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**VOLATILE FATTY ACID EFFECTS ON NITRITE REMOVAL AND
NITRATE FORMATION DURING ACTIVATED SLUDGE
TREATMENT**

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Merve Oguz

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DEDICATION

This dissertation is dedicated to
my beautiful daughter ZEYNEP
and
my baby son EDIZ

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ABSTRACT

Reaction sequences of nitrifying and denitrifying bacteria are widely used to eliminate nitrogenous compounds from wastewater. During nitrification, ammonia is oxidized to nitrite by autotrophic ammonia oxidizing bacteria and nitrite is further oxidized to nitrate by nitrite oxidizers. Subsequently, nitrate or nitrite is reduced by denitrifying bacteria to gaseous nitrogen compounds. It is common knowledge that nitrification is an aerobic process and denitrification an anaerobic process. However, recent research has shown that denitrification can occur under aerobic conditions in pure cultures. Volatile fatty acids (VFAs), produced during anaerobic treatment processes, can affect both nitrite oxidation and aerobic denitrification. VFAs were shown to reduce nitrate formation via nitrite oxidation in activated sludge systems and to stimulate aerobic denitrification in pure cultures. Nitrite removal inhibition by VFAs observed in activated sludge systems may be due to the level of aerobic denitrification which occurs. Investigation of this possibility can provide a new insight for the removal of nitrogen from wastewater and possibly reduce the chemical and energy demand for nitrogen treatment. The overall goal of this research was to demonstrate that nitrification and denitrification could occur in the same reactor under aerobic conditions in the presence of VFAs.

The impact of VFAs on nitrite removal and nitrate formation in activated sludge systems was studied in batch and CSTR experiments. The experimental work included measurements of nitrite removal, nitrate formation and CO₂ fixation in the absence and presence of VFAs. In addition, molecular tools were applied to examine changes in microbial population density when the population was exposed to VFAs. Production of

N₂O and activity of periplasmic nitrate reductase enzyme (NAP) which catalyses the first step of aerobic denitrification were also analyzed.

Nitrite removal and nitrate formation rates were reduced in the presence of VFAs in batch experiments. Nitrate formation rate was reduced to a greater extent (74%) than nitrite removal rate (35%) indicating that products other than nitrate were formed during nitrite oxidation. The addition of VFAs into an activated sludge CSTR treating municipal wastewater resulted in a rapid decrease in nitrate formation rate (> 70% reduction); however, nitrite removal rate was not reduced. No nitrogen was discharged in the effluent of the CSTR indicating that nitrogenous compounds were completely removed from the wastewater. In contrast, VFAs were not found to impact carbon dioxide fixation efficiency in either batch or CSTR experiments although it is generally believed to be limited by the availability of energy derived from nitrite oxidation. Non-inhibitory effect of VFAs on carbon dioxide fixation implied that VFAs disturb nitrite removal and nitrate formation by a different system other than nitrite oxidation. Additionally, the number of nitrite oxidizing bacteria (NOB) remained relatively constant in the presence of VFAs indicating that any reduction observed was not due to a decrease in NOB. N₂O gas was produced in the presence of VFAs which was a clear indication that aerobic reduction of nitrite and/or nitrate occurred. It appeared that aerobic denitrification was responsible for the unbalanced nitrification conversions in the presence of VFAs. Also, the activity of NAP enzyme increased when VFAs were present suggesting a significant role of aerobic denitrification during nitrogen conversions.

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CHAPTER 1

INTRODUCTION

1.1 Background

The biological elimination of nitrogen in wastewater treatment plants generally results from the combined processes of nitrification and denitrification. Reducing sewage nitrogen levels is necessary since discharges containing nitrogen can be toxic to aquatic life, cause oxygen depletion and eutrophication in receiving waters, and affect chlorine disinfection efficiency (Metcalf and Eddy, 1991). Nitrification and denitrification processes are two separate reactions performed by different groups of microorganisms. However, nitrite and nitrate are intermediates of both nitrification and denitrification and, therefore, can link the two processes chemically.

Nitrification is the chemolithautotrophic oxidation of ammonia to nitrate, an aerobic biochemical process conducted in two sequential oxidative stages: ammonia to nitrite (1st stage) and nitrite to nitrate (2nd stage). Each stage is performed by different bacterial genera capable of either ammonia or nitrite oxidation. These microorganisms use ammonia and nitrite as an energy source. No known single autotrophic microorganism performs both ammonia oxidation and nitrite oxidation.

The major source of organic carbon for nitrifying bacteria is carbon dioxide that is fixed to glucose via the Calvin cycle. All the reactions of CO₂ fixation use ATP and reducing power from NADPH generated during oxidation of the inorganic nitrogen

compounds. In this cycle, CO_2 is converted into a set of more complex sugars (like glucose), which are then assembled into the macromolecules comprising proteins, carbohydrates, lipids, nucleic acids, and other cell components (Bock et al., 1991).

Denitrification is a microbial process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production. Nitrate is reduced to nitrogen gas by heterotrophic microorganisms that use nitrate and/or nitrite instead of oxygen as electron acceptors and utilize organic matter in the wastewater as the carbon and energy source. Because denitrification is considered to be an anaerobic process, it is generally performed in a secondary anaerobic reactor following nitrification. However, nitrified effluents to a secondary reactor are low in organic matter. This negatively impacts the overall nitrogen removal efficiency via denitrification because the denitrifying organisms are heterotrophic and require organic carbon. In most cases, biodegradable organic matter is added to solve this problem; however, such additions increase the cost of treatment. Thus, new technologies and processes that enhance the nitrification-denitrification cycle under the same operational conditions have been sought (e.g. single reactor).

Although denitrification is generally considered an anaerobic process, this biotransformation mechanism has been observed under aerobic conditions in bacteria from at least four different genera (Robertson and Kuenen, 1990; Robertson et al., 1989; Bonin and Gilewicz, 1991; and Thomas et al., 1994). Conceivably, nitrification and denitrification could occur concurrently in the same reaction vessel under identical aerobic operating conditions with nitrite as an intermediate of both nitrification and denitrification. It appears that the primary difference between aerobic and anaerobic

denitrification involves the enzymes catalyzing the first step of nitrate reduction to nitrite. Two types of nitrate reductases can be expressed in denitrifying bacteria. One is the respiratory membrane-bound nitrate reductase and the other is the periplasmic nitrate reductase. The membrane-bound enzyme has an active site in the cytoplasm whereas the periplasmic enzyme is located in the periplasmic compartment. The periplasmic enzyme catalyses aerobic nitrate respiration while expression and activity of the membrane-bound enzyme are normally restricted to anaerobic conditions (Bell et al., 1990; 1993; Richardson and Ferguson, 1992). Complete aerobic denitrification requires the sequential action of four enzymes, periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS). Recently, the *nap*, *nir* and *nor* genes have been found to be linked (Philippot, 2002). Once the NAP enzyme becomes activated, other denitrification enzymes become active resulting in denitrification products. Bell and Ferguson (1991) showed that NOR and NOS were also active in the presence of oxygen.

The introduction of volatile fatty acids (VFAs) into activated sludge treatment systems is of growing concern as more anaerobically treated waste streams containing VFAs are subsequently routed through aerobic treatment processes. Anaerobic processes are typically operated to convert biodegradable particulate organic matter into VFAs (Rittmann and McCarty, 2000). Additionally, nitrogen contained in the organic material is released as ammonium (Grady et al., 1999). Therefore, due to the high concentrations of VFAs and ammonia in the effluent, anaerobic treatment of wastewater must be followed by aerobic process to lower BOD₅ levels and biotransform ammonia prior to

environmental discharge. Since ammonia transformation is typically achieved via nitrification, VFA effects on the process can impact treatment performance.

Previous studies have shown that VFAs affect both nitrification in activated sludge systems and aerobic denitrification in pure cultures; however, the effects of VFAs on both processes during activated sludge treatment have not been addressed. In activated sludge systems, VFAs were found to reduce nitrate formation by 24 to 70% with no effect on ammonia oxidation (Eilersen et al., 1994; Takai et al., 1997; Gomez et al., 2000). In these studies, the activity of nitrite oxidizing bacteria (NOB) was assessed based on the rate of nitrate production. Because of the decline in nitrate formation, researchers concluded that VFAs inhibited nitrite oxidation via NOB. However, these studies did not directly measure the activity of NOB to confirm the inhibition of nitrite oxidation in the presence of VFAs, and thus, did not determine the exact reason behind the reduction in nitrate formation.

Additionally, VFAs have been shown to affect aerobic denitrification. In a separate line of research based on the study of pure cultures, neutralized VFAs, especially butyrate, were found to activate the periplasmic nitrate reductase enzyme, the enzyme catalyzing the first step of aerobic denitrification (i.e. reduction of nitrate to nitrite) (Richardson and Ferguson, 1992; Ellington et al., 2002, 2003; Sears et al., 1997, 2000). The level of enzyme activity was largely dependent on carbon source and was lowest on malate, intermediate on acetate and highest on butyrate and caproate (Richardson and Ferguson, 1992). In addition, Sears et al. (1997) found that in cultures exposed to butyrate, the expression of periplasmic nitrate reductase and the rate of nitrate reduction were all high at all oxygen concentrations evaluated. The implication of these findings is

that the corespiration of oxygen and nitrate may be particularly important environments which are rich in reduced carbon.

Based on results of the aforementioned studies, nitrite removal and the apparent nitrate formation inhibition observed in activated sludge studies assessing the effects of VFAs on nitrification may be due to the level of aerobic denitrification which occurs. If this is the case, this process can contribute substantially to overall nitrogen removal because it occurs directly in aerated bioreactors in the presence of VFAs. Although this system offers several potential advantages, it cannot be fully realized until the mechanism responsible for VFA effects on nitrite oxidation is better characterized. The focus of the present research is a novel pathway for nitrite removal which can be stimulated due to VFAs formed during the anaerobic process.

1.2 Hypothesis and Objectives

The primary hypothesis of this research was that VFAs do not inhibit the nitrite oxidation pathway as previously suggested (Eilersen et al., 1994; Takai et al., 1997; Gomez et al., 2000) but rather these compounds stimulate the heterotrophic aerobic denitrification pathway during activated sludge treatment. The secondary hypothesis was that nitrite oxidation and aerobic denitrification can occur concurrently in the presence of VFAs.

The specific objectives of this research were to

1. Study the effects of VFAs (using acetic acid, propionic acid, and butyric acid) on nitrite removal and nitrate formation during activated sludge treatment.
2. Assess CO₂ fixation in the presence of VFAs as a measure of NOB activity.
3. Examine changes in the number of NOB after the addition of VFAs to investigate whether any decrease in nitrate formation was due to a numerical decline of NOB.
4. Explore the production of gaseous nitrogen compounds in the presence of VFAs under aerobic conditions to determine if aerobic denitrification occurred.
5. Examine the activity of periplasmic nitrate reductase in the presence of VFAs to study whether VFAs stimulated this enzyme.
6. Analyze genes for periplasmic nitrate reductase in mixed liquor to examine the possibility of aerobic denitrification in the presence of VFAs.
7. Examine protein concentration in biomass in the presence of VFAs to investigate if nitrite and/or nitrate assimilation were the primary pathway of nitrogen removal by bacteria.

To evaluate these objectives, a range of methods was used including:

- Chemical analysis (measurement of nitrite and nitrate concentrations and determination of the nitrite removal and nitrate formation rates based on these concentrations)
- Radiolabeled ¹⁴C experiments (measurement of CO₂ fixation by NOB)

- Enzymatic assays (Periplasmic nitrate reductase enzyme)
- Gas measurement (N_2O)
- Molecular experiments (Real-time PCR to measure total bacteria and *Nitrospira*, Nested PCR to detect the genes of periplasmic nitrate reductase enzyme).

CHAPTER 2

LITERATURE REVIEW

2.1 The Activated Sludge Process

Activated sludge is the most widely used biological process for the treatment of municipal and industrial wastewaters. This process is utilized to convert organic wastes to more stable inorganic forms or to cellular biomass. The majority of the organic matter, after primary sedimentation of wastewater, is converted to carbon dioxide and water by a diverse group of microorganisms. The remaining organic compounds are converted to cellular mass that can be separated from the waste flow by gravity settling. In general, the activated sludge process consists of a reactor (aeration tank), a settling tank (clarifier), solids recycle from the clarifier to the aeration tank, and a sludge (biomass) wasting line (Figure 2-1). The aeration tank is a suspended-growth reactor containing microbial aggregates or flocs of microorganisms termed activated sludge (Grady et al., 1999).

Activated sludge is a heterogeneous microbial culture composed of bacteria, protozoa, rotifers, and fungi (Bitton, 1994). However, the bacteria are mainly responsible for degradation of organic and nitrogenous compounds in the activated sludge treatment process (Grady et al., 1999). Microorganisms consume and oxidize input organic electron donors collectively called the biochemical oxygen demand (BOD) which is the measurement of the dissolved oxygen used by microorganisms in the biochemical oxidation of organic matter (Rittmann and McCarty, 2001).

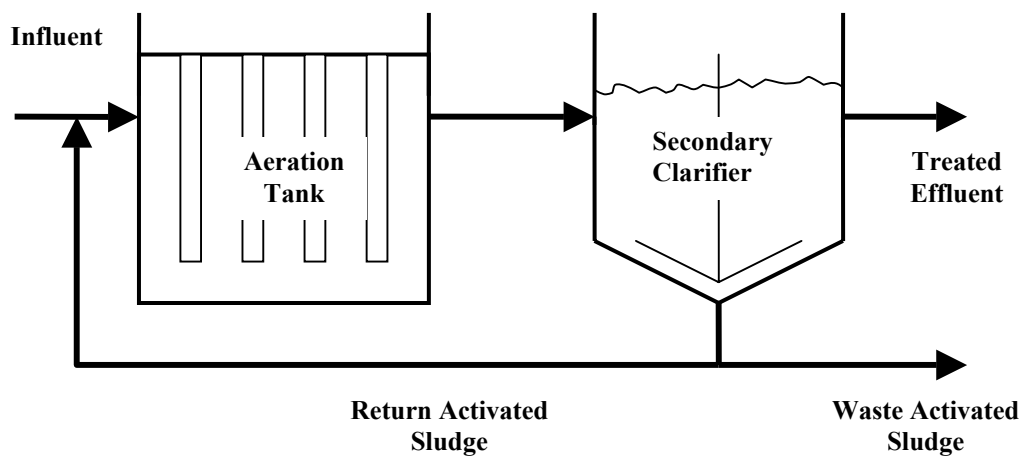


Figure 2-1. Typical activated sludge process (Adapted from Grady et al., 1999)

Heterotrophic bacteria use organic compounds as their electron donor and carbon source to synthesize new biomass in the presence of oxygen as an electron acceptor. Since removal of organic compounds is the primary purpose of activated sludge, heterotrophic bacteria predominate in the system. Microorganisms that use inorganic compounds as their electron donor and carbon dioxide as their carbon source are autotrophic bacteria. Nitrifiers are the most important autotrophic bacteria in activated sludge systems because they use ammonia and nitrite as electron donors and remove ammonia from the waste stream (Grady et al., 1999).

Activated sludge is maintained in suspension in the reactor through mixing by aeration or other mechanical means. After the slurry of treated wastewater and microbial flocs pass to the settling tank, the flocs are removed from the treated wastewater by settling and returned to the aeration tank or wasted to control the solids retention time (SRT). The clear effluent is discharged to the environment or sent for further treatment. The key factors of the activated sludge process are capturing the flocs in the settling tank and recycling them back to the reactor to maintain a high concentration of microorganisms in the reactor (Rittmann and McCarty, 2001).

2.2 Anaerobic Treatment and VFAs

Anaerobic technology is increasingly used for the treatment of wastewaters to hydrolyze and ferment a portion of biodegradable organic matter in wastewater solids. Complex organic waste components such as proteins, lipids and carbohydrates are degraded by fermentation to simple organic compounds, mainly short chain volatile fatty

acids (VFAs) and alcohols. Although the chemistry, biochemistry and microbiology of anaerobic treatment are more complex than for aerobic systems, it can be conceptualized as comprising the following steps: (1) hydrolysis of particulate organic matter to soluble substrates; (2) fermentation of those soluble substrates to produce VFAs, carbon dioxide and hydrogen (Grady et al., 1999).

The advantages of the anaerobic treatment process are numerous; however, a major disadvantage is that the effluent quality is generally not as good as that from aerobic processes and aerobic polishing is required to achieve effluent quality goals. The organic acid concentration is typically a key indicator used to measure anaerobic treatment performance. The key organic acids resulting from anaerobic treatment are the series of short chain fatty acids varying in chain length from formic acid with one carbon per mole to octanoic acid with eight carbon atoms per mole. These acids have been termed volatile fatty acids because in their unionized form, they can be distilled from boiling water. This meaning of the term “volatile” is different from its meaning in volatile organic compounds (VOCs), a term generally used to describe organic compounds that are readily removed from water by simple air stripping. The VFAs cannot be removed from water by air stripping (Rittmann and McCarty, 2001).

The VFAs generally found in highest concentrations during anaerobic treatment are acetic, propionic and butyric acids. Other nonvolatile organic acids are also formed as intermediates of organic waste degradation including lactic, pyruvic and succinic acids but their concentrations generally are much below those of the primary VFAs. In general, VFAs are quite soluble in their ionized and unionized forms and are present as dissolved species. At a normal pH (7-8) in WWTP operating systems, they are present in the

ionized (deprotonated) form. The pK_a values (the pH at which the acids are 50% in the acid form and 50% in the ionized form) are 4.75, 4.81 and 4.87 for acetic, butyric and propionic acids, respectively (Snoeyink and Jenkins, 1980).

2.3 Nitrification

Nitrification is used to eliminate the high nitrogenous biochemical oxygen demand (nBOD) of ammonium and organic nitrogen. This process usually is a required step in the removal of nitrogen because wastewaters almost always contain excess nitrogen in the reduced form. Nitrification is performed by autotrophic bacteria, which fix CO_2 as a source of carbon for cell material and obtain energy for the process by oxidizing inorganic substrates (Hagopian and Riley, 1998). The electron donor substrates are NH_4^+ and NO_2 . Autotrophs require large amounts of energy and electrons to reduce CO_2 to cellular carbon. Therefore, the yield of new cell material per unit of oxidized electron-donor substrate is much lower for an autotrophic organism than for a heterotrophic bacterium. For example, a typical f_s^o value (fraction of electron-donor electron equivalents invested in biomass) for aerobic heterotrophs is 0.6, while for nitrifying autotrophs the f_s^o is about 0.14 (Grady et al., 1999). Because of this lower yield, autotrophs also have a much lower maximum specific growth rate than heterotrophs (e.g., 0.76/day vs. 6/day) (Grady et al., 1999). This means that, under conditions highly favorable to both types of microorganisms, the autotrophs grow and accumulate more slowly than the heterotrophs. Because of the nitrifiers' slow growth rates, any nitrification system must maintain a relatively long SRT to prevent washout; even when

environmental conditions are favorable for the nitrifiers. For example, heterotrophic systems can operate with SRTs of only a few days, while nitrification systems generally require an SRT of at least 7.5 days (Grady et al., 1999).

2.3.1 Physiology of Nitrifying Bacteria

Nitrification is a two-step process, consisting of the conversion of ammonium to nitrite and its subsequent conversion to nitrate. These two steps are carried out by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively. The energy liberated during the oxidation of inorganic N compounds can be used by these organisms as the sole source of energy for carbon dioxide fixation and growth (Hooper et al., 1997). Chemolithoautotrophic nitrification is confined to a relatively narrow phylogenetic range of bacteria. Originally, the chemolithoautotrophic nitrifying bacteria were comprised within one family, *Nitrobacteraceae* (Koops and Pommerening-Rosser, 2001). However, phylogentic investigations made evident that many distinct groups of nitrifying organisms exist. Much of our knowledge about the physiology of AOB and their growth requirements has come from pure culture studies performed with a single species, *Nitrosomonas europaea*, primarily because of its availability in culture collections and the ease with which it can be grown (Prosser, 1989). Similarly, much of our knowledge of NOB has come from studies with *Nitrobacter winogradskyi*. However, the knowledge of nitrifying bacteria has evolved rapidly with the development of modern molecular techniques allowing discrimination among bacteria on the basis of their 16S rDNA sequences and the investigation of currently unculturable microorganisms.

At present, two phylogenetically distinct groups of AOB have been identified (Figure 2-2). One group is located within the gamma subclass of Proteobacteria. The second group belongs to the beta subclass of Proteobacteria. Based on ultrastructural properties, AOB have been divided into five genera, *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosovibrio*, and *Nitrosolobus* (Koops and Pommerening-Roser, 2001). It appears that *Nitrosomonas* is the most important genus in activated sludge and trickling filter systems receiving relatively high specific loadings of ammonia-N (≥ 20 mg/L), whereas *Nitrospira* is the most important genus in biofilm systems receiving very low ammonia-N (≤ 1 mg/L) concentrations (Grady and Filipe, 2000).

Four phylogenetically distinct groups of NOB have been described (Figure 2-3). The major group, which belongs to the alpha subclass of Proteobacteria, is represented by a single genus, *Nitrobacter*. Two marine species, *Nitrococcus* and *Nitrospina*, have been assigned to the gamma and the delta subclasses of Proteobacteria, respectively. *Nitrospira* are members of a distinct phylum close to the delta subclass of Proteobacteria (Koops and Pommerening-Roser, 2001). It appears that members of the genus *Nitrospira* are the most important NOB in wastewater treatment systems, although *Nitrobacter* have also been found (Schramm et al., 1998; Juretschko et al. 1998; Wagner et al. 1996). During their analysis of activated sludge from nitrifying full-scale systems, Wagner et al. (1996) could not detect *Nitrobacter*, using a general probe, suggesting that this group of organisms was either absent or present at levels below the detection limit. In their follow-up study on biomass from an industrial wastewater treatment plant, Juretschko et al. (1998) found that *Nitrospira*-like cells were the only NOB detected using molecular techniques. Further investigations, based on a 16S rDNA clone library from a nitrifying

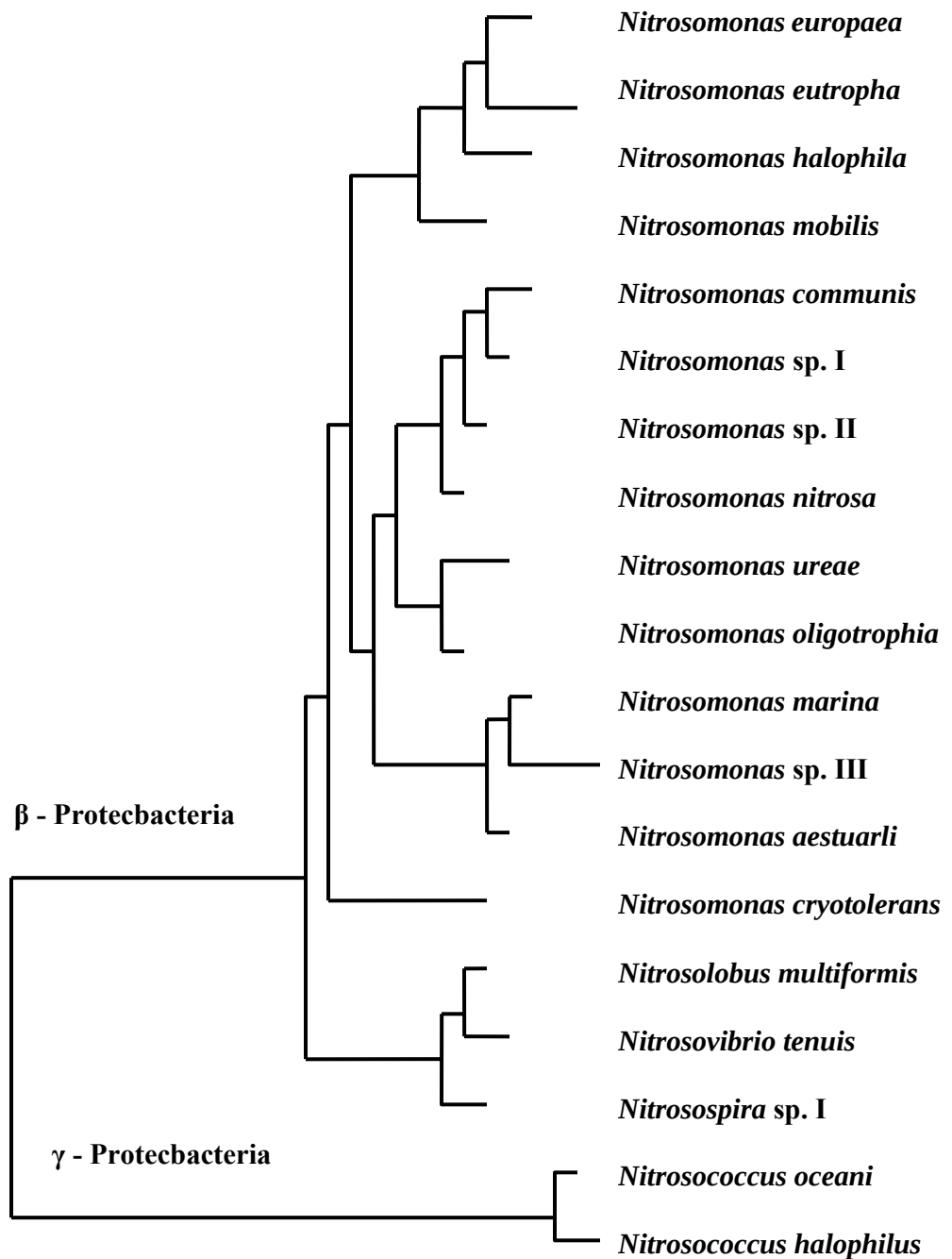


Figure 2-2. Dendrogram based on 16S rDNA sequences showing the phylogenetic interrelationship among cultured AOB (adapted from Koops and Pommering-Roser, 2001)

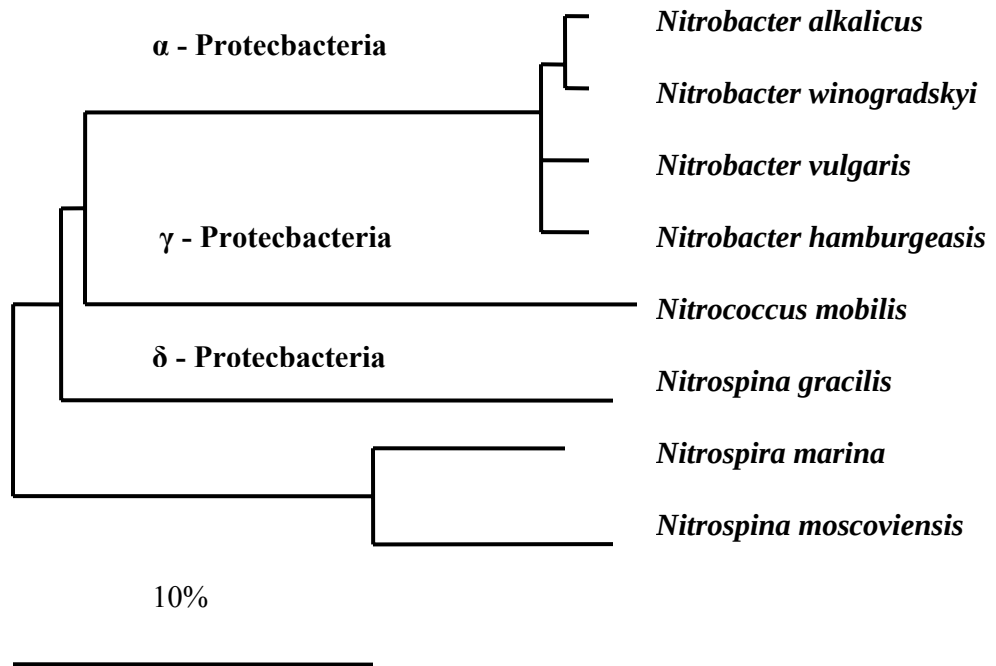
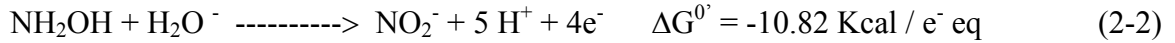
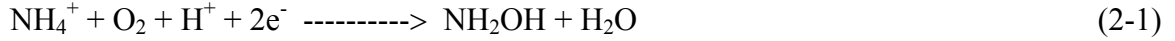


Figure 2-3. Dendrogram based on 16S rDNA sequences showing the phylogenetic interrelationships among cultured NOB (adapted from Koops and Pommering-Roser, 2001)

fluidized bed reactor (Schramm et al., 1998) showed that 16S rDNA sequences phylogenetically related to *Nitrospira moscoviensis* rather than to the genus *Nitrobacter* were present in this reactor. Based on the sequences obtained, a set of nested probes with different specificities for these *N. moscoviensis*-related organisms was constructed. Dense clusters of *Nitrospira*-like organisms were detected in this system. Therefore, the authors concluded that *Nitrospira*-related organisms, rather than organisms of the genus *Nitrobacter*, may play a major role in nitrifying freshwater systems (Hovanec et al., 1998). Studies in Australia also found *Nitrospira* to be the most abundant NOB in samples from a municipal full-scale WWTP, as well as from an enrichment culture derived from it (Burrell et al., 1998). Okabe et al., (1999) identified *Nitrospira*-like cells as important populations of nitrifying bacteria in both a domestic wastewater biofilm and an autotrophic biofilm. In addition, Kloop et al. (2000) could not measure *Nitrobacter* spp., the expected major NOB, in a nitrifying fluidized-bed biofilm reactor used for wastewater treatment. Daims et al., (2000, 2001b) found *Nitrospira*-like bacteria in nitrifying biofilms from two reactors receiving wastewater with different ammonia and salt concentrations. More recently, *Nitrospira* spp. were detected in both municipal and industrial wastewater treatment plants using cPCR and real-time PCR (Dionisi et al., 2002; Harms et al., 2002).

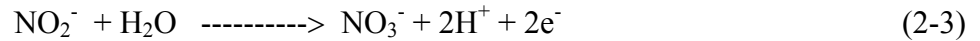
2.3.2 Biochemistry of Nitrifying Bacteria

In the first step of nitrification, AOB obtain energy by oxidizing NH_4^+ to NO_2^- according to the following reaction:



$\Delta G^{0'}$ is the standard free energy to transform each electron equivalent for the reaction when the pH is set to 7.0. The reaction, as written, contains 6 electron equivalents. Besides consuming oxygen, the oxidation of ammonium produces five equivalents of hydrogen per mole of ammonium (Madigan et al., 1997).

The second step of the nitrification reaction is the oxidation of NO_2^- to NO_3^- by NOB using the nitrite oxidoreductase enzyme in the presence of oxygen. Nitrite oxidation results in equal molar accumulation of nitrate (i.e. nitrite oxidation equals nitrate formation). The nitrite oxidation reactions are shown in the following equations:



Nitrification reactions occur in a stepwise fashion, each step involving removal of two electrons. Nitrogen species formed and their oxidation states are provided below (de Silva, 1997).



Oxidation State	-3	-1	+1	+3	+5
Species name	Ammonia	Hydroxylamine	Nitrooxyl	Nitrite	Nitrate

Except for the first step, the two-electron transfer in each step generates reduced internal electron carriers (such as NADH) that are capable of transferring electrons to the cytochromes. Therefore, electrons flow through the cytochromes and ultimately to the primary electron acceptor (O_2), a process called respiration, to generate the proton-motive gradient that enables the nitrifiers to generate energy stored in the form of ATP (Madigan et al., 1997). Oxidation of NO_2^- is the source of energy used for synthesis of energy-rich compounds (typically ATP) and of reduced cofactor production (typically NADH, H^+ and NADPH, H^+) needed in the Calvin-Benson pathway (Poughon et al., 2001). These oxidations are closely linked to the membrane electron transport chain. The proteins involved in the respiratory chain, composed of complexes I, II, III, and IV (Nicholls and Ferguson, 1992) have been detected in *Nitrobacter* (Bock et al., 1991).

The biochemical pathway of nitrification is illustrated in Figure 2-4 (de Silva, 1997). In the first step, ammonium monooxygenase, two electrons from a reduced electron carrier and molecular oxygen are required to convert NH_4^+ to NH_2OH (hydroxylamine). During the step 2, called dehydrogenation or dehydrogenolysis, NH_2OH is oxidized to NHO (nitrooxyl), producing 2 electrons that are transferred to NADH. NHO is further oxidized to NO_2^- (nitrite) in the step 3, producing the second acid equivalent and two more electrons. The step 4 is believed to be the most common reaction to produce NO_3^- (nitrate) from NO_2^- . The step 5 reaction requires another electron-donor substrate to supply electrons for the monooxygenase; therefore, this step will only occur if the NOB are not strict chemolithautotrophs. Energy is derived from the oxidation of nitrite to nitrate providing NADPH and ATP for CO_2 fixation via the Calvin cycle (Figure 2-5).

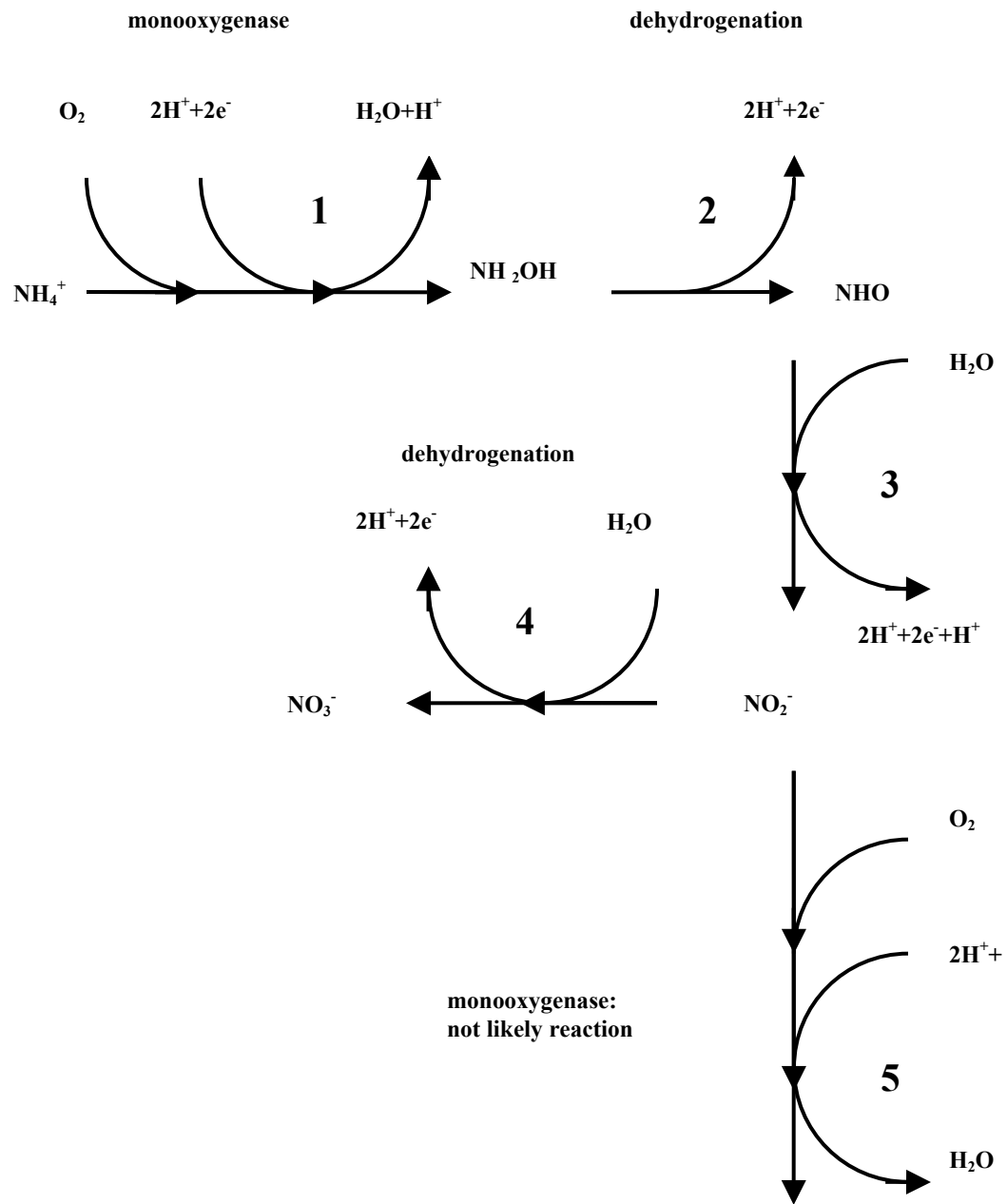


Figure 2-4. Biochemical pathway of nitrification (adapted from de Silva, 1997)

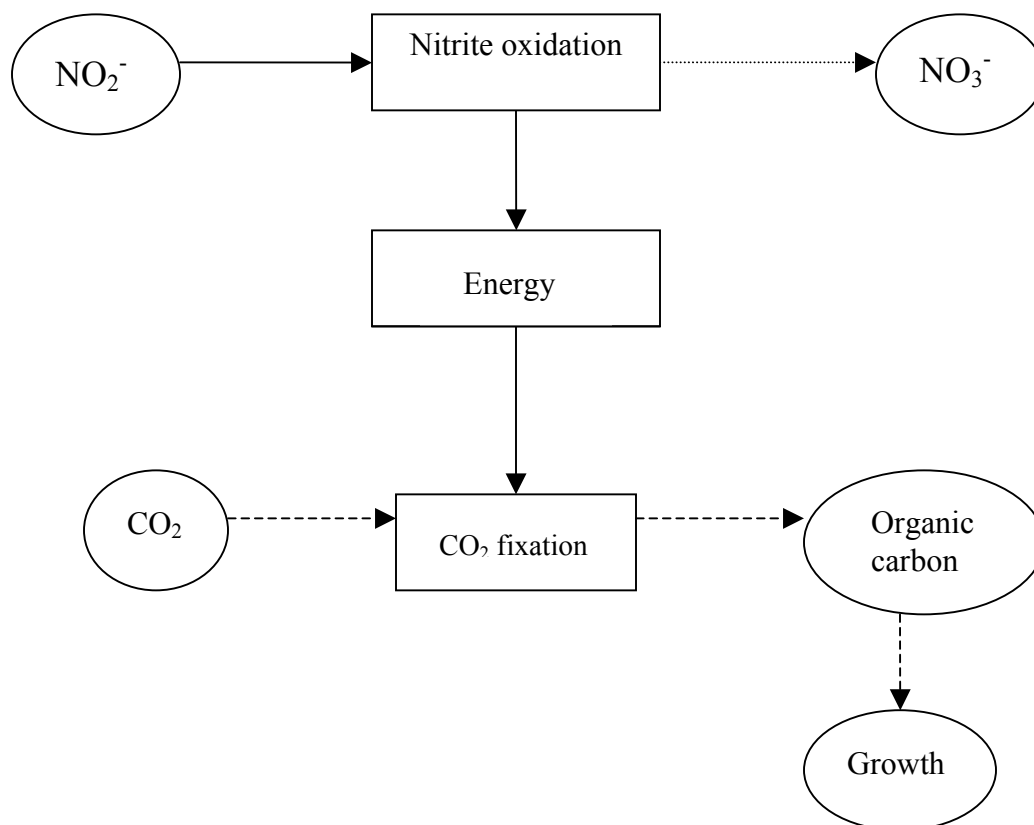


Figure 2-5. Relationship between nitrite oxidation and CO₂ fixation

2.3.3 Carbon Metabolism in Nitrifying Bacteria

The major source of organic carbon for nitrifying bacteria is from carbon dioxide fixation via the Calvin cycle (Figure 2-6). In this cycle, CO_2 is converted into a set of more complex building blocks (like glucose), which are then assembled into the macromolecules that comprise proteins, carbohydrates, lipids, nucleic acids, and other cell components (Hagopian and Riley, 1998). All CO_2 fixation reactions can occur in complete darkness using ATP and reducing power (NADPH) generated during oxidation of inorganic compounds (McFadden and Shively, 1991).

Carbon dioxide is brought into the cycle where it is joined to ribulose 1, 5-bisphosphate to form 3-phosphoglycerate (PGA). This reaction is catalyzed by the enzyme ribulose bisphosphate carboxylase (RuBisCO). PGA constitutes the first identifiable intermediate in the CO_2 reductive process. The new carbon atom in PGA, derived from CO_2 , remains at the same oxidation level as it was in CO_2 . The next two steps involve reduction of PGA to the oxidation level of a carbohydrate. In these steps, both ATP and NADPH are required. The ATP requirements of this process place additional burdens on an already inefficient energy-generating system in nitrifying bacteria. The average carbon atom in glyceraldehydes 3-phosphate is now at the reduction level of carbohydrate. Only one of the carbon atoms in glyceraldehydes 3-phosphate has been derived from CO_2 , the other two originated from the ribulose bisphosphate. In the next step, 6-carbon fructose 6-phosphate is formed and can be used for biosynthesis. In the cycle, glyceraldehydes 3-phosphate is formed as well, and is converted, with the addition of ATP, to the ribulose 1, 5-bisphosphate that is needed to again react with carbon dioxide, thereby, completing the cycle.

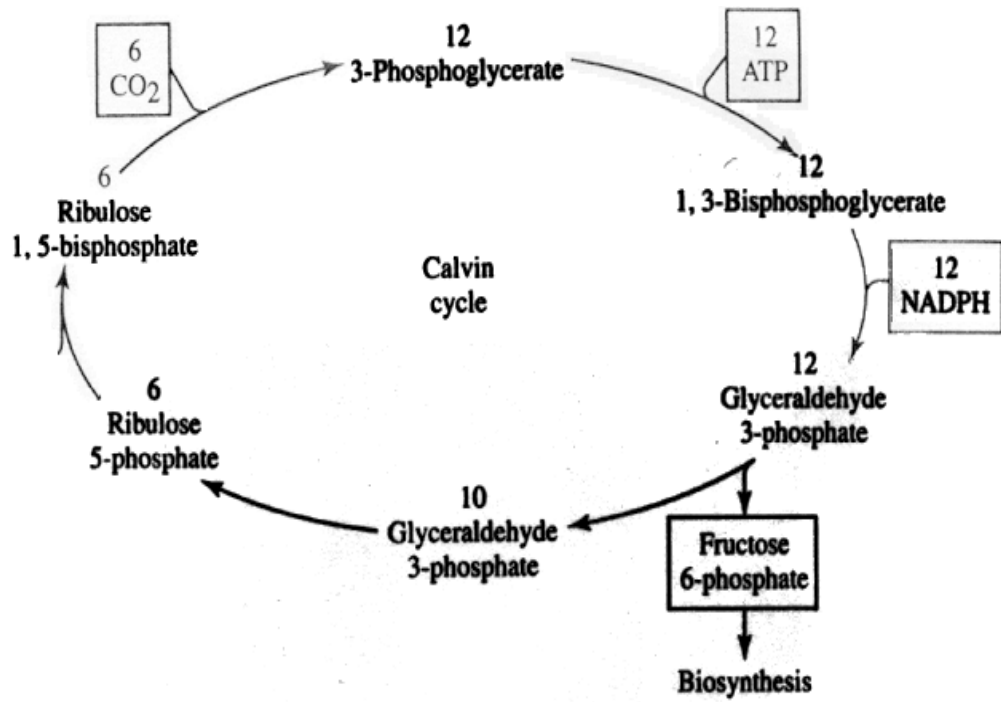
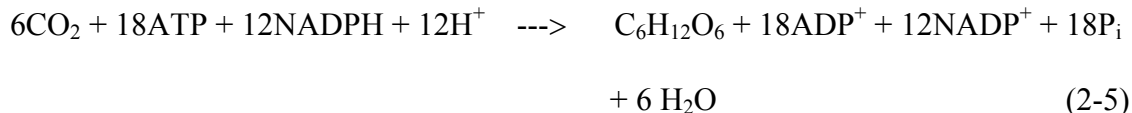


Figure 2-6. The Calvin cycle in autotrophic bacteria (Rittmann and McCarty, 2001)

The net reaction of the Calvin cycle is:



The fixation of three molecules of CO_2 by the Calvin cycle requires the investment of nine molecules of ATP and six of NADPH to obtain one molecule of glucose. Thus, this is an energy intensive process that has to be carefully regulated. Two physiological parameters, the availability of carbon sources (including CO_2) and reduced organic substrates supporting autotrophic growth control the expression of the Calvin cycle (Shively et al., 1998). A reduced metabolite originating from the oxidation of reduced compounds is most likely responsible for the induction of the Calvin cycle. For example, the activity of the Calvin cycle is higher during growth on butyrate than on the less- reduced substrate malate.

Although AOB are strict chemolithoautotrophs, studies on *Nitrobacter* showed that this bacterium can grow mixotrophically (defined as the ability to simultaneously incorporate inorganic and organic carbon sources) (Bock et al., 1991; Prosser 1989). Daims et al. (2001) studied inorganic carbon fixation in the recently discovered *Nitrospira*-like bacteria to investigate if this organism can also grow mixotrophically. Substrate uptake experiments using radioactively labeled [^3H] acetate, [^{14}C] pyruvate, [^{14}C] butyrate, [^{14}C] propionate and [^{14}C] bicarbonate were performed with living activated sludge and biofilm samples. The incubation conditions applied were aerobic, anoxic (i.e. anaerobic in the presence of nitrate) and anaerobic. Under aerobic conditions, the *Nitrospira*-like bacteria in bioreactor samples took up inorganic carbon (as HCO_3^- or

as CO₂) and pyruvate but not acetate, butyrate, and propionate suggesting that these bacteria can grow mixotrophically in the presence of pyruvate. This diverse ability to grow might contribute to the competitiveness of the *Nitrospira*-like bacteria in WWTPs. In contrast to the aerobic incubation experiments, no uptake of organic or inorganic carbon sources by the *Nitrospira*-like bacteria was observed under anoxic or anaerobic conditions (Daims et al., 2001). Based on these experiments, *Nitrospira*-like bacteria seemingly do not use acetate, propionate, butyrate or pyruvate in the presence of nitrate and the absence of oxygen. Thus, these results provide no indication that *Nitrospira*-like bacteria respire anaerobically with nitrate, but they might employ other strategies to survive periods of limited oxygen availability.

2.3.4 Activated Sludge Nitrification

In nitrifying activated sludge systems, ammonia-nitrogen is converted to nitrate-nitrogen, which is then released in the effluent, or subjected to a denitrification step if complete nitrogen removal is desired (Rittmann and McCarty, 2001). A successful nitrification process must account for the reality that heterotrophic bacteria always are present and competing with the nitrifying bacteria for dissolved oxygen and space. The nitrifying bacteria's high half-saturation coefficient for oxygen (K_O) puts them at a competitive disadvantage for oxygen (Rittmann and McCarty, 2001). Additionally, their slow growth rate is a disadvantage when competing for space. These two disadvantages can be overcome by ensuring that the nitrifiers have a long SRT, typically greater than 10 d, although larger values may be needed in the presence of toxic materials, a low dissolved oxygen (D.O.) concentration or low temperature (Grady et al., 1999).

Nitrification can be achieved either in the same reactor used in the treatment of carbonaceous organic matter or in a separate reactor following conventional activated-sludge treatment. The goal is to reconcile nitrogen oxidation by the autotrophic nitrifiers with the oxidation of organic matter by the faster-growing heterotrophs.

One-stage nitrification is the process configuration in which heterotrophic and nitrifying bacteria coexist in a single mixed liquor that simultaneously oxidizes ammonium and organic BOD. The one-stage reactor contains both nitrifiers and heterotrophs, which carry out their metabolism simultaneously. The key characteristic of one-stage nitrification is that the nitrifiers are usually only a small fraction of the biomass since organic compound oxidation produces a large yield of heterotrophic biomass. In order to ensure stable nitrification, the entire community has a long SRT.

Two-stage nitrification reduces the competition between heterotrophs and nitrifiers by oxidizing most of the organic BOD in a first stage, while the ammonium is oxidized in a second stage. Because the biomass in each stage is captured and recycled solely within that stage, each step develops its own microbial population. The first stage is essentially free of nitrifiers, while the second stage microbial population has a major fraction of nitrifiers since all nitrification and only a small amount of BOD oxidation occurs there. A major advantage of the two-stage process is that potential toxic effects to nitrifiers by biodegradable organic materials can be minimized in the second stage because they are removed in the first stage.

2.4 Denitrification

Denitrification is a microbial process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production. Nitrogen is reduced primarily to nitrogen gas (N_2), although other gaseous end products can occur. In wastewater treatment, denitrification is applied when complete removal of nitrogen from the water column is required. For denitrification to occur, nitrogen must be in one of its oxidized forms, nitrite or nitrate. Because many wastewaters contain reduced nitrogen (e.g. ammonia), denitrification frequently is coupled to nitrification, which is needed to generate oxidized nitrogen in the form of nitrite or nitrate.

Bacteria capable of denitrification are frequently isolated from soil, sediment and aquatic environments. Additionally, this alternative respiratory process is widespread among microorganisms belonging to phylogenetically distinct groups of bacteria such as *Pseudomonas*, *Alcaligenes*, *Paracoccus*, and *Thiobacillus* and Archae (Zumft, 1997).

Denitrification consists of four reaction steps (see below) by which nitrate is reduced to dinitrogen gas by the metalloenzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. These enzymes are usually induced sequentially (Philippot, 2002).

	$\text{NO}_3^- \rightarrow$	$\text{NO}_2^- \rightarrow$	$\text{NO} \rightarrow$	$\text{N}_2\text{O} \rightarrow$	N_2
	Nitrate reductase (NAP, NAR)	Nitrite reductase (NIR)	Nitric oxide reductase (NOR)	Nitrous oxide reductase (NOS)	
Oxidation State	+5	+3	+2	+1	0
Species name	Nitrate	Nitrite	Nitric oxide	Nitrous oxide	Nitrogen

All four products can be measured in some cases and the last three compounds are gaseous products that can be released to the atmosphere. For treatment applications, the desired product is N_2 because this molecule is innocuous in the atmosphere and results in the maximum number of electrons being accepted per mole of N reduced.

The biochemical pathway of denitrification is summarized in Figure 2-7. Each step of the reaction is associated with the gain of electrons carried by a reduced electron carrier. Therefore, an electron donor is required to generate the reduced carriers. In sewage treatment, these reactions usually are performed primarily by heterotrophic bacteria using organic matter as the electron donor. When the organic matter in wastewater itself is inadequate to reduce the nitrate, a supplemental source of carbon can be provided (de Silva, 1997).

2.5 Conceptual Model for Nitrogen Removal Focusing on Nitrite as the Central Compound

Nitrogen removal from wastewater is a process in which ammonium is oxidized to nitrate and then reduced to dinitrogen gas. Large amounts of oxygen (i.e. energy) and electron donors (i.e. chemicals) are required. Therefore, elimination of nitrogenous compounds by simultaneous nitrification and denitrification leading to a production of N_2 (or N_2O) is of great economic interest for wastewater treatment plants. The costs of the process may be greatly reduced if less oxidized intermediates could be used for nitrogen removal.

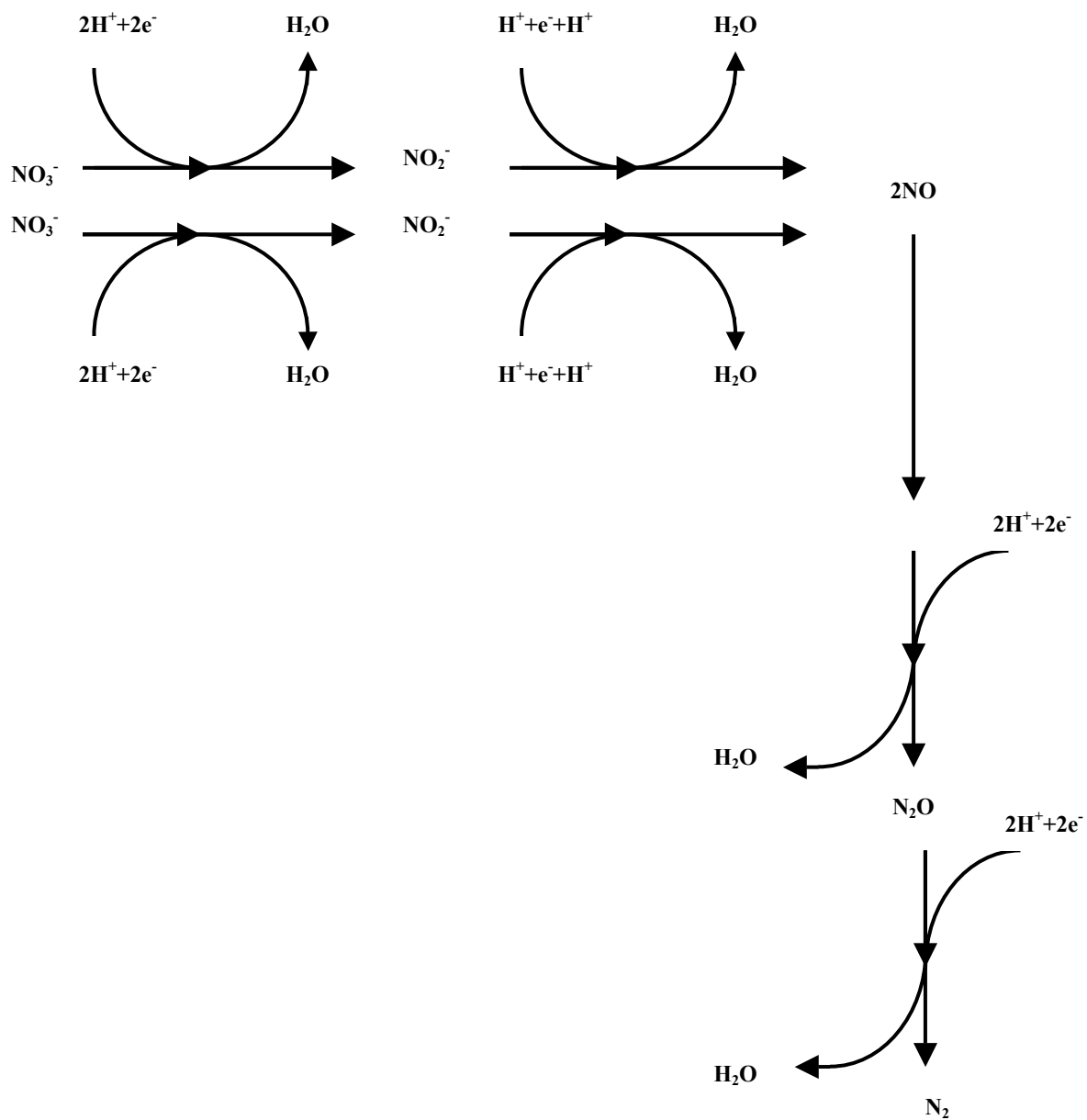


Figure 2-7. Biochemical pathway of denitrification (adapted from de Silva, 1997)

Nitrogen can occur in many forms in wastewater and undergo numerous transformations in wastewater treatment. These transformations allow the conversion of nitrogen to products that can more easily be removed from wastewater and are essential for most wastewater treatment systems. However, the large number of nitrogen compounds involved, the numerous reactions that can occur and the low growth rate of many of the bacteria involved make the study of nitrogen conversions difficult. Based on observations in practical systems and reports in the microbiological literature, several authors claim occurrence of 'non-conventional' N-elimination in the studied treatment processes. These claims are usually complicated to verify since proper mass balances (including the nitrogen gas produced) are difficult to make during wastewater treatment. Frequently, to establish a nitrogen balance in a WWTP, nitrification and denitrification are considered independently and calculations are made without considering interactions between these two reactions. However, for the past ten years, many studies have reported the existence of atypical behavior for nitrogen fixing, nitrifying, ammonifying and denitrifying bacteria. This unusual and unexpected behavior could explain some disturbances and nitrogen imbalances observed in WWTPs and in soils. A range of new microbial processes have been reported in WWTPs e.g. aerobic denitrification and heterotrophic nitrification (Robertson and Kuenen, 1990; Muller et al., 1995), anaerobic ammonia oxidation (Anammox) (Jetten et al., 1999), and denitrification by autotrophic nitrifying bacteria (Bock et al., 1995; Schmidt and Bock, 1997). These new reactions allow the design of new nitrogen removal systems in addition to explaining nitrogen imbalances.

A conceptual model for nitrogen removal based on nitrite as the central compound was developed to investigate possible pathways that nitrite can be converted to other nitrogenous compounds (Figure 2-8). This model differs from other nitrogen cycles (Jetten, 2001; Van Loosdrecht and Jetten, 1998; Stolz and Basu, 2002) because it focuses on nitrite rather than ammonia. Nitrification, denitrification, aerobic denitrification, Anammox and autotrophic denitrification are all potential pathways for nitrite removal in this model.

A key interconversion in the nitrite cycle is nitrification, an aerobic, oxidative reaction that sequentially produces nitrite and nitrate. Nitrification is promoted during wastewater treatment to remove nitrogenous oxygen demand and toxicity and as the initial step in biological nitrogen removal. The end products of nitrification serve as an alternative terminal electron acceptor for heterotrophs in denitrification in which nitrate is anaerobically converted to a gaseous product for removal. These reactions also occur under aerobic conditions which is called aerobic denitrification. Anammox is a biological process performed by autotrophic Planctomycetales using ammonia as the electron donor and nitrite as the terminal electron acceptor. In autotrophic denitrification, ammonia oxidizing bacteria (AOB) can perform denitrification of nitrite with ammonium or hydrogen as electron donor.

2.6 Aerobic Denitrification

Until about 10 years ago, denitrification was assumed to only occur under anoxic conditions. Increasing the DO supplied to anoxic denitrifying cells was shown to be

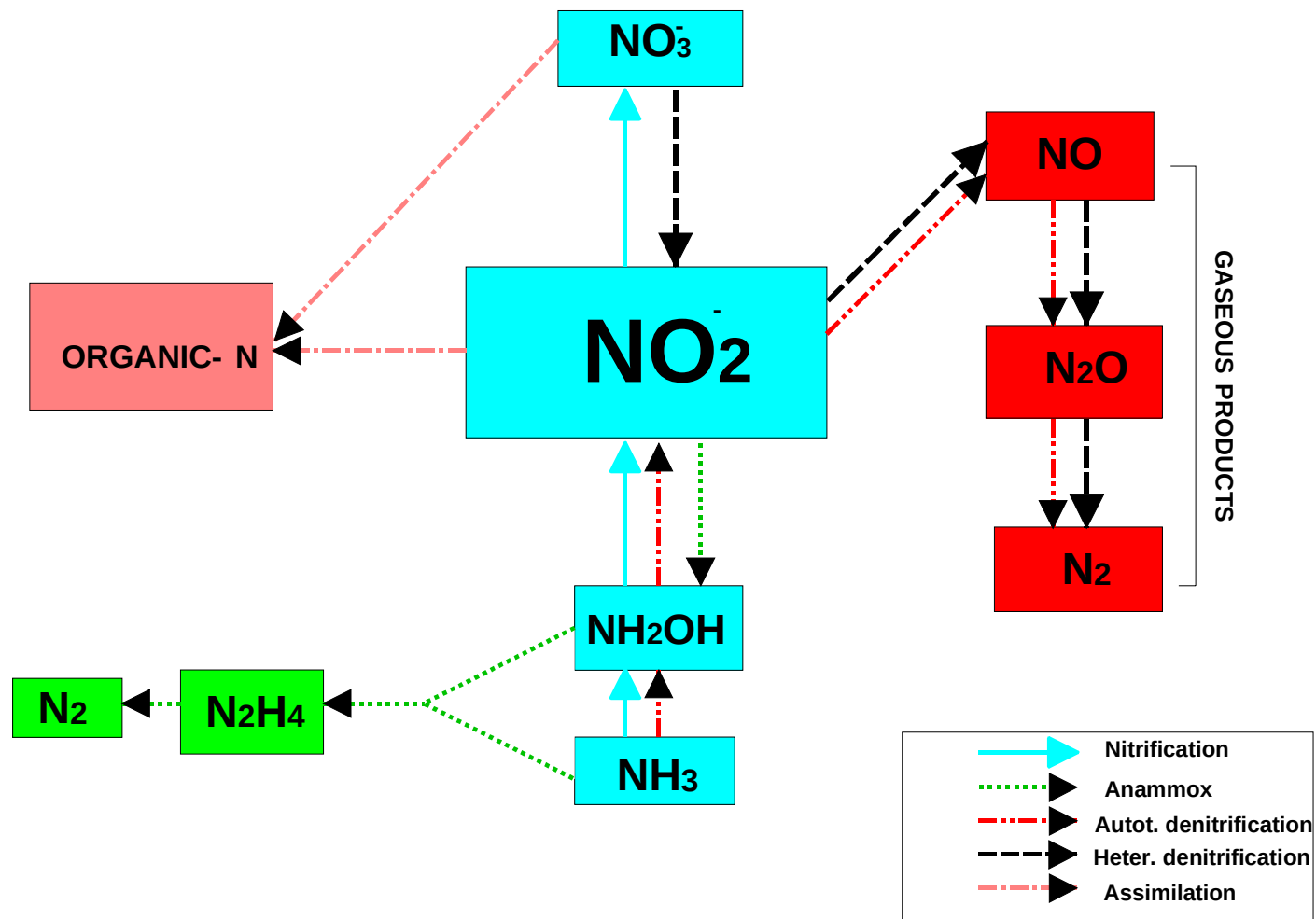


Figure 2-8. Conceptual model for nitrogen removal with nitrite as the central compound

accompanied by a decrease in denitrification rates and accumulation of nitrous oxide, nitric oxide, nitrite and then nitrate. However, other experiments showed that activity and synthesis of denitrifying enzymes could occur under oxic conditions (Robertson et al. 1989;; Carter et al. 1995; Frette et al. 1997; Lukow and Diekmann 1997; McDevitt et al. 2000; Patureau et al. 2000; Huang and Tseng 2001). Heterotrophic denitrifiers can produce N_2 from ammonium and/or nitrite in well-mixed aerobic suspensions.

The phenomenon of complete denitrification at high DO concentrations was first observed in *Paracoccus* species. While debate exists over which species and strains of *Paracoccus* denitrify aerobically, the ability to reduce nitrate to nitrite under aerobic conditions is widely accepted. Aerobic nitrate reduction has recently been demonstrated in a variety of other bacteria (Ellington et al., 2003).

Batch culture experiments confirmed that *Thiosphaera pantotropha* (*Paracoccus dentrifican*) produced N_2 from ammonium and/or nitrite and nitrate in well-mixed, aerobic suspensions (Robertson et al., 1995). Moreover, N_2 production by other heterotrophic denitrifiers under the same conditions was also confirmed (Robertson et al., 1995). In addition, *T. pantotropha* generated N_2O and N_2 in well-mixed chemostat cultures fed acetate. As the DO increased, N_2O and N_2 increased (Arts et al., 1995). *Pseudomonas stutzeri* SU2, which was isolated from the activated sludge of a sequencing batch reactor treating piggery wastewater, rapidly reduced nitrate to nitrogen gas under aerobic conditions without nitrite accumulation (Su et al., 2001).

Since molecular oxygen was previously shown to repress denitrification enzymes, the mechanism of aerobic denitrification received considerable attention. Elucidation of this process started with the discovery that in *T. pantotropha* two different nitrate

reductases were present. Fractionation of cells grown aerobically or anaerobically in the presence of nitrate revealed an anaerobically active membrane bound nitrate reductase (NAR) and an aerobically active periplasmic nitrate reductase (NAP) (Figure 2-9). The NAP was suggested to play a role in aerobic denitrification when *T. pantotropha* was grown with highly reduced carbon sources under aerobic conditions. The more reduced the carbon source (butyrate or caproate compared to acetate), the higher the NAP activity, confirming its possible function as a redox valve during aerobic growth (Richardson and Ferguson, 1992). NAP was assayed in intact cells of *T. pantotropha*, after aerobic growth with malate, succinate acetate, butyrate or caproate as the sole carbon source (Richardson and Ferguson, 1992). The level of enzyme activity was largely dependent on carbon source and was lowest on malate, intermediate on acetate and highest on butyrate and caproate. These results indicate that, during aerobic growth, activity of the periplasmic nitrate reductase enzyme increases with the extent of reduction of the carbon source.

The expression and *in-situ* activity of the NAP was also assayed in *Paracoccus denitrificans* Pd1222 grown under a range of aeration regimes in chemostat cultures with either malate or butyrate as the carbon source (Sears et al., 1997). Expression was observed at all oxygen concentrations tested between 0 and 100% air saturation. In cultures containing butyrate, the expression of periplasmic nitrate reductase and the rate of nitrate reduction were high at all oxygen concentrations. In contrast, for cultures with malate, expression of NAP was low. In response to aerobic growth on the reduced carbon substrate butyrate, NAP activities increase by at least an order of magnitude, relative to levels measured with more oxidized substrates such as malate or succinate, demonstrating a significant role for this process during butyrate metabolism (Richardson and Ferguson,

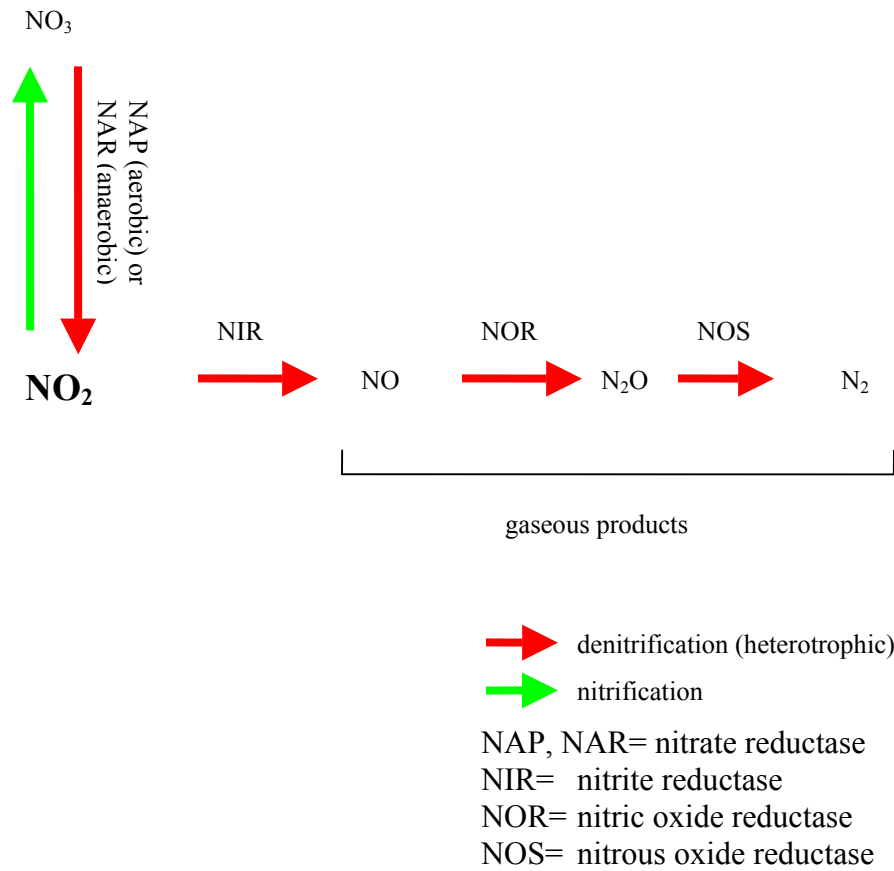


Figure 2-9. Aerobic and anaerobic denitrification steps and the enzymes catalyzing these processes

1992; Sears et al., 1993, 1997). NAP was not present to any extent when the organism was grown anaerobically under denitrifying conditions, regardless of the carbon source (Sears et al., 2000).

Sears et al., (2000) investigated the organization and transcriptional regulation of the *nap* operon in *Paracoccus pantotrophus*. The researchers demonstrated that expression of the *nap* operon was subject to induction aerobically, but only when the organism was growing on highly reduced carbon substrates, such as butyrate. Remarkably, operon expression was also negatively regulated in response to anaerobic conditions.

In another study, quantification of *nap* expression in batch cultures of *P. pantotrophus* (*pnap-lacZ*) demonstrated that expression responded positively to oxygen and was tightly regulated in response to the carbon substrate (Ellington et al., 2002). Correlation between NAP activity and *nap* gene expression demonstrated that transcription from the *nap* promoter was the major point of regulation for cellular NAP activity (Richardson and Ferguson, 1992; Ellington et al., 2002).

The conditions for maximal expression of the periplasmic nitrate reductase and aerobic nitrate respiration can be rationalized in terms of a role for the periplasmic nitrate reductase in dissipating excess reductant generated during oxidative metabolism of reduced carbon substrates (Sears et al., 1997). During chemoheterotrophic growth on reduced carbon sources, the carbon substrate must be oxidized to a level at which it can be assimilated. If oxidation results in the release of more reductant than needed for the generation of ATP required for metabolism of carbon, then a means of disposing of the excess reductant must be available. If excess reductant is not removed, the growth rate will be slowed to allow for reoxidation of NADH by cell maintenance reactions

(Richardson et al. 2001). The NAP-dependent pathway allows the cell to dissipate excess reductant under aerobic growth conditions. Such excess reductant can be generated during oxidative metabolism of highly reduced carbon substrates such as butyrate (Richardson and Ferguson, 1992; Sears et al., 1993, 1997; Ellington et al., 2002). As a consequence, the role of aerobic nitrate respiration may be to provide a poorly coupled route for oxidation of excess reducing equivalents to maintain the cellular redox balance during oxidative metabolism of highly reduced carbon substrates (Sears et al., 2000).

The aerobic denitrification process is now seen as a variant represented by several denitrifiers rather than the extremely rare exception. The range of oxygen concentrations in which N oxide respiration was found is broad, and the onset of N oxide utilization differs from one denitrifier to another. Aerobic denitrification results when activation of denitrification genes occurs at a high DO level. It was shown that genes encoding all the different classes of denitrifying reductases are genetically linked. For example, the *nos*, *nir* and *nor* genes in *P. stutzeri* and more recently the *nor*, *nir* and *nap* genes in G-179 were found to be linked (Bedzyk et al., 1999; Philippot, 2002). Regulatory control usually operates in favor of channeling electrons toward aerobic respiration. For instance, phase shifts between oxygen respiration and denitrification can be established as autonomous oscillations in a culture supplied with low oxygen concentrations (Zumft, 1997). If oxygen does not suppress N oxide utilization, aerobic denitrification manifests itself as the co-respiration of the two substrates (Zumft, 1997). No fundamental aspect that would make the aerobic variant a different type of denitrification has been uncovered. The enzymes found in aerobically denitrifying bacteria are the same as those of their anaerobic counterparts. The complete aerobic denitrification pathway can work in

the presence of oxygen with the following combination of enzymes: the periplasmic nitrate reductase, cytochrome *cd*₁ nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Ferguson, 1994). Purified cytochrome *cd*₁ nitrite reductase and nitrous oxide reductase from *Paracoccus denitrificans* GB17 both have properties identical to the enzymes from two other, anaerobically denitrifying strains of *Paracoccus*. Aerobic denitrification is not a question of the oxygen sensitivity of the reductases but, rather, one of regulation at the enzyme and genetic level.

Bacteria which co-respire nitrate and oxygen are widespread in the environment. This respiratory pathway may, therefore, make a significant, but little-studied, contribution to the nitrogen cycle. Table 2-1 shows the extent of heterotrophic organisms capable of aerobic denitrification. This reaction may be responsible, to a great extent, for the loss of nitrogen oxides in aquatic aerobic environments and needs to be accounted for in nitrogen balance calculation in soils or wastewater treatment processes.

2.7 Anaerobic Ammonia Oxidation (ANAMMOX)

Anaerobic ammonia oxidation (Anammox) is one of the most recently described nitrogen cycle reactions. This process was initially identified when ammonium removal was observed in an anaerobic fluidized denitrifying bed treating effluent from a methanogenic reactor (Mulder et al., 1995). A novel metabolic pathway for Anammox (shown in Figure 2.8) with nitrite as the electron acceptor was elucidated using ¹⁵N-labelled nitrogen (van de Graaf et al., 1997).

Table 2-1. Bacteria which have the NAP enzyme (adapted from Philippot, 2002; Richardson et al., 2001)

Alpha Proteobacteria	Beta Proteobacteria	Gamma Proteobacteria	Delta Proteobacteria	Epsilon Proteobacteria
<i>Paracoccus pantotrophus</i>	<i>Bordetella</i>	<i>Escherichia Coli</i>	<i>Desulfovibrio</i>	<i>Campylobacter</i>
<i>Rhodobacter capusulatus</i> AD2	<i>bronchiseptica</i>	<i>Haemophilus actinomycetemcomitons</i>	<i>desulfuricans</i>	<i>jejuni</i>
<i>Rhodobacter sphaeroides</i> f sp. denitrificans	<i>Ralstonia eutropha</i>	<i>Haemophilus ducreyi</i>		
<i>Sinorhizobium meliloti</i>	<i>Ralstonia metallidurans</i>	<i>Haemophilus influenzae</i>		
<i>Mantetospiruillum</i>		<i>Legionella pneumophila</i>		
<i>magnetotacticum</i>		<i>Pasteurella multocida</i>		
<i>Magnetococcus</i>		<i>Pseudomonas aeruginosa</i>		
		<i>Salmonella paratyphimurium</i>		
		<i>Salmonella typhimurium</i>		
		<i>Shewanella putrefaciens</i>		
		<i>Vibrio cholerae</i>		
		<i>Yersinia pestis</i>		

This autotrophic process reduces oxygen requirements by 50% and requires no supplemented organic carbon source. In addition, the biomass yield is very low so that little sludge is produced. *Planctomycetales* catalyse this process under chemolithotrophic conditions using ammonia as an electron donor and nitrite as an electron acceptor. The reaction occurs at extremely low growth ($0.1\text{--}0.05\text{ day}^{-1}$) and substrate utilization rates (Jetten et al., 1999). In addition to the fluidized bed systems, a sequencing batch reactor (SBR) was applied and optimized to quantify the microbial community that anaerobically oxidized ammonium (Strous et al., 1998). Important physiological parameters such as the maximum specific rate of ammonium consumption ($45 \pm 5\text{ nmol min}^{-1}\text{ mg protein}^{-1}$), the maximum specific growth rate (0.0027 h^{-1} , doubling time 11 days) and the biomass yield ($0.066 \pm 0.01\text{ mol C mol ammonium}^{-1}$) were determined in the SBR study. The temperature range for Anammox was $20\text{--}43^{\circ}\text{C}$ (with an optimum at 40°C). The process functioned well at pH 6.7–8.3 (with an optimum at pH 8). However, nitrite concentrations higher than 5 mM, reversibly inhibited the Anammox activity. During conversion of ammonia and nitrite no other intermediates or products, such as NO or N_2O , could be detected.

The influence of oxygen on the Anammox process was investigated in both batch and continuous flow systems. Initial batch experiments showed that oxygen completely inhibited Anammox activity when it was introduced into enrichment cultures (van der Graaf et al., 1996; Jetten et al., 1997). In a follow-up study, an intermittently oxic (2h) and anoxic (2h) reactor system was used to study the reversibility of oxygen inhibition (Strous et al., 1997). Results indicated that ammonium was not oxidized in the oxic periods, but that the Anammox activity during the anoxic periods remained constant

throughout the experiment, providing evidence that the inhibitory effect of oxygen was reversible.

The Anammox process has not yet been applied in a full-scale WWTP. Extreme sensitivity to oxygen appears to limit its practical implementation, although the Anammox reaction may explain some nitrogen loss during anaerobic treatment of highly nitrogenous wastewater.

2.8 Autotrophic Denitrification

In autotrophic denitrification, oxidation of ammonia to nitrite is followed by a reduction of nitrite to N_2O and N_2 . Ammonia acts as an electron donor and nitrite acts as an electron acceptor under oxygen limitation (Colliver and Stevenson, 2000). Molecular hydrogen may also serve as an electron donor for nitrite reduction by *Nitrosomonas eutropha* under anoxic conditions (Bock et al., 1995). The main end product is dinitrogen gas, but small amounts of NH_2OH and N_2O are also detected. Nitrate is not produced in autotrophic denitrification. The sequence of reactions in autotrophic denitrification is catalyzed by ammonia oxidizers (Wrage et al., 2001). The enzymes required for autotrophic denitrification are believed to be similar to those found in denitrifying bacteria.

The overall mechanism of autotrophic denitrification remains unclear. To date, two possible hypotheses have been proposed. First, N_2O and N_2 are produced as the result of biological activity by ammonia oxidizers. Second, these compounds are produced as a result of chemical reactions involving unstable nitrification intermediates. However, most

of evidence reported appears to support the first hypothesis (Anderson et al., 1993; Bock et al., 1995).

Oxygen concentration appears to be the single most important factor affecting the production and relative abundance of N_2O and N_2 generated. Muller et al. (1995) showed that autotrophic denitrification was the source of N_2 produced by sewage sludge cultured in recycling containers with a mixture of pure argon and low DO. Additionally, the researchers found that autotrophic denitrification only occurred after addition of ammonium and ceased when sufficient nitrite was present. The authors argued that N_2 production by ammonia oxidizers had not been observed earlier because either the background levels of N_2 in air were too high for any newly formed N_2 to be detected or the concentration of bacteria was too low. These results indicate that autotrophic nitrifiers might be responsible for a range of 'strange' nitrogen conversions in wastewater treatment processes. However, autotrophic denitrification rates are much slower than those for conventional nitrification and denitrification reactions. Reported conversion rates include 5×10^{-4} gN/gVSS-hr (Anderson and Levine, 1986) and 1.4×10^{-5} gN/gVSS-hr (Bock et al., 1995). These rates are more than an order of magnitude slower than conversion rates for conventional nitrification and denitrification reactions (1-5 gN/gVSS-hr). Therefore, the activity of autotrophic denitrifiers may only be important under special conditions and may not play a significant role in wastewater treatment processes for nitrogen removal.

2.9 Organic Assimilation

Inorganic compounds such as nitrite and nitrate are reduced by many organisms as sources of nitrogen. The products of such reductions are primarily amino groups ($-\text{NH}_2$). Assimilative metabolism of nitrogen is quite different from the use of these compounds as electron acceptors for energy metabolism (Madigan et al., 1999). In assimilative metabolism, only enough of the compound is reduced to satisfy nutrient needs for growth. The reduced atoms are eventually converted to cell material in the form of macromolecules. In dissimilative metabolism, a comparatively large amount of the electron acceptor is reduced. Nutrient uptake of nitrogen by activated sludge occurs preferentially with ammonia; nitrate-N is incorporated in the absence of reduced nitrogen (Grady et al., 1999). Nutrient uptake is a minor nitrogen removal mechanism via sludge wastage during wastewater treatment.

Microorganisms prefer to use ammonium nitrogen as an inorganic nitrogen source for cell synthesis because it already is in the (-III) oxidation state, the status of organic nitrogen within the cell. However, when ammonium is not available for synthesis, many prokaryotic cells can use oxidized forms of nitrogen as alternatives. Included here are nitrate, nitrite and dinitrogen gas. When an oxidized form of nitrogen is used, the microorganisms must reduce it to the (-III) oxidation state of ammonium, a process that requires electrons and energy, thus reducing their availability for synthesis. Using an oxidized nitrogen source affects the energy cost of synthesis. Reducing nitrogen for synthesis is not part of respiration and it does not yield any energy. Therefore, reducing the nitrogen source is an energy cost in that electrons which otherwise could have been

transferred to the acceptor to obtain energy are diverted to nitrogen reduction for synthesis.

2.10 Inhibition of Nitrite Oxidation and Nitrite Accumulation

The bacteria responsible for nitrification in treatment plants are very susceptible to inhibition due to process variables such as temperature, pH and DO. Even when these parameters are optimal and a sufficiently large nitrifying population is maintained, considerable disturbances may occur in the presence of small concentrations of inhibitory substances. Under inhibitory conditions, the nitrifying bacteria are not capable of oxidizing all of the nitrogen introduced into the wastewater treatment plant and much of it passes through the system untreated or partially treated.

It is well known that nitrifying microorganisms are sensitive to several inhibitors and toxic substances. Considerable effort has been made in monitoring and measurement of inhibition in recent years. The inhibition of ammonia oxidation has been studied intensively, although the nitrite oxidation step in nitrification could also be affected (Grunditz et al., 1998).

Under optimal conditions, nitrite does not typically accumulate in aerobic treatment systems (Philips and Verstraete, 2000). However, nitrite buildup can occur during full or bench-scale biological wastewater treatment. Several factors can induce nitrite accumulation in nitrification systems: low D.O. (Bernet et al., 2001; Pollice et al., 2002), pH (Suthersan and Ganczarczyk, 1986; Balmelle et al., 1992; Villaverde et al., 1997; Bae et al., 2002), high concentrations of free ammonia and free nitrous acid (FNA)

(Anthonisen et al., 1976; Cecen et al., 1995; Cecen and Ipek, 1998; Villaverde et al., 2000), free hydroxylamine (Yang and Alleman, 1992), heavy metals (Randall and Buth, 1984a; Lee et al., 1997; Madoni et al., 1999) and temperature ((Randall and Buth, 1984a; Randal and Buth, 1984b; Bae et al., 2002).

Early study of nitrite buildup due to free ammonia inhibition (Anthonisen et al., 1976) led to research that identified notable benefits for nitrite based denitrification. Thus, great effort was paid to the development of new technology processes (such as SHARON, ANAMMOX, OLAND) (Verstraete and Philips, 1998) that enable the short-cut of the nitrification-denitrification cycle by avoiding oxidation of nitrite to nitrate. Nitrification of ammonia only to nitrite, with subsequent reduction to gaseous nitrogen, offers several advantages such as: a 25% oxygen requirement reduction in nitrification, a 40% organic carbon requirement reduction in denitrification, a 40% higher denitrification rates, and less biomass production during anoxic growth. Nitrification is stopped at nitrite, by either wash out of nitrite oxidizers with a short SRT (SHARON) or oxygen control (ANAMMOX, OLAND), after which the nitrite is used to oxidize the remaining ammonium (ANAMMOX, OLAND). Another possibility to stimulate the nitrite route is based on the different sensitivity of nitrifiers to substrate-product inhibition, where nitrite oxidizers should be selectively more inhibited at lower NH_3 concentrations than ammonia oxidizers. In substrate-product inhibition, an accumulation of substrate or product of an enzyme slows the activity of that enzyme. Anthonisen et al. (1976) found that a nitrite-oxidizing population was very sensitive to free ammonia, with an inhibition threshold between 0.1 and 1.0 mg N/L. Whereas for *Nitrosomonas*, the inhibition threshold was as high as 10 to 150 mg N/L. Suthersan and Ganczarczyk (1986) later exposed a nitrifying

sludge to free ammonia in an attempt to promote nitrite buildup. Their effort garnered an increase in free ammonia tolerance by NOB up to 2.5 mg/L. The researchers identified free ammonia concentration, pH, time of exposure and biomass concentration as important factors in determining the time for acclimation. Another study of nitrite buildup through free ammonia exposure ended with NOB acclimation to free ammonia up to 20-40 mg/L (Turk and Mavinic, 1989).

Buday et al. (1999) found that non-dissociated NH_3 , HNO_2 , and HNO_3 inhibited nitrite oxidation although these compounds were expected to inhibit ammonia oxidation (Anthonisen et al., 1976). However, the non-dissociated NH_3 and the dissociated NO_2^- and NO_3^- were found to inhibit ammonia oxidation. These experiments were performed as batch tests, so the long-term behavior of the nitrifying microorganisms may differ after acclimatization.

Accumulation of nitrite was observed during the aerobic phase of a sequencing batch reactor (SBR) (Rhee et al., 1997). During SBR operation, no harsh conditions or compounds toxic to nitrite oxidizers were used except ammonia. Therefore, the authors concluded that the presence of free ammonia (0.35 mg-N/L) could be a major factor in causing nitrite build-up. However, Liu and Capdeville (1994) showed that an NOB population was capable of rapidly recovering its lost metabolic activity once the free ammonia concentration became less than 0.1 mg N/L and complete oxidation of nitrite to nitrate was achieved. Rhee et al. (1997) also found that nitrite buildup could be controlled by changing the period of the aerobic phase. Nitrite accumulation was greater when short aerobic periods (2 hr) were used compared to longer aerobic periods (4 hr). Nitrite

accumulation was presumed to be caused by a decrease in the growth of NOB in the short aerobic period.

The influences of pH, temperature and initial ammonia concentration on ammonia removal and nitrate production rates were studied in a continuous pilot unit containing a bacterial population enriched in autotrophic microorganisms (Mauret et al. 1996). The authors intended to demonstrate the possible effect of an interaction between these three parameters on nitrite oxidation and nitrite accumulation. For nitrite accumulation, the temperature effect (15°C - 25°C) was negligible and a strong interaction between initial ammonia concentration and pH was established. Regardless of temperature, a high ammonium level (between 6.6 and 8.9 mg N-NH₃/L) and a pH of 8.5 caused inhibition of nitrite oxidation (50% of the nitrogen introduced accumulated in the form of nitrite).

In another study, an enriched nitrifying culture was used to investigate the cause of nitrite build-up by controlling DO and total ammonia concentrations, as well as pH level in a batch system (Yang and Alleman, 1992). At pH 7.5, nitrite build-up was observed in the system with a low DO level (0.5 mg/L), while no nitrite accumulation was found in the system with a high DO level (6.0 mg/L) even though both of these tests had the same free ammonia levels. A high free ammonia level was, therefore, not considered to be the sole factor causing nitrite build-up. However, a low DO level was also inferred not to be the dominant factor for nitrite build-up since nitrite build-up was found in the high DO level systems at both pH values (8.0 and 8.5) while no nitrite accumulation was observed in either of the systems at a pH of 7.

2.10.1 Inhibition of Nitrite Oxidation by Azide

Azide was shown to be a selective bacteriostatic agent active against gram-negative bacteria and an inhibitor of nitrite oxidation in activated sludge (Tomlinson et al., 1966). Additionally, Ginestet et al., (1998) and Chandran and Smets (1999) used azide as a selective inhibitor of nitrite oxidation and showed that azide quickly inhibited NOB. The inhibition was independent of the nitrite concentration and was reversible after azide was removed by biomass washing. Chandran and Smets (1999) found that a sodium azide concentration of 24 μM selectively inhibited NOB without adversely affecting the maximum specific rate of ammonia oxidation. Ginestet et al. (1998) also noted inhibitory effects of azide on NOB. A 75% reduction in the specific oxygen uptake rate was achieved at 0.80 μM azide for NOB. An azide concentration of 24 μM instantly and completely inhibited NOB with no apparent effect on ammonia oxidizers. Ginestet et al. (1998) also confirmed that 24 μM azide had no effect on heterotrophic activity. Thus, it was concluded that azide can be used as a selective inhibitor of nitrite oxidation in a mixed bacterial population. The authors speculated that azide could act by complexation with the molybdenum atoms of the nitrite oxidoreductase, the enzyme catalyzing nitrite oxidation.

2.10.2 Inhibition of Nitrite Oxidation by Volatile Fatty Acids

Gomez et al. (2000) studied the effects of several volatile organic compounds such as ethanol, acetic acid, butyric acid and propionic acid ranging from 0 to 2000 mg/L as well as a mixture of VFAs at different concentrations (at a ratio of 4:1:1, acetate: propionate: butyrate) on the kinetics of nitrification in stable nitrifying activated sludge in

batch cultures. Inhibition effects were based on measurements of nitrate formation. At a concentration of 500 mg/L, the rate of nitrification inhibition differed for each compound with propionate and butyrate being the most inhibitory. Nitrate formation was completely inhibited in the presence of 1000 mg/L of either propionate or butyrate. At 2000 mg/L, inhibition was 80% with ethanol and 100% with acetate, propionate, and butyrate. The effect of VFA mixtures showed different degrees of inhibition in the nitrate formation process, suggesting that the type of compound played an important physiological role in the kinetics of nitrite oxidation. Ethanol and acetate were less inhibitory than propionate and butyrate. The authors suggested that respiration of the microorganisms might be influenced by the C:N ratio. Since the levels of inhibition in the nitrate formation rate were different for each substrate tested, the C:N ratio alone did not explain the physiological differences in the nitrifying sludge. The results showed that even at the same concentration, the microbial growth and respiration profiles could be influenced by the type of substrate in the nitrifying culture. When a mixture of VFAs at different concentrations was assayed, profiles similar to that of acetate were observed. At a mixture of VFAs concentration of 750 mg/L, nitrate formation decreased to 40% relative to the control; however, no nitrite accumulation was measured. These results indicated that the nitrifying sludge was very sensitive to the presence of these compounds, contrary to previous reports. The effect of VFA mixture pulses (at a ratio of 4:1:1, acetate: propionate: butyrate, over 14 hours) on the performance of continuous nitrifying process was also studied. No inhibition of the nitrification process was observed with VFA mixture pulses of 750 to 3000 mg/L. The researchers concluded that the non-inhibitory effect of organic matter on nitrification efficiency might be related to the feeding pattern.

In another study, Takai et al. (1997) monitored toxic and inhibitory effects of VFAs on nitrification activity in an aerobic biofilm. The effects of VFAs (acetic, propionic, butyric, iso-butyric, n-valeric, iso-valeric acid) on nitrification were examined based on batch experiments. VFA concentration ranges were set to 0-3000 mg/L. The researchers found that each VFA did not inhibit ammonia oxidation, but did affect nitrite oxidation. From the result of batch experiments, inhibition constants (γ) for 3000 mg/L acetic and propionic acid at pH 7.5 were 0.24 and 0.29, respectively. Effects of VFAs produced in biofilm reactors under various flow rates and recirculation ratios on nitrifying bacteria were also investigated using laboratory-scale treatment systems composed of an anaerobic biofilm reactor, an aerobic biofilm reactor and a sedimentation tank. High concentrations of VFAs were found in anaerobically treated effluent (with the average values of 56.6 mg/L acetic acid, 18.4 mg/L propionic acid, 5.7 mg/L butyric acid, 5.9 mg/L iso-butyric acid, 2.5 mg/L n-valeric, 5.6 mg/L iso-valeric acid) and nitrite accumulation was observed and assumed to be due to inhibition of nitrite oxidation in the aerobic biofilm reactor.

The effects of VFAs on nitrification activity of activated sludge were also studied in laboratory batch experiments by Eilersen et al. (1994). The concentration range of the various compounds used were: acetic acid, 0-2400 mg/L; formic acid, 0-1380 mg/L; propionic acid, 0-2220 mg/L; butyric acid, 0-2640 mg/L; n-valeric, n-caproic, isobutyric and isovaleric acid, 0-30 mM. Formic, acetic, propionic and butyric acids all affected nitrite oxidation, but exhibited no significant effect on ammonia oxidation. Formic acid was found to be the fatty acid most inhibitory to nitrite oxidation, whereas acetic acid was the least. There was a 40% inhibition in nitrite oxidation in the presence of 1.2 g/L of

acetate. The researchers did not indicate which concentrations of the other acids were inhibitory for ammonia and nitrite oxidation. Fatty acids of the iso-configuration were more inhibitory than fatty acids of the n-configuration (Eilersen et al., 1994) indicating that the structural formula of the compound played a decisive role in inhibition by these acids. Also, there was no correlation between the number of C-atoms and inhibition. In addition, no correlation between the degree of inhibition of nitrification by VFAs and liposolubility was observed, indicating that additional mechanisms, such as enzyme interference, may be involved.

In summary, previous studies found that VFAs affected nitrite removal and nitrate formation at different degrees (acetic acid was less inhibitory) although some inconsistencies exist. For instance, Eilersen et al. (1994) reported that there was a 40% reduction in nitrite oxidation in the presence of 1.2 g/L of acetate with no significant effect on ammonia oxidation, while another study indicated that nitrite oxidation was affected by 70% at 1.0 g/L of acetate (Gomez et al., 2000). However, Takai et al. (1997) found that acetic acid reduced nitrite oxidation by 24% at 3g/L. In addition, previous research suggests that the chemical structure of VFAs played an important role in nitrite removal and nitrate formation.

2.11 Summary

Nitrite and nitrate are intermediates of both nitrification and aerobic denitrification in the conceptional model for nitrogen removal. Combining nitrification

and denitrification in a single aerobic reactor appears to be a promising technique to remove nitrogen from wastewater as N_2O and N_2 .

Aerobic nitrate reduction was shown to occur in a variety of bacteria (Carter et al., 1995). The periplasmic nitrate reductase is responsible for the aerobic reduction of nitrate to nitrite. It was demonstrated that aerobic expression of the periplasmic nitrate reductase was largely influenced by more reduced carbon sources such as butyrate in pure culture experiments. In addition, it was found that VFAs, like butyrate and propionate produced during anaerobic treatment of wastewater, decreased nitrate formation in activated sludge systems. However, the impact of VFAs on nitrite removal in activated sludge was not evaluated. The mechanism of action for VFAs was assumed to be inhibition of nitrite oxidation via enzyme interference. Additionally, information related to the influence of VFAs on both nitrite removal and aerobic denitrification is not currently available. The nitrate produced as a consequence of nitrite oxidation is presumably further reduced via catalysis by periplasmic nitrate reductase. If this is the case, then estimations of nitrification rates based on measurements of nitrate could be lower than the true rate of nitrification. Further research is required to obtain a better insight into the complex cooperative effects in mixed cultures of nitrifying and denitrifying bacteria. New technological applications and novel microbial processes like aerobic denitrification can further enhance the removal of nitrogen from wastewater and reduce the chemical and energy demand for this process.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Approach

The effects of VFAs on nitrite removal during nitrification and the NOB activity of activated sludge were studied in laboratory batch and reactor experiments. A CSTR was selected to provide activated sludge samples for a comprehensive study of VFA impacts. Nitrite and nitrate were measured during VFA exposure to examine effects on nitrite removal and nitrate formation. Activity of NOB was investigated based on the measurements of CO₂ fixation and the number of *Nitrospira* and total bacteria. Since it was suspected that aerobic denitrification occurred in the presence of VFAs, N₂O gas production, NAP enzyme activity and the presence of the genes of NAP enzyme were also investigated.

A CSTR was established to provide an activated sludge representative of a mixed culture treating municipal wastewater. Mixed liquor suspended solids samples were retrieved from the CSTR to perform the batch experiments in which nitrite, nitrate, CO₂ fixation, the number of *Nitrospira* and total bacteria, N₂O gas production and NAP enzyme activity were measured. Furthermore, VFAs were spiked into the CSTR to investigate the effects of VFAs on nitrogen removal in a continuous flow activated sludge system. Figure 3-1 outlines the approach utilized in this research. A more detailed description of each method is given in the following sections of this Chapter.

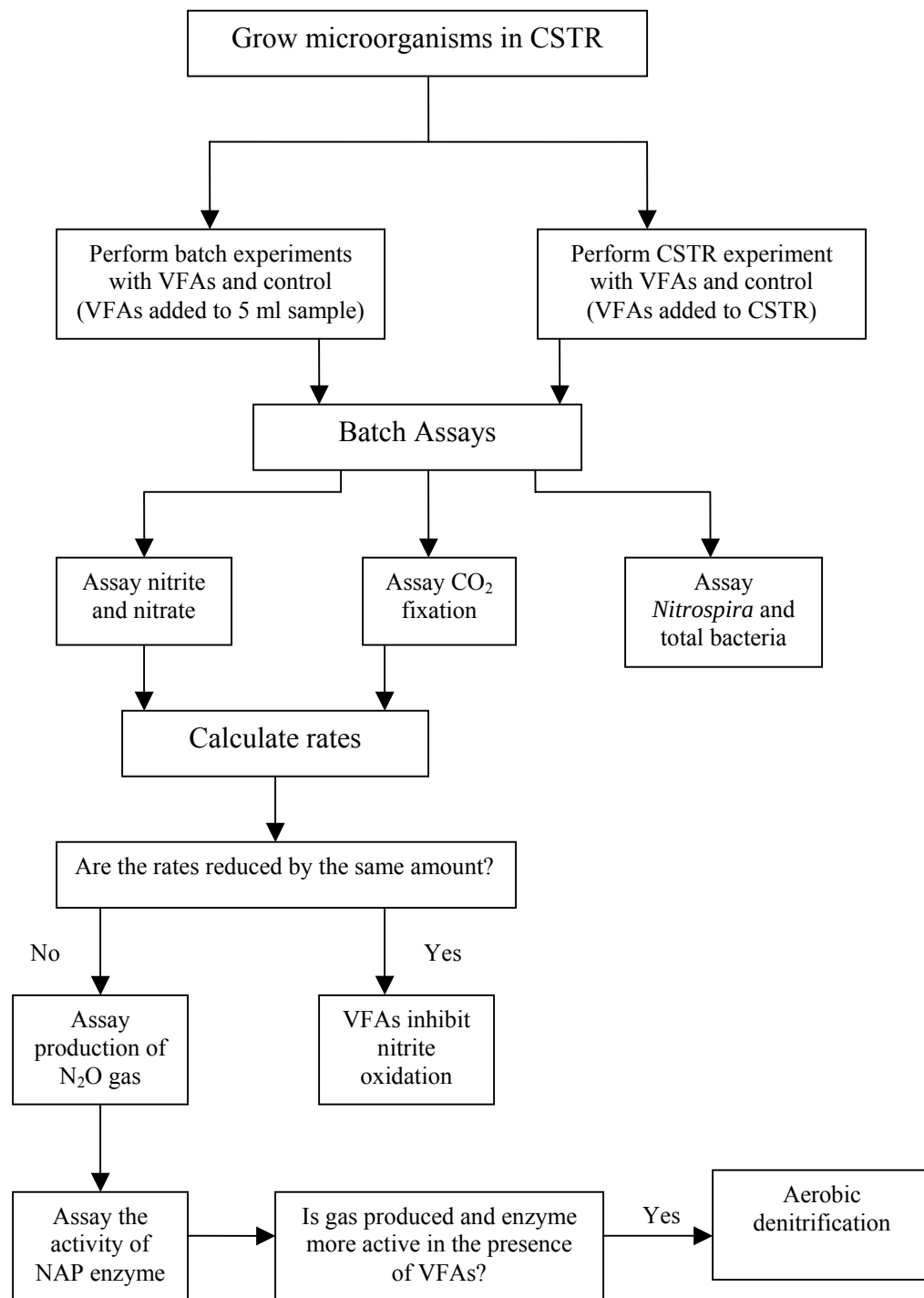


Figure 3-1. Outline depicting the experimental approach used in the evaluation of VFA impacts on nitrite removal and nitrate formation in activated sludge systems

3.2 Experimental Treatment System

3.2.1 Reactor Design

The bench-scale treatment system consists of a complete mix reactor and an external secondary clarifier with biomass recycle. The unit was designed to simulate a conventional activated sludge wastewater treatment process (Hawkins, 2000). The reactor illustrated in Figure 3-2 was constructed entirely of Plexiglas. The reactor consists of a 30.48 cm (12") section of 25.4 cm (10" diameter) Plexiglas tube with 30.48 cm (12") square, 1.27 cm (½") thick top and bottom plates of Plexiglas. The bottom and top plates each had a 25.4 cm (10") circular etching to provide a better fit for the sidewall tube section. The tube was bonded to the bottom plate to prevent leakage. The top plate was removable for sampling, cleaning and maintenance purposes. The reactor contents were mixed by placing the reactor on top of a 30.48 cm (12") square magnetic stir plate (model 547035, Barnstead / Thermolyne, Dubuque, IO) that turned a 5.08 cm (2") Teflon® coated stir bar inside the reactor. During reactor operation, the stir plate was operated at one-half its maximum turning speed to keep the activated sludge in suspension and the reactor contents completely mixed. At this speed, the stir plate was able to provide a volumetric power input of approximately 1500 kW/1000 m³ (Parker, 2001) well above the required 14 kW/1000 m³ to ensure complete mixing in an activated sludge CSTR using mechanical mixing devices (Grady et al., 1999).

The reactor contents drained continuously, via gravity overflow, to an external secondary clarifier through a 1.27 cm (½") diameter port. The drain port was positioned to provide a liquid volume of 10-L in each reactor. Sludge recycle and influent flows

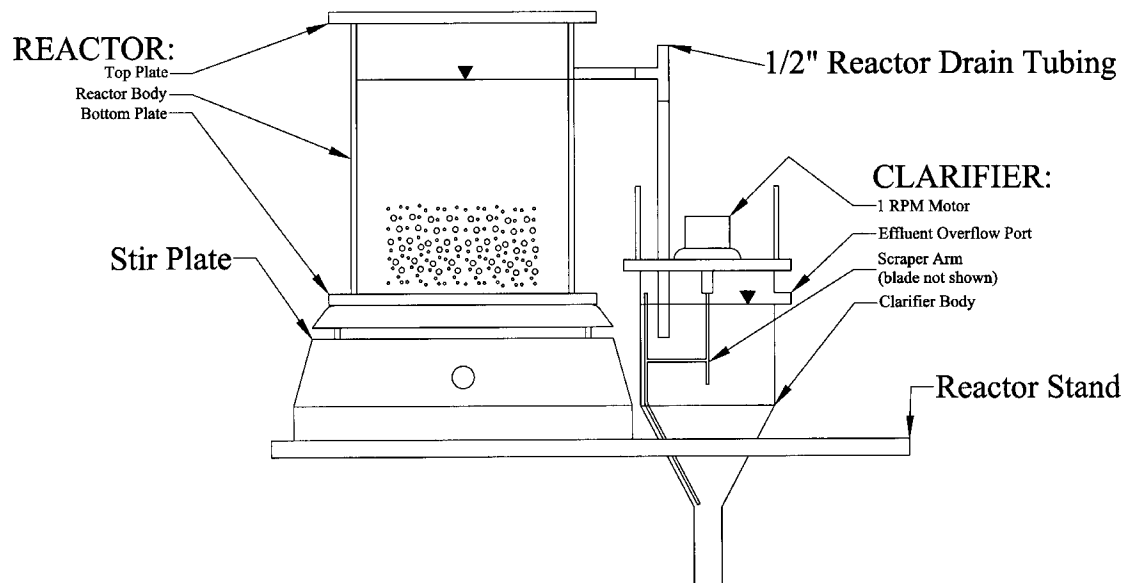


Figure 3-2. Diagram of the treatment unit, which consisted of a continuous-stirred tank reactor and an external secondary clarifier (Parker, 2001)

were introduced continuously into the reactor through 0.635 cm (1/4") ports located 5.08 cm (2") above the surface level of the mixed liquor and extending approximately 3.81 cm (1.5") from the sidewall into the reactor.

Aeration was provided to the reactor through two 15.24 cm (6") long non-clogging porous aquarium aerator tubes mounted to the bottom plate of the reactor via suction cups. A laboratory compressed air valve provided the air supply for the reactor. Air from this valve passed into a pressure regulator, a filter assembly, and into a 20-L carboy fitted with an airtight cap. Airflow was directed to the bottom of the carboy where it bubbled up through a water column. This process served to both humidify the air and equalize the air temperature, preventing excessive evaporation from the reactor during aeration. From the carboy, air traveled through a condensate trap to a 2-way control metering assembly.

3.2.2 Clarifier Design

The clarifier was composed of two components, a main body and a scraper arm assembly. The main body was constructed from a 15.24 cm (6") diameter glass tube. One end was heated and drawn down from the original 15.24 cm (6") diameter to a 3.18 cm (1 1/4") diameter, forming a conical bottom section. A 7.62 cm (3") section of 3.18 cm (1 1/4") glass tube was then welded onto the main body to serve as a reservoir for sludge scrapings. The conical section sloped approximately 70° from horizontal. The effluent drain consisted of a 0.953 cm (3/8") diameter 2.54 cm (1") long glass tube welded over a

precut hole in the side of the clarifier. The drain was located to provide a liquid volume of 2.90 ± 0.05 liters within the clarifier's main body.

A scraper arm was added to the clarifier to prevent sludge from accumulating on the clarifier walls. The scraper arm assembly was mounted on top of the clarifier's main body via a grooved circular trough cut into a 17.78 cm (7") square, 1.27 cm ($\frac{1}{2}$ ") thick Plexiglas base plate. A 10.16 cm (4") long section of 15.24 cm (6") diameter Plexiglas tube was bonded to this base plate and enclosed the scraper arm motor. This 1-rpm motor was bolted to the base plate with the motor shaft penetrating into the clarifier's main body through a hole drilled in the base plate. The sludge scraper arm consisted of 0.318 cm ($\frac{1}{8}$ ") diameter stainless steel wire that was shaped to conform to the side of the clarifier wall and mounted to the motor shaft with setscrews. An automobile wiper blade was placed on the scraper arm to provide a snug fit against the clarifier wall. A timer was used to engage the scraper arm motor every five minutes for the length of time required to rotate the scraper arm approximately one-half turn.

3.2.3 Collection and Storage of Influent Wastewater

Influent wastewater was obtained from the Knoxville Utilities Board (KUB) Kuwahee WWTP. The facility receives mostly municipal wastewater from the city of Knoxville, but also receives commercial and industrial wastewater inputs. The wastewater was collected in batches using three 25-L (6.5-gallon) and two 20-L (5.5-gallon) carboys which were filled using a sump pump submerged in the plant's primary clarifier effluent stream. Upon collection of the wastewater, the carboys were transported

to the laboratory and immediately placed in a 5°C constant temperature room where the wastewater was allowed to cool overnight before transfer to the experimental treatment system feed tank, a steel 208.2 L (55-gallon) drum. A small submersible pump was placed at the bottom of the feed tank to gently circulate the wastewater in order to minimize solids settling and maintain a homogeneous waste feed. At an influent flow rate of 19 mL/min to the reactor, the total volume of the five carboys was sufficient to feed the reactor for four days. Therefore, this procedure was repeated every four days throughout the treatment study.

3.2.4 Operating Conditions

The continuous-flow complete mix reactor at an SRT of 10 days was operated in a constant temperature room at 20°C. DO was maintained at 3 mg/L using a control system. SRT, temperature, and DO values were chosen based on optimum conditions for nitrification (Bitton, 1994) so as avoid any additional limitations on nitrification other than those due to presence of VFAs.

Influent was pumped from the feed tank to the reactor via a hole that connected two constant temperature rooms. The influent line to the reactors was placed at the bottom of the feed tank, which was maintained at 5°C to minimize degradation of the wastewater during storage. The influent and recycle flows were conveyed to the reactor using a Cole Parmer, Inc. continuous duty Standard Drive[®] peristaltic pump. The pump rotors were fitted with two pump heads (one for influent and one for recycle) so that the reactor would receive influent and recycle flows at exactly the same rate. The influent and recycle flow rates were set to 19 ml/min, which provided an HRT of 8.8 hours in the

reactor and 2.5 hours in the clarifier (excluding the recycle flow). An influent sampling port was plumbed into the influent line to the reactor with a tee. The placement of this sampling port effectively eliminated concerns about degradation in the storage tank and influent line. The effluent grab sample port was located immediately following the clarifier overflow. This placement avoided collection of the effluent after it had passed through several feet of tubing which was prone to photosynthetic growth.

3.2.5 DO Control System

A control system (Great Lakes Instruments) was installed to monitor and regulate the DO concentration in the reactor. The control system consisted of one Model 5500-Series DO probe, one Model D53 DO analyzer, and one solenoid valve unit. The probe was calibrated periodically throughout the study to ensure accuracy of measurements. Prior to calibration, the probe was removed from the reactor and rinsed with deionized water and gently wiped with a damp cloth to remove any attached bacterial growth on the membrane. The probe was then placed in a sealed plastic calibration bag and allowed to equilibrate to the conditions inside the bag. The analyzer computed the DO concentration based on the atmospheric pressure and temperature of the air surrounding the probe and made the necessary adjustments to the probe's internal calibration curve.

The control system's DO analyzer accepted input from the probe and converted the magnitude of the current produced by a probe into DO concentration in mg/L. The analyzer compared the reading with a previously specified set point concentration (3 mg/L). If the DO reading exceeded 3 mg/L, the analyzer switched a relay closing a solenoid valve, which stopped the airflow to the reactor until the analyzer read a value

below 3 mg/L. Once the DO concentration fell below 3 mg/L, the analyzer opened the solenoid valve, allowing airflow to enter the reactor. In this manner, tight control of the DO concentration in the reactor was maintained.

3.3 Batch Experiments

The effects of VFAs on nitrite removal, nitrate formation, CO₂ fixation, the number of total bacteria and *Nitrospira*, N₂O gas production and the periplasmic nitrate reductase enzyme activity were studied in laboratory batch experiments performed with fresh mixed liquor suspended solids samples obtained from the CSTR (Figure 3-3). Nitrite removal, nitrate formation, CO₂ fixation, N₂O gas production and the activity of NAP enzyme assays were carried out in triplicate in 40-ml serum vials containing 5 ml of mixed liquor samples and a mixture of VFAs (acetate, propionate, and butyrate at a ratio of 1:1:1). Sample vials were prepared by adding 5 ml of mixed liquor samples taken from the CSTR operating at room temperature into 40-ml serum vials. 25 mg-N/L of nitrite as sodium nitrite (NaNO₂) (final concentration) was used as the primary electron donor for the NOB. The final concentrations of mixture of VFAs were as follows: either 1500 mg/L (500 mg/L acetate, 500 mg/L propionate, and 500 mg/L butyrate) or 3000 mg/L (1000 mg/L acetate, 1000 mg/L propionate, and 1000 mg/L butyrate). This concentration range was chosen based on previous research findings (Eilersen et al., 1994; Takai et al., 1997; Gomez et al., 2000). Before the addition of VFAs, pH was adjusted to levels found in the CSTR (7.2-7.5) using 3 M sodium hydroxide (NaOH) added in 100 µL aliquots to neutralize the VFA mixtures. Control samples, in the absence of VFAs, were also

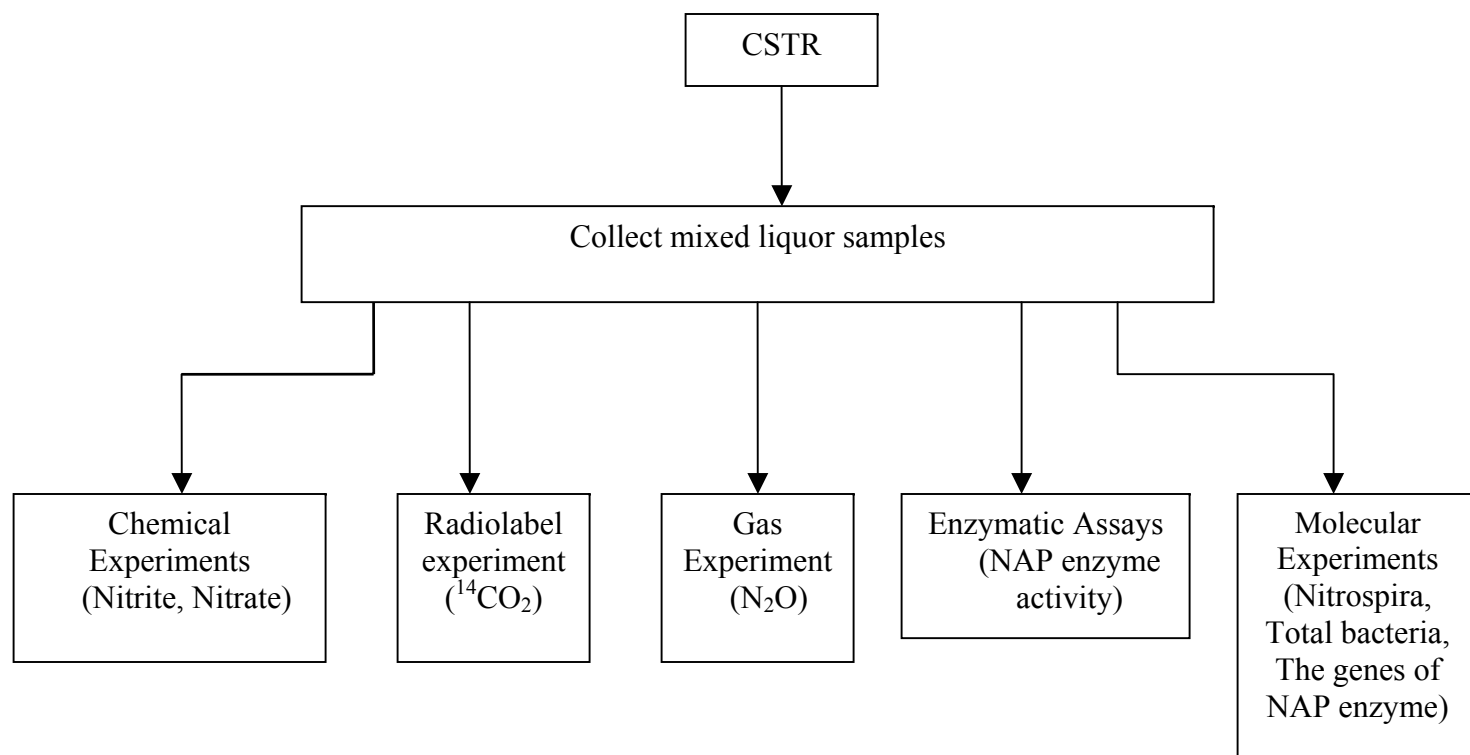


Figure 3-3. Schematic diagram showing chemical and microbiological analysis performed during batch experiments in the absence and in the presence of VFAs

analyzed. Additionally, 84 μM ATU (a specific ammonia oxidizer inhibitor) and 50 μM azide (a specific nitrite oxidizer inhibitor) (Ginestet et al., 1998) were used as inhibitors in some of the experiments to determine how nitrite removal, nitrate formation and CO_2 fixation were affected by typical nitrification inhibitors.

All assays were terminated after 5 h of incubation at 20°C on a shaker (200 rpm). Daims et al. (2001) demonstrated that 5 h of incubation was more suitable than 3 h for monitoring of the uptake of inorganic carbon by *Nitrospira*-like bacteria. In addition, Feliatra and Bianchi (1993) found that the best incubation time for inorganic carbon incorporation by NOB was 5 hr and that incubations of 12 or 24 h were too long and lower amounts of carbon were incorporated.

Batch samples were established as follows (Figure 3-4). 100 ml of mixed liquor for experimental samples and 100 ml of mixed liquor for control samples were removed from the CSTR. pH adjusted VFAs were dispensed into the experimental samples. Then, 25 mg N/L (final concentration) as sodium nitrite (NaNO_2) was added to both the experimental and control samples as a substrate and an energy source for the nitrite oxidizers. Samples were subdivided into 5 ml samples using 40-mL serum vials. Radiolabelled H^{14}CO_3 was also added to vials used in the CO_2 fixation experiments at a concentration of 20 μM (final concentration). Vials were shaken by hand and initial samples were taken for nitrite and nitrate measurements from the liquid phase using a 0.45 μM Gelman glass fiber syringe filter and dispensed into auto-sample vials (Figure 3-5). N_2O gas samples were also removed from the head space using an air-tight syringe. Two mL subsamples acidified with 2N H_2SO_4 were initially collected from the vials containing H^{14}CO_3 . The acidified samples were transferred to 20 mL scintillation vials

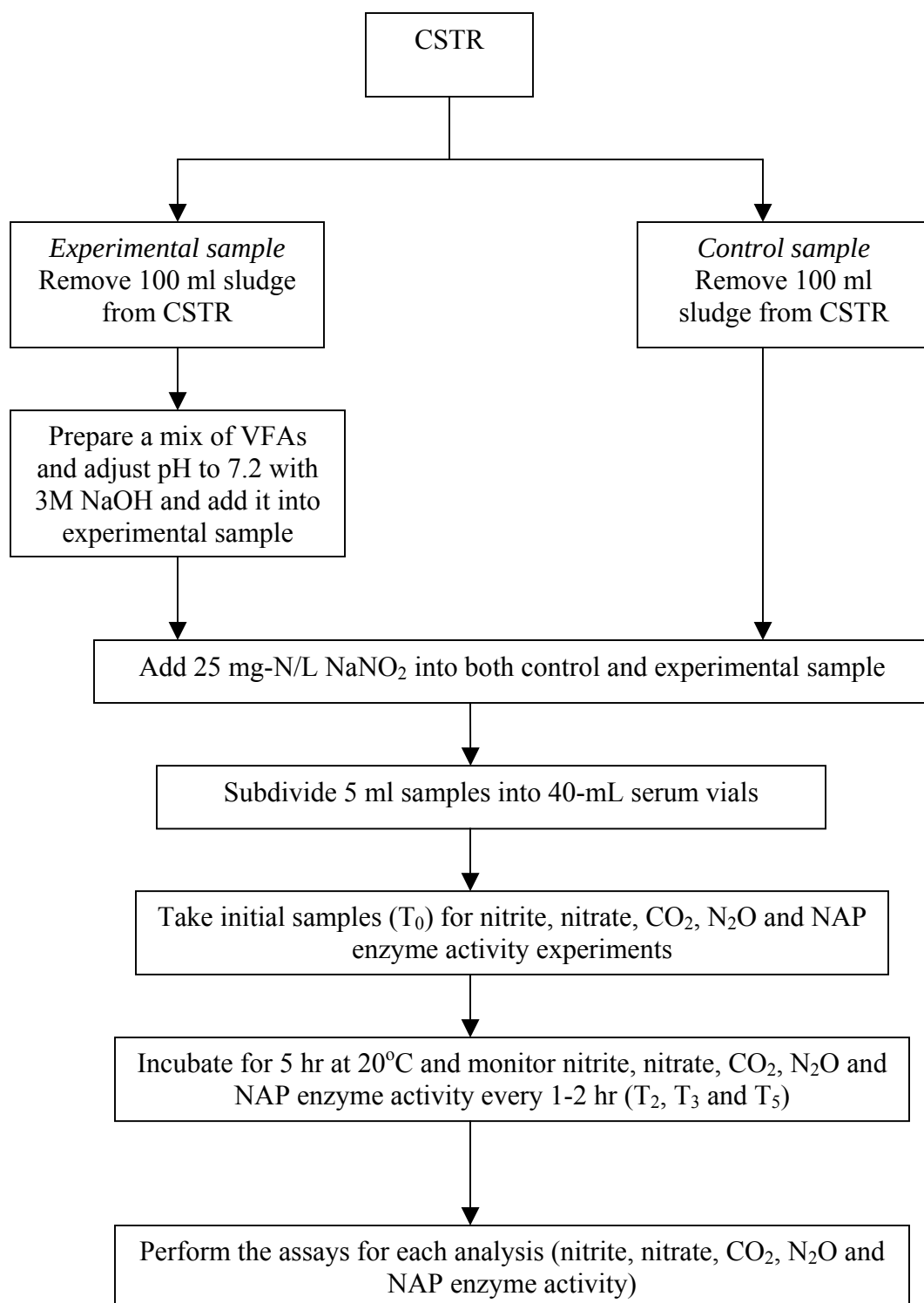


Figure 3-4. Schematic diagram showing various steps performed in batch experiments

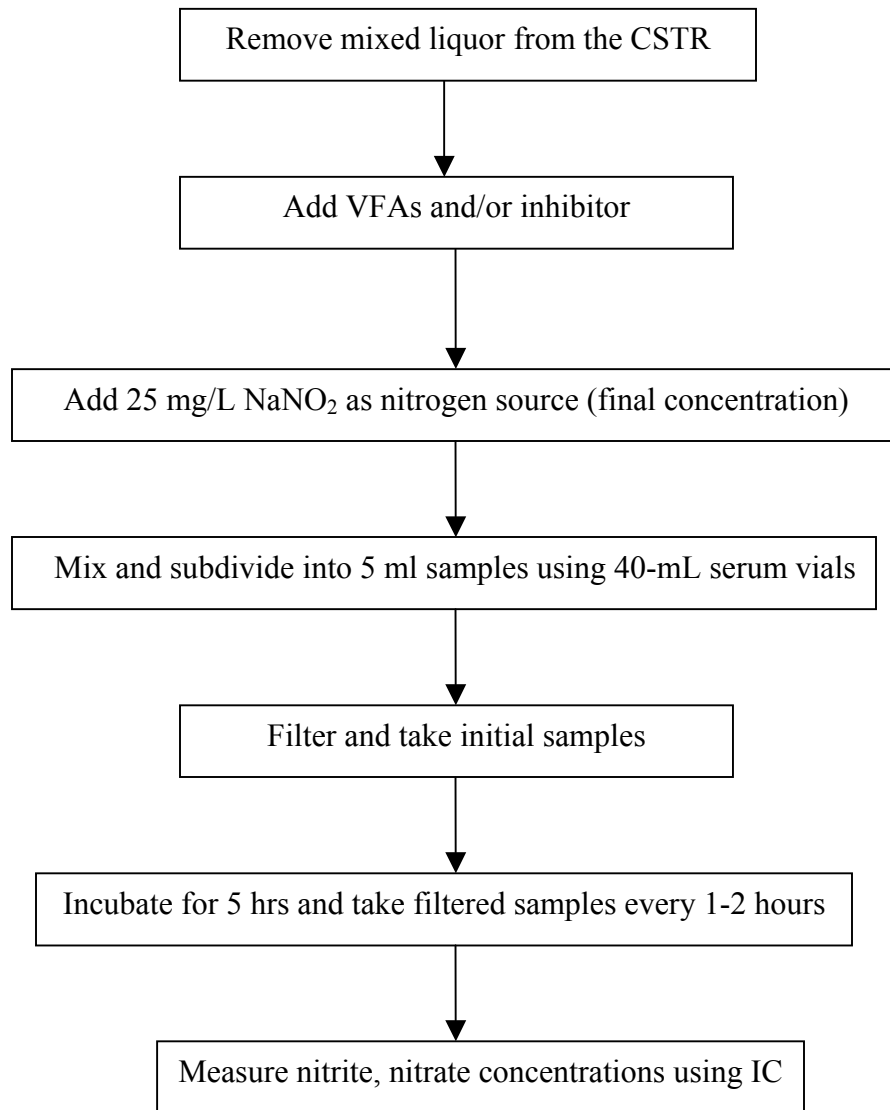


Figure 3-5. Schematic diagram showing the steps in batch experiments for analysis of nitrite and nitrate concentrations

and flushed with nitrogen gas using an UHP grade nitrogen tank for 30 min to remove inorganic ^{14}C (Figure 3-6). After initial samples were taken, all remaining vials were incubated at 20°C on a shaker (200 rpm) for 5h. Nitrite, nitrate and CO_2 samples were taken every 1 to 2 hours and at the end of the experiments using the same procedures described above. N_2O gas samples were taken only at the end of the incubation. The analyses performed in batch experiments are summarized in Table 3-1 and explained in detail in the Analytical Methods section.

3.4 Analytical Methods

3.4.1 Measurement of Nitrite-N and Nitrate-N Concentrations

The effects of VFAs on nitrite oxidation were assessed based on the rate of nitrite removal and nitrate production. It was, therefore, necessary to measure nitrite and nitrate concentrations over time in both control and experimental samples. Concentrations of nitrite-N and nitrate-N were determined in triplicate according to Standard Method 4110 B, Ion Chromatography with Chemical Suppression of Eluent Conductivity (APHA, 1998) using a Dionex DX 500 Ion Chromatograph (IC) outfitted with an Ionpac® AS4A 4mm anion exchange column. 1.5 mL of the sample was taken from the serum vials with a syringe and filtered using a $0.45\ \mu\text{m}$ Gelman glass fiber syringe filter into auto-sample vials. For each batch of samples, the IC was calibrated with six standards ranging from 0.1 mg-N/L to 150 mg-N/L. Additionally, a quality control sample was analyzed during each IC run. A calibration plot was generated to calculate the concentration of nitrite and nitrate in the samples.

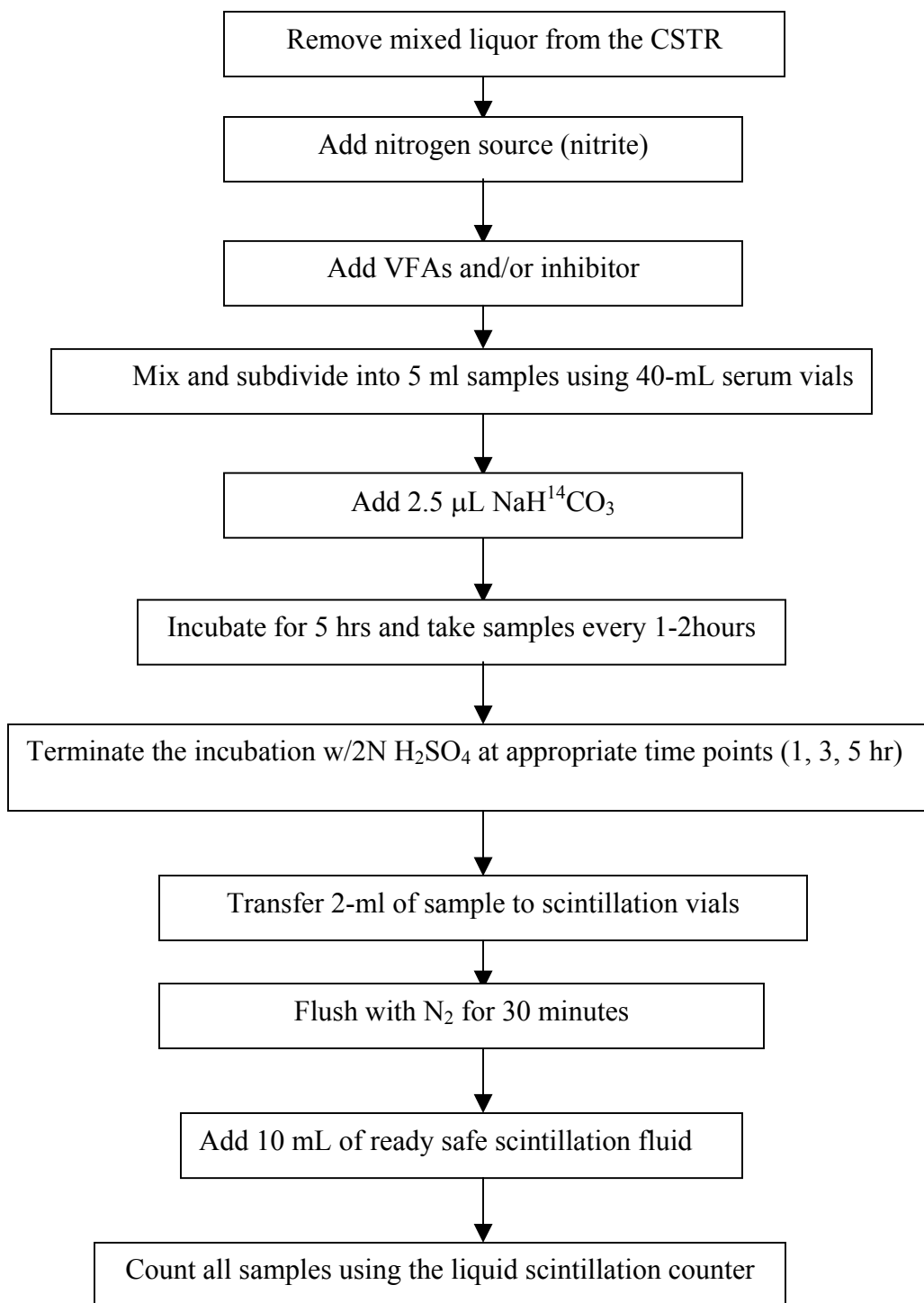


Figure 3-6. Schematic diagram showing the steps in radiolabeled CO_2 experiment

Table 3-1. Summary table of analytical parameters and methods performed in batch experiments

Parameter	Method	Units
Nitrite	Ion chromatography	mg/L
Nitrate	Ion chromatography	mg/L
CO ₂ fixation	NaH ¹⁴ CO ₃ incorporation	μg/L
N ₂ O	IR spectrometry	mg/L
NAP enzyme	Methylviologen assay	nmol/min/mg protein
<i>Nitrospira</i>	Real-time PCR	cells/L
Total bacteria	Real-time PCR	cells/L
Genes for NAP enzyme	Nested PCR	-

3.4.2 Measurement of CO₂ Fixation by NaH¹⁴CO₃ Incorporation

The activity of NOB was assessed based on the measurements of CO₂ fixation. Mixed liquor samples removed from the reactor, using a serological pipette, were utilized in CO₂ uptake experiments. 5 mL samples were placed into sterile 40-ml serum vials. 2.5 μL (1,375,000 dpm or 0.625 μCi per vial) NaH¹⁴CO₃, specific activity 6.3 mCi/mmol (Sigma) was added to each sample vial (final concentration 20 μM NaH¹⁴CO₃). The same quantity of NaH¹⁴CO₃ was also added to three separate scintillation vials, containing 1 mL of water and 10 mL of ready safe scintillation liquid, to determine the initial radioactive counts. All vials were sealed with a teflon/silica septum and cap and incubated at 20°C on a shaker (approx. 200 rpm). Incubation was terminated with 0.5 mL

of 2N H₂SO₄, added through the septum using a 1 mL syringe, for each time point. 2-ml subsamples were transferred to 20-mL scintillation vials and flushed with N₂ for 30 minutes to remove inorganic ¹⁴C as ¹⁴CO₂. The radioactive bicarbonate in the biomass was quantified by a Packard liquid scintillation counter Model 2900 TR (Packard Instrument Company, IL) using 10 ml of Ultima Gold XR (Packard Instrument Company, IL) as the scintillation cocktail (Daims et al., 2001; Brandt et al., 2001).

3.4.3 Determination of Nitrite Removal, Nitrate Formation and CO₂ Fixation Rates

The nitrite removal and nitrate formation rates were calculated from the concentrations of nitrite and nitrate in samples taken initially and every 1 to 2 hours in batch experiments. The CO₂ fixation rates were determined from the amounts of radiolabel incorporated into bacterial biomass during the 5-hr incubation period in batch experiments. The rates of nitrite removal, nitrate formation and CO₂ fixation were determined based on least squares regression analysis (linear fit) using the Sigma Plot computer program. Measured concentrations were plotted over time and the slope was equal to the rate.

% Inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \left[\frac{\text{control rate} - \text{inhibited rate}}{\text{control rate}} \right] \times 100 \quad (3.1)$$

3.4.4 Volatile Fatty Acids Analysis Procedure

This analysis was performed to document removal of VFAs in the batch experiments. A small aliquot of the sample was taken from the serum vials with a syringe and filtered through a 0.45 μm Gelman glass fiber syringe filter into auto-sample vials and then acidified with 10 μL of 6 N phosphoric acid. VFA analysis was performed using an HP 5890 II gas chromatograph equipped with an autosampler, a flame ionization detector (FID) and 30 m J&W DB-FFAP column (0.25 mm i.d., and 0.25 μm film thickness). The temperature of the column started at 80°C for 1 min and then increased to 160°C for 10°C/min. The injector and detector were maintained at 175°C and 300°C, respectively. Helium was employed as carrier gas at a flow rate of 1.5 mL/min which kept constant by the electronic pressure control (EPC). The concentration of VFAs was determined by comparison with a standard curve. The detection limits for the method were between 1 and 2 mg/L for each VFA.

3.4.5 Quantification of Total Eubacteria and Nitrite-Oxidizers (*Nitrospira*) in Mixed Liquor

3.4.5.1 DNA Extraction

Genomic DNA was extracted from 2 mL suspended solid samples by using a Fast DNA kit (Bio 101, Vista, CA), with minor modifications as described by Dionisi et al. (2002). Samples were centrifuged at 3000 rpm for 15 min at room temperature. Pellets were resuspended in 800 μL of CLS-TC buffer, and transferred to a lysing tube (1/4" sphere + Garnet Matrix). Samples were homogenized in FastPrep Instrument for 20 sec at a speed of 4.0, then chilled on ice for 5 min, and finally centrifuged at 10,000 rpm for 15

min at 4°C. Supernatant was transferred to a clean microcentrifuge tube. Afterwards 600 µL of binding matrix was added and mixed for 10 min at room temperature. The samples were centrifuged at 10,000 rpm for 2 min and the pellet was resuspended with 500 µL of 80% EtOH and transferred to a spin module. The samples were spun for 1 min and contents were discarded into a catch tube. This procedure was repeated twice. DNA was eluted from by resuspending the binding matrix in 100 µl of 10 mM Tris-HCl (pH 8), followed by 5 min incubation at room temperature and centrifuged 1 min at 14,000 rpm to transfer DNA to the catch tube. DNA concentration will be measured using a DyNA Quant200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA).

3.4.5.2 Real-time PCR

Real-time PCR assays were used to quantify eubacterial 16S rRNA and *Nitrospira* spp. 16S rRNA genes in mixed liquor samples.

Amplification of total eubacterial 16S rRNA genes was performed using primers 1055f (5'-ATGGCTGTCGTCAGCT-3') (Ferris et al., 1996) and 1392r (5'-ACGGGCGGTGTGTAC-3') (Lane, 1991) and the TaqMan probe 16STaq1115f (5'-6-FAM-CAACGAGCGCAACCC-TAMRA-3') (Harms et al., 2003). Real time PCR assays for quantification of total eubacteria were performed in a total volume of 25 µl with 5mM of MgCl₂, 12.5 µl Platinum[®] Quantitative PCR SuperMix-UDG (Life Technologies, Inc., Gaithersburg, MD), 15 pmol of primers 1055f and 1392r, 6. 25 pmol TaqMan probe 16STaq1115f and 3.2 to 7.0 ng of sample DNA (Harms et al., 2003). The PCR program

consisted of 3 min at 50°C, an initial denaturation at 95°C for 30 sec, annealing at 50°C for 60 sec and extension at 72°C.

Real-time PCR assay using TaqMan probe for *Nitrospira* 16S rRNA genes was performed using the primers NSR1113f and NSR1264r with a Bio-Rad iCycler with the iCycler iQTM fluorescence detector (Bio-Rad, Hercules, CA). These assays were developed and optimized for the quantification of *Nitrospira* spp. in MLSS samples (Dionisi et al., 2002; Harms et al., 2002). The target molecule for detection of the nitrite-oxidizing bacteria *Nitrospira* was the 16S rRNA gene. The *Nitrospira* rRNA gene primers NSR1113f (5'-CCTGCTTTCAGTTGCTACCG-3') and NSR1264r (5'-GTTTGCAGCGCTTTGTACCG-3') were previously designed and tested using genomic DNA extracted from municipal and industrial MLSS as templates (Dionisi et al., 2002). The TaqMan probe NSR1143fTaq (5'-6-FAM-AGCACTCTGAAAGGACTGCCAGG-TAMRA-3') was designed for *Nitrospira* 16S rDNA (Harms et al., 2003).

Real-time PCR assays for quantification of *Nitrospira* 16S rRNA gene with NSR1143fTaq were performed in a total volume of 25 µl with 5 mM of MgCl₂, 12.5 µl Platinum[®] Quantitative PCR SuperMix-UDG (Life Technologies, Inc., Gaithersburg, MD), 15 pmol of primers NSR1113f and NSR1264r, 6.25 pmol TaqMan probe NSR1143fTaq, and 3.2 to 7.0 ng of sample DNA (Harms et al., 2003). The PCR amplification consisted of 2 min at 50°C, 10 min at 95°C, 55 cycles at 95°C for 30 sec and at 63°C for 60 sec. Positive controls were included in all sets of amplifications along with negative controls (no DNA added). The presence and size of the amplification products were determined by a run on MJ optical.

3.4.6 Measurement of N₂O in Batch Experiments

Biological denitrification is a respiratory process defined as the enzymatic stepwise reduction of nitrogenous oxides coupled to electron transport phosphorylation with concomitant evolution of the gases nitric oxide, nitrous oxide and dinitrogen (NO, N₂O and N₂). Therefore, a measurement of gaseous products is an appropriate way of determining whether denitrification has occurred during wastewater treatment. In this research, infrared radiation (IR) spectrometry was used to measure N₂O. Chemical bonds in different environments absorb infrared radiation at varying intensities and at varying frequencies. Thus, IR spectroscopy involves collecting absorption information and analyzing it in the form of a spectrum. The frequencies at which there are absorptions of IR radiation ("peaks" or "signals") can be correlated directly to bonds within the compound in question. Because each interatomic bond may vibrate in several different motions (stretching or bending), individual bonds may absorb at more than one IR frequency.

Batch experiments following the protocols described in section 3.3 were performed for both control and samples with 3000 mg/L of VFA. Gas samples were withdrawn from the headspace of the reaction vials using a gastight syringe and injected to the IR instrument at 0 hr, 2 hrs, 3 hrs and 5 hrs and analyzed for N₂O. Samples collected from the room air using the same syringe were also injected into the IR instrument to confirm that N₂O was produced during biological activity and therefore was not present in room air samples. The concentration of N₂O was calculated using the software for IR instrument and Beer's Law. Samples also were analyzed for nitrite and

nitrate concentrations in the liquid phase, using ion chromatography as described in section 3.3.1.

3.4.7 Measurement of Periplasmic Nitrate Reductase (NAP) Enzyme Activity

Periplasmic nitrate reductase (NAP) catalyzes the first enzymatic reaction of aerobic denitrification by reducing nitrate to nitrite. Sears et al. (1997) have shown that butyrate increased the activity of the NAP enzyme. Thus, an increase in NAP activity in the presence of VFAs would be indicative of aerobic denitrification. Measuring NAP is sufficient to indicate stimulation of aerobic denitrification since when NAP gets activated, rest of the enzymes get activated, as well.

A methylviologen (MV^+) assay specific for periplasmic nitrate activity was used to determine NAP enzyme activity (Lopez-Lozano et al., 2002). Batch experiments using mixed liquor samples from the CSTR were performed as described in section 3.3 in four parallel treatments (Figure 3-7). 25 mg-N/L nitrite was added to the samples in the first treatment. Both 25 mg-N/L nitrite and 3000 mg/L VFA were utilized in the second treatment. Only 3000 mg/L VFA were added in the third treatment. Finally, neither nitrite nor VFAs were used in the fourth experiment. The NAP activity experiment was repeated three times with three different sludge samples. In the first experiment, mixed liquor removed from the CSTR was used in batch experiment. For the second experiment, mixed liquor samples were obtained from the CSTR after it was spiked with VFAs. These samples were stored in -80°C until they were assayed for enzyme activity. Finally, mixed liquor from the full-scale WWTP with the same four treatments described above was utilized in another enzyme assay. Each of these experiments was performed in

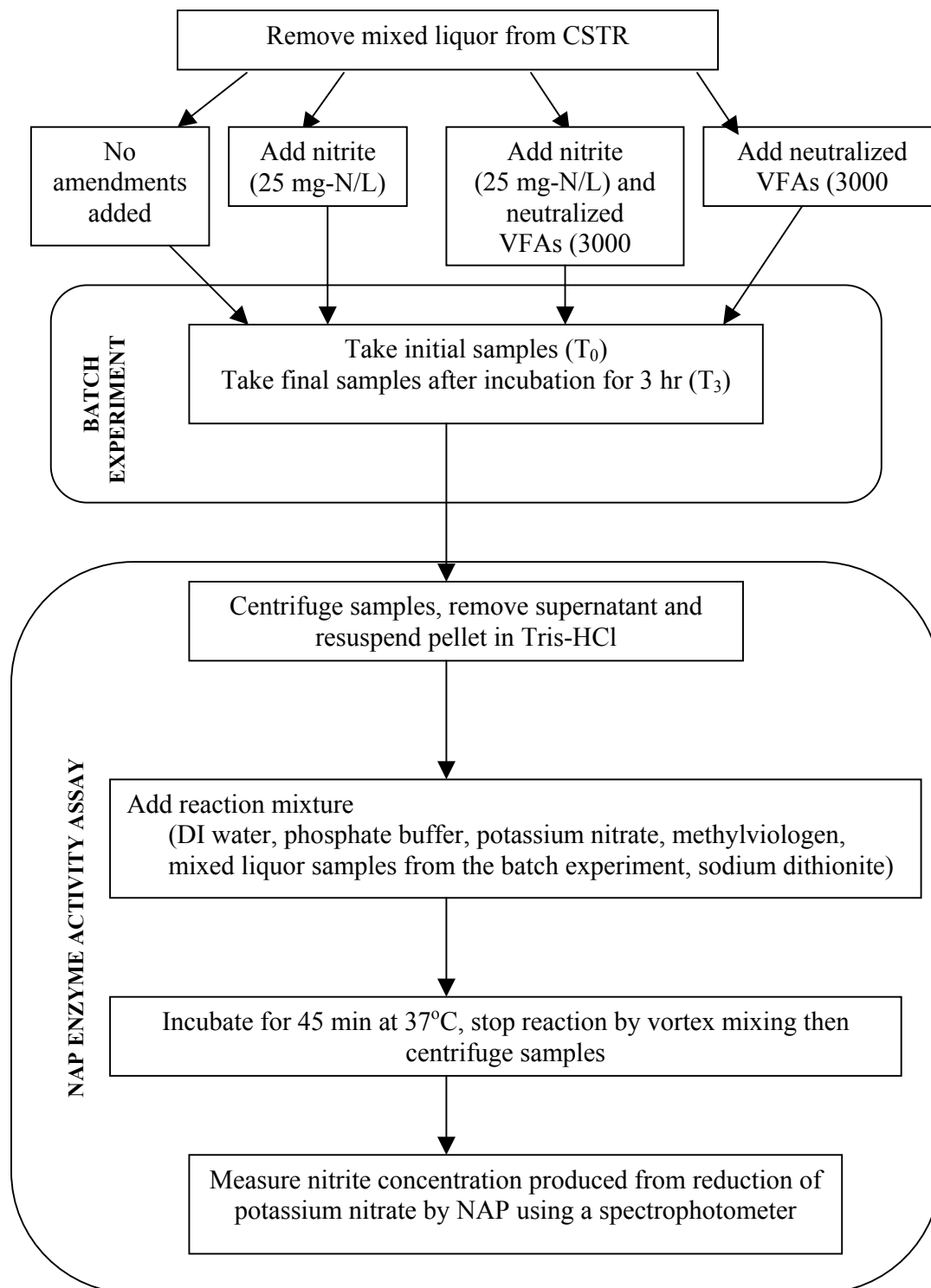


Figure 3-7. Schematic diagram showing the steps performed in the NAP enzyme assay experiment

triplicate. The samples were also assayed for nitrite and nitrate concentrations in the second and third experiments by ion chromatography (as described in section 3.3.1).

For the enzyme assay, 100 ml of mixed liquor suspended solids were collected in 500 mL centrifuge bottles for each treatment. Samples were centrifuged at 10,000 x g for 5 min after 0 and 5 hours of incubation. After pouring off most of the supernatant and carefully pipetting out the remaining medium, the pellet was directly resuspended in 1000 μ l of cold 50 mM Tris-HCl, pH 7.5 and homogenized in a Fast Preparation instrument to break the cell walls. The homogenates were immediately placed at -20°C and remained frozen until enzymatic analysis.

After thawing the samples, a reaction mixture was prepared as follows (in order of addition): 450 μ l of DI water; 200 μ l of 0.5 M phosphate buffer, pH 7.0; 100 μ l of 0.014 M KNO₃, 100 μ l of 40 mM methylviologen, 100 μ l of sludge sample, and 100 μ l of 0.1 M sodium dithionite (Na₂S₂O₄ prepared in 0.3 M NaHCO₃). Also, control samples were prepared without addition of sludge. After incubation for 45 min at 37 °C, the reactions were stopped by vigorous vortex mixing to oxidize the methylviologen and the tubes were centrifuged at 16,000 x g for 5 min. The supernatant was transferred to clean tubes and periplasmic nitrate reductase activity was assayed spectrophotometrically by measuring appearance of nitrite following EPA Method 354.1.

Total protein concentration of the mixed liquor samples was determined using the Modified Lowry Protein Assay Reagent Kit, following the instructions of the manufacturer (Pierce Biotechnology, Rockford, IL). The protein concentration was used to standardize enzymatic activities.

3.4.8 Inhibition of Periplasmic Nitrate Reductase (NAP)

Periplasmic nitrate reductase (NAP) catalyzes the first enzymatic reaction of aerobic denitrification reducing nitrate to nitrite. Butler et al. (2000) found that NAP is sensitive to thiocyanate (SCN) inhibition, with SCN acting as a competitive inhibitor of nitrate. In addition, Patureau et al. (1996) suggested that cyanide (CN) directly inhibits aerobic denitrifying enzymes. Thus, cyanide influences denitrification under aerobic conditions by totally inhibiting nitrate reduction, whereas under anaerobic conditions, no effect was observed on nitrate reduction (Patureau et al., 1996). Therefore, to inhibit aerobic denitrification, batch experiments (described in section 3.3) were performed in the presence of either SCN (10 mM) or CN (10 μ M) together with 3000 mg/L of VFAs. Additionally, a control sample containing neither CN nor SCN nor VFA was evaluated.

3.4.9 Detection of Genes for Periplasmic Nitrate Reductase (NAP)

The presence of the periplasmic nitrate reductase (NAP) gene was examined using molecular methods to demonstrate that the genetic potential for NAP activity exists. Nested PCR primers for the amplification of *napA* sequences have been developed and used to analyze DNA extracted from a freshwater community (Flanagan et al., 1999). McDevitt et al., (2000) used a similar method in the analysis of heterotrophic bacteria growing in a nitrite-oxidizing bioreactor. The presence of the periplasmic nitrate reductase in individual isolates and in a bacterial community has also been examined using biochemical and molecular methods, including analysis of *napA* sequences (McDevitt et al., 2000). The primer sequences used in this study are shown in Table 3-2.

Table 3-2. *napA* primer sequences used in this study (adapted from Flanagan et al., 1999 and McDevitt et al., 2000)

Primer sequence	Sequence	Position in <i>napA</i> nucleotide
V16	GCNCCNTGYMGNTTYTGYGG	136-156
V17	RTGYTGRTTTRAANCCCATNGTCCA	1131-1155
V66	TAYTTYTNNHSNAARATHATGTAYGG	256-282
V67	DATNGGRTGCATYTCNGCCATRTT	663-687
Ambiguity codes: N = G, A, T or C; M = A or C; R = G or A; H = A, C or T; S = G or C; D = G, A or T.		

Genomic DNA was extracted from 2 ml MLSS samples using a FastDNA kit (Bio101, Vista, Calif.) as described in section 3.3.4.1. For amplification of *napA* DNA two-rounds of PCR were used. Lyophilized primers were resuspended in 10 mM TE, pH 8 to a concentration of 1 mM, then 5 µl were transferred into 245 µl H₂O for a final concentration of 20 µM. A single reaction for part 1 consisted of 1 Amersham RTGTM PCR Bead, 0.625 µL V16 primer (500 nM), 0.625 µL V17 primer (500 nM), 22.75 µl fHPLC H₂O and 1 µl template. In the first round, DNA was denaturated at 96°C for 120s and the samples were then amplified for 36 cycles of 60 s at 55°C, 120 s at 72°C and 60 s at 94°C, before a final extension step of 600 s at 72°C. Part two of the nested PCR was performed using primers V66, V67 and 2 µl of reaction 1 product as template in each respective reaction. DNA was denaturated for 120 s at 96°C and amplified for 42 cycles of 60 s at 60°C, 120 s at 72°C and 60 s at 94°C before a final extension step of 600 s at 72°C. PCR products were visualized by gel electrophoresis (1% low EEO Agarose gel, 15 min at 75 V and 1 hr at 100 V).

PCR product (1 μ l) from reaction 1 or reaction 2 was used as template for TA cloning reaction (carried out per Invitrogen instructions). 20 colonies of reaction 1 and 30 colonies of reaction 2 from each time point were placed into 0.5 ml LB-Kan₅₀ and shaken overnight horizontally at 37°C. Filter-sterilized 40% glycerol in water (0.5 ml) was added to each of the 100 cultures which were then frozen at –80°C. Twenty 15 ml culture tubes, each containing 5 ml of LB-Kan 50 μ l/ml were inoculated with frozen cultures from reaction 2, T₀ #1-10 and reaction 2, T₂₄ #1-10. Clones were grown overnight at 37°C, while shaken. 4.5 ml of each culture were centrifuged at 8000 x g for 5 min for cell harvest and minipreps were performed following Promega instructions. DNA concentrations were measured using a Hoefer Fluorimeter (MBRF) to verify that sufficient plasmid DNA (150-200 ng/ μ l) was available for restriction digests and sequencing. *Eco*R1 digest was performed with the delineated components as follows per reaction: 15.5 μ l H₂O, 2 μ l BufferH (FisherScientific), 0.5 μ l *Eco*R1 and 2 μ l DNA template. *Eco*R1 digests were incubated overnight at 37 °C. Plasmid digests were run on a 1.5 % low EEO Agarose Gel stained with ethidium bromide in 0.5 x TBE at 75 V for 15 minutes, then at 100 V for 1 hour. T₀, 2, #1-5 and T₂₄, 2, #1, 2, 7-10 were sequenced at MBRF using primers M13f. The *napA* sequences were compared with those in accessible databases using the program basic local alignment search tool (BLAST) and a dendrogram was constructed using the CLUSTAL program from NCBI (<http://www.ncbi.nlm.nih.gov/>).

3.4.10 Ammonia Analysis

Ammonia concentrations in the influent and effluent samples were measured using Standard Method 4500 D, Ammonium Selective Electrode Method (APHA, 1998) to examine the performance of the CSTR for nitrogen treatment. Following sample collection, a volumetric pipette was used to transfer 100 mL of mixed liquor to a clean 150 mL Erlenmeyer flask. When ammonia analysis could not be performed immediately following sample collection, the samples were preserved by adding 200 μ L of concentrated H_2SO_4 , then stoppered and stored at 5°C. Four standards, 0.1, 0.5, 5 and 50 mg/L ammonia-N, and a quality control sample were prepared and used to construct the standard curve, which was utilized to determine ammonia-N concentrations in the samples.

3.4.11 Solids Analysis

3.4.11.1 Mixed Liquor Suspended Solids (MLSS) and Effluent Suspended Solids (SS_{eff})

To maintain 10-day SRT in the reactor, a controlled solids wasting policy was utilized. The amount of solids removed each day to maintain a constant SRT was determined using the following equation:

$$Wastage\ volume = \left(\frac{MLSS * Volume}{SRT} - Q * SS_{eff} \right) * MLSS \quad (3.2)$$

where:

Wastage volume = mixed liquor volume to be wasted (L)

MLSS = mixed liquor suspended solids (mg/L)

Volume = reactor volume (L) = 10 L

SRT = solids retention time (days) = 10 days

SS_{eff} = effluent suspended solids (mg/L)

Q = influent flow rate (L/day) = 27.36 L/day

This calculation required that solids analysis be performed daily to determine the MLSS, and SS_{eff} values. A-10 mL sample of mixed liquor collected from the center of the CSTR using a wide-mouth serological pipette for MLSS analysis. Additionally, a sample was taken from the 40-L carboy for SS_{eff} determination after gently stirring the effluent wastewater to obtain a representative sample. The 300 mL SS_{eff} sample was withdrawn using a pump with nylon tubing, into a 500-mL Erlenmeyer flask. The samples collected were gently poured through 47 mm diameter 1.5-micron glass fibers filters (Proweigh® by Environmental Express) mounted on a vacuum filter flask and washed down with DI water. All filters were pre-dried and weighed the day before usage, and pre-wetted with DI water just prior to use. The samples were analyzed to determine the MLSS and SS_{eff} concentrations according to Standard Method 2540 D, Total Suspended Solids Dried at 103-105 °C (APHA, 1998). MLSS and SS_{eff} concentrations were then calculated using the following equation:

$$mg \text{ suspended solids} / L = \frac{W_{FF} - W_I}{V} * 1000 \quad (3.3)$$

where:

mg suspended solids/L = MLSS or SS_{eff} (mg/L)

W_{FF} = final weight of filter (mg)

W_I = initial weight of filter (mg)

V = volume of sample (L)

3.4.11.2 Mixed Liquor Volatile Suspended Solids (MLVSS)

MLVSS values were determined to obtain a closer approximation of the biological component in the reactor. Standard Method 2540 E, Fixed and Volatile Solids Ignited at 550 °C (APHA, 1998) was conducted daily. The filter containing dried solids used for MLSS analysis was placed into a small muffle furnace and combusted for a minimum of 15 minutes at 550°C. The filter was then removed from the oven, reweighed and the MLVSS calculated using the following equation:

$$mg \text{ volatile suspended solids} / L = \frac{W_{FF} - W_{FV}}{V} * 1000 \quad (3.4)$$

where:

mg volatile suspended solids/L = MLVSS (mg/L)

W_{FF} = final weight of filter from the MLSS analysis (mg)

W_{FV} = final weight of filter after MLVSS analysis (mg)

V = volume of sample (L)

3.4.12 Statistical Analysis

General linear model (GLM) univariate analyses were performed on the data generated in this study, using the SPSS 12.0 (SPSS, Inc., Chicago, IL) statistical analysis software package, to determine statistical differences. GLM univariate assessment provides regression analysis and analysis of variance for one dependent variable by one or more factors and/or variables. Using this GLM procedure, one can test null hypotheses concerning the effects of other variables on the means of various groupings of a single dependent variable and investigate interactions between factors as well as the effects of individual factors, some of which may be random. In addition, the effect of covariates and covariate interactions with factors can be included. For regression analysis, the independent (predictor) variables are specified as covariates.

In this study, the independent variable was the controlled parameter, time, while the dependent variables were the measured parameters, nitrite, nitrate and carbon dioxide concentrations. The rates of nitrite removal, nitrate formation and carbon dioxide fixation in the absence of inhibitors (controls) and in the presence of inhibitors (experimental samples) were compared to determine statistical differences. In all analyses, a null hypothesis (H_0) that no differences exist in the rates between controls and experimental samples was tested. A failure of null hypothesis indicated a significant difference in the rates. In statistical analysis, the probability of rejecting the null hypothesis when it is true is often called the significance level (α) of the test. Unless indicated otherwise, the probability was maintained at 5% ($\alpha = 0.05$) in this research. SPSS analysis yielded the level of significance (p) at which values were significantly different. A value of $p < 0.05$ indicated that the rates were different at a 5% level of significance.

CHAPTER 4

RESULTS AND DISCUSSIONS

The major goal of this research was to study the impact of VFAs on the effectiveness of nitrite removal, nitrate formation and carbon dioxide fixation during activated sludge treatment of wastewater. To assess this goal, an activated sludge reactor with a 10-day SRT was operated at 20°C constant temperature and 3 mg/L of DO. The effects of a mixture of VFAs at a 1:1:1 (acetate: propionate: butyrate) ratio at the concentrations of 1500 mg/L and 3000 mg/L were studied in batch test using the activated sludge culture provided from the reactor. In addition, 3000 mg/L of VFAs were spiked into the reactor to evaluate the influence of VFAs on the continuous activated sludge culture.

In this chapter, results of various experiments conducted to evaluate the impact of VFAs on nitrite removal, nitrate formation and carbon dioxide fixation in both batch and continuous culture activated sludge systems are presented and interpreted. In addition, experiments performed to predict possible mechanisms of nitrate reduction are presented and discussed. The batch experiments are discussed first followed by the CSTR experiment. Operational data for the CSTR can be found in Appendix II.

4.1 BATCH EXPERIMENTS

Batch experiments were performed to investigate the impact of nitrification inhibitors and VFAs on nitrite removal via nitrification and/or aerobic denitrification. All

nitrite removal, nitrate formation, CO₂ fixation, N₂O gas production and the activity of periplasmic nitrate reductase enzyme tests were conducted as batch experiments.

4.1.1 Demonstration of Nitrite Oxidation in Batch Samples from the CSTR

Nitrification is the conversion of ammonia to nitrite then nitrate. During efficient nitrification, nitrite does not accumulate because it is oxidized to nitrate as soon as it is formed from ammonia. The accumulation of nitrite is generally assumed to result from the inhibition of NOB. Since mixed liquor samples collected from the CSTR contained less than 0.5 mg NO₂-N/L, it was necessary to supplement nitrite as the energy source for the NOB in subsequent batch experiments. Tests were performed using a range of nitrite-N concentrations to determine the optimum amount of nitrite-N to be used in the assays. Concentrations of nitrite-N and nitrate-N over a 5 h period were measured using IC as described in Chapter 3.

Essentially, all of the nitrite in both the 10 and 15 mg-N/L augmented samples was consumed in the first 3 hours during batch testing. This data could not be used to calculate nitrite removal and nitrate formation rates because it was unknown when concentrations dropped below the detection limit. Nitrite levels were measured in both 25 and 35 mg-N/L supplemented samples after 5 hrs of incubation (Figure 4-1). Thus, the rates could be calculated from the data obtained from these samples. The nitrite removal rates in the 25 mg-N/L and 35 mg-N/L samples were 4.04 mg/L-hr and 3.81 mg/L-hr, respectively. Initial nitrate levels in both samples were 23 mg-N/L due to effective nitrification in the CSTR used as the source of the mixed liquor seed for the batch tests. After 5 hr of incubation, nitrate concentrations rose to 41.41 mg-N/L and 42.48 mg-N/L

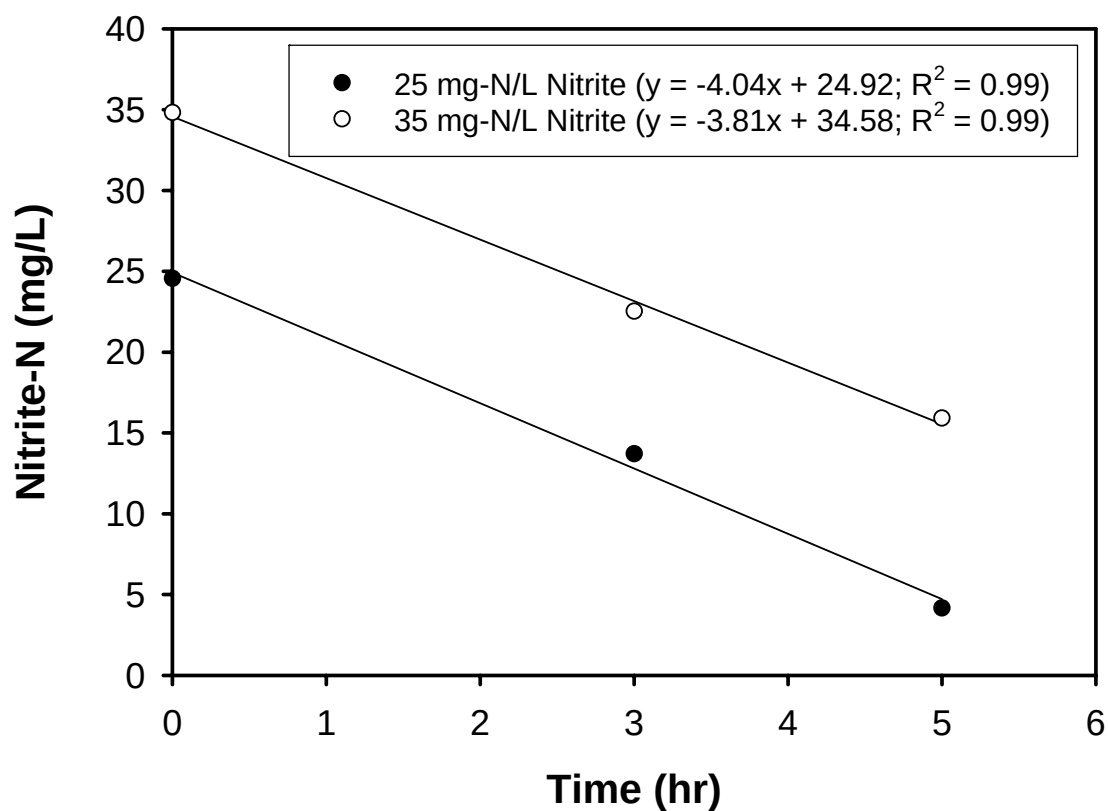


Figure 4-1. Nitrite removal rates at concentrations of 25 mg-N/L and 35 mg-N/L added to the mixed liquor batch samples obtained from the CSTR. The slopes represent the nitrite removal rates.

in the 25 mg-N/L and 35 mg-N/L samples, respectively, indicating that significant conversion occurred in the batch samples (Figure 4-2). The nitrate formation rates were calculated as 3.69 mg/L-hr in the 25 mg-N/L and 4 mg/L-hr, in the 35 mg-N/L samples.

The nitrite and nitrate data showed that rates were similar at both 25 and 35 mg-N/L indicating that nitrite removal and nitrate formation were independent of substrate concentration for the 25 mg-N/L and 35 mg-N/L NO_2^- levels added in this study. Therefore, 25 mg N/L was chosen as the optimum concentration to be used in all assays since 10 and 15 mg-N/L concentrations were insufficient to accurately determine rates and 25 and 35 mg-N/L concentrations gave similar results demonstrating that it was unnecessary to use more than 25 mg-N/L nitrite.

4.1.1.1 Control Data for Nitrite Removal and Nitrate Formation in Nitrite Oxidation

In the batch experiments, control samples without added inhibitors or VFAs were analyzed to demonstrate nitrite oxidation. Two separate calculations were performed. First, the total mass of nitrite removed and nitrate formed were calculated by subtracting the final concentrations of nitrite and nitrate from the initial concentrations. Second, nitrite oxidation and nitrate formation rates were determined based on the slope of a linear regression analysis of the time dependent data.

Nitrite oxidation in control batch samples was consistent throughout the course of this study. An initial nitrite concentration of 22.4 ± 1.68 mg-N/L was lowered to 8.23 ± 3.15 mg-N/L after 5 hours of incubation in 13 separate control experiments. As a result, 14.2 ± 3.18 mg-N/L of nitrite was removed, on average, representing a 63% decrease in concentration. The rate of nitrite removal was calculated as 2.85 mg-N/L-hr (Figure 4-3).

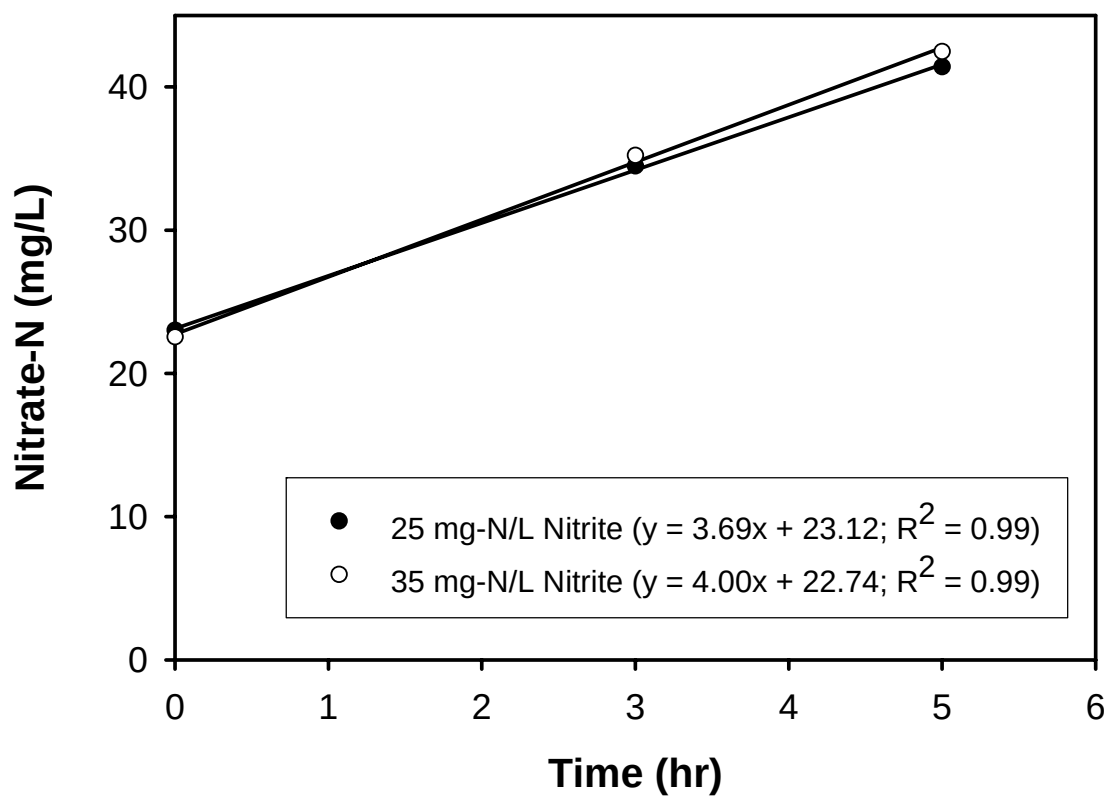


Figure 4-2. Nitrate formation for initial nitrite concentrations of 25 mg-N/L and 35 mg-N/L in mixed liquor batch samples obtained from the CSTR. The slopes represent the nitrate formation rates.

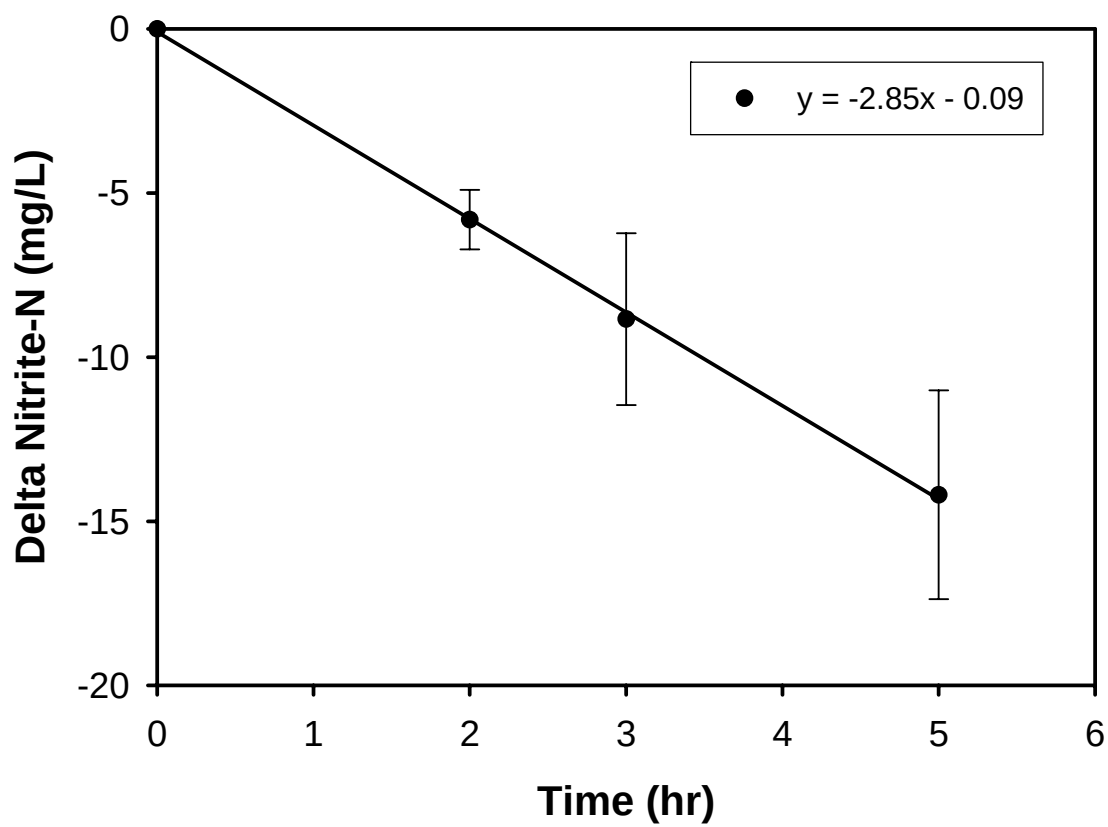
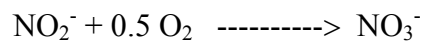


Figure 4-3. Average nitrite removal in control samples over time in batch samples (n = 13). The slope represents the average nitrite removal rate.

Alternatively, initial nitrate levels varied throughout each experiment because concentrations were dependent upon ammonia levels entering the CSTR and nitrification efficiency of the 10-day SRT reactor. The mean initial nitrate concentration was 8.98 ± 3.53 mg-N/L and the mean amount of nitrate accumulated after 5 hours was 22.8 ± 4.26 mg-N/L. As a result 13.82 ± 3.8 mg-N/L of nitrate was formed, on average, during the control experiments. The average nitrate formation rate was 2.79 mg-N/L-hr (Figure 4-4).

The stoichiometric relationship for the oxidation of nitrite to nitrate indicates that 1 mol of nitrite-N is converted to 1 mol of nitrate-N:



Thus, if efficient nitrite oxidation occurs, equal molar removal of nitrite and formation of nitrate would hold. Although there was slight variability in the starting concentration of nitrite, the mean nitrite decrease was 14.2 ± 3.18 mg-N/L while the mean nitrate increase was 13.82 ± 3.8 mg-N/L suggesting the 1:1 ratio expected when nitrite is oxidized to nitrate by NOB. Additionally, the nitrite removal and nitrate formation rates were similar in the control experiments (2.85 mg-N/L-hr and 2.79 mg-N/L-hr, respectively) (Figures 4-3 and 4-4).

In summary, nitrite oxidation resulted in a 1:1 ratio (nitrite removal to nitrate formation) with equal rates in the control batch samples. This ratio suggests conversion of all consumed nitrite to nitrate based on the stoichiometry of the reaction of nitrite oxidation in which 1 mol of nitrite-N is converted to 1 mol of nitrate-N.

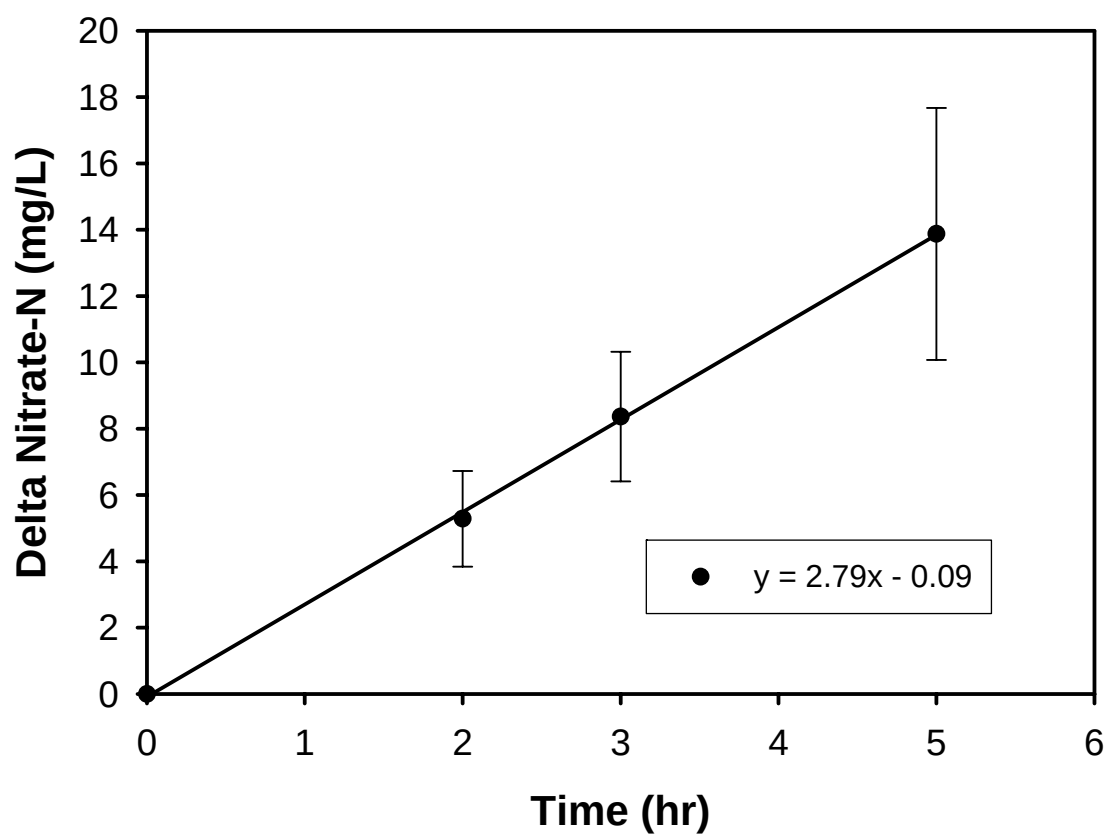


Figure 4-4. Nitrate formation in control samples over time in batch experiments (n = 13). The slope represents the average nitrate formation rate.

4.1.2 Demonstration of CO₂ Fixation in Batch Samples from the CSTR

The major source of organic carbon for NOB is carbon dioxide which is fixed via the Calvin cycle. It was expected that CO₂ fixation would be proportional to both nitrite removal and nitrate formation because NOB use energy gained from nitrite oxidation to fix CO₂. It was proposed by Billen (1976) and Somville (1978) that nitrification can be estimated indirectly by measuring CO₂ uptake by NOB, the underlying assumption being that there is a constant stoichiometric ratio between the rate of substrate oxidation and the rate of CO₂ uptake for a given species of nitrifier. Billen (1976) found that there was a constant relationship between nitrite oxidation and chemoautotrophic CO₂ fixation for organic carbon production. The relationship between substrate oxidation and CO₂ fixation was studied by Glover (1985) who found that the stoichiometric relationship between nitrification and carbon yield is dependent on the availability of the inorganic nitrogen substrate.

Similar to the nitrite oxidation experiments, tests were performed using a range of nitrite-N concentrations to determine the optimum amount of nitrite-N to be used in the CO₂ fixation assays. CO₂ fixation rates were calculated using 4 different nitrite-N concentrations: 10, 15, 25 and 35 mg-N/L. The uptake of CO₂ by NOB was studied by incubation of activated sludge with a radioactive carbonate source as described in Chapter 3. For the 10 and 15 mg-N/L nitrite concentrations, the amount of inorganic carbon incorporated in the biomass declined after 3 hours indicating that these levels were not adequate for a 5 hr experiment. However, CO₂ fixation rates were 2.21 µg/L-hr in the 25 mg-N/L sample and 2.22 mg/L-hr for the 35 mg-N/L sample indicating that the rate of CO₂ fixation was constant in both samples (Figures 4-5 and 4-6).

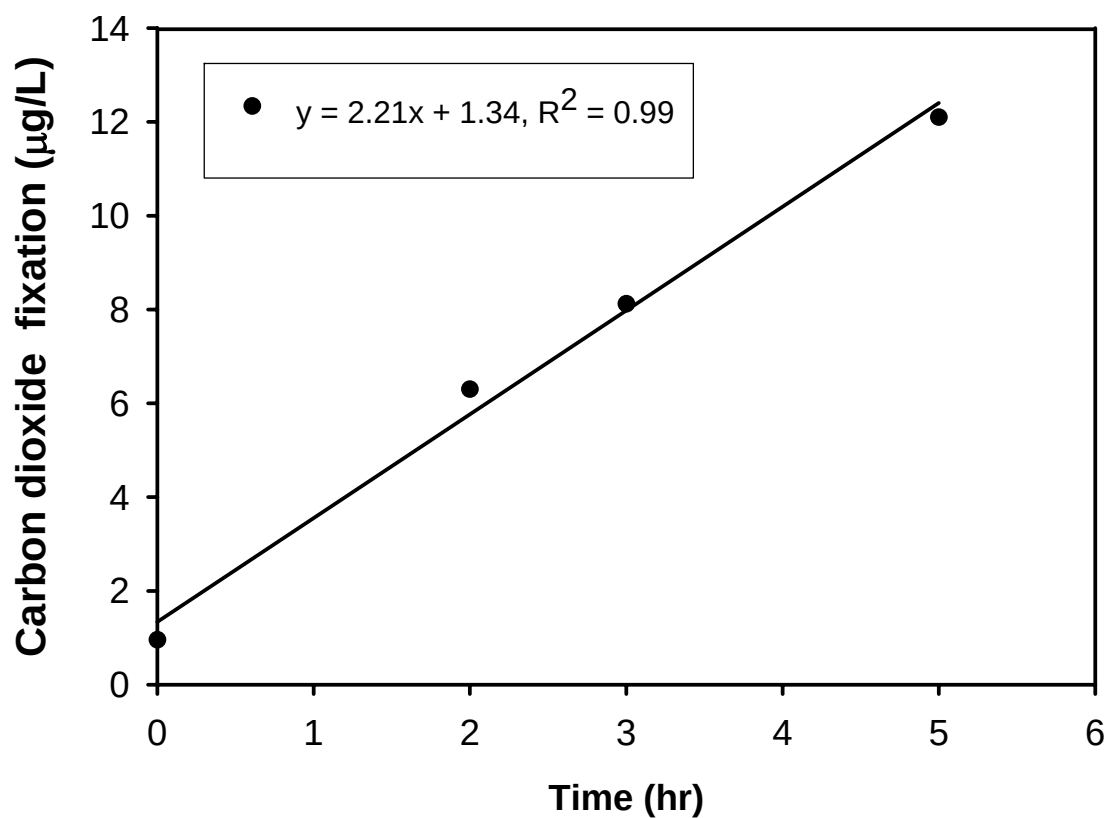


Figure 4-5. Carbon dioxide fixation over time in the 25 mg-N/L initial nitrite-N concentration sample. The slope represents the carbon dioxide fixation rate.

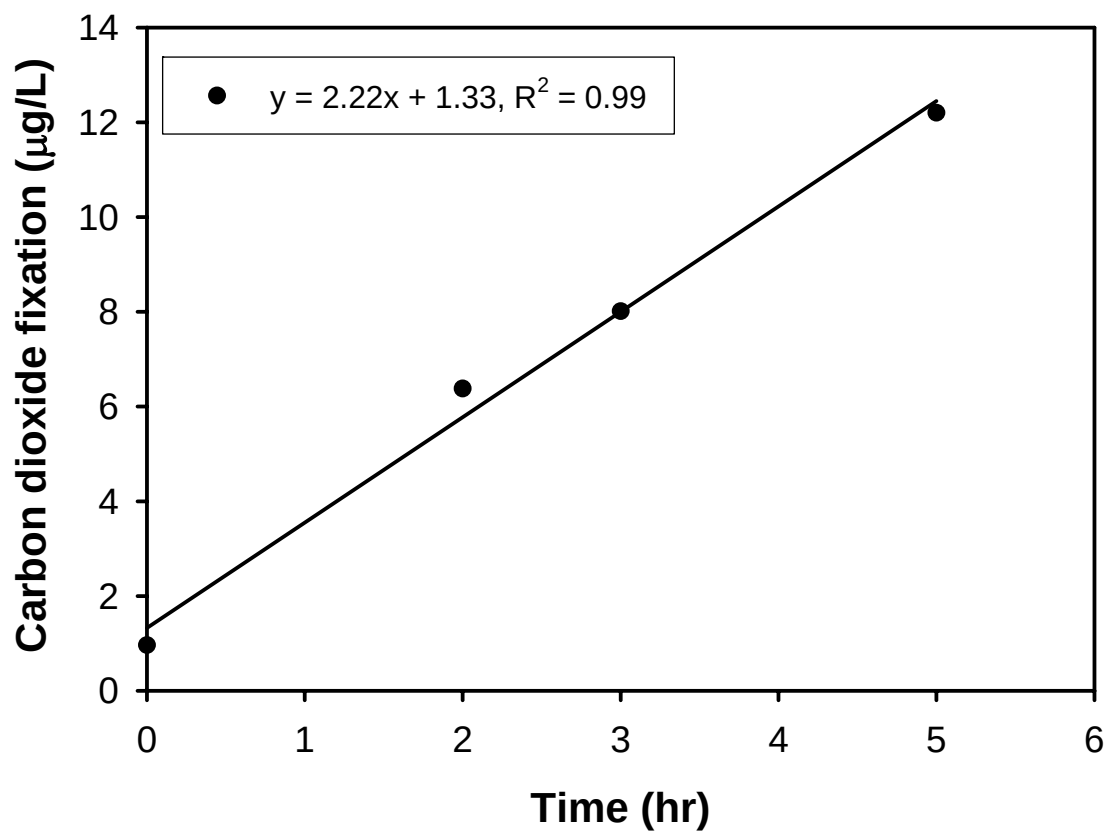


Figure 4-6. Carbon dioxide fixation over time in the 35 mg-N/L initial nitrite-N concentration sample. The slope represents the carbon dioxide fixation rate.

In addition, the carbon yield from nitrite oxidation (the ratio of carbon fixed to nitrogen oxidized) remained constant in both of the 25 and 35 mg-N/L samples, 0.00059 and 0.00056, respectively. Therefore, since the 10 and 15 mg-N/L were not adequate for CO₂ fixation and the CO₂ fixation rates were identical in the 25 and 35 mg-N/L samples, 25 mg N/L was chosen as the test concentration to be used in the CO₂ fixation assay. This concentration was studied in all subsequent experiments to assess nitrification performance. Control experiments, without any added inhibitor or VFAs were performed with the addition of 25 mg-N/L of nitrite. All the control data indicated that the amount of inorganic carbon assimilated into the biomass was consistent in 9 separate experiments showing good reproducibility in the control samples. The mean assimilated inorganic carbon was 20.3 µg/L after 5 hours of incubation and the mean carbon dioxide fixation rate was calculated as 4.06 µg/L-hr (Figure 4-7). The ratio of carbon incorporated to nitrogen oxidized was 1.42×10^{-3} in the control experiments. There is only a limited amount of information in the literature dealing with the stoichiometry of carbon uptake and substrate oxidation. Additionally, no previous study showed the relationship between nitrite removal, nitrate formation and CO₂ fixation in activated sludge reactors. The ratio of CO₂ fixed to nitrate produced was shown in pure *Nitrobacter* sp. and *Nitrococcus mobilis* cultures. Helder and de Vries (1984) and Belser (1984) found a ratio of 1 to 0.02 with *Nitrobacter* sp. In a study with *Nitrococcus mobilis*, the ratio was between 0.014-0.031 (Glover, 1985). The ratio found in this study was much lower than those, but note that it was expected that lesser amounts of carbon may be fixed because these experiments were performed using a mixed culture. Heterotrophic bacteria generate CO₂ through respiration which would provide a source of unlabeled CO₂ for NOB.

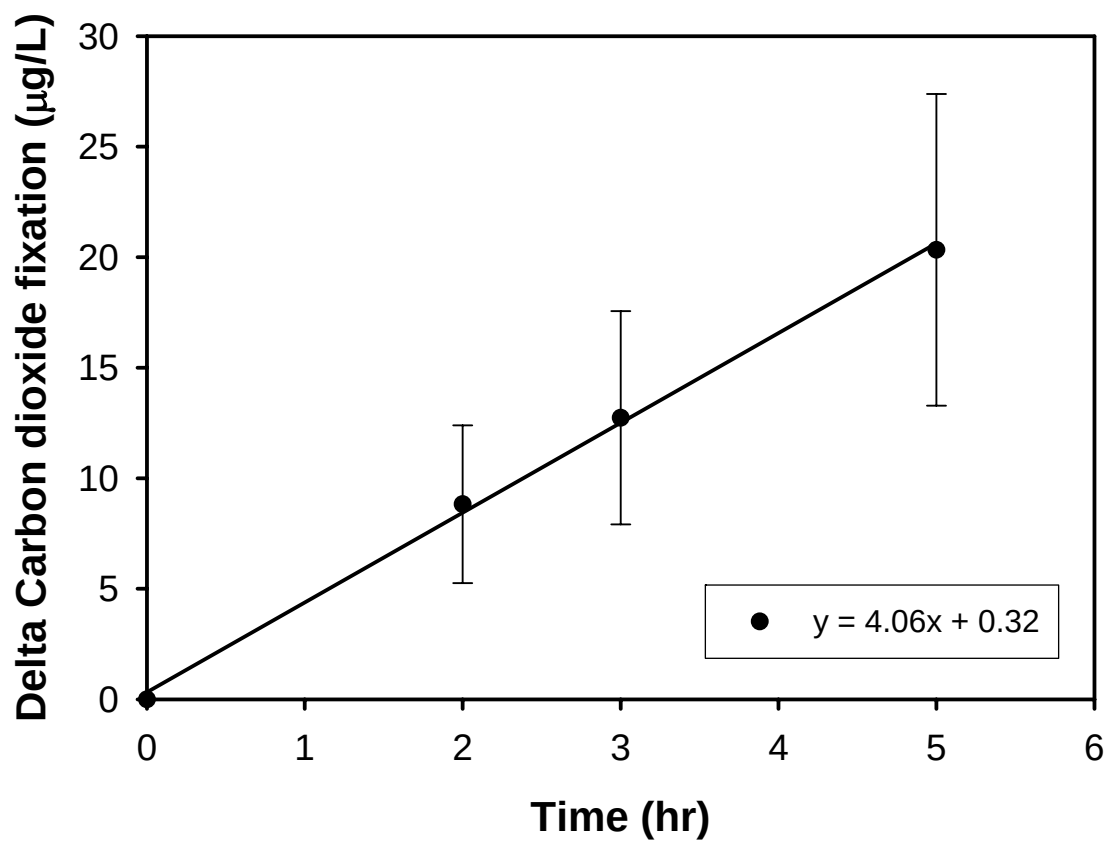


Figure 4-7. Carbon dioxide fixation in control experiments over time in batch experiments (n = 9). The slope represents the average carbon dioxide fixation rate.

4.1.3 Effect of Allylthiourea and Azide on Nitrite Oxidation

Traditionally, nitrification has been mathematically treated as one composite biochemical process, with the assumption that $\text{NH}_4^+\text{-N}$ to $\text{NO}_2^-\text{-N}$ oxidation limits the overall transformation of $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$ (Grady et al., 1999). However, if $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ oxidation both limit overall nitrification (at different stages of the process), the single-step representation is inadequate. Under such circumstances, individual characterization of both $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ is necessary. Thus, for mixed cultures, such as activated sludge, selective inhibitors that allow separation of the different activities are needed. Ideally, inhibition should be instantaneous and complete for the targeted population and should not affect other populations. Three compounds (allylthiourea, chlorate and azide) have been extensively used to study nitrification inhibition.

Allylthiourea [ATU ($\text{C}_4\text{H}_8\text{N}_2\text{S}$)] selectively inhibits ammonia oxidation at concentrations between 8 and 80 μM (Hooper, 1973; Sharma and Ahlert, 1977; Surmacz-Gorska et al., 1995). The mechanism for inhibition appears to be chelation of copper from the active site of the ammonia monooxygenase (Bédard and Knowles, 1989). Ginestet et al. (1998) performed a comprehensive investigation of ammonia oxidation using a mixed nitrifier culture from an activated sludge and found that instantaneous selective inhibition was achieved at an allylthiourea concentration of 86 μM .

Chlorate (ClO_3) has been used to inhibit nitrite oxidation in soils, sediments, and activated sludge systems (Belser and Mays, 1980; Hynes and Knowles, 1983; Surmacz-Gorska et al., 1995 and 1996). However, doubts concerning the slow and nonspecific action of chlorate limit its usefulness in discriminatory assays with mixed cultures (Belser and Mays, 1980; Hynes and Knowles, 1983; Ginestet et al., 1998).

Azide (N_3) is a selective bacteriostatic agent active in gram-negative bacteria and has been shown to inhibit nitrite oxidation in activated sludge. Azide is believed to complex with the molybdenum atoms of the nitrite oxidoreductase (Ginestet et al., 1998). Chandran and Smets (2000) found that an optimum azide concentration of 24 μM enabled selective inhibition of nitrite oxidation without affecting ammonia oxidation. This optimum concentration was also consistent with a previous report by Ginestet et al. (1998).

Based on literature results, ATU and azide were chosen to study ammonia and nitrite oxidation inhibition in this research. These compounds were used to distinguish biological activities performed by ammonia oxidizers, nitrite oxidizers and heterotrophs and to determine how nitrite removal, nitrate formation and CO_2 fixation rates were affected during nitrite oxidation. Inhibitor concentrations utilized in this study (ATU = 86 μM ; azide = 24 μM) were similar to those used in experiments performed by Ginestet et al. (1998) and Chandran and Smets (2000). However, initial experimental results indicated that azide did not completely inhibit nitrite oxidation at a concentration of 24 μM , therefore, a level of 50 μM was utilized in all subsequent experiments. ATU was not expected to inhibit nitrite removal and nitrate formation rates because ammonia oxidizers do not play a direct role in nitrite oxidation. However, it was anticipated that CO_2 fixation rates would be reduced since ammonia oxidizers also fix CO_2 . Azide was predicted to inhibit nitrite removal, nitrate formation and CO_2 fixation rates since this compound is a specific inhibitor for NOB.

Concentrations of nitrite, nitrate and carbon incorporated into biomass during each 5 hr batch incubation experiment were measured in the presence of either ATU or

azide. These experiments were repeated six times on different days to determine the reproducibility of the results and then the data was averaged. Total nitrogen mass after 5 hrs was summed and the rates of nitrite removal, nitrate formation and CO₂ fixation were determined via least squares regression analysis (linear fit). Statistical significance of the slopes was performed using the GLM as described in Chapter 3 (3.4.11).

4.1.3.1 Effect of Allylthiourea and Azide on Nitrite Removal and Nitrate Formation

a. Allylthiourea

The mean changes in nitrite concentrations over time in the 2 separate control and ATU experiments were plotted to determine the impact of ATU on nitrite removal (Figure 4-8). After 5 hr of incubation, the mean nitrite decrease was 11.71 ± 1.25 mg-N/L in the control and 11.50 ± 0.86 mg-N/L in the ATU experiments. Approximately 50% of the initial nitrite added was removed in both experiments indicating that the inhibitor had no effect on nitrite oxidation. Additionally, the average rates of nitrite removal were 2.35 mg-N/L-hr and 2.33 mg-N/L-hr in the controls and in the samples with added ATU, respectively. Again, these results implied that the presence of ATU had no negative impact on nitrite removal. Statistical analysis of the data also indicated no significant difference in nitrite removal rates between the control and ATU tests after five hours of incubation ($\alpha = 0.05$).

The impact of ATU on nitrate formation was also examined. After 5 hours of incubation, 11.23 ± 1.33 mg-N/L nitrate accumulated in the controls and 11.88 ± 0.52 mg-N/L nitrate was formed in the experimental tests (Figure 4-9). The mean nitrate formation rates were calculated as 2.24 mg-N/L-hr and 2.12 mg-N/L-hr in the control and

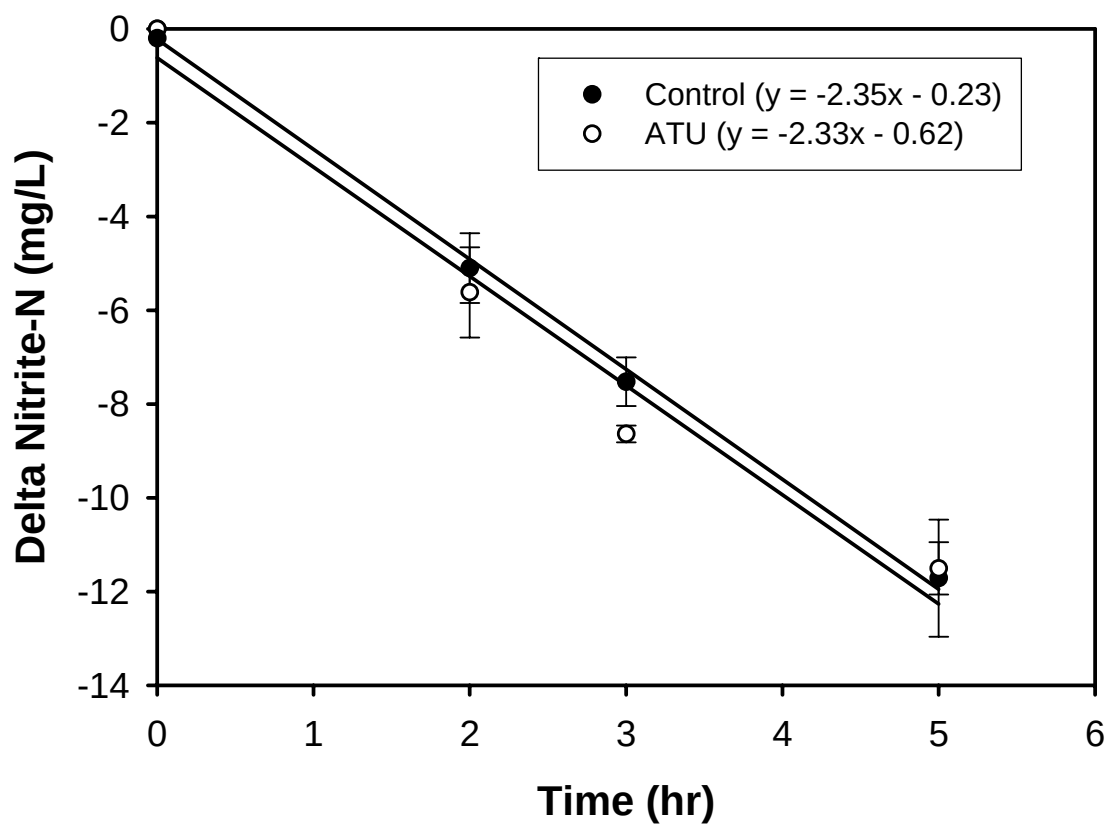


Figure 4-8. Nitrite removal in the control and ATU experimental samples over time in batch experiments ($n = 2$). The slopes represent the average nitrite removal rates.

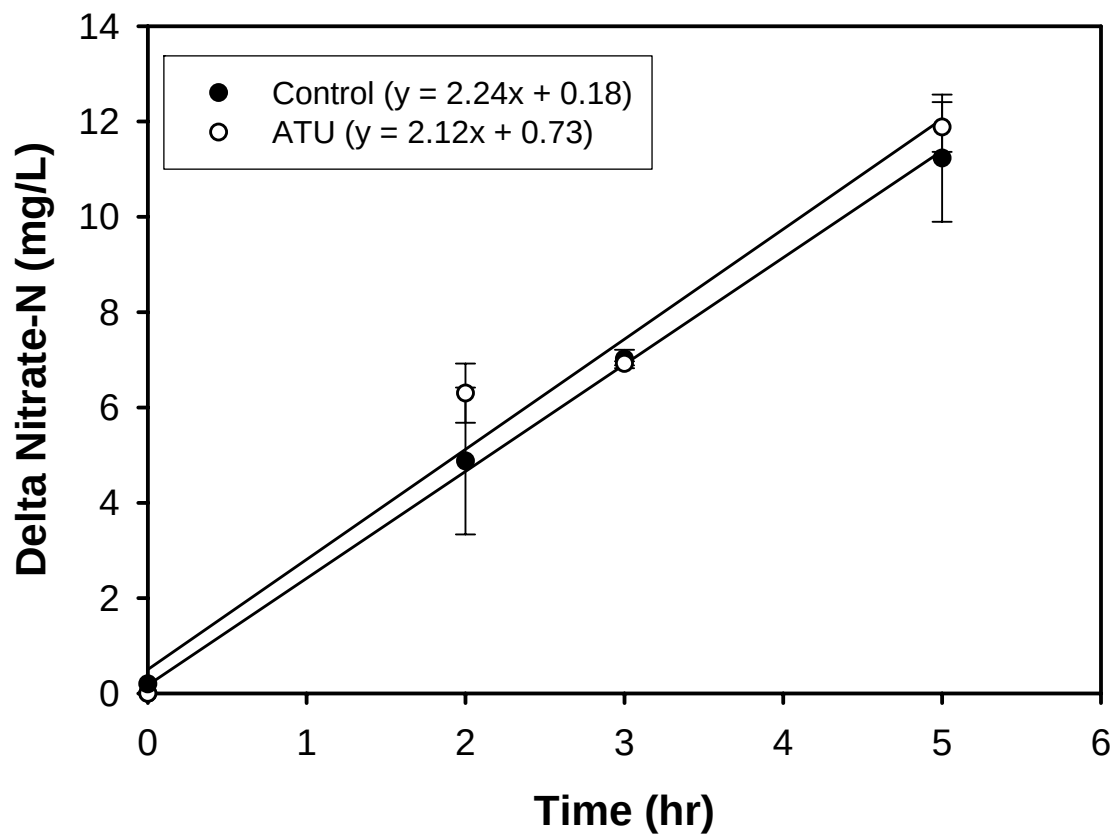


Figure 4-9. Nitrate formation in the control and ATU experimental samples over time in batch experiments ($n = 2$). The slopes represent the average nitrate formation rates.

ATU experimental samples, respectively. The similar rates and extent of the reactions suggested that ATU had little effect on nitrate formation, as well. Statistical analysis of the data also showed that ATU did not significantly impact nitrate formation ($\alpha = 0.05$).

In control experiments, the mean nitrite decrease and the mean nitrate increase were comparable (11.71 ± 1.25 mg-N/L and 11.23 ± 1.33 mg-N/L, respectively) confirming the stoichiometric 1:1 nitrite to nitrate ratio. Similar to the controls, the mass of nitrite removed (11.50 ± 0.86 mg-N/L) equaled the mass of nitrate formed in ATU experimental samples (11.88 ± 0.52 mg-N/L) also yielding a 1:1 ratio. Additionally, the rates of nitrite removal and nitrate formation were similar in both control and experimental samples. It can be concluded that nitrite was oxidized to nitrate in both control and ATU samples (Figures 4-8 and 4-9).

These results indicated that NOB in the ATU experimental samples behaved similarly to NOB in the controls, as anticipated, since ATU does not affect nitrite oxidation (i.e. nitrite removal = nitrate formation). These findings are in agreement with Ginestet et al. (1998) and Chandran and Smets (2000) who illustrated that ATU selectively inhibited ammonia oxidizers without affecting nitrite oxidizers.

b. Azide

The impact of azide on nitrite removal and nitrate formation was investigated to study inhibition of nitrite oxidation. Six separate experiments were performed using azide. Figure 4-10 represents the mean decrease in nitrite removal in both control and azide samples. After 5 hr of incubation, the mean decrease in nitrite concentration was 12.24 ± 2.04 mg-N/L in the control experiments representing 54% nitrite removal. In

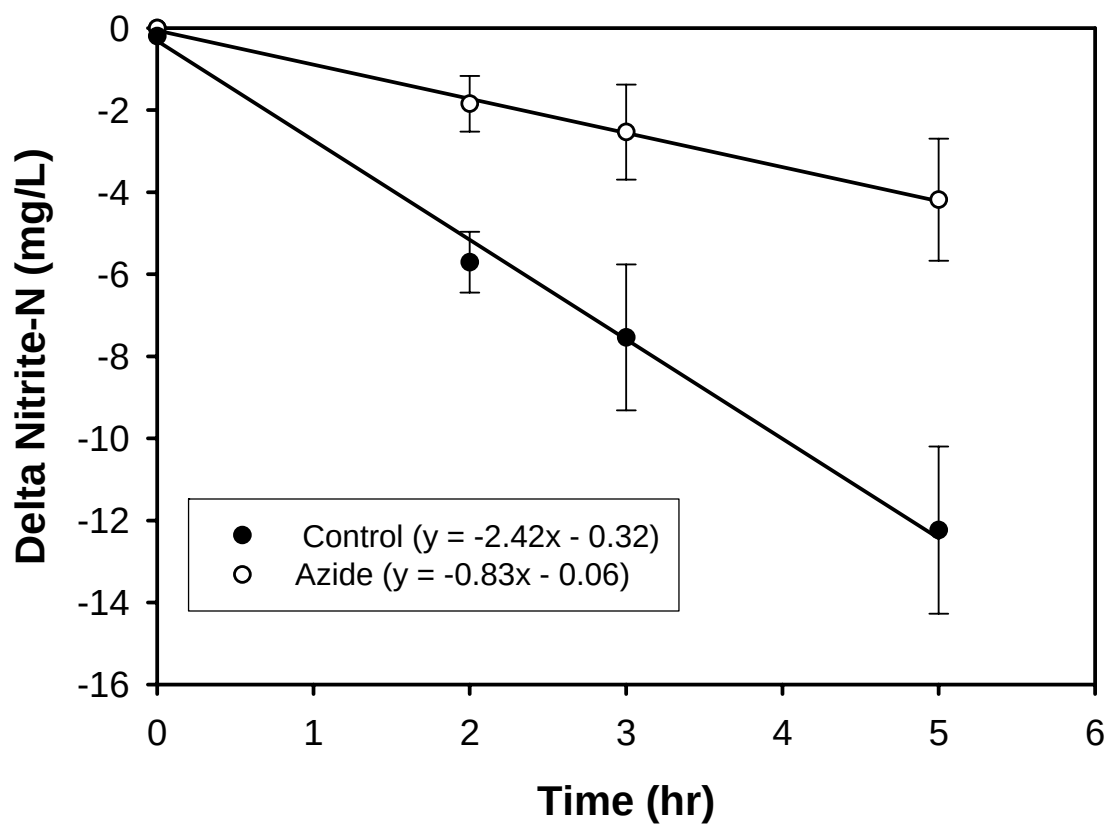


Figure 4-10. Nitrite removal in the control and azide experimental samples over time in batch experiments ($n = 6$). The slopes represent the average nitrite removal rates.

contrast, only 4.18 ± 1.48 mg-N/L of nitrite was removed in the presence of azide (19% decrease) indicating azide inhibition. The rates of nitrite removal averaged 2.42 mg-N/L-hr and 0.83 mg-N/L-hr in the controls and azide samples, respectively. These results indicate a 66% inhibition of the nitrite removal rate by azide. Statistical analysis of the data confirmed a significant difference in nitrite removal rates between the control and azide tests after five hours of incubation ($\alpha = 0.05$).

Nitrate accumulated in the controls (11.40 ± 1.67 mg-N/L) and formed in the azide tests (4.99 ± 0.75 mg-N/L) also indicated inhibition of nitrate formation (Figure 4-11). The mean rates of nitrate formation were 2.29 mg-N/L-hr in the absence of azide and 1.00 mg-N/L-hr in the presence of azide. Thus, the nitrate formation rate was reduced 56% percent indicating, as expected, the negative impact of azide on nitrate formation. Statistical analysis confirmed that the difference in rates between the control and tests was significant ($\alpha = 0.05$).

In control samples, the mean nitrite decrease and the mean nitrate increase were comparable (12.23 ± 2.04 mg-N/L and 11.40 ± 1.67 mg-N/L, respectively) and close to the predicted 1:1 stoichiometric ratio. In the presence of azide, the mass of nitrite removed (4.18 ± 1.48 mg-N/L) was similar to the mass of nitrate formed (4.99 ± 0.75 mg-N/L) also indicating a 1:1 ratio. In spite of the inhibition of nitrite removal and nitrate formation, the amount of nitrite consumed in the experiments was nearly equal to the amount of nitrate accumulated indicating nitrite removal and nitrate formation were inhibited to the same degree by azide. Additionally, the rates of nitrite removal and nitrate formation were similar for each of the control and experimental samples. The nitrite removal rate was 2.42 mg-N/L-hr and the nitrate formation rate was 2.29 mg-N/L-

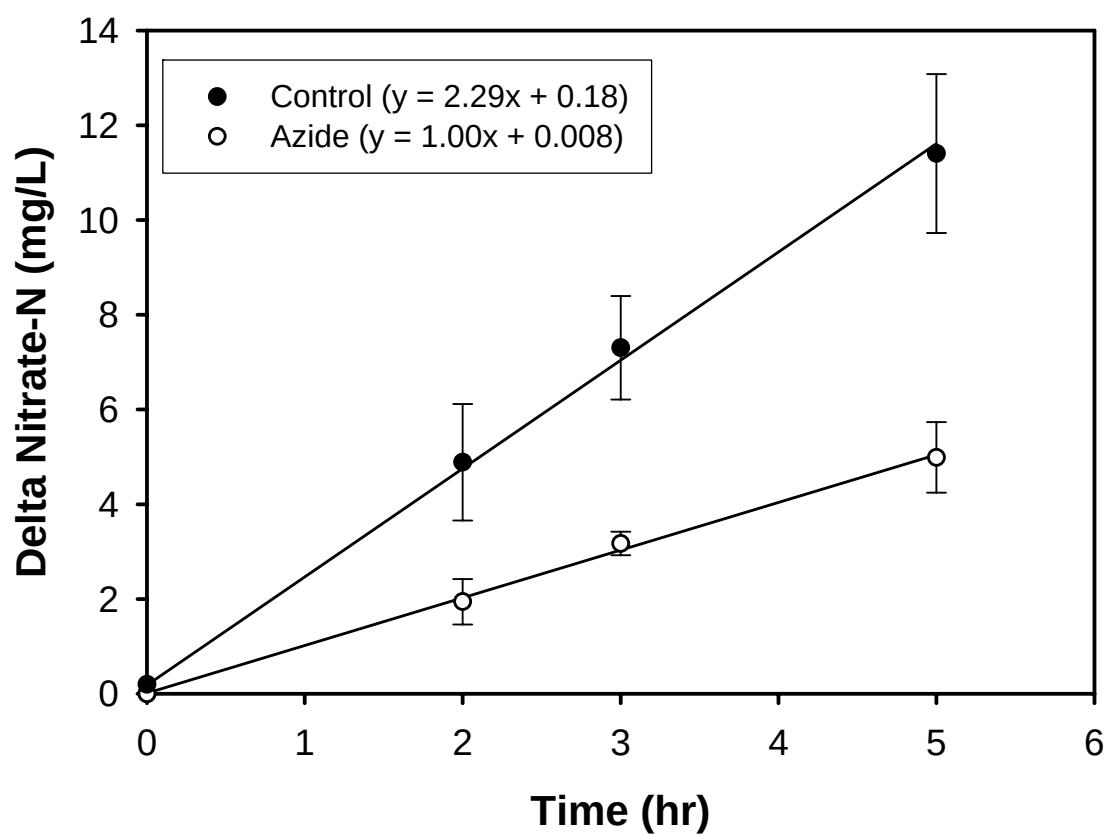


Figure 4-11. Nitrate formation in the control and azide experimental samples over time in batch experiments ($n = 6$). The slopes represent the average nitrate formation rates.

hr in the control samples. The rates of nitrite removal and nitrate formation in the azide samples were 0.83 mg-N/L-hr and 1.00 mg-N/L-hr, respectively. Thus, it can be concluded that nitrite was oxidized to nitrate in both control and azide samples, although nitrite removal and nitrate formation were similarly inhibited by azide.

Additionally, total nitrogen calculations were performed to determine if nitrogen was conserved during these experiments. Total nitrogen was calculated by adding the nitrite-nitrogen and nitrate-nitrogen concentrations measured in the liquid phase for each time point. In the controls, mean total nitrogen remained constant (32.60 ± 0.37 mg-N/L) throughout the experiment, suggesting that all nitrite consumed was converted to nitrate. In the azide experimental samples, mean total nitrogen values also did not change during the 5 hr incubation (33.11 ± 0.47 mg-N/L). Thus, although the rates of nitrite removal and nitrate formation were lower in the azide samples than those in the control, total nitrogen concentration remained constant over time indicating that no nitrogen was lost from this system. However, Ginestet et al. (1998) and Chandran and Smets (2000) illustrated complete inhibition of nitrite oxidation by azide which was not demonstrated in this research.

c. ATU and azide

ATU and 24 μ M azide were used together in one experiment to investigate the changes in nitrite removal and nitrate formation in the presence of both inhibitors. Figure 4-12 depicts changes in nitrite concentrations with and without inhibitors.

In the control samples, 10.83 mg-N/L of nitrite was consumed over 5 hours and the removal rate was calculated as 2.18 mg-N/L-hr. In the presence of ATU, nitrite

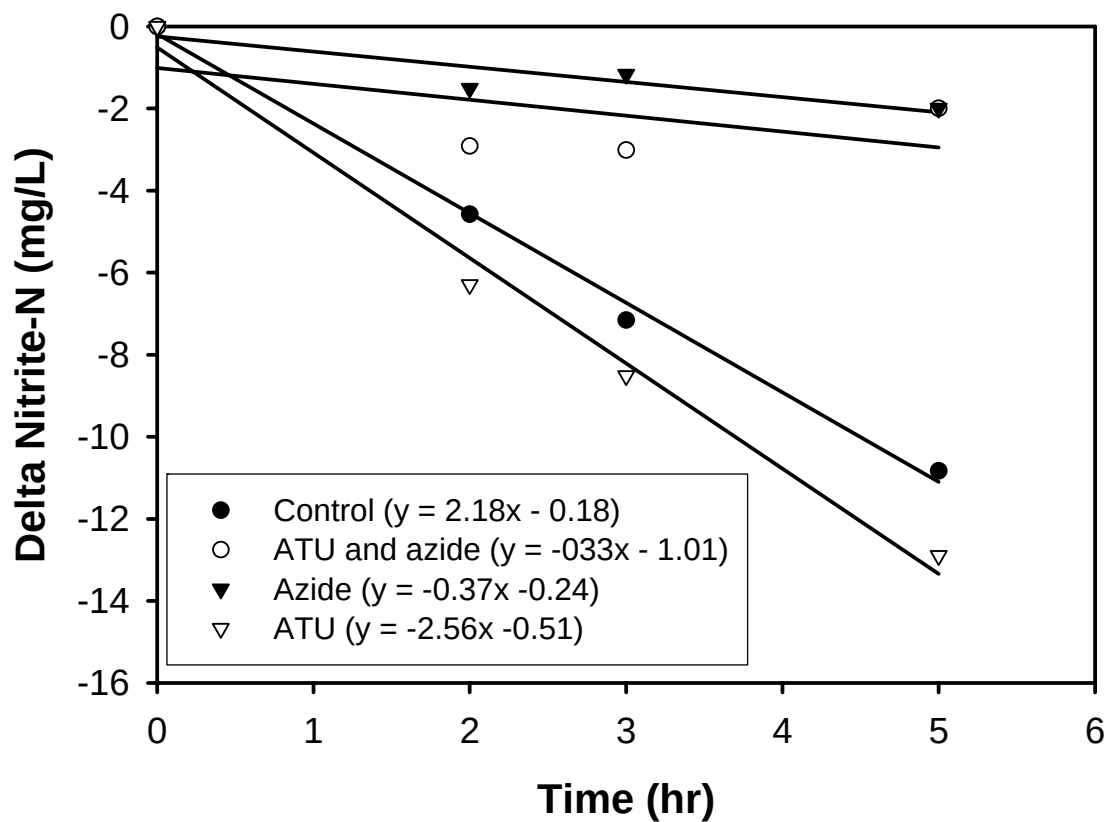


Figure 4-12. Nitrite removal in the control, ATU, azide, ATU and azide experimental samples over time in batch experiments. The slopes represent the average nitrite removal rates.

decrease over 5 hours was 12.99 mg-N/L and the rate of nitrite removal was 2.56 mg-N/L-hr, similar to results obtained in the control. These results are also similar to findings reported earlier in this Chapter and, again, indicate that ATU did not significantly affect nitrite removal. However, when azide was added, only 1.99 mg-N/L of nitrite was removed over time at a rate of 0.37 mg-N/L-hr, representing 83% inhibition of nitrite removal. As anticipated, the impact of both ATU and azide was similar to the impact of azide alone. The nitrite decrease over 5 hours was 1.99 mg-N/L and the rate of nitrite removal was 0.33 mg-N/L-hr. Results were almost identical to those obtained from the azide alone experiment indicating that AOB did not affect nitrite removal.

With respect to nitrate formation, results were similar in the control and ATU tests, 10.29 mg-N/L and 11.25 mg-N/L, respectively (Figure 4-13). However, only 4.43 mg-N/L of nitrate was formed when azide only used. Not surprisingly, the same amount of nitrate (4.44 mg-N/L) was formed when ATU and azide were utilized together. The rates of nitrate formation were 2.11 mg-N/L-hr in the control and 2.20 mg-N/L-hr in the ATU experimental samples again indicating no inhibition in the presence of ATU. However, the nitrate formation rate declined in the presence of azide and azide/ATU to 0.92 mg-N/L-hr, representing a 56% reduction. The rate of nitrate formation and the extent of inhibition when both ATU and azide were used indicate that ATU did not affect nitrate formation.

4.1.3.2 Effect of Allylthiourea and Azide on CO₂ Fixation

Chemical compounds inhibiting the activity of certain groups of chemoautotrophic bacteria are widely used for assessment of the intensity of

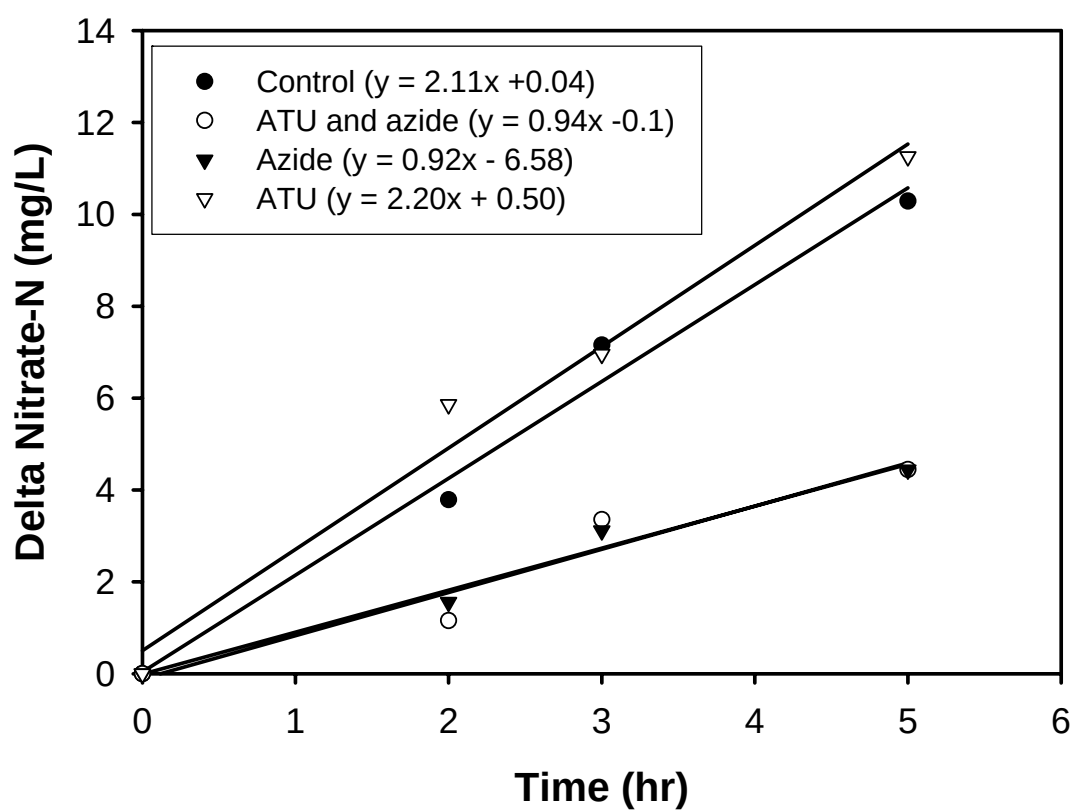


Figure 4-13. Nitrate formation in the control, ATU, azide, ATU and azide experimental samples over time in batch experiments. The slopes represent the nitrate formation rates.

microbiological processes. The use of the radioisotope method for determination of CO₂ fixation together with the inhibitor analysis allows one to quantitatively assess the contribution of individual groups of chemoautotrophic bacteria to the total CO₂ assimilation by bacterial groups. The level of nitrite oxidation in the activated sludge treatment was determined from the decrease in ¹⁴CO₂ assimilation in the presence of allylthiourea and azide inhibitors.

a. Allylthiourea

The mean increases in the amount of inorganic carbon assimilated into biomass by NOB over time in the 2 separate control and ATU experiments were plotted to determine the effect of ATU on CO₂ fixation and to investigate the relationship between nitrite oxidation and CO₂ uptake (Figure 4-14). After 5 hr of incubation, the mean amount of carbon fixed was 12.97 ± 1.05 µg/L in the control and 7.56 ± 1.38 µg/L in the ATU experiments. Compared to the controls, 41% less CO₂ was incorporated into the biomass in the presence of ATU suggesting that AOB contribute approximately half of the measured CO₂ uptake. The mean rates of CO₂ fixation were found to be 2.62 µg/L-hr and 1.55 µg/L-hr in the controls and in the ATU samples, respectively. A 41% inhibition in the rate was measured indicating, once again, that about half of the CO₂ was fixed by AOB. Statistical analysis of the data also demonstrated that there was a significant difference in the CO₂ fixation rates between the control and ATU tests after five hours of incubation ($\alpha = 0.05$).

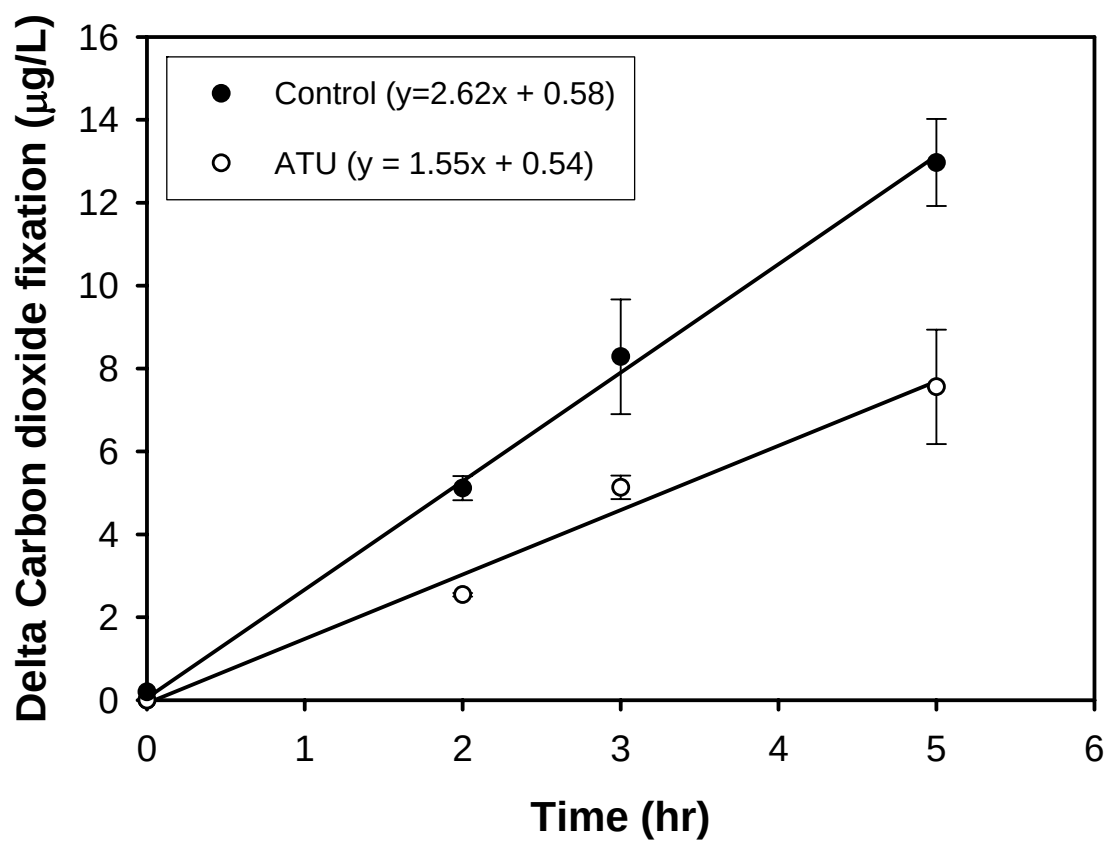


Figure 4-14. Carbon dioxide fixation in the control and ATU experimental samples over time in batch experiments ($n = 2$). The slopes represent the carbon dioxide fixation rates.

b. Azide

The effect of azide on CO₂ assimilation by NOB was investigated in 6 separate experiments. Figure 4-15 represents the mean amount of inorganic carbon assimilated into biomass by NOB during both control and azide experiments over time. After 5 hr of incubation, inorganic carbon incorporated into the biomass was 16.29 ± 4.58 µg/L in the control and 9.12 ± 3.96 µg/L in the azide experiments indicating 44% less assimilation of CO₂ in the presence of azide. The mean rate of CO₂ fixation was 3.25 µg/L-hr in the controls and 1.82 µg/L-hr in the azide samples which represents a 44% reduction in the rate when azide was present. These results indicated that about half of the CO₂ was fixed by NOB. Statistical analysis of the data also demonstrated that there was a significant difference in the CO₂ fixation rates between the control and azide tests ($\alpha = 0.05$).

The reduction in the CO₂ fixation in the presence of azide was not surprising since azide reduced nitrite removal (54%) and nitrate formation (56%). The NOB lacked the energy required to fix inorganic carbon to organic carbon which is typically obtained from the oxidation of nitrite to nitrate. The results showed that azide reduced nitrite removal, nitrate formation and CO₂ fixation as expected.

According to Saralov et al. (1984), it is possible to distinguish between CO₂ fixation by chemoautotrophic and by heterotrophic bacteria, using azide. This is due to the fact that generation of ATP in chemoautotrophs in the terminal sections of the respiratory chain, the c-type and α -type cytochrome oxidases, have a high sensitivity to azide and other respiratory inhibitors. Saralov et al. (1984) showed that 15 µM azide completely inhibited CO₂ fixation in the *Nitrobacter* sp. Also, Pimenov et al. (1990) found that azide exerted a negligible effect on CO₂ fixation by AOB and heterotrophs.

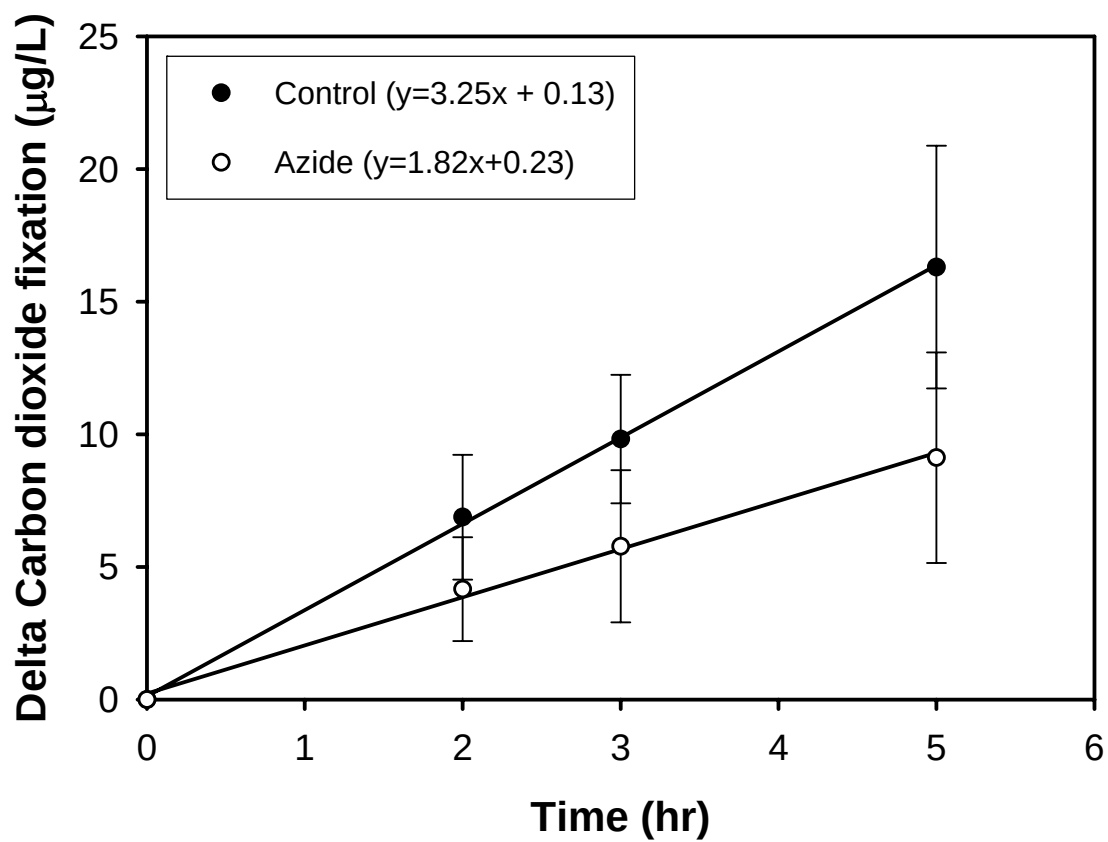


Figure 4-15. Carbon dioxide fixation in the control and azide experimental samples over time in batch experiments ($n = 6$). The slopes represent the carbon dioxide fixation rates.

c. ATU and azide

CO₂ fixation was investigated in the presence of both 80 µM ATU and 24 µM azide in one experiment in an attempt to distinguish other bacteria able to assimilate CO₂ from AOB and NOB. Figure 4-16 depicts changes in CO₂ fixation with and without inhibitors.

In the controls, 13.72 µg/L CO₂ was assimilated over 5 hrs and the fixation rate was 2.79 µg/L-hr. In the presence of ATU, the amount of CO₂ fixed decreased to 6.58 µg/L and the uptake rate declined to 1.32 µg/L-hr indicating that ATU reduced CO₂ fixation by approximately 52%. When azide was added, similar results to the ATU experiment were obtained; 6.05 µg/L CO₂ was fixed over 5 hrs and the CO₂ uptake rate was 1.21 µg/L-hr, a 56% inhibition. However, when ATU and azide were used together, only 4.23 µg/L of carbon was fixed after 5 hrs. Compared to the control, a 3-fold reduction in carbon incorporated into the biomass was noted. Additionally, the CO₂ fixation rate was reduced to 0.84 µg/L-hr, a 70% reduction in the rate of CO₂ fixation. This data indicate that AOB and NOB fixed 70% of the biomass carbon in the activated sludge.

4.1.3.3 Summary

Nitrite removal rates were equal to nitrate formation rates in the control experiments which indicated that all of the nitrite removed was converted to nitrate. ATU did not inhibit nitrite removal or nitrate formation rates, but did inhibit CO₂ fixation rates by approximately 50%. Since ATU primarily inhibits AOB, it can be concluded that ammonia oxidizers have little effect on nitrite removal and nitrate formation. Azide did

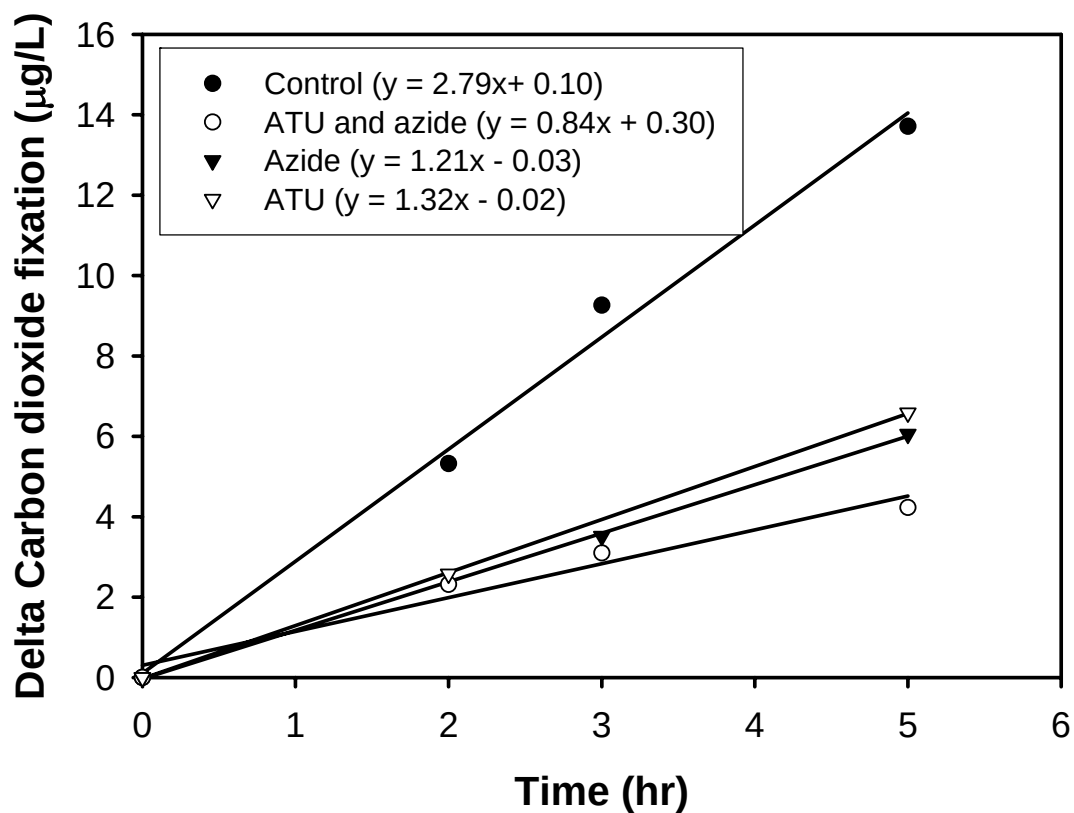


Figure 4-16. Carbon dioxide fixation in the control, ATU, azide, ATU and azide experimental samples over time in batch experiments. The slopes represent the carbon dioxide fixation rates.

inhibit nitrite removal, nitrate formation and CO₂ fixation rates, as expected. Although the rates of nitrite removal and nitrate formation were lower than those in the control, the ratio of nitrite removal to nitrite formation remained at 1:1 indicating a direct link between the two processes. Inhibition of CO₂ fixation by azide also indicated that the activity of NOB was inhibited since the bacteria were not able to fix inorganic carbon. Additionally, it was demonstrated that there was a connection between nitrite removal, nitrate formation and CO₂ fixation. Inhibition during nitrite oxidation results in low CO₂ fixation which is likely due to the reduced energy available.

4.1.4 Effect of VFAs on Nitrite Oxidation and CO₂ Fixation

The impact of VFAs on nitrite removal, nitrate formation and CO₂ fixation in activated sludge was of primary interest in this study. A mixture of VFAs (acetate, propionate and butyrate [1:1:1]) at two different concentrations (1500 mg/L and 3000 mg/L) was added to mixed liquor samples collected from the CSTR. Concentrations of nitrite, nitrate and inorganic carbon incorporated into biomass were measured in the presence of VFAs. These experiments were repeated several times to determine the reproducibility of the results.

4.1.4.1 Effect of VFAs on Nitrite Removal and Nitrate Formation

The mean decrease in nitrite concentration for 3 separate experiments in the absence (controls) and in the presence of 1500 mg/L of VFAs were plotted over time to determine the impact of VFAs on nitrite removal (Figure 4-17). Data from each experiment can be found in Appendix I. After 5 hr of incubation, the mean decrease in

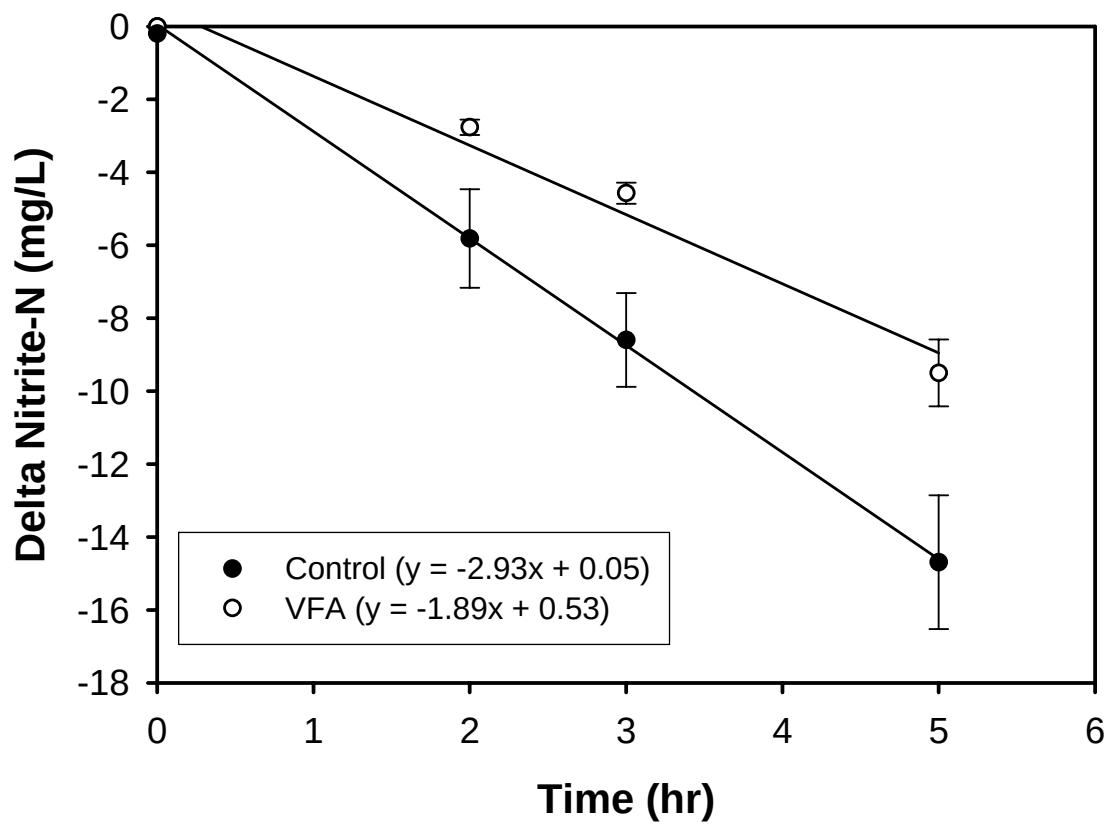


Figure 4-17. Nitrite removal in the control and 1500 mg/L VFAs experimental samples over time in batch experiments (n = 3). The slopes represent the nitrite removal rates.

nitrite concentration was 14.68 ± 1.83 mg-N/L in the control experiments, representing a 66% nitrite removal efficiency. However, only 9.49 ± 0.91 mg-N/L of nitrite was eliminated in the presence of 1500 mg/L VFAs indicating only 42% nitrite removal. Less nitrite was removed when VFAs were added. The data was further analyzed based on rates calculated from the slopes in Figure 4-17. Nitrite removal rate averaged 2.93 mg-N/L-hr and 1.89 mg-N/L-hr in controls and in samples containing 1500 mg/L VFAs, respectively. Nitrite removal rate was reduced by 35% in the VFA samples relative to the controls. Statistical analysis also suggested that there was a significant difference in nitrite removal rates between control and VFA samples after five hours of incubation ($\alpha = 0.05$).

The impact of 1500 mg/L VFAs on nitrate formation was also examined in 3 separate experiments. After 5 hours of incubation, 14.73 ± 2.98 mg-N/L nitrate accumulated in the controls. The mean nitrate increase in the presence of 1500 mg/L VFAs was lower than the level in the controls. Only 2.20 ± 0.35 mg-N/L nitrate was formed in samples containing the VFAs (Figure 4-18). The lower level of nitrate accumulation demonstrated that VFAs affected nitrate formation. The data was further analyzed based on the rates. The mean nitrate formation rates were calculated as 2.93 mg-N/L-hr and 0.45 mg-N/L-hr in controls and in samples containing 1500 mg/L VFAs, respectively. These results suggest that nitrate formation rate was reduced by 85% compared to the controls at a VFAs concentration of 1500 mg/L. A significant difference in nitrate formation rate was noted between controls and samples containing 1500 mg/L VFAs ($\alpha = 0.05$).

Comparison of nitrite removal and nitrate formation revealed that the mean nitrite

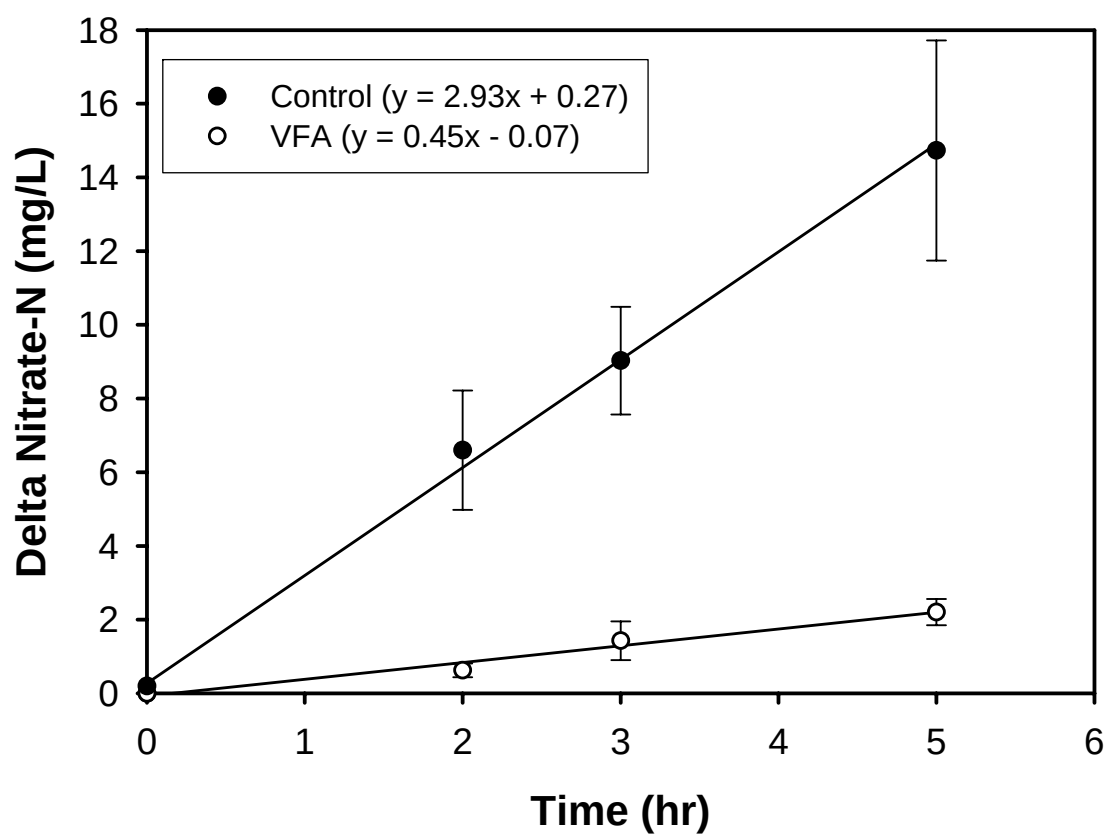


Figure 4-18. Nitrate formation in the control and 1500 mg/L VFAs experimental samples over time in batch experiments ($n = 3$). The slopes represent the nitrate formation rates.

decrease and the mean nitrate increase were similar in the controls, 14.68 ± 1.83 mg-N/L and 14.73 ± 2.98 mg-N/L, respectively and followed the expected 1:1 stoichiometric ratio. However in the 1500 mg/L VFAs samples, the mass of nitrite removed (9.49 ± 0.91 mg-N/L) was not equal to the mass of nitrate formed (2.20 ± 0.35 mg-N/L) indicating that more nitrite was removed than nitrate formed. Additionally, a simple mass balance calculation indicated that 7.29 mg-N/L nitrogen was unaccountable in the test vials. The ratio of nitrite removal to nitrate formation changed from 1:1 in the controls to 1:0.23 in the VFA samples indicating an imbalance between nitrite removal and nitrate formation. For every 1 mol of nitrite-N removed, only 0.23 mol nitrate-N was formed. Additionally, the nitrate formation rate in the VFAs samples (0.45 mg-N/L-hr) was lower than the nitrite removal rate (1.89 mg-N/L-hr) suggesting that nitrate formation was affected more than nitrite removal by the VFAs.

The impact of VFAs on nitrite oxidation was also investigated using a higher concentration of VFAs. 3000 mg/L of VFAs was utilized in 8 separate experiments. In the controls, a 14.63 ± 3.75 mg-N/L nitrite decrease was obtained after 5 hr of incubation whereas only 9.00 ± 2.79 mg-N/L of nitrite was removed in the presence of 3000 mg/L VFAs (Figure 4-19). Comparison of the control rate and the rate in the presence of 3000 mg/L VFAs showed that VFAs also had an impact on the nitrite removal rate. The nitrite removal rate in the controls (2.94 mg-N/L-hr) was much higher than that in the VFA containing samples (1.83 mg-N/L-hr), a 37% reduction in the rate. Statistical analysis showed that 3000 mg/L VFAs also affected nitrite removal rate significantly ($\alpha = 0.05$).

Nitrate concentrations measured at the end of the VFA experiments increased; however, they were lower than levels measured in the controls. The mean nitrate increase

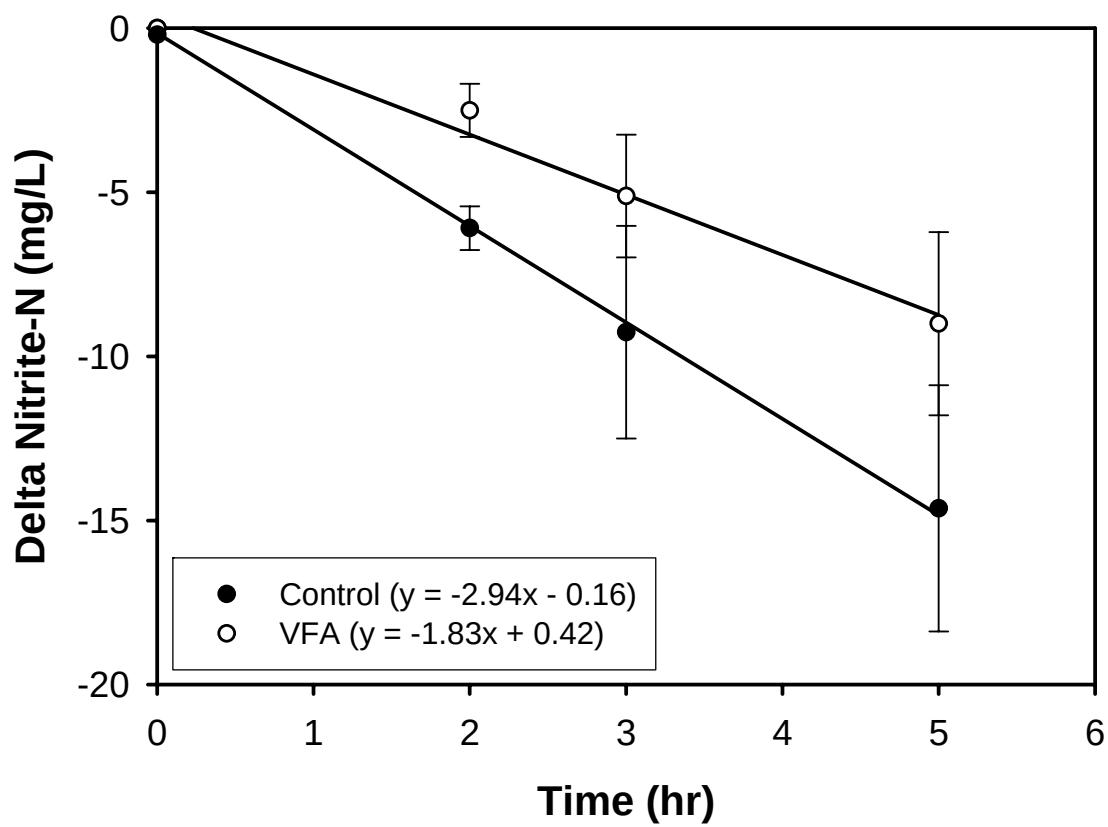


Figure 4-19. Nitrite removal in the control and 3000 mg/L VFAs experimental samples in batch experiments ($n = 8$). The slopes represent the nitrite removal rates.

was 14.33 ± 2.39 mg-N/L in the controls and 3.80 ± 1.13 mg-N/L nitrate accumulated in the presence of 3000 mg/L VFAs (Figure 4-20). The mean nitrate formation rates were 2.91 mg-N/L-hr and 0.77 mg-N/L-hr in controls and 3000 mg/L VFAs containing samples. Compared to the controls, nitrate formation rate was reduced by 74% by the VFAs. Statistical analysis showed that the reduction was significant ($\alpha = 0.05$).

The relationship between nitrite removal and nitrate formation was also investigated in the experiments performed with 3000 mg/L of VFAs. In the controls, nitrite consumed was 14.63 ± 3.75 mg-N/L whereas nitrate produced was 14.33 ± 2.39 mg-N/L (a 1:1 ratio). This was not the case in samples containing 3000 mg/L VFAs. Although 9.00 ± 2.79 mg-N/L of nitrite was removed, only 3.80 ± 1.13 mg-N/L nitrate accumulated. Thus, the ratio of nitrite removal to nitrate formation changed from 1:1 in the controls to 1: 0.42 in the VFAs samples implying that nitrite removal and nitrate formation were not affected to the same extent. Additionally, 5.20 mg-N/L of nitrogen was unaccountable (based on a simple mass balance) in the presence of 3000 mg/L VFAs. In the presence of 3000 mg/L VFAs, the nitrite removal rate (1.83 mg-N/L-hr) was different from the nitrate formation rate (0.77 mg-N/L-hr) also suggesting that nitrate formation was affected more than nitrite removal.

The effects of 1500 mg/L and 3000 mg/L VFAs on nitrite oxidation were comparable. Given that the mean decreases in nitrite removal were close to each other (9.49 ± 0.91 mg-N/L and 9.00 ± 2.79 mg-N/L for 1500 mg/L and 3000 mg/L VFAs), it appears that the two different concentrations had a similar impact on nitrite removal. Nitrite removal rate reduction was 35% and 37%, in the presence of 1500 mg/L and 3000 mg/L VFAs, respectively. Similarly, reduction of nitrate formation rate was 85% in the

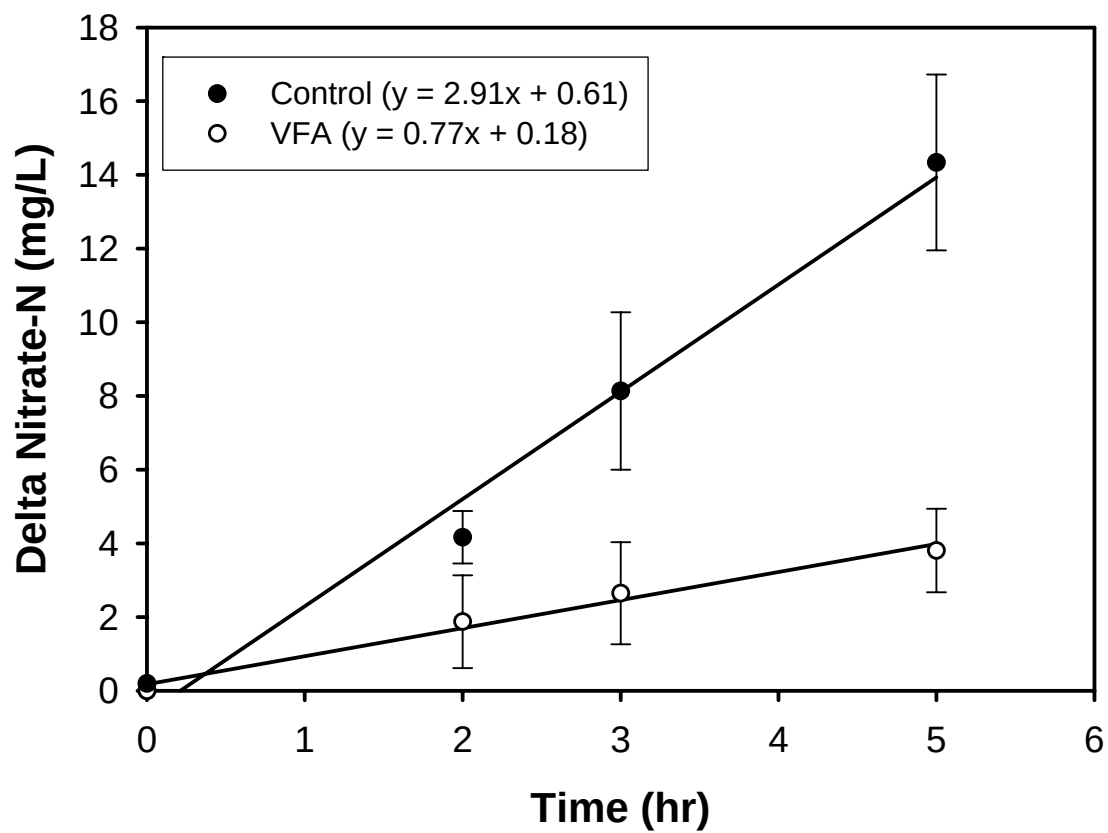


Figure 4-20. Nitrate formation in the control and 3000 mg/L VFAs experimental samples in batch experiments ($n = 8$). The slopes represent the nitrate formation rates.

presence of 1500 mg/L VFAs and 74% in the presence of 3000 mg/L VFAs.

In summary, nitrite removal and nitrate formation were both affected by VFAs. However, nitrate formation decreased more than nitrite removal resulting in an unbalance nitrite oxidation reaction.

4.1.4.2 Effect of VFAs on CO₂ Fixation

The impact of VFAs on CO₂ fixation was also investigated to examine the activity of NOB during exposure to 1500 mg/L and 3000 mg/L VFAs. During the 1500 mg/L VFAs treatment experiments, the mean amount of inorganic carbon assimilated into biomass by NOB in the controls was 28.39 µg/L after 5 hr (Figure 4-21). The mean inorganic carbon fixed in the presence of 1500 mg/L VFAs in 5 hr was measured as 31.45 µg/L indicating slightly higher amount of carbon was incorporated when 1500 mg/L VFAs were present. CO₂ fixation rates were found to be 5.68 µg/L-hr and 6.26 µg/L-hr, in the controls and in the 1500 mg/L VFAs experimental samples, respectively. The rate of CO₂ fixation in samples containing 1500 mg/L VFAs was higher than in the controls and no inhibition of CO₂ fixation was observed. Statistical analysis of this data also demonstrated that there was no significant difference between the rates ($\alpha = 0.05$).

As the experiments were repeated using 3000 mg/L of VFAs, the mean amount of carbon assimilated was found to be 20.38 µg/L in the controls and 23.91 µg/L in the experimental samples indicating that similar amounts of carbon were fixed in both controls and in the samples with VFAs (Figure 4-22). CO₂ fixation rates were 4.03 µg/L-hr and 4.75 µg/L-hr, in the controls and in the presence of 3000 mg/L of VFAs, respectively, implying that the rate of CO₂ uptake was not reduced by 3000 mg/L VFAs.

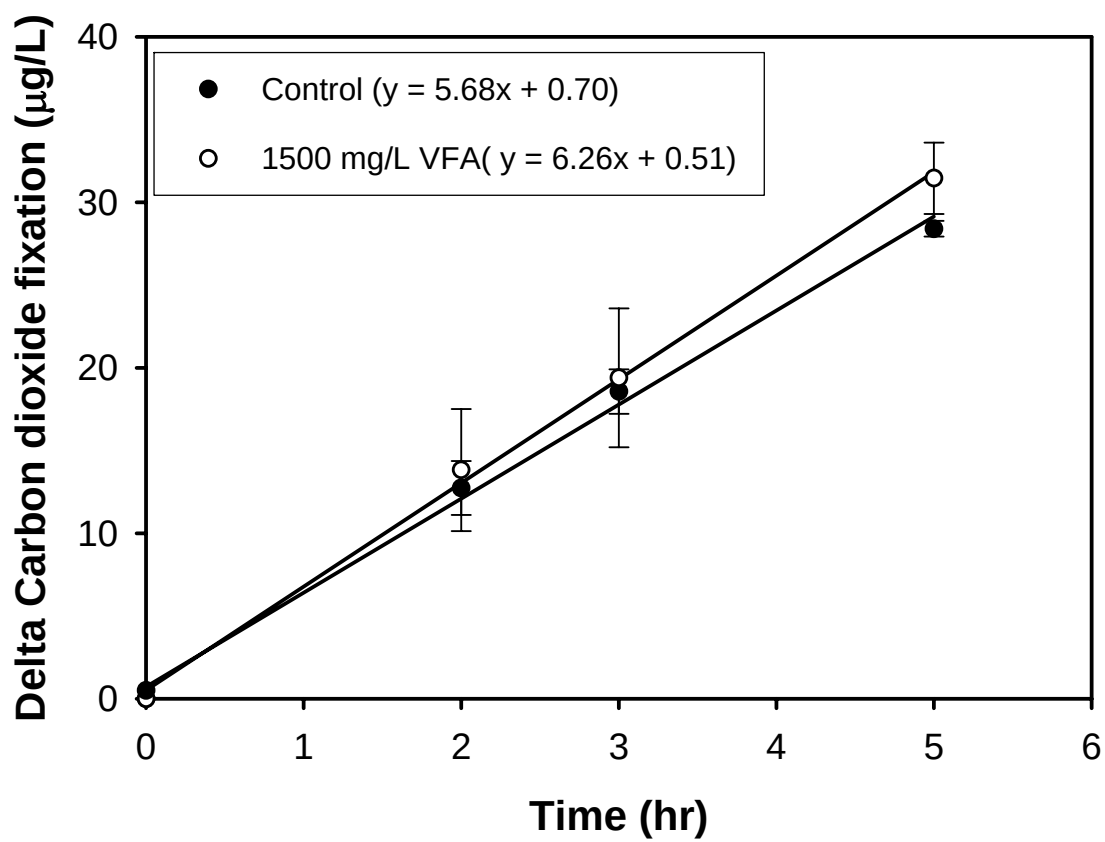


Figure 4-21. Carbon dioxide fixation in the control and 1500 mg/L VFAs experimental samples over time in batch experiments ($n = 3$). The slopes represent the carbon dioxide fixation rates.

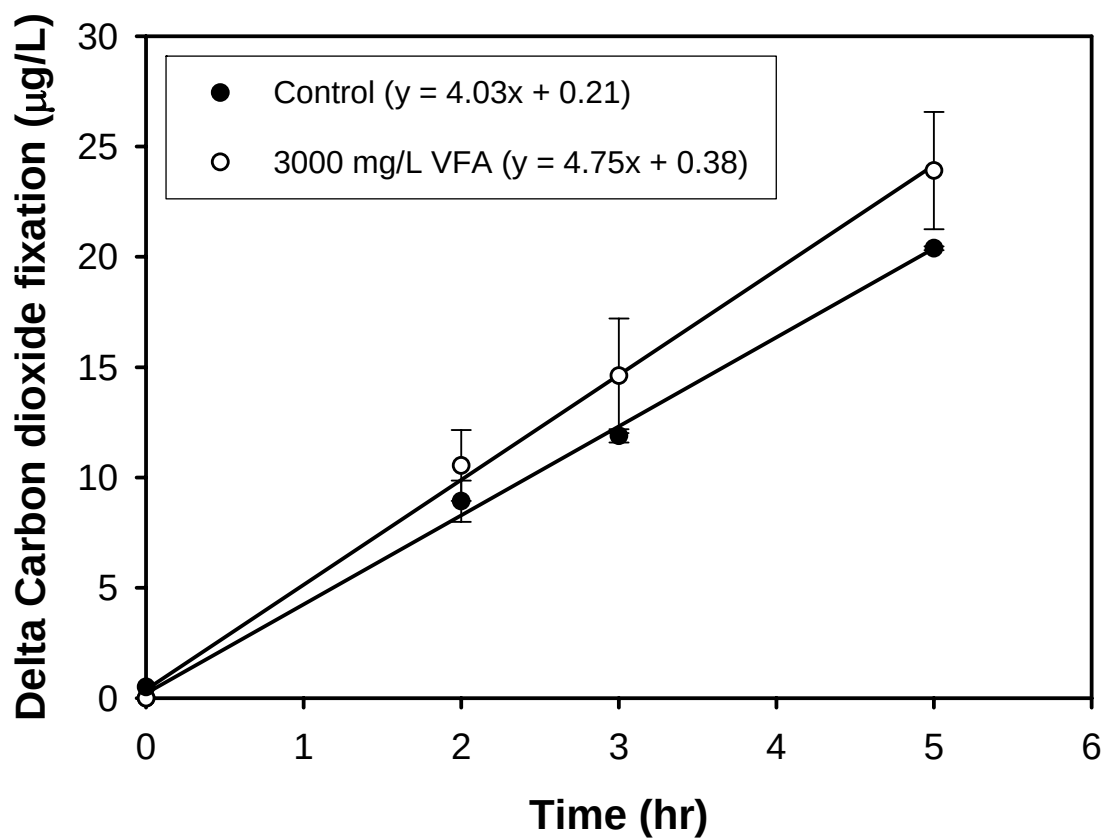


Figure 4-22. Carbon dioxide fixation in the control and 3000 mg/L VFAs experimental samples over time in batch experiments ($n = 3$). The slopes represent the carbon dioxide fixation rates.

Additionally, no statistically significant differences were observed between CO₂ fixation rates for the control and experimental samples ($\alpha = 0.05$).

In conclusion, the two different concentrations of VFAs had a similar impact on CO₂ fixation. The amount of inorganic carbon assimilated was comparable in the presence of either 1500 mg/L or 3000 mg/L VFAs. These concentrations were slightly higher than those obtained from the control experiments indicating that VFAs did not reduce CO₂ fixation.

4.1.4.3 Summary

The results showed that although nitrite removal and nitrate formation were reduced by VFAs, CO₂ fixation was not. It is apparent that VFAs do not affect nitrite removal, nitrate formation and CO₂ fixation in the same manner as azide. Although azide inhibited nitrite removal and nitrate formation, the 1 to 1 ratio between those two compounds remained unchanged. All nitrite removed was converted to nitrate even when rates decreased due to the presence of azide.

CO₂ fixation was reduced by azide but VFAs had no affect on CO₂ fixation. Results obtained in this study differed from the literature (Eilersen et al. 1994) and were surprising since it is generally thought that CO₂ fixation and nitrite oxidation are tightly coupled (McFadden and Shively, 1991). If the rate of one decreases, the other should decline, also. CO₂ fixation is generally believed to be limited by the availability of reducing power derived from nitrification (Prosser, 1989; Bock et al., 1991). However, VFAs did not reduce the CO₂ uptake rate although they had an impact on nitrite removal and nitrate formation rates. In past studies, CO₂ fixation in the presence of VFAs was not

investigated; thus, it was assumed that VFAs inhibited the nitrite oxidation pathway because of decreases measured in nitrate formation. Previous researchers have typically assessed the activity of nitrite oxidizing bacteria based on nitrate production. For example, Eilersen et al. (1994) concluded that acetic, propionic and butyric acids all inhibited nitrite oxidation since a lesser amount of nitrate was produced in the presence of VFAs compared to control experiments. Also, Gomez et al. (2000) observed that nitrate was produced in lesser amounts when VFAs were present although nitrite was not detected in their 80-hr batch experiments. The authors assumed that nitrite and/or nitrate were consumed for microbial protein formation by the heterotrophic microorganisms present in the activated sludge.

This study also indicated that a lesser amount of nitrate was produced in the presence of VFAs. However, nitrite removal, nitrate formation and CO₂ fixation results imply that VFAs impact nitrite removal and nitrate formation via a different mechanism. If VFAs had negatively affected the nitrite oxidation pathway, the CO₂ fixation pathway should also have been reduced since the NOB would not get the energy needed to fix CO₂. Since the oxidation of nitrite provides energy for CO₂ fixation and given that CO₂ fixation was not reduced, nitrite oxidation apparently occurred. Nitrate produced from the oxidation of nitrite may have been reduced to other reaction products via an alternative pathway such as denitrification. If so, gaseous nitrogen compounds such as nitric oxide (NO), nitrous oxide (N₂O) or dinitrogen gas (N₂) would be produced; however, these compounds were not measured and would appear as a nitrogen loss from the system. Thus, VFAs might stimulate aerobic denitrification which would result in altered levels of nitrite and nitrate relative to the controls.

4.1.5 Total Bacteria and *Nitrospira* Concentrations in the Batch Experiments

In addition to chemical analysis, genetic analyses were performed to examine the effects of VFAs on the bacterial content in batch experiments. Total bacterial concentration was used as an overall measure of biomass to determine if VFAs increased microbial content due to the additional carbon present. Additionally, *Nitrospira* concentrations were measured to determine if the decline in nitrate formation in the presence of VFAs was due to a numerical reduction of NOB.

Real-time PCR assays were used to quantify eubacterial 16S rRNA and *Nitrospira* spp. 16S rRNA in mixed liquor samples after the 5 hr incubation period in the presence and absence of VFAs in batch experiments. To allow for better comparison with traditional measures of biomass, the real-time PCR gene copy data was converted to a cell per liter basis. A value of 3.6 16S rRNA gene copies per cell was assumed for the calculation of total bacterial concentrations (Harms et al., 2003). Since the number of *Nitrospira* 16S rRNA copies per cell is unknown, it was assumed to be one based on average copies of 16S rDNA per cell in other nitrifying bacteria, such as *Nitrosomonas* and *Nitrobacter* (Harms et al., 2003).

Total bacteria concentrations remained constant for samples with VFAs and without VFAs (Figure 4-23) based on ANOVA analysis ($\alpha = 0.05$). This suggested that the decline in the rates of nitrite removal and nitrate formation was likely not due to heterotrophic growth and subsequent uptake of nitrite and/or nitrate. It was anticipated that some heterotrophic growth would occur when VFAs were added due to the additional carbon which could be used by the bacteria. However, little, if any, additional growth occurred.

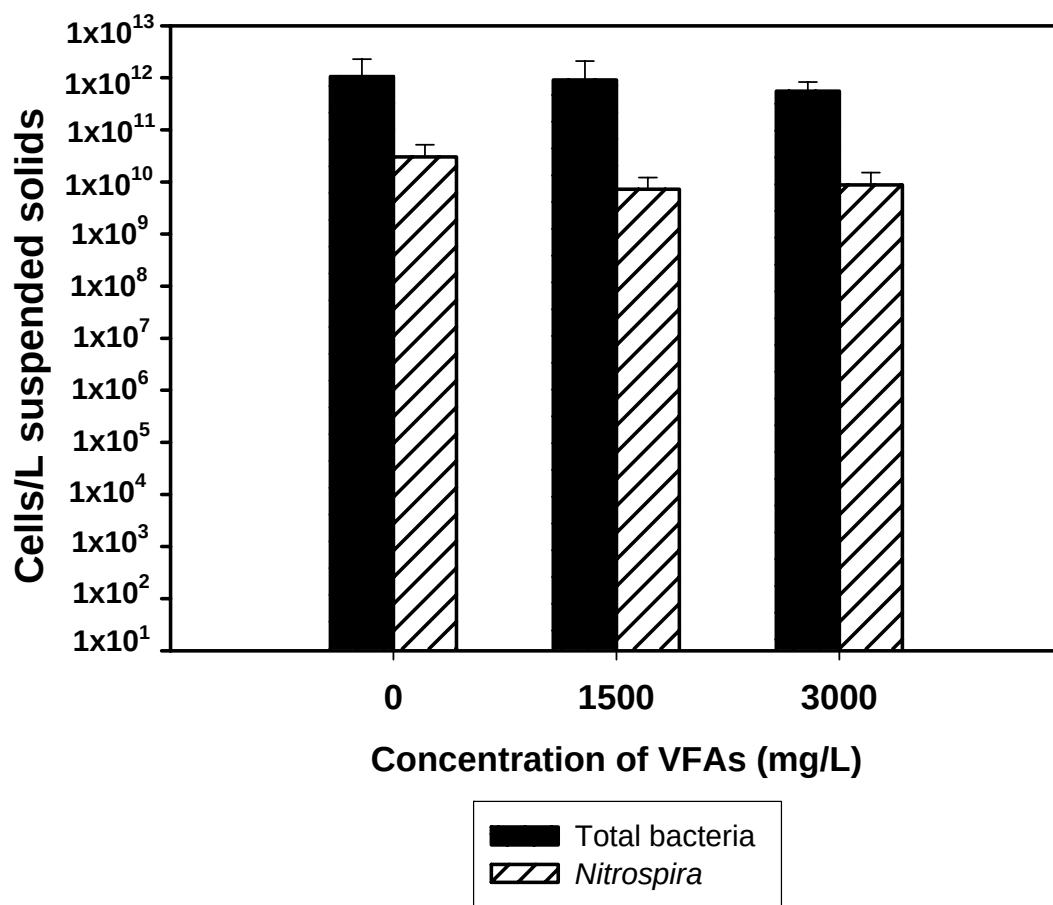


Figure 4-23. Number of *Nitrospira* and total bacteria in the absence and the presence of VFAs after 5 hours of incubation in batch experiments.

The total bacterial cell densities measured in this study ($8.41 \pm 2.60 \times 10^{11}$ cell/L) were consistent with those reported in the literature. Brown (2003) found a total eubacterial concentration of $7.0 (\pm 3.7) \times 10^{11}$ cell/L during operation of a CSTR (SRT = 10 d) treating municipal wastewater at 20°C. Harms et al. (2003) reported an average value of 4.3×10^{11} cells/L when applying the same real-time PCR assay used in the current study to 12 monthly samples from the full-scale municipal WWTP used to seed the CSTR (SRT = 10 d). Lee et al. (2002) reported biomass densities of 2.32×10^{11} to 6×10^{11} cells/L for a bench-scale activated sludge reactor operated at an SRT of 10 days and a temperature of 20°C.

Nitrospira concentrations were measured as $3.03 (\pm 3.86) \times 10^{10}$ cell/L in the control, $7.25 (\pm 1.17) \times 10^9$ cell/L in the 1500 mg/L VFAs and $8.77 (\pm 1.40) \times 10^9$ cells/L in 3000 mg/L VFAs samples (Figure 4-23). Based on ANOVA analyses ($\alpha = 0.05$), no significant change in the numbers of *Nitrospira* as a function of VFA concentration was observed.

Nitrospira numbers remained relatively constant in all samples indicating that this bacterium did not grow significantly during the batch experiments in the presence of VFAs. Higher levels of *Nitrospira* were not expected after only 5 hours of incubation since the growth rates of NOB are generally very low. For example, generation times vary from 10 to 140 hr for different strains of *Nitrobacter* (Bock et al., 1991). Additionally, the cell yield of all NOB is low (Bock et al., 1991). Since *Nitrospira* levels remained relatively constant during experiments with VFAs, it can be concluded that the reduction in nitrite removal and nitrate formation rates observed in the presence of VFAs was not due to a decrease in the number of NOB.

Nitrospira concentrations were very similar to values reported in the literature. Brown (2003) found a *Nitrospira* concentration of $2.9 (\pm 1.0) \times 10^{10}$ cell/L during operation of a municipal treatment CSTR (SRT = 10 d) at 20°C. Harms et al. (2003) observed an average *Nitrospira* concentration of 3.7×10^{10} cell/L for 12 monthly mixed liquor samples from the full-scale municipal WWTP used to seed the CSTR operated in this study. Additionally, Yuan and Blackall (2002) reported *Nitrospira* concentrations of approximately 3×10^9 cell/L in lab-scale activated sludge reactors treating municipal wastewater, using a real-time PCR assay.

4.1.6 Transition to the Conceptual Model

Mass balance analysis of the batch experiment data, with regard to nitrite and nitrate concentrations, indicated that some nitrogen could not be accounted when VFAs were present. These results, again, suggested that nitrogen was removed from the VFA containing systems and that the effects of VFAs were different from those of azide. Ginestet et al. (1998) showed that azide, a selective inhibitor of NOB, lowers nitrite oxidation via inhibition of the nitrite oxidoreductase enzyme through complexation with the molybdenum atoms. Thus, if VFAs had affected the nitrite oxidation pathway, results obtained would be similar to those observed with azide. However, results in the current study indicated that nitrite was removed from the VFA containing systems via a pathway other than nitrification because:

- In nitrite oxidation, equal molar conversion of nitrite to nitrate is expected. As anticipated, control experiments with no inhibitor added (azide or VFAs) showed equal changes in nitrite and nitrate concentrations over time. The sum of nitrite

and nitrate remained constant for each time point indicating that no nitrogen was lost in the control experiments. Additionally, the nitrite removal rate was equal to the nitrate formation rate and an equal amount of nitrite was converted to nitrate. Therefore, it was clearly shown that the nitrite removed was converted to nitrate both on a rate and a mass basis.

- Azide, a selective inhibitor of nitrite oxidation, behaved as expected since nitrite removal, nitrate formation and CO₂ uptake rates were reduced. Also, total nitrogen remained constant over time during the experiments.
- VFAs did not behave as a selective inhibitor because a greater reduction of nitrate formation than that of nitrite removal was observed yet the CO₂ uptake rate remained unchanged (or slightly increased). In addition, the total nitrogen measured in systems containing VFAs decreased over time.

Based on these findings, it appears that VFAs may stimulate another pathway for nitrogen removal. Experiments were performed to investigate possible pathways induced by the presence of VFAs.

4.1.7 Production of N₂O in Batch Experiments

Although denitrification preferably takes place under anoxic conditions, aerobic denitrification has been described by Robertson et al. (1989). N₂O is produced during denitrification as an intermediate in the reduction of nitrite to nitrogen gas (NO₂⁻ → NO → N₂O → N₂) by heterotrophic bacteria grown on reduced carbon sources such as butyrate (Anderson et al., 1993; Richardson et al., 2001). Therefore, to investigate whether aerobic denitrification possibly led to the lesser amount of nitrate produced in

the presence of VFAs (compared to controls), batch tests were performed to quantify N₂O production.

Experiments using 0 mg/L (control) and 3000 mg/L VFAs were designed to assess levels of N₂O gas over time. 25 mg N/L as sodium nitrite (NaNO₂) was added to these samples as a substrate and an energy source for the nitrite oxidizers. The parallel assays were terminated after 5h of incubation at 20°C (shaken at 200 rpm). Gas samples were withdrawn from the headspace using a gastight syringe and injected into an IR spectrometer at 0 hr, 2 hrs, 3 hrs and 5 hrs and analyzed for N₂O. Background air samples were also analyzed to confirm that N₂O was produced biologically from the samples and was not present in the ambient air. Concentrations of N₂O were calculated using instrumentation software, Beer's Law and the ideal gas law. The concentrations of N₂O were then normalized (on a nitrogen basis) for comparison with nitrogen species (nitrite and nitrate in the liquid phase) for each sample.

In the control samples, N₂O was not detected in the gas phase. Since little nitrogen was lost from the liquid phase in the control samples, these results were not surprising. In the first control experiment, 17.54 mg-N/L nitrite was removed and 17.55 mg-N/L nitrate was formed in the liquid phase. Additionally, nitrite removal and nitrate formation rates were 3.46 mg-N/L-hr and 3.46 mg-N/L-hr, respectively (Figure 4-24A). In the second control experiment, 11.4 mg-N/L nitrite was consumed and 11.4 mg-N/L nitrate accumulated. Similarly, a 2.28 mg-N/L-hr nitrite removal rate and a 2.3 mg-N/L-hr nitrate formation rate were measured (Figure 4-24B). The ratio of nitrite removal to nitrate formation was 1:1 in both experiments suggesting complete conversion of nitrite to nitrate. Additionally, in the first experiment, initial and final concentrations of total

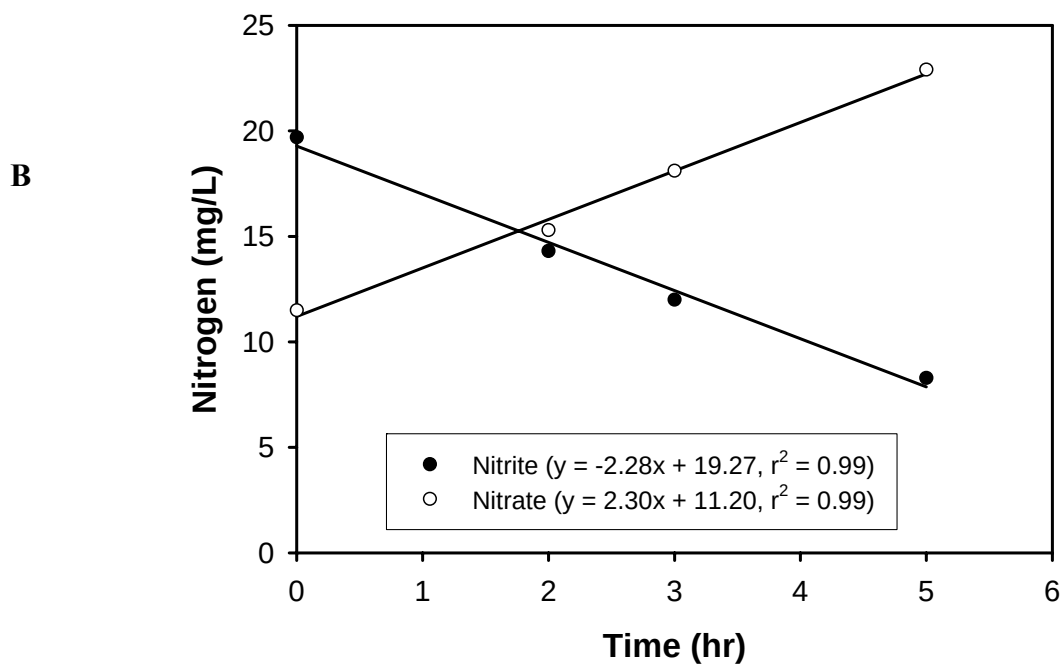
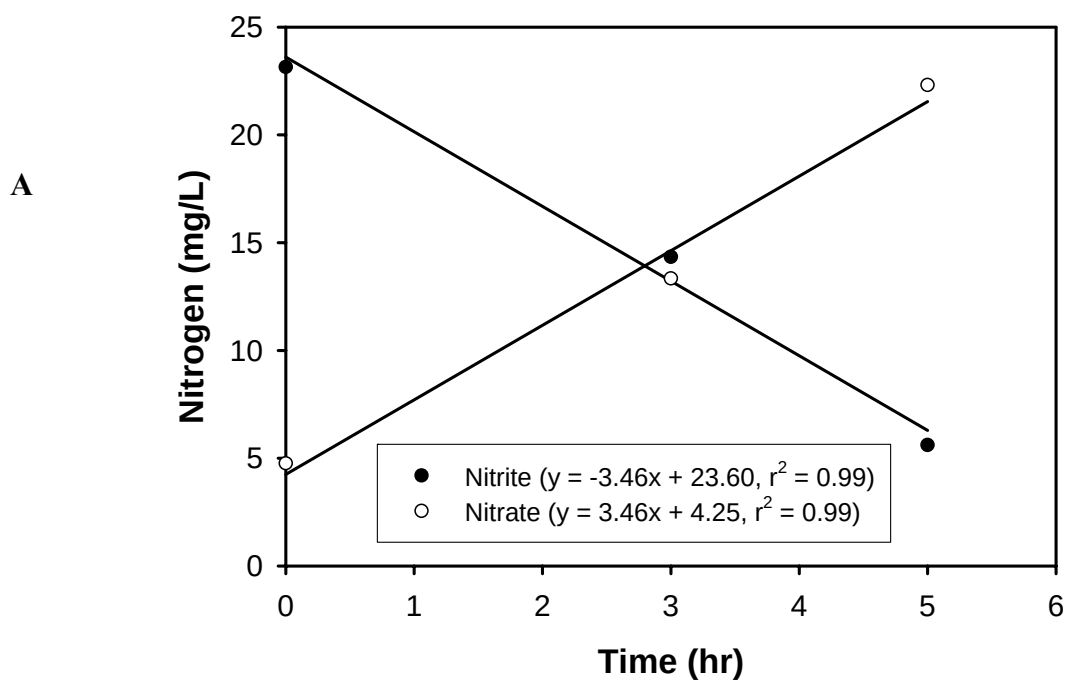


Figure 4-24. Liquid phase nitrite removal and nitrate formation in the two control experiments (A = first experiment, B = second experiment).

nitrogen were calculated as 27.91 mg-N/L and 27.92 mg-N/L, respectively. For the second experiment, the initial total nitrogen concentration was 31.19 mg-N/L and the final total nitrogen concentration was 31.19 mg-N/L. Therefore, no nitrogen was lost in the control samples in these two experiments.

When VFAs were added, although 14.53 mg-N/L nitrite was removed, only 4.41 mg-N/L of nitrate was formed indicating the ratio of nitrite removal to nitrate formation changed from 1:1 (control) to 1: 0.3 in the first experiment. The nitrite removal rate decreased from 3.46 mg-N/L-hr to 2.91 mg-N/L-hr and the rate of nitrate formation changed from 3.46 mg-N/L-hr to 0.88 mg-N/L-hr in the liquid phase during the first experiment (Figure 4-25A). Similarly, the amount of nitrite removed and the amount of nitrate formed were 10.21 mg-N/L and 2.53 mg-N/L, respectively, in the second experiment. The ratio of nitrite removal to nitrate formation decreased from 1:1 to 1: 0.25. Nitrite removal and nitrate formation rates also dropped to 1.99 mg-N/L-hr and 0.53 mg-N/L-hr, respectively, in the second experiment (Figure 4-25B).

Total nitrogen concentrations decreased over the 5 hour test period in both VFA tests. In the first experiment, initial and final total nitrogen concentrations were 29.28 mg-N/L and 19.16 mg-N/L, respectively. For the second experiment, the initial total nitrogen concentration was 36.15 mg-N/L and the final total nitrogen concentration was 28.47 mg-N/L. Therefore, 10.12 mg-N/L and 7.68 mg-N/L nitrogen was lost from the aqueous phase in the presence of VFAs during the first and second experiments, respectively. In other words, 34.5% of the nitrogen mass in the first and 21.2% of the nitrogen mass in the second experiment could not be accounted.

The role of biomass uptake of nitrogen in the unbalanced nitrogen concentrations

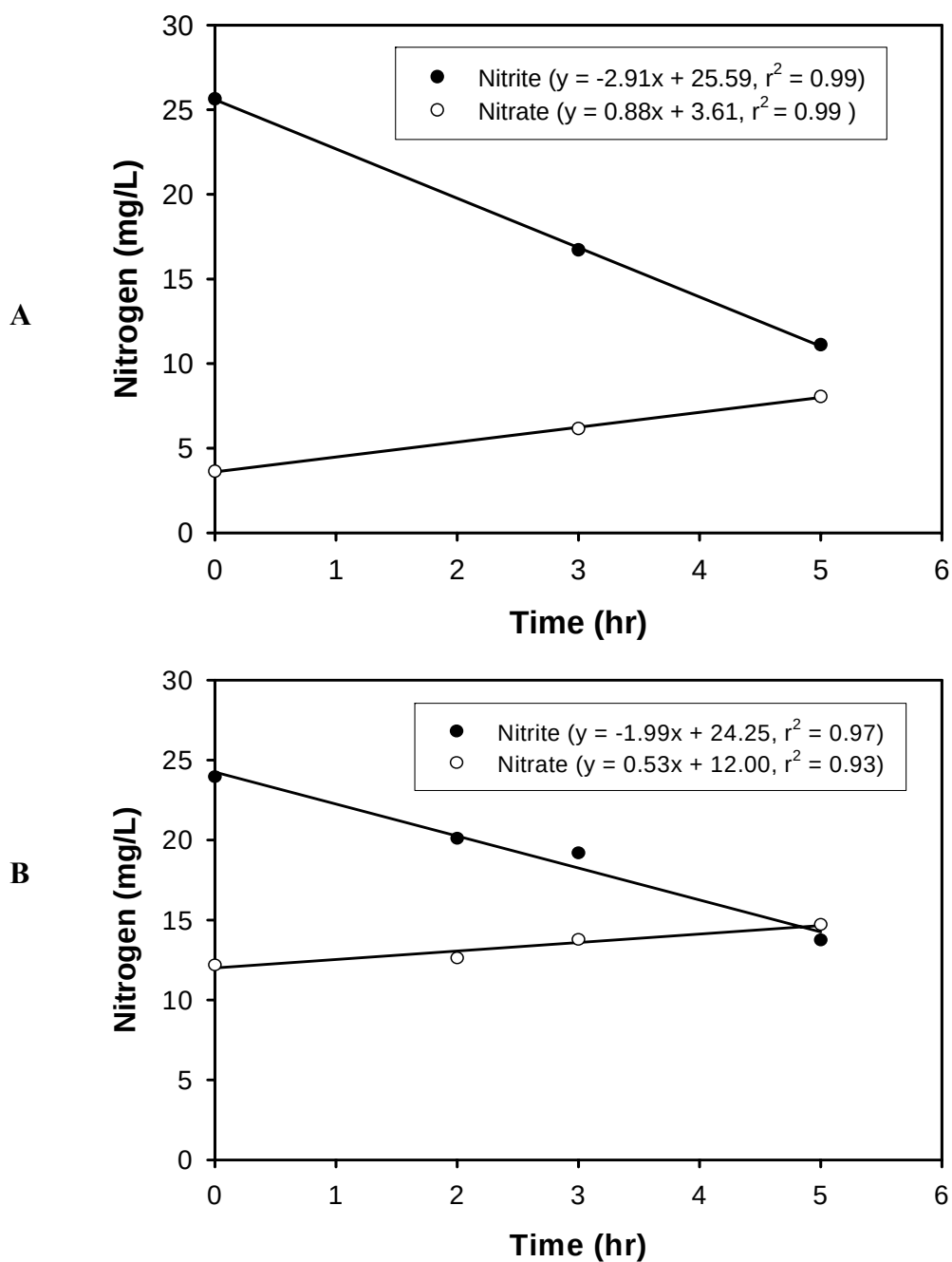


Figure 4-25. Liquid phase nitrite removal and nitrate formation in the two 3000 mg/L VFAs experimental samples (A = first experiment, B = second experiment)

during the batch tests in the presence of VFAs was investigated by measuring the protein concentration and the number of total bacteria to quantify bacterial growth. The protein concentration (measured by the Lowry assay) was 675 µg/ml at time zero and 685 µg/ml after 6 hr. Similarly, the total number of bacteria measured by real-time PCR was 1.09×10^{12} cells/L at the beginning of the experiment and 1.04×10^{12} cells/L at the end of the experiment. Both measures indicated no change in microbial growth due to the presence of VFAs. Therefore, the organic assimilation pathway was not a likely route for the nitrogen losses noted in the presence of VFAs.

IR spectrometry of gas products from batch cultures after 5 hrs of incubation demonstrated that N₂O was produced in the presence of VFAs. In these experiments, N₂O accounted for 54% (5.5 mg-N/L) to 58% (4.45 mg-N/L) of the missing nitrogen in the liquid phase. Additionally, some nitrogen may have been converted to N₂ gas and not detected by IR spectrometry. Jetten et al., (1997) found that in chemostat cultures with *Alcaligenes faecalis*, 25% of the NO₂⁻ - N was lost as N₂O and the remaining 75% went to nitrogen gas. In another study, *Alcaligenes faecalis* showed significant aerobic denitrifying activity, producing almost equivalent amounts of N₂ and N₂O (Robertson et al., 1995). Also, Arts et al. (1995) studied aerobic denitrification in chemostat cultures with *Thiosphaera pantotropha* and found that both N₂ and N₂O were produced by all of the cultures. Although only half of the missing nitrogen was recovered in the work reported here, these results clearly illustrate that N₂O was produced in biologically active samples containing VFAs. Based on these results, additional experiments were performed to determine if the biologically active mixed liquor culture contained the enzyme necessary to convert nitrate to nitrite and then N₂O.

4.1.8 Periplasmic Nitrate Reductase (NAP) Enzyme Activity

4.1.8.1 NAP Enzyme Activity in CSTR Mixed Liquor Samples in Batch Experiments

Although denitrification is generally believed to be an anoxic process, nitrate respiration has been observed under aerobic conditions in a number of bacteria, including *Escherichia coli* (Carter et al., 1995), *Paracoccus denitrificans*, (also known as *Thiosphaera pantotropha*) (Ludwig et al., 1993) and *Pseudomonas aeruginosa* (Robertson and Kuenen, 1990). It was demonstrated that synthesis and activity of denitrifying enzymes could occur under aeration conditions. For example, the denitrifying bacterium *Paracoccus denitrificans* can express two types of respiratory nitrate reductases which catalyze the reduction of nitrate to nitrite: a membrane-bound nitrate reductase (NAR) and a periplasmic nitrate reductase (NAP) (Berks et al., 1995). The periplasmic enzyme catalyses aerobic nitrate respiration while expression and activity of the membrane-bound enzyme are normally restricted to anaerobic conditions (Richardson and Ferguson, 1992). Previous studies have shown that aerobic growth on reduced carbon substrates, such as butyrate, produced and enhanced expression of the periplasmic nitrate reductase enzyme. It was also demonstrated that NAP activity correlates with the oxidation state of the carbon source in pure cultures of *Paracoccus denitrificans*. Lower NAP activities occurred during growth on more oxidized carbon substrates like succinate and malate than during growth on highly reduced carbon substrates such as caproate and butyrate (Sears et al., 1997). In the current study, greater decrease in nitrate formation than nitrite removal was observed. Additionally, N₂O was detected in the presence of VFAs. Thus, it was suspected that nitrate might be used as an electron acceptor by bacteria in the presence of oxygen. Therefore, to assess if the NAP

enzyme was present in the aerobic batch cultures (which could account for the measured reduction in nitrate formation rates in the presence of VFAs), a methylviologen assay specific for NAP activity was utilized. Methylviologen can act as an electron donor to the NAP enzyme. Since this compound is not permeable for membrane-bound nitrate reductase, methylviologen donates electrons predominantly to the periplasmic enzyme (Carter et al., 1995).

Batch experiments, consisting of four parallel treatments, were performed to assess the NAP activity in mixed liquor obtained directly from the CSTR. Treatments consisted of: 1) addition of nitrite (25 mg-N/L), 2) addition of both nitrite (25 mg-N/L) and neutralized VFAs (3000 mg/L), 3) addition of neutralized VFAs (3000 mg/L) and 4) no addition of nitrite or VFAs. Samples were initially collected (T_0) and again after 3 hr (T_3) of incubation on a shaker table. Mixed liquor samples were added to a methylviologen reaction mixture and incubated for 45 min at 37°C as described in Chapter 3 (Lopez-Lozano et al., 2002). Nitrate reduction was measured by production of nitrite under each set of test conditions (expressed as nmol of nitrite produced per minute per milligram of proteins) (Patureau et al., 1996; Bedzyk et al., 1999).

The enzyme activities in batch samples with no amendments were 0.330 nmol/min/mg protein and 0.368 nmol/min/mg protein, for T_0 and T_3 , respectively, (Figure 4-26). In samples with added nitrite, the activities of NAP enzyme were 0.495 nmol/min/mg protein at T_0 and 0.522 nmol/min/mg protein at T_3 . These results suggested that NAP activity remained relatively constant over time in the samples with no amendments and with added nitrite during the batch experiment. Statistical analysis indicated no significant difference in NAP activity between T_0 and T_3 in these samples.

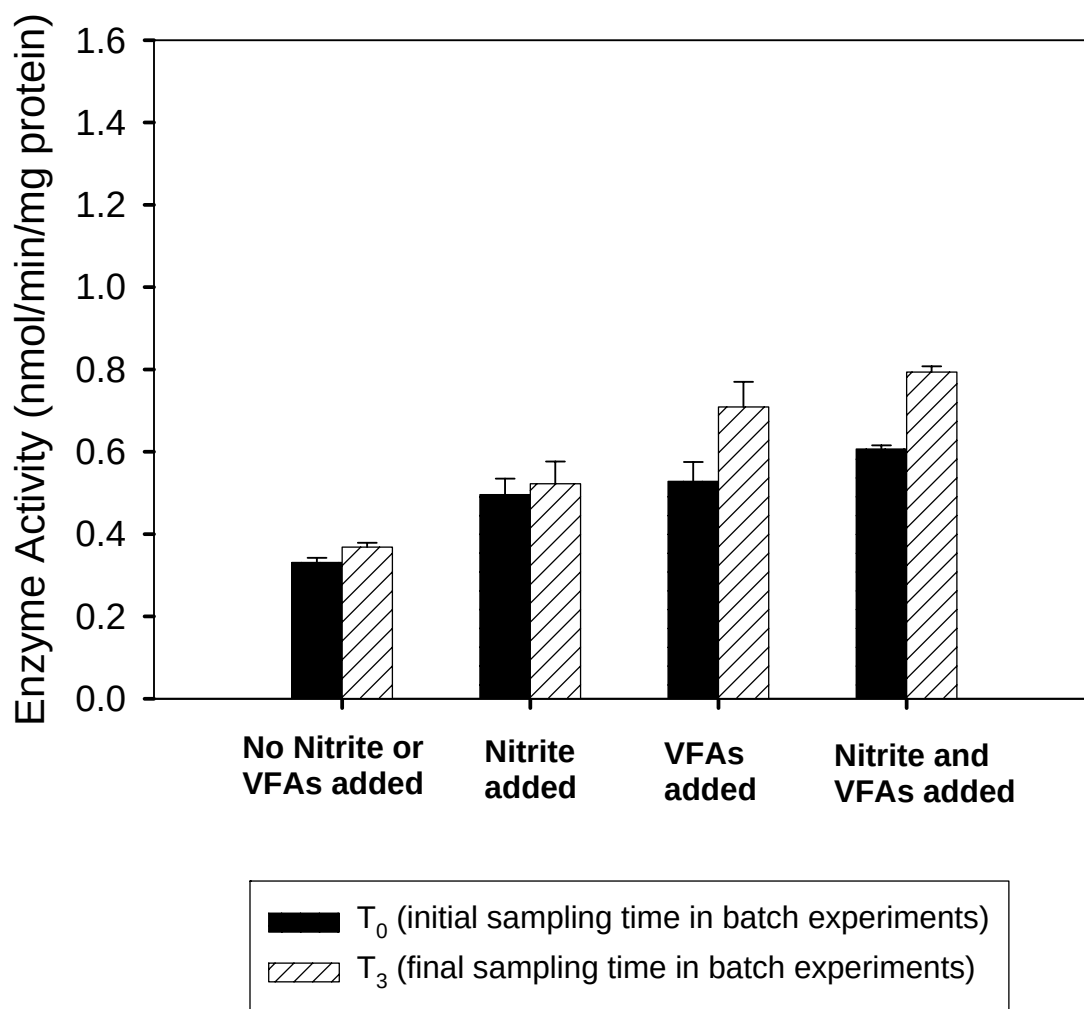


Figure 4-26. Periplasmic nitrate reductase enzyme activity in batch experiment performed using mixed liquor from the CSTR for each treatment and time points (Nitrite was 25 mg-N/L and added VFAs were 3000 mg/L).

The difference in the enzyme activity at T_0 between the control and nitrite added sample was due to a sampling time variation. Enzyme activity levels at T_0 were higher in the treatment than the control (no amendment) due to the delay encountered when nitrite was added to the batch sample. The time lag between addition of nitrite and T_0 analysis for this sample (≈ 10 min) apparently stimulated enzyme activity relative to the control. The same increase in enzyme activity was also noted at T_0 when VFAs were added. Madigan et al., (1997) stated that the formation of enzyme increases with increasing time. The enzyme activity was 0.527 nmol/min/mg protein and 0.708 nmol/min/mg protein in samples with only VFAs added at times 0 and 3 hrs, respectively. A statistically significant difference was found between T_0 and T_3 for the VFA amended samples. The samples containing both nitrite and VFAs produced enzyme activities of 0.606 nmol/min/mg protein at T_0 and 0.793 nmol/min/mg protein at T_3 . Statistical analysis confirmed that there was a significant difference between T_0 and T_3 in these samples. Statistical analysis between the four treatments demonstrated that NAP enzyme activity was significantly different in the non amended sample from the amended samples indicating that addition of nitrite and VFAs increased the enzyme activity. Significant difference was also observed between the treatments without VFAs and the treatments containing VFAs after incubation. Additionally, no significant difference was noted between the treatment with only VFAs and the treatment with both nitrite and VFAs. In other words, the presence of VFAs rather than nitrite increased the periplasmic nitrate reductase activity. The addition of nitrite in the presence of VFAs did not make any significant difference in the activity of NAP enzyme. These findings indicated that both

nitrite and VFAs stimulated the activity of the enzyme, but VFAs had a larger affect than nitrite.

The activity of NAP has previously been shown to be 60-fold higher in *P. denitrificans* cultures grown on butyrate (2800 nmol MV oxidized min⁻¹ mg protein⁻¹) than in those grown on malate (45 nmol MV oxidized min⁻¹ mg protein⁻¹) (Sears et al., 1993). Ellington et al., (2003) found that the activity of the periplasmic nitrate reductase of *Paracoccus pantotrophus* was 40 nmol MV oxidized (mg dry weight⁻¹) mg⁻¹ during growth with butyrate and 7.5 nmol MV oxidized (mg dry weight⁻¹) mg⁻¹ during growth with succinate. These activity values are higher than those measured in the current study; however, it should be noted that the literature studies were performed on pure cultures. Additionally, in bacteria, expression of denitrification genes is coordinated (Philippot, 2002). Then an increase in NAP would also increase the nitrite reductase enzyme. Since the activity measurements were based on the accumulated nitrite, removal of nitrite by nitrite reductase would result in apparently lower rates.

The results obtained in this study demonstrate that the activity of periplasmic nitrate reductase can be influenced by VFAs. Thus, it is possible that oxygen insensitive nitrate respiration may make a significant and previously unrecognized contribution to the flux from nitrate to nitrite in aerobic environments. The nitrite produced as a consequence of nitrate reduction (catalyzed by periplasmic nitrate reductase) is presumably further reduced by nitrite-respiring bacteria. If this is the case, the increased NAP enzyme activity in the aerobic activated sludge culture could account for the lower nitrate levels measured in this study when VFAs were present.

4.1.8.2 NAP Enzyme Activity in Mixed Liquor Samples from the Kuwahee Wastewater Treatment Plant

The activity of NAP enzyme was also investigated in mixed liquor samples collected from the full-scale municipal WWTP. It was anticipated that enzyme would be present in the WWTP since the CSTR was seeded with activated sludge from this facility. The experiment was performed to demonstrate that the activity of NAP enzyme can be increased by VFAs and that the potential for aerobic denitrification exists in the full-scale WWTP. Batch experiments described in section 4.1.8.1 were performed to measure NAP activity. Samples were incubated for 5 hr rather than the 3 hr time period initially used to assess NAP activity to better compare results from previous 5 hr batch experiments. Nitrite removal and nitrate formation were also measured such that a possible relationship between enzyme activity and the missing nitrogen in the liquid phase could be developed.

For samples with no amendments, the enzyme activity was initially 0.78 nmol/min/mg protein and increased slightly to 0.88 nmol/min/mg protein after the 5-hr incubation period (Figure 4-27). Similarly, enzyme activities in the nitrite amended samples remained constant over time (0.85 nmol/min/mg protein and 0.88 nmol/min/mg protein, at T_0 and T_5 , respectively). The NAP activity showed an increase from 0.92 nmol/min/mg protein to 1.37 nmol/min/mg protein in samples amended with VFAs. When both nitrite and VFAs were added, the activity of the NAP enzyme increased from 0.99 nmol/min/mg protein to 1.42 nmol/min/mg protein. Statistical analysis showed that there was a significance difference in NAP activity between the initial and final times in the treatments with VFAs only and both nitrite and VFAs added. Additionally, treatments in which VFAs were added differed significantly from those with no VFAs after

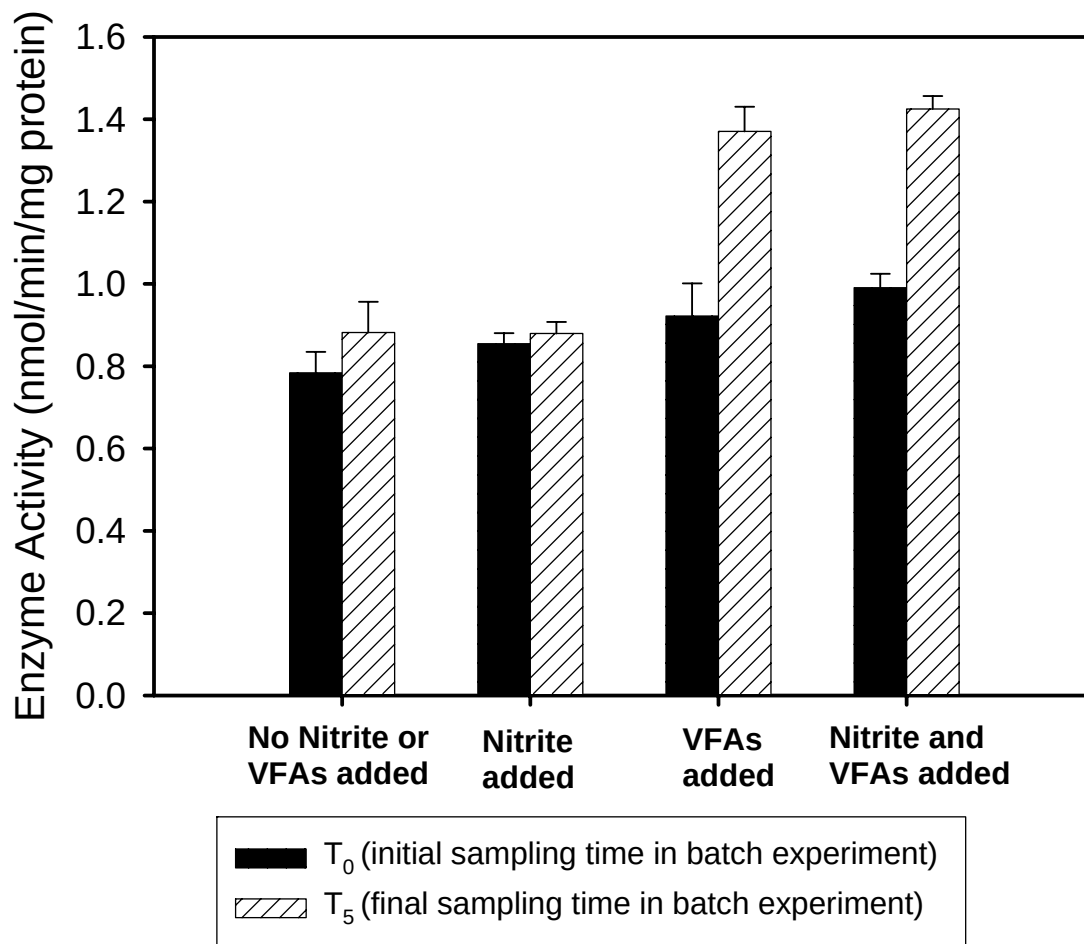


Figure 4-27. The periplasmic nitrate reductase enzyme activity in batch experiment performed using mixed liquor from the Kuwahee WWTP for each treatment and time points (Nitrite was 25 mg-N/L and added VFAs were 3000 mg/L).

incubation. These results clearly show that VFAs enhance the activity of NAP enzyme.

Nitrite and nitrate nitrogen concentrations in the liquid phase were measured at the beginning and at the end of the batch experiment to calculate the missing nitrogen (Table 4-1). In the samples with no added nitrite or VFAs, nitrite concentrations were 0.26 mg-N/L at T_0 and not detectable at T_5 . Nitrate concentrations remained constant during the experiment because there was no oxidizable nitrite in this sample. Thus, the total nitrogen concentration remained constant indicating no nitrogen was lost. In samples amended with 25 mg-N/L, nitrite concentrations were 22.61 mg-N/L and 14.88 mg-N/L at initial and final sampling times, respectively. Nitrate concentrations increased from 4.73 mg-N/L to 12.35 mg-N/L over the 5 hr incubation period. Thus, 7.73 mg-N/L nitrite was removed and 7.62 mg-N/L nitrate was formed. The total nitrogen concentrations were 27.34 mg-N/L and 27.23 mg-N/L showing there was no nitrogen lost. When both nitrite (25 mg-N/L) and VFAs (3000 mg-N/L) were added, the nitrite concentration decreased from 22.8 mg-N/L to 14.34 mg-N/L and the nitrate concentration increased from 4.53 mg-N/L to 5.89 mg-N/L. Although 8.46 mg-N/L nitrite was removed from the system, only 1.36 mg-N/L of nitrate was formed indicating 7.1 mg-N/L of nitrogen was lost. When only VFAs (3000 mg-N/L) were added, nitrite concentration dropped from an initial 0.44 mg-N/L to undetectable after 5 hrs. The nitrate concentration was reduced from 4.19 mg-N/L to 1.26 mg-N/L, a 2.93 mg-N/L decrease. Due to a lack of nitrite in this sample, nitrate did not accumulate via oxidation of nitrite. Nitrate reduction may have occurred due to VFA stimulation of the aerobic denitrification process. The possibility of organic nitrate assimilation was ruled out since there was no increase in the

Table 4-1. Summary of nitrite and nitrate concentrations measured in the liquid phase in the beginning and at the end of the batch experiment performed using mixed liquor samples from the Kuwahee WWTP

Sample Name	Time (hours)	Nitrite-N concentration (mg/L)	Nitrate-N concentration (mg/L)	Total Nitrogen^(a) (mg/L)	Missing Nitrogen^(b) (mg/L)
without nitrite addition	0	0.26	4.67	4.93	0
	5	0	4.99	4.99	
with 25 mg-N/L nitrite addition	0	22.61	4.73	27.34	0
	5	14.88	12.35	27.23	
with 25 mg-N/L nitrite and 3000 mg/L VFAs addition	0	22.80	4.53	27.33	7.10
	5	14.34	5.89	20.23	
With 3000 mg/L of VFAs addition	0	0.44	4.19	4.62	3.37
	5	0	1.26	1.26	

(a) Calculated by adding nitrite-N and nitrate-N concentrations for each time point

(b) Calculated by subtracting total nitrogen at the beginning from total nitrogen at the end of the batch experiment

protein content for this sample (625 µg/ml at T₀ and 660 µg/ml at T₅).

Based on the enzyme activity measurements, the amount of nitrate reduced in a 5-hr batch experiment could be predicted. During the enzyme assay, nitrite-N was produced from the nitrate-N added to the reaction mixture; therefore, the amount of nitrite-N produced should equal the amount of nitrate-N reduced. Based on this relationship, the amount of nitrate which would have been reduced during a 5-hr batch experiment was predicted. The missing nitrogen values derived in Table 4-1 were compared to the predicted nitrate concentrations (Table 4-2). The idea was to predict how much of the missing nitrogen in the liquid phase could be attributed to nitrate reduced by the NAP enzyme. These calculations were performed using the samples with only VFAs added and both VFAs and nitrite added at T₅ because nitrogen was lost in the liquid phase in these two batch samples. It was found that 2.93 mg-N/L nitrate would have been reduced over 5 hrs in the sample with only VFAs added. In the same sample, 3.37 mg-N/L nitrogen was lost from the liquid phase during the 5 hr incubation period. Therefore, 87% of the missing nitrogen in the liquid phase during the batch experiment may have been reduced from nitrate to nitrite and further to gaseous nitrogen products. Similarly, in the sample with both nitrite and VFAs added, the missing nitrogen was 7.10 mg-N/L and the estimated amount of nitrate reduced based on the enzyme assay over 5 hrs was 3.86 mg-N/L which corresponds to 55% of the missing nitrogen. Sears et al., (1997) studied the activity of periplasmic nitrate reductase in *Paracoccus denitrificans* grown on butyrate and found that nitrite reductase reduced most of the nitrite produced by the nitrate reductase. So, this may be the reason that only 55% of missing nitrogen was recovered since the nitrite produced during the enzyme assay may have been converted to gaseous

Table 4-2. Comparison of nitrate consumption in the enzyme assays with nitrite removal-nitrate formation batch experiments

Sample	Measured nitrite production (nitrate reduction) in the enzyme assay (mg-N/L) ^(a)	Predicted nitrite production (nitrate reduction) (mg-N/L) ^(b)	Missing nitrogen mg-N/L/ in batch experiment ^(c) mg-N/L	% of nitrogen attributed to nitrate reduction in the batch experiment
T ₅ VFA	0.44	2.93	3.37	87
T ₅ VFA,N	0.58	3.86	7.10	58

(a) Measured amount of nitrite produced during the 45-min enzyme assay

(b) Predicted amount of nitrite which would have been produced during 5 hr period

(c) Missing nitrogen concentrations in the liquid phase during the batch experiment derived from Table 4-1

nitrogen compounds. Although predicted amounts of nitrite production were close in both samples, more nitrogen was lost in the sample in which both VFAs and nitrite were added. The additional nitrogen loss was likely due to the nitrite concentration differences between the two samples.

It appears that when VFAs are present, periplasmic nitrate reductase activity increases, thereby increasing the rate of conversion of nitrate to nitrite. Such a reaction would result in the apparent reduction in nitrate formation measured in the liquid phase during the batch experiments.

4.1.9 Inhibition of Periplasmic Nitrate Reductase (NAP)

Batch experiments were performed in the presence of 3000 mg/L of VFAs and with either SCN (10 mM) or CN (10 μ M) to inhibit the aerobic denitrification suspected

to occur in the presence of VFAs. SCN and CN were expected to inhibit only aerobic denitrification when used with VFAs. Under this scenario, nitrate would no longer be reduced to nitrite and the ratio of nitrite removal to nitrate formation should return to 1:1. Additionally, when the SCN or CN inhibitor was used alone (no VFAs), the rates should remain similar to those in the control experiments. Unfortunately, even small amounts of SCN and CN inhibited nitrification. The nitrite and nitrate concentrations remain constant over the 5-hr period when these inhibitors were added essentially acting like killed controls (Appendix III). Thus, the expected results were not observed and these experiments did not confirm the presence of an aerobic denitrification pathway.

4.2 CONTINUOUS-STIRRED TANK REACTOR EXPERIMENT

A continuous-stirred tank reactor (CSTR) experiment was performed to investigate the impact of VFAs on nitrite removal efficiency and to determine if nitrite was removed from wastewater via aerobic denitrification. A 3000 mg/L mixture of VFAs (1000 mg/L acetate, 1000 mg/L propionate, and 1000 mg/L butyrate) was spiked directly into the CSTR. The pH of the VFAs mixture was adjusted to 7.5 using 3 M sodium hydroxide (NaOH) before the VFAs were added to the CSTR. The pH of the CSTR remained near 7.5 during the experiment so the pH control system was never activated. Subsamples from the CSTR were taken immediately after spiking, every 2 hours during the first six hours and after 24 hours to examine the changes in nitrite removal, nitrate formation, CO₂ fixation and on the number of total bacteria and *Nitrospira* over time. Additionally, the activity of the periplasmic nitrate reductase and the presence of *nap*

gene were measured. A control sample was collected before addition of VFAs to determine nitrite removal, nitrate formation and CO₂ fixation in the absence of VFAs.

Batch experiments, described in section 4.1, were performed on each CSTR subsample to determine nitrite removal, nitrate formation and CO₂ fixation. A diagram outlining the procedure followed for the CSTR experiment appears in Figure 4-28. The nitrite removal and nitrate formation rates were calculated from nitrite and nitrate concentrations measured over time during each 5 hr batch experiment. The CO₂ fixation rates were determined from the amounts of radiolabelled carbon incorporated into bacterial biomass over the 5-hr incubation period. The number of total bacteria, *Nitrospira*, and activity of the periplasmic nitrate reductase enzyme were measured in the control sample and in CSTR samples collected over time after addition of VFAs. The presence of the *nap* gene was assayed in samples initially removed from the CSTR and in samples exposed to VFAs for 24 hours.

4.2.1 Effect of VFAs on Nitrite Oxidation and CO₂ Fixation in the CSTR

4.2.1.1 Effect of VFAs on Nitrite Removal and Nitrate Formation

The changes in nitrite concentrations over the 5 hour incubation period for each CSTR sample were plotted in Figure 4-29. The extent of the nitrite removal reaction can be assessed by comparison of data obtained after 5 hours of incubation. Addition of VFAs to the CSTR increased the amount of nitrite removed during batch testing. In mixed liquor samples collected from the CSTR before VFA addition and immediately after VFA addition, nitrite removal was similar, 12.55 mg-N/L and 10.20 mg-N/L, respectively. However, the amount of nitrite removed increased to 16.03 mg-N/L, 17.39

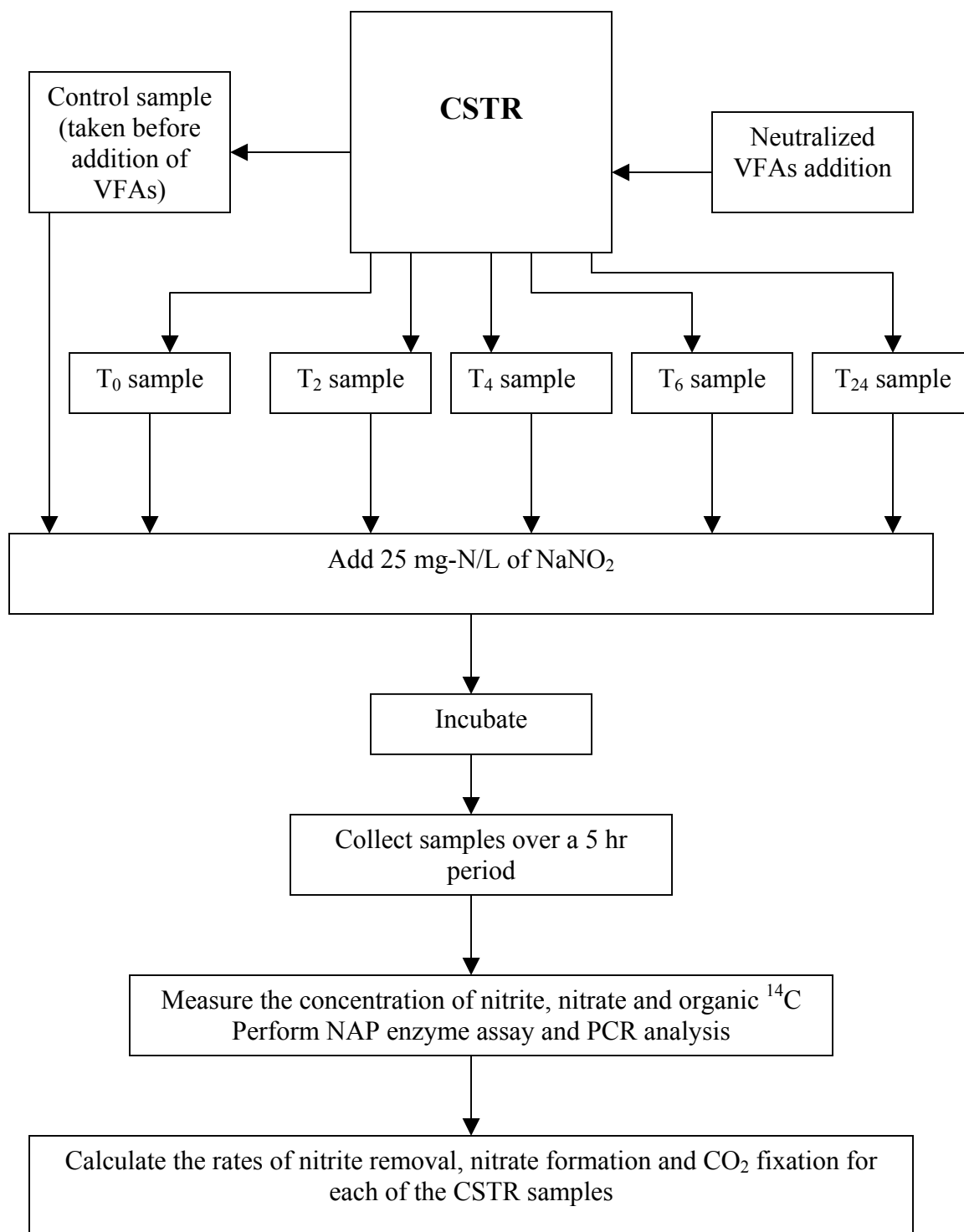


Figure 4-28. A schematic diagram describing the continuous flow reactor experiment during VFAs exposure.

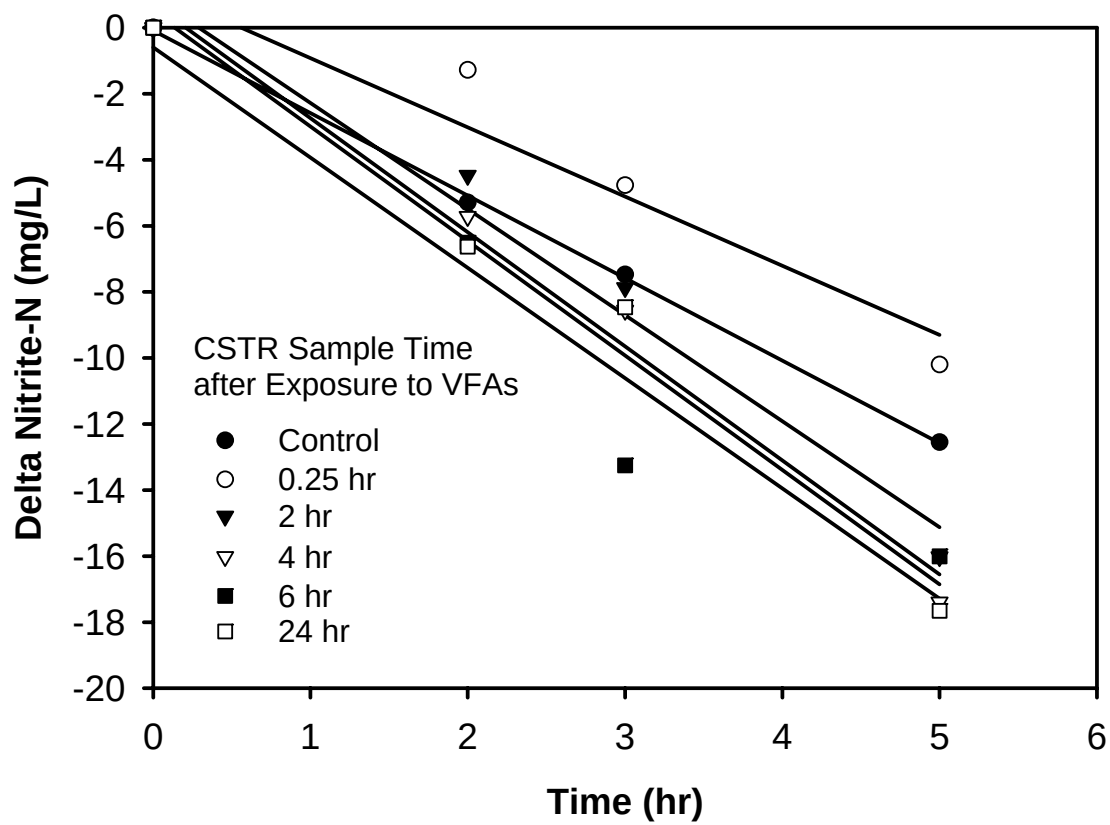


Figure 4-29. Changes in nitrite concentrations over a 5 hour period for each CSTR sample exposed to 3000 mg/L of VFAs

mg-N/L, 16 mg-N/L and 17.66 mg-N/L in batch samples taken 2, 4, 6 and 24 hours after VFAs addition, respectively. Nitrite removal increased in samples exposed to VFAs for more than 2 hrs clearly showing that nitrite oxidation was not reduced by the presence of VFAs in the CSTR.

Before addition of VFAs, the nitrite removal rate for CSTR samples was 2.49 mg-N/L-hr. After addition of VFAs, the nitrite removal rate dropped to 2.09 mg-N/L-hr, a decrease of 16%. Rates increased to 3.21 mg-N/L-hr, 3.45 mg-N/L-hr, 3.34 mg-N/L-hr and 3.47 mg-N/L-hr, at 2, 4, 6 and 24 hours after VFAs addition, respectively. The initial reduction of the nitrite removal rate ceased after 2 hr of exposure and, in fact, increased 29-40% over the 24-hr VFA exposure period. Statistical analysis indicated that there was no significant difference between nitrite removal rates demonstrating that nitrite removal was not reduced. These results differed from those obtained in the batch test in which nitrite removal was reduced by 37%. Although similar nitrite removal rates were obtained for the control samples in both batch and CSTR experiments (2.91 mg-N/L-hr and 2.49 mg-N/L, respectively), the rates of nitrite removal in the presence of VFAs were different in batch and CSTR experiments (0.77 mg-N/L-hr and 3.37 mg-N/L, respectively). This dissimilarity could result from the difference in the method of addition of VFAs. In the CSTR experiment, VFAs were spiked directly in the CSTR and batch assays were run at 2 to 24 hrs after addition at which VFA concentration was diluted. On the contrary, VFAs were added directly into vials during batch experiments.

In contrast to the effect of VFAs on nitrite removal, addition of VFAs reduced nitrate formation in the CSTR (Figure 4-30). In mixed liquor samples taken from the CSTR before addition of VFAs, 12.02 mg-N/L of nitrate had accumulated. However,

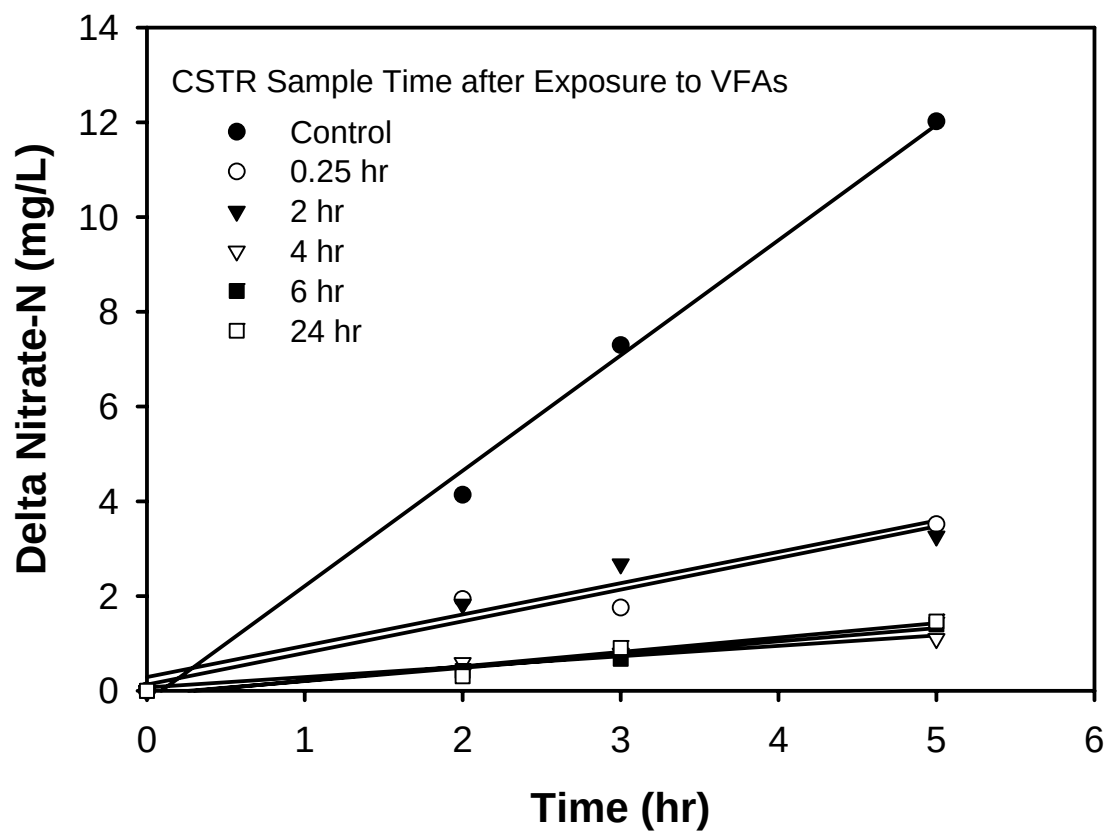


Figure 4-30. Changes in nitrate concentrations over a 5 hour incubation period for each CSTR sample exposed to 3000 mg/L of VFAs

only 3.51 mg-N/L and 3.26 mg-N/L of nitrate was formed, at 0.25 hr and 2 hr after VFAs addition, respectively. The amount of nitrate formed continued to drop to 1.10 mg-N/L, 1.39 mg-N/L and 1.46 mg-N/L in 4 hr, 6 hr and 24 hr VFA exposure periods, respectively, suggesting that the extent of nitrate formation was reduced by the presence of VFAs in the CSTR. This impact on nitrate formation was also evident in the rate reduction from 2.43 mg-N/L-hr before VFAs addition to 0.67 mg-N/L-hr representing 73% decrease in the CSTR after addition of VFAs. The nitrate formation rate for CSTR mixed liquor samples exposed to VFAs for 2 hours was similar to that measured immediately after VFAs addition (0.66 mg-N/L-hr). Nitrate formation rates in CSTR samples exposed to VFAs for 4, 6 and 24 hr decreased to 0.22 mg-N/L-hr, 0.28 mg-N/L-hr and 0.30 mg-N/L-hr, respectively. In general, the nitrate formation rate (based on measured NO_3^-) was reduced by at least 85% in mixed liquor samples exposed to VFAs for more than 4 hours. Statistical analysis of the nitrate data demonstrated that the rate of nitrate formation was significantly reduced by the VFAs over the duration of the 24 hr exposure experiment. Reduction of nitrate formation in the CSTR was similar to that in the batch experiments in which a 74% of reduction in nitrate formation was observed over 5 hrs.

Before addition of VFAs, the amount of nitrite removed was equal to the amount of nitrate formed in the CSTR. However, upon VFA addition, all nitrite was not converted to nitrate. Although more nitrite was consumed after the addition of VFAs, lesser amounts of nitrate accumulated. A significant amount of nitrogen was converted to a form other than the measured chemical species (nitrite and nitrate).

During normal operation of the CSTR, effluent nitrite concentrations were between 0.5 mg-N/L and 0.9 mg-N/L while effluent nitrate concentrations ranged from 6 mg-N/L to 25 mg-N/L (See Appendix II). At 4 hr after addition of VFAs into the CSTR, neither nitrite nor nitrate was detected in the effluent indicating complete nitrogen removal from the aqueous phase of the wastewater. Although nitrite and nitrate levels were below detection limits within hours after VFA addition, concentrations in the CSTR returned to normal effluent levels after 48 hrs. The impact of VFAs on activated sludge with an SRT of 10 days appeared to be short lived.

4.2.1.2 Effect of VFAs on CO₂ Fixation

The impact of VFAs on CO₂ fixation by CSTR mixed liquor samples was also investigated due to the linkage of CO₂ fixation and nitrite oxidation in NOB. The amount of CO₂ fixed before VFAs addition was 17.74 µg/L whereas levels fixed after VFAs addition were 24.59 µg/L, 31.95 µg/L, 31.58 µg/L, 34.28 µg/L and 16.95 µg/L at 0.25, 2, 4, 6 and 24 hours, respectively (Figure 4-31). More CO₂ was fixed in the presence of VFAs than in the control; however, CO₂ fixation returned to background level 24 hr after exposure.

The rate of CO₂ fixation was 3.56 µg/L-hr prior to VFA addition and 4.89 µg/L-hr after VFAs were initially added. The rate of CO₂ uptake increased 2, 4 and 6 hrs after VFA exposure to approximately 6.4 µg/L-hr. After 24 hrs, the CO₂ fixation rate returned to background levels (3.34 µg/L-hr). These results showed that CO₂ fixation rates were not reduced but rather stimulated (up to 90%) by the one time addition of VFAs. Statistical analysis also indicated that the CO₂ fixation rates at 2, 4 and 6 hrs after VFA

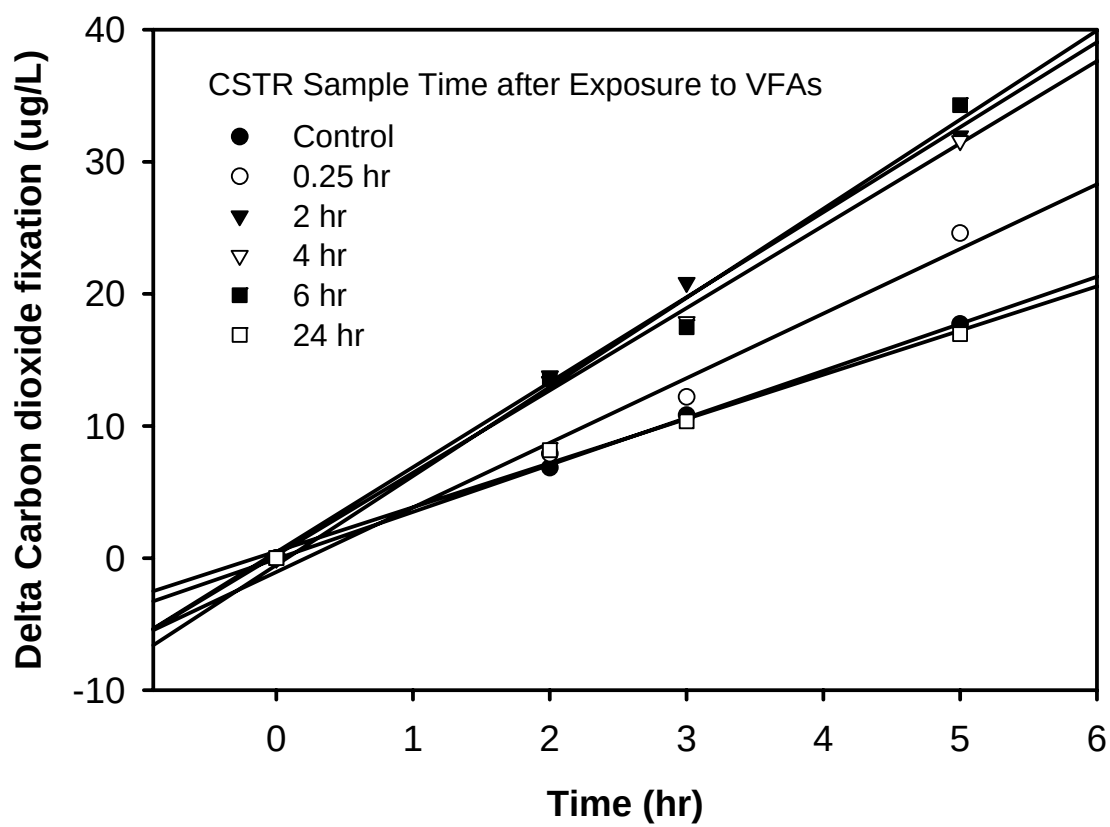


Figure 4-31. Changes in carbon dioxide fixation over a 5 hour incubation period for each CSTR sample exposed to 3000 mg/L of VFAs

addition were significantly different from the control rate. Shively et al. (1998) found that a reduced metabolite originating from the oxidation of reduced compounds is most likely responsible for the induction of the Calvin cycle. For example, the activity of the Calvin cycle is higher during growth on butyrate than on the less- reduced substrate malate. Therefore, the increase in CO₂ fixation after exposure to VFAs may be due to the same induction potential as that noted for butyrate.

The behavior of activated sludge after exposure to VFAs was different in batch than in the CSTR cultures. In the former, the nitrite removal rate was negatively affected (37% reduction) whereas in the latter, the nitrite removal rate increased after VFA exposure (Table 4-3). In contrast, nitrate formation rates were more negatively affected by the presence of VFAs in the CSTR experiment (82% reduction) than in the batch experiments (74% reduction). CO₂ fixation rates were higher in the CSTR experiment than in the batch experiments in the presence of VFAs. Figure 4-32 depicts the relative ratio of reduced rate to control rate for nitrite removal, nitrate formation and CO₂ fixation in the CSTR experiment. This figure clearly shows that although nitrate formation rates were reduced by VFAs, nitrite removal and CO₂ fixation rates, in general, were not. In contrast, the removal of nitrite and the fixation of CO₂ increased in the presence of VFAs.

The results obtained from the CSTR experiment were different from those found in the literature. To date, only one study was conducted which evaluated the effects of VFAs on a CSTR (Gomez et al., 2000). The addition of 750-3000 mg/L VFA pulses (at a ratio of 4:1:1) over 14 hr into a CSTR indicated that no inhibition of the nitrification process occurred. The authors found that the rate of nitrate formation remained constant with or without VFAs. These results differ from those obtained in this study in which

Table 4-3. Summary of nitrite removal, nitrate formation and CO₂ fixation rates in batch and CSTR experiments (VFAs were 3000 mg/L)

	Nitrite Removal		Nitrate Formation		CO ₂ Fixation	
	Batch	CSTR	Batch	CSTR	Batch	CSTR
^a Rates in the absence of VFAs (controls) (mg/L-hr)	2.94 ± 0.78	2.48	2.91 ± 0.87	2.43	4.03 ± (0.04) x 10 ⁻³	3.56 x 10 ⁻³
^b Rates in the presence of 3000 mg/L of VFAs (mg/L-hr)	1.83 ± 0.55	3.08 ± 0.56	0.77 ± 0.23	0.44 ± 0.21	4.75 ± (0.47) x 10 ⁻³	6.08 ± (1.41) x 10 ⁻³
% Reduction	37	-24	74	82	-18	-71

a: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite without VFAs

b: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite and 3000 mg/L of VFAs

c: Negative values reflect an increase

d: Calculated using the following equation: $\{(\text{control rate} - \text{reduced rate}) / \text{control rate}\} \times 100$

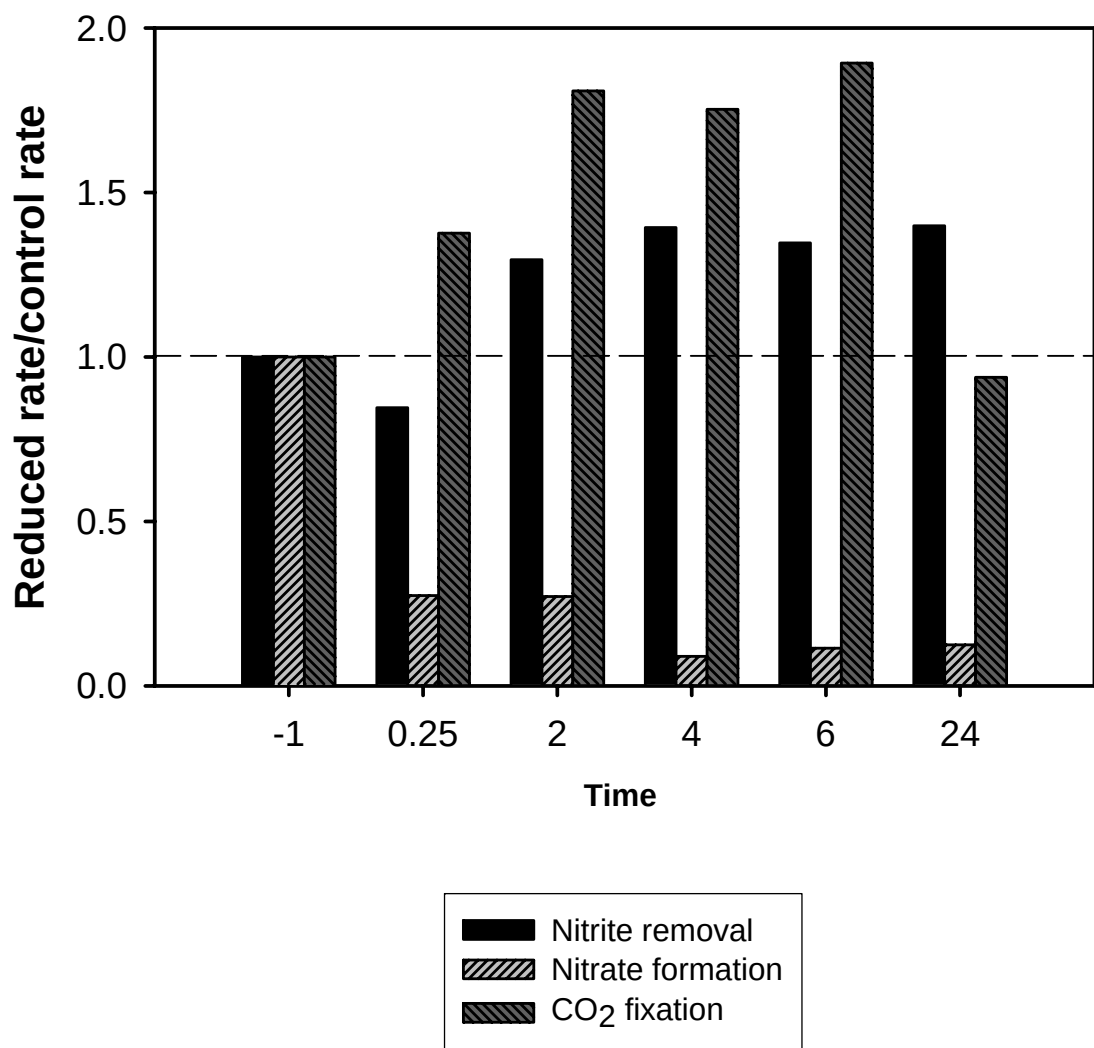


Figure 4-32. Relative ratio of reduced rates/control rates for nitrite removal, nitrate formation and CO₂ fixation after addition of 3000 mg/L VFAs into the CSTR. A ratio of one indicates no reduction whereas ratios below one indicate reduction and ratios above one indicate stimulation of rates.

reduction in nitrate formation was observed in the presence of VFAs. Gomez et al. (2000) feed the CSTR using a synthetic influent which contained no organic carbon other than the VFAs. In this study, municipal wastewater served as the organic carbon source; therefore, it is likely that the microbial population which developed in each reactor differed considerably. It may be that an aerobic denitrification population did not develop in the Gomez et al. (2000) study. Additionally, Gomez et al. (2000) only added 780 mg VFAs/day whereas 3000 mg VFAs/day were added in this study. The authors suggested that the noninhibitory effect of VFAs on the nitrification efficiency in their system might be related to the feeding pattern.

4.2.2 NAP Enzyme Activity in Mixed Liquor Samples during Exposure to VFAs in the CSTR Experiment

NAP activity was also measured in the CSTR experiment to investigate if the addition of VFAs stimulated the activity of this enzyme in activated sludge and, as a result, enhanced aerobic denitrification. NAP enzyme activity was assayed in mixed liquor samples obtained directly from the CSTR before and after addition of VFAs. Biomass samples were added to a methylviologen reaction mixture and incubated for 45 min at 37°C. The supernatant of centrifuged samples was assayed for nitrate reductase activity by measuring the appearance of nitrite as described in Chapter 3 (Lopez-Lozano et al., 2002) (Figure 4-33). Total protein concentration in mixed liquor samples was also measured.

The periplasmic nitrate reductase enzyme activity increased from 0.37 nmol/min/mg protein (prior to VFAs addition) to 2.09 nmol/min/mg protein (6 hr after

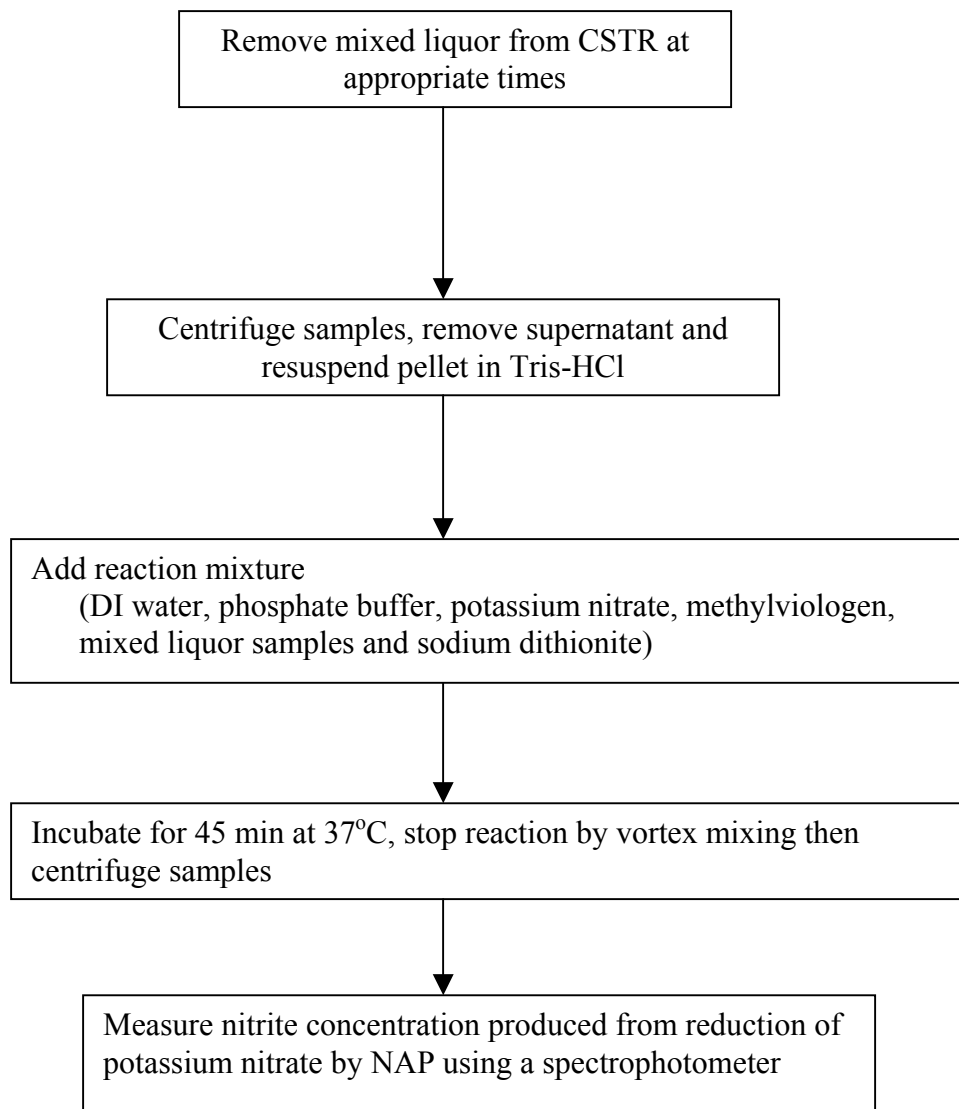


Figure 4-33. Schematic diagram showing the steps performed in the NAP enzyme assay in the CSTR experiment with 3000 mg/L of VFAs

VFAs addition) (Figure 4-34). Enzyme levels dropped over time to 0.85 nmol/min/mg protein after 48 hrs of VFA exposure. The five fold increase after 6 hrs clearly shows enhanced enzyme activity resulting from VFA exposure. Statistical analysis confirmed the significant difference between pre VFA contact and 6-24 hr exposure.

An attempt was made to determine if there was a relationship between enzyme activity and missing nitrogen in the CSTR experiment discussed in Section 4.2.1.1. Nitrite and nitrate concentrations were measured over time and total $\{\Sigma (\text{NO}_3^- + \text{NO}_2^-)\}$ and missing nitrogen levels were calculated (Table 4-4). Before addition of VFAs into the CSTR, total nitrogen concentrations remained constant throughout the experiment. After addition of VFAs, total nitrogen levels decreased in all samples collected over time. The amount of missing nitrogen increased with increasing VFAs contact time. As much as 16 mg-N/L of the 21 mg-N/L initially present could not be detected in CSTR samples exposed to VFAs for 24 hours. Enzyme activity measured in the 24 hr sample was elevated by a factor of five over that without VFA addition (Figure 4-34).

Based on the enzyme activity measurements, the amount of nitrate which would have been reduced in a 5-hr batch experiment was determined. The missing nitrogen values derived in Table 4-4 were compared to the predicted nitrate concentrations (Table 4-5). It was found that 34% to 45% of the unaccounted nitrogen may have been converted from nitrate to nitrite by periplasmic nitrate reductase. The nitrite may have subsequently been transformed to other products such as NO, N₂O or N₂. Since only nitrite (produced from nitrate) can be measured in the enzyme activity assay, alternative end products were not quantified. Because nitrite reductase is also active when NAP enzyme is stimulated by butyrate (Sears et al., 1997), nitrite may have been lost during the enzyme assay

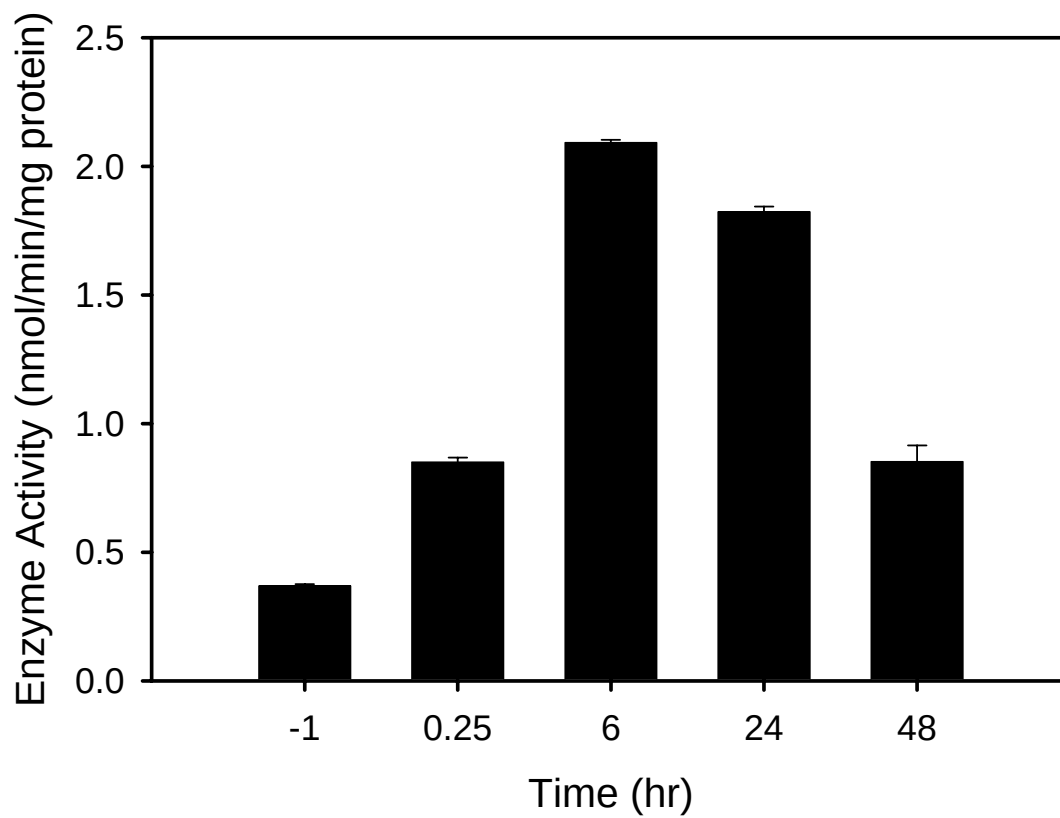


Figure 4-34. The periplasmic nitrate reductase enzyme activity for each CSTR sample exposed to 3000 mg/L of VFAs

Table 4-4. Summary of nitrite and nitrate concentrations measured over time in the continuous-flow reactor VFA inhibition experiment.

Sample ^(a)	Time (hours)	Nitrite-N concentration (mg/L)	Nitrate-N concentration (mg/L)	Total Nitrogen ^(b) (mg/L)	Missing Nitrogen ^(c) (mg/L)
-1	0	23.79	7.46	31.25	0
	2	18.5	11.60	30.1	
	3	16.31	14.76	31.07	
	5	11.23	19.48	30.71	
0.25 hr	0	24.03	5.63	29.66	6.69
	2	22.75	7.56	30.31	
	3	19.26	7.38	26.64	
	5	13.83	9.14	22.97	
6 hr	0	23.01	0.33	23.34	14.62
	2	16.45	0.75	17.2	
	3	9.75	1.00	10.75	
	5	6.99	1.73	8.72	
24 hr	0	20.87	0.2	21.07	16.19
	2	14.24	0.51	14.75	
	3	12.40	1.10	13.50	
	5	3.21	1.66	4.87	

(a) Hours before or after addition of 3000 mg/L of VFAs

(b) Calculated by adding nitrite-N and nitrate-N concentrations at each time point

(c) Calculated by subtracting total nitrogen at the beginning from total nitrogen at the end of the batch experiment

Table 4-5. Comparison of nitrate consumption in NAP enzyme assay and in the continuous-flow reactor VFA inhibition experiment

Sample	Measured nitrite production (nitrate reduction) in the enzyme assay (mg-N/L)^(a)	Predicted nitrite production (nitrate reduction) (mg-N/L)^(b)	Missing Nitrogen mg-N/L/ in CSTR experiment ^(c) (mg/L)	% of Nitrogen attributed to Nitrate reduction by NAP
0 hr	0.360	2.70	6.69	40
6 hr	0.884	6.63	14.6	45
24 hr	0.773	5.79	16.9	34.3
48 hr	0.361	2.71	N/A	N/A

(a) Measured amount of nitrite produced during the 45-min enzyme assay

(b) Predicted amount of nitrite which would have been produced during 5 hr period

(c) Missing nitrogen concentrations in the liquid phase during the batch experiment derived from Table 4-4

resulting in an under estimation of NAP activity.

4.2.3 Total Bacteria and *Nitrospira* Concentrations

Genetic analyses were performed to investigate the impact of VFAs on the bacterial content of the CSTR. Total bacterial concentration was used to determine if VFAs increased biomass content because of the addition of carbon in the CSTR. Additionally, *Nitrospira* concentrations (as a measure of NOB) were measured to determine if the decline in nitrate formation in the presence of VFAs was due to a numerical reduction of *Nitrospira*.

Real-time PCR assays were used to quantify eubacterial 16S rRNA genes and *Nitrospira* spp. 16S rRNA genes in mixed liquor samples collected from the reactor before and after addition of VFAs. Real-time PCR data was collected as the number of gene copies in a sample. The real-time PCR data for total bacteria was converted to a cell per liter basis assuming a value of 3.6 16S rRNA gene copies per cell (Harms et al., 2003). Since the number of *Nitrospira* 16S rRNA copies per cell is unknown, one copy was assumed based on the average 16S rRNA gene copies per cell in other nitrifying bacteria, such as *Nitrosomonas* and *Nitrobacter* (Harms et al., 2003).

The total bacterial cell densities were $1.17 (\pm 1.85) \times 10^{12}$ cells/L before the addition of VFAs, and $1.09 (\pm 0.9) \times 10^{12}$, $1.04 (\pm 0.65) \times 10^{12}$, $1.75 (\pm 1.87) \times 10^{12}$ and $1.90 (\pm 1.96) \times 10^{12}$ cells/L at 0.25, 6, 24 and 48 hrs after VFAs addition, respectively (Figure 4-35). These values are similar to those reported by Harms et al (2003) for the activated sludge used to seed the CSTR. Based on ANOVA analysis, differences between total bacteria concentrations in each set of samples were not significant ($p = 0.05$). Since

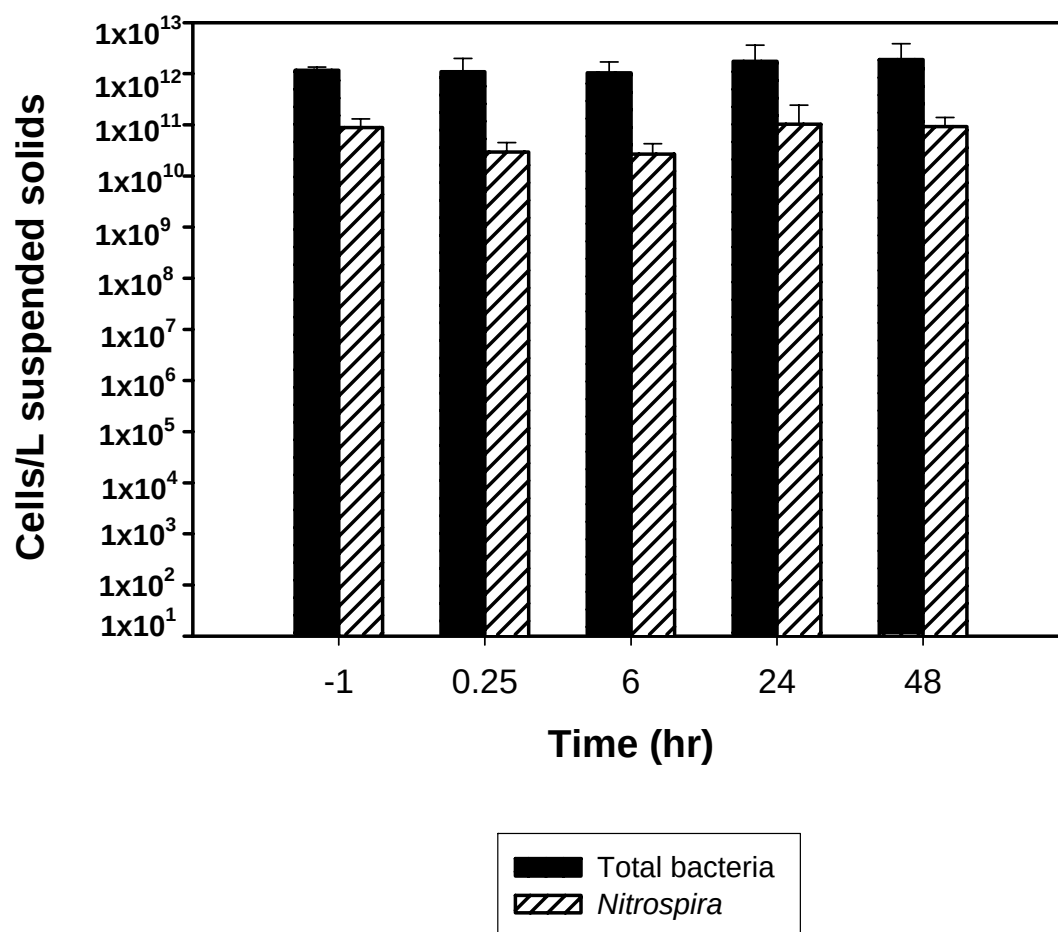


Figure 4-35. Number of total bacteria and *Nitrospira* before and after a single addition of 3000 mg/L of VFAs into the CSTR.

the number of total bacteria remained relatively constant, the reduction in nitrate formation noted was probably not due to consumption by heterotrophic bacteria. An increase in heterotrophic growth was expected when VFAs were spiked into the reactor due to the increase in carbon; however, this was not the case. Richardson et al. (2001) reported that reduced carbon sources, such as propionate and butyrate, were not used for growth by aerobic denitrifying bacteria but were utilized as a redox valve. This mechanism may also control VFA utilization in this study.

Results of this study indicated that *Nitrospira* were present at high levels throughout the reactor experiment with VFAs. Concentrations were measured as $8.8 (\pm 4.2) \times 10^{10}$ cells/L before the addition of VFAs, and $2.9 (\pm 1.6) \times 10^{10}$, $2.67 (\pm 1.6) \times 10^{10}$, $1.03 (\pm 1.4) \times 10^{11}$ and $9.19 (\pm 4.7) \times 10^{10}$ cells/L at 0.25, 6, 24 and 48 hrs after VFAs addition, respectively (Figure 4-35). These values are similar to those reported by Harms et al. (2003) (3.7×10^{10} cells/L) and Yuan and Blackall (2002) (3×10^9 cells/L). Based on ANOVA analyses, differences between the numbers of *Nitrospira* as a function of VFA contact time in each set of samples were not significant ($p = 0.05$) indicating little, if any, growth occurred.

4.2.4 Detection of Genes for Periplasmic Nitrate Reductase (NAP)

The presence of the periplasmic nitrate reductase gene in the CSTR bacterial community was assayed to determine whether the heterotrophic bacteria had the genetic potential to utilize nitrite as an electron acceptor via aerobic denitrification. A nested PCR approach for the amplification of a specific *napA* sequence was used to analyze DNA extracted from the mixed liquor. This analysis was performed in samples removed

from the reactor before the addition of VFAs (T_0) and 24 hrs after the addition of VFAs (T_{24}).

PCR products were visualized by gel electrophoresis. The predicted size of this product, based on the sequence of *P. denitrificans napA* was 430 bp (McDevitt et al., 2000). A PCR product corresponding approximately to the predicted size of the *napA* PCR product was observed in both T_0 and T_{24} samples in reaction 2 (Figure 4-36). These products were cloned into *E.Coli* and individual plasmid preps yielded inserts of two different sizes, 350 and 450 bp (Figures 4-37 and 4-38). The inserts from 5 plasmids from each of the T_0 and T_{24} libraries were sequenced and compared with those in a publicly accessible database using the program basic local alignment search tool (BLAST) (Table 4-6). All sequences had homology to NAP sequences contained in heterotrophs. The DNA sequences were also translated to putative amino acid sequences and had 74% to 83% identities to the *napA* protein (Table 4-6). The dendrogram derived from the DNA or amino acid sequence alignment indicated that five sequences were closely related to one another and to *M. magnetotacticum* and five other sequences were more closely related to each other and to *A. brasilense* (Figure 4-39). The identification of *napA* genes in mixed liquor samples indicated that bacteria with the potential for aerobic denitrification were present in the CSTR. Over the last decade, the ability to respire using nitrate as an electron acceptor under aerobic conditions has been found to be widespread among bacteria. Carter et al., (1995) found that 10^4 to 10^7 bacteria per gram of soil and sediment were able to respire with nitrate under aerobic conditions. In another study, approximately 70% of 1000 heterotrophic isolates obtained from a nitrite-oxidizing reactor were able to express periplasmic nitrate reductase (McDevitt et al., 2000). Thus, it

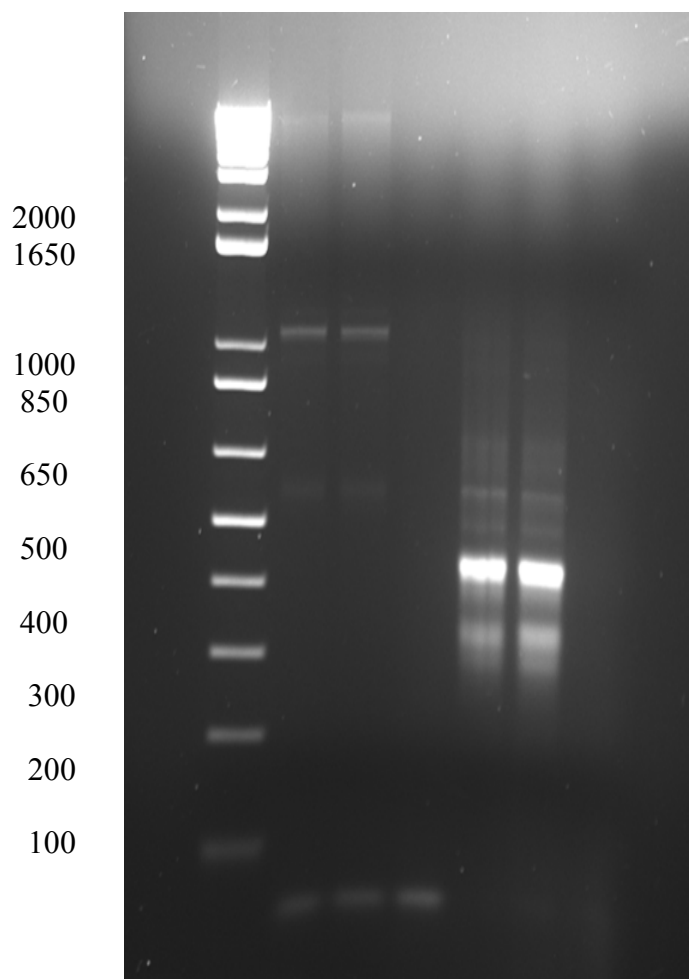


Figure 4-36. Products arising from two-rounds of PCR amplification of the *napA* gene on a 1 % low EEO Agarose gel. Lane 1 is the 1 kb ladder; lane 2, T₀ rxn 1; lane 3, T₂₄, rxn 1; lane 4, negative control, rxn 1; lane 5, T₀, rxn 2; lane 6, T₂₄, rxn 2; lane 7, negative control, rxn 2

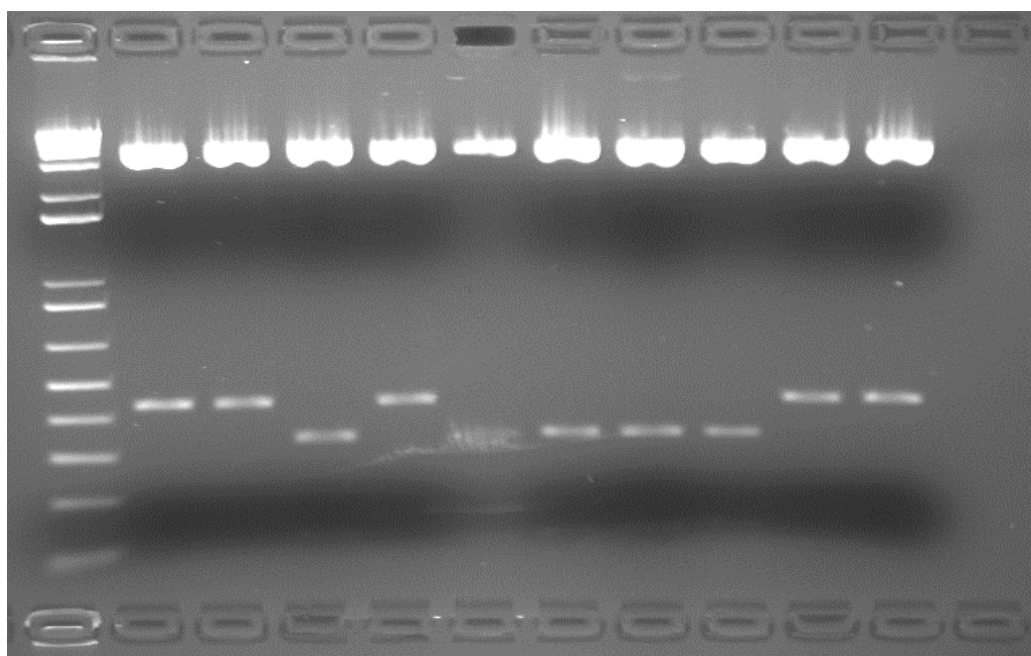


Figure 4-37. EcoRI digests of plasmids second reaction PCR products from the T_0 sample on a 1.5 % EEO agarose gel in 0.5X TBE. First lane is the 1 kb ladder; lane 2 to lane 11 ranges from plasmids numbers 1 to 10

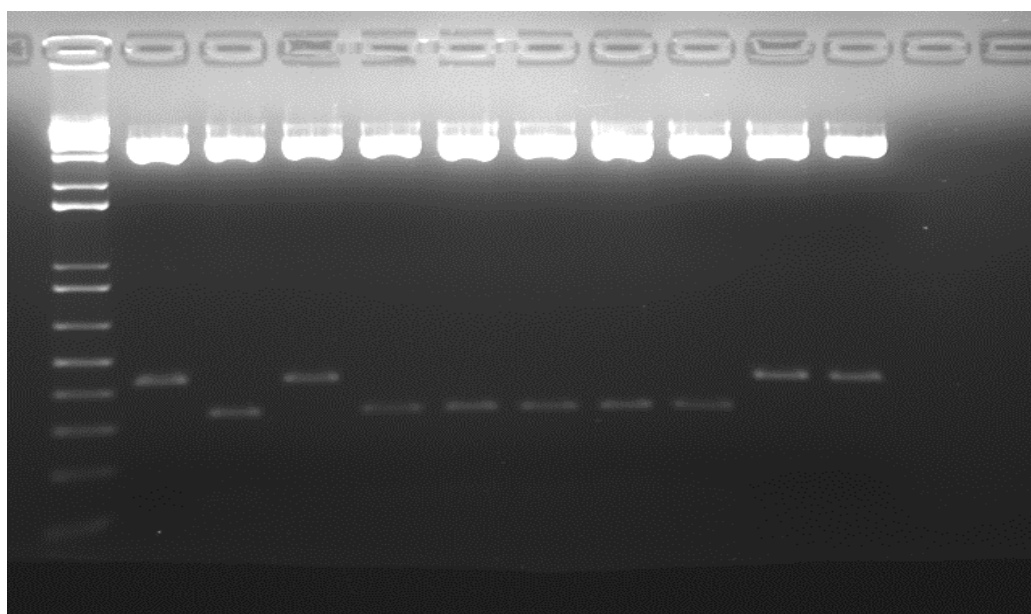


Figure 4-38. EcoR1 digests of plasmids of second reaction PCR products from the T24 sample on a 1.5 % EEO agarose gel in 0.5X TBE. First lane is the 1 kb ladder; lane 2 to lane 11 ranges from plasmids numbers 1 to 10

Table 4-6. Identification of plasmids from the bench-scale reactor by BLAST

Clone Name	Best Match Nucleotide	Periplasmic Nitrate Reductase Genes	Best Match Translated Protein Blastx
T0-2-1	AF245096 <i>Azospirillum brasilense</i> 87 % forward	Periplasmic nitrate reductase large subunit (<i>napA</i>) gene, partial cds; Periplasmic nitrate reductase small diheme cytochrome c subunit (<i>napB</i>); Membrane-anchored tetraheme c-type cytochrome (<i>napC</i>) genes, complete cds	CAA06231.1 <i>Rhodopseudomonas</i> sp. Identities 83 % Positives 90 %
T0-2-2	AJ004933.1 <i>Rhodopseudomonas</i> sp. 87 % forward	<i>napE</i> , <i>napD</i> & <i>napA</i> genes of <i>nap</i> operon, 5' region	AAG31648.2 / AF314590.2 <i>Bradyrhizobium japonicum</i> Identities 74 % Positives 81 %
T0-2-3	AF245096 <i>Azospirillum brasilense</i> 84 % forward	Periplasmic nitrate reductase large subunit (<i>napA</i>) gene, partial cds; Periplasmic nitrate reductase small diheme cytochrome c subunit (<i>napB</i>); Membrane-anchored tetraheme c-type cytochrome (<i>napC</i>) genes, complete cds	BAB59022.1 <i>Magnetospirillum magnetotacticum</i> Identities 82 % Positives 90 %
T0-2-4	X71385.1 <i>A. eutrophus</i> 88 % forward	Periplasmic nitrate reductase genes <i>napA</i> and <i>napB</i>	CAA06231.1 <i>Rhodopseudomonas</i> sp. Identities 83 % Positives 92 %
T24-2-1	AE004547.1 <i>Pseudomonas aeruginosa</i> PA01 83 % reverse	Periplasmic nitrate reductase gene <i>napC</i> , <i>napB</i> , <i>napA</i> , <i>napD</i> , <i>napF</i> , <i>napE</i>	BAA15989.1 <i>Escherichia coli</i> Identities 79 % Positives 88 %

Table 4-6. Continued

Clone Name	Best Match Nucleotide	Periplasmic Nitrate Reductase Genes	Best Match Translated Protein Blastx
T24-2-2	AJ004933.1 / RSAJ4933 <i>Rhodopseudomonas</i> sp. 83 % forward	<i>napE</i> , <i>napD</i> & <i>napA</i> genes of <i>nap</i> operon, 5' region	ZP_00021142.1 <i>Ralstonia metallidurans</i> Identities 85 % Positives 902 %
T24-2-7	AE004547.1 <i>Pseudomonas aeruginosa</i> PA01 82 % reverse	Periplasmic nitrate reductase gene <i>napC</i> , <i>napB</i> , <i>napA</i> , <i>napD</i> , <i>napF</i> , <i>napE</i>	BAA15989.1 <i>Escherichia coli</i> Identities 82 % Positives 92 %
T24-2-8	AB055444.2 <i>Magnetospirillum magnetotacticum</i> 86 % forward	Periplasmic nitrate reductase gene cluster (<i>napF</i> , <i>napD</i> , <i>napA</i> , <i>napG</i> , <i>napH</i> , <i>napB</i> , <i>napC</i>), complete cds	BAA15989.1 <i>Escherichia coli</i> Identities 78 % Positives 86 %
T24-2-9	X71385.1 <i>A. eutrophus</i> 88 % forward	Periplasmic nitrate reductase genes <i>napA</i> and <i>napB</i>	NP_288786.1 <i>Escherichia coli</i> O157 Identities 84 % Positives 91 %
T24-2-10	AF245096 <i>Azospirillum brasilense</i> 87 % forward	Periplasmic nitrate reductase large subunit (<i>napA</i>) gene, partial cds; Periplasmic nitrate reductase small diheme cytochrome c subunit (<i>napB</i>); Membrane-anchored tetraheme c-type cytochrome (<i>napC</i>) genes, complete cds	NP_233066.1 <i>Vibrio cholerae</i> Identities 80 % Positives 89 %

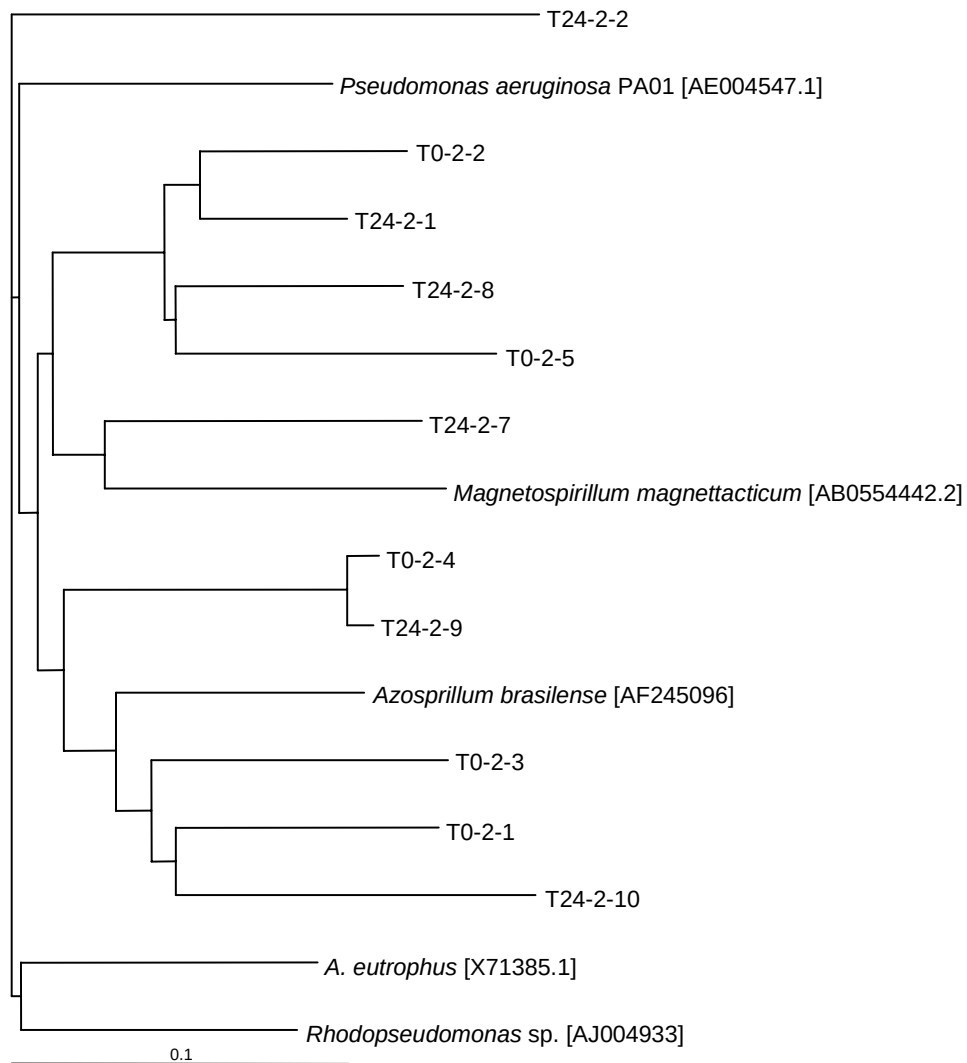


Figure 4-39. Phylogenetic NAP dendrogram showing the relationship of cloned NAP sequences from the reactor. The numbers in brackets are accession numbers for the NAP sequences.

has become clear that the periplasmic nitrate reductase respiratory system is a significant bioenergetic system in cells growing in aerobic environments.

4.3 Linkage to the Conceptual Model

In this section, an attempt was made to investigate possible pathways in which nitrite and/or nitrate could be converted based on the conceptual model developed for this research (Figure 2-2). As discussed in Chapter 2, a range of microbial processes for nitrogen conversion has been reported which may occur in soil, aquatic systems and wastewater treatment plants. Examples of such reactions are aerobic denitrification (Robertson et al., 1995), anaerobic ammonium oxidation (Anammox) (Mulder et al., 1995) and autotrophic denitrification (Bock et al., 1995). These possible pathways in the conceptual model were assessed based on the data from this research to determine which pathway was responsible for the lost nitrogen.

In the current study, the Anammox process was not likely responsible for the nitrogen losses noted because of the low ammonia and high DO concentrations utilized in this research. The influence of oxygen on the Anammox process has been studied in both batch and continuous systems (van der Graaf et al., 1996; Jetten et al., 1997). Reported results showed that oxygen completely inhibited Anammox activity when it was deliberately introduced into enrichment cultures. Additionally, Strous et al., 1997 reported that ammonium was not oxidized during oxic periods, but that Anammox activity in anoxic periods remained constant throughout the experiment. Dissolved oxygen levels measured during the batch experiments in the current study were greater

than 2.0 mg/L which would have inhibited Anammox activity. Additionally, Anammox bacteria need ammonia as an electron donor; however, ammonia concentrations in this research were consistently below 0.1 mg-N/L during all experiments. Therefore, there was not enough ammonia available for use as an energy source by the bacteria. Ultimately, it was concluded that the Anammox pathway was not a primary mechanism for nitrogen removal in this research.

Similarly, autotrophic denitrification was not a possible pathway in which nitrite could be converted to other nitrogenous compounds. Muller et al., (1995) found that autotrophic denitrification only occurred after addition of ammonium and ceased when sufficient nitrite was present. In this study, ammonia concentrations were consistently below 0.1 mg-N/L and nitrite was supplied (25 mg-N/L) throughout the study. Additionally, Wrage et al., (2001) reported that autotrophic denitrification occurred only under certain circumstances, such as low organic carbon content, low dissolved oxygen concentrations (0.2 mg-L) and low pH. In this study, dissolved oxygen levels were maintained above 2.0 mg/L and the pH was adjusted near 7.5. Also, in autotrophic denitrification, the conversion rates were much slower than those for conventional nitrification and denitrification reactions. Conversion rates of 0.042 mg-N/L-hr (Anderson and Levine, 1986) and 0.012 mg-N/L-hr (Bock et al., 1995) have been reported. The rates obtained in the current research averaged 1.94 ± 0.47 mg-N/L-hr which represents a 50-150-fold increase over rates published in the literature. Based on the numerous dissimilarities between test parameters and those required for autotrophic denitrification, it is unlikely that this process was responsible for the nitrogen losses observed during the current study.

The role of organic assimilation in the unbalanced nitrogen levels noted during batch experiments was investigated by measuring bacterial growth (protein concentration and number of total bacteria) over time in the presence of VFAs (Table 4-7). Protein levels were initially 675 µg/ml (time = 0) and remained close to that level (685 µg/ml) after the 6 hr incubation time. Similarly, total bacterial numbers, measured by real-time PCR, were 1.09×10^{12} cells/L at the beginning of the experiment and 1.04×10^{12} cells/L at the end of the experiment. Based on both measures, there was no change in microbial growth due to the addition of VFAs. Therefore, the organic assimilation pathway was eliminated as a significant mechanism for the nitrogen losses noted after addition of VFAs.

Based on the overall results obtained in all experiments, aerobic denitrification was the most likely pathway for the nitrogen imbalance noted in VFAs containing tests. As discussed earlier, Richardson and Ferguson (1992) found that the level of the periplasmic nitrate reductase enzyme activity was largely dependent on carbon source and was highest on butyrate, one of the VFAs used in the current study. Sears et al. (1997) also demonstrated that the expression of periplasmic nitrate reductase and the rate

Table 4-7. Changes in total number of bacteria and protein concentration in the presence of VFAs

Time (hrs)	Total number of bacteria (cells/L)	Protein concentration (µg/ml)
0	1.09×10^{12}	675
6	1.04×10^{12}	685

of nitrate reduction were high at all oxygen concentrations in cultures containing butyrate. In response to aerobic growth on butyrate, NAP activities increase by at least an order of magnitude demonstrating a significant role for the aerobic denitrification during butyrate metabolism (Richardson and Ferguson, 1992; Sears et al., 1993, 1995, 1997). In the current study, the activity of NAP enzyme also increased in the presence of VFAs indicating that the aerobic denitrification pathway was possible. Additionally, the detection of N_2O gas supported the idea that aerobic denitrification was the likely mechanism responsible for the nitrogen losses.

A simple nitrogen mass balance was performed to determine nitrogen speciation during nitrite oxidation in biologically active systems. Mass balances with control (no VFAs) and VFAs samples were calculated based on measurements of N_2O in the gas phase and nitrite and nitrate in the liquid phase for the experiment described in section 4.1.7. In the control experiment, initial nitrite and nitrate concentrations in the liquid phase were 23.15 mg-N/L and 4.76 mg-N/L, respectively. Therefore, the initial total nitrogen concentration was 27.91 mg-N/L. At the end of the control experiment, the nitrite concentration dropped to 5.62 mg-N/L while the nitrate concentration increased to 22.31 mg-N/L. The final total nitrogen concentration after 5 hours was 27.92 mg-N/L which clearly showed that no nitrogen was lost in this experiment. Additionally, it was demonstrated that all nitrite-N consumed (17.53 mg/L) was converted to nitrate-N (17.55 mg/L) and that N_2O was not produced in the control experiment. Essentially, all of the consumed nitrite-N (76% of NO_2^- initially present) was converted to nitrate-N via nitrification (Figure 4-40).

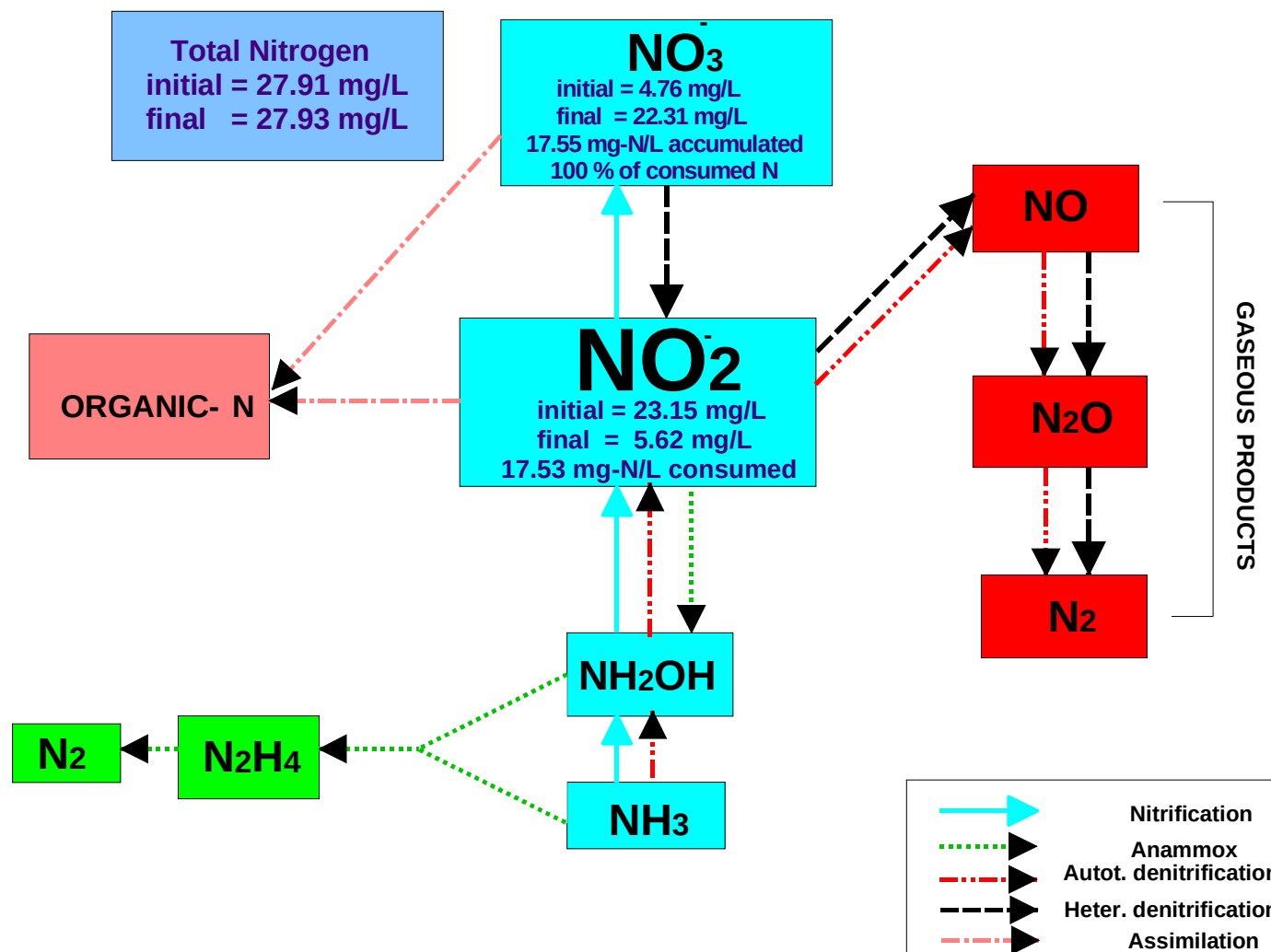


Figure 4-40. Nitrogen mass balance in the control (no VFAs) experiment

In the presence of VFAs, the initial nitrite concentration was 25.64 mg-N/L and the nitrate-N concentration was 3.64 mg-N/L. The initial N₂O concentration was below the detection limit of 1 mg/L. Therefore, the initial total nitrogen concentration was 29.28 mg-N/L. At the end of the 5 hour incubation period, the nitrite concentration decreased to 11.11 mg-N/L which was almost twice as high as that in the control and the nitrate concentration increased to 8.05 mg-N/L. Additionally, 5.5 mg-N/L of N₂O gas was measured. Therefore, the final total nitrogen concentration was summed as 24.66 mg-N/L indicating that nitrogen was lost in the presence of VFAs. Overall, 14.53 mg/L of nitrite-N was consumed and 4.41 mg/L of nitrate-N accumulated (Figure 4-41). In other words, only 30.35% of the consumed nitrite-N was converted to nitrate suggesting another pathway other than nitrification was active. Due to the increase in periplasmic nitrate reductase enzyme activity (Section 4.1.8), it could be assumed that a portion of nitrate-N was converted back to nitrite-N. Additionally, 37.85% of the consumed nitrite-N was accumulated as N₂O strongly indicating that denitrification had occurred. The remaining 31.8% of nitrogen may have been converted to N₂ gas based on the literature reports, which was not detectable by IR spectrometry. Jetten et al., (1997) stated that in chemostat cultures with *Alcaligenes faecalis*, 25% of the NO₂⁻-N was lost as N₂O and the remaining nitrogen went to nitrogen gas. In another study, *Alcaligenes faecalis* showed significant aerobic denitrifying activity, producing almost equivalent amounts of N₂ and N₂O (Robertson et al., 1995). Additionally, Arts et al. (1995) studied aerobic denitrification in chemostat cultures with *Thiosphaera pantotropha* and found that both N₂ and N₂O were produced by all of the cultures.

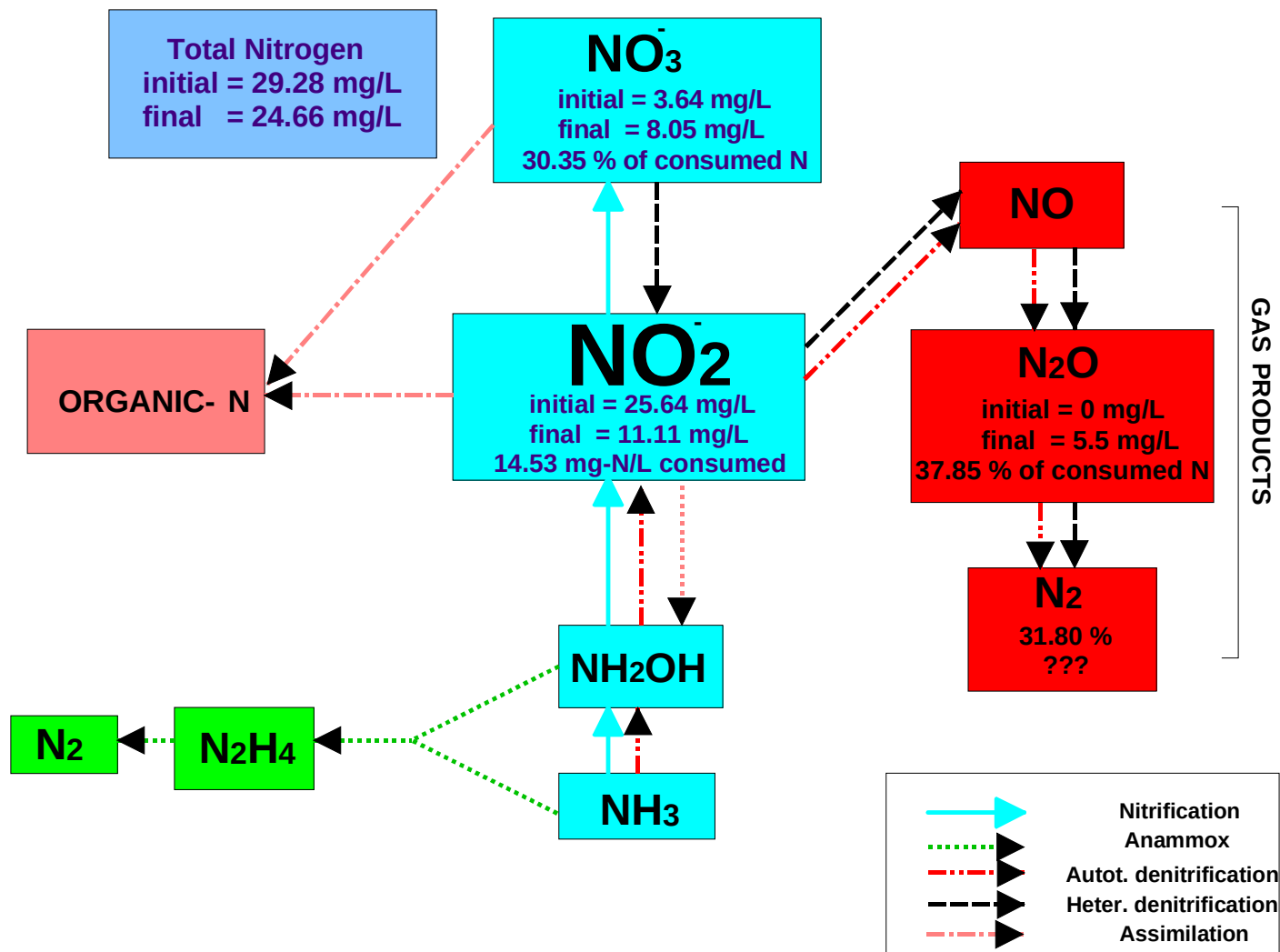


Figure 4-41. Nitrogen mass balance in the VFAs experiment

The increase in NAP enzyme activity in the presence of VFAs observed during the current study strongly suggested that N_2O production was due to the aerobic reduction of nitrogen oxides by the microbial population. Complete denitrification requires the sequential action of four enzymes, nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS). Recently, the *nap*, *nir* and *nor* genes have been found to be linked (Philippot, 2002). Once the NAPenzyme becomes activated, other denitrification enzymes becomes active resulting in denitrification products. Bell and Ferguson (1991) showed that NOR and NOS were also active in the presence of oxygen.

Aerobic denitrification is a rather newly discovered metabolic activity which can be carried out by many different heterotrophic bacteria. The results obtained indicated that aerobic denitrification is responsible, to a great extent, for the loss of nitrogen oxides noted in this study and that this reaction should be considered in any nitrogen balance calculations used to assess wastewater treatment performance.

CHAPTER 5

CONCLUSIONS AND IMPLICATIONS

5.1 Conclusions

The following conclusions can be drawn from this study.

1. VFAs decreased the nitrite removal and the nitrate formation rates in batch experiments

Nitrite removal and nitrate formation rates were reduced in the presence of VFAs in batch experiments. Reduction of the nitrate formation rate was greater than the nitrite removal rate suggesting an imbalance between those rates and that they were not affected to the same extent by the presence of VFAs. Additionally, nitrite consumption was higher than nitrate accumulation and the total measured nitrogen concentration decreased. This unbalanced reaction suggested that processes other than nitrification occurred in the presence of VFAs.

2. VFAs reduced the nitrate formation rate, but did not affect the nitrite removal rate in the CSTR experiment.

After VFAs addition, a rapid decrease in nitrate formation rate ($> 70\%$ reduction) was obtained; however, nitrite removal rate was not reduced in the CSTR. The total nitrogen concentration also decreased. Neither nitrite nor

nitrate could be detected in the CSTR effluent 4 hours after addition of VFAs into the reactor indicating that nitrogen was completely removed from the wastewater. Thus, in the presence of VFAs, both nitrification and denitrification occurred concurrently. No nitrogen (ammonia, nitrite and nitrate) was discharged from the CSTR indicating excellent treatment performance for nitrogen removal compared to normal operation which generally contains nitrate.

3. No decrease in CO₂ fixation rates by VFAs occurred in either batch or CSTR systems

CO₂ fixation rates were not reduced in either batch or CSTR experiments by VFAs. These results implied that VFAs disturb nitrite removal and nitrate formation rates by a different mechanism than nitrite oxidation. If VFAs had affected the nitrite oxidation pathway, the CO₂ fixation pathway would also have been reduced since the energy needed in CO₂ fixation was derived from the oxidation of nitrite. Therefore, VFAs do not inhibit nitrite oxidation via inhibition of CO₂ fixation.

4. *Nitrospira* numbers remained relatively constant

The number of NOB (*Nitrospira*) did not change in the presence of VFAs indicating that *Nitrospira* were present throughout all batch and CSTR experiments. Any reduction observed was not due to a decrease in the number of *Nitrospira*.

5. N₂O gas was produced in the presence of VFAs in batch experiments

It was demonstrated that N₂O was produced in batch samples containing VFAs. No N₂O was detected in any control samples. The N₂O production observed during experiments was likely due to aerobic reduction of nitrogen oxides such as nitrite and nitrate. Detection of N₂O was a clear indication that aerobic denitrification was responsible for the unbalanced nitrification conversions in the presence of VFAs.

6. The activity of periplasmic nitrate reductase enzyme (NAP) increased by VFAs

The activity of periplasmic nitrate reductase enzyme (NAP) both in batch and CSTR experiments increased when VFAs were present. Aerobic nitrate reduction increased with increasing activity of NAP enzyme suggesting a significant role of aerobic denitrification during nitrogen conversions. The activation of NAP enzyme catalyzes nitrate reduction. Since the genes for periplasmic nitrate reductase enzyme (NAP), nitrite reductase enzyme (NIR) and nitric oxide reductase (NOR) are genetically linked, the other steps of denitrification were likely stimulated as a result of NAP activation.

7. The periplasmic nitrate reductase enzyme gene (*napA*) was present in activated sludge biomass

The periplasmic nitrate reductase enzyme gene (*napA*) was detected in mixed liquor samples taken from the CSTR suggesting that aerobic denitrification

via the periplasmic nitrate reductase enzyme (NAP) had the potential to take place in the CSTR. The results showed that the ability to respire with nitrate as an electron acceptor under aerobic conditions is present among the heterotrophic population of the CSTR. It is apparent that many bacteria capable of aerobic nitrate respiration contain *napA* genes, which is consistent with the physiological evidence for the presence of periplasmic nitrate reductase in these organisms.

5.2 Implications

Conventional nitrogen removal WWTPs typically combine nitrification (aerobic oxidation of ammonia to nitrate) and denitrification (anoxic conversion of nitrate into nitrogen gas) to remove aqueous phase nitrogen from wastewater streams. Because denitrification has been thought to be an anaerobic process, it is performed in a separate reactor following nitrification. However, nitrified effluents are low in organic matter because organic compounds are biodegraded in upstream unit processes. Since organic matter serves as the electron donor during denitrification, low levels can negatively impact overall nitrogen removal efficiency. In most cases, biodegradable organic matter is added to resolve this problem; however, this step increases the overall cost of the treatment. New technologies and/or processes that enable the nitrification-denitrification cycle to occur under the same operational conditions have been sought to reduce treatment costs. Elimination of nitrogenous compounds by simultaneous nitrification and

denitrification, leading to the production of N_2O and N_2 gases, is of great economic interest for wastewater treatment plants.

There has been considerable speculation concerning the physiological role and the ecological implications of aerobic denitrification. The nitrite produced as a consequence of nitrate reduction catalyzed by periplasmic nitrate reductase in aerobic conditions was presumably further reduced by nitrite respiring bacteria or used as a substrate by nitrite oxidizing organisms. If the latter is the case, then estimations of nitrification rates based on measurements of nitrate could be lower than the true rate of nitrification.

It was suggested that aerobic denitrification might offer a physiological advantage in environments which are rich in reduced carbon such as VFAs. The accessibility of VFAs produced during anaerobic processes is a very important factor during nitrogen removal by the combination of nitrite oxidation and aerobic denitrification since VFAs were confirmed to stimulate aerobic nitrate reduction. Anaerobic processes are operated to convert biodegradable particulate organic matter into VFAs, which are subsequently separated from the particulate matter and fed to biological nutrient removal systems to enhance their performance. The objective of fermentation processes is conversion of biodegradable organic matter to VFAs and the harvesting of those VFAs for addition to biological nutrient removal systems. Conversion of biodegradable organic matter to VFAs is accomplished by operation of SRTs that allow the growth of hydrolytic and acidogenic bacteria but preclude the growth of methanogens which convert VFAs to methane. The latter is necessary because VFAs are desirable for nutrient removal systems, and thus one does not want those to be converted to methane. Operation at reduced temperature also makes it easier to limit the growth of methanogens and

maximize the production of VFAs. Reduced pH values also aid in controlling the growth of methanogens. Thus, it is not difficult to accumulate VFAs for aerobic denitrification.

Results obtained in this research demonstrated that nitrite oxidation and aerobic denitrification can be combined in a single aerobic CSTR in the presence of VFAs. Nitrate disappearance during the aerobic phase was due to aerobic denitrification stimulated by VFAs and no nitrite accumulated in the system. Successful development of this process could eliminate the need for separate reaction tanks or separate zones within tanks for nitrogen removal. To achieve this process, anaerobic processes can be followed by an aerobic CSTR to perform nitrification-aerobic denitrification simultaneously in WWTPs.

To conclude, aerobic denitrification has important implications in wastewater treatment processes. Stimulation of oxygen insensitive nitrate respiration may make a significant and previously unrecognized contribution to the flux of nitrate to nitrite in oxic and micro-oxic environments. Because most of the conventional nitrogen-removal systems are based on either the recirculation of biomass between aerobic and anoxic zones, or the application of alternate aerobic and anoxic conditions, the existence of this flexible nitrate–oxygen metabolism contributes to rapid acclimatization and effective response with non-negligible nitrate removal rates. It is essential to obtain more physiological and kinetic insight both into the mechanisms of action and regulation of the enzymes implied and into the environmental factors affecting their function. The application of aerobic denitrification is highly promising with regard to the potential savings in energy. New process technological applications and novel microbial processes

like aerobic denitrification will further enhance the removal of nitrogen from wastewater and reduce the chemical and energy demands for this removal.

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APPENDICES

**APPENDIX I. Tables That Summarize Nitrite Removal,
Nitrate Formation and Carbon Dioxide Fixation Rates in
Controls and Samples during Batch Experiments**

Table I-1. Nitrite removal, nitrate formation and CO₂ fixation rates in the absence and in the presence of ATU in the batch experiments

	Nitrite removal	Nitrate formation	CO ₂ fixation	Experiment No
^aRates in the absence of ATU (mg/L-hr)	2.50	2.37	2.44×10^{-3}	1
	2.18	2.10	2.78×10^{-3}	2
	mean 2.35 ± 0.23	mean 2.24 ± 0.19	mean $2.62 (\pm 0.24) \times 10^{-3}$	
^bRates in the presence of ATU (mg/L-hr)	2.47	2.03	1.73×10^{-3}	1
	2.19	2.20	1.32×10^{-3}	2
	mean 2.33 ± 0.19	mean 2.12 ± 0.13	mean $1.55 (\pm 0.29) \times 10^{-3}$	
% Reduction^d	1.2	15	30	1
	-0.46 ^c	-5	53	2
	mean 0.37	mean 5	mean 42 ± 16.26	

a: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite without ATU

b: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite and ATU

c: Negative values reflect an increase

d: Calculated using the following equation: $\{(\text{control rate}-\text{reduced rate})/\text{control rate}\} \times 100$

Table I-2. Nitrite removal, nitrate formation and CO₂ fixation rates in the absence and in the presence of azide in the batch experiments

	Nitrite removal	Nitrate formation	CO ₂ fixation	Experiment No
^aRates in the absence of azide (mg/L-hr)	2.50	2.37	2.44×10^{-3}	1
	2.18	2.10	2.78×10^{-3}	2
	2.78	2.80	2.15×10^{-3}	3
	1.73	1.94	4.02×10^{-3}	4
	2.77	2.05	4.00×10^{-3}	5
	2.66	2.48	4.08×10^{-3}	6
	mean 2.42 ± 0.41	mean 2.29 ± 0.26	mean $3.25 (\pm 0.89) \times 10^{-3}$	
^bRates in the presence of azide (mg/L-hr)	1.19	1.75	1.40×10^{-3}	1
	0.37	0.91	1.21×10^{-3}	2
	1.02	1.12	0.82×10^{-3}	3
	0.84	0.84	2.12×10^{-3}	4
	0.97	1.15	2.43×10^{-3}	5
	0.58	1.60	2.94×10^{-3}	6
	mean 0.83 ± 0.30	mean 1.23 ± 0.37	mean $1.82 (\pm 0.87) \times 10^{-3}$	
% Reduction	52	26	42	1
	83	60	57	2
	63	60	63	3
	51	56	47	4
	65	44	40	5
	78	30	28	6
	mean 65 ± 13.12	mean 46 ± 15.18	mean 46 ± 12.54	

a: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite without azide

b: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite and azide

Table I-3. Nitrite removal, nitrate formation and CO₂ fixation rates in the absence and in the presence of 1500 mg/L of VFAs and % reduction of nitrite removal, nitrate formation, CO₂ fixation in the batch experiments

	Nitrite removal	Nitrate formation	CO ₂ fixation	Experiment No
^a Rates in the absence of VFAs (mg/L-hr)	3.32	3.54	5.58 x 10 ⁻³	1
	2.72	2.37	5.70 x 10 ⁻³	2
	2.78	2.80	5.77 x 10 ⁻³	3
	mean 2.94 ± 0.33	mean 2.90 ± 0.62	mean 5.68 (± 0.09) x 10 ⁻³	
^b Rates in the presence of 1500 mg/L of VFAs (mg/L-hr)	2.05	0.42	6.75 x 10 ⁻³	1
	1.70	0.42	5.91 x 10 ⁻³	2
	1.94	0.50	6.12 x 10 ⁻³	3
	mean 1.89 ± 0.18	mean 0.44 ± 0.03	mean 6.26 (± 0.43) x 10 ⁻³	
% Reduction	38	88	-20.96 ^c	1
	37	82	-3.7 ^c	2
	30	82	-0.06 ^c	3
	mean 35 ± 4.36	mean 84 ± 3.47	mean -8.24 ± 11.16	

a: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite without VFAs

b: Calculated from the slopes of plots after 5 hr incubation with 25 mg-N/L nitrite and 1500 mg/L of VFAs

c: Negative values reflect an increase in CO₂ fixation

Table I-4. Nitrite removal, nitrate formation and CO₂ fixation rates in the absence and in the presence 3000 mg/L of VFAs and % reduction of nitrite removal, nitrate formation, CO₂ fixation in the batch experiments

	Nitrite removal	Nitrate formation	CO ₂ fixation	Experiment No
^aRates in the absence of VFAs (mg/L-hr)	1.73	1.94	4.02 x 10 ⁻³	1
	2.77	2.05	4.00 x 10 ⁻³	2
	2.66	2.48	4.08 x 10 ⁻³	3
	3.99	4.38	n.a	4
	3.46	3.46	n.a	5
	2.28	2.3	n.a	6
	2.73	2.61	n.a	7
	3.85	3.62	n.a	8
	mean 2.94 ± 0.78	mean 2.91 ± 0.87	mean 4.03 (± 0.04) x 10 ⁻³	
^bRates in the presence of 3000 mg/L of VFAs (mg/L-hr)	1.35	0.96	4.26 x 10 ⁻³	1
	1.64	0.52	4.80 x 10 ⁻³	2
	1.69	0.93	5.20 x 10 ⁻³	3
	2.20	0.96	n.a	4
	2.91	0.88	n.a	5
	1.99	0.53	n.a	6
	1.36	0.90	n.a	7
	1.29	0.43	n.a	8
	mean 1.83 ± 0.55	mean 0.77 ± 0.23	mean 4.75 (± 0.47) x 10 ⁻³	
% Reduction	22	50	-5.97 ^c	1
	40	75	-20 ^c	2
	36	60	-27.45 ^c	3
	45	78	n.a	4
	16	75	n.a	5
	13	77	n.a	6
	50	66	n.a	7
	66	88	n.a	8
	mean 36 ± 18.2	mean 72 ± 11.9	mean -18 ± 10.90	

Table I-5. Rates and % reduction of nitrite removal, nitrate formation, CO₂ fixation in the CSTR experiment performed with the addition of 3000mg/L of VFA

Time (hrs)	Nitrite removal		Nitrate formation		CO ₂ fixation	
	Rate (mg/L-hr)	Reduction (%)	Rate (mg/L-hr)	Reduction (%)	Rate (µg/L-hr)	Reduction (%)
-1	2.48	0	2.43	0	3.56	0
0	2.1	16	0.67	73	4.9	-38
2	3.21	-30	0.66	73	6.44	-81
4	3.45	-39	0.22	91	6.24	-75
6	3.19	-29	0.27	88	6.74	-89
24	3.47	-40	0.37	85	3.34	0

Percent reduction values were calculated using the following equation: $\{(\text{control rate}-\text{reduced rate})/\text{control rate}\} \times 100$
 Minus reduction values show the rates increased compared to control

APPENDIX II. Operational Data for the CSTR

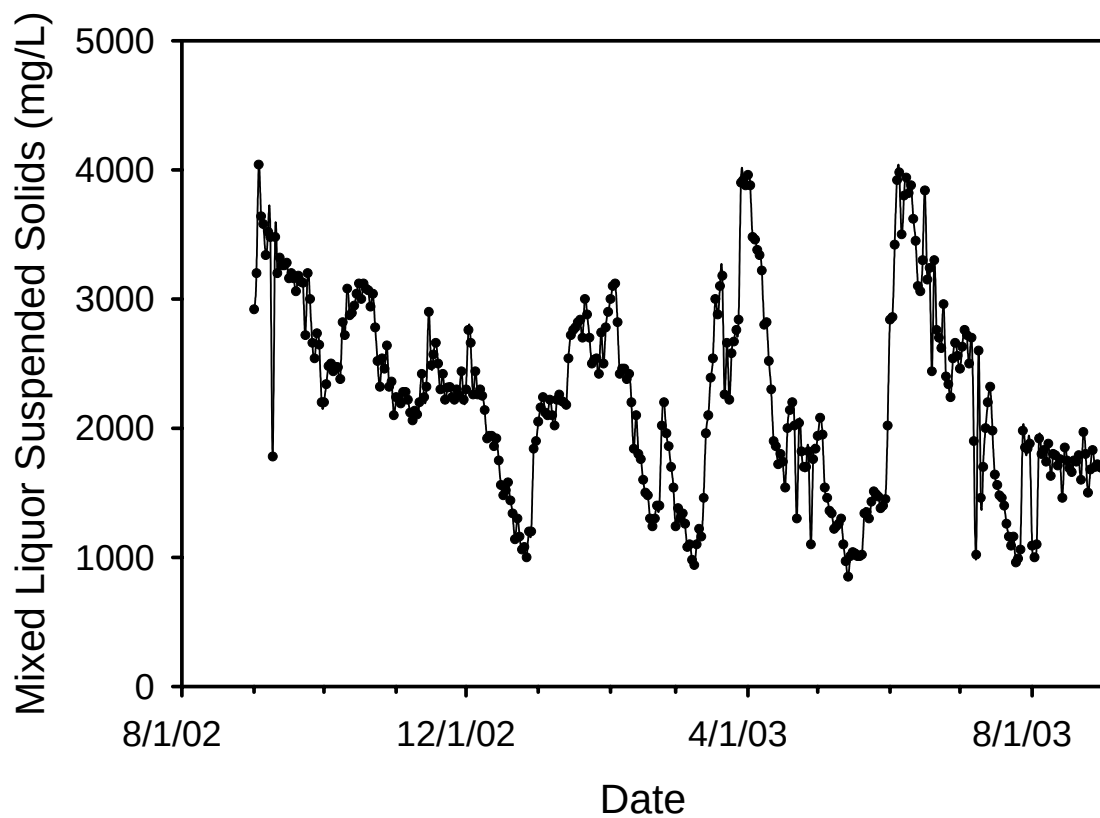


Figure II-1. MLSS concentration in the CSTR throughout the study

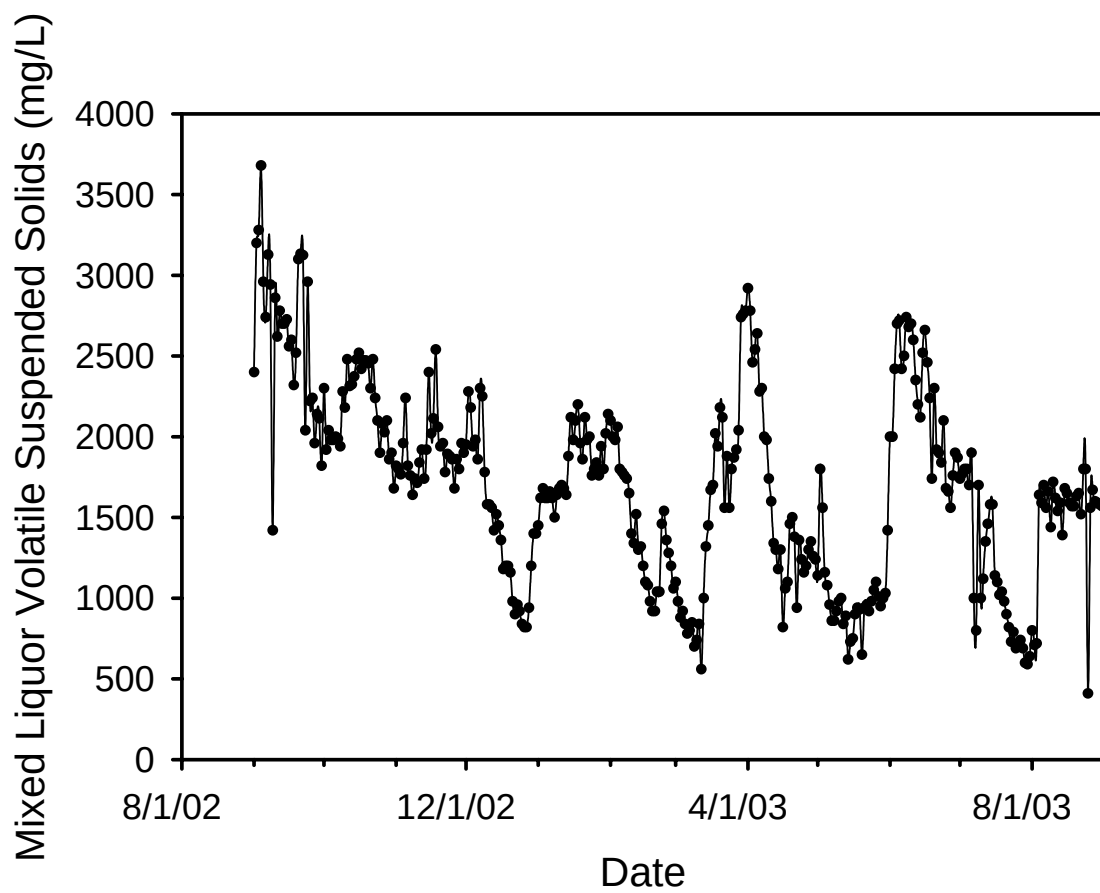


Figure II-2. MLVSS concentration in the CSTR throughout the study

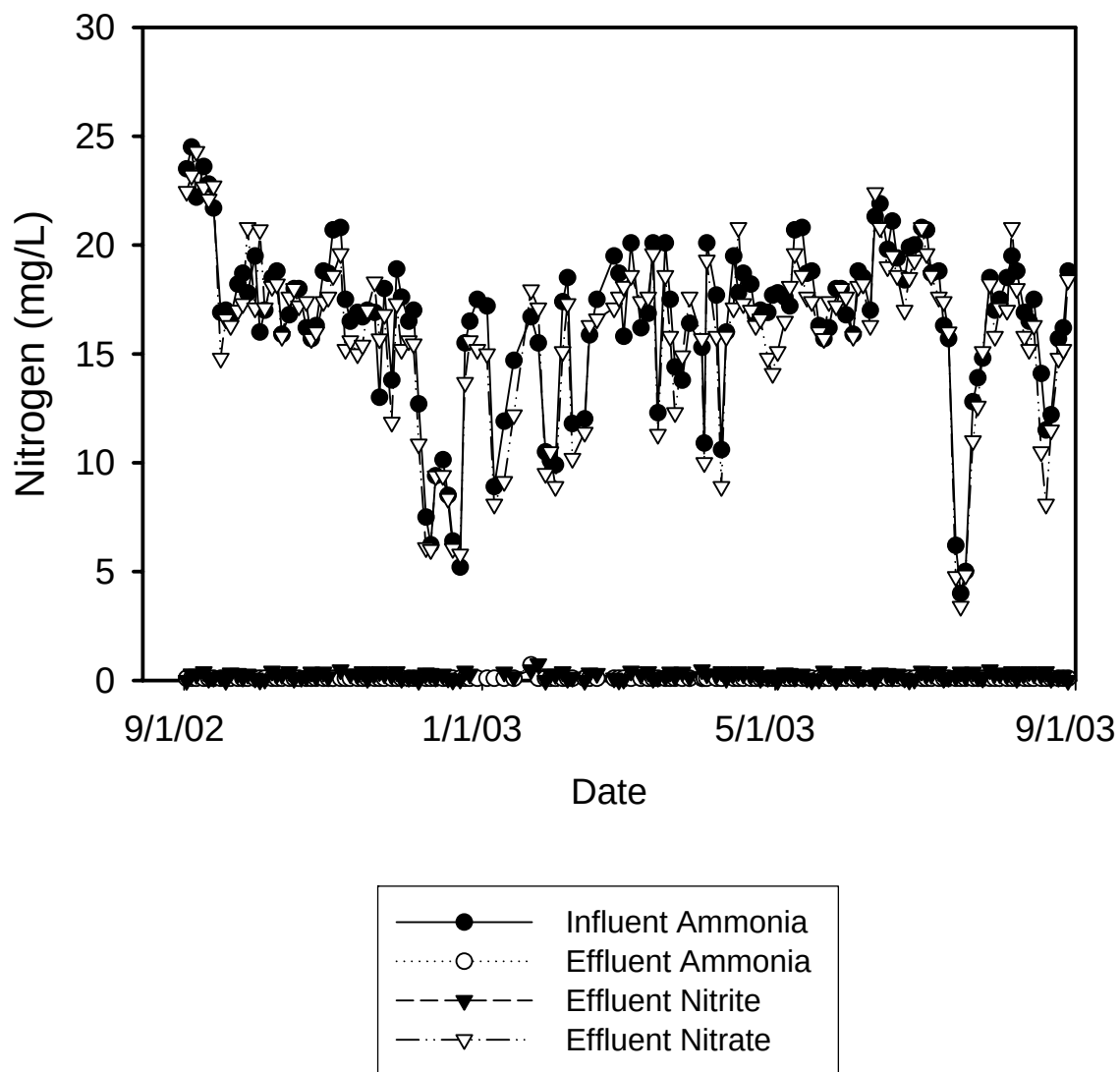


Figure II-3. Nitrogen concentration in the CSTR throughout the study

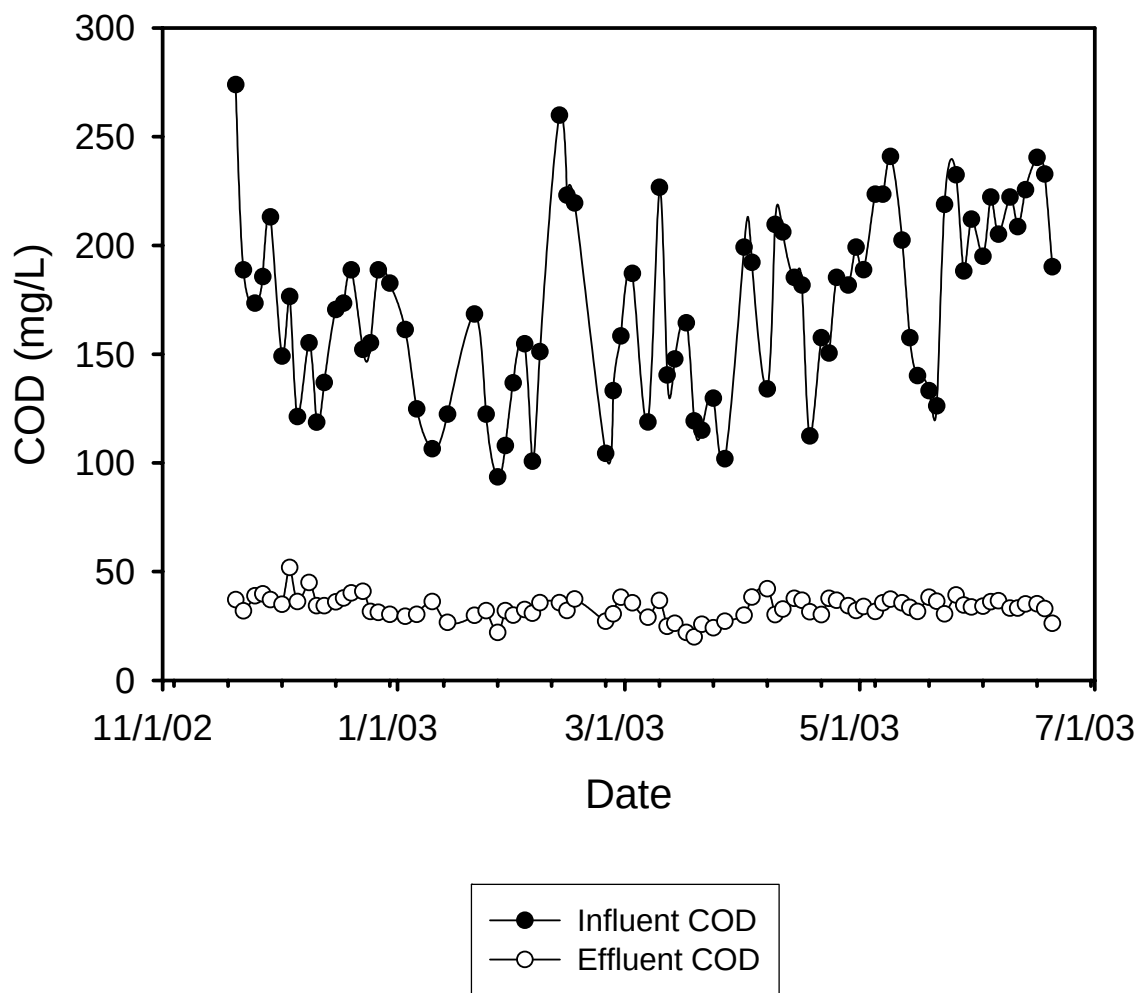
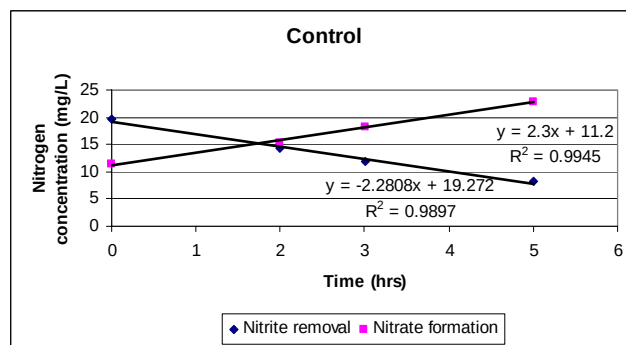


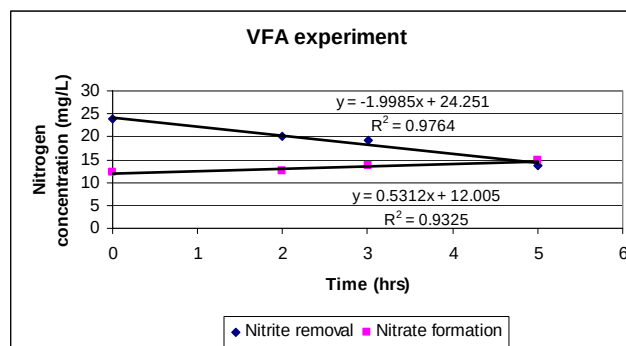
Figure II-4. COD treatment performance of the CSTR

APPENDIX III. Inhibition of NAP Enzyme by SCN and CN

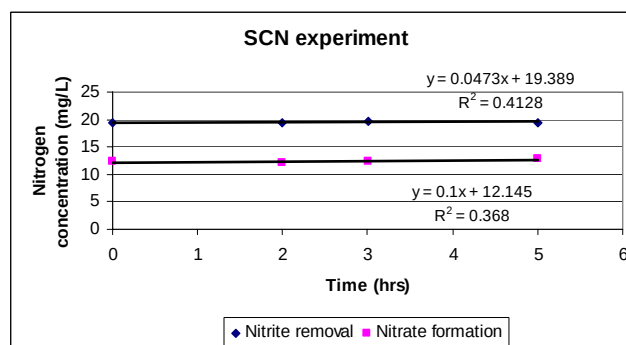
A



B



C



D

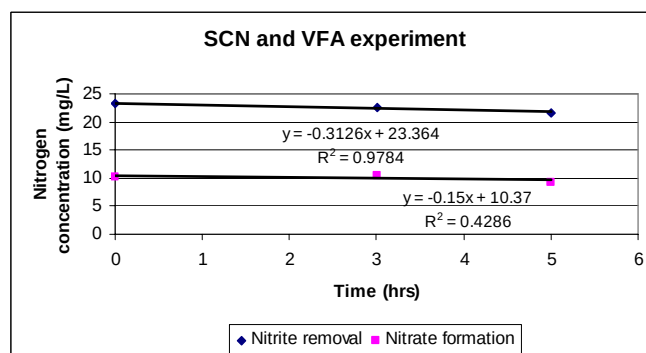
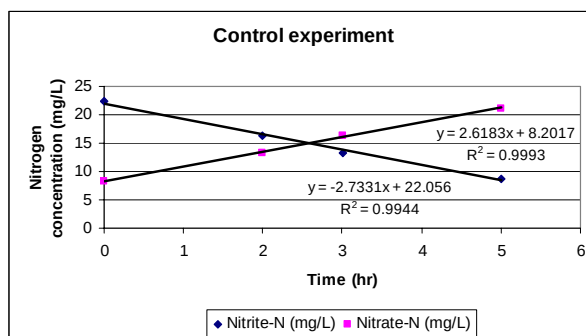
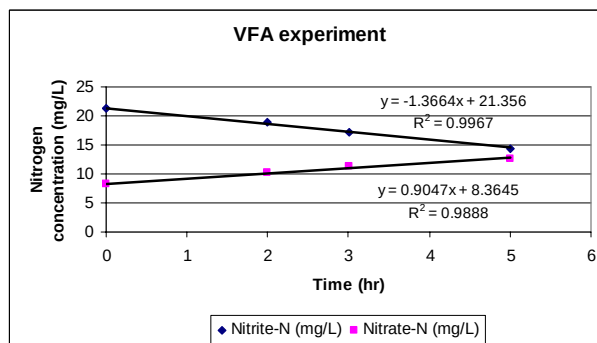


Figure III-1. Nitrite removal and nitrate formation in SCN inhibition experiment (A) control, (B) 3000 mg/L of VFAs, (C) 10 mM of SCN, (D) 10 mM of SCN and 3000 mg/L of VFAs.

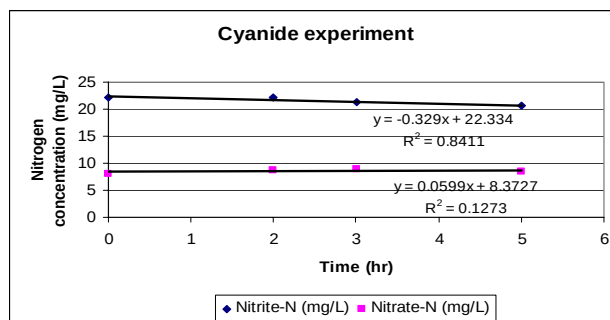
A



B



C



D

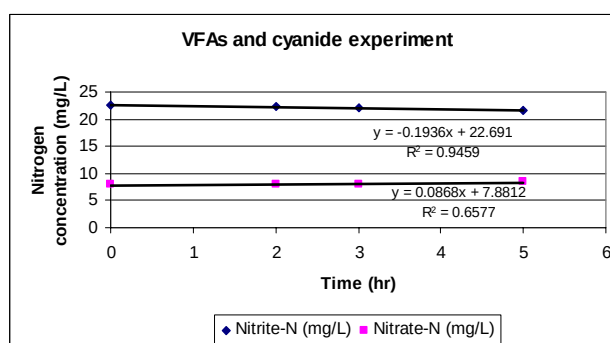


Figure III-4. Nitrite removal and nitrate formation in CN inhibition experiment (A) control, (B) 3000 mg/L of VFAs, (C) 10 µM of CN, (D) 10 µM of CN and 3000 mg/L of VFAs.

VITA

Merve Temizer Oguz was born in Istanbul, Turkey on June 5, 1970. She graduated with a Bachelor of Science degree in Environmental Engineering from Yildiz Technical University, Istanbul in 1993. She was awarded a full scholarship by Turkish Ministry of Education to pursue higher education in the United States. She entered Colorado State University in 1996 and received a Master of Science degree in Civil Engineering in 1998. Then, she pursued a Doctorate of Philosophy degree in Civil Engineering at the University of Tennessee, Knoxville and graduated in 2004.