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Ecological Factors in Design of a Two-Sludge Nitrifying Activated Sludge System Incorporating Side-Stream Treatment of Anaerobic Digester Supernatant

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ABSTRACT

Reject water comprised of anaerobic digester supernatant and the liquid reject from sludge dewatering operations (filtrate and centrate) is a problematic recycle stream for municipal biological wastewater treatment facilities. The high ammonia loadings exert a substantial oxygen demand to the front end of nitrification facilities. The situation is compounded by imported nitrogen to centralized sludge processing facilities and intermittent operation of dewatering facilities. A number of side-stream reject water treatment configurations have emerged in recent years. In addition to reducing the impact of recycle loadings, a number of the configurations tout the potential for cultivating a stable source of nitrifying microorganisms to bioaugment the main-stream.

This research investigated the impact of engineering decisions, specifically the choice of a plug flow reactor (PFR) versus a continuously stirred tank reactor (CSTR) configuration of the side-stream reject water treatment bioreactor, on the structure and function of ammonia-oxidizing bacteria (AOB) communities. The objective of this research was to assess the extent and impact from bioaugmentation of side-stream biomass into the main-stream process. A two-sludge configuration in which the side-stream biomass is isolated from the main-stream biomass was selected to facilitate the development of unique populations in the side-stream bioreactor in order to further allow evaluation of the impact of biodiversity on nitrification function. The goal was to show that input biomass from side-stream reactors allows main-stream reactors to maintain performance under conditions that cause failure in control reactors not receiving input biomass. Chemical analysis of bioreactor effluent was used to characterize bioreactor function. Biomass samples collected from the experimental bioreactors were used in molecular biology assays to
characterize microbial structure and to use in respirometric assays to further characterize nitrification function.

Molecular analysis of experimental and full-scale systems revealed that the AOB community structure in side-stream reject water treatment bioreactors matches the AOB community structure in the main-stream bioreactors in which the side-stream biomass is input. This was true for either a two-sludge configuration in which side-stream biomass is isolated from the main-stream or for a single-sludge system with continuous re-inoculation of the side-stream bioreactor from the main-stream RAS. It is also evident that bioaugmentation of main-stream bioreactors with biomass from side-stream reject water treatment increases AOB diversity in the main-stream. Furthermore, a main-stream bioreactor with biomass input from a PFR side-stream exhibited greater AOB diversity than a main-stream bioreactor with biomass input from a CSTR side-stream. A potential benefit of increased AOB diversity due to bioaugmentation from the side-stream system is increased system reliability in the face of short-term toxicity events. Furthermore, side-stream bioreactors would be beneficial as a source of nitrifiers to re-seed the main-stream bioreactor following a seasonal transition to cooler wastewater temperatures or short-term peak flows during wet weather conditions. In this research, bioaugmentation may have prevented washout of critical populations in the main-stream bioreactor coupled to a PFR side-stream bioreactor thereby stabilizing nitrification.
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“Nothing in the world can take the place of perseverance...Persistence and determination alone are omnipotent. Press On!” -Calvin Coolidge
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NOMENCLATURE AND LIST OF SYMBOLS

AMO – ammonia monooxygenase
AOB, *ns* – ammonia oxidizing bacteria
NH₄–N ammonium–nitrogen
BLAST – basic local alignment search tool
BNR – biological nutrient removal
CaCO₃ – calcium carbonate
COD – chemical oxygen demand
CSTR – continuously stirred tank reactor
DNA – deoxyribose nucleic acid
*f* – biomass yield
FISH – fluorescence in situ hybridization
HRT – hydraulic retention time
IFAS – integrated fixed film activated sludge
k – pseudo first order rate coefficient
Ks – substrate half-saturation coefficient
MLSS – mixed liquor suspended solids
MLVSS – mixed liquor volatile suspended solids
NCBI – national center for biotechnology information
NO₃–N nitrate–nitrogen
NO₂–N nitrite–nitrogen
NOB, *nb* – nitrite oxidizing bacteria
NOD – nitrogenous oxygen demand
OUR – oxygen uptake rate
PBS – phosphate buffered saline
PFR – plug flow reactor
PCR – polymerase chain reaction
POTW – publicly owned treatment works
RAS – return activated sludge
RFLP – restriction fragment length polymorphism
rRNA – ribosomal ribonucleic acid
$R^2$ – coefficient of determination.
SRT – solids retention time
TKN – total kjehldahl nitrogen
TSS – total suspended solids
VSS – volatile suspended solids
$X$ – biomass concentration
$\mu$ - biomass growth rate
Chapter 1
Overview

1.1 Introduction and Problem Statement

Ecological engineering is defined as the act of designing a bioreactor to foster the development of a specific microbial community (Thiele and Zeikus 1988). Activated sludge—where input dissolved oxygen and engineered settling zones foster the development of fast-growing, flocculent biomass is a 100-year old example of ecological engineering. The concept of bioaugmentation was established in the latter half of the nineteenth century and was brought to the wastewater treatment industry over 50 years ago (Schelling et al. 1987). That notwithstanding, it is easy to understand how the significance of these early advances into engineering bacterial populations and their functions could have been overlooked given the crude analytical techniques available to characterize microbial communities compared to relatively advanced techniques for measuring chemical constituents. Furthermore, even simplistic mechanistic descriptions of microbial functions can be quite complex. It is impossible to engineer something you can’t measure or describe so, instead, design emphasis was placed upon materials balances, hydraulics and transport, and kinetics and stoichiometry (Grady et al. 1999). However, recently, there has been a renewed interest ecological engineering (Thiele and Zeikus 1988, vanLoosdrecht et al. 1997, Grady and Filipe 2000) stemming from multiple factors. For one thing, greatly improved analytical techniques and investigative methods now exist to characterize microbial community structure and function. Also, greater emphasis is being placed on advanced biological treatment techniques to meet strict effluent nutrient limits. Last but not least, many POTWs built using the generous subsidies made available via the Clean Water Act
Construction Grants program are approaching or have exceeded their design life. Sewer agencies now seek to put off capital improvements projects by squeezing more capacity and function from existing infrastructure as opposed to funding 100% of capital improvements through loan programs paid off through user fees. A conspicuous target for attacking capacity issues is biological nitrification because the bacteria that perform this transformation are generally considered the most critical populations.

Biological nitrification, first described more than 100 years ago (Schloesing and Muntz 1877), is the step-wise oxidation of ammonia-nitrogen (NH$_4$-N) to nitrate-nitrogen (NO$_3$-N) through a nitrite-nitrogen (NO$_2$-N) intermediate. It is a function performed exclusively by autotrophic microorganisms capable of using reduced nitrogen as an energy source. This capability has been harnessed very successfully for wastewater treatment, providing an effective means of mitigating toxicity due to effluent ammonia, but it is not without problems. For example, the efficiency of the nitrification process is affected by several factors including temperature, pH, dissolved oxygen, carbon to nitrogen ratio in the raw wastewater, and the presence of any of a number of organic and inorganic inhibitory compounds (USEPA 1993). In fact, ammonia and nitrite are both inhibitory to ammonia-oxidizing bacteria (AOB) and particularly, nitrite-oxidizing bacteria (NOB), at relatively low concentrations (Anthonisen et al. 1976, Gee et al. 1990b). The above factors notwithstanding, even under ideal conditions, biological nitrification is a slow process due to the slow growth rate that is characteristic of the metabolism of nitrifying microorganisms. This leads to several drawbacks related to construction, design, and operation of municipal wastewater treatment plants. The process requires a long solids retention time (SRT), in excess of 15 days are often required for reliable nitrification in cold climates, requiring large basins,
larger than would be necessary for carbon oxidation alone. Accumulation of inert solids is a significant disadvantage of long SRT systems. The volume of reactors and settlers and energy consumption increase concomitantly (Yuan et al. 1998), thereby increasing construction and operating costs. Furthermore, a long SRT increases the risk of settling problems. *Microthrix parvicella*, a filamentous microorganism that is commonly observed in high SRT biological nutrient removal (BNR) plants (Jenkins et al. 2003) reduces sedimentation capacity due to sludge bulking. Another significant disadvantage is that long SRT systems limit options for future upgrades to biological nutrient removal. Thus, difficulties in maintaining nitrification not only impact the ability to meet discharge limits for ammonia, it also interferes with other functions of municipal wastewater treatment. The result is increased costs for municipal wastewater treatment and lower quality treated effluent. Naturally, then, engineers and practitioners view with great interest any modification that allows nitrification with a shorter sludge age.

One way to overcome limitations imposed by a long SRT is to utilize attached growth processes such as trickling filters. However, nitrification in trickling filters is particularly sensitive to cold weather. A more recent development is the development of hybrid suspended growth-attached growth processes that utilize floating attached growth media, so-called integrated fixed-film activated sludge (IFAS). The technology has been effective but the floating media complicates handling and disposal of waste Biosolids.
1.2 Literature Review

Nitrification has been the target of numerous bioaugmentation projects designed to uncouple nitrifier SRT from bulk SRT. Introduction of bacterial cultures to activated sludge wastewater treatment systems (bioaugmentation) can be considered for increasing growth rates, increasing tolerance to toxic material, increasing the spectrum of bacteria available for natural selection, and increasing the concentration of desirable bacteria and thus reducing their response time (lag phase) to specific substrates (Stevens 1989). All four circumstances apply to nitrification. Rittmann (1996) stated that bioaugmentation has its greatest applicability when the added microorganisms are slow growers such as nitrifiers and Rittmann (1996) and modeled the effect of input biomass on main-stream treatment. The model predicted that at an SRT greater than 15 days, the input biomass reduced the minimum ammonia concentration below $S_{\text{min}}$. The model also predicted that the input biomass would allow stable nitrification at an SRT less than 1.5 days, below $S_{\text{RT,min}}$. However, as is often the case, the real world has failed to meet the expectations of model predictions. In one study, the input of select strains of nitrifying microorganisms into laboratory-scale reactors under a washout stress was studied by Abeysinghe, et al (2002). Bioaugmentation was beneficial but substantial maintenance doses were necessary. Another study found that warm input AOB could induce nitrification in cold non-nitrifying reactors, although at reduced rates. Average nitrification rates for biomass cooled to 10°C decreased 58%, 71%, and 82% when the biomass was acclimated to 20°C, 25°C, and 30°C. (Head and Oleszkiewicz 2004). Authors have suggested a number of reasons for the inability of input biomass to do in nature what they do in axenic culture (Stephenson and Stephenson 1992). Among the explanations for reduced activity is that the introduced bacteria
were unable to compete and survive the harsh conditions of activated sludge reactors. Hence, alternatives for generating endogenous biomass were sought. One novel approach was to introduce a storage tank for waste sludge into the flow stream thereby providing a bank of biomass that could be withdrawn in the case of shock loads or toxicity incidents (Yuan et al. 1998).

Another approach involves recovery and enrichment of nitrifying microorganisms in side-stream reactors treating anaerobic digester supernatant. Ammonia-nitrogen in recycled digester supernatant may comprise up to 20% of total influent ammonia loading (Siegrist 1996)—more if it is a centralized solids handling facility—but is generally less than 1% of the flow (Mossakowska et al. 1997). In addition to reducing the ammonia-nitrogen load to the main-stream process by 5 to 25% (Berends et al. 2003), a side-stream reactor can serve as an enriched source of ammonia-oxidizing bacteria (AOB) and/or nitrite-oxidizing bacteria (NOB) depending on system operation. In the past decade multiple side-stream treatment configurations have been developed in an attempt to capture both benefits, including BABE, In-Nitri, Bioaugmentaion R, and MAUREEN. The existence of multiple systems could be an indication that the most efficient configuration for generating nitrifying biomass in side-stream reactors has not been identified.

In simple terms, suspended growth systems may be designed for either continuous (CSTR) or plug-flow (PFR) configurations. Past research (Chudoba, et al. 1985) has shown that a plug-flow configuration provides higher rates of nitrification and is less susceptible to substrate inhibition. On the other hand, kinetic theory explains how a CSTR configuration could provide resistance to wastewater toxicity (Weber 1972) from high-strength reject water. Toxic inhibition due to
elevated concentrations of ammonia and nitrite-nitrogen forms is a well-known phenomenon (Anthonisen et al. 1976) (Gee et al. 1990a). However, kinetic theory cannot explain how configuration impacts the diversity and physiology of the microorganisms.

1.2.1 Reject Water Treatment Technologies

Generally speaking, the composition of recycle sidestreams poses several challenges for treatment:

- High concentrations of nutrients. NH$_3$-N may be as high as 1,500 mg/L and orthophosphate as high as 20 mg/L (USEPA 1987). Thus despite the fact that the daily recycle flow is typically less than 1% of the daily influent wastewater flow, the daily recycle nutrient load as a result of the processing of solids from a typical wastewater treatment plant is 10 to 15% of the total influent nutrient load. Intermittent digester decanting or sludge dewatering results in recycle over a shorter period causing short-term load peaks possibly leading to break-through and a reduction in effluent quality.

- High concentrations of ammonia. Recycle nitrogen is primarily in the form of ammonia. Free ammonia, as well as intermediate products of ammonia oxidation including hydroxylamine and nitrite can severely inhibit nitrification (Mossakowska et al. 1997). Furthermore, accumulation of nitrite to the detriment of main-stream treatment.

- Insufficient alkalinity for nitrification. The alkalinity present is typically only one-half what is required for complete nitrification (Water Environment Federation 2010). As a result, some chemical addition to provide alkalinity is typically required for sidestream nitrification.
- Insufficient BOD for heterotrophic denitrification (Water Environment Federation 2010). Supplemental carbon addition is required for sidestream denitrification depending on the process.

- Low concentration of readily biodegradable COD. For this reason chemical phosphorus removal from sidestream treatment is generally more practical.

- Elevated temperature. Mesophilic anaerobic digesters typically operate at 35°C. The rate of sidestream nitrification is maximized at 30°C but becomes inhibited above 35°C. Therefore, temperature control (cooling in the summer; heating in the winter) is an essential component of some sidestream treatment processes.

The three strategies for managing recycle nutrient loads from reject water are depicted in Figure 1-1 (Constantine et al. 2007). Export refers to transfer of untreated sludges to a regional biosolids treatment facility or to a nearby plant with less stringent nutrient limits that is better equipped to handle additional nutrient loadings.

**Manage Sidestream Recycle Load**

The manage option refers to alternatives for coping with recycle loads without actually reducing the daily recycle load. The manage option involves modification or expansion of the mainstream biological treatment system to handle the recycle streams or equalization of recycle streams to reduce peak loads and spread out loads over the course of one week.
**Treatment of Sidestream Recycle Load**

Sidestream treatment alternatives reduce recycle nutrient loads. Three categories of side-stream treatment are presented:

- **Bioaugmentation** processes are activated sludge processes that produce an enriched biomass that is input to the mainstream to support nitrification. A secondary benefit of some bioaugmentation processes is that nitrate-laden effluent can be discharged to the headworks for odor control. The bioaugmentation processes can also be operated for denitrification with external carbon addition but the odor control benefit is lost;

- **Non-bioaugmentation** processes utilize shortcuts in the nitrogen pathway to remove nitrogen more efficiently than can be achieved in the mainstream activated sludge system. No bioaugmentation or odor control benefits are realized;

- **Physical-chemical** nutrient removal and recovery processes remove nutrients from the sidestream producing a beneficial reuse product.
A diagram showing the sidestream treatment alternatives reviewed in this report is shown in Figure 1-2.

Figure 1-2 – Sidestream Treatment Alternatives

**Bioaugmentation processes**

Side-stream bioaugmentation processes nitrify the sidestream and bioaugment the main-stream with nitrifiers from the side-stream mixed liquor. The intent of bioaugmentation is to boost the effective solids retention time (SRT) in the main-stream to allow nitrification to proceed at otherwise unfavorable conditions. Bioaugmentation is adopted primarily to save space. The processes may be manipulated to stop at nitrite at elevated temperatures or with the incorporation of pH control. This is referred to as the “nitrite shunt”.

Two approaches to bioaugmentation have been defined: in situ and external (Parker and Wanner 2007). In situ bioaugmentation provide internal process enhancements to enrich or increase the
activity of endogenous nitrifier populations. The advantage of in situ processes is said to be that the bioaugmented biomass is already adapted to the mainstream. Cultivation *in-situ* prevents predation or wash-out of nitrifiers because the nitrifiers are incorporated into the flocs of the existing mainstream activated sludge system. External bioaugmentation is the addition of nitrifiers from external activated sludge reactors treating reject water, parallel treatment trains, or downstream processes. The advantage of external bioaugmentation is that nitrification in the mainstream is uncoupled from the aerobic SRT. The amount of nitrifying organisms needed to be grown in the main treatment stream is reduced by the amount of new nitrifying organisms supplied from the sidestream treatment system. Since a smaller amount of nitrifiers need to be grown in the mainstream treatment plant, the nitrification section (oxic section) can be smaller and the MLSS concentration lower since higher SRT is not required.

The characteristics, advantages, and disadvantages of the following bioaugmentation processes are presented:

- **AT-3**;
- **In-Nitri**;
- **B.A.B.E.**;
- **STRASS**;
- **BAR**.

**AT-3**

The AT-3 process is a flow-through process without sludge retention. A blend of 67% reject water and 33% RAS from the main-stream is fed to the AT-3 reactor (Stinson et al. 2007). Nitrified sidestream mixed liquor is discharged to the main-stream aeration tanks. Alkalinity in
the RAS provides some of the supplemental alkalinity required to for nitrification. Physical and operational characteristics of the AT-3 process are as follows:

- Aeration tank;
- No clarifiers;
- HRT = SRT = 1 to 3 days;
- Chemical feed system for alkalinity;
- In-situ bioaugmentation.

A schematic of the process is shown in Figure 1-3. The AT-3 process has been operational at New York City’s 26th Ward WPCP for many years. The process, as practiced at 26th Ward is a nitritation process meaning that ammonia oxidation is stopped at nitrite using DO and pH control.

Mainstream Autotrophic Recycle Enabling Enhanced N-removal (MAUREEN) is a modified version of AT-3 that incorporates an anoxic compartment for denitrification and recycle of effluent biomass from the effluent end of the tank to the influent of the tank (Stensel 2006). Methanol or other source of organic carbon is required for denitrification. A flow diagram for the MAUREEN process is shown in Figure 1-4.
In-Nitri® process is a self-contained activated sludge treatment system. Feed to the InNitri® reactor is primarily reject water, although a small slip-stream of primary effluent is sometimes added to acclimate side-stream biomass to the main-stream. The volume of primary effluent added is small so as not to lower the reactor temperature. The warm temperature of the reject water (typically 25 °C to 35°C) allows nitrification at a significantly lower SRT than the main-stream process (Kos 1998). Liquid nitrate-rich effluent can be discharged to the headworks or primary treatment for odor control. Waste sludge which contains a high percentage of nitrifiers, is discharged into the main-stream aeration tank for bioaugmentation. Physical and operational characteristics of the InNitri® process are as follows:

- Aeration tank;
- Clarifier;
- Chemical feed system for alkalinity;
- HRT = 1 to 2 days;
- SRT = 3 to 5 days;
- External bioaugmentation.
A flow diagram of the InNitri® process is shown in Figure 1-5. The process is classified as external bioaugmentation because the biomass is from an activated sludge reactor external to the mainstream biomass (Parker and Wanner 2007).

Figure 1-5 – Flow Diagram for the InNitri® Process

B.A.B.E

The Bio-Augmented Batch Enhanced (B.A.B.E.) process is a sequencing batch reactor (SBR) which is fed reject water and a slip-stream RAS from the main-stream. RAS flow is up to 25% of the total mainstream RAS flow. Effluent from the B.A.B.E. process is discharged to the mainstream aeration tanks. Physical and operational characteristics of the B.A.B.E process are as follows:

- SBR;
- HRT < 1 day;
- SRT = 1 to 2 days;
- Source of biomass is main-stream RAS;
- In-situ bioaugmentation.
A flow diagram of the B.A.B.E. process is shown in Figure 1-6. The technology has been operated full-scale at multiple locations in the Netherlands (Van Der Zandt et al. 2006).

![Figure 1-6 – Flow Diagram for the B.A.B.E.® Process](image_url)

**STRASS**

The STRASS process is a high-sludge SBR process for nitritation and denitrification of reject water (Wett et al. 1998). Denitrification requires an external carbon source. Alternatively, nitrite-laden decant can be discharged to headworks for odor control. Waste sludge is discharged to the mainstream aeration tanks. Distinguishing features of the STRASS process are as follows:

- Sequencing batch reactor;
- pH controlled intermittent aeration;
- D.O. less than 0.5 mg/L;
- SRT > 20 days;
- External bioaugmentation.
In the Bioaugmentation R (BAR) process, reject water is recycled to the reaeration portion of a contact stabilization activated sludge system (Parker and Wanner 2007). The stream is fully nitrified and the nitrifiers are carried forward to the portions of the mainstream aeration tank receiving primary effluent. Physical and operational characteristics of the BAR process are as follows:

- Nitrification in main-stream sludge reaeration (no additional reactors or clarifiers);
- In situ bioaugmentation;

The technology known as the Regeneration-DeNitrification (R-DN) process in the Czech Republic.

**Non-bioaugmentation processes**

The properties of biomass from sidestream non-bioaugmentation process are such that decay and predation limit the effectiveness of bioaugmentation. Two non-bioaugmentation processes are described: SHARON and DEMON.

**SHARON**

The Single reactor, High-activity Ammonia Removal Over Nitrite (SHARON) process is a nitritation-denitrification process wherein ammonia is oxidized to nitrite and nitrite is converted to nitrogen gas (Hellinga et al. 1998). The process consists of one or two continuous-flow completely-mixed reactors without sludge retention. Reject water ammonia is oxidized to nitrite in the first stage; nitrite is reduced to nitrogen gas in the second stage. The reactions can be spatially separated using two reactors or separated in time by cycling aeration. Denitrified reject water is discharged to the mainstream. A flow diagram of the SHARON® process is shown in Figure 1-7. Physical and operational characteristics of the SHARON® process are as follows:
- Temperature controlled to inhibit nitrite oxidation;
- Once-through process - no sludge retention;
- Insulated reactor with heat exchanger;
- Denitritation to nitrogen gas with methanol addition;
- HRT = SRT = 1 to 2 days;
- Two separate reactors or one reactor with cycling of air.

![Flow Diagram for the SHARON® Process](image)

**DEMON**

The DEamMONification (DEMON) process is a sequencing batch reactor process for deammonification, the anaerobic oxidation of ammonia. In the first step, ammonia is partially oxidized to nitrite. In the second step, the remaining ammonia is oxidized to nitrogen gas with nitrite as the terminal electron acceptor (anammox reaction) (Wett et al. 2007). Effluent containing a small amount of nitrite is discharged to the mainstream. A flow diagram for the DEMON® process is shown in Figure 1-8. Distinguishing features of the process are as follows:
- Sequencing batch reactor;
- Completely autotrophic – no external carbon source required;
- D.O. less than 0.5 mg/L;
- pH-controlled intermittent aeration;
- Insulated reactor with heat exchanger;
- SRT is 30-50 days.

![Flow Diagram for the DEMON® Process](image)

**Figure 1-8 – Flow Diagram for the DEMON® Process**

**Physical-Chemical processes for nutrient recovery**

**Struvite precipitation**

Controlled struvite precipitation processes are typically used to remove and recover phosphorus. Effluent from the process will contain ammonia because phosphorus is the limiting reactant to produce struvite from reject water (Water Environment Federation 2010). A flow diagram for the Ostara struvite precipitation process is shown in Figure 1-9. Physical and operational characteristics of the Ostara process are as follows:

- Fluidized bed reactor
- Magnesium feed required

![Flow Diagram for the OSTARA® Process](image)

Figure 1-9 – Flow Diagram for the OSTARA® Process

**Ammonia stripping**

Multiple technologies are available for removal and recovery of ammonia from sidestreams including air stripping, steam stripping, and vacuum distillation. Ammonia removal is facilitated by raising the pH to 10 or 11 to convert all the ammonia to the NH₃ form and then stripping the gas (Water Environment Federation 2010). Physical and operational characteristics of ammonia stripping are as follows:

- Pre-treatment to remove solids;
- Heat exchanger;
- Distillation column;
- Chemical addition: caustic to raise pH of untreated stream and acid to readsorb ammonia after stripping.

A flow diagram for the CAST® process is shown in Figure 1-10.
1.3 Research Objectives

The objective of this study was to evaluate how engineering decisions, specifically, configuration of a suspended growth side-stream bioreactor, impacts the following:

1) Structure of the side-stream nitrifier community,

2) Efficiency of bioaugmentation efficiency from side-stream bioreactor to main-stream bioreactor, and

3) Functional stability of main-stream nitrification.

This research is designed to yield multiple outcomes. First, it will provide mechanisms by which engineers can enhance species diversity in activated sludge systems. Second, it will show the impact of diversity on functional stability of activated sludge nitrification systems. Collectively, these outcomes will establish the basis for reducing minimum solids retention times for nitrification. This is expected to provide options to extend capacity at existing treatment plants.
and provide alternative, cost-effective treatment plant designs for new or expanded treatment plants. At the same time the fundamental new knowledge that will be obtained about the impact of diversity on functional stability would advance the field of biological wastewater treatment systems.

The approach is to operate laboratory-scale bioreactors for the purpose of analyzing reactor function by comparing treatment performance and generating biomass to use in molecular assays characterizing microbial community structure and respirometric assays characterizing biomass function.

1.4 Specific Aims

The central hypothesis is for the research is that “Biodiversity enhances function.”

Specific Aim #1: Specific Aim #1 was designed to test the hypothesis that identical AOB communities would form in side-stream reactors operated in different configurations.

Specific Aim #2: Specific Aim #2 was designed to test the hypothesis that main-stream nitrification stability is not enhanced by bioaugmentation.

Specific Aim #3: Develop a novel mechanistic model of nitrification based on the macromolecular composition of nitrifying populations.
1.5 Layout of Dissertation

The dissertation is sub-divided into five chapters: Chapter 1 is the overview and includes the problem statement, literature review, research objectives and specific aims of the project. Chapter 2 is an evaluation of the structure of AOB communities utilizing molecular biology techniques for a laboratory-scale main-stream activated sludge system with bioaugmentation from side-stream reject water reactors. Chapter 3 is a comparison of AOB community structure utilizing molecular biology techniques applied to laboratory-scale and full-scale mixed liquor samples for two different side-stream reject water treatment configurations. Chapter 4 is a an evaluation of AOB community function utilizing batch respirometry for a laboratory-scale main-stream activated sludge system with bioaugmentation from side-stream reject water reactors. Chapter 5 summarizes the finding, significance, and future research topics. Appendix A describes the development of a novel quantitative assay for AOB which was used to evaluate bioaugmentation of main-stream bioreactors with biomass from side-stream bioreactors. Appendix B describes the materials and equipment used to conduct the research. Appendix C provides amoA genetic sequence information generated for the manuscript presented in Chapter 3.

Chapters 2, 3, and 4 each consist of a manuscript published in the peer-reviewed journal Water Science and Technology (Smith and Oerther 2006, Smith et al. 2008, Smith and Oerther 2009).
1.6 References


Parker, D., and J. Wanner. 2007. Improving Nitrification through Bioaugmentation. in Nutrient Removal 2007, Baltimore, MD.


Chapter 2

Microbial community development in a laboratory-scale nitrifying activated sludge system with input from a side-stream bioreactor treating digester supernatant

2.1 Abstract

Three laboratory-scale activated sludge treatment trains were operated to investigate the effect on biodiversity in plug flow (PFR) main-stream sewage treatment from input of biomass from side-stream reactors treating anaerobic digester supernatant. One train had a completely mixed (CSTR) side-stream reactor, one a plug flow (PFR) side-stream reactor, and the third train was a control that did not receive input from a side-stream reactor. Restriction endonucleases were used to digest PCR-amplified ammonia monooxygenase subunit A (amoA) genes in monthly samples from each reactor. Restriction fragment banding patterns from polyacrylimide gel electrophoresis indicated that the structure of the ammonia oxidizing bacteria (AOB) populations in all five reactors stabilized by the fourth month of operation and then did not vary subsequently. Furthermore, a dendrogram generated using the Jaccard distance showed that the AOB in each side-stream reactor was most similar to the main-stream reactor in the same train indicating that the AOB population in the side-stream reactor exerts a strong influence on the population in the main-stream reactor. Sequencing results indicated that *Nitrosomonas europea*, an r-strategist, was the dominant AOB in the PFR side-stream reactor, while *Nitrosomonas europea* and *Nitrosomonas marina*, a marine bacterium, were strongly represented in the CSTR side-stream reactor.

* This manuscript was published in Water Science and Technology (2006) 54(1): 209-216
2.2 Introduction

Biological nitrification, the step-wise oxidation of ammonia-nitrogen to nitrate-nitrogen, is performed by nitrifying microorganisms that use reduced nitrogen as an energy source. It is a very successful wastewater treatment technology that provides an effective means of mitigating toxicity due to effluent ammonia and is a necessary first step in removal of total nitrogen. However, the slow growth rate that is characteristic of nitrifying microorganisms interferes with other functions of municipal wastewater treatment and increases costs for treatment. The process requires a long solids retention time (SRT) requiring large basins, much larger than would be necessary for carbon oxidation alone, thereby increasing construction costs. A long SRT may also cause excessive growth of undesirable microorganisms such as Microthrix parvicella, a filamentous microorganism that is commonly observed in high SRT biological nutrient removal (BNR) plants (Jenkins et al. 2003). One consequence is reduced sedimentation capacity due to sludge bulking.

Recovery and enrichment of nitrifying microorganisms in side-stream reactors treating anaerobic digester supernatant is one potential means of reducing solids retention time in main-stream reactors. Ammonia-nitrogen in recycled digester supernatant is a significant load comprising up to 20% of total influent loading (Siegrist 1996). A side-stream reactor would oxidize the recycled nitrogen and provide a stable input of microorganisms to the main-stream system. One potential limitation is the survival of the input microorganisms as the substrate conditions in digester supernatant are unique. However, the development and input of unique microbial communities to main-stream reactors is also a potential benefit. Increased biodiversity from
input of microorganisms possessing desirable properties may create a more robust system less susceptible to inhibition or upsets.

The objectives of this research were to characterize the structure of microbial communities in laboratory-scale side-stream and main-stream reactors using molecular biology tools and to evaluate whether a plug flow reactor (PFR) or completely stirred tank reactor (CSTR) configuration in the side-stream produces a more diverse biomass in main-stream reactors allowing for more stable nitrification. This study was designed to test the hypothesis that identical AOB communities would form in side-stream reactors operated in different configurations. In other words, do engineering or operating decisions regarding reactor configuration have a profound impact on biodiversity which, in turn, may impact reactor performance. If the hypothesis is rejected, what impact does greater diversity have on the performance of main-stream nitrification systems.

Two techniques were primarily used to achieve the objectives: construction of 16S ribosomal ribonucleic acid (rRNA) gene libraries and molecular fingerprinting using Restriction Fragment Length Polymorphism (RFLP). An inventory of gene sequences from environmental samples can be obtained by extracting community DNA, amplifying specific genes using the polymerase chain reaction (PCR), and inserting those sequences into plasmids that are maintained in competent cells of *Escherichia Coli* to create a gene library (Wilderer et al. 2002). The 16S rRNA gene is a popular target for gene libraries because its size, structural organization, and evolutionary rate of change make it a powerful tool for phylogenetic analysis (Olsen and Woese 1993). However, in recent years the use of fingerprinting methods has become a popular
alternative to the laborious task of constructing 16S rRNA gene libraries (Wilderer et al. 2002). One such method consists of subjecting PCR amplicons to enzymatic digestion with restriction endonucleases creating fragments of various lengths depending on gene sequence. The restriction fragments are separated by length using gel electrophoresis and visualized as bands. The length of the DNA fragments representing each band can be estimated by comparison with a DNA step ladder containing multiple fragments of known sizes run on the same gel alongside the samples. The procedure allows a quick comparison of biodiversity by simply counting bands and comparing fragment lengths. This technique was recently applied to analysis of the $amoA$ gene as part of an investigation of ammonia oxidizing bacteria (AOB) populations (Egli et al. 2003). The $amoA$ gene was also chosen for this study in order to provide specific resolution of the diversity of the AOB population. The low variability of 16S rRNA genes from AOB makes it difficult to identify different strains. Furthermore, PCR primers targeting 16S rRNA genes may cross-react with members of other phylogenetic and physiological groups, especially when they are used with environmental samples containing complex microbial gene pools (Rotthauwe et al. 1997). On the other hand, the $amoA$ gene is unique to AOB and therefore has been used by several researchers as a function-based specific target for the detection, quantification, and phylogenetic analysis of AOB (Aoi et al. 2004).
2.3 Methods

2.3.1 Laboratory-scale bioreactors.

Two sets of laboratory-scale bioreactors were operated for a period of eight months. Set-one was composed of two aerated, stirred, 1-Liter chemostat reactors (A1 and B1) operated at 35°C treating a synthetic wastewater designed to mimic recycle-streams returning from anaerobic digesters containing 1,000 mg/L NH₄-N, 50 mg/L Phosphorous, between 6,000 and 7,500 mg/L alkalinity as CaCO₃, plus other micro-nutrients. The pH was controlled at a low set-point between 7.0 and 7.2 using controllers (Cole Parmer, Vernon Hills, IL) and pH meters (Thermo Electron, Beverly, MA). Set-two was composed of three aerated, 6-Liter sequencing batch reactors (A2, B2, and C2) operated at 20°C treating a synthetic domestic wastewater containing 30 mg/L NH₄-N, 400 mg/L acetate-COD, 5 mg/L phosphorous, plus alkalinity and other micro-nutrients. All five reactors were started on February 7, 2004 with seed waste activated sludge obtained from the Muddy Creek WWTP in Cincinnati, Ohio.

Reactor A1 was operated to mimic a completely mixed configuration with a fill and react phase of 24 hrs. Reactor B1 was operated to mimic a plug flow condition with feed added in one dose per 24 hrs. Daily, 60-mL of biomass was transferred from A1 to A2 and from B1 to B2 in a single dose. Reactor C2 did not receive bio-augmentation. Reactors A2, B2, and C2 were operated to mimic plug flow conditions with a fill phase of 15 min, a react phase of 7 hrs, and a settle and decant phase of 1 hr. The hydraulic retention time (HRT) and SRT in Reactors A1 and
B1 were identical with a value of 5 days. The HRT in Reactors A2, B2, and C2 was 12 hrs. The SRT in Reactors A2, B2, and C2 was 12 days.

Routine process parameters were measured including chemical oxygen demand (COD), pH, ammonia-nitrogen, nitrite-nitrogen, nitrate-nitrogen, and phosphorus. COD, nitrate, nitrite, and phosphorous were measured colorimetrically using a spectrophotometer and Hach (Loveland, CO) Test’n Tube reagents. Ammonia and pH were measured using combination electrodes. Nitrate and nitrite were also measured using ion-selective electrodes. The experimental reactor configuration is shown in Figure 2-1.

![Experimental Reactor Configuration](image)

**Figure 2-1 - Experimental Reactor Configuration**

2.3.2 Extracting Genomic DNA.

Genomic DNA was extracted from samples collected from each of the reactors using the Soil DNA Extraction kit (MoBio, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, proteins and cell materials were physically and chemically separated from nucleic acids
following chemical lysis and mechanical disruption to release cell contents. Purified genomic DNA was collected from the spin filters in 50 μl of sterile RNase-free water.

2.3.3 Polymerase Chain Reaction.

PCR to generate products for RFLP was performed using oligonucleotide primers targeting conserved regions of the amoA gene as previously described to amplify a 453 bp fragment: amoA1F (5’ ggg gTT TCT ACT ggT ggT 3’); and amoA2R (5’ CCC CTC KgS AAA gCC TTC TTC 3’, where K = g or T and S = g or C)(Rotthauwe et al. 1997). Each reaction had a total volume of 50-μl containing: 1X PCR buffer; 0.025 U/μl DNA polymerase; 2 mM magnesium chloride; 200 μM of each primer; 1-2 μL of genomic DNA; and sterile water for the balance. Conditions for PCR included: initial denaturation and enzyme activation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 45 seconds, and extension at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 3 min and a quench to 4°C. The presence and size of PCR products was verified on a 1% agarose gel cast with ethidium bromide at a concentration of 0.5 ug/mL and visualized under ultraviolet light. PCR products were stored at –20°C.

PCR for cloning was performed using oligonucleotide primers targeting the 16SrRNA gene: S-D-Bact-0011 and S-D-Bact-1492. Each reaction had a total volume of 50-μl containing: 1X PCR buffer; 0.025 U/μL DNA polymerase; 2 mM magnesium chloride; 200 μM of each primer; 1-2 μL of genomic DNA; and sterile water for the balance. Conditions for PCR included: initial denaturation and enzyme activation at 94°C for 5 min; 35 cycles of denaturation at 95°C for 1
min, annealing at 52°C for 30 seconds, and extension at 72°C for 1 min. Amplification was followed by a final extension at 72°C for 7 min and a quench to 4°C. The presence and size of PCR products was verified as before for amoA fragments and used immediately in cloning reactions.

2.3.4 Restriction Fragment Length Polymorphism.

Five units each of the restriction endonucleases Rsal and HaeIII (Promega, Madison, WI) added to 5 - 15 μL of amoA PCR products (total reaction volume = 20μL) to digest samples for restriction fragment length polymorphism (RFLP) analysis. Restriction digests were incubated at 37°C for four hours followed by 15 minutes at 80°C to inactivate the enzyme. Digest products and DNA size markers were separated on 10% polyacrylimide gels run in 1X TBE buffer for 75 minutes at a constant voltage of 75 volts on a mini-gel apparatus by BioRad. The gels were post-stained with SYBR green (10 μL of concentrated stock / 100 mL 1X TBE buffer) (Molecular Probes, Eugene, OR) for 40 minutes. Images were recorded and processed using Kodak 1D (Rochester, NY) software from images recorded using a Kodak EDAS 290 camera and Spectroline ultraviolet transilluminator (Westbury, NY).

2.3.5 Cloning 16SrRNA Gene Fragments.

The TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s instructions. Briefly, fresh PCR product was ligated with the pCR®2.1-TOPO® vector by incubating at room temperature for 5 min. Chemically competent Escherichia coli DH5α were incubated on ice for 10 min with 2 μl of the ligation reaction. The cells were transformed by
heating to 42°C for 30 sec, immediately placing the sample on ice, and adding 250 μl of room temperature SOC. After recovery for 1 hr at 37°C, 5 to 250 μl of the cell suspension was plated by spreading on petri dishes containing Luria-Bertani agar with 50 μg/ml ampicillin (1 L of LB agar [pH 7.0] contains: 10 g tryptone, 10 g sodium chloride, 5 g yeast extract, and 15 g agar) and coated with 40 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) (40 mg-Xgal/ml-dimethylformamide [DMSO]).

2.3.6 Sequence Analysis of Clones.

Transformed, white colonies were picked from petri dishes containing LB agar with ampicillin using sterile toothpicks. The toothpicks were incubated overnight in broth tubes containing LB media. The cell cultures were transferred to 1.5 mL centrifuge tubes and centrifuged to generate a cell pellet. Following decant of the broth, plasmids were extracted and purified using the Qiagen (Valencia, CA) QIAprep® Spin Miniprep kit according to the manufacturer’s instructions. Purified plasmids were submitted to the University of Cincinnati DNA Core Lab for sequencing. Sequencing results were compared to an existing database online using the Basic Local Alignment Search Tool (BLAST) utility by NCBI.

2.3.7 Data Analysis:

RFLP images were compared using the Jaccard distance (1-Jaccard coefficient). The Jaccard coefficient considers the presence or absence of restriction fragments and is equal to the ratio of the number of restriction fragments in common between two profiles to the total number of
restriction fragments present in both profiles. The agglomerative hierarchical clustering developed by Ward (1963) was applied to obtain dendrograms using the Community Analysis Package software 3.0 (Pisces Conservation Ltd, Lymington, UK).

2.4 . Results and Discussion

2.4.1 Nitrification:

Influent and effluent nitrogen concentrations for each reactor during the first eight months of operation are shown in Figure 2-2. Ammonia oxidation has been nearly complete in all reactors during the entire course of the study, but nitrite oxidation has not been stable. Complete oxidation of nitrite to nitrate in the PFR and CSTR side-stream reactor configurations was achieved after two and four months, respectively. The prolonged startup in the side-stream reactors was attributed to inhibition due to free ammonia and/or free nitrous acid because the synthetic wastewater medium did not contain organic or inorganic compounds known to cause inhibition of nitrification. Air stripping of ammonia from the PFR side-stream reactor (B1) may have alleviated inhibition due to ammonia allowing for a shorter start-up. Although pH was controlled it was not the same in both reactors. The pH in this reactor (data not shown) generally varied between 7.5 and 8.5 but sometimes exceeded 9.0 before pH control was instituted. Following depletion of the ammonia in the PFR, CO₂ stripping put a continuous upward pressure on pH. Conversely, the pH in the CSTR side-stream reactor was commonly less than 7.5. The volume and mass of chemical that would have been required to suppress the pH to the level of
the CSTR was deemed excessive and detrimental to assay of the biology, which was the main purpose of the experiment.

The main-stream reactors occasionally experience periods of nitrite accumulation for reasons yet unexplained. Significant nitrate would have been carried over into the main-stream system with side-stream biomass (about 10 mg/L). However, higher effluent nitrate concentrations were not observed in bioaugmented reactors. This phenomenon was acknowledged early in the research but defied a satisfying explanation. Generation of gaseous compounds of nitrogen associated with denitrification or nitrite oxidation is a possibility but cannot explain why that would not have occurred to an equal extent in the control reactor. In any case, closing the nitrogen balance on reactor operation was not deemed critical for achieving the specific aims of the dissertation because respirometry was proposed to be utilized in a later study to characterize nitrification function in the main-stream reactors.

2.4.2 RFLP:

The results from RFLP analysis targeting the *amoA* gene for each reactor during the first eight months of operation are shown in Figure 3. In general, the analysis indicates that reactor populations stabilized by the fourth month of operation and did not vary subsequently. It was also evident that the control reactor (C2) contains the least complex banding pattern. The stable population in C2 was characterized by bands at approximately 170 bp, 100 bp, 75 bp, 50 bp, and 45 bp. The RFLP pattern for the stable community in main-stream reactor A2 contains these bands and an additional band at approximately 90 bp which also appears in the pattern for side-stream reactor A1 from where it likely originated. The RFLP pattern for the control reactor also
appears in the pattern for the stable community in main-stream reactor B2. In addition, the pattern for B2 includes bands at approximately 240 bp, 190 bp, 150 bp, and 90 bp. These additional bands can also be identified in the pattern for the stable community in side-stream reactor B1. Thus it appears that the ammonia-oxidizing population in the side-stream reactor exerts a strong influence on the population in the main-stream reactor. It was also very clear that a plug flow configuration in the side-stream reactor creates a more complex banding pattern than a completely mixed configuration. A plausible explanation for this observation was that the higher substrate gradient present in a plug flow configuration permits the proliferation of more
species. It was interesting to note from comparison of Figures 2 and 3, that the function of the AOB community (ammonia oxidation) did not follow structure. Despite the variations in the structure of the ammonia oxidizer community in the reactors between start-up and stable population (2 – 4months), ammonia oxidation was nearly complete from the beginning of the experiment for all five reactors.

![Figure 2-3 - Polyacrylimide gel electrophoresis showing RFLP Patterns following digestion of the amoA gene from the communities present in each experimental reactor]

The RFLP patterns shown in Figure 2-3 are compared in the dendrogram shown in Figure 2-4. The dendrogram confirms the visual observations of RFLP patterns. According to the dendrogram, stable AOB populations existed in each reactor following startup. Samples from April through September were clustered closely together for each reactor. Also, AOB populations in the main-stream reactors were more closely related to the side-stream reactors from which they receive biomass than they were to the other main-stream reactors. Furthermore, the AOB populations in the CSTR side-stream train were more closely related to the main-stream...
control reactor than were the populations in the PFR side-stream train. This seems logical since reduced nitrogen concentrations in the main-stream reactors would more closely resemble the conditions in the CSTR side-stream reactor than in the PFR side-stream. In addition to these observations, the dendrogram portrays the evolution of the AOB populations in the reactors. For instance, samples from the main-stream reactors were clustered closely together in February and March but diverged beginning in April. Conversely, samples from the side-stream reactors were not closely related to the main-stream reactors, or each other, during startup. This was likely due to strong selective pressure associated with the substrate conditions in these reactors.

![Figure 2-4 - Dendrogram comparing similarity among RFLP patterns over eight months of operation](image-url)
2.4.3 Cloning and Sequencing

Sequences retrieved from the CSTR side-stream reactor were mainly associated with the *Nitrosomonas* lineage. *N. europea* and *N. marina* had the strongest representation but *N. aestuarii*, *N. mobilis* were also indicated. *N. europea* is a classic r-strategist that would thrive under the high gradients in the side-stream reactors. However, *N. marina* and *N. aestuarii* are both marine organisms. It is suspected that the high ionic strength of the side-stream reactors due to the added alkalinity made the habitat suitable for these organisms. Heterotrophic sequences matching marine bacteria were also indicated. The lack of sequences matching known nitrite oxidizers was noteworthy.

Nearly half of the sequences retrieved from the PFR side-stream reactor most closely matched *N. europea*. This was not surprising. As previously mentioned, the high gradients in the side-stream reactors, in particular the PFR reactor, would likely provide a competitive advantage an r-strategist like *N. europea*. However, it was surprising that no other ammonia oxidizers were indicated. Furthermore, like the CSTR, no nitrite oxidizers were indicated in the PFR reactor.

The remainder of the sequences from both reactors matched phototrophic species from the green sulfur bacteria and green non-sulfur bacteria.

2.5 Conclusions
The hypothesis that identical AOB communities would form in side-stream reactors operated in is rejected. Distinct communities formed in each side-stream reactor based on RFLP banding patterns. Other findings were as follows:

1. Nitrite accumulation in reactors treating high-strength ammonia side-streams negatively impacts startup, particularly in a CSTR reactor configuration.

2. The function of the AOB population in side-stream reactors treating high-strength ammonia wastewater does not necessarily follow structure. Ammonia oxidation was complete during startup despite variations in diversity as measured by RFLP banding patterns.

3. AOB populations in side-stream reactors treating high-strength ammonia wastewater dominate community structure in main-stream systems to which they were input.

4. *N. europea* was the most common species identified in side-stream reactors treating high-strength ammonia wastewater, particularly for the reactor in a PFR configuration.

5. Marine AOB were significant in side-stream reactors treating high-strength ammonia wastewater in a CSTR configuration.

2.6 References


Chapter 3

Ecological engineering of bioaugmentation from side-stream nitrification

3.1 Abstract

Wastewater treatment relies on careful integration of environmental engineering with microbial ecology. This would seem to be particularly the case when attempting to enhance survivability of organisms introduced from outside the main-stream reactor, i.e., bioaugmentation. Molecular biology tools were utilized in this study to assist in understanding the mechanisms of successful bioaugmentation. Molecular fingerprinting showed that side-stream reactor configuration strongly influenced ammonia-oxidizing community structure. In both lab-scale and full-scale systems, AOB communities in the side-stream and main-stream were identical. The experimental systems revealed that a batch side-stream produced greater diversity of AOB than a continuously-fed side-stream in a batch main-stream system whereas the full-scale side-stream resulted in essentially an AOB monoculture. Phylogenetic analysis revealed less diversity than molecular fingerprinting perhaps due to biases in the cloning/transformation procedure.

* This manuscript was published in its entirety in Water Science and Technology (2008) 57(12): 1927-1933

3.2 Introduction

Recently, there has been a renewed interest ecological engineering (Grady and Filipe, 2000; Thiele and Zeikus, 1988; vanLoosdrecht et al., 1997) stemming from multiple factors. For one thing, greatly improved analytical techniques and investigative methods now exist to characterize
microbial community structure and function. Another reason is that greater emphasis is being placed on advanced biological treatment techniques to meet strict effluent nutrient limits. One of the greatest challenges of biological nutrient removal (BNR) is maintaining nitrification. The low specific growth rates of nitrifying bacteria relative to the organisms that catalyze denitrification and biological phosphorous removal causes nitrification to be the rate-limiting step of BNR-without nitrification there can be no denitrification. For years, bioaugmentation has been sought as a means of stabilizing nitrification performance (Schelling et al., 1987) because bioaugmentation has its greatest applicability when the added microorganisms are slow growers such as nitrifiers (Rittmann, 1996). A potential source of nitrifiers for bioaugmentation is a sidestream treatment system for nitrogen-rich anaerobic digester supernatant.

AOB catalyze the oxidation of ammonia to nitrite, the first step in the process of nitrification. Because of their global importance, understanding of their ecology and physiology has become a subject of intense research in recent years (Kowalchuk and Stephen, 2001). A total of 16 species of AOB have been described and named. The taxonomy of the AOB includes one genus in the Gamma-subclass of the Proteobacteria (Nitrosococcus) and two genera in the Beta-subclass of the Proteobacteria (Nitrosomonas and Nitrosopira). The Beta-subclass Proteobacteria are further subdivided into three “clusters” of Nitrosospira (including “Nitrosospira”, “Nitrosolobus”, and “Nitrosovibrio”), and six “lineages” of Nitrosomonas (including “Nitrosomonas europaea/Nitrosococcus mobilis”, “Nitrosomonas communis”, “Nitrosomonas marina”, “Nitrosomonas oligotropha”, “Nitrosomonas cryotolerans”, and “Nitrosomonas sp. Nm143”) (Koops et al., 2006). The subdivisions are significant for AOB because of their strong structure-
function relationship, that is, their classification is closely related to their ecophysiological properties and, hence, their distribution in nature (Koops and Pommerening-Roser, 2001).

Molecular biology techniques are utilized in this study to examine nitrifying biomass in lab-scale and full-scale bioreactors bioaugmented from a side-stream nitrification system treating ammonia-laden digester reject water. The primary objective is to demonstrate how engineering decisions, particularly reactor configuration, influence microbial ecology and can thus be used to engineer microbial communities. A second objective is to compare AOB populations that develop in a lab-scale system compared with a full-scale system in a similar configuration.

3.3 Methods

3.3.1 Reactor operation

3.3.1.1 Full-Scale Treatment Plant.

The process diagram for the New York City 26th Ward Water Pollution Control Plant (WPCP), a 322 ML/d biological nutrient removal (BNR) activated sludge plant, is shown in Figure 3-1. The numbers represent the three biomass sampling locations. The centrate nitrification system is a four-pass, step-feed, full-scale aeration tank formerly used to treat primary effluent, but retrofitted to a plug-flow configuration to treat approximately 3.8 ML/d of centrate generated from dewatering anaerobically digested sludge. Reported centrate characteristics are shown in Table 3-1. Biomass samples were taken at a time when the effluent from the centrate tank
reactor was redirected to the main plant’s RAS stream, thus “seeding” the aeration tanks with nitrifiers. This period showed significant nitrogen removal for the plant ranging between sixty to seventy percent of the influent TKN. This could be attributed partially to “seeding”. Nitrification in the centrate reactor ranged between 80-100% of the influent TKN. The pH was controlled at a target of 7.5 with the addition Sodium hydroxide with the majority of the effluent being in the form of nitrite rather than nitrate. This fluctuated significantly as a result of influent ammonia concentrations and free-ammonia inhibition of nitrite oxidizing bacteria. Historically, the system has shown to be capable of oxidizing 70% – 90% of the centrate ammonia load when sufficient alkalinity is provided (Stinson et al., 2007).

![Flow sheet for the New York City 26th Ward Water Pollution Control Plant showing the 3 biomass sampling locations](image)

**Figure 3-1** - Flow sheet for the New York City 26th Ward Water Pollution Control Plant showing the 3 biomass sampling locations

3.3.1.2 Laboratory-Scale Reactors.

Two sets of laboratory-scale bioreactors were operated for a period of eight months. Set-one was composed of two aerated, stirred, 1-Litre chemostat reactors (A1 and B1) operated at 35°C treating a synthetic wastewater designed to mimic recycle-streams returning from anaerobic digesters containing 1,000 mg/L NH₄-N, 50 mg/L Phosphorous, between 6,000 and 7,500 mg/L alkalinity as CaCO₃, plus other micro-nutrients. The pH was controlled at a low set-point using
controllers (Cole Parmer, Vernon Hills, IL) and pH meters (Thermo Electron, Beverly, MA). Reactor A1 was operated to mimic a completely mixed configuration and reactor B1 was operated to mimic a plug-flow configuration. Set-two was composed of three aerated, 6-Litre sequencing batch reactors (A2, B2, and C2) operated in a plug flow configuration at 20°C treating a synthetic domestic wastewater containing 30 mg/L NH₄-N, 400 mg/L acetate-COD, 5 mg/L Phosphorous, plus alkalinity and other micro-nutrients. Daily, 60-mL of biomass was transferred from A1 to A2 and from B1 to B2 in a single dose. All influent ammonia was oxidized to nitrate at the time reactor biomass from the laboratory-scale reactors was sampled. The experimental reactor configuration is shown in Figure 3-2.

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<th>Maximum</th>
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<tr>
<td>TSS, mg/L</td>
<td>80</td>
<td>980</td>
<td>2,990</td>
</tr>
<tr>
<td>Total BOD – filtered, mg/L</td>
<td>12</td>
<td>68</td>
<td>966</td>
</tr>
<tr>
<td>COD – filtered, mg/L</td>
<td>116</td>
<td>548</td>
<td>3,000</td>
</tr>
<tr>
<td>Alkalinity as CaCO₃, mg/L</td>
<td>1,190</td>
<td>2,011</td>
<td>2,640</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
<td>7.6</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Table 3-1 - Characteristics of New York City WPCP centrate (Mishalani and Husband, 2001)
3.3.2 Molecular Techniques

3.3.2.1 Restriction Fragment Length Polymorphism (RFLP).

Chromosomal DNA was isolated from 2 mL biomass samples and the amoA gene was amplified using the polymerase chain reaction (PCR) with a primer set previously developed to target a broad range of AOB (Rotthauwe et al., 1997). Amplified amoA was digested using restriction endonucleases Rsal and HaeIII simultaneously (Promega, Madison, WI). The restriction fragments were separated by molecular weight using gel electrophoresis and visualized as bands.
3.3.2.2 Phylogenetic Analysis.

PCR was utilized to amplify amoA as before. Fresh PCR product was ligated with the pCR®2.1-TOPO® vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent *Escherichia coli* according to manufacturer’s instructions. Transformed, white colonies were picked from petri dishes containing LB agar with ampicillin using sterile toothpicks. The toothpicks were incubated overnight in broth tubes containing LB media. The cell cultures were transferred to 1.5 mL centrifuge tubes and centrifuged to generate a cell pellet. Following decant of the broth, plasmids were extracted and purified using the Qiagen (Japan) Mini-preps plasmid extraction kit according to the manufacturer’s instructions. Purified plasmids were submitted to the University of Cincinnati DNA Core Lab for sequencing. Nucleic acid sequences were converted to FASTA format and imported to BioEdit v5.0.9 (Hall, 1999). Preliminary proofreading was performed by manual inspection, and low quality sequences were discarded. Very similar sequences that exhibited greater than 95% nucleic acid similarity were discarded to yield a set of unique sequences per sample. Reference sequences were imported from GenBank and aligned with the unique environmental sequences using ClustalX using default parameters (Thompson et al., 1997). Thereafter, the sequences were trimmed to 414 nucleotides and realigned. The alignment was imported into PAUP 4.0b10 and a phylogenetic tree was created using the distance optimality criterion with the neighbor-joining clustering option. A bootstrap tree was then created using the full heuristic search option with 100 replicates and the Kimura-2 evolutionary model.
3.4 Results and Discussion

3.4.1 RFLP

Little variability in the RFLP banding pattern between sample locations was observed for the 26th Ward samples as indicated in Figure 3-3(a). All 3 samples (including duplicates) produced fragments at or around 40 bp, 50 bp, 150 bp, 170 bp, 200 bp, and 250 bp. Within samples, the bands representing fragment lengths of 150 bp and 170 bp produced very strong signals in all samples. The band representing 200 bp had the weakest signal in all samples. A possible explanation for the low variability observed despite the vastly different physico-chemical conditions between the sampling locations is the enrichment of certain AOB populations in the nitrogen-rich centrate nitrification tank leading to dominance in the main-stream.

The banding patterns for the laboratory-scale systems indicate much higher diversity than the 26th Ward as more complex RFLP banding patterns are produced (Figure 3-3b). It is also apparent that reactor configuration profoundly impacts the structure of the AOB community. The biomass from the plug-flow treatment train (train “B”) generated fragments at 200 bp, 225 bp, and 250 bp which were clearly absent from the continuous flow train (train “A”). Furthermore, the 150 bp fragment is observed in plug-flow train at a much higher intensity indicating greater mass.

An in silico digestion was performed in an attempt to identify the AOB represented in the restriction diagrams. The restriction map utility available in BioEdit (Hall, 1999) was used to
generate theoretical fragments from reference *amoA* sequences retrieved from GenBank. A comparison of the experimental fragment sizes observed with theoretical fragment sizes from selected reference species is shown in Table 3-2. The data indicate that multiple AOB species are present in the 26th Ward samples as there are no perfect matches between samples and reference species. Further analysis of band presence/absence reveals the likely presence of organisms C-91 (*N. eutropha*) and Nm2 (*N. communis*). C-91 was the only organism found to produce a fragment around 250 bp in length. Nm2 (*N. communis*) was the only organism found to produce a fragment around 200 bp in length. Nm2 would have also generated a 90 bp fragment which was not observed in the sample. One possible explanation is that the band was below the detection limit. The intensity of the 200 bp band is already quite weak so the 90 bp, with a lower molecular weight, would have been even weaker and perhaps below detection.
Figure 3-3 - RFLP of amoA from full-scale (a) and experimental bioreactor (b) systems. L1 = 100 bp DNA ladder, L2 = 25 bp DNA ladder, 1 = 26th Ward mixed liquor, 2 = 26th Ward return activated sludge, 3 = 26th Ward centrate nitrification tank, A1, B1, A2, B2, C2 lab bioreactor samples

RFLP suggested that multiple AOB were also present in each lab-scale reactor system. The banding pattern from control reactor C2 includes a band near 250 bp which would indicate the presence of organism C-91. Bands near 105-bp and 175-bp would only be produced if organism Nm103 (*N. europaea*) were present. A 75 bp fragment would be observed in the sample if either Nm90 (*N. nitrosa*) or Nm22 (*N. marina*) were present but both organisms would produce a 90-bp fragment, and *N. marina* would produce a 120-bp fragment as well, neither of which were observed. Thus the control reactor samples show evidence for up to three types of AOB, although the biodiversity in the sample cannot be completely accounted for by the reference species analyzed. The sample from treatment train “A” (continuous flow) also shows evidence of three AOB. In addition to C-91 and Nm103, the observance of a 75-bp along with a 90-bp fragment implies the presence of organism Nm90. However, bands at 150-bp and 250-bp are weak suggesting that organism C-91 is less prevalent compared to the control reactor. The “B”
train (plug flow) appeared to harbor the highest diversity. In addition to the bands observed in the control reactor, bands were observed at 90-bp, 200-bp, and 225 bp. A 200-bp fragment is characteristic of organism type Nm2. A 225-bp fragment is characteristic of organism Nc2 (*N. mobilis*). Hence, the plug flow treatment train shows evidence for the existence of five types of ammonia-oxidizing organisms including Nm103, C-91, Nm2, Nc2, and Nm90.

Thus two clusters, or phylogenetic groups, of organisms were found to exist in the treatment systems incorporating side-stream treatment: one representing *N. europaea* (Nm103), *N. eutropha* (C-91), and *N. mobilis* (Nc2); and the other representing *N. nitrosa* (Nm90) and *N. communis* (Nm2). Although phylogenetically separated, these organisms are similarly distributed in more or less eutrophicated environments. All have high substrate affinity and thus favor high ammonia concentrations, such as in wastewater treatment plants (Koops et al., 2006).

<table>
<thead>
<tr>
<th>Sample</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>105</th>
<th>120</th>
<th>135</th>
<th>150</th>
<th>175</th>
<th>200</th>
<th>225</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>26&lt;sup&gt;th&lt;/sup&gt; Ward</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>A1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>B1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Nc2</td>
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<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>C-91</td>
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<td>-</td>
</tr>
<tr>
<td>Nm51</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>N. nitrosa</em></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td><em>N. marina</em></td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
</tr>
</tbody>
</table>

Table 3-2 - Band presence/absence from RFLP of environmental samples and in silico digestion of reference sequences. A “+” indicates that a band was observed at the indicated fragment size (sample) or that the indicated fragment size is predicted from an in silico digestion (reference sequence).
3.4.2 Phylogenetic Analysis

In order to obtain higher resolution of AOB communities, amoA clones retrieved from the full-scale and laboratory-scale reactors were cloned and sequenced. Cloned amoA sequences exhibited remarkably low species diversity in the AOB community, in modest contrast with RFLP. Preliminary screening revealed that the majority of clones within each sample were identical. Out of a total of ten clones for each sample, four from laboratory-scale reactors A2 and B2 and one from laboratory-scale reactor C2 were deemed unique (<95% nucleic acid similarity) and prepared for further analysis. Seven of the ten clones from a sample of mixed liquor taken at the full-scale treatment facility were exactly identical the other three were greater than 95% identical, so only one clone was prepared for further analysis. The RFLP analysis revealed that AOB populations were identical throughout the full-scale treatment plant so the samples taken from the return activated sludge and centrate nitrification tank were not cloned and sequenced.

The ten unique amoA sequences and sequences from 22 reference organisms were aligned and compared. RFLP analysis revealed that the sampled AOB communities were dominated by N. europaea-like, N. eutropha-like, and N. communis-like organisms so reference organisms representing clusters 7 and 8 (Pommerening-Roser et al., 1996) were selected. Two of the four sequences retrieved from reactor A2 were most similar (100% and 96% similarity) to the amoA sequence described for Nitrosomonas europaea strain Nm50. The other two were most similar to the sequence from Nitrosomonas species ENI-11 (98% similarity each). Likewise, two of the four sequences retrieved from reactor B2 were most similar (97% similarity each) to the amoA
sequence described for *Nitrosomonas europaea* strain Nm50. The other two were most similar to the sequence from *Nitrosomonas* species ENI-11 (98% and 88% similarity). The unique sequence from reactor C2 was 100% identical to the sequence obtained for *Nitrosomonas europaea* strain C-31. The unique sequence from the full-scale reactor was most similar (85% similarity) to the sequence obtained for *Nitrosomonas* GH22.

A phylogenetic tree showing the relationships between the unique environmental sequences and several reference sequences is shown in Figure 3-4. In addition to the sequences from the above reference species, *amoA* sequences from Gamma-AOB and the corresponding gene fragment of the particulate methane monooxygenase (pmo) gene from select methane oxidizers were included for tree-rooting purposes. The tree shows, as expected, that all *amoA* sequences retrieved from the laboratory-scale reactors are most closely related to strains of *N. europaea* within cluster 7. The clone from the New York 26th Ward WPCP, on the other hand appears to be more closely related to *N. eutropha*, also within cluster 7.
Figure 3-4 – Consensus phylogenetic amoA tree constructed using 414 aligned nucleotides from reference organisms and unique sequences retrieved from full-scale and lab-scale reactors. The bootstrapped neighbor-joining tree was created in PAUP 4.0b10 using full heuristic search option along with the Kimura-2 distance parameter. A bootstrap tree was then created using the full heuristic search option with 100 replicates and other default parameters.

3.5 Conclusions

Molecular fingerprinting using RFLP shows that AOB community structures of varying composition and complexity form without obvious differences in nitrification performance. This observation is in line with previous research work (Egli et al., 2003).

Molecular fingerprinting results also show that side-stream reactor configuration strongly influences AOB community structure in the main-stream. AOB communities in the side-stream and main-stream were identical.
The experimental systems revealed that sidestream bioaugmentation did increase AOB diversity in the main-stream and that a batch side-stream produces greater diversity of AOB than a continuously-fed side-stream in a batch main-stream system. On the other hand, the full-scale side-stream system selected for essentially, a monoculture. This was likely the result of a feed-forward mechanism resulting from system configuration. The side-stream enriched endogenous AOB populations in the main-stream giving them a competitive edge over invaders. An important implication of this result is how it impacts system reliability. It has been postulated that biodiversity enhances ecosystem reliability (Naeem and Li, 1997). If this is the case, blending of RAS with centrate in side-streams may not increase system reliability as much as a side-stream treatment system isolated from the endogenous biomass.

Phylogenetic analysis of cloned sequences from laboratory-scale reactors did not suggest as much diversity as RFLP. All sequences closely matched sequences in the *N. europaea* lineage. The cause of this discrepancy is not known. One possible explanation is bias resulting from the cloning/transformation procedure but this possibility was not investigated. Relatively speaking, however, more unique sequences were retrieved from bio-augmented main-stream reactors compared to the control suggesting that bioaugmentation from side-streams may increase biodiversity.

3.6 References


Chapter 4

Respirometric evaluation of side-stream treatment of reject water as a source of nitrifying bacteria for main-stream activated sludge bioreactors

4.1 Abstract

A laboratory-scale bioreactor study was conducted to characterize differences in nitrification function in main-stream reactors due to bioaugmentation from side-stream reactors treating reject water. The objective was to evaluate how configuration of a suspended growth side-stream bioreactor impacts nitrification function in the main-stream bioreactor. A bioaugmentation effect was not observed in main-stream reactors operated at warm temperatures. Complete oxidation of ammonia to nitrate was observed in the bioaugmented and control main-stream reactors although nitrite accumulation was observed in each case. Furthermore, respirometry did not reveal superior kinetics in bioaugmented reactors operated at warm temperatures. At cold temperatures bioaugmentation may have stabilized ammonia oxidation in main-stream reactor B2 bioaugmented from a PFR side-stream. Complete ammonia oxidation was observed for most of cold period of operation in the main-stream bioreactor B2. Furthermore, respirometry revealed a higher rate of ammonia oxidation and more stable nitrite oxidation compared with the control bioreactor.

* This manuscript was published in its entirety in Water Science and Technology (2009) 60(10): 2677-2684
4.2 Introduction

Anaerobic digester reject water may comprise up to 20% of the total reactive nitrogen load influent to a municipal wastewater treatment plant—even greater proportions in centralized solids handling facilities—but is generally less than 1% of wastewater flow (Mossakowska et al., 1997). Thus treatment of digester reject waters can benefit operation by eliminating a troublesome recycle stream. Furthermore, bioaugmentation of main-stream bioreactors with nitrifying biomass generated in a side-stream treatment system can increase main-stream solids retention time (SRT) and release reactor volume for other purposes thereby also providing a significant economic benefit to treatment plants needing to add treatment capacity or nutrient removal capability. The development in recent years of multiple side-stream treatment configurations means that the engineer has many choices when it comes to designing a side-stream treatment system. One of the most fundamental decisions engineers make is the choice between a completely stirred tank reactor (CSTR) configuration and a plug flow reactor (PFR) configuration. The potential for inhibition due to toxicity from high concentrations of ammonia nitrogen in reject water would be the classic case for a CSTR configuration. On the other hand, past research (Chudoba et al., 1985) has shown that a plug-flow configuration provides higher rates of nitrification and is less susceptible to substrate inhibition. The objective of this study was to evaluate how configuration of a suspended growth side-stream bioreactor impacts nitrification function in the main-stream bioreactor.
4.3 Methods

4.3.1 Bench-Scale Reactor Operation

Two sets of laboratory-scale bioreactors were operated. Set one was composed of two aerated, stirred, chemostats (A1 and B1) treating synthetic anaerobic digester reject water. A CSTR or PFR configuration was simulated by feeding hourly or in one batch each day, respectively. Set two was composed of three aerated, 6-Liter sequencing batch reactors (A2, B2, and C2) treating a synthetic domestic wastewater and operated in a PFR configuration by feeding once per cycle. A portion of the waste biomass (60 mL) from the side-stream reactors was transferred from A1 to A2 and from B1 to B2 in a single dose each day. The experimental reactor configuration is described in Figure 4-1 and Table 4-1.

Reactor operation was divided into two periods: warm and cold. During the warm period the reactors were operated at 20°C for 8 SRT’s following start-up. A transition period followed during which the main-stream reactors were moved into a temperature-controlled room and temperature was gradually reduced down to 10°C for the cold period of operation. The reactors remained at 10°C for 32 SRTs. Reactor nitrification performance was evaluated during both warm and cold periods by routine analysis of ammonia-nitrogen (NH$_3$-N), nitrite-nitrogen (NO$_2$-N), and nitrate-nitrogen (NO$_3$-N).
### Table 4-1 – Laboratory-Scale Bioreactor System Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Side-Stream Reactors</th>
<th>Main-Stream Reactors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (L)</td>
<td>CSTR (A1)</td>
<td>PFR</td>
</tr>
<tr>
<td>Configuration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>35</td>
<td>Variable</td>
</tr>
<tr>
<td>Hydraulic Residence Time (days)</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>Solids Residence Time (days)</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>

![Figure 4-1 Laboratory-Scale Bioreactor Configuration](image)

#### 4.3.2 Respirometry

Biomass wasted from the main-stream reactors was recovered for use in batch respirometry. Two types of respirometric assays were conducted. Oxygen uptake assays using a commercial respirometer were conducted for the purpose of estimating kinetic coefficients. A second type of assay was conducted by measuring inorganic nitrogen concentrations for the purpose of
characterizing ammonia and nitrite oxidation to improve estimation of kinetic coefficients from the oxygen uptake assays.

4.3.2.1 Oxygen uptake assays

After washing residual oxygen demand from the biomass using a phosphate buffered saline (PBS) solution biomass was diluted with PBS, combined with nutrients and substrate in 250-mL media bottles, and placed into a commercial oxygen replenishment type respirometer (Challenge Environmental Systems) set up in a room with a controlled temperature of 26°C. A concentrated solution of ammonium chloride was injected into each assay to provide an initial substrate concentration equal to 46 mg/L nitrogenous oxygen demand (NOD). The endpoint of the assay was the point at which cumulative oxygen uptake reached the NOD of the input substrate or leveled off without increasing for an extended period. Assays were conducted daily or semi-daily for one SRT each during the warm and cool period of operation. One or more assays with biomass but not exogenous substrate (seed) were conducted with each batch of assays.

The Equations representing biomass growth, substrate consumption, and oxygen uptake for a two-step nitrification model developed by others (Chandran and Smets, 2000) are summarized in the Petersen mantrix shown in Table 4-2. Differential equations for each species can be obtained by multiplying the kinetic term in the last column of Table 4-2 with the appropriate stoichiometric term in columns 2 through 5. $S$ is the reduced nitrogen concentration (mg NOD L$^{-1}$), $f_s$ is the biomass yield coefficient, $OU$ is the cumulative oxygen uptake, $\mu_{max}$ is the maximum specific growth rate (h$^{-1}$), and $K_s$ is the half-saturation coefficient. The subscript $ns$ is
used to denote ammonia oxidizing bacteria (AOB) activity and the subscript \( nb \) is used to denote nitrite oxidizing bacteria (NOB) activity.

Table 4-2 – Petersen Matrix Summarizing the equations for substrate consumption, oxygen consumption, and biomass synthesis for two-step nitrification model

<table>
<thead>
<tr>
<th>Oxidation by</th>
<th>( S_{nh} )</th>
<th>( S_{no2} )</th>
<th>( OU_{ns} )</th>
<th>( OU_{nb} )</th>
<th>( X_{ns} )</th>
<th>( X_{nb} )</th>
<th>Rate Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X_{ns} )</td>
<td>( 1 )</td>
<td>( 1 - \frac{X_{ns}}{K_{ns}} )</td>
<td>( 1 - \frac{X_{ns}}{K_{ns}} )</td>
<td>+1</td>
<td>( \mu_{max_{ns}} = \frac{X_{ns} + X_{nb}}{K_{ns} + S_{ns}} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( X_{nb} )</td>
<td>( 1 + \frac{(S_{no2} - S_{ns})}{K_{no2}} )</td>
<td>( 1 - \frac{X_{nb}}{K_{nb}} )</td>
<td>( 1 - \frac{X_{nb}}{K_{nb}} )</td>
<td>+1</td>
<td>( \mu_{max_{nb}} = \frac{X_{nb} + S_{nb} + S_{ns}}{K_{nb} + S_{nb}} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The kinetics of NH\(_4\)\(^+\)-N to NO\(_2\)\(^-\)-N oxidation and NO\(_2\)\(^-\)-N to NO\(_3\)\(^-\)-N oxidation were estimated by fitting the numerical solutions of differential equations described in Table 4-2 to oxygen uptake profiles obtained in response to NH\(_4\)\(^+\)-N pulses. The differential equations were solved simultaneously using a fourth-order Runge-Kutta method. Biokinetic parameters for each oxidation step were mathematically estimated by minimizing the sum of squared errors (SSE) between the solution of the appropriate differential equation and the experimental oxygen-uptake data using the SOLVER utility in MS Excel. Substrate and biomass concentrations were expressed in terms of oxygen demand such that each mg of ammonia nitrogen is equal to 3.5 mg NOD, each mg of nitrite nitrogen is equal to 1.1 mg NOD, and each mg biomass volatile suspended solids is equal to 1.42 mg chemical oxygen demand (COD). The values for yield coefficients were assumed as reported in the literature (Chandran and Smets 2000).

On account of correlation between \( \mu_{max} \), \( X \), and \( f_{s} \), the maximum specific NH\(_4\)\(^+\)-N and NO\(_2\)\(^-\)-N oxidation activities were expressed as pseudo first order rate coefficients, \( k \) as follows:

\[
k = \frac{\mu_{max}}{f_{s}} \times \frac{X}{K_{s}}
\]
4.3.2.2 Nitrogen assays

500 mL of waste biomass from each of the main-stream reactors was directly placed into a 1 L open-top beaker. Each beaker was mixed mechanically using a magnetic stir bar, flushed with air supplied through an aquarium diffuser stone, and buffered with bicarbonate. Assay duration was chosen to match the length of the aeration cycle in the laboratory-scale reactors. A concentrated solution of ammonium chloride was injected into each assay to provide an initial substrate concentration equal to 46 mg/L nitrogenous oxygen demand (NOD). A small sample was taken from each beaker at routine intervals to measure pH, NH$_3$-N, NO$_2$-N, and NO$_3$-N.

4.4 Results and Discussion

4.4.1 Bench-Scale Bioreactor Operation

Nitrification performance for the laboratory-scale bioreactors is shown in Figure 4-2. Nitrification was complete and stable in all five reactors during the entire period of main-stream reactor operation near 25°C. Periods of instability were experienced in all reactors thereafter. Two brief periods of persistent elevated effluent nitrite occurred in the CSTR side-stream reactor (A1). Complete nitrite oxidation resumed after each period. On the other hand, nitrite oxidation was very unstable in the PFR side-stream reactor (B1) about two-thirds of the final 350 days of the experiment. The instability of the PFR side-stream may have been due to inhibition from free ammonia which would have been at a concentration of around 5 mg/L as N at the beginning of each cycle. Inhibition to NOB is thought to occur at a free ammonia concentration of 0.1 to
1.0 mg/L as N (Anthonisen et al., 1976). Although, complete and stable nitrite oxidation did occur at the beginning of the experiment, the reaction could be prone to upset from the slightest perturbation. On the other hand, ammonia oxidation may be all that is desired if denitrification is practiced.

Nitrification became unstable in all three main-stream reactors after the operating temperature reached the minimum of 10°C on Day 128. Both ammonia oxidation and nitrite oxidation were unstable in reactor A2, which receives biomass input from the CSTR side-stream. Effluent nitrite peaked at 23.8 on Day 167; effluent ammonia peaked at 16.5 mg/L on Day 273. Complete nitrification did not resume until after Day 388, following 260 days of operation at 10°C. Although ammonia oxidation remained strong throughout the experiment, nitrite accumulation did occur in reactor B2, input from the plug flow side-stream. Effluent nitrite from reactor B2 peaked at 19.5 mg/L on Day 273. Nitrate became the dominant inorganic nitrogen species again on Day 358. A period of partial ammonia oxidation occurred in reactor C2 from Day 240 through Day 290, otherwise ammonia oxidation was strong. However, nitrite oxidation was very weak during the initial 300 days of cold-temperature operation in reactor C2 with no biomass input. Nitrate became the dominant effluent inorganic nitrogen species after Day 388.

Performance results from bench-scale reactor operation do not indicate a benefit from bio-augmentation for main-stream reactor operation at warm temperatures. At cold temperatures bioaugmentation may have stabilized ammonia oxidation during the transition from warm to cold temperatures and shortened the recovery period for nitrite oxidation in main-stream reactor B2. A similar bioaugmentation effect was not observed in main-stream reactor A2. One possible
explanation is that AOB from the PFR side-stream biomass was acclimated to a high substrate gradient and, hence could have been expected to more successfully incorporate into a PFR main-stream. Conversely, AOB from the CSTR side-stream would have acclimated to a low gradient and may have been sensitive to relatively high concentrations of ammonia.

4.4.2 Respirometry Nitrogen Assays

A set of nitrogen assays was conducted on day 18 during the warm period of operation at 25°C (Figure 4-3). Nitrification was complete and stable in the main-stream reactors at the time the assays were conducted as indicated in Figure 4-2. Despite the sampling interval, characteristics of nitrification function were evident. All three assays exhibited substantial nitrite accumulation indicating that both ammonia oxidation and nitrite oxidation were rate-limiting. Nitrite accumulation appeared to be most severe for bioaugmented biomass from reactor B2 and least severe for control reactor biomass. Nitrite accumulation in all three main-stream reactors is probably attributable to a relatively higher growth rate for AOB compared to NOB at temperatures in excess of 20°C (Hellinga et al., 1998). Aside from that, control reactor biomass (C2) appeared to exhibit a higher initial rate of ammonia oxidation and a lower half-saturation coefficient for nitrite oxidation than bioaugmented biomass (A2 and B2). Thus, carryover of factors from the side-stream reactors may have been detrimental to main-stream nitrification.

A second set of nitrogen assays was conducted on day 223 during the cold period of operation at 10°C. The data presented in Figure 4-4 reflect the observations in Figure 4-2. Ammonia oxidation was incomplete in reactor A2 resulting in nearly equal concentrations of NH₃-N and
NO$_2$-N in the effluent. Ammonia oxidation was strong in reactor C2 but it appears that NOB were nearly completely washed out resulting in an effluent NO$_2$-N concentration equal to the input NH$_3$-N. In general, persistence of reduced forms of nitrogen in the effluent from reactors A2 and C2 was believed to be attributable to low concentrations of AOB and NOB. On the other hand, nitrification was complete and stable in reactor B2 resulting in nearly complete oxidation of ammonia to nitrate.

4.4.3 Respirometry Oxygen Uptake Assays

Typical experimental seed-corrected cumulative oxygen uptake (markers) and modeled total cumulative oxygen uptake (dashed lines) for assays conducted during the warm period of reactor operation between Day 20 and Day 27 are shown in Figure 4-5. Modeled oxygen uptake associated with ammonia and nitrite oxidation is also shown in Figure 4-5. A two-step model was utilized on account of the significant nitrite accumulation observed from each of the nitrogen assays presented in Figure 4-3. The initial slope of each respirogram represents the maximum oxygen uptake rate (OUR) which is directly proportional to the maximum growth rate and the initial active biomass concentration. The model indicates that this portion of each assay was dominated by ammonia oxidation for each assay. Thus it appears that AOB biomass from reactors A2 and C2 had superior growth properties. On the other hand, a more abrupt transition from the maximum rate of ammonia oxidation to completion of the reaction indicates higher substrate affinity in the biomass from reactor B2 compared with the biomass from reactors A2 and C2. Comparison of the slope for nitrite oxidation according to the model indicates that NOB exhibited the highest growth rate in reactor C2. The model indicates that nitrite oxidation did not
complete in reactor A2 during the course of the assay. Variation in the tail end of each assay was attributed to heterotrophic endogenous activity and not to nitrifying bacteria.

Figure 4-2 - Influent and effluent nitrogen for bench-scale bioreactors: (a) A1, (b) B1, (c) A2, (d) B2, (e) C2
Figure 4-3 - Ammonia and nitrite profiles for main-stream reactors acclimated to $T = 25^\circ C$: (a) A2, (b) B2, (c) C2
Figure 4-4 - Ammonia and nitrite profiles for main-stream reactors acclimated to T = 10°C: (a)A2, (b)B2, (c)C2

No attempt was made to characterize nitrification kinetics during the cold period of operation because of the instability of the reaction in the bench-scale reactors experienced during that phase of the experiment.
The pseudo first order rate coefficient, \( k \), and half-saturation coefficient, \( K_s \), for ammonia oxidation and nitrite oxidation for the biomass from each main-stream reactor are shown in 4-3. The estimated biokinetic coefficients for ammonia oxidation for control reactor biomass were superior to the coefficients for bioaugmented biomass with a higher \( k \) and a lower \( K_s \). As for nitrite oxidation, the estimated kinetics of nitrite oxidation were least favorable for biomass bioaugmented from a CSTR side-stream and most favorable for biomass bioaugmented from a PFR side-stream. Thus, the respirograms and the estimates of biokinetic properties made from the respirogram data for nitrification at warm temperatures confirm the findings of bench-scale reactor operation, specifically that there is no clear benefit to nitrification from bioaugmentation.
Table 4-3 - Kinetic Parameter Estimates for Main-Stream Reactor Biomass Acclimated to 25°C

<table>
<thead>
<tr>
<th></th>
<th>Ammonia Oxidation</th>
<th>Nitrite Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k ( (1/\text{hr.}) )</td>
<td>( K_s ) ( (\text{mg NOD/L}) )</td>
</tr>
<tr>
<td>A2</td>
<td>11.9</td>
<td>1.56</td>
</tr>
<tr>
<td>B2</td>
<td>5.50</td>
<td>2.71</td>
</tr>
<tr>
<td>C2</td>
<td>44.7</td>
<td>0.339</td>
</tr>
</tbody>
</table>

4.5 Conclusions

Ammonia oxidation and nitrite oxidation are both rate-limiting in bioreactors with a plug flow configuration and operated at temperatures of 25°C and higher.

A CSTR side-stream reactor configuration provided more stable nitrite oxidation in side-stream reactors treating reject water.

There was no apparent benefit to bioaugmentation for main-stream bioreactors operated at warm temperatures. In fact, carryover of factors from the side-stream reactors may have been detrimental to main-stream nitrification.

Bioaugmentation may have prevented washout of critical populations in the main-stream bioreactor coupled to a PFR side-stream bioreactor thereby stabilizing nitrification. The potential benefit to municipal wastewater treatment is faster recovery of nitrification following a seasonal deterioration due to reduced wastewater temperature.
4.6 References


Chapter 5
Conclusions, significance, and future research

5.1 Conclusions

This research investigated the influence of reject water side-stream bioreactor configuration on AOB diversity and nitrification performance in side-stream and main-stream bioreactors. The major findings of this research in relation to side-stream and main-stream bioreactor AOB community structure and nitrification function are summarized in the sections that follow. The central hypothesis is for the research is that “Biodiversity enhances function”.

5.1.1 Side-stream bioreactor AOB community structure

Specific Aim #1 was designed to test the hypothesis that identical AOB communities would form in side-stream reactors operated in different configurations. This hypothesis is rejected. Distinct communities formed in each side-stream reactor based on RFLP banding patterns. Also,

- *N. europea*, an ecological r-strategist, was the most common species identified in side-stream reactors treating reject water.
- Marine AOB were significant in side-stream reactors treating high-strength ammonia wastewater in a CSTR configuration.
5.1.2 Side-stream bioreactor nitrification function

Nitrite accumulation in reactors treating high-strength ammonia side-streams negatively impacts startup, particularly in a CSTR reactor configuration. However, once startup completed, a CSTR side-stream reactor configuration provided more stable nitrite oxidation in side-stream reactors treating reject water.

AOB community function does not necessarily follow structure in side-stream bioreactors treating reject water. Molecular fingerprinting using RFLP shows that AOB community structures of varying composition and complexity form without obvious differences in nitrification performance.

5.1.3 Main-stream bioreactor biodiversity

AOB populations in side-stream reactors treating reject water dominate community structure in main-stream systems to which they are input. Molecular fingerprinting results revealed that AOB communities in the side-stream reject water reactors and their associated main-stream reactors were identical.

A greater number of unique sequences were retrieved from bio-augmented main-stream reactors compared to the control suggesting that bioaugmentation from side-streams increases biodiversity.
5.1.4 Main-stream bioreactor nitrification function

Specific Aim #2 was designed to test the hypothesis that main-stream nitrification stability is not enhanced by bioaugmentation. This hypothesis was accepted for conditions in which nitrification was already stable in the main-stream reactor. Some evidence was observed that bioaugmentation provides stable nitrite oxidation in a main-stream bioreactor under temperature stress.

Ammonia oxidation and nitrite oxidation are both rate-limiting in bioreactors with a plug flow configuration and operated at temperatures of 25°C and higher.

Nitrification function in main-stream bioreactors operated at warm temperatures was equally effective with or without side-stream bioaugmentation.

Nitrification performance of a main-stream bioreactor under temperature stress with bioaugmentation from a PFR side-stream was found to be more stable than nitrification performance of a main-stream bioaugmented from a CSTR side-stream or not bioaugmented at all.

5.2 Significance

Molecular analysis of experimental and full-scale systems revealed that the AOB community structure in side-stream reject water treatment bioreactors matches the AOB community structure
in the main-stream bioreactors in which the side-stream biomass is input. This was true for either a two-sludge configuration in which side-stream biomass is isolated from the main-stream or for a single-sludge system with continuous re-inoculation of the side-stream bioreactor from the main-stream RAS. It is also evident that bioaugmentation of main-stream bioreactors with biomass from side-stream reject water treatment increases AOB diversity in the main-stream. Furthermore, a main-stream bioreactor with biomass input from a PFR side-stream exhibited greater AOB diversity than a main-stream bioreactor with biomass input from a CSTR side-stream. A potential benefit of increased AOB diversity due to bioaugmentation from the side-stream system is increased system reliability in the face of short-term toxicity events. Furthermore, side-stream bioreactors would be beneficial as a source of nitrifiers to re-seed the main-stream bioreactor following a seasonal transition to cooler wastewater temperatures or short-term peak flows during wet weather conditions. In this research, bioaugmentation may have prevented washout of critical populations in the main-stream bioreactor coupled to a PFR side-stream bioreactor thereby stabilizing nitrification.

5.3 Future Research

This study focuses on measuring structural diversity of AOB and functional stability of nitrification in main-stream activated sludge systems bioaugmented from side-stream reject water treatment systems. Future work should focus on measuring functional diversity using molecular biology assays such as FISH to identify and quantitate 16S rRNA and precursor 16S rRNA from AOB in order to determine which species are successfully bioaugmented and understand the mechanisms that enable them to incorporate into main-stream biomass. The
objective is to begin to develop a model for populations of ammonia-oxidizing bacteria (AOB) based on their macromolecular composition, specifically 16S ribosomal ribonucleic acid (rRNA) levels. This was the original intent of Specific Aim #3 (Chapter 1), but significant progress was not made. Instead, a novel assay using quantitative PCR was developed (Appendix A). The molecular target of the quantitative assay, the gene encoding for the red copper protein nitrosocyanin, \( ncyA \), could also be utilized in a macromolecular composition model. The useful characteristics of the \( ncyA \) gene are that it occurs only as a single copy in all genomes of aerobic AOB, and no homologous sequences have been identified in other organisms. Thus it is a more specific identifier of AOB than 16S which occurs in all bacteria, and AMO which is homologous to methane monooxygenase found in methane oxidizing bacteria.

Also, measurement of the structural diversity of the nitrite oxidizing bacteria would provide useful insights into second step of the nitrification process since the results from this study suggest that the nitrite oxidation step may be less stable than ammonia oxidation for side-stream reject water treatment systems.

Molecular characterization of samples from full-scale systems, especially In-Nitri, BABE, Bioaugmentation R and AT-3 in order to contrast the relative bioaugmentation efficiencies of one-sludge and two-sludge systems.
Appendix A

Evaluation of activated sludge bioaugmentation using real-time PCR for quantitation of ammonia oxidizing bacteria

A.1 Abstract

Quantitative real-time PCR was utilized to evaluate the efficiency of bioaugmentation from side-stream treatment in a laboratory-scale activated sludge system. Total ammonia-oxidizing bacteria (AOB) were quantified using a primer set developed previously targeting the gene encoding for ammonia monooxygenase (AMO), amoA. A novel target, the gene encoding for the red copper protein nitrosocyanin, ncyA, was used to monitor *N. europaea*-like AOB using primers developed in this study. This target is ideal for quantitative PCR applied to activated sludge because the ncyA gene occurs only as a single copy in all genomes of aerobic AOB, and no homologous sequences have been identified in other organisms. In general, AOB were found in greater abundance in the main-stream reactor bioaugmented from a plug-flow side-stream reactor compared to the reactor bioaugmented from a continuous-flow side-stream reactor and a control reactor not bioaugmented, in particular during the initial period of reactor operation at warm temperatures. Consistently low occurrence of *Nitrosomonas*-like sequences was observed in all reactors utilizing the new primers developed for the novel target sequence.

* This manuscript was submitted for publication in its entirety to Water Research.

A.2 Introduction

Reliable quantification of ammonia-oxidizing bacteria (AOB) is needed to permit optimal design and operation of activated sludge reactors. The present status of wastewater system design is
characterized by routines that predict nitrifier biomass based on measurements of raw wastewater characteristics, estimates of mixed liquor volatile suspended solids (MLVSS), and presumed biokinetic parameters (Sedlak 1991). However, respirometry data indicate that VSS measurements overestimate total active biomass (Young and Cowan 2004). Furthermore, measurements of yield coefficients are unreliable and highly dependent upon culture conditions and history (Grady et al. 1999). As a result, activated sludge nitrifier biomass may be overestimated and many wastewater bioreactors may be operating suboptimal. It has been proposed that such overestimates can lead to unstable operation and excessive capital and operating costs (Dionisi et al. 2002, Coskuner et al. 2005).

In the last decade, new methods for quantitating AOB have been developed based on molecular biology techniques. The two most common approaches for estimating absolute numbers of AOB in activated sludge are fluorescence in situ hybridization (FISH) (Daims et al. 2001, Coskuner et al. 2005) and quantitative polymerase chain reaction (qPCR) (Dionisi et al. 2002, Harms et al. 2003, Araki et al. 2004). FISH has been called the “gold standard” for quantitation of bacterial cells in the environment because it allows enumeration of individual cells (Coskuner et al. 2005), nevertheless, the method has important disadvantages. Manual counting techniques are arduous and time-consuming, and results are not statistically significant (Daims et al. 2001). Furthermore, the relatively high detection limit of FISH can lead to undercounting or non-detection of important populations if local cell densities are not sufficient to generate signal (Rittmann et al. 1999). Other authors suggest that PCR-based quantitation is more appropriate because of its high analytical sensitivity and precision (Dionisi et al. 2002). Furthermore, compared to other PCR-based assays, real-time qPCR is rapid, has a very large dynamic range of
starting target molecule determination (Heid et al. 1996), and is simple to conduct, particularly using pre-mixed ingredients available from many vendors. A disadvantage of PCR-based methods is that they do not provide an absolute cell number. This may be significant in cases where microorganisms contain more than one copy of a particular target gene.

So far, qPCR assays have been developed for two loci, genes encoding for 16S ribosomal RNA and the A subunit of the enzyme ammonia monooxygenase (AMO), \textit{amoA}. The fact that all known genomes of betaproteobacterial AOB (Beta-AOB) including the nitrosomonads contain only one copy of the 16S rRNA gene (Arp et al. 2007) makes this locus an ideal target for quantitative assays. However, due to the highly conserved nature of the molecule and restrictions on the size of the amplicon for optimal real-time PCR (RT-PCR), primer specificity is a troublesome problem (Utaker and Nes 1998). In contrast, the \textit{amoA} gene is a highly specific target that exhibits a high degree of similarity among distantly-related AOB while discriminating against homologues that reside in genomes of gammaproteobacterial AOB and methane-oxidizing bacteria (Utaker and Nes 1998). Unfortunately, the \textit{amoA} gene exhibits a variable copy number in the genomes of Beta-AOB (Mctavish et al. 1993, Norton et al. 1996) which complicates interpretation of qPCR results.

Other promising targets for quantitation of AOB, which have not yet been tested, include the hydroxylamine oxidoreductase (HAO) gene cluster which encodes for the proteins involved in oxidation of hydroxylamine to nitrite (Bergmann et al. 2005). The \textit{hao} gene cluster is likely subject to similar limitations as \textit{amoA}, namely multiple copies and orthologous sequences in the genomes of non-AOB (Bergmann et al. 2005, Arp et al. 2007). Therefore, this research
evaluated yet another target gene for quantitation of AOB in environmental samples, namely the gene encoding for the red copper protein nitrosocyanin (ncyA) whose function is yet unknown (Whittaker et al. 2000). While the ncyA gene exists as a single copy in the genomes of all AOB, the ncyA gene has not been observed in anaerobic ammonia-oxidizing (anammox) bacteria or ammonia-oxidizing crenarchaea and it does not have any known homologues (Arp et al. 2007). Therefore, qPCR targeting the ncyA gene has the potential to be a very specific target for quantitation of Beta-AOB which have been shown repeatedly to be the most abundant and functionally significant AOB in engineered wastewater treatment facilities. PCR primers were designed to target the nitrosocyanin gene in *N. europaea* ATCC19718 (NE0143). Reaction kinetics using the new ncyA primers were compared with previously developed primers for amplification of amoA in a broad range of organisms.

A.3 Materials & Methods

A.3.1 Laboratory-scale bioreactors

Two sets of laboratory-scale bioreactors were operated for a period of 20 months. Set-one was composed of two aerated, stirred, 1-Liter chemostat reactors (A1 and B1) operated at 35°C treating a synthetic wastewater designed to mimic recycle-streams returning from anaerobic digesters containing 1,000 mg/L NH₄-N, 50 mg/L Phosphorous, between 6,000 and 7,500 mg/L alkalinity as CaCO₃, plus other micro-nutrients. The pH was controlled at a low set-point between 7.0 and 7.2 using controllers (Cole Parmer, Vernon Hills, IL) and pH meters (Thermo Electron, Beverly, MA). Set-two was composed of three aerated, 6-Liter sequencing batch
reactors (A2, B2, and C2) operated at 25°C treating a synthetic domestic wastewater containing 30 mg/L NH₄-N, 400 mg/L acetate-COD, 5 mg/L Phosphorous, plus alkalinity and other micro-nutrients. Biomass from the previous laboratory-scale reactor experiment in the same configuration was salvaged and blended with mixed liquor from the Northern Kentucky Sanitation District #1 (SD1) Dry Creek Treatment Plant in Kenton County, Kentucky.

Reactor A1 was operated to mimic a completely mixed configuration with a fill and react phase of 24 hrs. Reactor B2 was operated to mimic a plug flow condition with feed added in one dose per 24 hrs. Daily, 60-mL of biomass was bioaugmented from A1 to A2 and from B1 to B2 in a single dose. Reactor C2 did not receive bioaugmentation. Reactors A2, B2, and C2 were operated to mimic plug flow conditions with a fill phase of 15 min, a react phase of 7 hrs, and a settle and decant phase of 1 hr. The hydraulic retention time (HRT) and solids retention time (SRT) in Reactors A1 and B1 were identical with a value of 5 days. The HRT in Reactors A2, B2, and C2 was 12 hrs. The initial SRT in Reactors A2, B2, and C2 was 12 days.

Following start-up and 100 days of operation of the main-stream reactors near 25°C, temperature was gradually reduced to 10°C in order to evaluate the impact of input biomass on nitrification at reduced temperature. The main-stream reactors were maintained for 32 SRT’s at 10°C before SRT was gradually reduced to further stress nitrification and cause washout.

Routine process parameters were measured including chemical oxygen demand (COD), pH, NH₄-N, NO₂-N, NO₃-N, and phosphorus. COD, NO₂-N, NO₃-N, and phosphorus were measured colorimetrically using a spectrophotometer and Hach (Loveland, CO) Test’n Tube reagents.
NH₄-N and pH were measured using combination electrodes. NO₂-N and NO₃-N were also measured using ion-selective electrodes. The experimental reactor configuration is shown in Figure A-1.

Figure A-1 - Experimental reactor configuration

A.3.2 Sample collection and nucleic acid extraction

At regular intervals, 2 mL biomass samples were collected. Samples were pelleted by centrifugation, decanted, and preserved at -80°C. Genomic DNA was extracted from biomass samples using the Ultraclean soil DNA extraction kit (Mo Bio laboratories, Inc., Carlsbad, CA) according to the manufacturer’s instructions. The genomic DNA was used as template material for PCR.
A.3.3 Real-Time PCR

Quantitative RT-PCR (real-time PCR) assays targeting two loci in *N. europaea* were developed and optimized for mixed liquor samples using SYBR-Green chemistry. Real-time PCR reactions were conducted on an Applied Biosystems Model 7500 Real-Time PCR system using Applied Biosystems software. Assays were performed in 25 µL reactions using 12.5 µL of SYBR Green PCR Master Mix (an optimized mixture of polymerase, PCR buffer, dexoynucleotide triphosphates, reference dye, and SYBR Green I supplied at 2X concentration) (Applied Biosystems, Foster City, CA), 1 µL (10-30 ng) of template DNA, a 50 nM concentration of each primer, and water. PCR conditions were as follows: 2 minutes at 50°C, 10 min at 95°C, followed by 40 cycles each of 15 s at 94°C and 15 s at 60°C. A dissociation stage consisting of 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C was run at the end of the amplification stage to identify non-specific binding.

The *amoA* gene encoding the A protein subunit of ammonia monooxygenase was amplified using primers targeting a broad range of Beta-AOB (Rotthauwe et al. 1997). The *ncyA* gene encoding the functional protein nitrosocyanin in *N. europaea* was amplified using a new set of primers developed for this study and designed according to guidelines set by Applied Biosystems (Foster City, CA). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). Primer names and sequences are shown in Table A-1.
Table A-1 - PCR primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
<th>Use</th>
<th>Reference</th>
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<tbody>
<tr>
<td>A189</td>
<td>GGHGACTGGGAYTTCTGG</td>
<td>151-168</td>
<td>Clone amoA</td>
<td>(Holmes et al. 1995)</td>
</tr>
<tr>
<td>amoA-2R’</td>
<td>CCTCKGSAAGCTTCTTC</td>
<td>802-820</td>
<td>Clone amoA</td>
<td>(Okano et al. 2004)</td>
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<tr>
<td>amoA-1F</td>
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<td>Quantify amoA</td>
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<td>205-224</td>
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<td>This study</td>
</tr>
<tr>
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<td>TCCTTGCCTGATGGGTATT</td>
<td>255-236</td>
<td>Quantify NE0143</td>
<td>This study</td>
</tr>
</tbody>
</table>

External standard curves for quantitation of *amoA* were developed for the range $1 \times 10^4$ – $1 \times 10^{10}$. Briefly, PCR primers targeting *amoA* were used to amplify DNA extracted from *N. europaea* ATCC 19718. The PCR product was cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted with a Plasmid Mini-Preps Spin Kit (Qiagen, Valencia, CA), and the plasmid concentration was measured with a spectrophotometer measuring absorbance at 260 nm. The sequences of the vector and PCR insert were known allowing a calculation of the copy numbers of *amoA* from molecular weights. Serial dilutions of plasmid DNA were run in triplicate alongside samples.

External standard curves for quantitation of nitrosocyanin were developed for the range $1 \times 10^3$ – $1 \times 10^8$. Serial dilutions of genomic DNA extracted from pure cultures of *N. europaea* were run in triplicate alongside samples.
A.4  Results & Discussion

A.4.1  Operation of laboratory-scale bioreactors

Effluent nitrogen concentrations from the three main-stream reactors are shown in Figure A-2. Day 0 is the beginning of steady-state operation following a start-up of three SRT’s. Nitrification was complete and stable during the period of operation near 25°C. However, periods of incomplete ammonia and nitrite oxidation were experienced in all three reactors as the operating temperature was reduced. Nitrification instability was particularly pronounced in reactor A2, which receives biomass input from the continuous-feed side-stream, where a prolonged period of poor ammonia oxidation (160 days) occurred. Meanwhile, NH$_4$-N oxidation remained stable in main-stream reactor B2 input with biomass from a batch side-stream and main-stream reactor C2 with no biomass input. However, NO$_2$-N accumulation was observed in reactors B2 and C2.
The “B” train was shut down on Day 500 due to failure of side-stream reactor B1. The “A” and “C” trains remained in operation for the remainder of the experiment.

A.4.2 Quantitation of amoA in laboratory main-stream bioreactors

Our results indicate that the reliability of this primer set for qPCR is questionable. The amplification efficiency is low as indicated by the slope (-4.9) and intercept (60 cycles) of the standard curve. The slope and intercept for a 100% efficient reaction is -3.32 and 33-37 cycles, respectively. Based on a melting curve analysis the poor efficiency may be due to heterogeneity in the 491-bp amplicon. Such heterogeneity can be a problem for amplicons larger than 250 bp (Wang and Seed 2006). Additionally, the $R^2$, which is a measure of the accuracy of the dilutions was 0.86. Previous communications reported an $R^2$ of 0.99 using this primer set for qPCR. although the slope and intercept for the standard were not reported (Aoi et al. 2004).
Given the poor efficiency of the reaction, AOB biomass concentration would be greatly misjudged based on the amoA qPCR assay. Nevertheless, assuming that all reactions were subject to the same conditions and causes for error, the data reported in Figure A-3 reveal that amoA occurred in higher concentrations in main-stream reactor B2 (biomass input from a PFR side-stream) than in reactors A2 (biomass input from a CSTR side-stream) and C2 (control), at least up until Day 429. This is particularly evident during the initial 100-day period of warm temperature operation when values differed by nearly an order of magnitude (Figure A-3). Variability in gene copy number and sequence cannot explain such a large difference as all Beta-AOB have 2 or more nearly identical copies of the amo operon (Norton et al. 2002). Coincidentally, nitrification was more stable in reactor B2 during this period as shown in Figure A-2. Side-stream reactor B1 began to fail on Day 358 which might explain the lower relative numbers of amoA in B2 on Days 429 and 484.
A.4.3 Quantitation of *N. europaea* in laboratory main-stream bioreactors

The kinetics of RT-PCR using the newly developed primer set targeting *N. europaea ncyA* gene were much better than the kinetics of RT-PCR targeting the *amoA* gene; the slope, intercept, and $R^2$ for the *ncyA*-standard curve were -3.2, 39 cycles, and 0.99, respectively. To our surprise, however, Figure A-4 indicates that the nitrosocyanin target was present only at relatively low levels in the reactor samples. For instance, assuming 1.2 genomes per cell, a mass of $1 \times 10^{-14}$ g/cell, and a 50% DNA extraction efficiency, target AOB would be less than 10 µg/L in all samples, representing less than 0.001% of the total biomass (MLVSS = 2,000 mg/L). This difference in quantitation was very surprising because previous data obtained from FISH targeting *N. europaea/N. eutropha* and genetic sequencing of *amoA* clone libraries (not shown)
indicated that the *N. europaea*-like AOB were very significant in the main-stream reactors. We hypothesize that the observed variation in AOB numbers is due to both an overestimation based on *amoA* gene targeting and an underestimation based on the *ncyA* gene. The latter could have been caused by variation in sequence of the nitrosocyanin gene at the strain level. Ongoing studies are aimed at establishing a clone library to assess sequence diversity in *ncyA* genes of environmental isolates.

Figure A-4 - Quantitation of nitrosocyanin present in main-stream bioreactors

A.5 Conclusions
The RT-PCR-based assay presented in this study is a viable approach for quantification of *N. europaea*, a predominant Beta-AOB, in activated sludge. Furthermore, the experiments were performed with relatively inexpensive SYBR green chemistry without the need for costly dual-labeled TAQMAN probes. One limitation for broad application of this assay at the present stage is the lack of a library of nitrosocyanin sequences; however, many more sequences will become available soon as genome and metagenome projects progress. Should sequence diversity prohibit the design of a primer set that is universally applicable to assess diversity and abundance of all Beta-AOB, a combination with full-cycle 16S methodology will be valuable for targeting various species/lineages necessary to sufficiently characterize AOB populations in a given activated sludge system.

A.6 References


Appendix B
Materials and Methods

C.1 Laboratory-Scale Reactors

C.1.1 Side-Stream Reactors

C.1.1.1 Synthetic Wastewater Storage Vessel

Instrument/Apparatus: Storage vessel
Purpose: Synthetic reject water storage
Type: Erlenmyer Flask
Construction: Borosilicate glass
Capacity: 250 mL
Manufacturer: Kimax
Model:
Accessories: elastomeric plug with holes for tubing in/out

C.1.1.2 Feed Pump

Instrument/Apparatus: Pump
Purpose: Synthetic reject water feed
Type: Peristaltic
Construction: plastic/steel
Capacity: 250 mL
Manufacturer: Cole Parmer
Model: Masterflex L/S
Accessories: Masterflex neoprene tubing, Tygon tubing, Easy-Load pump heads

C.1.1.3 Reactor Vessel

Instrument/Apparatus: Reactor vessel
Purpose: contact wastewater with mixed liquor
Type: annular jacketed
Construction: borosilicate glass
Capacity: 2,000 mL
Manufacturer: Schott
Model:
Accessories: Tygon tubing, aquarium diffuser stone, aquarium air pump

C.1.1.4 pH/ORP Controller

Instrument/Apparatus: pH/ORP controller
Purpose: maintain pH of side-stream reactors
Type: on/off
Construction:
Capacity: 2 setpoints
Manufacturer: Cole Parmer
Model: 5656-00
Accessories:

C.1.1.5 Chemical Feed Pump

Instrument/Apparatus: Pump
Purpose: chemical (acid/base) feed
Type: tubing pump
Construction: plastic/steel
Capacity: 0.002 to 43 mL/min
Manufacturer: Cole Parmer
Model: Masterflex C/L
Accessories: Nalgene bottles for chemical reservoir

C.1.1.6 Water Bath

Instrument/Apparatus: Water bath
Purpose: Maintain temperature of side-stream reactors
Type: Recirculating; Immersion thermostat
Construction: Stainless steel tub
Capacity: 18 l
Manufacturer: Lauda
Model: Ecoline E-119
Accessories: Tygon tubing

C.1.1.7 Stir plate

Instrument/Apparatus: Stir plate
Purpose: Suspending biomass
Type: Magnetic
Construction:
Capacity: 1,100 W
Manufacturer: Thermolyne
Model: Cimarec 2
Accessories: magnetic stir bars
C.1.1.8 Timer
Instrument/Apparatus: Timer
Purpose: pump/blower sequencing
Type: Tabletop w/AC outlets
Construction:
Capacity: 4 circuits/40 programs
Manufacturer: ChronTrol
Model: XT
Accessories:

C1.2 Main-stream reactors

C.1.2.1 Synthetic Wastewater Storage Vessel
Instrument/Apparatus: Storage vessel
Purpose: Synthetic domestic wastewater storage
Type:
Construction: plastic
Capacity: 40 L
Manufacturer: Sterilite
Model:
Accessories: modified with holes for feeding chemical

C.1.2.2 Feed / Decant Pump
Instrument/Apparatus: Pump
Purpose: Domestic wastewater feed, treated effluent discharge
Type: Peristaltic
Construction:
Capacity: 0.001 mL/min to 3,400 mL/min
Manufacturer: Cole Parmer
Model: Masterflex L/S
Accessories: Masterflex L/S pump heads, Masterflex neoprene tubing, Tygon tubing

C.1.2.3 Reactor Vessel
Instrument/Apparatus: Reactor vessel
Purpose: contact wastewater with biomass
Type:
Construction: polycarbonate
Capacity: 6 L
Manufacturer:
Model:
Accessories: Tygon tubing, aquarium diffuser strips, laboratory air pump
C.1.2.4 Stir plate

Instrument/Apparatus: Stir plate  
Purpose: Suspending biomass  
Type: Magnetic  
Construction:  
Capacity: 1,100 W  
Manufacturer: Thermolyne  
Model: Cimarec 2  
Accessories: magnetic stir bars

C.1.1.5 Timer

Instrument/Apparatus: Timer  
Purpose: pump/blower sequencing  
Type: Tabletop w/AC outlets  
Construction:  
Capacity: 4 circuits/40 programs  
Manufacturer: ChronTrol  
Model: XT  
Accessories:

C.1.1.6 Air Pump

Instrument/Apparatus: Air Pump  
Purpose: mixing/oxygenating reactor biomass  
Type: Diaphragm  
Construction:  
Capacity: 1,050 cu. in. / min./ 12 psig  
Manufacturer: Air Cadet  
Model: 7530-40  
Accessories: Tygon tubing (3/8 in.)

C.2. Batch Respirometry

C.2.1. Respirometer

Instrument/Apparatus: Respirometer  
Purpose: measure biomass respiration  
Type: Headspace oxygen replenishment  
Construction:  
Capacity: 8 vessel  
Manufacturer: Challenge  
Model: AER-8  
Accessories: 250 mL serum bottles with rubber septum screw caps, syringes, oxygen tank, online computer, magnetic stir bars,
C.3 Molecular Biology

C.3.1 PCR machine

Instrument/Apparatus: Thermal cycler
Purpose: nucleic acid amplification
Type:
Construction:
Capacity:
Manufacturer: Applied Biosystems
Model: GeneAmp 2700/9400
Accessories: microvials

C.3.2 BeadBeater

Instrument/Apparatus: Mini-beadbeater
Purpose: disrupt cells
Type:
Construction:
Capacity: 8 vials
Manufacturer: Biospec
Model: MiniBeadbeater-8
Accessories: 2-ml screwcap vials

C.3.3 centrifuge

Instrument/Apparatus: tabletop centrifuge
Purpose: nucleic acid separation
Type:
Construction:
Capacity: 24 tubes
Manufacturer: Eppendorf
Model: 5415 D
Accessories: 2 ml screwcap vials

C.3.4 Spectrophotometer

Instrument/Apparatus: Spectrophotometer
Purpose: reactor effluent characterization
Type: laboratory
Construction:
Capacity:
Manufacturer: Hach
Model: DR/2500
Accessories: Hach test kits, pipettes, syringe filters, syringes
C.3.5 Mixer/Vortexer

Instrument/Apparatus: Mixer/vortexer
Purpose: mix samples/molecular biology reagents
Type: tabletop / variable-speed
Construction:
Capacity:
Manufacturer: Scientific Industries
Model: Vortex-Genie 2
Accessories:

C.3.6 Electrophoresis unit

Instrument/Apparatus: Slab Gel Apparatus
Purpose: electrophoresis of nucleic acids
Type: turn & cast submarine
Construction: Plastic
Capacity: 24 lanes
Manufacturer: E-C Apparatus Corp.
Model: CSSU1214
Accessories: power supply, microwave, Erlenmeyer flask

C.3.7 Electrophoresis

Instrument/Apparatus: Slab Gel Apparatus
Purpose: electrophoresis of nucleic acids
Type: vertical
Construction: plastic
Capacity: 2 slab gels
Manufacturer: Bio-Rad
Model: Mini Protean 3
Accessories: precast polyacrylimide gels

C.3.8 DNA extraction kit

Instrument/Apparatus: DNA extraction kit
Purpose: isolate genomic DNA
Type: physical/chemical
Construction:
Capacity:
Manufacturer: Mo Bio
Model: UltraClean Soil DNA
Accessories: bead beater, -80°C freezer, pipettes
C.3.9 PCR purification kit

Instrument/Apparatus: PCR purification kit
Purpose: DNA purification
Type: chemical/physical
Construction:
Capacity: 10 ug
Manufacturer: Qiagen
Model: QIAquick
Accessories: vortexer/mixer

C.3.10 DNA cloning kit

Instrument/Apparatus: DNA Cloning kit
Purpose: Cloning isolated DNA
Type: TA
Construction:
Capacity:
Manufacturer: Invitrogen
Model: TOPO TA Cloning
Accessories: vortexer/mixer, pipettes, water bath

C.3.11 UV Illuminator

Instrument/Apparatus: UV Illuminator
Purpose: slab gel visualization
Type: Laboratory / variable intensity
Construction:
Capacity:
Manufacturer: Spectroline
Model: TVR-312
Accessories: Imaging cabinet

C.3.12 Electronic Gel Imager

Instrument/Apparatus: Electronic gel imager
Purpose: Electrophoresis documentation
Type:
Construction:
Capacity:
Manufacturer: Kodak
Model: EDAS 290
Accessories: computer
Appendix C

amoA sequence data

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>NYC Clone10

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>NYC Clone02

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