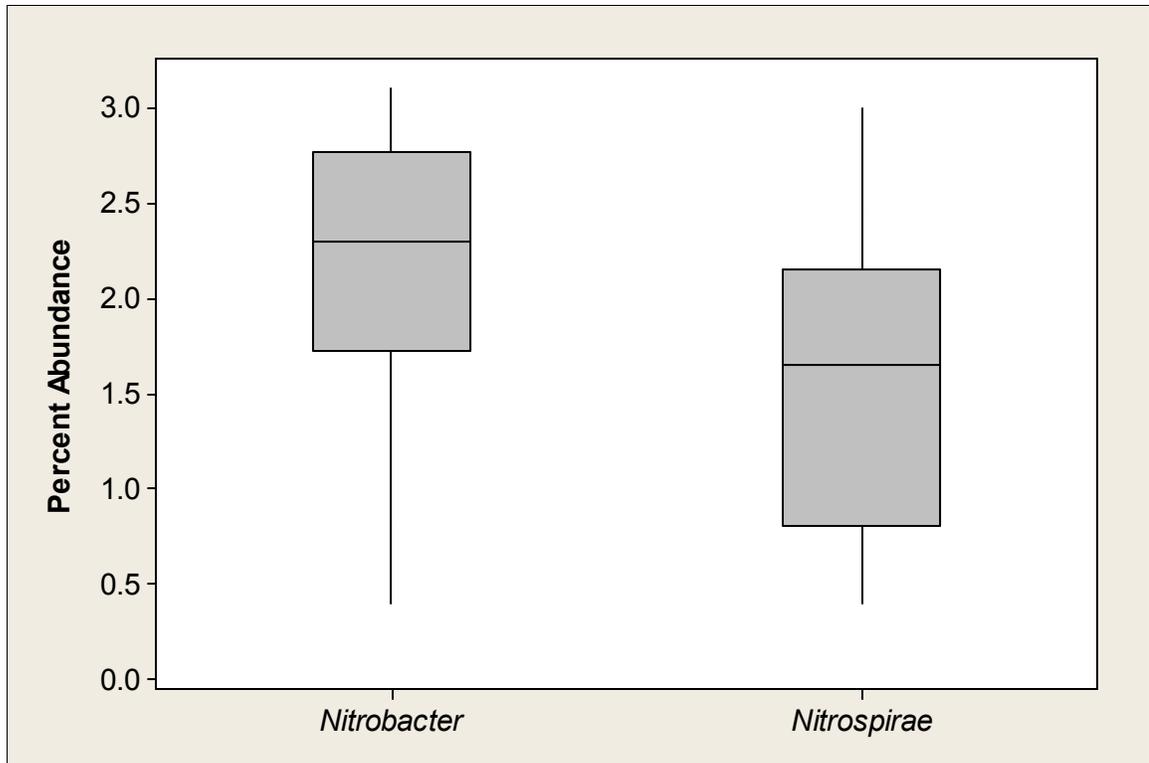


Figure 6-7: Percent Abundance of NOB Bacteria from Activated Sludge

Descriptive statistics for the NOB experimental results are presented (Tables 6-6 and 6-7).

Table 6-6: *Nitrobacter* Percent Abundance Results

Dissolved CO ₂ Conc	pH	μ_{\max}	Average Microbe Conc	Standard Deviation	Range	Coefficient of Variation	Images w/ High Accum
34	6.86		2.1	0.87	2.7	0.41	0/10
5	6.5	0.42	16.9	6.1	20.9	0.36	5/10
5	7.0		17.3	6.8	20.1	0.39	5/10
5	7.5	0.77	24.5	11.0	31.7	0.45	8/10
5	8.0	0.59	5.7	1.2	3.9	0.21	1/10
10	6.5	0.55	9.6	4.8	9.5	0.50	1/10
10	7.0	0.52	13.9	5.1	17.6	0.37	3/10
10	7.5	0.52	16.6	8.7	20.8	0.52	2/10
10	8.0	0.77	13.9	7.2	18.6	0.52	3/10
15	6.5	0.58	23.9	8.4	26	0.35	8/10
15	7.0	0.57	22.2	8.4	27.4	0.38	8/10
15	7.5	0.57	14.7	3.9	11.3	0.27	2/10
15	8.0	0.5	7.0	2.2	7.8	0.31	3/10

Table 6-7: *Nitrospirae* Percent Abundance Results

Dissolved CO ₂ Conc	pH	μ_{\max}	Average Microbe Conc	Standard Deviation	Range	Coefficient of Variation	Images w/ High Accum
34	6.86		1.6	0.83	2.6	0.52	0/10
5	6.5	0.42	20.5	6.6	19.3	0.32	3/10
5	7.0		32.7	7.6	25.4	0.23	5/10
5	7.5	0.77	22.8	7.5	24.7	0.33	5/10
5	8.0	0.59	7.7	2.5	8.4	0.32	2/10
10	6.5	0.55	17.8	3.6	10.2	0.20	3/10
10	7.0	0.52	19.9	5.8	14.0	0.29	3/10
10	7.5	0.52	21.6	3.8	13.0	0.18	4/10
10	8.0	0.77	19.4	6.3	18.6	0.32	5/10
15	6.5	0.58	15.4	6.8	18.4	0.44	3/10
15	7.0	0.57	19.1	4.0	13.2	0.21	4/10
15	7.5	0.57	13.8	4.0	13.1	0.29	3/10
15	8.0	0.5	9.8	3.3	11.4	0.34	2/10

Results show the blocking variable, NOB Type, as well as all treatment effects to be significant with *Nitrospirae* being predominant (Table 6-8). The *Nitrospirae* had a percent abundance across all treatment combinations of 18.4 percent compared to 15.5 percent for the *Nitrobacter*. As a significant interaction is present, analysis of the main effects is not appropriate. Due to the complexity of

the interaction, treatment effects will be evaluated for each NOB type microbe separately.

Table 6-8: ANOVA of Percent Abundance of NOB Bacteria

Source	DF	SS	MS	F	P-Value
NOB Type	1	491.06	491.06	11.56	0.001
CO ₂	2	319.44	159.72	3.76	0.025
pH	3	3610.68	203.56	28.34	0.000
CO ₂ *pH	6	2767.74	461.29	10.86	0.000
Error	227	9641.51	42.47		
Total	239	16830.44			

S = 6.51718 R-Sq = 42.71% R-Sq (adj) = 39.69%

6.3.2.1 *Nitrobacter*

Analysis of the *Nitrobacter* bacteria shows all treatment effects to be statistically significant (Table 6-9). As the interaction is significant, analysis of the main effects is not appropriate. A graphical display of the interaction is provided (Figure 6-8).

Table 6-9: ANOVA of Percent Abundance of *Nitrobacter* Bacteria

Source	DF	SS	MS	F	P-Value
CO ₂	2	259.04	129.52	3.16	0.046
pH	3	1825.30	608.43	14.85	0.000
CO ₂ *pH	6	2051.02	341.84	8.35	0.000
Error	108	4423.92	40.96		
Total	119	8559.28			

S = 6.40017 R-Sq = 48.31% R-Sq (adj) = 43.05%

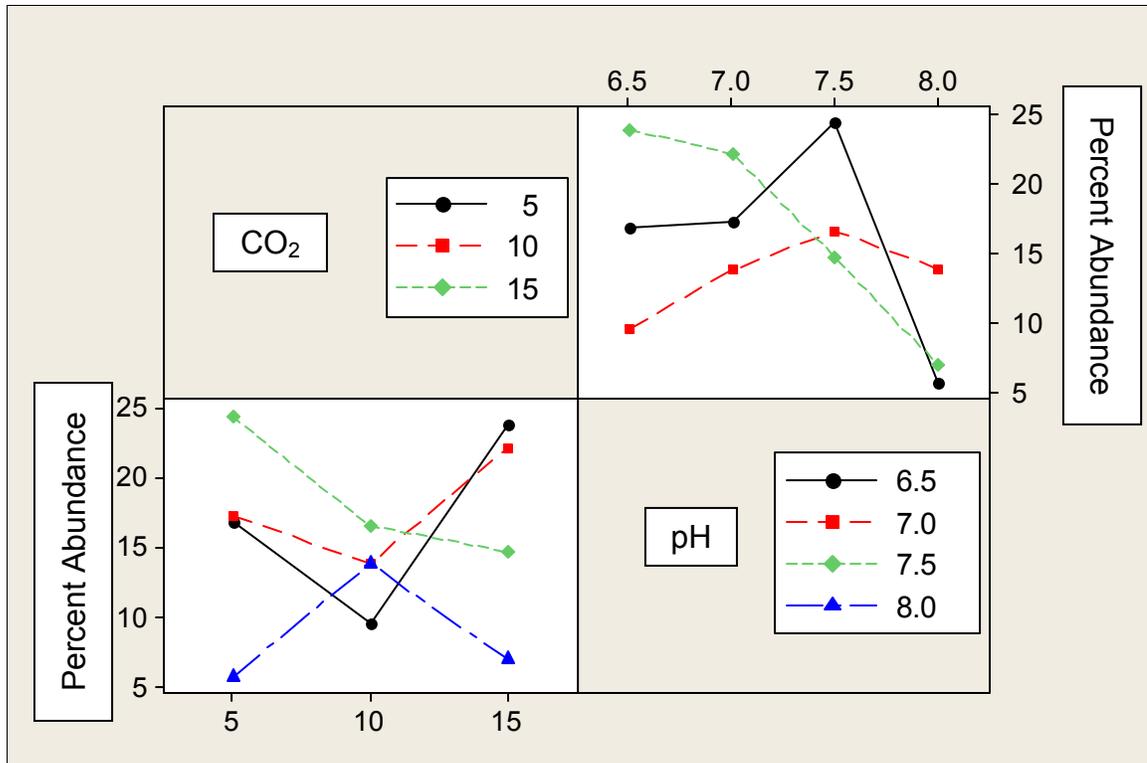


Figure 6-8: Interaction Effect of *Nitrobacter* Bacteria

Results are mixed depending on the dissolved CO₂ concentration and the pH level. The general trend shows that as pH increases, the percent abundance of *Nitrobacter* decreases. Results indicate two species of *Nitrobacter* exist. This is observed by reviewing figure 6-10. Dissolved CO₂ concentrations at 5 and 15 mg/l and at pH levels of 7.5 and 6.5, respectively, provide the greatest percent abundance. The high abundance level at a pH of 7.5 can be explained as this is the preferred pH range of this microbe (Table 2-3). The pH level of 6.5 but with a higher dissolved CO₂ concentration suggests the interrelationship of CO₂ with pH on microbe growth of another species of *Nitrobacter*.

6.3.2.2 Nitrospirae

Analysis of the *Nitrospirae* bacteria shows all treatment effects to be statistically significant (Table 6-10). As the interaction is significant, analysis of the main effects is not appropriate. A graphical display of the interaction is provided (Figure 6-9).

Table 6-10: ANOVA of Percent Abundance of *Nitrospirae* Bacteria

Source	DF	SS	MS	F	P-Value
CO ₂	2	915.27	457.64	15.57	0.000
pH	3	2059.90	686.63	23.36	0.000
CO ₂ *pH	6	1630.17	271.70	9.24	0.000
Error	108	3174.75	29.40		
Total	119	7780.10			

S = 5.42179 R-Sq = 59.19% R-Sq (adj) = 55.04%

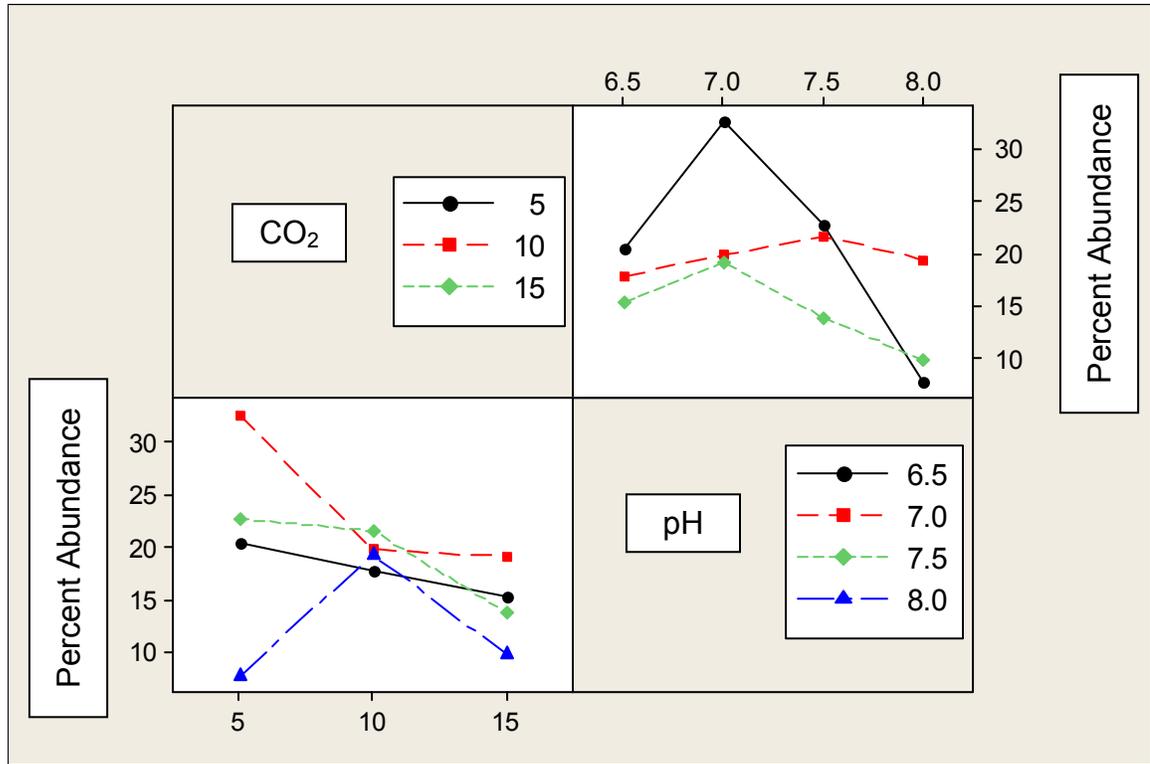


Figure 6-9: Interaction Effect of *Nitrospirae* Bacteria

Results for the *Nitrospirae* microbes are mixed. The microbial concentration appears to increase and then decrease as pH goes from 6.5 to 8.0. The percent abundance at specific dissolved CO₂ concentration is dependent on pH but at 10 mg/l their concentrations appear similar. The greatest *Nitrospirae* abundance occurs at a pH of 7.0 and a dissolved CO₂ concentration of 5 mg/l. This is not in agreement with published data for this microbe showing an optimum pH of 8.0 - 8.3 (Table 2.3). This elevated percent abundance at this dissolved CO₂ concentration/pH combination may suggest a species of *Nitrospirae* not previously identified.

6.3.3 Validation Study of FISH Results

As many of the results of the dissolved CO₂ concentration/ pH combinations exhibited high percent abundances, a study was conducted to determine if the digital image results reflected high microbial concentrations (percent abundance). Some concern existed in the digital image areas identified as high accumulation that these could be phosphate crystals or perhaps some other contaminant fluorescing and giving false readings. Six slides were prepared that included representative AOB and NOB that exhibited high abundance (Table 6-11). *E. coli* was used as a negative control and *Bacillus subtilis* was used as a positive control.

Table 6-11: Slide Preparation for Validation Study

		Slide						Comment
		1	2	3	4	5	6	
Samples	CO ₂ = 5 mg/l pH = 8.0	X	X				X	High levels of <i>Nitrosomonas</i>
	CO ₂ = 15 mg/l pH = 7.0	X		X			X	High levels of <i>Nitrospira</i>
	CO ₂ = 5 mg/l pH = 7.5	X			X		X	High levels of <i>Nitrobacter</i>
	CO ₂ = 5 mg/l pH = 7.0	X				X	X	High levels of <i>Nitrospirae</i>
	SCBWWTP	X	X	X	X	X	X	Seed material
	<i>E. coli</i>	X	X	X	X	X	X	Negative control
	<i>Bacillus subtilis</i>						X	Positive control
Fish Probes	No probe	X						Hybridization Buffer
	NSM156		X					<i>Nitrosomonas</i> probe
	Nsv433			X				<i>Nitrospira</i> probe
	NIT3				X			<i>Nitrobacter</i> probe
	Ntspa0712					X		<i>Nitrospirae</i> probe
	LGC353b [88]						X	<i>Bacillus</i> probe

Evaluation of the digital images (40X objective) for each slide revealed that the high percent abundances do reflect high AOB or NOB (Figure 6-10 to 6-15). Except for the *Bacillus subtilis* which fluoresces as it is the positive control, only the AOB and NOB nitrifying probes display fluorescence for the nitrifying bacteria.

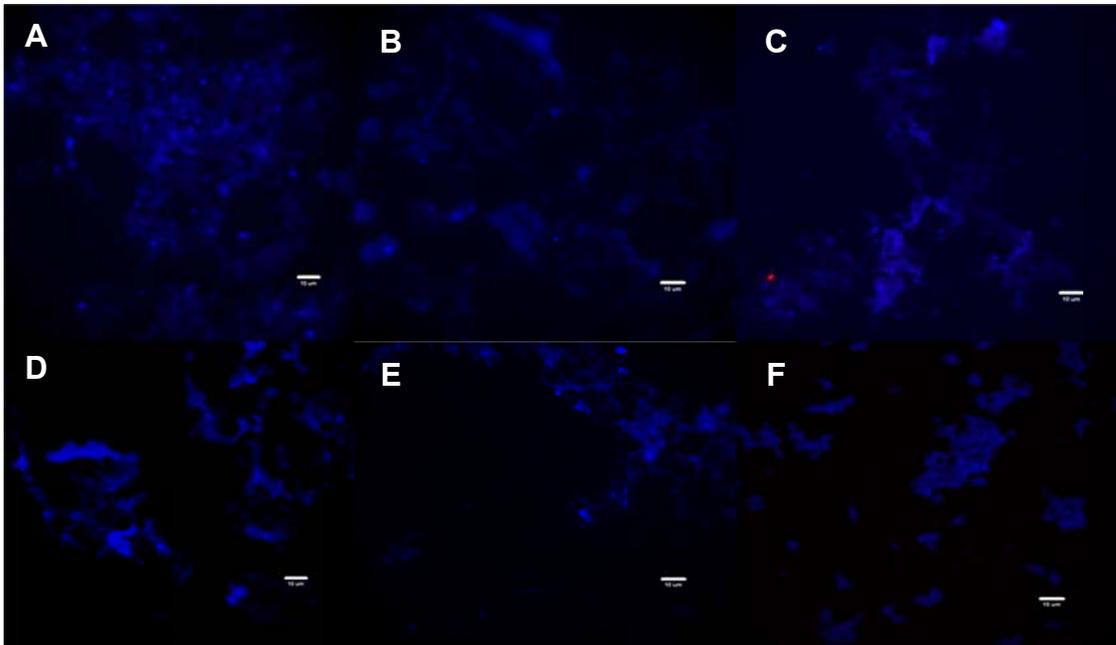


Figure 6-10: Slide 1 No Probe with (A) *Nitrosomonas*, (B) *Nitrosospira*, (C) *Nitrobacter*, (D) *Nitrospirae*, (E) Seed Material, and (F) *E. coli*. Scale bars equal 10 μm

No auto fluorescence is observed in Figure 6-10 indicating no fluorescence effect with the hybridization buffer.

The digital images in figures 6-11 through 6-14 were adjusted to be consistent with the results from the daime analysis. The DAPI and Cy3 images were evaluated to determine their brightness by evaluating the pixel value range. A ratio was calculated, termed multiplicative brightness adjustment factor, and this was used to adjust the images on each slide. On each set of slides, the DAPI image (blue) was adjusted and its multiplicative effect is listed in the figure legends. For the bioreactor and seed material merged images, a region of the image has been magnified to show the cell abundance for each AOB or NOB. The high abundance of cells for the bioreactor images (Image A of Figures 6-11

through 6-14) is evident with reduced cell abundance for the seed material image (Image B of Figures 6-11 through 6-14). The same exposure times were used on all images to maintain consistency.

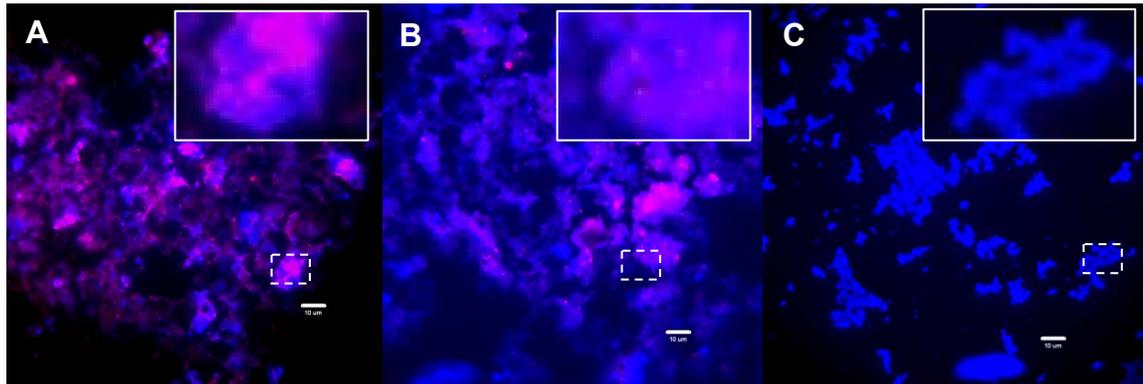


Figure 6-11: Slide 2 NSM156 Probe with (A) *Nitrosomonas*, (B) Seed Material, and (C) *E. coli* using a Multiplicative Factor of 2.6. Scale bars equal 10 µm

Some non-specific binding fluorescence is observed from the images (A and B) but this is not unexpected as FISH probes do bind to extracellular polymeric substances, which gives low level fluorescence in parts of the flocs. The small, discrete objects are cells and appear to be pear shaped indicative of the *Nitrosomonas* morphology (Table 2-3). *E. coli* is a negative control and does not exhibit any fluorescence due to this FISH probe. A magnified section can be observed in image C. Micro-colonies of cells are observed in higher levels in the bioreactor compared to the seed material. Furthermore, the micro-colonies in the bioreactors have higher levels of *Nitrosomonas* and are brighter, which indicates higher ribosome content and therefore, a higher specific growth rate [89].

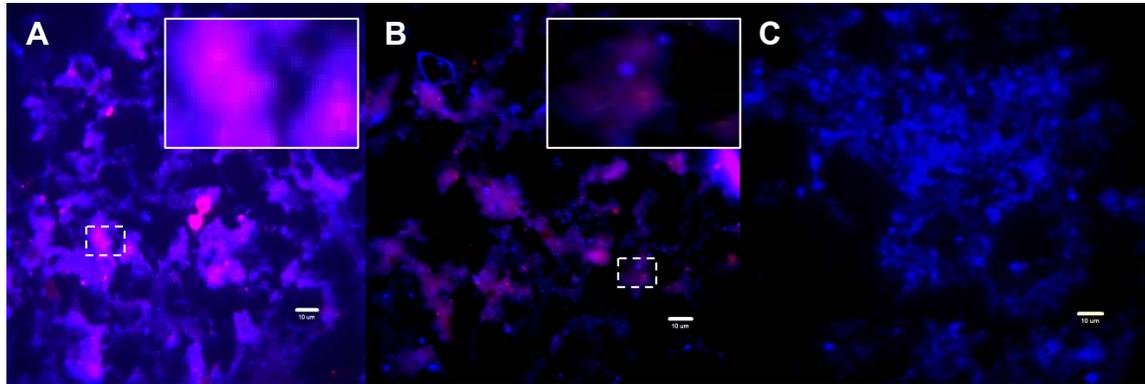


Figure 6-12: Slide 3 Nsv433 Probe with (A) *Nitrosospira*, (B) Seed Material, and (C) *E. coli* using a Multiplicative Factor of 2.3. Scale bars equal 10 µm

Some non-specific binding fluorescence is observed from the images (A and B) but this is not unexpected as FISH probes do bind to extracellular polymeric substances, which gives low level fluorescence in parts of the flocs. The small, discrete objects observed in these images should have a spiral appearance indicative of *Nitrosospira* morphology but are too small at this magnification to be positively identified (Table 2-3). *E. coli* is a negative control and does not exhibit any fluorescence due to this FISH probe. Micro-colonies of cells are observed in higher levels in the bioreactor compared to the seed material. Furthermore, the micro-colonies in the bioreactors have higher levels of *Nitrosospira* and are brighter, which indicates higher ribosome content and therefore, a higher specific growth rate [89].

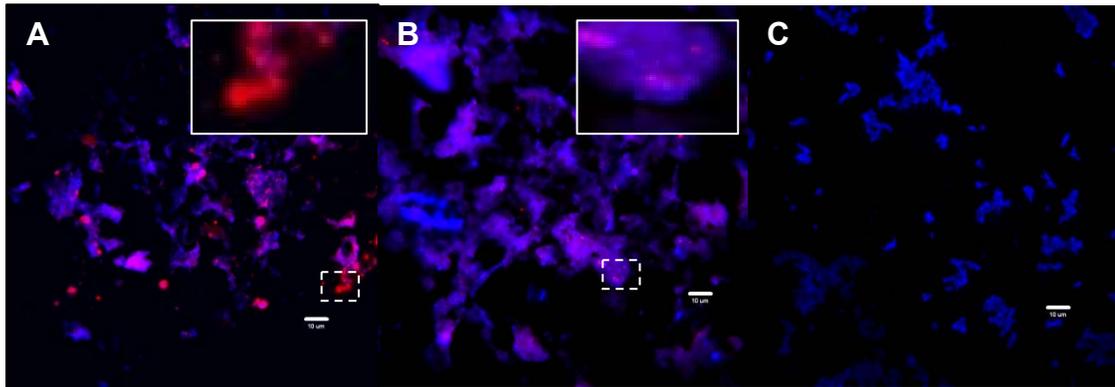


Figure 6-13: Slide 4 NIT3 Probe with (A) *Nitrobacter*, (B) Seed Material, and (C) *E. coli* using a Multiplicative Factor of 1.6. Scale bars equal 10 µm

Some non-specific binding fluorescence is observed from the images (A and B) but this is not unexpected as FISH probes do bind to extracellular polymeric substances, which gives low level fluorescence in parts of the flocs. The small, discrete objects observed in these images should have a rod shaped appearance indicative of *Nitrobacter* morphology but are too small at this magnification to be positively identified (Table 2-3). *E. coli* is a negative control and does not exhibit any fluorescence due to this FISH probe. Micro-colonies of cells are observed in higher levels in the bioreactor compared to the seed material. Furthermore, the micro-colonies in the bioreactors have higher levels of *Nitrobacter* and are brighter, which indicates higher ribosome content and therefore, a higher specific growth rate [89].

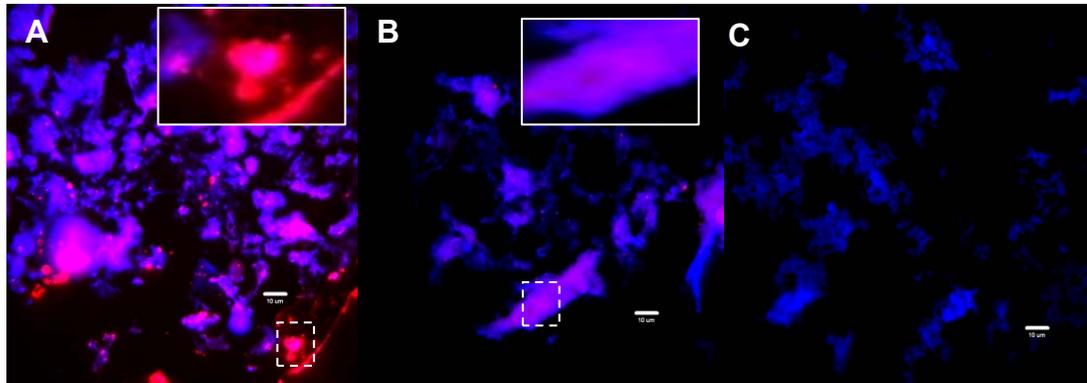


Figure 6-14: Slide 5 Ntspa0712 Probe with (A) *Nitrospirae*, (B) Seed Material, and (C) *E. coli* using a Multiplicative Factor of 1.9. Scale bars equal 10 µm

Some non-specific binding fluorescence is observed from the images (A and B) but this is not unexpected as FISH probes do bind to extracellular polymeric substances, which gives low level fluorescence in parts of the flocs. The small, discrete objects observed in these images should have a long slender rod appearance indicative of *Nitrospirae* morphology but are too small at this magnification to be positively identified (Table 2-3). *E. coli* is a negative control and does not exhibit any fluorescence due to this FISH probe. Micro-colonies of cells are observed in higher levels in the bioreactor compared to the seed material. Furthermore, the micro-colonies in the bioreactors have higher levels of *Nitrospirae* and are brighter, which indicates higher ribosome content and therefore, a higher specific growth rate [89].

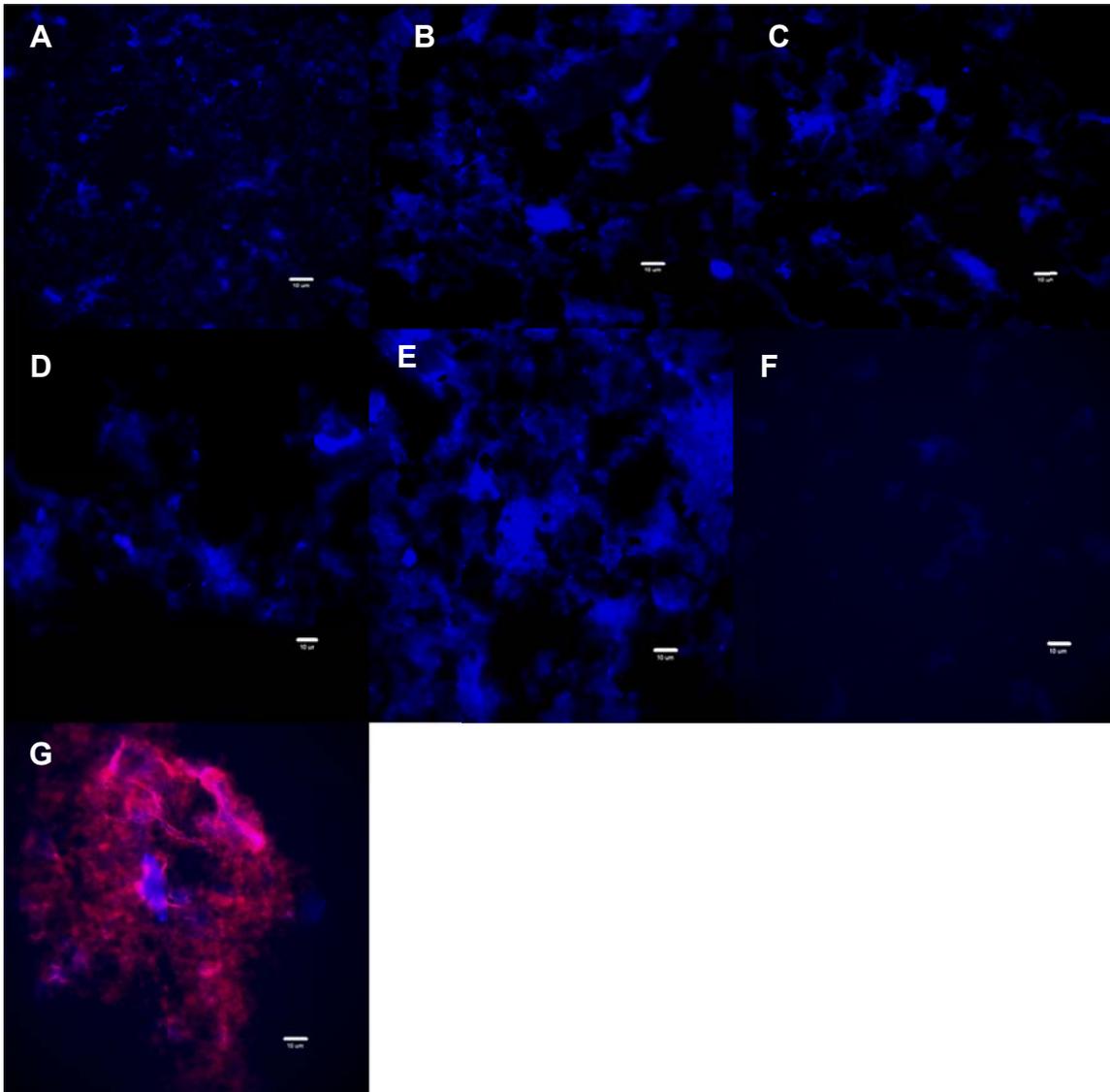


Figure 6-15: Slide 6 LGC353b Probe with (A) *Nitrosomonas*, (B) *Nitrospira*, (C) *Nitrobacter*, (D) *Nitrospirae*, (E) Seed Material, (F) *E. coli*, and (G) *Bacillus subtilis*. Scale bars equal 10 μm

Micro-colonies of cells are observed in the *Bacillus subtilis* image. This result is expected as this microbe was used as a positive control. The other images do not show any fluorescence indicating that the observed high abundance reported during the daime analysis do represent high accumulations of AOB and NOB.

During this study, an image of *Nitrospirae* was taken showing high abundance of individual cells and high accumulation (Figure 6-16). This digital image is provided as an example of nitrifiers under conditions highly conducive to growth.

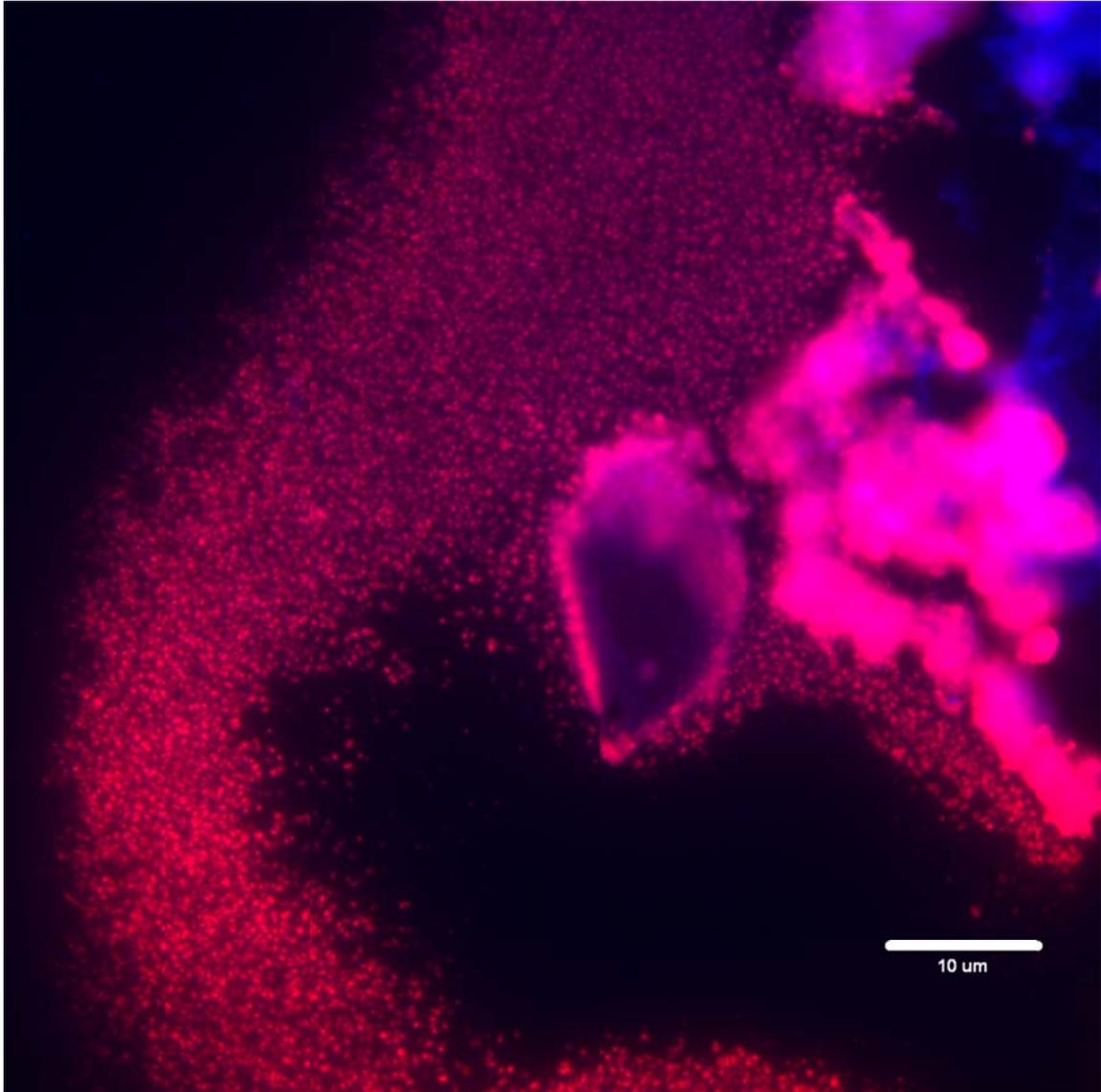


Figure 6-16: *Nitrospirae* Shown with High Accumulation of Cells (100X Objective)

6.3.4 Biomass Growth Determination

A model was developed to ascertain the abundance of the nitrifiers at the conclusion of a reactor study. This was done as a check to ensure that the

reported percent abundance could be achieved. Two simultaneous equations, the substrate utilization rate $\left[\frac{dS}{dt} = -\left(\frac{\hat{q}S}{K+S}\right) \cdot X_a\right]$ and the biomass growth rate $\left[\frac{dX_a}{dt} = \left(\hat{\mu} \frac{S}{K+S} - b\right) \cdot X_a\right]$, were evaluated using an iterative approach. Both heterotrophic and autotrophic bacteria were evaluated using MATLAB (Natick, MA) and a ratio of their biomass concentrations was calculated. Growth was based on Monod kinetics and standard kinetic coefficients were utilized [2, 12, 63]. After 10 days of reaction, the autotrophs are approximately 85% of the biomass (Figure 6-17). The biomass concentrations, depicted on the left vertical axis, show the microbe growth and decay over the 10 day period. The percent of autotrophic biomass to total biomass is shown on the right vertical axis.

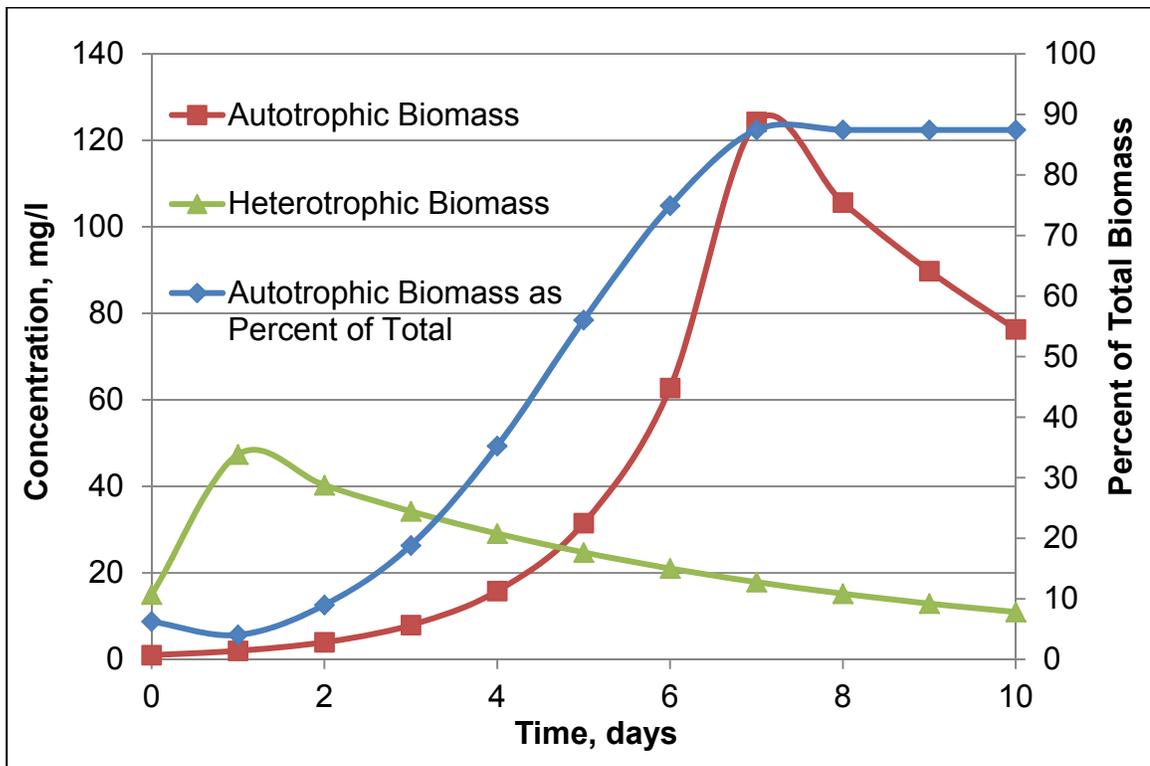


Figure 6-17: Autotrophic Biomass as Percent of Total Biomass to Confirm High Percent Abundance Measurements

6.4 Discussion

Before reviewing and commenting on the results of this study, a proper microbial assessment could not have been undertaken without knowledge and expertise on the use of the daime software. Appropriate time was spent (six hours) in developing the expertise to ensure proper segmentation was conducted. Interpreting the digital images prior to segmentation can be considered “artsy” and therefore care was taken to ensure consistent results were attained.

Statistical model assumptions were generally satisfied but some departures in normality of error terms and differences in dissolved CO₂ concentrations/ pH combination variances were observed. This is not unexpected given the range of microbe percentages seen during the evaluation. As these deviations were not severe and statistical models are robust, data transformations were not undertaken. In addition, outliers were not removed as they added to model understanding.

The activated seed sludge was obtained from the discharge side of the aeration basin of a modified Ludzack-Ettinger wastewater facility. Process measurements reported in this study, 34 mg/l dissolved CO₂ concentration and a pH of 6.86, remained constant from samples obtained over several years from this plant location. The four microbes evaluated in this study were all identified in this activated sludge. *Nitrosospira* was the dominate AOB and statistically greater

than *Nitrosomonas* while the NOB microbes, *Nitrobacter* and *Nitrospirae*, exhibited similar abundances. These abundances are invariably due to influent and plant operating conditions.

Analysis of the dissolved CO₂ concentration/ pH combinations does present challenges. Those combinations with the higher microbe abundances could be said to predominate and thus be the preferred set of operating conditions. This interpretation is too simple. Interpretation of the results requires that the maximum specific growth rate as well as optimum growth conditions suggested from literature be used in conjunction with the dissolved CO₂ concentration/ pH combinations. Achieving a high abundance of a particular microbe at a high growth rate may not coincide. In fact, this occurred during this study.

The AOB microbes exhibit their greatest maximum specific growth at a pH of 8.0 for each dissolved CO₂ concentration (figure 5-6). This is in agreement with literature for optimum pH growth conditions for *Nitrosomonas* (7.9 – 8.2). *Nitrosospira* exhibited its greatest microbe percentage at a pH of approximately 7.0 and a dissolved CO₂ concentration of 15 mg/l. Other high abundance occurred at a pH of 7.5 for the 5 and 10mg/l dissolved CO₂ concentration. This may suggest a shift in ideal growth conditions as dissolved CO₂ concentrations change or the optimum growth conditions may exist in the pH range of 7.0 to 7.5. Literature provides an optimum pH range for *Nitrosospira* of 7.5 to 8.0 but cited references are not available.

Evaluation of the seed material showed the predominance of the *Nitrosospira* microbe. However, at a pH of 8.0 and across all dissolved CO₂ concentrations, *Nitrosomonas* was dominant. Based on ratios when compared to *Nitrosospira*, this was 11.3, 5.3 and 2.6 times greater at 5, 10 and 15 mg/l, respectively.

Nitrosospira was dominant at a pH of 7.5; and when compared to *Nitrosomonas*, averaged approximately 23 percent greater microbe abundance. However, this concentration difference does not compare to the predominance of *Nitrosomonas* at a pH of 8.0. Even though the highest abundance of *Nitrosomonas* was observed at a dissolved CO₂ concentration of 5 mg/l, the highest maximum specific growth rate occurred at a dissolved CO₂ concentration of 10 mg/l. Thus, suggesting the synergistic effect of dissolved CO₂ concentration with pH on microbe growth and a combination of AOB at these conditions.

Although the NOB microbes were found to be statistically different, their numerical differences in percent abundance were not pronounced (18.4 versus 15.5 for *Nitrospirae* and *Nitrobacter*, respectively). At a pH of 7.0 and a dissolved CO₂ concentration of 5 mg/l, the *Nitrospirae*, which has an optimum growth pH of 8.0 – 8.3, was approximately twice the percent abundance as the *Nitrobacter*. Thus, suggesting that another *Nitrospirae* species may exist not previously identified. At a pH of 7.5, where *Nitrobacter* has an optimum growth pH of 7.2 – 7.6, the percent abundance of the *Nitrobacter* and *Nitrospirae* microbes were approximately equal. And at a pH of 8.0, where *Nitrospirae* has an optimum

growth pH of 8.0 – 8.3, *Nitrospirae* exhibits approximately a forty percent greater abundance than *Nitrobacter*. Although this result is to be expected, the percent abundance of the *Nitrospirae* at the pH 8.0 level across all dissolved CO₂ concentrations was still lower than at other pH levels. Interestingly, some NOB results were expected but others did not match expected optimum pH based on microbial percent abundances.

The highest maximum specific growth rates for the NOB microbes occurred at dissolved CO₂ concentrations and pH values of 5 mg/l and a pH of 7.5; and 10 mg/l and a pH of 8.0, respectively (Figure 5-8). Each shows a maximum specific growth rate of 0.77 with approximately the same percent microbial abundance. This suggests that neither NOB microbe is predominant; with both contributing to conversion of nitrite to nitrate.

Figures 5-6 to 5-9 in chapter 5 show the effect of μ_{max} at the dissolved CO₂ concentration/ pH level combinations. It is evident that differences do exist but identifying specific microbes that are predominant at these dissolved CO₂ concentration/ pH level combinations has not been successful in all cases. As the aeration basin of a WWTP has been said to be a complex microbial community probably containing several different genera of microbes capable of nitrification [23]; a combination of different AOB/ NOB appears a more likely scenario in assessing the relationship between dissolved CO₂ concentration/ pH level combinations, microbe abundance and nitrification growth rates.

Of particular interest is the effect of dissolved CO₂ concentration on the abundance of the *Nitrosomonas* microbe. The percent abundance values are much greater than for the *Nitrosospira*, *Nitrobacter* and *Nitrospirae* microbes which exhibit equivalent abundances. This may suggest that these microbes are more sensitive to the effects of carbon dioxide.

It has been shown that μ_{\max} is affected by dissolved CO₂ concentration and pH but which effect, if any, dominates at these experimental conditions (Figures 5-6 to 5-9 in chapter 5). A partitioning of the treatment sums of squares was undertaken (Table 6-11). The AOB and NOB were compared to the designed experiment that optimized the dissolved CO₂ concentration/ pH combination (experiment three from chapter 5). Percent contribution for the dissolved CO₂ concentration, pH, and the dissolved CO₂ concentration/pH interaction were calculated. These are compared to the main effects previously reported as well as the R² generated from their ANOVA analysis (Figures 5-6, 6-4, and 6-10).

Table 6-12: Partitioned Treatment Sums of Squares by Treatment Effect

Microbe	R²	CO₂	pH	CO₂/pH Interaction
Chapter 5 Experiment 3	92.5	17.4	82.6	NA
<i>Nitrosomonas</i>	42.7	26.1	49.2	24.7
<i>Nitrosospira</i>	48.9	4.3	72.2	23.5
<i>Nitrobacter</i>	48.3	6.3	44.1	49.6
<i>Nitrospirae</i>	59.2	28.2	63.4	8.4

With one exception, pH dominated the maximum specific growth rate of the microbes. Only for the *Nitrobacter* microbe did the CO₂*pH interaction show a

higher percent contribution. This is due to increased variation seen across treatment combinations. Even so, it was still significantly greater than the percent contribution of the dissolved CO₂ contribution.

The validation study showed that the high percent abundance measurements were genuine when using these experimental reactor operating conditions. The initial concerns of phosphate crystals generated during the experiment or other material fluorescing were not warranted. Calculation of autotrophic biomass as a percent of total biomass confirmed that high levels of percent abundance measurements are possible when reactor operating conditions select for AOB or NOB.

6.5 Conclusions

The maximum specific growth rate of nitrifying bacteria is influenced by dissolved CO₂ concentration and pH. Though each contributes to enhancing the nitrification rate, pH has a more pronounced influence. This is evidenced by larger F values for the pH effect from ANOVA source tables, the percent influences of the treatment sums of squares, and the largest nitrification rates occurring at pH values of 7.5 and 8.0, depending on the microbe.

The AOB bacteria appear to have the most influence on nitrification rates with dissolved CO₂ concentrations of 10 mg/l or 15 mg/l at a pH of 8.0 providing the

highest nitrification rates. Based on literature and statistical analysis of this research, *Nitrosomonas* is the predominant AOB microbe at these dissolved CO₂ concentrations and pH combinations although its percent microbial abundance was not pronounced at 15 mg/l. As both *Nitrosomonas* and *Nitrospira* had low percent microbial abundance at this dissolved CO₂ concentration, a third unidentified AOB may be present. Optimizing conditions for the growth of AOB microbes is necessary if maximum specific growth rates are to be realized.

The NOB microbes are statistically different based on their percent concentrations across the dissolved CO₂ concentration and pH combinations with *Nitrospirae* being dominant. However, at many combinations to include those conditions that provide the maximum specific growth rate, $\mu_{\max} = 0.77$ (Figure 5-9), their concentrations are equivalent. Thus, suggesting that both NOB microbes, *Nitrobacter* and *Nitrospirae*, contribute to the nitrification rate.

This study was based on dissolved CO₂ concentrations ranging from 5 to 15 mg/l, pH levels from 6.5 to 8.0, and non-limiting substrate (ammonium) and dissolved oxygen levels. These combinations provided for optimum growth conditions based on many previously conducted experiments. As it has been suggested that activated sludge is comprised of a diverse microbial ecology [23], similar results should be achieved using seed material from other wastewater treatment processes. However, whether similar percent abundance results will be achieved using experimental conditions specific to WWTP's other than a

Modified Ludzack-Ettinger process, which this experiment is based upon, is unknown. Appropriate experimentation would need to be conducted to determine optimum dissolved CO₂ concentration and pH levels for process condition typical of other treatment processes.

Chapter 7

Conclusions

The original hypothesis stated that nitrification was limited due to reduced levels of carbon dioxide in the aeration basin of a wastewater treatment facility. This was based on the premise that the aeration basin is in equilibrium with the atmosphere. Subsequent field testing revealed this assumption to be incorrect with elevated levels of carbon dioxide found throughout a wastewater treatment facility.

Research focused on understanding the effects of carbon dioxide and pH on nitrification and determining if an optimum dissolved CO₂ concentration/ pH combination exists that maximizes nitrification. Experimentation revealed that at low (< 5 mg/l) and high (> 30 mg/l) dissolved CO₂ concentrations inhibition effects are apparent. Further research found a dissolved CO₂ concentration of 10-15 mg/l and a pH of 8.0 to provide for optimum nitrification.

Microbial studies were conducted on the designed experiment that determined the optimum dissolved CO₂ concentration/ pH combinations using the two most common AOB and NOB. Results were mixed depending on the dissolved CO₂

concentration/ pH combinations, but across all levels *Nitrosomonas* was the dominant AOB with *Nitrospirae* being the dominant NOB. Additionally, high abundance measurements for some dissolved CO₂ concentration/pH combinations that were not at optimum pH suggest that these genera have multiple members (i.e., species) with different growth sensitivities.

Based on these results, future research should focus on the following items:

- Pilot Plant or Full-Scale Demonstration: Evaluation of the optimal dissolved CO₂ concentration/pH on the rate of nitrification at a WWTP will validate this research.
- Elevated pH Operating Protocol: Establishing an effective pH control methodology that adjusts and maintains an appropriate pH is not without challenges. Treatment of the influent and sequential metering locations would need to be established. In addition, the effluent pH may need adjustment to a lower pH in order to comply with an existing permit. As there are many different WWTP configurations, a customized approach for each facility would probably be necessary. This should be conducted prior to a pilot plant or full-scale demonstration.
- Treatment of High Ammonium Levels in Anaerobic Digester Supernatant: Several WWTPs that operate anaerobic digesters were found to contain

ammonium levels from 600-1,000 mg/l and had dissolved CO₂ concentrations of 100 mg/l. The treated solids of these digesters were disposed by application onto agricultural land, removal of the solids for disposal in a sanitary landfill or drying of the solids for subsequent sale as a fertilizer. In processes where the solids are removed leaving a liquid supernatant high in ammonia and dissolved CO₂ concentration, the supernatant is returned to the head works of the WWTP for treatment. For WWTP's treating their supernatant, this liquid is mixed with the influent at approximately 15 percent of the influent flow rate. A strategy to treat the supernatant would benefit the WWTP by removing this nitrogen source and improve their treatment capabilities.

- Low F/M Experimentation: Research in this study focused on high F/M in experimentation. Evaluation of the low F/M should produce similar results as found in this study, but confirmation is needed.

The current regulations for nitrate concentrations in drinking water have been set to 10 mg/l in the USA, Japan and Korea and 11.3 mg/l for the European Union. Levels may be permitted lower at the state or local level [90-92]. With the recent proposals by the Environmental Protection agency (EPA) to establish nutrient criteria for the State of Florida which in many cases are much lower than currently permitted, this research could prove very beneficial in meeting these proposed standards [93].

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