INTEGRATED BIOLOGICAL TREATMENT OF SWINE WASTE FOR NITROGEN REMOVAL AND ENERGY RECOVERY

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering in the UNC Gillings School of Global Public Health.

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ABSTRACT

Eric Timothy Staunton: Integrated Biological Treatment of Swine Waste for Nitrogen Removal and Energy Recovery
(Under the direction of Michael D. Aitken)

Nitrogen emissions associated with swine waste management have been identified as sources of several adverse public health and environmental effects, including: contamination of drinking water, respiratory diseases, production of ground level ozone, depletion of stratospheric ozone, release of greenhouse gases, and acidification of soils and watersheds. These adverse effects make the technologically and economically feasible removal of nitrogen highly desirable. To understand the technological feasibility of nitrogen removal from swine waste, two biological systems were designed and operated: a traditional, pilot-scale, Modified Ludzack-Ettinger (MLE) process; and a lab-scale, single-reactor, nitritation/anaerobic ammonium oxidation (anammox) process.

The MLE process removed ~98% influent ammonium-N and ~83% influent total-N. Approximately 75% of the chemical oxygen demand (COD) was oxidized; the majority of COD was utilized as a source of electron equivalents for denitrification. The concentration of COD varied seasonally and full-scale N removal is expected to vary seasonally as well. There was only enough COD for complete denitrification in winter months. Alkalinity in the waste is insufficient to meet the demand associated with nitrification, although the extent of external alkalinity addition is expected to vary seasonally. Of the removed nitrogen, ~8% was released as nitrous oxide, primarily as a side-product of nitrification.

The single-reactor nitritation/anammox process removed up to 96% of influent ammonium-N and up to 90% influent total-N. Several observations indicated anammox as the primary N removal pathway though the relative contribution of denitrification is unknown. This system required at least 49% less oxygen than a conventional nitrogen removal system and required no external alkalinity which should minimize the cost associated with nitrogen removal. Of the removed N, 11% was converted to nitrous oxide.

The microbial community associated with nitritation/anammox was examined by quantitative PCR and bar-coded amplicon sequencing. The microbes known to perform anammox were found to comprise a small fraction of the total biomass. A significant shift in the dominant anammox bacteria was observed, from a seed culture dominated by *Candidatus Brocadia* to *Candidatus Kuenenia* dominating in the reactor biomass. An uncharacterized *Planctomycete* was identified as a dominant member of the community, though it is unknown if this microbe performs anammox.

To my husband, Brad, whose love and fortitude have been a source of never ending motivation, driving me to complete this work. Thank you!

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$\hat{\mu}$	maximum specific growth rate
anammox	anaerobic ammonium oxidation
AOA	aerobic ammonium oxidizing archaea
AOB	aerobic ammonium oxidizing bacteria
BOD	biochemical oxygen demand
$CaCO_3$	calcium carbonate
CAFO	combined animal feeding operation
CANON	completely autotrophic nitrogen removal over nitrite
CCW	counter clockwise
CH_4	methane
CO_2	carbon dioxide
COD	chemical oxygen demand
DEAMOX	denitrifying ammonium oxidation
DEMON	deammonification
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EPA	Environmental Protection Agency
GC	gas chromatograph/chromatography
GF	glassfiber
HDPE	high-density polyethylene
HRT	hydraulic retention (residence) time
hzsA	hydrazine synthase

ICP-MS	inductively coupled plasma-mass spectrometry
ID	inside diameter
IFAS	integrated fixed film activated sludge
LAB	loosely associated biofilm
LPM	liters per minute
MBBR	moving-bed biofilm reactor
MLE	modified Ludzack-Ettinger
n	sample size
N	nitrogen
N_2	nitrogen gas
N_2O	nitrous oxide
NC	North Carolina
NH_4^+-N	ammonium-nitrogen
NO	nitric oxide
NO_2^--N	nitrite-nitrogen
NO_3^--N	nitrate-nitrogen
NOB	aerobic nitrite oxidizing bacteria
nosZ	nitrous oxide reductase
O_2	oxygen gas
OLAND	oxygen limited autotrophic nitrification and denitrification
OM	organic matter
PCoA	principal coordinate analysis
PCR	polymerase chain reaction

PTFE	polytetrafluoroethylene
PVC	polyvinyl chloride
$\hat{q}$	maximum biomass specific rate of substrate utilization
qPCR	quantitative polymerase chain reaction
R	ideal gas constant
RBC	rotating biological contactor
RFU	relative fluorescence units
RPM	revolutions per minute
SBR	sequencing batch reactor
SBBR	sequencing batch biofilm reactor
SHARON	single reactor high activity ammonium removal over nitrite
SNAD	simultaneous partial-nitrification anammox and denitrification
SNAP	single-stage nitrogen-removal using anammox and partial nitritation
SRT	solids retention (residence) time
SS	stainless steel
T	temperature
TDN	total dissolved nitrogen
TIN	total inorganic nitrogen
TN	total nitrogen
TSS	total suspended solids
UHP	ultra-high purity
USP	United States Pharmacopoeia
VOC	volatile organic compound

VSS	volatile suspended solids
X	biomass concentration
Y	yield coefficient

Chapter 1: Introduction

North Carolina (NC) is currently home to ~8.4 million swine (*1*), most of which are raised in confined animal feeding operations (CAFOs). These swine collectively produce more nitrogen in their waste than can be assimilated at agronomic rates in the entire region of swine production (*2*), creating substantial waste management issues. Waste from swine farms is typically stored in large open-air lagoons and periodically applied to sprayfields to fertilize crops (*2*). Use of Open-air lagoons lead to several environmental consequences and public health problems, such as emission of ammonia, methane and other greenhouse gases to the atmosphere (*3*, *4*).

It has been estimated that a 50% reduction in ammonia emissions would lead to $189 million year^{-1} in health benefits in North Carolina alone (*5*). Because of serious environmental issues with a rapidly growing swine production industry, in 2007 North Carolina banned construction of new lagoons and sprayfields that could not meet new, stricter environmental performance standards, which has led to interest in developing new waste management systems (*6*).

As much as 4% of total greenhouse gas emissions (as CO_2 equivalents), mostly in the form of methane (CH_4), are estimated to be due to waste management (*7*). There is much interest in producing and capturing methane from swine manure and other animal wastes as a means of producing power. For example, legislation in North Carolina authorizes higher payment for electricity generated on swine farms from captured methane. This has caused an increasing interest in covering swine waste lagoons to preclude gaseous emissions to the

atmosphere and allow for anaerobic digestion of waste with capture of the produced methane for power generation. Although this system would reduce the carbon content associated with swine waste, doing so would not address the substantial impacts of the waste on nitrogen releases to the environment.

I explored integrating nitrogen removal by nitrification and denitrification with anaerobic digestion (Chapter 4), however, this method was found to be economically infeasible (*8*). A major factor in the estimated cost of nitrification/denitrification is the cost of aeration for nitrification. Therefore, I explored coupling anaerobic digestion for methane production (and ultimately power generation) with nitrogen removal through the anaerobic ammonium oxidation ("anammox") pathway. Successful implementation of this process should allow for a substantial reduction in operating cost as compared to a nitrification/denitrification system.

The specific aims of this dissertation are outlined below:

1.1 Specific Aims

Aim 1: Design, construct and operate a lab-scale system to evaluate the technical feasibility of anaerobic ammonium oxidation for nitrogen removal from anaerobically digested swine waste

The growth of anammox organisms is very slow and therefore a reactor configuration that has high biomass retention is desired. For this reason, a sequencing batch reactor (SBR) was selected to perform the anammox process. This reactor can minimize the growth of nitrite-oxidizing bacteria by up-to three mechanisms of inhibition to favor conditions for anammox. Further, this reactor configuration should allow for the aerobic consumption of the majority of the influent organic matter, which would limit the activity of heterotrophic denitrifiers and hopefully stabilize nitrogen removal by anammox.

Aim 2: Evaluate the influence of design variables (e.g. total cycle time, loading rates, timing of periods, temperature, DO) on performance and stoichiometry of the anammox system.

In an effort to minimize the cost of a full-scale anammox system, the effect of design variables was evaluated. Parameters such as total cycle time and loading rate have an effect on the kinetics of anammox treatment, which influences the size of a full-scale anammox reactor.

Aim 3: Examine the effect of design variables on the stability of the anammox community

For biological treatment to be successful, an established microbial community must be present in the reactor. The microbial community associated with the anammox process was evaluated utilizing various molecular methods. Quantitative PCR was coupled with high-throughput amplicon sequencing to explore absolute and relative abundance of microbes of interest.

1.2 Dissertation Organization

This dissertation is a compilation of three manuscripts intended for publication in peer-reviewed journals. These manuscripts (Chapters 4-6) are draft versions to be submitted at a later date. All tables in the manuscript chapters are configured for journal submission. In general, supporting information is included as appendices to this dissertation although organization has been modified for clarity. Occasionally, tables or figures from the manuscript supporting-information were included in the chapters; this is indicated by a superscript dagger (†). Chapter 2 is a review of relevant literature, Chapter 3 discusses theory of biological reactor design, and Chapter 7 provides conclusions and recommendations for future research. The first manuscript chapter represents a significant joint effort of multiple authors. My role was to perform nitrogen analysis, assist with system operation and

troubleshooting, to construct cumulative mass loading and discharge diagrams, and to prepare the draft manuscript.

Chapter 2: Literature Review

2.1 Background

Anthropogenic inputs of fixed nitrogen to the environment, particularly from intensive agriculture, have led to substantial pollution of both air and water globally (*9*). North Carolina is currently home to approximately 8.4 million swine (*1*), most of which are raised in confined animal feeding operations (CAFOs). Waste from CAFOs is typically stored in large, open-air lagoons and periodically applied to sprayfields to fertilize crops (*2*). There are currently ~4,000 active and ~650 inactive lagoons in NC (*2*), mostly in the eastern half of the state (Figure 2.1). The scale of waste production produces more nutrients (mainly nitrogen) than can be assimilated at agronomic rates in the entire region of swine production (*2*).

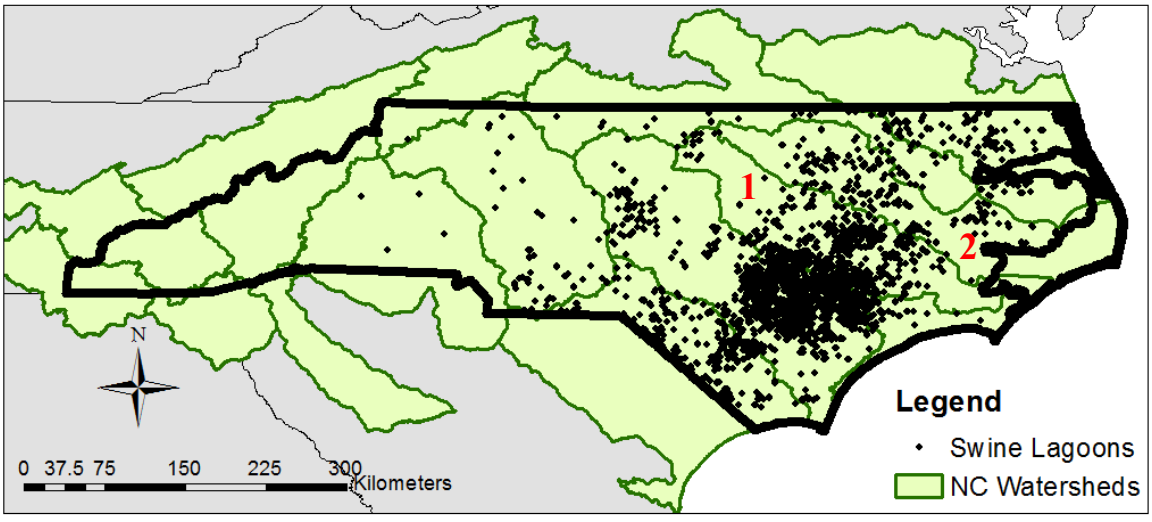

Figure 2.1: Swine lagoons in NC and regional watersheds (*10–13*). Neuse River Basin is denoted by 1, Tar-Pamlico by 2.

Open-air lagoons are the source of adverse public health effects , such as respiratory ailments (*14*), and the source of several environmental consequences, such as emission of

ammonia, methane and other greenhouse gases to the atmosphere (*3*, *4*). These are explored in greater depth below (Section 2.3 and 2.4).

2.2 Composition of Swine Waste

Swine waste is a complex mixture of feces, urine and water used to wash the barns. The complex matrix of naturally occurring organic matter (OM), such as that which occurs in swine waste, makes it convenient to measure organic content as the amount of oxygen needed to oxidize the sample, or the oxygen demand. This can be measured as either chemical oxygen demand (COD) when a chemical oxidizing agent is used (e.g. dichromate, permanganate), or biochemical oxygen demand (BOD) when a biological catalyst is used. Due to the recalcitrant nature of some organic matter, COD is typically greater than BOD. It is important to note that BOD and COD are simply representative *measures* of organic matter concentration. These measures do not explicitly imply that biological conversion of organic matter necessarily consumes oxygen. Other reactions such as denitrification (Section 2.5.2) and anaerobic digestion (Section 2.8.1Anaerobic Digestion) consume oxygen demand but do not consume oxygen.

Organic matter and nitrogenous species are of primary concern in this dissertation. Due to how a farm is operated and the type of farm in consideration, it is impossible to state with certainty a "typical" concentration of organic matter and nitrogen in a waste storage lagoon. However, results from the pilot scale study at Butler Farms in Lillington, NC (referred to as the pilot study; chapter 4) can provide an estimated concentration of these species for the farm of interest. From 9/10/2010 through 5/27/2011 the composition of the liquid waste storage lagoon was analyzed at least twice per week. Results are shown in Table 2.1 (Chapter 4).

Table 2.1: Characteristics of lagoon effluent at Butler Farms.

Parameter	Mean	St Dev	n
Ammonium-N (mg L^{-1})	2,340	160	99
Total-N (mg L^{-1})	2,750	230	84
Total Dissolved-N (mg L^{-1})	2,590	220	80
Total COD (mg L^{-1})	7,550	2,240	38
Soluble COD (mg L^{-1})	5,370	1,820	37
TSS (mg L^{-1}) [a]	1,570	260	24
VSS [b]/TSS	0.39	0.02	12
pH	7.71	0.23	27
Total Alkalinity (mg $CaCO_3$ L^{-1})	12,200	400	24
Bicarbonate Alkalinity (mg $CaCO_3$ L^{-1})	10,600	500	24
Temperature (°C)	5.1 - 30.5	N/A	240

[a] Total Suspended Solids: The mass of dried solids filterable from a given volume of liquid.
[b] Volatile Suspended Solids: The mass of dried solids from a given volume of liquid that burn at 550°C.

During the course of the study period there was significant variation of the lagoon organic matter (measured as COD) as a function of time. This variation closely matched the observed variation in temperature. Below ~15°C (winter months), the concentration of COD started climbing, and above ~15°C the concentration of COD decreased (Figure 2.2; top). No apparent variation was observed in the concentration of ammonium-N or total-N (Figure 2.2; bottom).

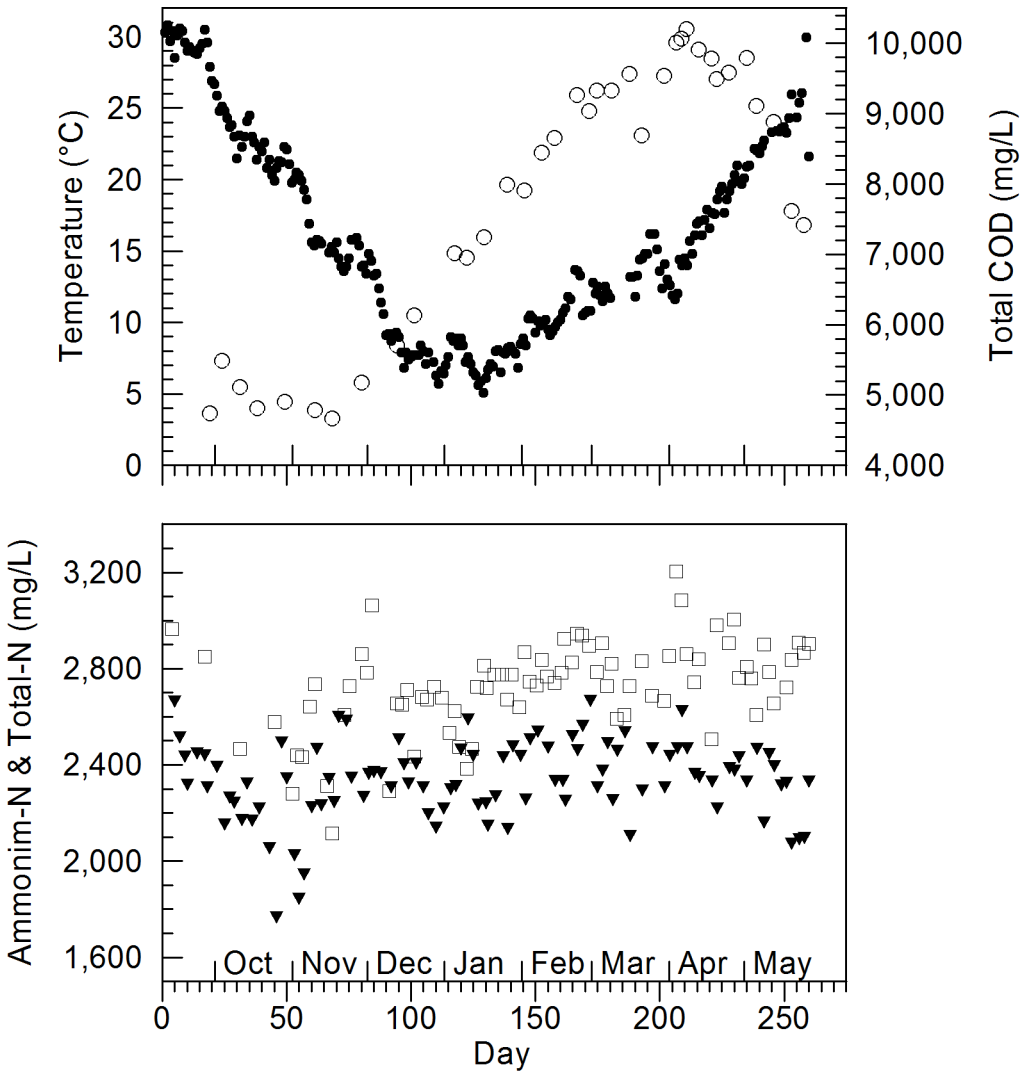

Figure 2.2: Lagoon temperature (●), concentration of lagoon total COD tCOD (○), total-N (□) and ammonium-N (▼) for the duration of the pilot project at Butler Farms. When the temperature is below approximately 15°C there is limited methane production leading to a COD increase in the lagoon. When temperature rises above 15°C, lagoon COD starts dropping as gas production increases. Data are means; standard deviation is not reported though was typically less than 10%.

Other studies that report values for the characteristics of anaerobically digested swine waste are compiled in Table 2.2. Svoboda et al. (*15*) reported that since no two farms are the same, waste management practices for swine farms need to be considered individually. It has also been found that the type of farm (i.e. sow, nursing, or finishing) can have an impact on

the composition of waste produced (*16*). Despite the fact that the farm type in the studies cited in Table 2.2 is unknown it can be seen that parameters reported at Butler Farms are typical of anaerobically digested swine waste, especially in terms of ammonium-N.

Anaerobic digestion affects several key characteristics (e.g. organic matter, pH, ammonium content, and alkalinity) of a given waste. However, assuming there is little oxidized nitrogen in the influent waste, anaerobic digestion should have minimal impact on total-N. Therefore, measurements of total-N from studies on raw waste can be used to determine "typical" values for the concentration of total-N. A concentration of total-N ranging from 1,100-3,800 mg N L^{-1} (*17–20*) is typically reported. Several thousand mg N L^{-1} is not uncommon for swine waste, making nitrogen removal prior to waste discharge a highly desirable goal.

Table 2.2: Characteristics of anaerobically digested swine waste from the literature. References (*21*) and (*22*) reported a range of values instead of mean ± standard deviation. N/R=Not Reported. N/D=Non-detectable. Units are mg L^{-1} unless noted.

Item	(*21*)	(*22*)	(*23*)	(*24*)	(*25*)
Ammonium-N	2,000-4,000	500 - 800	418 ± 10	3,808 ± 98	970 ± 50
Nitrite-N	N/D	0.05 - 1.0	N/R	N/R	<2
Nitrate-N	N/D	1.0 - 3.0	5.8 ± 2.4	N/R	<0.5
Total-N	3,000-5,000	N/R	472 ± 6	4,041 ± 59	1,550 ± 160
Total COD	8,000-17,000	800 – 1,000	437 ± 6	11,540 ± 860	2,940 ± 1,100
TSS	5,000-10,000	N/R	1,255 ± 9	N/R	N/R
pH [a]	8.3	7.2-8.5	7.61	N/R	8.1 ± 0.1
Bicarbonate Alkalinity	N/R	N/R	N/R	N/R	6,780 ± 580

[a] standard units

2.3 Public Health Impact of Swine Waste

When lagoon waste is sprayed on fields, nitrifying bacteria in the soil can oxidize ammonium to nitrate, which can penetrate into aquifers (*26*); several sites across North Carolina have been found to contain nitrate concentrations in excess of EPA's drinking water standard of 10 mg $NO_3^- L^{-1}$ (*27*), which has been identified as a possible cause of methemoglobinemia, especially in infants (*28*). Animal and epidemiological studies have also linked increased concentrations of nitrate in drinking water to reproductive and developmental toxicity (*29*).

There have been reports of pathogen contamination of ground-waters due to sprayfield irrigation (*30*). Due to stormwater runoff as well as discharge from groundwater into receiving waters, swine waste has also led to the contamination of surface waters with pathogens (*31*).

2.3.1 Air Pollution

High concentrations of ammonium in open-air lagoons leads to significant concentrations of atmospheric ammonia in nearby communities (*32*). Volatilized ammonia has been linked to respiratory problems among those exposed to the gas, including populations near (e.g. residents and students at nearby schools) and working at swine farms (*33–35*). Further, ammonia reacts in the atmosphere to form fine particles that can cause respiratory disease (*36*, *37*).

Equal production of nitrous and nitric oxide (NO) have been observed for the microbial processing of organic wastes (*38*) and therefore, I expect anaerobic lagoons to be sources of NO emissions. This gas reacts in the troposphere to produce ground-level ozone (*39*), which has been associated with increased risk of mortality (*40*). Odors associated with emissions from anaerobic lagoons have severely impacted the quality of life for those living near farms

(*41*). After accounting for other factors (e.g. proximity to schools, agricultural fields, and sewage treatment plants), the median purchase price of homes within 1 mile of a 10,000-head farm was found to decrease by as much as 8% (*42*).

It has been estimated that a 50% reduction in ammonia emissions would lead to $189 million $year^{-1}$ in health benefits in North Carolina alone (*5*). Swine farming has been estimated to emit 20-46% of total gaseous N emissions in North Carolina (*43*, *44*), indicating that N removal from swine waste would have a significant impact on the health of North Carolinians.

2.4 Environmental Impact of Swine Waste

Open-air lagoons are sources of several gaseous pollutants of environmental concern, including methane (*45*), nitrous oxide (*46*), volatile organics (e.g. paraffins, olefins, aromatics, ethers, and aldehydes) (*47*), ammonia (*4*), and hydrogen sulfide (*48*). Of these, methane and nitrous oxide are greenhouse gases with global warming potentials ~25 and ~300 times greater than that of carbon dioxide on a per molecule basis, respectively (*49*). In 1990, 4% of all greenhouse gas emissions in NC were attributed to methane emissions from waste management (*7*). One study estimated the emissions of methane from a lagoon at 5.6 kg CH_4 $animal^{-1}$ $year^{-1}$ (52 kg ha^{-1}) (*3*). The emission of nitrous oxide from sprayfields has been estimated at 1.4% of applied N (*50*). This gas has also been linked to stratospheric ozone depletion (*51*).

Volatile organic compounds (VOCs) are known greenhouse gases and contribute to ground-level ozone production (*52*). VOCs also include classes of compounds related to nuisance odors (e.g. dimethyl sulfide) (*47*).

Atmospheric transport and subsequent deposition of volatilized ammonia can also cause acidification of watersheds and soils, noxious algal blooms, bottom-water hypoxia and other

environmental consequences (*53*). Due to regional transport of ammonia, these effects are felt throughout the state and in neighboring states (*54*).

These adverse environmental effects of current swine waste management practices make the technologically and economically viable removal of nutrients (mainly nitrogen) highly desirable.

2.4.1 Environmental Performance Standards

In an effort to mitigate the adverse environmental effects of swine waste, the 2007 General Assembly of North Carolina enacted the 2007 Swine Farm Environmental Performance Standards Act (Senate Bill 1465). This bill banned the construction of new swine farms that employ anaerobic lagoons and sprayfields as the primary waste-treatment/disposal technology. New farms must employ technologies for improved environmental performance standards (*6*). These standards were updated in 2009 to the following (*55*):

1. Eliminate the discharge of animal waste to surface waters and groundwater through direct discharge, seepage, or runoff
2. Substantially eliminate atmospheric emission of ammonia
3. Substantially eliminate the emission of odor that is detectable beyond the boundaries of the parcel or tract of land on which the swine farm is located
4. Substantially eliminate the release of disease-transmitting vectors and airborne pathogens
5. Substantially eliminate nutrient and heavy metal contamination of soil and groundwater

The 2007 legislation also created a methane capture pilot program which authorizes the higher payment for electricity generated on swine farms from anaerobic digestion. It should

be noted that participants in the pilot program are not required to meet environmental performance standards.

2.5 Conventional Biological Processes to Remove Ammonium

When municipal wastewater treatment plants are required to remove total-N before discharge of treated waste to receiving waters, a two-step process is used. In the first step, nitrification, ammonium is oxidized to nitrate under aerobic conditions. In the second step, denitrification, the produced nitrate is reduced to N_2, an innocuous gas that comprises 80% of the atmosphere.

The most expensive part of nitrogen removal has been reported as provision of oxygen for removal of ammonium (*56*) and provision of external carbon for denitrification (*57*). In terms of power demand, an economic analysis of the pilot project at Butler Farms found aeration to be the most expensive portion of nitrogen removal (aeration consumed 63% of total power requirements) (*8*). Another significant operating cost for a nitrification/denitrification system is the provision of an alkaline agent (such as sodium hydroxide) to maintain a neutral pH in the nitrification reactor; providing an alkaline agent for nitrification accounted for 12.4% of the 10-Year annualized cost for a full-scale nitrogen removal facility based on the pilot study (*8*).

2.5.1 Nitrification

Nitrification is used extensively at municipal wastewater treatment plants that are required to remove ammonium-nitrogen (NH_4^+-N). This is a two-step process carried out by different groups of autotrophic bacteria. *Nitroso-* species (e.g. *Nitrosomonas*) oxidize ammonium to nitrite (ammonium oxidizing bacteria; AOB) and *Nitro-* species (e.g. *Nitrobacter*) oxidize the produced nitrite to nitrate (nitrite oxidizing bacteria; NOB).

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \qquad \text{ammonium oxidation to nitrite} \qquad [2.1]$$

$NO_2^- + O_2 \rightarrow NO_3^-$	nitrite oxidation to nitrate	[2.2]
Net: $NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O$	nitrification	[2.3]

This process requires 2 moles of O_2 per mole of ammonium-N (equation 2.3); on a mass basis, this translates to 4.57 g O_2 (g NH_4^+-N)$^{-1}$. Nitrification also consumes two equivalents of alkalinity per mole of ammonium-N oxidized; on a mass basis, this is 7.14 g alkalinity as $CaCO_3$ per g NH_4^+-N oxidized. If there is insufficient alkalinity in the influent waste, it must be supplemented via an external source to ensure the process operates at the desired pH.

2.5.1.1 Optimal Conditions for Nitrification

Due to the relatively long doubling time and high oxygen demand of nitrifying bacteria, nitrification typically occurs under moderate solids retention time (SRT; the average time it takes to replace the solids in a reactor) and in a well-aerated reactor. Further, to ensure sufficient alkalinity to neutralize the produced acid, nitrification reactors are typically operated with an external source of alkalinity added to maintain neutral pH (*58*).

2.5.2 Denitrification

When municipal wastewater treatment plants are required to remove total-N, denitrification is coupled to nitrification. Denitrification involves the reduction of nitrate-nitrogen (NO_3^--N) or nitrite-nitrogen (NO_2^--N) produced during nitrification at the expense of an electron donor, often organic matter (equation 2.4). Organic matter could be from the wastewater, provided though endogenous decay of microorganisms, or supplied externally in the form of an inexpensive and readily available source of organic carbon such as methanol or acetate.

NO_3^- + organic matter $\rightarrow 0.5N_2 + xCO_2 + yH_2O + OH^-$ heterotrophic denitrification [2.4]

From equation 2.4, it is apparent that denitrification from nitrate produces alkalinity. Typically, one equivalent of alkalinity is produced per mole of nitrate-N reduced; on a mass

basis, 3.57 g alkalinity as $CaCO_3$ per g nitrate-N reduced (*58*). This is half the alkalinity consumed by nitrification, which if recovered, could reduce the requirement of external alkalinity addition for biological nitrogen removal relative to nitrification alone.

It should be noted that NO_2^- can be used in place of NO_3^- for denitrification. This process has been referred to as "short-cut" denitrification, or denitritation, and has been much less well studied than conventional denitrification with nitrate. The effect of nitrite reduction on alkalinity is not well-addressed in the literature, although it is reported that nitrite reduction to nitric oxide is the step that produces alkalinity (*59*). This implies that denitrification from nitrite would produce alkalinity.

The balanced chemical reaction for denitritation using acetic acid as an electron donor and ignoring cell synthesis is shown below (equation 2.5). This reaction shows that reduction of nitrite to N_2 produces one equivalent of alkalinity per mole of nitrite reduced or 3.57 g alkalinity as $CaCO_3$ per g nitrite-N reduced.

$$\frac{3}{4}CH_3COOH + NO_2^- \rightarrow \frac{3}{2}CO_2 + H_2O + OH^- \qquad \text{nitrite reduction to } N_2 \qquad [2.5]$$

Nitrate reduction to nitrogen gas follows the pathway below (*60*):

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

Each step in this pathway uses a different enzyme to carry out the reduction of nitrogen. This knowledge is useful when quantifying denitrifying bacteria in microbial ecosystems (section 2.9.1 qPCR).

2.5.2.1 Autotrophic Denitrification

Autotrophic denitrification using alternate electron donors (e.g. molecular hydrogen or elemental sulfur) has been reported (*61*, *62*). This process is not as well studied as the heterotrophic case, but is important to consider for the current research. In regards to alkalinity, autotrophic denitrification using sulfur species as an electron donor results in a net consumption of alkalinity due to produced sulfate (*62*, *63*). This information is especially important when trying to understand microbial N transformations and differenting denitrification (heterotrophic or autotrophic) from anammox.

2.5.3 By-Products and Intermediates

Nitrification and denitrification produce nitric oxide (NO) and nitrous oxide (N_2O) as by-products and intermediates, respectively. The deleterious environmental effects of NO and N_2O have been discussed previously (Section 2.3.1). The exact mechanism of by-product formation is unknown, but several factors have been associated with increased NO and N_2O production. Factors affecting nitrous oxide production from nitrification include low dissolved oxygen, high nitrite or ammonium concentration, low SRT, low temperature, and the presence of toxic compounds (*64*). Release of nitrous oxide as an end product from denitrification has been linked to low concentration of dissolved oxygen, and limited availability of COD amendable to denitrification (*64*).

2.5.4 Application of Nitrification/Denitrification to Anaerobically Digested Swine Waste

Conventional biological nitrogen removal was applied to anaerobically digested swine waste from Butler Farms in Lillington, NC (Chapter 4). From this study I found high nitrite accumulation in the nitrification reactor (average NO_2^--N was ~76% of total NO_x^--N),

although this was more likely to have resulted from operational upsets than from an inherent characteristic of the digested swine waste. Even after accounting for alkalinity production from denitrification, there was insufficient alkalinity for oxidation of influent total nitrogen (6.46 kg alkalinity as $CaCO_3$ (kg TN)$^{-1}$). Most (80%) of the influent organic matter was removed and ~7% of the influent TN was converted to N_2O; >99% of N_2O production occurred in the nitrification reactor. Nitric oxide production was not measured.

2.6 Anaerobic Ammonium Oxidation

Anaerobic ammonium oxidation (anammox) is a recently discovered pathway in the global biogeochemical nitrogen cycle. Under anoxic conditions, ammonium is used as an electron donor to reduce nitrite to nitrogen gas (equation 2.6) through hydrazine as an intermediate (*65*). This reaction is thought to be performed in a novel "anammoxosome", a membrane-bound region within anammox bacteria that is thought to contain the highly toxic intermediates of the anammox pathway (*66*). The stoichiometry of anammox metabolism has been found to vary from 1.11-2.00 nitrite-N reduced per ammonium-N oxidized (*67–70*) and the variation is thought to be due to the effect of SRT; with a longer SRT the stoichiometry approaches 1:1 (*71*). For simplicity, the one to one stoichiometry is shown in equation 2.6.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \text{anammox} \qquad [2.6]$$

Nitrite consumption in excess of the 1:1 ratio is typically ascribed to the oxidation of nitrite to nitrate anaerobically with the generated reducing equivalents used for carbon dioxide fixation, likely via the acetyl-CoA pathway (*72*). In the context of biological nitrogen removal from wastewater using anammox, it has been estimated that 11% of the ammonium-N load is converted to nitrate (*67*, *73*). However, this would only be true if conditions were maintained that prevent denitrification and anammox from occurring co-currently.

Equation 2.6 is a low energy-yielding reaction (-357 kJ) which implies a low growth yield (per mol N) and, correspondingly, a slow overall growth rate. A low specific growth rate (0.0027 h^{-1}) for anammox bacteria is reported (*67*) but a higher specific growth rate (0.016 h^{-1}) has also been observed (*74*). This difference is likely due to different anammox species and/or different reactor conditions.

Most species of anammox bacteria studied thus far have an optimum temperature for growth in the range of 30-35°C (*75–77*). These studies have been done in the context of sidestream treatment at wastewater treatment plants (e.g. treatment of ammonium-rich supernatant from anaerobic digestion of sludge); however, anammox is an important part of the global biogeochemical nitrogen cycle, contributing to as much as half of the total nitrogen removal from natural environments (*78*). These bacteria have been found in almost every aquatic environment, including groundwater (*79*), freshwater (*80*), saltwater (*78*), estuaries (*81*), hot springs (*82*), hydrothermal vents (*83*), wastewater treatment plants (*84*), and arctic ice (*85*). One community studied from Young Sound (Northeast Greenland) had a temperature optimum of 12°C (*86*).

2.6.1 Anammox History

For many years, oxidation of ammonium was thought to be a strictly aerobic process. However, in 1977, Broda postulated the existence of ammonium oxidation using nitrite or nitrate as the electron acceptor based on thermodynamic calculations (*87*). It was not until 1995 that anaerobic ammonium oxidation was discovered in a denitrifying fluidized bed reactor treating supernatant from a methanogenic reactor with supplemental nitrate (*84*). These bacteria were initially thought to oxidize ammonium with nitrate as the electron acceptor, but future work illustrated that nitrite was the oxidant (*69*).

It was not until 2007 that the first full-scale anammox system was reported in Rotterdam, Netherlands (*88*). This system treated supernatant from an anaerobic digester that had been oxidized prior to undergoing the anammox process. To date, there are ~100 full-scale nitritation/anammox treatment systems worldwide, mostly (75%) treating side streams from wastewater sludge treatment, landfill leachate, or food processing digestate (*89*, *90*).

2.6.2 Anammox Mechanism

Anaerobic ammonium oxidation is carried out by several enzymes. The first step is to reduce nitrite to nitric oxide. Nitric oxide reacts with ammonium to form hydrazine, which is then oxidized to nitrogen gas. To date, anammox bacteria are the only known organisms that produce hydrazine as an intermediate (*91*). Nitrite is also oxidized to nitrate to generate reducing equivalents to fix carbon dioxide (*72*).

2.6.3 Nitritation

From the stoichiometry of anammox (equation 2.6) it is obvious that if most of the influent N is present as ammonium, an anammox system requires partial oxidation of influent ammonium to nitrite. This process has been termed "nitritation" to distinguish it from conventional nitrification as used in conventional biological processes to remove ammonium (Section 2.5.1 Nitrification). Though given a different name, nitritation is identical to the first step of nitrification (equation 2.1), aerobic ammonium oxidation. The net reaction of equations 2.1 and 2.6 is given below (equation 2.7).

$$2\mathrm{NH}_4^+ + 1.5O_2 \rightarrow N_2 + 3H_2O + 2H^+ \qquad \text{nitritation/anammox} \qquad [2.7]$$

From equation 2.7 it can be calculated that this process requires 0.75 moles of O_2 per mole of ammonium-N; on a mass basis, this translates to 1.71 g O_2 (g NH_4^+-N)$^{-1}$. This is a savings of 62.5% when compared to full nitrification. Nitritation/anammox also consumes

one equivalent of alkalinity per mole of ammonium-N oxidized; on a mass basis, this is 3.57 g alkalinity as $CaCO_3$ per g NH_4^+-N oxidized, half that consumed in conventional nitrification.

2.6.3.1 Substrate Inhibition

It is well agreed upon in the literature that nitrite is inhibitory for anammox bacteria; however, there is some debate concerning the reversibility of nitrite inhibition (*70*, *92*) and the concentration of nitrite that causes inhibition. Inhibitory concentrations have been reported from 5-420 mg NO_2^--N L^{-1} (*70*, *92–96*). To ensure stability of the anammox process, the instantaneous concentration of nitrite needs to remain relatively low (below the inhibitory concentration) during treatment, which places constraints on reactor design and operation.

2.6.3.2 Control of Nitrite Oxidizing Bacteria (NOB) Activity

The success of a nitritation/anammox process to treat waste that contains ammonium as the principal form of inorganic N depends on minimizing the oxidation of nitrite by NOB. In an effort to selectively inhibit the activity of NOB, several control mechanisms have been proposed based on physiological differences between AOB and NOB. A commonly used mechanism of control is the use of low dissolved oxygen (*97–100*), which takes advantage of the higher affinity for oxygen of AOB over NOB (*97*). Typically, nitritation is achieved with a dissolved oxygen concentration near 0.5 mg DO L^{-1} (*73*, *97*, *101*, *102*), although some studies have shown nitrite accumulation at high DO (5.0-6.6 mg DO L^{-1}) (*21*, *103*). This approach has been demonstrated at full-scale (*100*).

At elevated temperature, AOB grow faster than NOB (Figure 2.3) (*104*). By controlling the solids retention time, NOB can be washed out of the nitritation reactor. Heating the influent wastewater can be cost-prohibitive, so this technology is often employed to treat

side-streams of municipal treatment, such as supernatant from a mesophilic anaerobic digester. This approach has also been demonstrated at full scale (*88*).

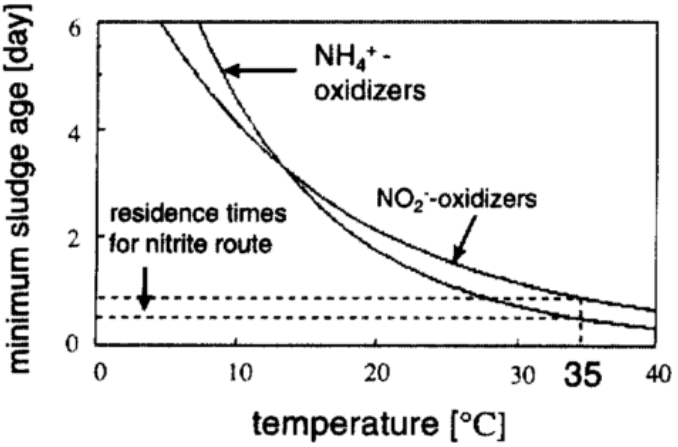

Figure 2.3: AOB and NOB growth rate as a function of temperature. Figure reproduced from Hellinga et al. (*104*)

Other mechanisms for NOB control have been proposed, but none have been demonstrated at full-scale. Although AOB and NOB are both autotrophic species (*58*), the concentration of carbonate has been found to have an effect on activity (*105*). Using sodium carbonate as a source of alkalinity for nitrification resulted in high nitrite accumulation, whereas switching the alkalinity source to sodium hydroxide resulted in nitrate accumulation. This suggests NOB could be more susceptible to substrate inhibition by carbonate than AOB.

Ammonium and nitrite have been proposed as candidates for suppression of NOB activity (*101*, *103*, *106*). There is still significant debate in the literature if the ionized or non-ionized form of these compounds is inhibitory, and the inhibitory concentration range is still under determination.

Isaka et al. (*107*) have found that NOB are more susceptible to heat shock than AOB. This study used nitrifying bacteria that were suspended in a gel carrier and heated to 60-90°C for one hour. However, this study found that nitritation was unstable and repeated heat

shocking of the gel carrier was necessary to keep the effluent nitrate concentration low. Such a process would increase operational complexity and cost significantly and, therefore, is undesirable in a full-scale system.

It has been determined that NOB take longer to resume activity after transient anoxia than AOB (*108*). This implies that in a reactor design where biomass is exposed to alternating anoxic/aerobic conditions, AOB should dominate the nitrification community and significant nitrite accumulation should occur.

The last mechanism to inhibit NOB activity, anaerobic digestion followed by biological nitrogen removal, is not understood well enough to use as a designed method of control, but is still important to consider in the context of this research. In one study,Anceno et al. studied two systems for swine waste treatment in parallel: one system that was designed for biological nitrogen removal and the other with anaerobic digestion prior to biological nitrogen removal (*18*). In the system that involved anaerobic digestion, AOB dominated the community and significant nitrite accumulation occurred. In the system without anaerobic digestion, nitrate was the principle nitrification end-product. This implies that preceding nitrogen removal by anaerobic digestion selected against NOB, although no mechanism has been proposed for the observation.

2.6.4 Biomass Retention

Due to the slow growth rate of anammox biomass (*67*), a reactor configuration with efficient solids retention is required to prevent washout of the bacteria. This is often accomplished by growing a biofilm on a supporting medium (*73*, *109*), using granular biomass (*77*, *110*, *111*), or a combination (*112*).

The principle behind a biofilm system is relatively straightforward. An easily separable growth substrate (e.g. expanded clay or Kaldnes® carrier) is added to a bioreactor and the active bacteria colonize the media. By retaining the media in the reactor, the bacteria are easily contained.

Granule based systems are a bit more complicated than biofilm based systems. Granules are small, dense clusters of bacteria that settle very quickly in a reactor, which allows for high biomass retention. Several factors have been observed to affect granulation. Mechanical stress was observed to favour compact granule formation until an upper threshold was reached, at which point increased stress caused breakup of the granules and cell lysis (*110*). Mechanical stress can be provided by mixing (*110*), gas flow (*113*), or both. Anammox granules with an average diameter of 0.2-2.39 mm have been reported (*68*, *110*, *112*, *114*, *115*).

Although the mechanism that causes granulation remains unknown, data from Clippeleir et al. (*114*) suggest that granulation is a function of nitrogen removal rate. Since substrate removal rate is based upon biomass concentration, this implies that granulation is a function of biomass concentration. Granule formation has been observed to be positively correlated with calcium addition to an anammox reactor (*116*) and apatite has been found to increase granule strength (*117*). The addition of calcium can stabilize the negative charge on bacteria from extracellular polymeric substances allowing the microbes to bind together. However, calcium addition has also been found to lead to the precipitation of calcium phosphate salts (e.g. $Ca_4H(PO_4)_3$) and accumulation of inorganic matter in a reactor (*116*, *118*). Metal sulfide salts have also been hypothesized to contribute to granulation (*119*).

In one study, co-granulation of nitrifying and anammox bacteria was reported (*118*). This study was performed using synthetic media and the applicability to real waste is unknown.

2.6.5 Advantages and Disadvantages of Anammox Treatment

Nitrogen removal by anammox has several distinct advantages over conventional biological nitrification/denitrification. The following list was compiled by Gao & Tao (*120*).

- Anammox bacteria are typically viewed as autotrophic, although some species of anammox bacteria have been shown to utilize organic matter, especially volatile fatty acids (*121–123*). Assuming the more-generally accepted fully-autotrophic case, organic matter is not needed for nitrogen removal and, theoretically more organic matter could be used for biogas production. This fact suggests an economic advantage of anammox over conventional nitrogen removal since external carbon addition has been cited as one of the most expensive parts of nitrogen removal via denitrification (*57*). For anaerobically digested swine waste, the implication is that overall nitrogen removal may increase since denitrification was limited by the supply of biodegradable COD at Butler Farms (*8*).
- From the stoichiometry of equation 2.7 versus equation 2.3 it can be calculated that anammox requires 1.71 g O_2 (g NH_4^+-N)$^{-1}$ as compared to 4.57 g O_2 (g NH_4^+-N)$^{-1}$ for nitrification, a savings of 62%. This also provides an economic advantage for anammox treatment, as provision of oxygen has been cited as one of the most expensive parts of nitrogen removal (*56*). Equation 2.7 also shows that anammox consumes 3.57 g alkalinity as $CaCO_3$ per g ammonium-N oxidized. Cumulative mass loading diagrams from the pilot project (Chapter 4) show that 4.21 g of alkalinity as $CaCO_3$ was supplied to the reactor in lagoon effluent per g of total-N. This indicates there should be sufficient alkalinity for nitrogen removal by anammox and no external alkalinity source needs to be added.

- Nitrification and denitrification both produce N_2O as a side-product or intermediate, whereas it is unclear if N_2O is a direct side-product of anammox. This may result in a net decrease of N_2O production, but this advantage would need to be confirmed experimentally.
- The low growth yield of anammox bacteria results in lower sludge production, which lowers the cost associated with sludge handling.

There are also significant disadvantages associated with nitrogen removal by anammox.

- Nitrite (a substrate of anammox) is easily oxidized to nitrate by NOB and easily reduced to nitrogen gas by denitrifiers. Both groups bacteria need to be selectively inhibited via reactor design and control. Further, nitrite is fairly toxic to anammox bacteria and as such, the instantaneous concentration needs to be controlled.
- The low specific growth rate of anammox bacteria can lead to several operational problems, including biomass washout or out-competition by other bacteria (i.e. denitrifiers).
- Incomplete nitrogen removal by anammox due to the formation of nitrate has been cited as a disadvantage; however, depending on reactor operation and influent characteristics, this may not be a significant problem, as nitrate could be consumed by limited denitrification activity.

2.6.6 Temperature Effects

Most systems for nitrogen removal by anammox operate at elevated temperatures (30-35 °C) (*88*, *100*, *124*, *125*). This is likely due to the long doubling time of anammox bacteria and the desire to grow the bacteria as quickly as possible by utilizing their optimum temperature. Reject-water from a mesophilic digester is often at a temperature conducive to the optimum

growth temperature of anammox bacteria and is often used as influent to an anammox process.

2.7 Recently Proposed Processes for Nitrogen Removal

Recently, several alternative nitrogen removal systems have been proposed. For an in-depth discussion of reactor design theory and proposed systems for nitrogen removal via the anammox pathway see Chapter 3.

2.8 Coupling Anammox to Anaerobic Digestion of Swine Waste

2.8.1 Anaerobic Digestion

Under strict anaerobic conditions, biochemical oxygen demand can be converted to methane as shown in reaction 2.8.

$$BOD \rightarrow vCH_4 + wCO_2 + xH_2 + yNH_3 + zH_2O \qquad \text{anaerobic digestion} \qquad [2.8]$$

Typically, not all of the organic matter that is present in a waste is converted to methane during anaerobic digestion. Anaerobic reactions besides methanogenesis also tend to produce organic products, such as volatile fatty acids (e.g. acetate and propionate) that partially accumulate in the digested waste. This residual organic matter would be potentially available for denitrification in a nitrification/denitrification system for removing nitrogen from the effluent of a covered lagoon performing anaerobic digestion. These organic products could also interfere with a downstream anammox process (*115*).

If the COD $(NH_4^+\text{-}N)^{-1}$ ratio is too high, anammox bacteria could be unable to out-compete heterotrophic denitrifiers for nitrite (*126*). Anaerobic digestion is a mechanism to remove organic matter from a waste and make that waste more amenable to anammox. It is important to note that methane production was substantially reduced during the winter at Butler Farms indicated by the increase in COD (Figure 2.2). This observation is typical of winter at Butler Farms as indicated by historical data on methane production (Figure 2.4).

These data show significant temperature effects on anaerobic digestion, which could impact the stoichiometry and therefore, performance, of a downstream nitrogen removal system depending on the season. Since the pilot project was completed, Butler Farms has implemented a full-scale mesophilic anaerobic digester that should maintain anaerobic digestion throughout the winter. The impact this system has had on the year-round concentration of COD is unknown.

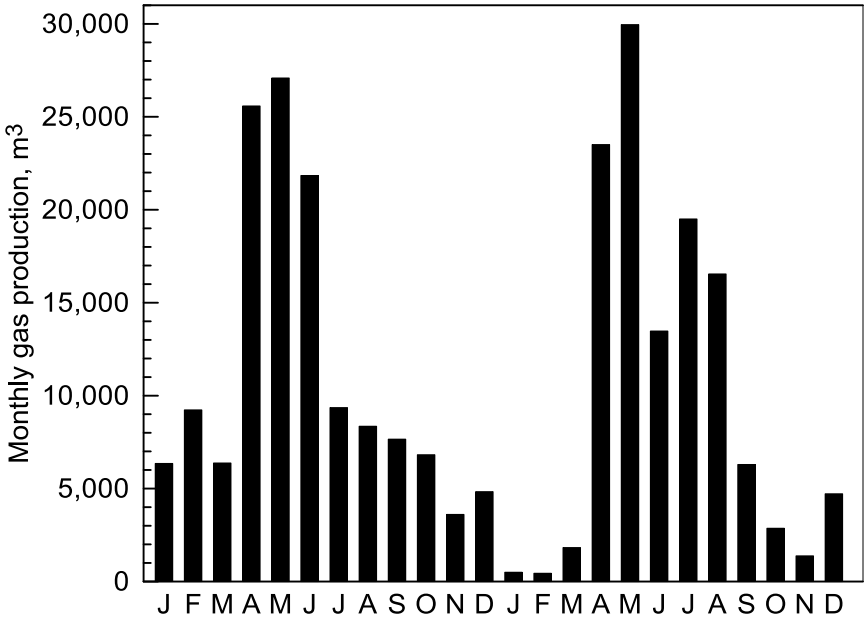

Figure 2.4: Methane production at Butler Farms since installation of the lagoon cover (*8*).

2.8.2 Studies Combining Anammox with Anaerobic Digestion of Swine Waste

To date, there have been limited studies that combine anaerobic digestion of swine waste with nitrogen removal by anammox (*21*, *23*, *25*, *115*, *127–129*).

The reactors in most of the cited studies were operated at elevated temperature (30-37°C). This is well known to be the optimum temperature for anammox bacteria that have been studied in terms of wastewater treatment, however, this is significantly higher than the minimum temperature of the lagoon liquid observed at Butler Farms during winter 2010-

2011. One of the cited studies (*128*) performed anammox in a sequencing batch reactor (SBR) at ambient temperature (15-30°C) with little activity below 20 °C.

Many of the previous studies used a two-reactor, continuous flow, single-reactor high-activity ammonium removal over nitrite (SHARON)/anammox system for nitrogen removal (*21*, *25*, *115*, *127*). Three used SBR systems (*23*, *128*, *129*). The latter studies also had fairly low ammonium-N in the influent (400-700 mg N L^{-1}; 15-30% the ammonium-N concentration from Butler Farms).

Two of the previous studies diluted influent waste extensively; in one case, the waste was diluted 12-40 times with tap water (*21*) and in the other, the waste was diluted 10-50 times with synthetic media (*115*). This was done to prevent inhibition from COD, nitrite or other compounds and to account for the high suspended solids content in the influent. This is not a cost-effective option for full-scale treatment.

The total-N, ammonium-N and COD concentration in the influent for these studies is summarized in Table 2.3. These values bracket the concentration of these parameters observed at Butler Farms.

Table 2.3: Total-N, ammonium-N, and total COD (mg L^{-1}) concentration in influent to anammox processes treating anaerobically digested swine waste.

Reference	Total-N	Ammonium-N	Total COD
(*23*)	472 ± 6	418 ± 10	437 ± 6
(*25*)	1,550 ± 160	970 ± 50	2,940 ± 1,100
(*21*)	3,000-5,000	2,000-4,000	8,000-17,000
(*115*)	N/R [a]	3,780 ± 460	4,740 ± 1,050
(*127*)	1,310 ± 160	960 ± 160	N/R
(*128*)	662 ± 190	519 ± 134	387 ± 145
(*129*)	N/R	245 ± 16	420 ± 60

[a] Not Reported

Analysis of the microbial community in the previous studies has been done by fluorescent *in situ* hybridization (*23*, *25*, *115*), scanning electron microscopy (*25*, *127*), and denaturing gradient gel electrophoresis (*23*).

None of the above studies evaluated the stoichiometry of a one-reactor anammox system nor the effect of changing design variables on the microbial community via quantitative polymerase chain reaction (qPCR) or bar-coded pyrosequencing. Further, none of the cited studies examined the effect of changing reactor conditions on the stability of the anammox process.

2.9 Microbiology

All 5 *Candidatus* genera of anammox bacteria (*Brocadia, Kuenenia, Scalindua, Anammoxoglobus,* and *Jettenia*) (*130*) are members of the deeply branching phylum *Planctomycetes*. These organisms are unusual among the *Bacteria* in that they contain an intracellular membrane and lack peptidoglycan in their cell walls (*65*). Each ecosystem tends to be dominated by a single anammox genus, suggesting that each of these bacteria is highly adapted to its ecological niche (*131*).

There is no pure isolate of any known anammox organism. Due to their slow-growing nature, traditional microbiological methods are not feasible and newer molecular microbiology methods need to be employed to study the bacteria involved in this process.

2.9.1 qPCR

Quantitative polymerase chain reaction (qPCR) is a method that counts the number of copies of a gene of interest in a system. This method works by incorporating a fluorescent label into newly synthesized DNA. When the fluorescent signal exceeds a critical threshold during the PCR reaction, the signal can be compared to a standard curve to determine the gene copy number. In the context of engineered anammox systems, three groups of bacteria

are of primary importance for quantification; anammox bacteria, denitrifying bacteria, and NOB. Each of these three processes consumes nitrite and understanding their relative abundances is important for understanding the fate of nitrite in a nitritation/anammox system.

Typically, 16S rRNA genes are used for quantification of bacterial concentration. This method relies on the bacteria of interest being closely related to each other, but unrelated to other organisms. This degree of relation allows for specific primers to be developed for the bacteria of interest. However, anammox and denitrifying bacteria are phylogenetically diverse; as such, functional gene analysis can be much more informative when quantifying the bacteria capable of performing the respective function. Recently, the apparently conserved gene for hydrazine synthase has been used to quantify anammox populations (*91*).

A good candidate gene for qPCR is unique to the pathway that performs the process of interest. To quantify the number of bacteria performing denitrification, a gene unique to the denitrification pathway would be ideal. Nitrate reduction to nitrite is carried out by denitrifiers and by bacteria using nitrate as a nitrogen source. Nitrite reduction to nitric oxide is performed by anammox bacteria and denitrifiers. Nitric oxide reduction is performed by several pathogens to survive an immune response. Since these enzymes are found in multiple pathways, they are not ideal candidates for qPCR to determine the relative contribution of denitrification to nitrogen removal. However, nitrous oxide reduction is performed only by denitrifiers and has been used to quantify denitrifying bacteria (*132*, *133*).

Unfortunately, aerobic nitrite oxidation by NOB uses the same enzyme as nitrite oxidation by anammox; therefore a functional gene approach is not a viable option to quantify NOB in the proposed system. In this case, 16S rRNA genes must be used to quantify

the number of NOB in the system. Primer sets for NOB 16S rRNA genes have been developed (*134*).

2.9.2 High-Throughput Sequencing

So called "next-generation" or high-throughput sequencing techniques allow for the determination of thousands to millions of short DNA sequences simultaneously. This technology is used to sequence very large stretches of DNA (e.g. chromosomes) by aligning overlapping regions or generating large numbers of short sequences such as in microbial community analysis. These systems typically utilize a sequencing-by-synthesis approach rather than older technologies, such as Sanger sequencing, which work by chain termination. Multiple high-throughput platforms have been developed including the Illumina MiSeq platform that I used in this study. The MiSeq platform works as follows (*135*):

1. The target region is amplified using PCR with primers that contain the Illumina adapter sequence, an index sequence, a 10-nt pad (to prevent hairpin formation and control primer melting temperature), a 2-nt linker, and the gene-specific primer (*136*).
2. The PCR amplicon is applied to a lane on a glass flow cell that is coated with a "lawn" of two types of Illumina adapter oligonucleotides; a forward adapter, and a reverse adapter. The adapter incorporated into the amplicon in step 1 hybridizes to its complementary adapter on the flow cell.
3. A polymerase creates a strand of DNA complementary to the amplicon. The template is denatured and washed away leaving a strand of DNA attached to the flow cell.
4. Each strand is amplified through "bridge amplification" to create a cluster or colony of DNA fragments. The reverse strands are cleaved and washed away leaving only the forward strands.

5. A sequencing primer is added to the flow cell and mixtures of 3`-blocked fluorescently-tagged nucleotides are added. Nucleotides are incorporated by basepair matching and mismatched nucleotides are washed away. The DNA is excited by a laser which releases the fluorescent signal and removes the 3` blocking.
6. Step 5 is repeated until the fragment of DNA has been sequenced. The read product is denatured and washed away.
7. The DNA fragment is allowed to bind to the opposite oligonucleotide, amplified, and denatured. The forward strands are cleaved and washed away leaving only the reverse strands.
8. Step 5 is repeated to generate a sequence of the reverse read. In this way a "paired-end" read is generated.
9. Up to 15 gigabases with 25 million 300-basepair paired-end sequences can be generated per flow cell (*137*).

This technology produces tremendous amounts of data, more than is needed by most researchers for most purposes. As such, methods have been developed that allow for combining multiple samples onto the same sequencing run and sequencing multiple samples in parallel. This is known as "bar-coding", "indexing", or "multiplexing" and is performed as follows (*138*):

1. A short, sample-specific stretch of DNA (barcode) is introduced to the amplicon during Step 1 of the sequencing preparation above. In this way, every amplicon associated with that sample has the same barcode.
2. Samples are pooled together and loaded onto the sequencing platform and the sample is sequenced.

3. *In silico* analysis is performed to associate barcodes with the sample from which the sequence originated.

High-throughput sequencing systems have been applied to diverse ecosystems such as marine water (*139*), soil (*140*), human intestines (*141*), wastewater treatment plant influent (*142*), and wastewater activated sludge (*143*). This technology has also been used in the identification of human pathogens in wastewater treatment plants (*144*), however, only anammox reactors treating synthetic media have been sequenced using these techniques (*145*, *146*) and no studies have been performed on anaerobic digesters treating swine waste.

Chapter 3: Biological Reactor Design

3.1 Introduction

Design of reactors, particularly biological reactors, is part science, part engineering, and part art. This chapter will explore the fundamental science and engineering behind design of biological systems and review some common systems for nitrogen removal via nitrification/denitrification and nitritation/anammox. Specific reactor configurations such as a sequencing batch reactor and a moving bed biofilm reactor are also discussed.

3.2 Reactor Design Equation

The reactor design equation can be derived from a fundamental mass balance (Equation 3.1).

$$\frac{dV_{reactor}C_{i,out}}{dt} = Q_{in}C_{i,in} - Q_{out}C_{i,out} + V_{reactor}r_i \quad (3.1)$$

Where:

$V_{reactor}$	=	volume of the reactor
i	=	a species of interest
$C_{i,out}$	=	the concentration of species i leaving the reactor
$C_{i,in}$	=	the concentration of species i entering the reactor
Q_{in}	=	the flow rate into a reactor
Q_{out}	=	the flow rate out of a reactor
r_i	=	the rate of reaction on species i; positive indicates i is produced

Assuming the following:

- Steady-state
  - $dV_{reactor}C_{i,out}/dt = 0$
  - $Q_{in} = Q_{out} = Q$
- Ideal continuously-fed stirred tank reactor

Equation 3.1 simplifies to:

$$V_{reactor} = \frac{Q(C_{i,in} - C_{i,out})}{-r_i} \quad (3.2)$$

The flow rate and the influent concentration of species i is characteristic of the system being studied and the concentration of species i in the effluent is part of the design objectives. By knowing r_i the volume of the reactor can be determined. Unfortunately, r_i is rarely known precisely and must be determined though batch experiments, lab and pilot scale studies, or modeling.

3.3 Biological Reactor Design

In biological systems the rate expression is often given by:

$$-r_i = qX_i \quad (3.3)$$

Where:

q = biomass specific rate of substrate utilization

X_i = concentration of biomass active on species i

The substrate utilization rate can be related to more fundamental properties of the system and the biomass by Monod kinetics:

$$q = \frac{\hat{q}\, C_i}{K_i + C_i} \quad (3.4)$$

Where:

$\hat{q}$ = maximum biomass specific rate of substrate utilization

K_i = half saturation constant (i.e. concentration of component i at which the substrate is utilized at half its maximum rate)

The concentration of component i is part of the design equation and $\hat{q}$ and K_i can be determined empirically. There are additional relationships that be used to predict X_i, but they will not be explored here.

Design of biological reactors involves ensuring the reactor has the conditions required for the bacteria that perform a desired reaction. These include: temperature, pH, dissolved oxygen, substrate, micronutrients, and sufficient solids retention time (SRT; i.e. the bacteria are retained in the system long enough to become important members of the microbial community).

3.3.1 Reactor Conditions Necessary for Biological Nitrification

As discussed in Chapter 2, biological nitrification is a two-step process where ammonium is oxidized to nitrite and subsequently oxidized to nitrate. Full nitrification of ammonium to nitrate is typically performed at ambient temperature, neutral pH, 2-4 mg L^{-1} dissolved oxygen, and a 7-18 day SRT.

3.3.2 Reactor Conditions Necessary for Biological Denitrification

Denitrification primarily occurs under anoxic conditions when nitrate/nitrite and an electron donor (typically organic matter) is present in a system. The bacteria that facilitate this reaction thrive under a range of temperatures, typically near neutral pH, and a 2-10 day SRT. A typical design parameter for the ratio of COD to N is ranges from 4-5.5.

3.3.3 The Modified Ludzack-Ettinger Process (Pre-denitrification)

The Modified Ludzack-Ettinger (MLE) process is one of the most commonly used systems for biological nitrogen removal via nitrification/denitrification. This process has denitrification preceding nitrification, which allows the heterotrophic organisms to utilize organic matter in the influent as an electron donor for denitrification. Once the waste has

been denitrified, it is treated aerobically to remove additional organic matter (if present) and nitrify the influent ammonium. An internal recycle supplies nitrate to the pre-denitrification zone. This process also allows for the recovery of alkalinity produced by denitrification to be consumed by nitrifying bacteria. This configuration minimizes the need for external alkalinity and electron donor addition.

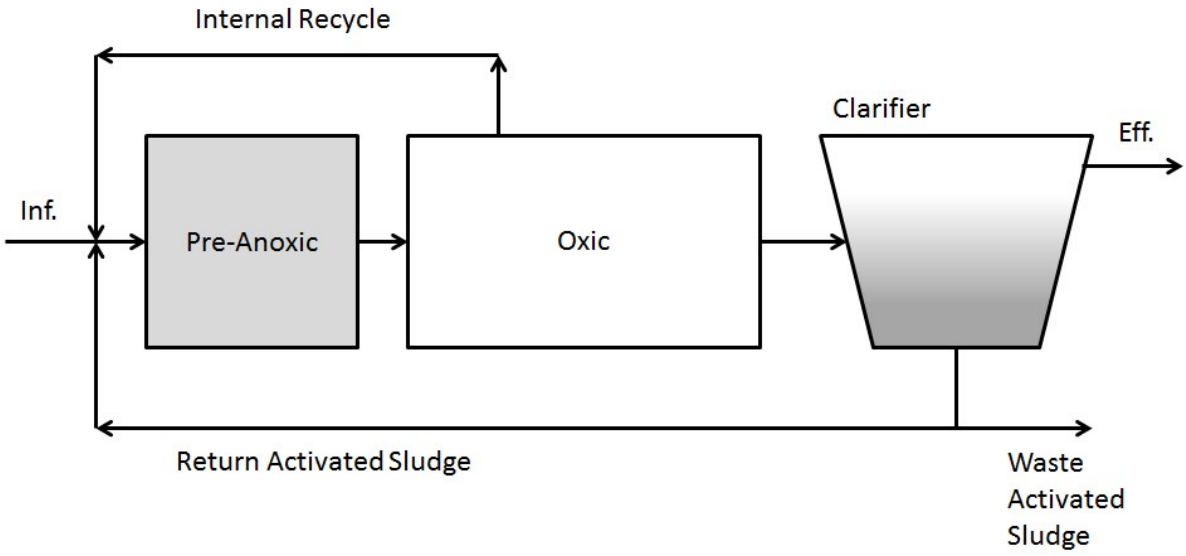

Figure 3.1: Block flow diagram of the MLE process. Inf. – Influent. Eff. – Effluent.

3.4 Reactor Configurations

Thus far, the primary consideration has been ideal continuously-fed stirred tank reactors. The research presented in Chapter 4 utilizes real continuously-fed stirred tank reactors and the research presented in Chapters 5 and 6 utilizes a sequencing batch-moving bed biofilm reactor. This is a hybrid system between a sequencing batch reactor and a moving bed biofilm reactor; these configurations are explained in this section.

3.4.1 Sequencing Batch Reactors

An SBR performs the functions of a continuous-fed biological system but in a single tank. Treatment in an SBR involves discrete cycles in which new waste is added to the reactor (fill period); biological reactions are carried out under either oxic or anoxic

conditions (react period); the contents of the reactor are allowed to settle, allowing for retention of the biomass (settle period); and supernatant is then decanted (draw period); the reactor can then be allowed to sit without waste if needed (idle period). These periods are rough guidelines and can be modified/omitted to meet design specifications (e.g. waste can undergo reactions during the fill period).

Some of the settled biomass can also be removed, or "wasted", from the system as well. After decanting the treated waste, a new cycle is then initiated. A treatment cycle can be designed to last from several hours to several days. Parallel reactors can be used so that different parts of a cycle are carried out at different times; this permits continuous treatment of the waste by letting one reactor undergo the react period while the other reactor(s) are settled, decanted and filled. The hydraulic retention time of an SBR (in days) is equal to the full volume of the reactor divided by the volume of waste decanted per day. The solids retention time (in days) is equal to the amount of biomass in the system divided by the amount of biomass wasted per day. The hydraulic and solids retention times would be equal if decanting were performed with unsettled waste.

It is important to note that SBRs are inherently unsteady systems. Although the literature often refers to an SBR at "steady-state" this is incorrect terminology. Therefore, this research refers to an "equivalent steady-state" where effluent quality is consistent between cycles. In continuous-flow reactors, it is typically considered that steady-state takes 3 SRT to reach. I assume a similar period of operation to achieve equivalent steady-state in an SBR.

3.4.2 Moving Bed Biofilm Reactors

The moving bed biofilm reactor (MBBR) utilizes pieces of floating plastic media with a high protected surface area (500-1200 $m^2 m^{-3}$) that are physically moved through the reactor

(typically by blowers or impellers) and provide a large protected surface area for bacteria to colonize. Typical media used in MBBRs is shown in Figure 3.2**Error! Reference source not found.**.

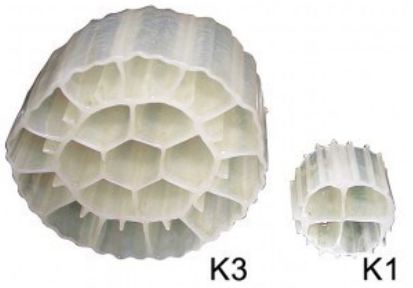

Figure 3.2: Two types of media used in MBBRs (*147*).

Integrated Fixed Film Activated Sludge (IFAS) processes are a hybrid between conventional suspended growth systems and MBBRs. IFAS systems include a biomass separation and recycle step to increase the concentration of suspended active biomass in the MBBR. However, any process will have biomass growing in the liquid phase; the function of that biomass is dependent upon retention time, substrate availability, and other environmental factors. This implies there is little to no difference between an MBBR and an IFAS system.

3.5 Recently Proposed Processes for Nitrogen Removal via Nitritation/Anammox

Recently, several alternative nitrogen removal systems have been proposed to remove nitrogen via the anammox pathway. Many of these processes have a large degree of similarity and can be divided into two broad categories: oxygen-limited systems where ammonium oxidation and nitrogen removal occur simultaneously, and two-stage systems were oxidation and anammox occur in separate reactors.

3.5.1 OLAND, CANON, DEMON and SNAP

Oxygen Limited Autotrophic Nitrification and Denitrification (OLAND), Completely Autotrophic Nitrogen removal Over Nitrite (CANON), DEamMONification (DEMON), and

Single-stage Nitrogen-removal using Anammox and Partial nitritation (SNAP) systems are virtually indistinguishable from each other and illustrated in Figure 3.3.

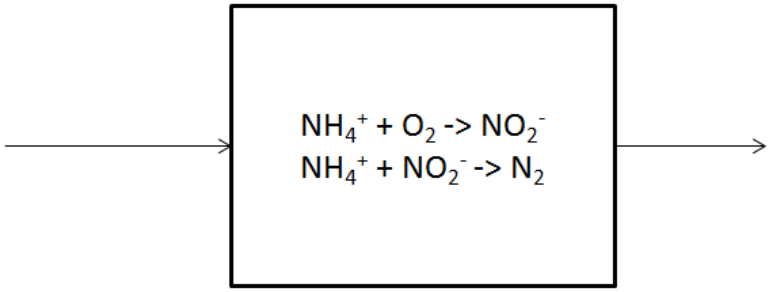

Figure 3.3: One-reactor systems for nitrogen removal by anammox.

In the first description of OLAND systems (*148*) it is quite clear that this system makes use of the ability for ammonium oxidizing bacteria to use nitrite/nitrate as a terminal electron acceptor under low-DO conditions. When nitrifiers do this, they reportedly use hydrogen or ammonium as the electron donor (*149*) with normal denitrification intermediates (NO and N_2O). However, an NCBI protein BLAST search of nitrous oxide reductase from *Thiobacillus denitrificans*, a known autotrophic denitrifier (*150*), against *Nitroso-* genera (the *Nitrosomonas*, *Nitrosolobus*, *Nitrosococcus*, *Nitrosocystis*, *Nitrosopumilus*, and *Nitrosospira* databases) did not result in any significant matches (*151*). Moreover, recent papers on OLAND systems specify that known anammox bacteria are responsible for nitrogen removal (*152*, *153*).

CANON, DEMON, and SNAP systems function under virtually identical conditions to OLAND systems: a low-DO environment is maintained to encourage nitritation and anammox to occur in one reactor (*100*, *154–156*). Low DO conditions can be maintained either through continuous or intermittent aeration. So long as DO does not increase to a level that will inhibit anammox bacteria, nitrite will be consumed as it is produced, leading to nitrogen removal.

Most studies on these processes have been done using synthetic media. However, when real wastewater is treated, a source of alkalinity typically needs to be added. Sodium bicarbonate (*152*) and reject water from sludge dewatering (*100*) have been used as a source of alkalinity. In some studies, no external alkalinity is needed (*23*).

Though most of the academic literature on DEMON systems focuses on using synthetic media, there has been significant work in the private sector bringing DEMON to full-scale market. DEMON is currently licensed for US distribution through World Water Works. The first US-based full-scale DEMON system was brought online in October 2012 at the Hampton Roads Sanitation District in Virginia. To date, DEMON is only recommended for high-strength (>200 mg NH_4^+-N L^{-1}) waste (*157*), though there is significant interest in using DEMON systems for mainstream treatment at municipal wastewater treatment plants (*158*).

3.5.2 SNAD

Simultaneous partial-Nitrification Anammox and Denitrification (SNAD) incorporates conventional denitrification into the oxygen-limited systems described above (*159–161*). The authors claim that the nitrate produced by anammox is reduced by denitrifiers. However, bioreactors are typically complex systems and any energy-yielding reaction that can occur, will occur. This implies that if oxidized N and organic matter co-exist in an anammox reactor, some portion of the microbial community will consist of conventional denitrifiers, making this system no different than a CANON/OLAND/DEMON system treating real waste.

3.5.3 ANITA™ Mox

ANITA™ Mox is very similar to the OLAND/CANON/DEMON/SNAP systems described above; a single reactor system for nitritation and anammox. The primary and major

difference between ANITA^{TM} Mox and other systems is the addition of plastic carriers designed to support biofilm growth of microorganisms as they move through the reactor. ANITA^{TM} Mox is marketed by AnoxKaldnes at Kruger Inc., a subsidiary of Veolia Water Solutions and Technology for treatment of anaerobic digester centrate, industrial wastewaters, and landfill leachate. To support biofilm growth, these systems typically rely on AnoxKaldnes plastic carriers; typically K3, Anox K5, or BiofilmChip^{TM} M (*162*).

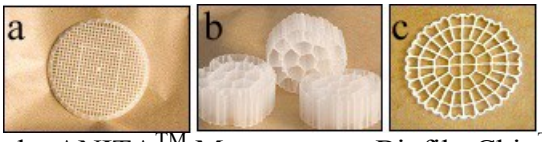

Figure 3.4: Media used in the ANITA^{TM} Mox process. BiofilmChip^{TM} M (a), K3 (b), Anox K5 (c).

This system is advertised to achieve an ammonium removal efficiency greater than 90% and total nitrogen removal in the range of 75-85% (*163*). ANITA^{TM} Mox systems are started using 3-15% mature biofilm-coated media from The BioFarm in Malmö, Sweden to shorten start-up time (*164*).

It is unclear from Veolia's literature the exact process configuration of ANITA^{TM} Mox. The system is known to operate at relatively high dissolved oxygen (0.5-1.5 mg L^{-1}) with continuous aeration (*164*). The method of reactor operation (i.e. continuous flow or sequencing batch) is unknown.

ANITA^{TM} Mox is typically described as an MBBR although there has been work to develop an IFAS system. As described in section 3.5.2 there is little inherent difference between these two processes. By lengthening the suspended phase SRT to 5 days (from 1 day) an ANITA^{TM} Mox IFAS system was able to remove nitrogen ~4 times faster than an ANITA^{TM} Mox MBBR system (*165*).

3.5.4 SHARON/Anammox

The above systems describe nitritation and anammox occurring in one reactor. The other possible reactor configuration involves nitritation and anammox occurring in separate reactors. By maintaining high temperature (~30°C) and low SRT (1-2 days) NOB can be selectively removed from an aerobic nitrifying system (*104*). This so called "Single-reactor High-activity Ammonium Removal Over Nitrite" (SHARON) takes advantage of the higher growth rate of AOB at elevated temperatures to selectively oxidize the influent ammonium-N to nitrite-N.

When this process is not coupled to denitrification, effluent from the SHARON process typically has the proper stoichiometry for nitrogen removal by anammox (*166*). This effluent can then be fed to a separate anaerobic reactor in which the anammox reaction occurs. One of the assumptions of SHARON is that there is sufficient alkalinity in the influent waste so that complete alkalinity consumption produces effluent with the proper stoichiometry for anammox. However, the amount of alkalinity in a given waste is not necessarily correlated to the amount of ammonium-N.

3.5.5 DEAMOX

One of the primary concerns associated with anammox treatment of high ammonium-N wastewaters is that when half the influent ammonium-N is oxidized, the concentration of nitrite can be inhibitory to anammox bacteria. For example, the waste at Butler Farms had on average 2,340 mg NH_4^+-N L^{-1}. If half this was oxidized to nitrite, there would be ~1,170 mg NO_2^--N L^{-1}, which is far greater than the inhibitory threshold of any known anammox species. However, it is the concentration of nitrite in the anammox reactor that is most

important, and reactors can be designed to maintain relatively low instantaneous concentrations of reactants and intermediates such as nitrite.

One method for circumventing the potential for accumulation of inhibitory concentrations of nitrite is DEnitrifying AMmonium OXidation (DEAMOX) (*167*, *168*). This process oxidizes half the influent N to nitrate, which is not known to be toxic to anammox bacteria. The oxidized waste is then exposed to anoxic conditions where conventional denitrifying bacteria reduce nitrate to nitrite and the produced nitrite is consumed by anammox bacteria.

The authors who proposed DEAMOX do not propose a mechanism for how nitrite is released by denitrifying bacteria instead of being reduced through the normal denitrification pathway. It has been reported that when all of a cell's respiratory needs can be met by nitrate reduction to nitrite, denitrifiers have a tendency to release nitrite into the environment (*169*). As such, under high NO_3^- $(COD)^{-1}$ ratios, partial denitrification could serve as a nitrite source for anammox.

3.5.6 Summary of Loading Rates for Single-Reactor Anammox Systems Treating High-Strength Waste

Table 3.1: Summary of literature loading rates for single reactor anammox systems treating high-strength waste.

Reactor Configuration	NRR [a] ($kg\ N\ m^{-3}\ d^{-1}$)	Volume (m^3)	Feed	% TIN [b] Removal	Reference
MBBR	0.3	2.1	Sludge dewatering supernatant	57.5	(*73*)
DEMON	0.6	500	Sludge dewatering reject water	N/R	(*170*)
DEMON	0.4	134	Reject water	>80	(*100*)
DEMON	0.8	6	Digester centrate	75	(*171*)
OLAND-RBC [c]	1.3	0.0028	Digested black water	76	(*152*)
Granule	0.5	0.4	Digester supernatant	>90	(*172*)
ANITAMox MBBR	1.2	4*50	Digester supernatant	84	(*164*)
ANITAMox MBBR	0.7	0.007	Reject water	70	(*165*)
ANITAMox IFAS	2.4	0.007	Reject water	80	(*165*)
Granule	0.45	0.01	Sludge dewatering reject water	85	(*173*)
SNAD [d]	0.1	0.005	Anaerobic digester liquor of swine wastewater	80	(*128*)
CANON [d]	0.5	0.0015	Anaerobically pre-treated swine slurry	75	(*129*)
CANON SBBR [e]	0.06-0.16	0.0065	Swine digester liquor	53	(*23*)

[a] Nitrogen Removal Rate
[b] Total Inorganic Nitrogen
[c] Rotating Biological Contactor
[d] Ambient Temperature System
[e] Sequencing Batch Biofilm Reactor

3.6 Conclusion

Ultimately, successful biological treatment of any system involves the presence and activity of microbes expressing a desired phenotype and maintaining treatment conditions to support growth of those organisms. The design of biological systems requires the careful of control of environmental variables to maximize the contribution of desired pathways while minimizing the contribution of undesired but related pathways.

Numerous systems have been proposed for nitrogen removal and this chapter has explored some the most recent emerging systems. For a more comprehensive overview of biological systems the reader is referred to the literature.

Chapter 4: Nitrification/Denitrification of Anaerobically Digested Swine Waste

Eric T. Staunton[1], Sarah R. Bunk, Glenn W. Walters, Stephen C. Whalen,

Joseph Rudek, and Michael D. Aitken

4.1 Introduction

Anthropogenic inputs of fixed nitrogen to the environment, particularly from intensive agriculture, have led to substantial pollution of both air and water globally (*9*) . In the U.S., North Carolina is currently home to 8.5 million swine (*1*), most of which are raised in industrial-scale facilities (*174*). Waste from swine farms in North Carolina is typically stored in large, uncovered lagoons and periodically applied to sprayfields to fertilize crops (*2*). The scale of waste generation results in more nitrogen than can be assimilated at agronomic rates in the entire region of swine production (*2*).

High concentrations of ammonium in swine waste lead to emissions of ammonia to the atmosphere (*175*), which can cause significant concentrations of ammonia in air at nearby communities (*32*) as well as transport and subsequent deposition over longer distances (*54*). Volatilized ammonia has been linked to respiratory problems among exposed populations, including swine farm workers (*34*) and those living or attending school near the farms (*33*, *35*). Further, ammonia reacts in the atmosphere to form fine particles that can cause respiratory disease (*36*). In addition to ammonia, uncovered lagoons are sources of methane emission (*3*), with corresponding implications for climate change (*175*, *176*).

[1] Responsible for analysis of nitrogenous species, assist with system operation and troubleshooting, generation of cumulative mass loading and discharge diagrams, and preparation of draft manuscript

In an effort to mitigate the environmental and human health impacts of emissions from industrial-scale swine farms, the North Carolina legislature enacted the 2007 Swine Farm Environmental Performance Standards Act (*6*). This legislation banned the construction of new swine farms that employ open anaerobic lagoons and sprayfields as the primary methods of waste treatment and disposal (as crop fertilizer), respectively; instead, new farms must employ technologies that meet environmental performance standards (*6*, *177*). The standards require substantial reductions in emissions of various pollutants to soil, groundwater, surface water and air, including emissions of ammonia. The 2007 NC Renewable and Energy Efficiency Portfolio Standard Act also established a target for statewide energy production from swine waste, and it created a pilot program which authorized higher rates of payment for electricity generated from anaerobic digestion and methane capture systems on swine farms (*6*). However, the incentives for energy production are not coupled to requirements to meet environmental performance standards. In addition, there are no environmental performance standards for existing swine farms, so that the state of practice for waste management continues to be storage and treatment in open lagoons with spray irrigation on nearby land for disposal.

We conducted a pilot study to evaluate the technical and economic feasibility of coupling conventional biological nitrogen removal (nitrification and denitrification) with anaerobic digestion for methane capture and energy recovery at a swine farm. The study was conducted at one of the few swine farms in North Carolina practicing full-scale anaerobic digestion of waste in covered lagoons with a methane capture system. Of particular interest was an analysis of stoichiometric issues relevant to nitrogen conversions in waste from which a substantial fraction of organic matter would have been removed by anaerobic digestion,

including the availability of electron donors for denitrification, oxygen consumption, and net alkalinity demand of combined nitrification and denitrification. Production of nitrous oxide (N_2O) was also quantified. Details of the economic analysis are available elsewhere (*8*).

4.2 Materials and methods

4.2.1 Study Design and General Characteristics

The pilot-scale nitrogen removal system was installed in a trailer located at the edge of a covered lagoon at Butler Farms in Lillington, North Carolina, USA (Figure A.1). Butler Farms is an 8,000-head grow/finishing farm with 10 barns, each housing an approximately equal number of animals. The lagoon that served as the source of influent for this study was the larger of two at the farm, receiving waste from six of the barns. The lagoon is not mixed, has a maximum volume of 2.5×10^7 L, and maximum depth of 3.3 m. As is typical for swine farms in North Carolina, waste is flushed from the barns with liquid from the lagoons. Periodically the lagoon liquid is sprayed onto on-site fields in accordance with State agronomic regulations. The lagoon cover and methane collection system were installed approximately two years before this study was initiated.

Influent to the pilot system was pumped continuously from the lagoon through an opening in the lagoon cover at the opposite end of the lagoon from the barn discharge, from a depth of 1 m below the liquid surface. The lagoon liquid was pumped to a flow-through, sealed tank in the trailer, from which the influent to the pilot system was pumped via peristaltic pump. The remainder of the lagoon liquid flow was recirculated to the lagoon through a second opening in the cover, approximately 4.5 m from the intake.

The on-site trailer accommodated the reactors (Figure A.2), pumping equipment, associated instrumentation as described below, computer for system monitoring and control,

and an area for analytical equipment (analytical balance, drying oven, COD digester, filtration apparatus, titration apparatus, and benchtop pH meter; Figure A.2). It was heated and air-conditioned to maintain consistent inside temperature; over the course of the study, the temperature in the trailer was 23.2 ± 3.2 °C (n = 288).

Chemical characteristics of the lagoon liquid for the entire duration of continuous system operation are summarized in Table 4.1.

Table 4.1: Lagoon liquid characteristics over the duration of the pilot-scale MLE system operation. Units are mg L^{-1} unless otherwise noted.

Parameter [a]	Mean ± s.d. (n)	Range
NH_4^+-N	2,310 ± 280 (101)	1,770 - 2,670
Total-N	2,750 ± 230 (85)	2,120 - 3,740
TDN	2,610 ± 180 (81)	2,060 - 2,980
Total COD	7,550 ± 2,240 (38)	1,770 - 10,200
Soluble COD	5,370 ± 1,820 (37)	1,390 - 7,740
TSS	1,580 ± 260 (27)	1,180 - 2,180
VSS/TSS	0.40 ± 0.04 (15)	0.34 - 0.51
pH	7.71 ± 0.23 (27)	7.28 - 8.12
Total Alkalinity	12,200 ± 420 (27)	11,200 - 13,100
Bicarbonate Alkalinity	10,600 ± 460 (27)	9,840 - 11,300

[a] Units are mg L^{-1} except units for alkalinity (mg $CaCO_3$ L^{-1}), pH (unitless), and Volatile/Total (unitless).

4.2.2 Pilot System Overview

The nitrogen removal system was a Modified Ludzack-Ettinger (MLE) process without external solids recycle. Influent (covered lagoon liquid) was pumped to the denitrification reactor. Denitrified liquid was pumped to the nitrification reactor, to which pure oxygen was supplied as the oxygen source. Mixed liquor was internally recycled by pumping between the nitrification and denitrification reactors at a recycle ratio of 2.5 (recycle flow rate/system influent flow rate). Effluent from the nitrification reactor was pumped to the lagoon for discharge. A simplified process flow diagram is provided in Figure 4.1.

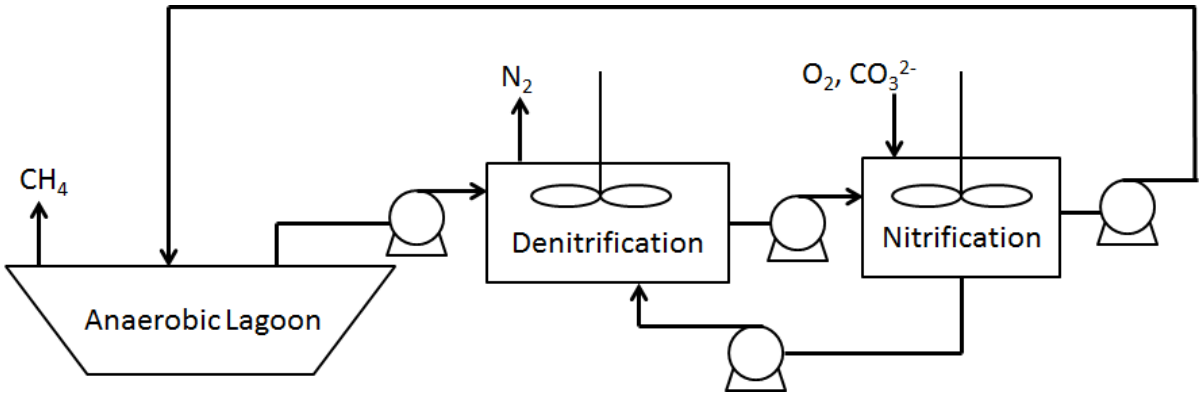

Figure 4.1: Schematic of the pilot-scale nitrogen removal system; not to scale.

The hydraulic retention times (HRT) were ~33 days for the nitrification reactor and ~9 days for the denitrification reactor, based on the system influent flow and mean volume of each reactor. The pilot system was operated continuously from 10 September, 2010 through 27 May, 2011. Over the first five months there were several issues with equipment failure and other operational problems that led to reactor upsets, usually manifested as nitrite accumulation in the nitrification reactor. Therefore, reactor operating and performance data are reported only for the period after which the final operating conditions were established (final 107 days; referred to below as the performance reporting period). Because the influent characteristics are important for stoichiometric analysis and did not depend on reactor performance, as noted above the influent properties are reported for the entire period of continuous operation in Table 4.1.

4.2.3 Reactor Design

The reactors were 5,000 L (nitrification) and 1,000 L (denitrification) high-density polyethylene septic tanks. Operating volumes were approximately 2,000 L and 500 L, respectively. The reactors were totally enclosed except for ports for pump tubing and off-gas; each reactor was operated with a headspace pressure ~0.5 kPa gauge. To prevent reactor short circuiting, liquid pumped into a reactor was discharged above the liquid surface and the

intakes for liquid pumped from the reactor were located near the bottom of the tank. Mixing in the denitrification reactor was provided by an internal recirculation line with intake and discharge located at opposite ends of the tank. Mixing in the nitrification reactor was provided by a submersible pump (190 watts, 70 LPM) located at the bottom near one end of the tank whose discharge was directed to the opposite end of the tank. Each reactor was assumed to be completely mixed, so that the effluent composition from each reactor was the same as its contents.

Pure oxygen (average flow ≈0.88 LPM) was provided to the nitrification reactor via fine-bubble diffuser (Western Outdoor Aquatics, Inc.; Frederick, Colorado, USA) through a mass flow controller (Omega; Stamford, Connecticut, USA). The pH in the nitrification reactor was adjusted by pumping a concentrated sodium carbonate solution into the reactor in response to continuous pH measurement using a proportional digital controller (Hannah Instruments; Woonsocket, Rhode Island, USA), with pH 6.8 as the minimum set-point. The majority of sodium carbonate required for pH control was consumed over the first few months of system operation, with relatively little consumed over the performance reporting period. Liquid volume in each reactor was measured by comparing the liquid level to a calibrated scale on the exterior of the tank; the volume in each reactor was recorded daily.

All pumping into and out of each reactor was with dedicated peristaltic pumps (MasterFlex computerized drive with Easy-Load II head and high-performance precision Norprene L/S 36 tubing; Cole-Parmer, Vernon Hill, Illinois, USA). Each pump was operated continuously with adjustment as needed to maintain the desired volume in each reactor. The pump drives were controlled with software (WinLIN; Cole-Parmer) installed on a personal

computer. Pump flow rates were periodically calibrated by timed delivery into a graduated cylinder.

4.2.4 Startup

The nitrification reactor was filled to the desired volume with tap water and ~200 L of return activated sludge from the Orange Water and Sewer Authority wastewater treatment plant (Chapel Hill, North Carolina, USA), which performs nitrification and biological phosphorus removal. The denitrification reactor was filled to the desired volume with lagoon liquid. The system was operated in batch mode with internal recycle between the nitrification and denitrification reactors for 12 days, then the internal recycle between reactors was turned off to allow strictly batch operation in the nitrification reactor for 4 weeks. Continuous operation was initiated after this period of batch operation.

4.2.5 Instrumented Measurements

The temperature of the lagoon liquid was measured continuously with a probe submerged in the lagoon near the intake for the pilot system influent. The temperature and pH of mixed liquor in the denitrification reactor were monitored continuously with probes mounted in the internal recirculation line used for mixing. Temperature, pH and dissolved oxygen (DO) of mixed liquor in the nitrification reactor were monitored continuously with probes similarly mounted in an internal recirculation line. Temperature probes were calibrated against an electronic thermometer (Cole-Parmer) that had been calibrated against a mercury thermometer whose calibration was traceable to the US National Institute of Standards and Technology; room-temperature deionized water was the calibration medium. The mass flow controller for oxygen delivery was calibrated using a Gilibrator automated bubble meter (Sensidyne; St. Petersburg, Florida, USA). Calibration of the DO and pH probes was checked

weekly according to manufacturers' instructions. For reporting purposes, temperature, pH and DO were recorded manually daily.

4.2.6 Sample Collection

Lagoon liquid was collected from the sealed tank inside the trailer used as the source of reactor influent. Samples from each reactor were obtained from a port located on the respective internal recirculation line. Samples of lagoon liquid, denitrification effluent, and nitrification effluent were collected at least twice weekly, but not all samples were analyzed for every parameter. Samples were immediately filtered through glass fiber filters (Whatman GF/B or GF/C) on-site using a filtration apparatus dedicated to each sampling location. Chemical oxygen demand (COD), total suspended solids (TSS), and alkalinity were measured on-site. Otherwise, filtered and unfiltered samples were frozen in an on-site freezer and transported weekly to the laboratory at the University of North Carolina-Chapel Hill campus for further analysis. Off-gas from the nitrification reactor and headspace gas from the denitrification reactor were collected weekly over the performance reporting period and stored in air-tight syringes for transport to the campus laboratory.

4.2.7 Analytical Methods

Filtered samples were analyzed for ammonium, nitrite, and nitrate according to standard methods 4500-NH_3F, 4500-NO_2^-B, and 4500-NO_3^-F, respectively (*178*). Each of duplicate dilutions (100×–5,000× as needed) were measured in duplicate. Ammonium chloride, sodium nitrite, and potassium nitrate were used to prepare standard curves. Concentrations are reported in mg N L^{-1}. In preliminary analyses of lagoon liquid, neither nitrite nor nitrate were detected.

Total nitrogen (TN) and total dissolved nitrogen (TDN) were analyzed on unfiltered and filtered samples, respectively, using a Shimadzu (Columbia, Maryland, USA) total organic carbon analyzer with total nitrogen attachment. Duplicate dilutions (500×–2,000× as needed) were each measured in duplicate or triplicate; triplicates were analyzed when the difference between duplicate measurements was > 2%. Disodium ethylene diamine tetraacetic acid was used to prepare standard curves for TN and TDN analyses.

Total COD (tCOD) and soluble COD (sCOD) were determined using CHEMetrics (Midland, Virginia, USA) COD digestion vials (20–1500 ppm range) on unfiltered and filtered samples, respectively, in duplicate. Samples were diluted 10× directly in the COD vials. Sodium acetate was used as the standard, on the assumption that the majority of sCOD in the anaerobically digested lagoon liquid comprised volatile fatty acids.

Alkalinity was measured using standard method 2320B (*178*). Fresh sulfuric acid solution was prepared as needed and samples were titrated to pH 4.3 as a measure of total alkalinity. The volume of acid required to reach pH 5.8 was recorded and used to calculate bicarbonate alkalinity. Acid equivalents required to reach the respective pH endpoints were converted to alkalinity in mg $CaCO_3$ L^{-1}. Total suspended solids (TSS) were measured using standard method 2540D (*178*).

Nitrous oxide was measured on a Shimadzu 14A gas chromatograph (GC) with electron-capture detector and 90% argon/10% methane as carrier gas. Methane (CH_4) was measured on a Shimadzu 8AIF GC with flame ionization detector and ultra-high-purity (UHP) N_2 as carrier gas. Carbon dioxide (CO_2) was measured with a Shimadzu 8AIT GC with thermal conductivity detector and UHP He as carrier gas. Gas-phase ammonia was captured in 0.2% (w/v) boric acid and measured as aqueous ammonium.

4.2.8 Data Analysis

Cumulative mass loading and mass discharge were calculated by multiplying the concentration of a constituent by the net pumping rate into or out of a reactor, respectively. Pumping rates were recorded daily; concentration data were linearly interpolated between measured values. Cumulative mass loading and discharge across the system were used to calculate removal efficiency over the performance reporting period.

Oxygen consumption in the nitrification reactor was assumed to equal the oxygen required to oxidize ammonium to nitrite and nitrate plus the net change in total COD across the reactor. The proportion of oxygen required for oxidation of ammonium to nitrite vs. nitrate was based on the ratio of nitrite and nitrate in the nitrification reactor effluent and the known stoichiometry of nitrification reactions (3.43 g O_2 (g NH_4^+-N)$^{-1}$ for ammonium oxidation to nitrite and 4.57 g O_2 (g NH_4^+-N)$^{-1}$ for ammonium oxidation to nitrate).

Cumulative off-gas flow from each reactor was combined with the mean gas-phase concentration of N_2O to estimate the yield of N_2O relative to ammonium-N removal (cumulative mass of N_2O-N production/cumulative mass of NH_4^+-N consumption) over the performance reporting period. The off-gas flow from the nitrification reactor was estimated based on the known mass of O_2 addition (converted to molar units based on the mean temperature in the trailer and the ideal gas law), mass of oxygen consumed, and the volumetric composition of measured gases (CO_2, CH_4, N_2O and NH_3), with the balance assumed to be O_2. The off-gas flow from the denitrification reactor was based on the mass of nitrogenous gases (N_2, N_2O and NH_3) produced and the measured gas composition, assuming the unmeasured balance to be N_2; the cumulative mass production of nitrogenous gases was

estimated from the net change of TN across the system minus the N_2O-N released from the nitrification reactor.

4.3 Results

Characteristics of the influent to the pilot system (lagoon liquid) over the entire project period are summarized in Table 4.1. The most variable influent parameter was COD, which resulted from the variability of temperature in the lagoon (Figure 4.2). Because the lagoon was not heated, the temperature of the lagoon liquid varied seasonally in accordance with the ambient temperature. Gas production in the lagoon declines with decreasing temperature in the winter months (Figure 2.4), corresponding to decreased anaerobic consumption of COD during the winter. COD concentrations in the lagoon liquid did not begin to decline from the peak until the lagoon temperature reached approximately 15 °C (Figure 4.2).

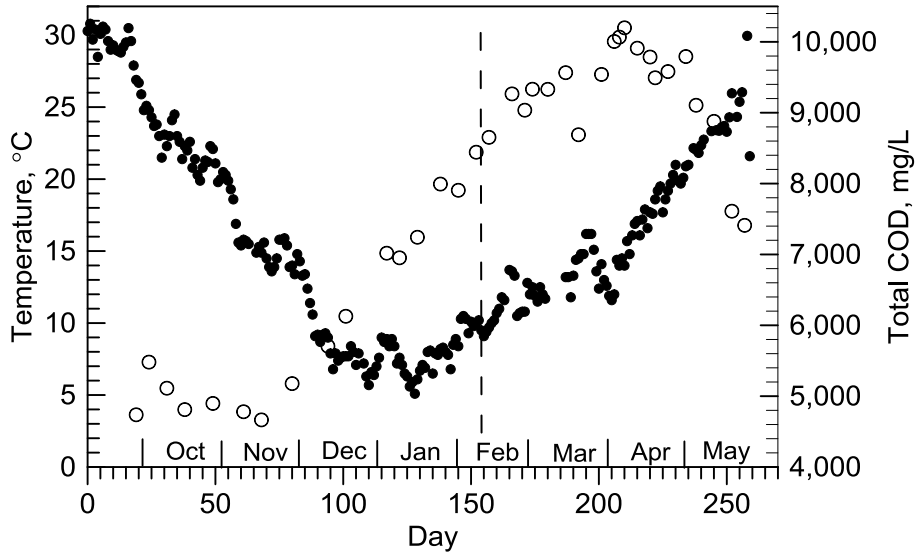

Figure 4.2: Lagoon liquid temperature (filled symbols) and total COD concentration (open symbols) over the duration of pilot-scale MLE process operation (day 0 was 10 September, 2010). The dashed vertical line indicates the beginning of the performance reporting period as defined in the text. Data are the mean of measured values.

The majority (95%) of TN in the lagoon liquid was dissolved, with ammonium-N accounting for 89% of the TDN. The majority of TSS appears to be inert, as the mean VSS/TSS ratio was only 0.4.

4.3.1 System Performance

Data on nitrogen species, COD and other characteristics of the influent and each reactor over the performance reporting period are summarized in Table 4.2. Based on cumulative mass loading and discharge over this period (Table 4.3), the system achieved 98% removal of NH_4^+-N, 83% removal of total-N, and 75% removal of total COD. Cumulative mass loading and discharge of TN and NH_4^+-N are plotted in Figure 4.3. Nearly all of the effluent nitrogen other than N_2O could be accounted for as nitrite and/or nitrate (Tables 4.2 and 4.3).

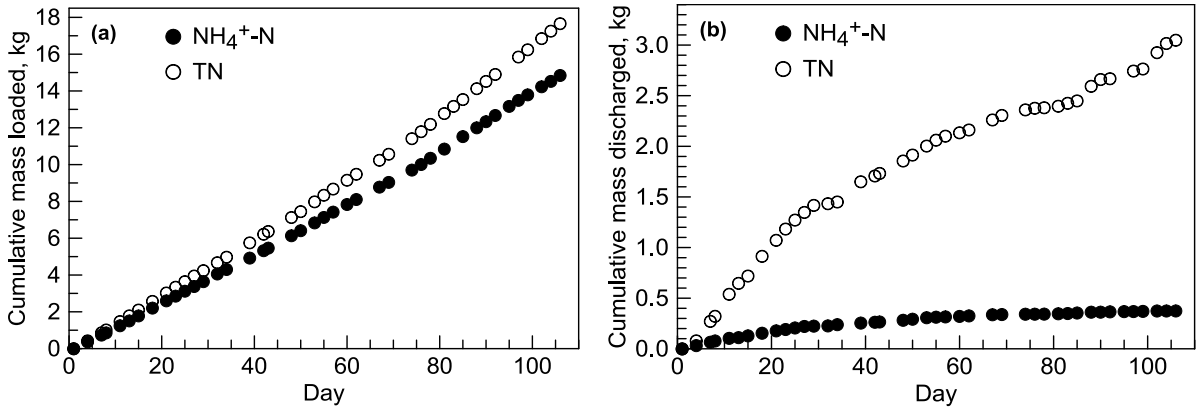

Figure 4.3: Cumulative mass of nitrogen (a) loaded to and (b) discharged from the pilot-scale MLE process over the performance reporting period.

Table 4.2: Influent (lagoon liquid) and reactor characteristics over the performance reporting period of the pilot-scale MLE system. [a]

Parameter [b]	Influent		Denitrification		Nitrification [c]	
NH_4^+-N	2,370 ± 140	(2,070 – 2,670; 41)	651 ± 86	(499 – 858; 42)	78 ± 53	(1 – 199; 42)
NO_2^--N	NA		32 ± 43	(ND – 140; 42)	484 ± 217	(3 – 779; 42)
NO_3^--N	NA		71 ± 75	(ND – 249; 42)	288 ± 234	(66 – 1,190; 42)
Total-N	2,820 ± 140	(2,510 – 3,200; 41)	836 ± 158	(609 – 1,250; 41)	681 ± 191	(439 – 1,080; 41)
TDN	2,690 ± 150	(2,340 – 2,980; 37)	794 ± 165	(565 – 1,180; 37)	682 ± 191	(450 – 1,090; 40)
Total COD	9,270 ± 740	(7,410–10,200; 20)	3,080 ± 210	(2,600–3,580; 31)	3,180 ± 460	(2,620–4,680; 32)
Soluble COD	6,660 ± 1,190	(3,900–7,740; 19)	2,240 ± 170	(1,890–2,450; 31)	2,390 ± 330	(1,610–2,940; 32)
Volume	NA		533 ± 59	(450 – 750; 86)	2,000 ± 80	(1,500–2,200; 99)
Temperature	16.3 ± 4.9	(9.1 – 30.0; 93)	22.3 ± 2.2	(16.5 – 26.3;100)	24.5 ± 2.7	(16.4 – 30.5; 100)
DO	NA		NA		10.9 ± 7.6	(0.8 – 27.8; 100)
pH	7.53± 0.17	(7.28 – 7.86; 13)	7.92±0.34	(7.01 – 8.56; 99)	6.97 ± 0.07	(6.84 – 7.43; 100)

[a] mean ± s.d. (range; n); ND, not detected; NA, not applicable

[b] Units are mg L^{-1} except units for Volume (L), Temperature (°C), and pH (unitless).

[c] Nitrification reactor characteristics = system effluent characteristics

Table 4.3[†]: Cumulative mass loading and discharge across the system over the performance reporting period.

Parameter	Loading (kg)	Discharge (kg) [a]	Removal (%)
NH_4^+-N	14.9	0.4	97.5
Total-N	17.7	3.1	82.7
TDN	16.9	3.2	81.4
NO_2^--N	NA	2.3	NA
NO_3^--N	NA	1.2	NA
N_2O-N	NA	1.2	NA
Total COD	55.2	13.9	74.7
Soluble COD	38.8	10.6	72.7

4.3.2 Consumption of COD

There was no measurable removal of total COD across the nitrification reactor (Figure 4.4), suggesting that virtually all of the total COD removal across the system occurred in the denitrification reactor; there was, however, a small amount of soluble COD removal in the nitrification reactor (Figure 4.4). Accordingly, the system effluent COD appears to comprise mostly non-biodegradable or very slowly biodegradable COD (*i.e.*, approximately 25% of the influent tCOD was non-biodegradable over the retention times used in this study).

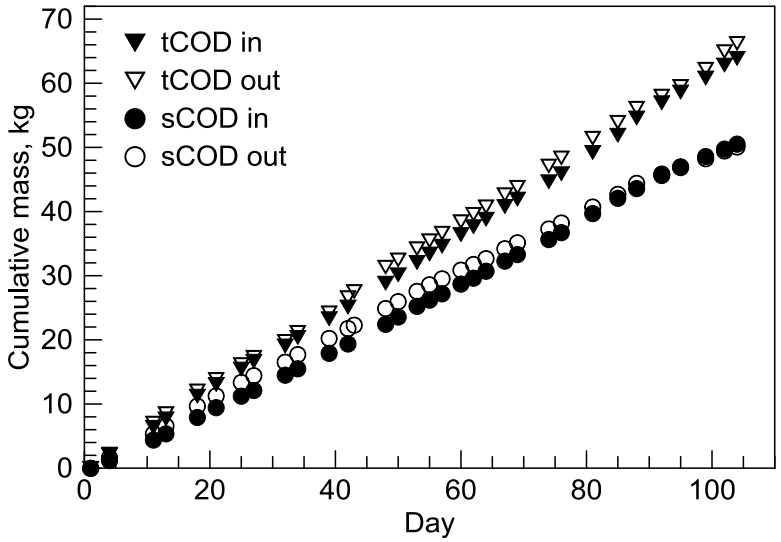

Figure 4.4: Cumulative COD loading and discharge across the nitrification reactor over the performance reporting period of the pilot-scale MLE system.

4.3.3 Gas-phase Measurements

Data on the composition of the gas phase in each reactor over the performance reporting period are summarized in Table 4.4. For stoichiometric analysis, the most important of these gases was N_2O. Based on the cumulative mass production of N_2O and the mass removal of ammonium across the system, 8.2% of the oxidized NH_4^+-N was converted to N_2O-N. Because the concentration of N_2O in the off-gas from the denitrification reactor was much lower than that from the nitrification reactor (Table 4.4), as well as the fact that there was a much greater flow of gas (primarily oxygen) through the nitrification reactor, the contribution of N_2O from denitrification was negligible (3.3% of the total N_2O produced).

Table 4.4: Reactor off-gas composition over the performance reporting period [a]

Gas	Denitrification Reactor			Nitrification Reactor		
CO_2 (%)	8.9 ± 2.7	(5.2 – 15.1; 13)		37.2 ± 8.9	(16.9 – 49.8; 13)	
CH_4 (%)	2.2 ± 0.8	(1.5 – 4.1; 14)		0.02 ± 0.05	(0.01 – 0.20; 14)	
N_2O (%)	0.13 ± 0.20	(0.01 – 0.67; 14)		0.78 ± 0.19	(0.43 – 1.11; 14)	
NH_3 (ppmv)	38 ± 21	(2 -82; 11)		5.0 ± 4.3	(<1 – 15; 11)	

[a] mean ± s.d. (range; n); ND, not detected;

With concentrations of off-gas ammonia in the ppmv range (Table 4.4), volatilization of ammonia was negligible (~0.01% of the ammonium removal across the system). Methane in the headspace of the denitrification reactor is assumed to represent volatilization of dissolved methane present in the influent (lagoon liquid).

4.4 Discussion

Swine waste is high-strength with respect to both biodegradable organic matter and ammonium-N. It is, therefore, a candidate for anaerobic digestion with energy recovery as well as a significant source of nitrogen pollution to the environment. There have been previous studies on nitrogen removal from swine waste not first subjected to anaerobic

digestion (*179*), but only studies that have focused on coupling anaerobic digestion with biological nitrogen removal are relevant to the present study. Most earlier work has included some combination of at least partial oxidation of ammonium with nitrogen removal by denitrification or anaerobic ammonium oxidation (anammox), although various schemes have been proposed. These include bypassing a fraction of the raw waste around the anaerobic digester to provide more electron donors for denitrification (*18*, *20*, *180*, *181*); recycling nitrified effluent to the anaerobic digester, which therefore would be responsible for both denitrification and methanogenesis in the same reactor (*19*, *20*, *182*); nitritation and anammox for nitrogen removal of anaerobically digested waste (*21*, *23*, *127*, *183*); and the concept evaluated in the present study, anaerobic digestion of the complete waste stream followed by nitrification/denitrification (*20*, *181*, *184*, *185*).

The previous studies in which either nitritation/anammox or nitrification/denitrification were evaluated on anaerobically digested swine waste have been conducted at small laboratory scale. In the study by Rajagopal et al (*20*), both the anaerobic digestion and nitrogen removal processes were operated at small pilot scale (~120 L). None of the studies on nitrification/denitrification utilized the MLE configuration, and in some cases a supplemental carbon source was added to maximize denitrification (*23*, *184*, *185*). Therefore, none of the previous studies on nitrogen removal from anaerobically digested swine waste is completely comparable to the present study.

4.4.1 Denitrification

Employing nitrification/denitrification on waste that has already been subjected to anaerobic digestion maximizes the amount of organic matter available for conversion to methane while minimizing the amount of oxygen required for subsequent aerobic treatment.

A drawback to this approach, however, is that the residual organic matter from anaerobic digestion might be insufficient to meet the electron donor demand for denitrification, thereby limiting the extent of nitrogen removal. Nitrate has the capacity to remove 2.86 g COD per g NO_3^--N (more if biomass growth is accounted for). Using data for the entire project (Table 4.1), the total COD in the lagoon liquid would not have met the demand for denitrification if all TN were converted to nitrate. As noted above, the residual COD in the lagoon varies seasonally in response to the extent of gas production in the lagoon, so that the extent of denitrification in a biological nitrogen removal system can be expected to vary seasonally as well.

In principle, the total COD in the lagoon liquid over the performance reporting period (Tables 4.2 and 4.3) should have been sufficient to completely remove nitrate if all of the TN in the lagoon liquid were converted to NO_3^--N. However, only 88% total nitrogen removal was achieved. The COD concentration in the denitrification reactor was high (Table 4.2), suggesting that residual COD not used for denitrification may not have been readily biodegradable. This is supported by the observations that there was little difference in the COD concentrations between the denitrification and nitrification reactors (Table 4.2) and that there was no removal of sCOD across the nitrification reactor (Figure 4.4), which suggests that most of the residual COD was not aerobically degradable either. The bioavailability of residual COD in anaerobically digested swine waste has been demonstrated to depend on the solids retention time of the digester (*186*).

As noted above, previous studies have explored either bypassing a fraction of the raw waste around the anaerobic digester, or recycling nitrified effluent to the anaerobic digester, to provide the necessary electron equivalents for denitrification. Both of these approaches

would reduce the amount of methane that could be generated from the waste. In addition, adding raw waste directly to the nitrogen removal process can substantially increase the oxygen consumption associated with the aerobic component of the process (*181*). Another proposed strategy, which simultaneously would decrease oxygen consumption, is to partially oxidize ammonium to nitrite (nitritation), relying on the produced nitrite for denitrification (*20*, *185*); the electron donor demand from denitrification with nitrite (1.71 g COD (g NO_2^- N)$^{-1}$) is less than that of nitrate. Although not intentional, in this study nearly two-thirds of the oxidized nitrogen in the effluent from the nitrification reactor (system effluent) was in the form of nitrite (Tables 4.2 and 4.3).

Unlike municipal wastewater treatment in which there may be upper limits on total nitrogen discharged to a receiving water, there are no such limitations on nonpoint nitrogen sources from agricultural waste management in the U.S. Nevertheless, extensive removal of ammonium-N by nitrification and even partial denitrification of the oxidized nitrogen would still have a substantial impact on human health and on reducing nitrogen loads to the environment, respectively. These factors should be taken into account when evaluating the impacts and limitations of coupling anaerobic digestion with nitrogen removal from swine waste.

4.4.2 Ammonium Oxidation

Throughout the study, nitrite tended to accumulate in the nitrification reactor in lieu of complete oxidation of ammonium to nitrate, even during most of the performance reporting period (Table 4.2). Accumulation of nitrite typically is observed under oxygen-limiting conditions, which in fact is the principal means of promoting nitritation over complete ammonium oxidation to nitrate (*90*). However, DO was not limiting in the present study

(Table 4.2), suggesting that other mechanisms were responsible for limiting nitrite oxidation. Because there were several process upsets early in the study, it is possible that nitrite-oxidizing bacteria (NOB) were selectively washed out of the system. Alternatively, it is possible that characteristics of swine waste adversely affect NOB. It is not possible from the available data to differentiate between these possibilities.

Although ammonium removal was excellent, it was necessary to add supplemental alkalinity (as carbonate) to the anaerobically digested lagoon effluent to maintain pH > 6.8 in the nitrification reactor. Most of the added alkalinity was required earlier in the project before stable reactor operation was achieved, so that the total carbonate requirement during the performance reporting period was not quantifiable.

Oxidation of ammonium to either nitrite or nitrate consumes 7.1 g alkalinity as $CaCO_3$ per g NH_4^+-N oxidized. Based on the lagoon liquid characteristics over the duration of the project (Table 4.1), the total alkalinity would be far less than required for oxidation of all the TN. However, denitrification from nitrate produces alkalinity to an extent that is nearly half of the alkalinity consumed from ammonium oxidation per unit nitrogen (*187*), so that there would be sufficient alkalinity in the lagoon liquid for complete oxidation of total nitrogen followed by denitrification of the nitrate produced. However, as noted above, the extent of denitrification would vary seasonally and also depends on the biodegradability of the residual COD after anaerobic digestion. Therefore, the need for additional alkalinity to maintain pH for nitrification can be expected to vary seasonally. Depending on how much alkalinity is required, this can represent a significant operating cost in a full-scale system (*8*). Providing alkalinity is one rationale for enhancing denitrification by adding undigested raw waste to the nitrification/denitrification process (*181*).

A strategy that would simultaneously reduce the alkalinity demand, oxygen demand and electron donor demand for nitrogen removal from anaerobically digested swine waste is to combine nitritation of a fraction of the ammonium with anammox to remove the remainder of the ammonium (*21*, *23*, *127*, *183*). Nitritation/anammox treatment is explored in Chapter 5.

4.4.3 Nitrous Oxide Production

The yield of N_2O-N per unit NH_4^+-N oxidized (8.2 %) obtained in this study is within the range reported in the literature for biological nitrogen removal systems (*188*), although it is higher than the range observed in a survey of municipal wastewater treatment plants in the U.S. (*189*). Production of N_2O tends to be higher in systems operated for nitritation rather than complete nitrification of ammonium to nitrate (*189*, *190*) and has generally been associated with low DO conditions (*191–193*). Ammonium oxidation to nitrite exceeded the complete oxidation to nitrate in this study, but the mean DO concentration in the nitrification reactor was generally quite high (Table 4.2). Ahn *et al*. (*189*) have suggested that a combination of high nitrite and high DO can also lead to increased rates of N_2O production in nitrifying systems. As noted above, it is also possible that the characteristics of swine waste may inherently influence the activity of NOB and/or N_2O production during ammonium oxidation.

Nearly all of the N_2O produced in this study was attributable to the nitrification process, rather than denitrification. A similar observation was made in a previous study on nitrification/denitrification of anaerobically digested swine waste (*194*), although the yield of N_2O per unit TN removed was much lower (0.07-0.15%) than in the present study.

4.5 Conclusions

Nitrification/denitrification is capable of achieving high extents of ammonium removal from anaerobically digested swine waste. The extent to which total nitrogen can be removed

depends on the biodegradable organic matter in the digested waste that is available for denitrification; the available organic matter in turn can depend on the extent of gas production during the anaerobic digestion process, which can vary seasonally. In the pilot system operated in this study, the majority of influent organic matter was consumed in the denitrification step. The extent of denitrification will also influence the stoichiometry of net alkalinity consumption across the system; the lower the extent of denitrification, the more likely supplemental alkalinity would be required to maintain neutral pH in the nitrification reactor. Production of N_2O from the nitrification/denitrification process can be significant, offsetting some of the greenhouse-gas benefit from anaerobic digestion with methane capture for energy recovery.

4.6 Acknowledgements

Financial support for this project was provided by Carolina Public Health Solutions (Gillings School of Global Public Health, University of North Carolina at Chapel Hill). I thank John McNeill for operating the pilot system and for performing several of the chemical and physical analyses. I also thank Tom Butler and other personnel at Butler Farms for providing access to the site, their hospitality, and their logistical assistance throughout the project.

Chapter 5: Nitritation/Anammox Treatment of Anaerobically Digested Swine Waste

Eric T. Staunton[2] and Michael D. Aitken

5.1 Introduction

Since the early 1990's, swine production in North Carolina (NC), USA has transitioned from smaller farms to large confined animal feeding operations (CAFOs) housing > 2,500 animals each. Typically, waste from these CAFOs is stored in uncovered lagoons and periodically applied to nearby sprayfields for crop irrigation (*2*). In 2007, due to the adverse environmental and public health effects of these waste management practices, NC passed legislation mandating environmental performance standards for new farms (*6*). This legislation also authorized higher rates of payment for any electricity generated from swine waste; however, existing farms were not required to couple energy generation to increased environmental performance standards. Because of ongoing concerns over nitrogen releases to the environment from current swine waste management practices, there has been growing interest in exploring economical means of removing nitrogen from waste at existing farms.

I have investigated the technological feasibility and Bunk explored the economic feasibility of coupling anaerobic digestion of swine waste for power generation with nitrogen removal via nitrification and denitrification (Chapter 4; *8*). That study was conducted in a pilot-scale nitrification/denitrification system at one of the few swine farms in NC performing full-scale anaerobic digestion in covered lagoons with methane capture. The

[2] Responsible for analysis of chemical species, reactor operation, generation of cumulative mass loading and discharge diagrams, and manuscript preparation

system was determined to be technologically feasible, but the operating cost might not be economically feasible primarily due to the oxygen and supplemental alkalinity required for complete nitrification.

In 1995, a new bacterium belonging to the phylum *Planctomycetes* was discovered that can oxidize ammonium anaerobically using nitrite as a terminal electron acceptor (*67*, *84*). The theoretical reaction is shown in Equation 2.6 (reproduced below), although the actual molar stoichiometry of nitrite removed per unit ammonium removed has been determined empirically to be approximately 1.3, and nitrate has also been observed as a product (*67*, *195*).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad [2.6]$$

The anaerobic ammonium oxidation (anammox) process has since been proven to be a cost-effective technology for treating wastewaters rich in ammonium-N and low in biodegradable organic matter (*196*). If the primary nitrogenous species in an influent waste is ammonium, anammox must be coupled to the first step of nitrification, the aerobic oxidation of ammonium to nitrite (nitritation) by ammonium-oxidizing bacteria (AOB). A system that utilizes nitritation/anammox as a primary nitrogen removal process should require less than half as much oxygen and reduced alkalinity compared to a nitrification/denitrification system, and no external electron donors would be required due to the autotrophic nature of the bacteria involved.

Despite the potential advantages, there have been limited studies that combine anaerobic digestion of swine waste with nitrogen removal by anammox (*21*, *23*, *25*, *115*, *127*, *128*, *183*). Of these, only three studies utilized one-reactor systems (*23*, *128*, *129*), with the others performing the aerobic and anaerobic reactions in separate reactors. None of these studies

provided an in-depth analysis of the stoichiometry of the anammox process when treating high strength nitrogenous waste (> 1,000 mg NH_4^+-N L^{-1}) with a high concentration of chemical oxygen demand (COD $(NH_4^+\text{-N})^{-1}$ > 1.0).

There are three key challenges involved with successful treatment of waste by nitritation/anammox in a single reactor: converting a fraction of the ammonium to nitrite in stoichiometric balance with the remaining ammonium; preventing out-competition of the anammox bacteria by heterotrophic denitrifiers capable of utilizing nitrite as an electron acceptor; and minimizing the oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). In addition, although nitrite is a necessary substrate its accumulation must be controlled because it is inhibitory to anammox bacteria (*96*). This is especially problematic in wastewaters that contain a high concentration of ammonium, which could lead to a high concentration of nitrite in the reactor.

These challenges could be addressed by utilizing an SBR under oxygen-limited conditions, with intermittent input of oxygen superimposed on otherwise anoxic conditions; such alternating conditions are analogous to how SBRs can achieve nitrification/denitrification in a single reactor (*197*). This reactor configuration should allow for controlled conversion of ammonium to nitrite and simultaneous or sequential conversion of the nitrite to nitrogen gas via anammox. Aerobic ammonium oxidizers have a higher affinity for oxygen than NOB (*198*), and oxygen limitation has been shown to be an effective technique for the inhibition of NOB at full scale (*199*). This setup should also allow for the majority of influent biodegradable COD to be consumed aerobically, minimizing the amount of COD available as an electron donor for denitrification and stabilizing nitrogen removal by the anammox process.

In this study, I evaluated the technical feasibility of a single-reactor nitritation/anammox system for removing nitrogen from anaerobically digested swine waste. Of particular interest were the operation of an SBR for controlled nitritation and a stoichiometric analysis of the relative contributions of anammox vs. denitrification for nitrogen removal.

5.2 Materials and Methods

5.2.1 Experimental Design and Overview

Anaerobically digested swine waste was obtained from a covered anaerobic lagoon at Butler Farms in Lillington, NC, USA. General characteristics of the farm and the digested waste composition from samples collected over a 9-month period as part of an on-site pilot study are provided elsewhere (Chapter 4). The present study was carried out in a laboratory system comprising a 20-L (working volume) SBR.

Because there is limited experience with nitritation/anammox treatment of digested swine waste in an SBR, I approached reactor design and process operation heuristically. Without *a priori* knowledge of the optimum loading rate, cycle time or frequency of intermittent oxygen addition, the reactor was operated for a long period (over 450 days) in which these variables were adjusted. The goal was to increase the loading rate while maximizing ammonium removal and minimizing nitrite accumulation, recognizing that the key groups of bacteria (AOB and anammox bacteria) grow very slowly.

Initially I used a perforated-rubber fine-bubble diffuser for aeration, but the diffuser rapidly became coated with a thick biofilm while little biomass accumulated in the mixed liquor or as granules. Because it was important that the laboratory reactor be as physically representative of full-scale conditions as possible, I replaced the fine-bubble diffuser (which provided a high surface area relative to total reactor volume) with a coarse-bubble diffuser

that had a much smaller surface area. In an effort to increase biomass retention in the reactor, I eventually added plastic media that has been used in moving-bed biofilm reactors at full-scale. To overcome oxygen-transfer limitations of the coarse-bubble diffuser, the mixing intensity in the reactor was increased and air was replaced with pure oxygen as the oxygen source. The increased mixing intensity had an adverse impact on the plastic media, as described in more detail below.

The SBR was initially seeded with a combination of granular anammox biomass from an anammox reactor operated by the City College of New York and return activated sludge from the Orange Water and Sewer Authority wastewater treatment plant (Chapel Hill, North Carolina, USA) as a source of AOB. After an episode of excess aeration in which nitrite accumulated to inhibitory levels and anammox activity did not resume, the reactor was re-seeded with ~900 mL of cyclone underflow from a full-scale sidestream deammonification system at the Hampton Roads (Virginia, USA) Sanitation District.

The final reactor design and operating conditions were established 65 days before the termination of the study. The design, operation, and performance information provided below are from this period only. A single batch of digested swine waste from Butler Farms was used over this period, and was stored at 4°C. The start of the final period of operation is defined as day 0, and any data shown in the appendices for periods preceding that day are shown as negative values.

5.2.2 Chemicals

All chemicals and standards were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise noted. Compressed air (breathing air, grade D), oxygen (USP grade), nitrogen (industrial grade), and helium (UHP, grade 5.0) were purchased from Airgas

(Durham, NC, USA). The standard for gas chromatographic (GC) analysis of the major gases was obtained from Scott Specialty Gases (Plumsteadville, PA, USA) and consisted of 20% oxygen (O_2), 1% carbon dioxide (CO_2), with the balance nitrogen (N_2). Nitrous oxide (N_2O, 99%) was obtained from Sigma-Aldrich (Allentown, PA, USA).

5.2.3 Reactor Design

General features of the reactor design are provided below, with a schematic shown in Figure 5.1. Other physical details of the reactor and its components are summarized in Appendix B.

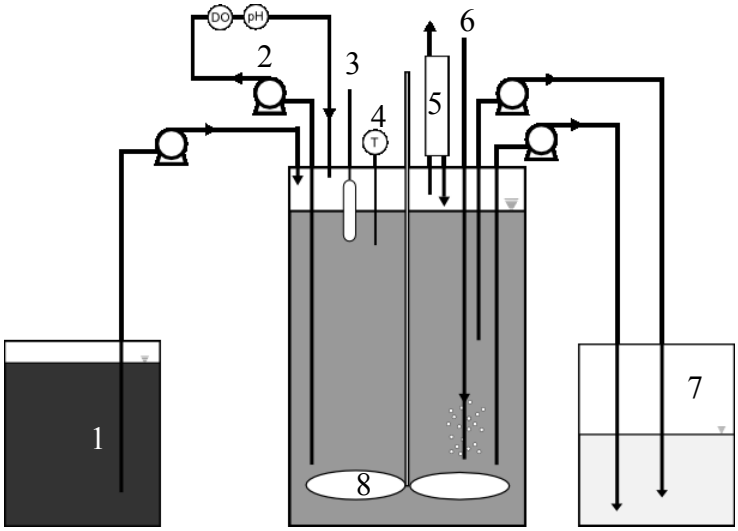

Figure 5.1: Schematic diagram of the lab-scale nitritation/anammox reactor. 1: Swine waste/system feed. 2: recirculation loop for monitoring pH and DO. 3: Floating level gauge. 4: Temperature probe. 5: Offgas condenser. 6: Oxygen diffuser. 7: Effluent storage tank. 8: Impeller mixer.

The reactor consisted of a 20-L (working volume) cylindrical, stainless-steel (SS) vessel with an inside diameter (ID) of 28.2 cm and a depth of 40.3 cm (Figure A.3); the liquid level in the reactor was 34.6 cm. The reactor vessel was flanged with a 1.3 cm-thick SS plate, to which the top headplate (also 1.3 cm-thick SS) was bolted. A 0.16 cm butyl rubber gasket between the headplate and flange provided a gas-tight seal. The upper ~55% of the liquid volume contained Kaldnes K1 media (Pentair Aquatic Eco-Systems Inc., Apopka, FL, USA),

which has a protected specific surface area of 500 $m^2\ m^{-3}$ (~6 cm^2 $piece^{-1}$). The reactor was suspended in an insulated water bath maintained at 35°C with a precision immersion circulator (Techne TU-20D, rated temperature stability 0.005°C; Cole Parmer instrument Co., Vernon Hills, IL, USA).

Tubing was inserted through the reactor headplate for influent, effluent, a recirculation loop for monitoring pH and dissolved oxygen (DO), temperature probe, O_2 supply, and offgas. The reactor headplate was also equipped with a 3.2-cm diameter sample port with a threaded nylon plug made gas-tight with a 0.16 cm-thick butyl rubber gasket. The reactor was vented such that headspace pressure was near atmospheric. The offgas line was equipped with a SS water-jacketed condenser (18.4 cm long, 2.8 cm ID); cold (6-8°C) water flowed through the jacket co-current with the offgas, and the condensate was returned to the reactor. All other openings in the headplate were sealed gas-tight with PTFE or nylon Swagelok® fittings.

Reactor pH and DO were measured with a Hanna Instruments (Woonsocket, RI, USA) BlackStone BL981411 pH Mini Controller and model HI8410 DO controller, respectively; both controllers were used as in-line meters rather than for process control. Temperature was measured with a ProSense RTD0100-06-030-H thermistor.

Pure oxygen was delivered to the reactor through a 3.2-mm ID SS perforated lance diffuser. The diffuser had two sets of five perforations (16 mm openings) on opposite sides of the lance at vertical intervals of 1.25 cm from the bottom of the lance. Oxygen flow was controlled at 1 L min^{-1} with an Omega (Stamford, CT, USA) FMA5400/5500 mass flow controller at a source pressure of 345 kPa gauge. Each pulse of oxygen was timed using an Auber (Alpharetta, GA, USA) ASL-51 timer.

Feed and reactor effluent were transferred with dedicated peristaltic pumps (MasterFlex computerized drive with Easy-Load II head and high-performance precision Norprene® L/S 36 tubing, 9.5 mm ID; Cole-Parmer). The feed and decant volumes were pumped from or into, respectively, a 2-L graduated cylinder until the target volume was delivered.

Mixing in the reactor was provided by a mechanical mixer suspended from the reactor headplate through gas-tight shaft housing with ceramic seals. The mixer was driven by a direct current motor with speed controller and was run continuously at 135 RPM except for settle and decant periods. It consisted of two impellers located at different depths, with the lower impeller providing most of the mixing of the fluid phase. The upper impeller was installed at the liquid surface to maximize oxygen transfer from the reactor headspace.

5.2.4 Operating Conditions

The reactor cycle was 2 days, with 1.0 L of digested swine waste fed per cycle. Each cycle was divided into four periods: feed (10 min), react, settle (1 min), and decant (5 min). All reported times are nominal; any time not accounted for in the feed, settle, and decant periods was utilized in the react period.

To promote oxygen-limited conditions, oxygen was provided with intermittent pulsing at a frequency determined empirically; the final condition was 6 s O_2 flow followed by 37 min without flow. The volume of O_2 delivered per pulse was measured independently (Figure C.2). An oxygen transfer efficiency study was conducted to assess diffuser performance (Figure C.3). Oxygen was also measured in the reactor offgas to assess system oxygen transfer (combined transfer from the diffuser and the reactor headspace) using a mass-balance analysis (not shown). Oxygen transfer efficiency through the diffuser was only

0.43%; the overall system transfer was 83 ± 7%, indicating that the majority of oxygen transfer was from the headspace.

5.2.5 Sample Collection

Samples were collected routinely from the reactor effluent or occasionally from the internal recirculation line (especially for intracycle sampling events). Samples were immediately filtered through Whatman GF/B glass fiber filters and frozen for subsequent analysis. Samples were analyzed less than one week after collection.

5.2.6 Physical and Chemical Analyses

Physical and chemical analyses were performed as previously described (Section 4.2.7 Analytical Methods) except for total nitrogen (TN) and total dissolved N (TDN). Typically, triplicate measurements were made from a single dilution (100×-2,000× as needed), but periodically a single measurement of each of triplicate dilutions was performed to ensure method accuracy.

Total-N and TDN were measured by oxidizing unfiltered and filtered samples (diluted 1,000×-10,000× as needed), respectively, with Fluka brand potassium persulfate (Sigma-Aldrich, Allentown, PA, USA) under alkaline conditions according to the method of Soloranzo and Sharpe (*200*). Ammonium chloride was used to prepare standard curves.

Shards of plastic from abraded K1 media were observed in the reactor effluent, which contributed to measured effluent total COD (tCOD). To account for the contribution of plastic tCOD during routine measurements, the actual mass of effluent tCOD was estimated from the ratio of consumed soluble COD (sCOD) to consumed tCOD excluding the plastic. At the termination of reactor operation, the reactor contents were passed through a 75 μm (Tyler Standard Mesh No. 200) sieve to separate the plastic shards from the majority of the

other solids; the sCOD and tCOD concentrations of the unretained volume were then measured and subtracted from the respective influent values to calculate consumption. The ratio of consumed sCOD to consumed tCOD was ~0.7 (Appendix C.11), which was combined with measured sCOD values to estimate the actual mass of non-plastic tCOD in the reactor effluent.

5.2.7 Offgas Composition and Flow

After passing through the condenser (Section 5.2.3 Reactor Design), reactor offgas was routed to a Gow-Mac series 350 GC with thermal conductivity detector (Gow-Mac Instrument Co., Bethlehem, PA, USA), fitted with a Valco injection valve and digital valve interface (Valco Instrument Co., Houston, TX, USA). Unless an offgas analysis was desired, the offgas flowed continuously through the injection valve and bypassed the GC. Periodically samples of offgas were diverted to the GC by automatic injection. Injections were timed to correspond with the end of a pulse of oxygen and halfway between pulses (every 18.55 min).

The GC was operated isothermally at 35°C with a detector temperature of 58°C and detector current of 110 mA. The column was a CTR-1 coaxial packed column (Alltech Associates, Deerfield, IL, USA), 2 m long x 0.6 cm diameter. The carrier gas was helium with a flow rate of 60 mL min^{-1} at 207 kPa gauge. Injection-valve control and data acquisition, integration and analysis were performed by an SRI Model 203 PeakSimple Chromatography Data System (SRI Instruments, Torrance, CA, USA). Concentrations of O_2, CO_2, N_2, and N_2O were quantified by comparison to standards of known composition. Offgas ammonia was captured in 0.2% boric acid and the concentration determined as aqueous ammonium.

To determine the offgas flow rate, flow was diverted to a 10-L Tedlar® bag and the bag filled for the duration of a reactor cycle. Volume of the collected gas was measured by water displacement.

5.2.8 Intracycle Analysis

To assess dynamic conditions in the reactor during a cycle, samples were taken 0.5, 1, 2, 4, 6, 12, 24, 32, 38, and 48 hours after the start of a cycle; the samples were analyzed for nitrogenous species and COD.

5.2.9 Maximum Rate Estimates

Near the end of reactor operation, the maximum rates of relevant biological processes were determined in two separate in-reactor experiments, each consisting of four consecutive periods. In both cases, the rate experiment was started immediately after a normal feeding. In period (i), strictly anoxic conditions were imposed by purging the reactor with N_2 followed by intermittent pulsing of N_2 at the pulsing frequency normally used to supply O_2; nitrite consumption was measured to estimate the combined rate of denitrification and anammox. In period (ii), the sparge gas was switched to O_2 for ~10 hr at the normal pulsing frequency, which allowed for nitritation, anammox, and denitrification. In period (iii), oxygen was bubbled continuously through the reactor with the sampling port in the headplate open (although no DO accumulation observed during this period); nitrite and ammonium consumption were measured to estimate the combined rate of nitritation and anammox. In period (iv), the sparge gas was switched to N_2, the headspace purged and the headplate closed to provide anoxic conditions; on the assumption that all biodegradable COD would have been consumed before period (iv), measuring nitrite and ammonium consumption allowed the maximum rate of anammox to be determined. The maximum rate of

denitrification was estimated by the difference in rates of nitrite consumption between period (i) and period (iv). The maximum rate of nitritation was estimated from the rates of change in ammonium and nitrite in periods (iii) and (iv).

5.2.10 Data Analysis

The mass of each constituent into or out of the reactor over a cycle was calculated by multiplying the concentration by the incremental volume fed (loading) or decanted (discharge). If a concentration was not measured over a given interval of cycles, it was assumed to equal the average of the concentrations from samples bracketing that interval. Cumulative masses were used to determine daily loading and removal rates for constituents of interest by linear regression. The r^2 for cumulative mass v. time was typically greater than 0.95, with the exception of effluent ammonium, for which r^2 was 0.85; this was largely a result of continual improvement in ammonium removal under the final operating conditions through the end of the study.

The first three TDN measurements were rejected because they were less than the sum of the independently measured total inorganic nitrogen (TIN) species. Other outliers were determined by a statistical method described previously (*201*). For gas-phase data, the sum of measured gases was rejected if either any individual component was rejected as an outlier or if the sum was determined to be an outlier. Statistical analyses were done with SAS v 9.3.

5.3 Results

5.3.1 Reactor Performance

Influent and effluent concentrations of each constituent of interest are summarized in Table 5.1. Ammonium-N was 85% of the influent TDN and 80% of the influent TN. The ratio of tCOD TN^{-1} was 1.8 and the ratio of alkalinity TN^{-1} in the influent was 3.8 mg $CaCO_3$

(mg N)$^{-1}$. Because the reactor pH remained near neutral (7.1-7.3, personal observation) without the addition of any external source of alkalinity, the alkalinity in the influent was sufficient to support the net demand associated with the various nitrogen transformation mechanisms occurring in the reactor.

Table 5.1: SBR influent and effluent characteristics. [a]

Parameter	Influent		Effluent	
Nitrogen				
Ammonium-N	2,080 ± 100	(32)	98.0 ± 80.4	(31)
Nitrite-N	N/D [b]		27.3 ± 7.7	(31)
Nitrate-N	N/D		24.5 ± 5.2	(30)
TIN	2,080 ± 100 [c]	(32)	147 ± 75	(30)
TDN	2,440 ± 400	(7)	188 ± 74	(11)
TN	2,610 ± 370	(11)	274 ± 84	(12)
COD				
Total	4,610 ± 240	(17)	2,510 ± 230	(16)
Soluble	2,440 ± 260	(17)	986 ± 93	(16)
Solids				
Total	2,480 ± 280	(8)	2,270 ± 120	(4)
Volatile	1,810 ± 150	(8)	1,690 ± 90	(4)
Volatile/Total	0.71 ± 0.03	(8)	0.74 ± 0.03	(4)
pCOD [d]/VSS	1.24 ± 0.14	(7)	1.54 [e]	(1)
Alkalinity				
Bicarbonate	8,430 ± 180	(31)	1,860 ± 170	(30)
Total	9,890 ± 155	(31)	2,380 ± 175	(30)

[a] Data represent means ± standard deviation. The number of samples analyzed is in parentheses. Units are mg L^{-1} except units for pCOD/VSS (mg COD (mg VSS)$^{-1}$), alkalinity (mg $CaCO_3$ L^{-1}), and Volatile/Total (unitless).

[b] N/D – not determined

[c] Assumes all inorganic-N in the influent is ammonium.

[d] particulate COD (sCOD subtracted from tCOD).

[e] Measured on sample passed through a 75 μm screen.

Cumulative mass loading and discharge diagrams generated to assess system performance are shown in Figure 5.2; rates calculated from these data are summarized in

Table 5.2. Under the final operating conditions represented by these data, the reactor removed 95% of the influent ammonium and 90% of the total-N. Dissolved nitrogen accounted for 66% of the effluent TN. Effluent TDN consisted of 49% NH_4^+–N, 16% NO_2^-–N, 13% NO_3^-–N, and 18% dissolved organic-N. Nitrate in the reactor effluent accounted for only 1.2% of the removed ammonium. Although difficult to discern in Figure 5.2, the concentrations of NH_4^+–N and TN in the reactor effluent continued to decline through the end of the study (Figure C.8).

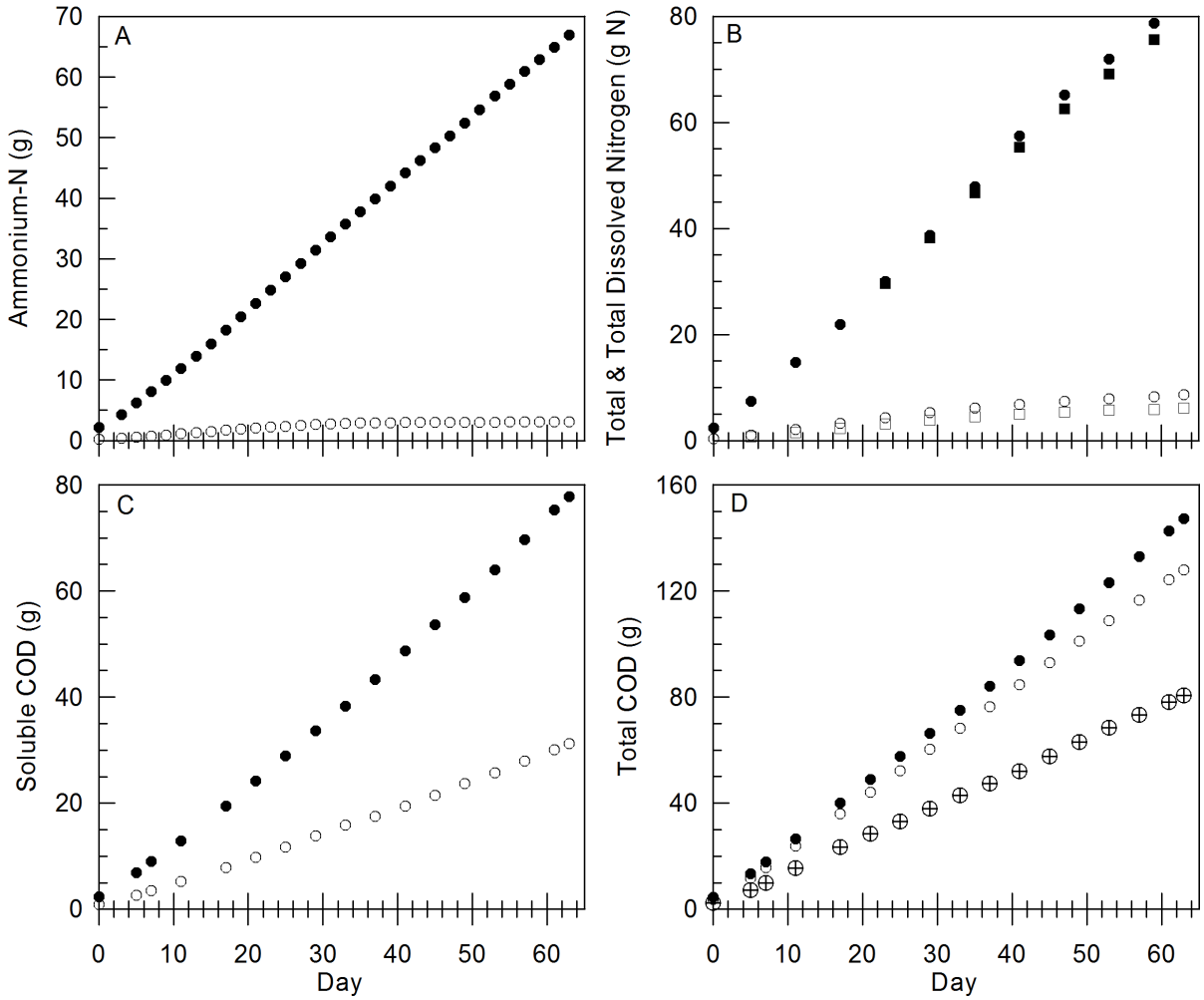

Figure 5.2: Cumulative mass loading and discharge rates for the anammox SBR for (A) ammonium-N (B) total nitrogen (circles) and total dissolved nitrogen (squares) (C) soluble COD (D) total COD. Solid and hollow symbols represent influent and effluent mass respectively. Circles with cross-hatches represent the estimated actual effluent mass of tCOD by subtracting the estimated plastic COD from the measured tCOD. Note differences in the y-axis scales among the graphs.

Table 5.2: Summary of loading and discharge rates of the nitration/anammox system based on cumulative mass data [a].

Parameter	Loading		Discharge		% Removal [b]
Ammonium-N	52.7 ± 0.0	(1.00)	2.30 ± 0.18	(0.847)	95.6 ± 7.4
TDN	64.2 ± 1.9	(0.996)	4.67 ± 0.33	(0.958)	92.7 ± 7.0
TN	67.0 ± 1.1	(0.998)	6.73 ± 0.34	(0.975)	90.0 ± 4.8
tCOD [c]	114 ± 1	(0.999)	62.7 ± 0.0	(1.00)	45.0 ± 0.4
sCOD	60.1 ± 8.0	(0.998)	24.4 ± 0.3	(0.998)	59.5 ± 1.0
Alkalinity	248 ± 0	(1.00)	59.7 ± 4	(0.999)	75.9 ± 0.5
Oxygen [d]	169		29		83 ± 7

[a] Slope of linear best fit divided by reactor volume (g m^{-3} d^{-1}) ± 95% confidence interval; r^2 is given in parentheses.
[b] 95% confidence interval determined by propagation of uncertainty (*202*).
[c] tCOD discharge rate and percent removal are based on estimated effluent mass.
[d] Oxygen loading based on measured volume of O_2 per pulse and known number of pulses per day. Oxygen discharge based on measured composition and offgas flowrate. Oxygen % removal indicates transfer efficiency. Error is based on offgas measurement error (Table 5.3).

5.3.2 Reactor Offgas

The sum of the measured gases (CO_2, N_2, O_2, and N_2O) accounted for an average of 94% of the total offgas (Table 5.3). Of these gases, the most important for stoichiometric analysis are N_2O, N_2, and O_2. On a mass basis, nitrogenous gases accounted for 75.0 ± 7.7% of removed TN, with 11.4 ± 1.0% of the removed TN converted to N_2O. Stripping of ammonia to the gas phase was negligible, accounting for < 0.01% of the removed NH_4^+-N (Appendix C.9).

Table 5.3: SBR offgas composition.

Component	Composition [a]	
CO_2	44.2 ± 4.9	(406; 1)
N_2O	4.27 ± 0.35	(402; 5)
O_2	15.5 ± 1.4	(406; 1)
N_2	27.0 ± 3.2	(404; 3)
Total [b]	94.4 ± 5.0	(389; 18)

[a] Data represent mean ± standard deviation (number of samples accepted; number of samples rejected as outliers). Units are mol%.
[b] Includes only the samples for which all 4 gases were quantified.

5.3.3 Intracycle Analysis

Intracycle sampling was used to estimate rates of change of individual constituents during the course of a cycle (Figure 5.3). Neither the rate of tCOD removal nor the rate of sCOD removal was significantly different than zero ($p > 0.05$; Table 5.4), but nitrogen was removed throughout the cycle. Solubilization of particulate N occurred within the first hour of the cycle, with most solubilization occurring before the first sampling event. Between the first two sampling events, all particulate N consumed in the reactor accumulated as dissolved organic N ($p > 0.05$) and both species remained constant for the duration of the cycle.

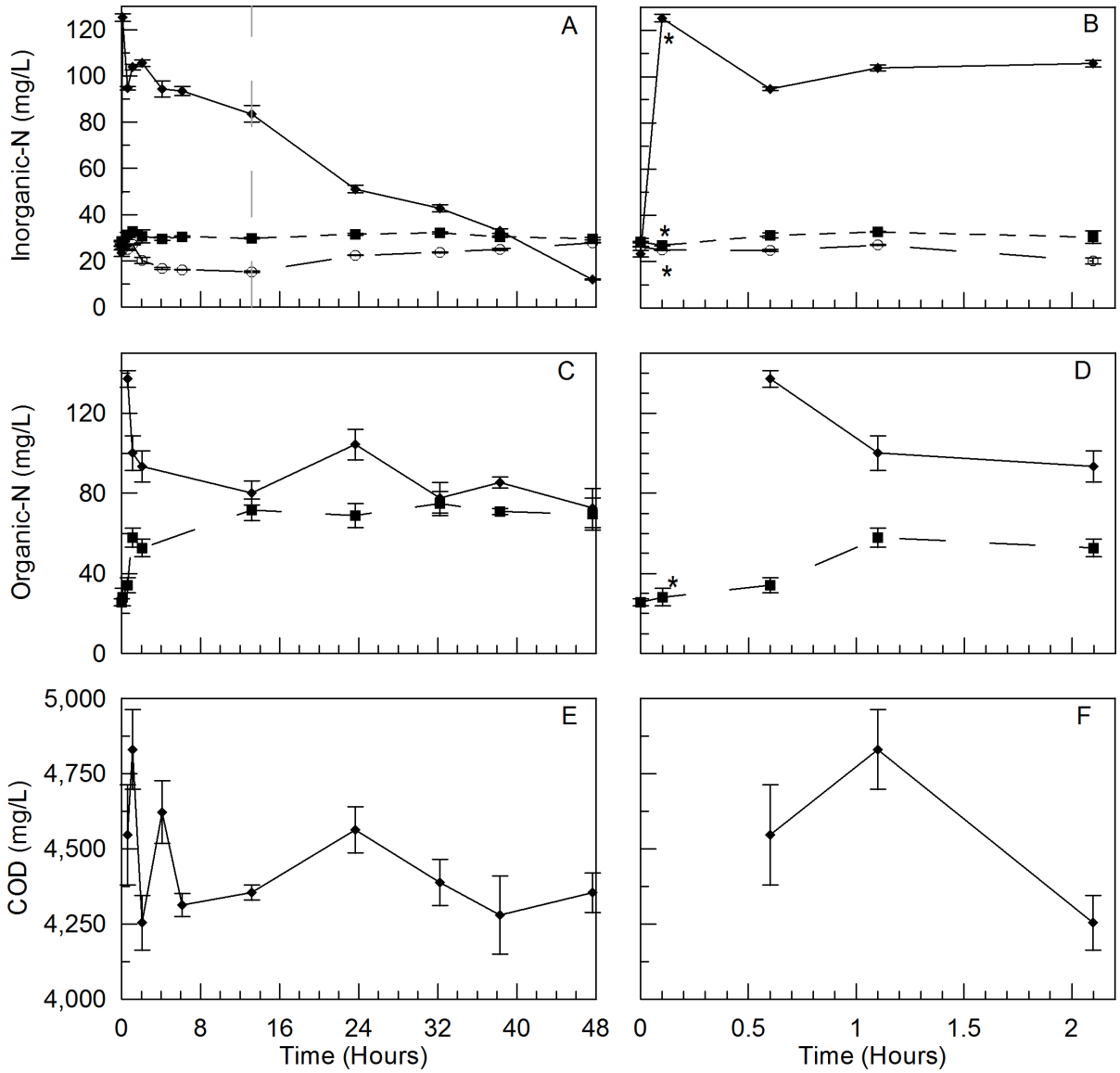

Figure 5.3: Concentration profiles of (A and B) ammonium-N (◆), nitrite-N (○), and nitrate-N (■) (C and D) particulate-N (◆), dissolved organic-N (■) and (E and F) total COD. Soluble COD had minimal variation across the cycle (1,060 ± 40 mg COD L^{-1}; Appendix C.12). Samples corresponding to t=0 represent effluent data from the previous cycle. Data points marked with an asterisk are calculated values based on the known mass added by the end of the feed period and the concentration at the end of the previous cycle; these values were not included in particulate analysis due to the settling period which caused non-representative sampling of solids in the effluent. Panels (B), (D), and (F) show detailed views of the first 2 hours of cycle operation. The dashed grey vertical line in panel (A) represents the transition from AOB-limited operation to anammox-limited operation. Data are means and standard deviations of replicate measurements (n=3); some error bars are smaller than the symbol.

Table 5.4: Comparison of SBR removal rates (mg L^{-1} d^{-1}) from intracycle analysis and cumulative mass analysis.

Constituent	Intracycle [a]	Cumulative Mass [b]
NH_4^+-N	50.2 ± 0.5	50.4 ± 0.2
TIN	47.7 ± 5.3	49.8 ± 0.1
TDN	33.2 ± 4.5	60.0 ± 1.9
TN	47.8 ± 3.9	60.3 ± 1.1
tCOD	104 ± 74 [c]	51.3 ± 0.9
sCOD	9.40 ± 18.4 [c]	35.7 ± 8.0

[a] Data represent slope of best-fit line ± 95% confidence interval.
[b] Final 25 days of operation.
[c] No significant difference from 0.

Comparing rates determined from intracycle sampling to the rates based on cumulative mass over the reported period of reactor operation (Table 5.4), the removal rates for TDN and TN were greater based on cumulative mass analysis than on intracycle sampling. The rates of removal for NH_4^+-N and TIN were similar in both methods.

5.3.4 Maximum Rates

The maximum rates of relevant biological processes were determined in duplicate in-reactor experiments (Figure 5.4). The conditions imposed over the course of each experiment are summarized in Table 5.5; note that the condition during period (ii) in each experiment was comparable to normal reactor operation. During the anammox-only period (period iv), TIN was removed at a rate of 69 and 51 mg L^{-1} d^{-1} for replicates A and B, respectively, in good agreement with the rates of N removal calculated from cumulative masses (Table 5.4). The stoichiometry of the anammox reaction estimated from these rates was 2.26 and 1.79 NO_2^--N $(NH_4^+\text{-N})^{-1}$, respectively.

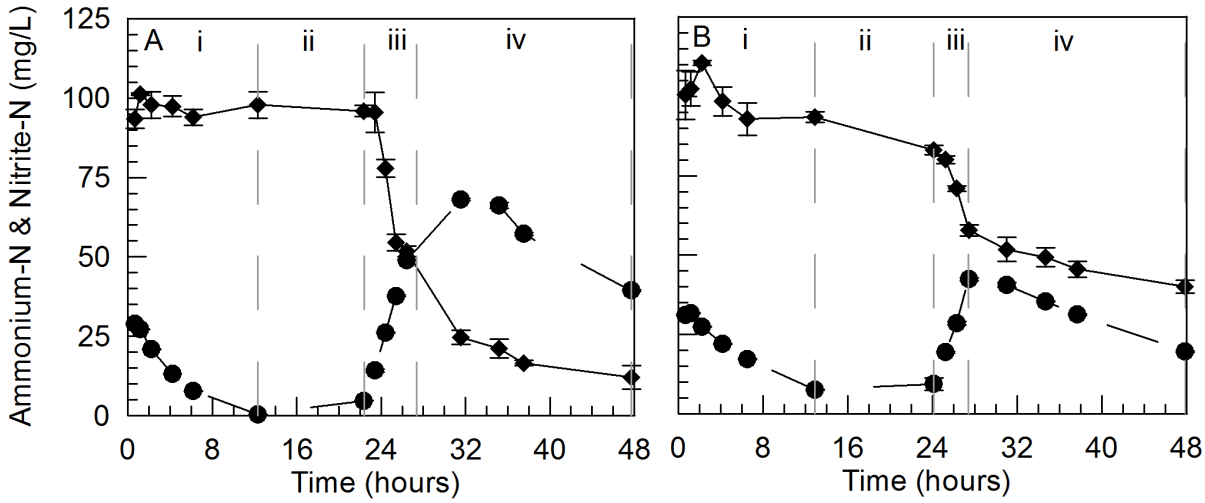

Figure 5.4: Duplicate (A,B) concentration profiles of ammonium-N (◆) and nitrite-N (●) during in-reactor rate experiments. Nitrate-N had little variation, with a mean and standard deviation of 26.3 ± 2.9 and 25.0 ± 2.1 mg L^{-1} for replicates A and B, respectively (not shown). Periods with distinct operating conditions (summarized in Table 5) are separated by dashed vertical lines. COD in periods ii, iii, and iv was not measured. The concentration of tCOD and sCOD in period i was 4,430 ± 200 and 1,090 ± 30 mg L^{-1}, respectively for replicate A (not shown). For replicate B, these concentrations were 4,170 ± 50 and 1,060 ± 20 mg L^{-1}, respectively (not shown).

Table 5.5: Conditions for maximum rate experiments.

Period	Sparge Gas	Gas Input [a]	Rxns [b]	pH (A)[c]	pH (B) [c]
i	N_2	I	D, An	7.1→7.6	8.1→8.3
ii	O_2	I	D, N, An	7.6→7.6	8.3→8.1
iii	O_2	C	N, An	7.6→8.0 [d]	8.1→8.0
iv	N_2	I	An	8.0→8.1	8.0→8.4

[a] Frequency of gas addition: I, intermittent; C, continuous.
[b] Biological reactions promoted: D, denitrification; N, nitritation; An, anammox.
[c] Beginning and ending pH values for replicate A and replicate B.
[d] Increase in pH was likely due to purging the headspace of CO_2 with the reactor top open.

After accounting for the contribution of anammox to nitrite consumption in period (i), denitrification consumed NO_2^--N at a rate of 16 mg L^{-1} d^{-1} in both experiments. In comparison to maximum rates of TN removal shown in Table 5.4, denitrification could account for no more than 30% of the observed TN removal during normal reactor operation.

5.4 Discussion

Previous studies investigating anammox treatment of swine waste have explored both one-reactor (*23*, *128*, *129*) and two-reactor (*21*, *25*, *115*, *127*, *183*, *203*, *204*) systems. Only studies in which the aerobic and anaerobic reactions were carried out in one vessel are applicable to this work. Previous one-reactor studies used swine waste with much lower ammonium (225 – 420 mg NH_4^+-N L^{-1}, COD (380 – 420 mg L^{-1}) and COD N^{-1} ratio (0.6 – 1.0) than the waste used in this study. Additionally, two of the previous studies (*128*, *129*) used extended feed periods (feeding occurred during 50-96% of the total cycle time), which avoided a high instantaneous concentration of ammonium and COD at the beginning of the reactor cycle. The only study that used a short feed period added ammonium chloride or sodium acetate to the influent waste to maintain a controlled COD N^{-1} ratio (*23*). All these studies were done at smaller scale than the work presented here (1.5 – 6.5 L). None of the previous studies maintained the very low DO conditions used in this study and none looked at N_2O emissions associated with nitritation/anammox treatment of anaerobically digested swine waste. Therefore, no previous study is exactly analogous to the present work.

Typical anammox stoichiometry has been reported to be 1.3 NO_2^--N $(NH_4^+\text{-N})^{-1}$, though earlier studies on anaerobically digested swine waste reported 1.7-2.1 NO_2^--N $(NH_4^+\text{-N})^{-1}$ (*21*, *25*, *203*). These systems were operated in such a way that the contribution of heterotrophic denitrification from nitrite should have been minimal so the reason for the deviation from "typical" anammox stoichiometry is unknown.

5.4.1 COD Removal

Installing an anaerobic digester upstream of a nitritation/anammox system allows for maximizing the amount of COD to be used for biogas production from swine waste and minimizing the COD load to the N removal system (Chapter 4). Minimizing the COD load

should help autotrophic N removal by minimizing the potential contribution of heterotrophic denitrification to net N removal; in turn, maximizing autotrophic N removal reduces the overall oxygen requirements. Earlier work on nitrification/denitrification of the same digested swine waste (Chapter 4) indicated seasonal variability of the COD N^{-1} ratio; sufficient COD for complete denitrification was available only in winter months, when gas production in the covered lagoon at the farm decreased. Because reactor performance in the present study was evaluated with a single batch of waste, COD variability was not an issue. To fully assess potential seasonal variability of COD content of the digested waste and its impact on a nitritation/anammox system, an on-site pilot study would need to be performed.

There was significant removal of COD across the reactor (Tables 5.2 and 5.3), with three potential mechanisms that could be responsible for the observed removal: aerobic oxidation, denitrification with nitrate, or denitrification with nitrite. The half-saturation coefficient for oxygen has been reported to be lower for aerobic heterotrophs than for AOB (*205*), suggesting that the heterotrophs would be more competitive for the limited O_2 supply under microaerobic (low-DO) conditions. It is likely, therefore, that aerobic activity was a significant COD removal mechanism in the SBR under the final operating conditions. Using data shown in Figure 5.2 and Table 5.4, the maximum aerobic COD removal rate would have been 0.03 g COD g VSS^{-1} d^{-1}; this rate is well below the typical value for activated sludge treatment of municipal wastewater (*58*). As noted below, denitrification also contributed to COD removal in the SBR.

The intracycle analysis (Figure 5.3) showed no significant change in the concentration of COD throughout a cycle, which indicates that any COD consumption primarily occurred prior to the first sampling event. Nitrogen removal was observed throughout the cycle,

indicating that autotrophic N removal, rather than heterotrophic denitrification, was the primary N removal mechanism. Further support for a primarily autotrophic mechanism of N removal is provided by the estimated rate of denitrification with nitrite (Section 5.3.4 Maximum rates).

5.4.2 Nitrogen Removal

The microaerobic conditions that existed in the reactor likely promoted the partial oxidation of ammonium to nitrite rather than complete nitrification to nitrate (*199*); due to the difference in the oxygen half-saturation coefficient between AOB and NOB (*198*), low-DO conditions generally favor nitritation and preclude competition for nitrite by NOB relative to anammox bacteria (*195*). Aerobic oxidation of nitrite was not observed even under fully aerobic conditions (Figure 5.4), suggesting that nitrate in the reactor effluent was produced as a side product of anammox (*67*).

Effluent nitrate was far below the amount predicted by anammox stoichiometry (*67*), which suggests nitrate consumption by denitrifiers. Recently, nitrate formation due to anammox was found to be inversely proportional to the solids retention time (*71*), and the retention time in the present study was quite long. However, even systems with indefinite retention of anammox biomass exhibited significant nitrate accumulation, whether the bacteria were grown in suspended culture (*195*) or as a biofilm (*165*).

The intracycle concentration of nitrite (Figure 5.3a) suggested that the reactor cycle consisted of two phases: an AOB-limited phase (decreasing concentration of nitrite) and an anammox-limited phase (increasing concentration of nitrite). This apparent biphasic behavior of nitrite throughout a cycle places constraints upon the effluent concentration of nitrite. To maintain maximum N removal, the concentration of nitrite must remain above the half-

saturation coefficient throughout most of the cycle; for the biomass in this study, the estimated half-saturation coefficient for NO_2^- was 2.7 mg N L^{-1} (Figure C.6). The AOB-limited phase was likely the result of the oxygen-limited nature of the system and the oxygen demand exerted by non-nitrogenous species.

Net nitrogenous oxygen consumption over the reported period of reactor operation (Appendix C.4) was between 1.47 g O_2 (g TN)$^{-1}$ (assuming aerobic oxidation of COD) and 2.33 g O_2 (g TN)$^{-1}$ (assuming removal of COD by denitrification), resulting in an anammox stoichiometry between 0.75 ± 0.06 and 2.1 ± 0.3 NO_2^--N (NH_4^+-N)$^{-1}$. However, anammox stoichiometry less than 1.0 NO_2^--N (NH_4^+-N)$^{-1}$ is not possible. Assuming typical anammox stoichiometry of 1.3-1.7 NO_2^--N (NH_4^+-N)$^{-1}$, the net nitrogenous oxygen demand would be 1.94-2.15 g O_2 (g N)$^{-1}$, which supports a conclusion that COD removal probably occurred by a combination of aerobic oxidation and denitrification.

The aerobic oxidation of ammonium requires 7.1 g alkalinity as $CaCO_3$ per g ammonium-N oxidized. Assuming a stoichiometry of 1.3-1.7 g NO_2^-N (g NH_4^+-N)$^{-1}$ for anammox and assuming that all organic N removed would have been ammonified first, the alkalinity required for net TN removal would have been ~9,500-11,700 mg L^{-1} as $CaCO_3$. The alkalinity in the digested swine waste would barely have met this minimal demand (Table 5.1), but denitrification would have contributed additional alkalinity. Regardless, the net alkalinity consumption was sufficient to maintain neutral pH in the reactor without any requirement for added alkalinity. Alkalinity addition can represent an expensive operating cost for full-scale nitrification/denitrification (*8*); eliminating the need for external alkalinity represents a significant operational savings.

Ammonium removal was excellent overall and reached 99% by the end of reactor operation (Table S3). Other studies on nitritation/anammox treatment of anaerobically digested swine waste reported 50-96% ammonium removal and 50-80% TN removal (*23*, *128*, *129*). Single-reactor nitritation/anammox systems treating municipal digester centrate report 70-90% TN removal (*100*, *206–209*). Limitations in the N removal capacity of an anammox system are generally associated with the production of nitrate due to anammox (*195*), and some authors (*210*) have stated that anammox can remove a maximum of 88-89% influent TN due to the produced nitrate. This study achieved a maximum TN removal of 93%, likely due to the relatively high COD content that promoted denitrification of the majority of nitrate produced by anammox.

5.4.3 Nitrous Oxide Production

Nitrous oxide yield is of increasing interest in biological nitrogen removal systems due to its significant global warming potential (*49*). The observed yield of 11% N_2O-N per unit TN removed is higher than typically observed (0.6-6%) in either one- or two-reactor anammox systems (*211*). One study has reported an N_2O yield of 10.9% during prolonged aeration of a nitritation/anammox system (*212*). Nitrous oxide is not a known side-product nor intermediate of anammox activity (*213*) and highly enriched anammox cells have been shown to produce very little N_2O (*214*). The stoichiometry of COD/N was insufficient for denitrification to be a dominant mechanism for the production of N_2O in the present study. Therefore, AOB are the most likely source of N_2O; this is consistent with what was found in the nitrification/denitrification system used to treat this waste in a previous study (Chapter 4).

Low dissolved oxygen and increased nitrite concentration have both been positively associated with N_2O production (*64*). These are the same conditions which existed in the

reactor in this study, so that the high yield of N_2O is not surprising. Overall, however, the inherent impact of nitrogen removal from anaerobically digested swine waste on N_2O production is not well characterized.

5.5 Conclusions

Partial nitritation and anammox were successfully applied to swine waste from a full-scale anaerobic digester. The main finding of this study was that a sequencing batch reactor can be operated under oxygen-limiting conditions to control the extent of AOB activity while suppressing NOB activity. Aerobic activity was the primary mechanism of COD removal, although denitrification contributed to overall N removal. Most of the COD was removed at the beginning of the SBR cycle.

Over 99% of influent ammonium and over 90% of influent TN can be removed in a one-reactor nitritation/anammox system, with the majority of N removal occurring via autotrophic mechanisms. N_2O emission accounted for 11% of the removed N and was likely a result of AOB activity.

5.6 Acknowledgements

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Chapter 6: Microbial Community Analysis of a Nitritation/Anammox Bioreactor Treating Anaerobically Digested Swine Waste.

Eric T. Staunton[3] and Michael D. Aitken

6.1 Introduction

North Carolina (NC) is currently home to approximately 8.4 million swine (*1*), most of which are raised in confined animal feeding operations (CAFOs). Waste from CAFOs is typically stored in large, open-air lagoons and periodically applied to sprayfields to fertilize crops (*2*). There are currently ~4,500 lagoons in NC which produce more nutrients (mainly nitrogen) than can be assimilated at agronomic rates in the entire region of swine production (*2*). Nutrient emissions associated with swine production are the cause of several public health and environmental concerns including respiratory ailments (*14*) and the emission of ammonia and various greenhouse gases to the atmosphere (*3*, *4*).

We previously investigated coupling anaerobic digestion of swine waste for energy recovery with nitrogen removal by nitrification/denitrification at an 8,000-head swine farm in NC (Chapter 4). While this scheme can achieve substantial reductions in nitrogen emissions to the environment, it was found to be cost-prohibitive (*8*). I subsequently conducted a lab-scale study on the digested waste to explore nitrogen removal by nitritation/anammox (Chapter 5), which should significantly lower the cost associated with requirements for both oxygen and supplemental alkalinity compared to nitrification/denitrification.

[3] Responsible for physical, molecular, and data analysis and manuscript preparation

To employ anammox as the predominant nitrogen removal mechanism in a system that receives ammonium as the primary N species in the waste, it is necessary to oxidize approximately half of the ammonium to nitrite (nitritation) via aerobic ammonium-oxidizing bacteria (AOB). Ultimately, successful biological treatment of any system involves the presence and activity of microbes expressing a desired phenotype and maintaining treatment conditions to support growth of those organisms. To this effect, it is important to understand the impact of design variables on the relative abundances of desired microbes.

Previous efforts to characterize anammox communities have involved the creation of clone libraries of 16S rRNA genes (*215–217*) or quantitative polymerase chain reaction (qPCR) to quantify gene targets of interest (*215*, *217*, *218*). The development of low-cost, high-throughput sequencing techniques has provided an alternative approach that allows sequencing of entire communities, with thousands to millions of sequences per sample (*219*). The resulting dataset can be probed both for known organisms of interest and for abundant organisms that would not otherwise be detected. High-throughput sequencing has seen application in numerous complex environmental samples, including anammox systems (*145*, *146*).

In the present study, I examined the microbial community of a lab-scale nitritation/anammox system treating anaerobically digested swine waste, whose performance has been described elsewhere (Chapter 5). Of significant interest were which anammox species dominated the community, how the mature reactor community compared to the seed and influent communities, and how granule size affected community composition.

6.2 Materials and Methods

6.2.1 Reactor Description

An in-depth description of the reactor, its operation, and its performance characteristics is provided elsewhere (Chapter 5). Briefly, digested swine waste was obtained from an 8,000-head farm in North Carolina; the waste contained approximately 2,200 mg L^{-1} NH_4^+-N and 2,600 mg L^{-1} total-N. A 20-L sequencing batch reactor was operated for more than 450 days while process operating variables were adjusted to optimize nitritation/anammox for N removal. The final set of operating conditions (final 65 days) comprised intermittent pulsing of pure oxygen for 6 sec every 37 min at continuously low dissolved oxygen concentration (< 0.1 mg L^{-1}). The hydraulic retention time in the reactor was 40 days.

At the beginning of the study, the reactor was seeded with granular anammox biomass provided by City College of New York (CC seed) and activated sludge as a source of AOB. After approximately 6 months of operation, the reactor was re-seeded with anammox biomass from a full-scale deammonification system operated by the Hampton Roads (Virginia, USA) Sanitation District (HRSD seed). Only the HRSD seed was included in the community analysis; previous work demonstrated that the dominant anammox genus in the CC seed was *Candidatus Brocadia* (*220*).

6.2.2 Sample Collection

When reactor operation was terminated, the entire reactor contents were passed through a column of autoclaved sieves consisting of mesh sizes described in Table D.1. To recover the solids, each sieve was first rinsed with 1-2 L autoclaved potassium phosphate buffer (5 mM, pH 7.3) with a total wash volume of 14 L. After rinsing, each sieve was backwashed with sterile N-free anammox media (*67*) with ammonium and nitrite omitted, and the backwash

volume was stored at 4°C for 24 hours prior to DNA extraction. No solids were retained by the largest (3.50 mm opening) sieve.

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined in triplicate by standard methods 2540D, and 2540E respectively (*178*). The ratio of VSS to TSS was determined on individual samples and the ratio averaged.

6.2.3 DNA Extraction

Five pieces of Kaldnes K1 media were collected from the reactor and washed twice in sterile DI water. Wash-water was centrifuged at 5,000×g for 5 min and the pellet collected; biomass collected from wash-water is referred to as the "loosely associated biofilm" (LAB). Due to lack of observable growth on the K1 media (Figure A.6), DNA extractions were not performed on the solid substrate. The pellet from the LAB was treated as described below.

DNA was extracted from 1.7 mg VSS from each of the size fractions, influent swine waste, and biofilm attached to the impeller above the liquid surface (Figure A.8); for the LAB, DNA was extracted from 2.6 mg VSS. DNA extractions were performed using the MoBio (Carlsbad, CA USA) Powerlyzer Powersoil® DNA extraction kit according to the manufacturer's directions. Bead beating was performed on a Fisher Scientific flatbed vortexer for 10 min. Final extract was eluted in 50 µL autoclaved, sterile-filtered (0.45 µm) TE buffer (pH=8.0). DNA extractions were performed in duplicate for each size fraction (designated as "A" and "B" for a given sample), with each extraction treated independently for subsequent analysis.

The concentration of DNA in each extract was measured using a NanoDrop ND-3300 Fluorospectrometer (ThermoScientific; Waltham, MA, USA) and Quant-iT Picogreen dsDNA Kit (Invitrogen; Carlsbad, CA, USA).

6.2.4 qPCR

Previously published primer sets (Table 6.1) were used to quantify genes for hydrazine synthase (*hzsA*, anammox functional gene), nitrous oxide reductase (*nosZ*, denitrifying functional gene), *Nitrobacter* 16S rRNA, and *Nitrospira* 16S rRNA. *Nitrobacter* and *Nitrospira* were evaluated as canonical nitrite-oxidizing bacteria (NOB) typically found in wastewater treatment systems. Primers were optimized with respect to $MgCl_2$ concentration and primer melting temperature using an Eppendorf Mastercylcer Gradient with 5Prime Mastermix (Gaithersburg, MD, USA). Template for the construction of standard curves was a plasmid clone linearized with EcoRI. Amplification efficiency was determined by the method of Pfaffl (*221*).

Table 6.1: Quantitative PCR primers used in this study.

Target	Primer Name	Primer Sequence (5`→3`)	Tm (°C)[a]	Amplicon Length	Amp. Eff.[b]	Detection Limit[c]	Reference
hzsA	*hzsA_1597F* *hzsA_1857R*	WTYGGKTATCARTATGTAG AAABGGYGAATCATARTGGC	55	260	1.91	3	(*91*)
nosZ	*nosZ1F* *nosZ1R*	WCSYTGTTCMTCGACAGCCAG ATGTCGATCARCTGVKCRTTYTC	60	259	1.88	2	(*132*)
Nitrobacter 16S rRNA	*FGPS 872f* *FGPS 1269r*	CTAAAACTCAAAGGAATTGA TTTTTTGAGATTTGCTAG	54	397	1.73	42	(*222*)
Nitrospira 16S rRNA	*Nspra675f* *Nspra746r*	GCGGTGAAATGCGTAGAKATCG TCAGCGTCAGRWAYGTTCCAGAG	60	71	1.96	3	(*223*)

[a] Empirically determined optimum melting temperature.
[b] Amplification efficiency.
[c] Detection limit of the qPCR assay expressed as copy number per µL DNA extract.

The qPCR reaction utilized QuantiTect SYBR Green PCR master mix (Qiagen, Valencia, CA, USA) with the SmartCycler platform (Cepheid, Sunnyvale, CA, USA) in 25 µl reactions. The reaction mixture consisted of 12.5 µL master mix, 10.5 µL deionized water, 1µL primer mix (final concentration 0.6 µM each of the forward and reverse primer), and 1 µL template DNA extract (20-100 pg genomic DNA per reaction). Amplification of denitrifying and anammox functional genes as well as the *Nitrobacter* 16S rRNA gene required the addition of 1 µL of 25 mM $MgCl_2$. This volume was subtracted from the amount of water used to maintain a 25 µL reaction.

The conditions for qPCR were as follows: 15 min at 95°C, 45 cycles of 15 s at 95°C, 30 s at the melting temperature (Table 6.1), and 30 s at 72°C (except for *Nitrobacter*, for which the elongation step was 45 s at 72°C). Fluorescence was measured during the elongation step and products analyzed by melt curve analysis from 60-95°C at 0.2 °C s^{-1}. The r^2 value for each qPCR standard curve (cycle threshold vs log gene copy number) was >0.995.

6.2.5 Illumina MiSeq sequencing and analysis

The V4 region of the 16S rRNA gene was amplified using PCR primers 515F/806R with barcode on the forward primer. The PCR reaction used HotStarTaq Plus MasterMix Kit (Qiagen, Valencia, CA, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 min, with a final elongation step at 72°C for 5 min. PCR products were confirmed on a 2% agarose gel. Multiple samples were pooled in equal proportions based on molecular weight and DNA concentration. Pooled samples were subsequently purified using calibrated Ampure XP beads.

The pooled and purified PCR product was used to prepare a DNA library by following the Illumina TruSeq DNA library preparation protocol. Amplification of sample DNA and sequencing were performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq following the manufacturer's guidelines.

Forward and reverse reads were joined, and exact barcode matches were culled from the full dataset and oriented to the 5`→3` direction at MR DNA labs. Libraries were further processed with mothur v.1.33.3 (*136*) and analyses followed the "Schloss MiSeq Standard Operating Procedure (SOP)" (http://www.mothur.org/wiki/MiSeq_SOP). Data processing and clean-up included demultiplexing the run, removing barcodes and primers, discarding short (less than 227 bp) and long (greater than 275 bp) reads, and discarding reads that contained ambiguous base calls or long (greater than 8) homopolymer runs. Sequences were checked for chimeras using the UCHIME implementation in mothur and chimeric sequences removed from analysis. The Greengenes database (*224*) was used for classification of representative sequences and *Archaea* were retained in the final dataset. Sequences were classified into operational taxonomic units (OTUs) based on 97% homology. OTUs that comprised a single read were removed from downstream analysis.

Community comparisons within mothur were processed using libraries randomly reduced to the smallest library (24,450 sequences); the calculation of relative abundance and quantification of selected bacterial groups was performed considering all available sequences. The abundance method was used within mothur to identify representative sequences of the determined OTUs, and a local BLAST search was performed against these sequences to quantify bacterial groups of interest (such as *Candidatus Brocadia* and *Nitrosomonas* spp.). Sequences with ≥97% similarity over the entire query sequence were

considered positive matches in the local BLAST. Abundant OTUs were identified with a BLAST search against the NCBI nonredundant database. Sequence similarity was determined using Clustal Omega (*225*).

Alignments for phylogenetic trees were generated in MEGA6 (*226*) with ClustalW and trimmed to allow direct comparison of GenBank sequences to the shorter MiSeq sequences. Bootstrapped neighbor-joining trees (10,000 replicates) were generated using MEGA6.

6.3 Results

6.3.1 Solids Recovery and Characterization

When operation of the nitritation/anammox bioreactor was terminated, the contents were passed through a series of sieves to characterize the biomass by size fraction. The sieving procedure recovered 70 % of the TSS measured in the mixed liquor before sieving, with the vast majority of solids passing through the smallest (75 μm opening) sieve (Table D.2). The largest size fractions (1.00-2.00 mm and 2.00-3.50 mm) morphologically resembled pieces of biofilm, likely from the interior of tubing used for mixed liquor recirculation (Figure A.9). The VSS/TSS ratio ranged from 0.33 to 0.9; smaller aggregates (75-500 μm) had the highest ratio (Table D.2).

6.3.2 qPCR

Anammox bacteria were quantifiable only in the material retained by the sieves (Figure 6.1), which collectively accounted for only 4.4% of the recovered VSS from the reactor. Interestingly, the melting temperature of the anammox qPCR product changed between the HRSD seed culture and the reactor samples, from ~84°C to ~80°C (Figure D.2). Denitrifiers were quantifiable in all samples, but neither *Nitrobacter* nor *Nitrospira* was detected by qPCR in any sample from the reactor. The abundance of anammox bacteria in the HRSD seed culture was greater than the abundance of anammox bacteria in any of the reactor

biomass size fractions, but generally within one order of magnitude (Figure 6.1). The abundance of denitrifying bacteria was similar among all reactor samples and higher than in both the influent swine waste and the HRSD seed culture (Figure 6.1).

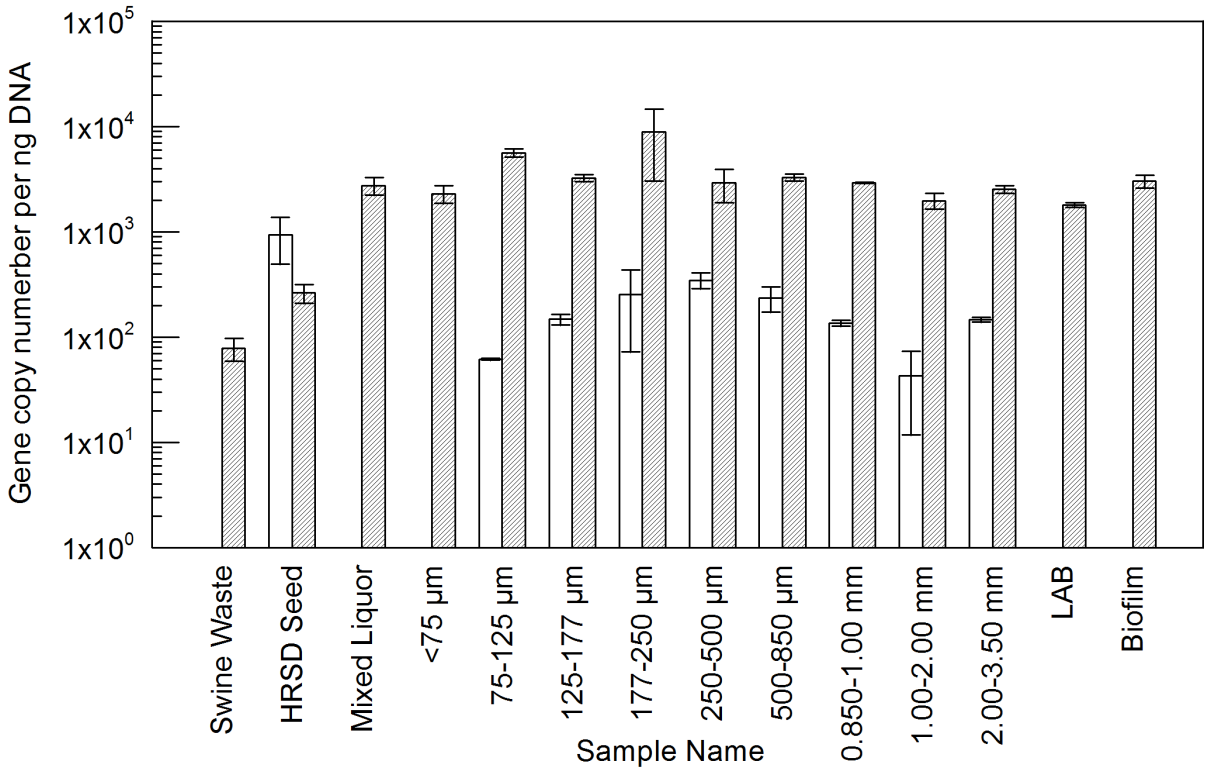

Figure 6.1: Gene copy number associated with size separated granules from the anammox reactor. *hzsA* (white bar; average limit of detection 10^2 copies (ng DNA)$^{-1}$); *nosZ* (shaded bar; average limit of detection 10^3 copies (ng DNA)$^{-1}$). Each bar represents the mean of single qPCR assays of duplicate DNA extractions and the error bar is the range. Absent bars indicate no detection.

6.3.3 Sequencing

A total of 4,732,736 sequences were obtained from the Illumina MiSeq sequencing run. Of 194,249 unique sequences analyzed, 88,882 potential chimeras were detected. After removing chimeras, a total of 1,180,694 sequences were retained for downstream analysis. The mean library size was approximately 42,000 sequences. The smallest library (replicate B of the 850 µm – 1.00 mm size fraction) contained 24,450 sequences, while the largest (replicate B of the influent swine waste) contained 59,174 sequences (Table 6.2). The most

abundant phyla (> 5% of individual libraries) represented were: *Proteobacteria, unclassified Bacteria, Firmicutes, Bacteroidetes, Chlorobi, Chloroflexi, Synergistetes, Planctomycetes, Verrucomicrobia, Actinobacteria,* and *Acidobacteria* (Table D.3).

Table 6.2: Summary of sequence data and diversity estimation.

Sample	No. of sequences	No. of OTUs[a]	Inv. Simpson[a,b]
Swine Waste A	58,048	1,450	73.5
Swine Waste B	59,174	1,450	77.0
Mixed Liquor A	37,793	622	8.02
Mixed Liquor B	51,190	723	6.26
<75 µm A	40,625	716	6.12
<75 µm B	40,241	710	6.14
75-125 µm A	42,643	696	38.3
75-125 µm B	35,782	684	41.1
125-177 µm A	36,717	643	41.2
125-177 µm B	34,172	636	39.4
177-250 µm A	41,215	646	37.5
177-250 µm B	28,745	574	37.7
250-500 µm A	41,252	697	42.6
250-500 µm B	40,796	685	39.9
500-850 µm A	41,818	716	43.5
500-850 µm B	38,856	699	42.3
850 µm-1.00 mm A	35,222	696	46.4
850 µm-1.00 mm B	24,450	581	48.1
1.00-2.00 mm A	44,520	753	50.4
1.00-2.00 mm B	47,303	783	48.1
2.00-3.50 mm A	49,199	736	38.0
2.00-3.50 mm B	45,673	769	49.2
LAB A	42,908	752	7.58
LAB B	37,216	967	7.94
Biofilm A	40,055	651	26.5
Biofilm B	49,325	733	29.5
HRSD Seed A	46,125	867	24.6
HRSD Seed B	49,632	865	24.2

[a] Values based on a random subsample of 24,450 sequences per library.
[b] Inverse Simpson Index – inverse of the Simpson diversity indicator.

6.3.4 Diversity Analysis of Sequence Libraries

The sequence libraries represent a variety of samples relevant to reactor operation, including influent swine waste, HRSD seed culture, size-fractionated aggregates, and two biofilms (LAB associated with the plastic media, and the biofilm that accumulated on the reactor impeller shaft). Bacterial diversity was highest in the influent swine waste and lowest in the reactor mixed liquor, LAB, and sieve column flow-through (*i.e.*, biomass < 75 μm). Generally, the reactor granules retained by the sieves had higher diversity than the HRSD seed culture (Table 6.2).

A phylogenetic tree of reactor communities (Figure 6.2) shows two dominant groups, with the granules from the reactor grouping together and the mixed liquor, LAB, and biomass < 75 μm forming another group. Principal coordinate analysis (PCoA) of the libraries showed no significant difference in any of the samples retained by the sieves (Figure D.3). Statistical analysis failed to find any significant difference between what was not retained by the sieves and the LAB. Accordingly, libraries from reactor samples are divided into two primary groups for further discussion: the reactor granules (any aggregate retained by the sieves) and the biomass not retained by the sieve column.

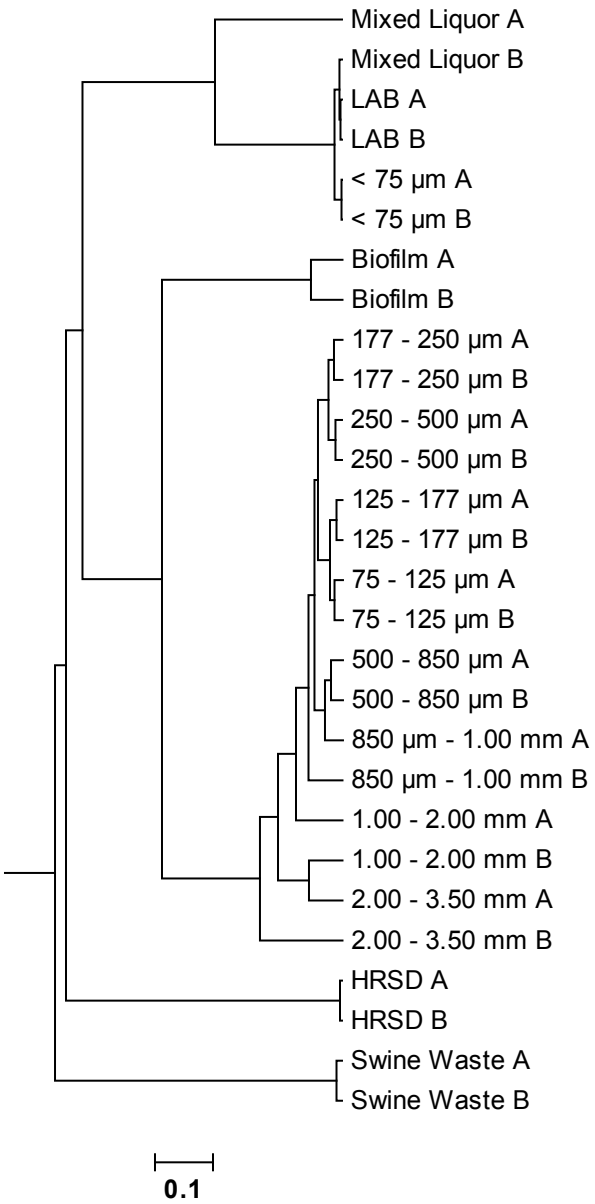

Figure 6.2: Phylogenetic tree showing the relationships of the sequenced microbial communities. All libraries were randomly reduced to the size of the smallest library (24,450 sequences) for comparison.

The phylogenetic tree and PCoA additionally indicated a significant difference between the HRSD seed and the mature granules that developed in the reactor over the course of its operation. Generally, the sequencing results indicated good reproducibility between duplicates (Figure 6.2). One exception is that the mixed liquor duplicates do not cluster (Figure 6.2); this is likely attributable to the large number of sequences associated with an

unclassified OTU within the order *Methylococcales* and an OTU most closely related to the genus *Methylotenera* within "Mixed Liquor A" but not associated with any other library (17.1% and 12.7% respectively; Table D.3).

6.3.5 Bacteria Associated with N Transformations

Aerobic nitrogen-oxidizing bacteria are well-established to belong largely to the genera *Nitrosomonas* (AOB) and the NOB *Nitrobacter* and *Nitrospira*. Anammox bacteria are associated with various genera within the phylum *Planctomycetes*. Denitrifying bacteria are much more phylogenetically diverse and cannot be identified easily from the 16S rRNA gene alone.

Sequences related to *Nitrosomonas* were generally evenly distributed at low relative abundance among all samples, with the exception of the influent swine waste (negligible abundance) and the impeller shaft biofilm, which contained 3-4% *Nitrosomonas* (Table 6.3). Sequences associated with *Nitrobacter* were not detected to a significant degree within any library, and sequences related to *Nitrospira* were detected only in the HRSD seed culture, suggesting that reactor operation suppressed the growth of NOB.

Table 6.3: Percent relative abundances of sequences of interest in libraries. Values ≥ 1% are in bold. Dashes indicate < 0.01%.

Sample	*Candidatus Brocadia* OTU-55	*Candidatus Kuenenia* OTU-3	*Nitrosomonas* OTU-34	*Nitrospira* OTU-119	*Nitrobacter* OTU-111
Swine Waste A	0.03	0.06	0.05	0.02	0.01
Swine Waste B	0.06	0.06	0.07	0.05	0.01
Mixed Liquor A	0.01	0.08	0.41	0.01	0.03
Mixed Liquor B	0.02	0.11	0.36	0.01	0.01
<75 µm A	0.02	0.10	0.22	0.02	0.02
<75 µm B	0.02	0.06	0.26	0.01	0.01
75-125 µm A	0.02	**6.29**	0.69	0.01	0.09
75-125 µm B	0.15	**4.32**	0.91	0.02	0.11
125-177 µm A	0.10	**5.09**	0.70	0.01	0.14
125-177 µm B	0.08	**4.80**	0.58	0.02	0.10
177-250 µm A	0.13	**6.68**	0.55	0.02	0.12
177-250 µm B	0.15	**5.89**	0.54	-	0.09
250-500 µm A	0.14	**5.55**	0.45	0.02	0.23
250-500 µm B	0.18	**6.57**	0.52	0.01	0.24
500-850 µm A	0.16	**6.28**	0.42	0.01	0.16
500-850 µm B	0.12	**5.90**	0.56	0.02	0.13
850 µm-1.00 mm A	0.11	**5.82**	0.56	0.01	0.05
850 µm-1.00 mm B	0.12	**5.49**	**1.04**	0.02	0.46
1.00-2.00 mm A	0.09	**2.89**	0.73	0.01	0.03
1.00-2.00 mm B	0.09	**2.42**	0.42	0.01	0.04
2.00-3.50 mm A	0.10	**4.65**	0.44	0.01	0.04
2.00-3.50 mm B	0.13	**6.82**	0.64	0.02	0.05
LAB A	0.01	0.08	0.36	0.01	0.01
LAB B	0.01	0.06	0.54	0.01	0.04
Biofilm A	-	0.05	**3.08**	0.02	0.07
Biofilm B	0.01	0.09	**4.19**	0.01	0.29
HRSD Seed A	**2.67**	0.31	0.80	**1.58**	0.04
HRSD Seed B	**2.81**	0.32	0.88	**1.03**	0.02

There was a significant shift in the anammox community from *Candidatus Brocadia* in the seed culture to *Candidatus Kuenenia* in the reactor granules (Table 6.3). Sequences related to *Candidatus Brocadia* accounted for 2.7% of the seed but only 0.12% of the reactor granules and 0.02% of the biomass < 75 μm. In contrast, sequences related to *Candidatus Kuenenia* accounted for an average of 5.2% of the reactor granules, but only 0.3% of the HRSD seed and 0.08% of the biomass < 75 μm. Both these anammox groups are poorly represented in the influent swine waste. Aggregates smaller than 75 μm were not further separated, so the smallest aggregate size associated with anammox microbes is unknown. Other anammox genera (*Candidatus Anammoxoglobus, Candidatus Jettenia,* and *Candidatus Scalindua*) were not detected in any library.

Sequences associated with an unclassified member of the *Planctomycetes* within the order *Phycisphaerales* (OTU-22; Table D.3) grew to 2.2% abundance within the reactor granules but had very low relative abundance in the seed culture and the influent swine waste.

6.3.6 Other Significant Bacterial Groups in the Reactor Samples

With the exception of OTU-6, every dominant (>2%) bacterial group within the seed culture was poorly represented in the reactor granules (Table D.4); similarly, every dominant bacterial group within the reactor granules had low relative abundance in the seed. The common OTU between the seed culture and the reactor granules was an uncharacterized member the *Ignavibacteriaceae*. The most abundant OTU in the seed culture was an uncharacterized *Sinobacteraceae* (13%), whereas the most abundant OTU in the reactor granules was an unclassified *Flavobacteriales* (6.0%).

A single OTU (OTU-1) comprised 26-38% of the mixed liquor, LAB and biomass < 75 μm, but only 0.29% of the swine waste and 0.24% of the HRSD seed culture. This OTU was also detected in the reactor granules but at a much lower, though still significant, relative abundance (Table D.3). Although abundant, this bacterial group belongs to an unknown phylum within the *Bacteria*.

6.4 Discussion

The quantification of anammox bacteria in a wastewater treatment facility has historically been done via 16S rRNA genes (*227*). However, when dealing with groups of phylogenetically diverse organisms it is often advantageous to employ a functional gene method utilizing a gene that is unique to the pathway of interest. There has been recent interest in using a functional gene approach to quantify anammox bacteria, typically using one of the subunits of hydrazine synthase (*218*, *228*, *229*), a unique gene in the anammox pathway (*91*). I used the functional gene approach in this study.

The majority of studies on anammox communities have utilized reactors treating synthetic media (*230–233*). There has been some prior research on the anammox community treating real waste (*88*, *170*) and specifically swine waste (*23*, *25*, *115*), but studies that have focused on anammox treatment of anaerobically digested swine waste are most relevant to the present study. Previous work on anammox bacteria in swine waste treatment systems has utilized PCR (*234*), clone libraries (*235*), denaturing gradient gel electrophoresis (*23*) or florescent *in situ* hybridization (FISH) (*23*, *25*, *115*) to characterize the microbial community.

Anammox bacteria are well-established to grow in granules, with reported diameters ranging from 0.2-2.4 mm (*68*, *110*, *112*, *114*, *115*). However, the impact of granule size on the microbial community is not well understood. The effect of granule size on the anammox

community has been explored in systems treating synthetic media (*218*, *236*, *237*) or reject water (*229*, *237*, *238*) using up to six size classes. These studies assessed the anammox communities by a combination of techniques, including construction of clone libraries (*236*), FISH (*237*, *238*), and qPCR (*218*, *229*). High-throughput sequencing techniques have been used to study anammox communities (*145*, *146*), but these systems were fed synthetic media and the sequencing was not coupled to size separation of the biomass. To our knowledge, no previous study has investigated an anammox community treating anaerobically digested swine waste by both qPCR and high-throughput sequencing nor the effect of granule size on the anammox community by high-throughput sequencing.

6.4.1 Anammox Bacteria

Sequences of known anammox species were primarily observed in libraries generated from the solids retained in the sieve column (Table 6.3); this is consistent with lack of detection of the *hzsA* gene in the material that passed through the sieves (Figure 6.1). Due to the typical growth of anammox bacteria as granules or in biofilms (*239*), this was not unexpected. Of significant interest was the shift in the dominant anammox organism from *Candidatus Brocadia sp.* in the seed culture to *Candidatus Kuenenia sp.* in the reactor at the end of the study, indicating the latter was better suited to the conditions in the reactor. Population shifts in anammox systems have been observed before (*240*), and understanding the conditions that select for dominant species is key to selecting an appropriate seed culture. Utilizing a seed that is enriched in the necessary microbes could significantly shorten reactor startup time, especially when dealing with very slow-growing bacteria.

Prior work on this reactor demonstrated that the concentration of nitrite varied between 15 and 30 mg NO_2^--N L^{-1} (Chapter 5), far below the inhibitory range typically reported for

anammox systems (*70*, *92–95*). The concentration of nitrite at the HRSD plant from which the seed culture was obtained is typically < 1 mg NO_2^-N L^{-1} (*208*). Though the reactor in the present study and the system located at HRSD both operated below inhibitory levels of nitrite, it is possible that the differences in nitrite concentration could have influenced anammox kinetics and, therefore, the competitiveness of the dominant anammox species in each system.

The concentration of ammonium in our system was 110-300 mg NH_4^+-N L^{-1} at the start of a cycle and 10-210 mg NH_4^+-N L^{-1} in the effluent (Chapter 5). Based on operating pH (7.1-7.3), free ammonia accounted for ~1% of total ammonium. The concentration of the ionized and non-ionized ammonia species is far below what has previously been reported as inhibitory of the anammox process (*96*). Therefore, ammonium inhibition is unlikely to have played a significant role in selecting the dominant anammox organism of the system.

Recently, various metals have been identified as inhibitory of the anammox process, including iron, copper, zinc, cadmium, and nickel (*173*, *241*, *242*). The swine waste treated in this study contained iron, copper, and zinc in the inhibitory range (Table D.5). It is important to note, however, that studies on the inhibitory effects of heavy metals have all involved communities dominated by *Candidatus Brocadia*. The effect of metals on *Candidatus Kuenenia sp.* is unknown; differences in tolerance to high concentrations of toxic heavy metals might explain the observed shift in the dominant anammox genus between the seed culture and the mature biomass in the reactor.

The doubling time of various anammox species is generally considered to be quite long (~11-14 days) (*67*), likely due to the low energy-yielding reaction and autotrophic nature of the bacteria involved. However, recently both *Candidatus Kuenenia* (*122*) and *Candidatus*

Brocadia (*243*) have been shown to utilize volatile fatty acids (VFAs) as electron donors. Although I did not measure VFA concentrations in the digested swine waste, I assume that much of the residual biodegradable organic matter comprised VFAs. Though the oxidation of organics in this system was likely to occur primarily aerobically (Chapter 5) it is possible that mixotrophic growth contributed to the community composition. The effect of mixotrophic growth on the maximum growth rate of anammox bacteria is unknown, but could provide a competitive advantage of one anammox genus over others.

Recently, a new genus within the family *Planctomycetaceae* was discovered to be capable of anaerobic ammonium oxidation and named *Candidatus Anammoximicrobium moscowii* (*244*). This was the first time a genus outside the *Brocadiaceae* was identified as performing anammox. In the present study, OTU-22 was identified as a *Phycisphaerales* within the *Planctomycetes* but clustered separately from both the *Brocadiaceae* and the *Planctomycetaceae* (Figure 6.3). Sequences similar to OTU-22 have previously been associated with coastal hot springs (*245*) and artificial microbialites (*246*) but never anammox systems. It should be noted that it is typically difficult to correlate function of a given microbe with taxonomic identity, but observing a second *Planctomycete* associated with granules performing anammox is an interesting finding.

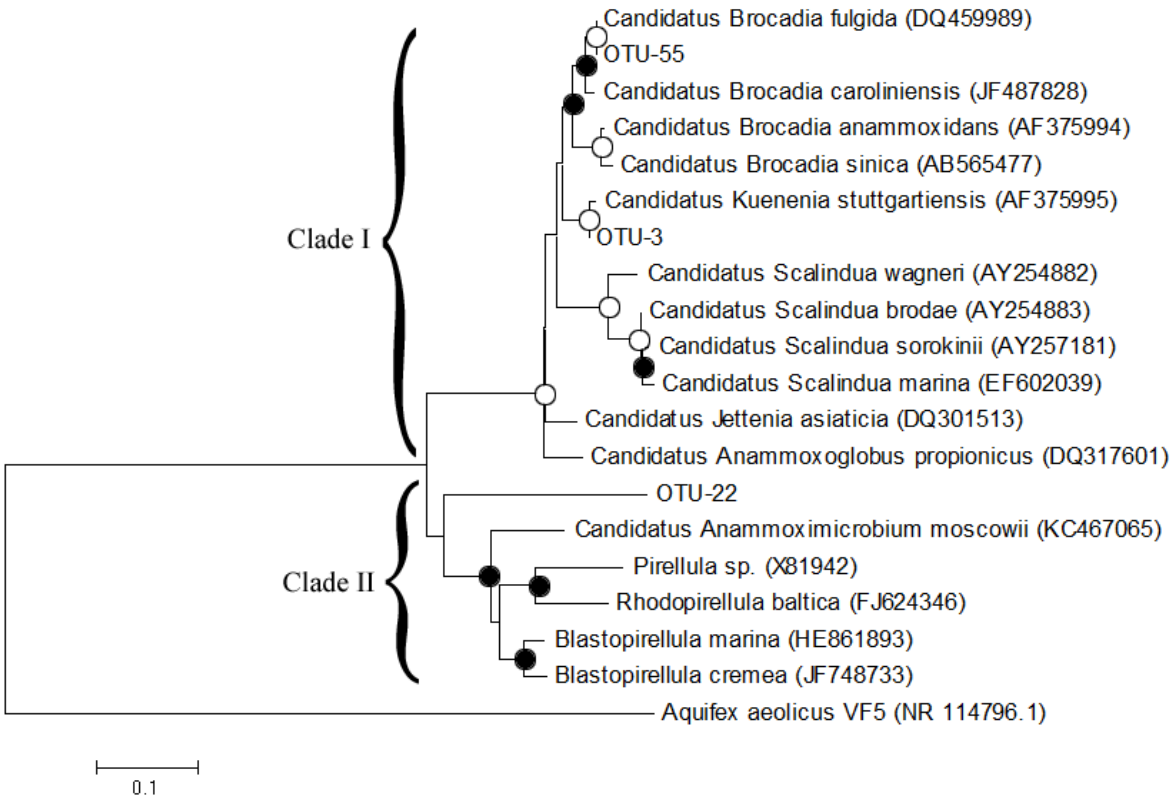

Figure 6.3: Neighbor-joining tree showing relationship of *Planctomycetes* OTUs from this study to known *Planctomycetes*. Clade I represents known species of anammox bacteria whereas Clade II is known to not perform anammox, with the exception of *Candidatus Anammoximicrobium moscowii*. The anammox ability of OTU-22 is unknown. *Closed* and *open* circles on nodes indicate ≥ 50% and ≥ 95% bootstrap values, respectively. GenBank accession numbers are in parentheses. The tree used *Aquifex aeolicus*, the deepest branching known bacterium, as an outgroup. OTU-3 and OTU-22 were primarily associated with the reactor granules; OTU-55 was primarily associated with the seed culture from HRSD.

6.4.2 Other Bacterial Species

Sequences related to *Nitrosomonas,* a genus of AOB, had surprisingly low relative abundance in all samples (Table 6.3). Although ammonium oxidation was a critical function of the bioreactor and the concentration of ammonium in the waste was high (Chapter 5), the energy yield from ammonium would have been much lower than that from the oxidation of organic matter in the waste; therefore, I expected that AOB would be a small fraction of the reactor biomass but I did not quantify them. Known ammonium-oxidizing archaea

(*Nitrosoarchaeum limnia* and *Nitrososphaera sp.; AOA*) amplify with the sequencing primers used in this study but were not detected in the sequence libraries.

Sequences associated with an uncharacterized *Bacteria* (OTU-1) accounted for 38% of the sieve column flow-through and most closely clustered with OTU-6 and OTU-9 (87-89% similarity; Figure D.1). Both the closely related relatives were identified as *Ignavibacteriaceae* within the *Chlorobi* (Table D.4). OTU-1 was ~90% similar to the closest cultured relative in the NCBI database, *Ignavibacterium album*. The most closely related sequences in the NCBI database are 93-94% similar to the representative sequence of OTU-1 and are typically found in wastewater systems, specifically those enriched in denitrifying bacteria (*247*, *248*). The relative abundance of this suspected denitrifier could explain the high gene copy number of *nosZ* (Figure 6.1). However, it is important to note that denitrifiers are a broad group of facultative microbes typically capable of growing under both aerobic and anoxic conditions. Presence of the denitrifying functional gene speaks only to the genetic potential of denitrification and does not necessarily indicate the contribution of this process to N removal.

6.5 Acknowledgements

The authors thank David Singleton, Jill Stewart, and Ann Matthysse for their assistance and insight into data analysis and troubleshooting.

Chapter 7: Conclusions and Recommendations

The overall objective of this dissertation was to explore coupling nitrogen removal to anaerobic digestion for treatment of swine waste. Performing N removal on swine waste that has already been subject to anaerobic digestion maximizes the amount of organic matter used for biogas production (and, as such, energy recovery) while allowing for removal of a significant fraction of nitrogen associated with the waste. Special attention was paid to stoichiometric parameters associated with the various pathways of N removal, with emphasis on the oxygen consumption and alkalinity demand as these represent potentially significant costs associated with full-scale treatment. Overall, this work has shown that N removal from anaerobically digested swine waste is technologically feasible at this time via two separate pathways. Additionally, it has been shown that the readily biodegradable fraction of COD is significantly smaller than total measured COD. Further conclusions and recommendations for each manuscript chapter are outlined below:

7.1 Nitrification/Denitrification of Anaerobically Digested Swine Waste

7.1.1 Conclusions

A conventional nitrification/denitrification system demonstrated very high (98%) ammonium removal and substantial removal (85%) of total nitrogen. Total COD in the digester effluent exhibited significant seasonal variation. Correspondingly, the extent of TN removal in a full-scale system is expected to vary seasonally, with the TN removal in the pilot-scale system corresponding to a “best-case” scenario. The extent of denitrification also affects the need for supplemental alkalinity in the nitrification process; lower COD leads to a

lower extent of denitrification which in turn necessitates increased alkalinity addition. No significant consumption of COD was associated with the aerobic process, which indicates that the vast majority of biodegradable COD in the digester effluent is amenable to denitrification. Nitrite accumulated in lieu of nitrate as a nitrification end-product, though it is unknown if this observation is an inherent property of anaerobically digested swine waste or an artifact of operational problems. Nitrous oxide emissions associated with N removal were primarily associated with the nitrification process and accounted for 8.2% of removed N. Parallel work demonstrated this system might be cost-prohibitive at full scale, although a significant uncertainty in the cost-benefit analysis is the requirement for addition of an external source of alkalinity (*8*).

7.1.2 Recommendations

Issues associated with the pilot-scale nitrification/denitrification system centered around the difficulty of measuring and controlling the reactor volumes and the abundance of foam produced in the nitrification reactor. Both these issues could be resolved by relying on an overflow system for level control which would also allow accumulated foam to exit the system. However, with the development of a nitritation/anammox system (Chapter 5) and potential cost savings, future work should focus on better understanding of anammox applied to anaerobically digested swine waste and bringing the technology to full-scale.

7.2 Nitration/Anammox Treatment of Anaerobically Digested Swine Waste

7.2.1 Conclusions

A single-reactor nitritation/anammox system can achieve very high ammonium (99%) and total-N (93%) removal. The oxygen demand associated with nitritation/anammox was significantly lower (~50%) than a nitrification/denitrification system, representing a potentially significant savings. Additionally, no external alkalinity was required at any point

throughout the duration of operation, demonstrating further savings as compared to a system that employs conventional nitrification/denitrification as a primary N removal mechanism. Heterotrophic denitrification accounted for a maximum of 30% of N removal, though the exact contribution of denitrification under standard operating conditions is unknown.

Oxygen limitation was able to control the activity of AOB and was likely one of the conditions that led to inhibition and washout of NOB. COD in a one-reactor nitration/anammox system was mainly degraded via an aerobic pathway, though low nitrate in the effluent suggests contribution of at least partial denitrification. Production of nitrous oxide accounted for ~10% of removed N and is likely the result of a side-product of nitrification, though the exact pathway of nitrous oxide production is unknown.

7.2.2 Recommendations

The nitration/anammox system experienced several issues related to design and operational challenges inherent in bench-scale systems. At several points in reactor life, significant information could have been gained by observing the contents of the reactor and as such I recommend future work is done with clear vessels (e.g. acrylic) with a darkening cover to prevent the growth of phototrophic organisms rather than inherently opaque reactors (e.g. stainless steel).

There were also significant challenges associated with the provision of oxygen for the aerobic part of the process. Gas transfer is a function of bubble size, and gas-liquid contact time. The diffusor used in this study had perforations 1.6 mm in diameter; using a diffusor with smaller holes would result in smaller bubbles translating to increased oxygen transfer. Commercially available ceramic air diffusers (e.g. aquarium airstones) meet this criterion and have been previously used in anaerobically digested swine waste (*129*). These diffusors are

prone to become brittle with age and should be inspected regularly. However, the use of a clear reactor should make inspection relatively straight forward. A biofilm will grow on this diffusor as it did on the perforated-rubber diffuser used in this study, but the small surface area should make biofilm contribution negligible. Additionally, I recommend installing diffusors below the plane of the impeller used for mixing; this creates a downward force on the bubbles and maximizes gas-liquid contact time as well as disrupts large bubbles and increase available surface area for mass transfer.

Additionally, larger-scale reactors with an increased liquid depth can further increase gas-liquid contact time as the bubble moves through the water column which will aid in mass transfer. Significant information could be gleaned from larger pilot-scale systems that use conventional full-scale equipment with well-characterized performance (e.g. diffusors, mixers, etc.).

The biological component of bioreactors adds significant complexity to the system. Future studies should fully assess physical performance of the reactor prior to addition of the biological seed culture. This allows for the use of coloured dyes (e.g. congo red) to be used as a tracer rather than chemical species (e.g. nitrate) such as was used in this study. Oxygen transfer must be assessed in the wastewater due to the effect of salts, particulates, etc on gas transfer.

Growth of biofilm is a known problem in small-scale systems due to the large surface area to volume ratio. Significant biofilm accumulation was observed on reactor walls and baffles but not on the moving biofilm support media. Lack of biofilm growth on the media may have been a result of the high mixing intensity used in the reactor. Future studies should

explore fixed biofilm support media to maximize available surface area for biofilm growth. Additional rationale for focusing on biofilm-based systems is provided in section 7.3.2.

7.3 Microbial Community Analysis of Nitritation/Anammox Biomass Treating Anaerobically Digested Swine Waste.

7.3.1 Conclusions

This work included the first investigation of size-fractionated nitritation/anammox biomass treating real waste by high-throughput sequencing techniques. The biomass retained by the screens accounted for ~4% of recovered solids. Sequences that correspond to known anammox organisms were only detected in aggregates retained by a 75 μm screen and were poorly represented in the aggregates not retained by the screens. Additionally, a shift in the dominant anammox genus was observed from *Candidatus Brocadia* in the seed culture to *Candidatus Kuenenia* in the reactor by the end of the study. Sequences associated with a second uncultured *Planctomycete* were also observed associated with the granules though it is unknown if this *Planctomycete* performs anammox.

NOB were successfully repressed and washed out of the reactor indicating nitrite removal was achieved only via anaerobic pathways. Although aerobic ammonium oxidation was observed in the reactor, sequences associated with AOB were a relatively small component of the sequence libraries, likely due to the low energy yield from ammonium oxidation as compared to heterotrophic activity. Sequences associated with AOA were not observed.

7.3.2 Recommendations

The bacteria in this study eventually granulated and though the steps of granulation have been postulated (*249*), the underlying mechanism of granulation is yet to be determined. Without knowing the mechanism of granulation it is impossible to be certain that granulation can be maintained. Significant biofilm growth was observed on static reactor walls and

baffles but no biofilm was observed on the moving K1 media. Therefore, I suggest focusing further development of a static biofilm system using a biofilm supporting media such as the BioWeb from Entex Inc. (http://entexinc.com/solutions/bioweb).

Additionally, if a seed culture from a smaller-scale system treating the target waste is not available, I suggest seeding a reactor with anammox sludges from multiple sources where the dominant species of interest is known and different in each source. This should maximize the likelihood of inoculating with an abundance of bacteria that are suited to the environment in the reactor and lead to rapid start-up.

APPENDIX A: REACTOR AND BIOMASS PHOTOS

Figure A.1: The trailer housing the pilot system adjacent to a covered anaerobic lagoon at Butler Farms. Liquid from the lagoon was pumped into the trailer from an opening in the cover and effluent was discharged into a second opening located approximately 4.5 m away.

Figure A.2: Interior of the trailer showing the reactors (left) and laboratory section (right). The nitrification reactor is the larger yellow tank.

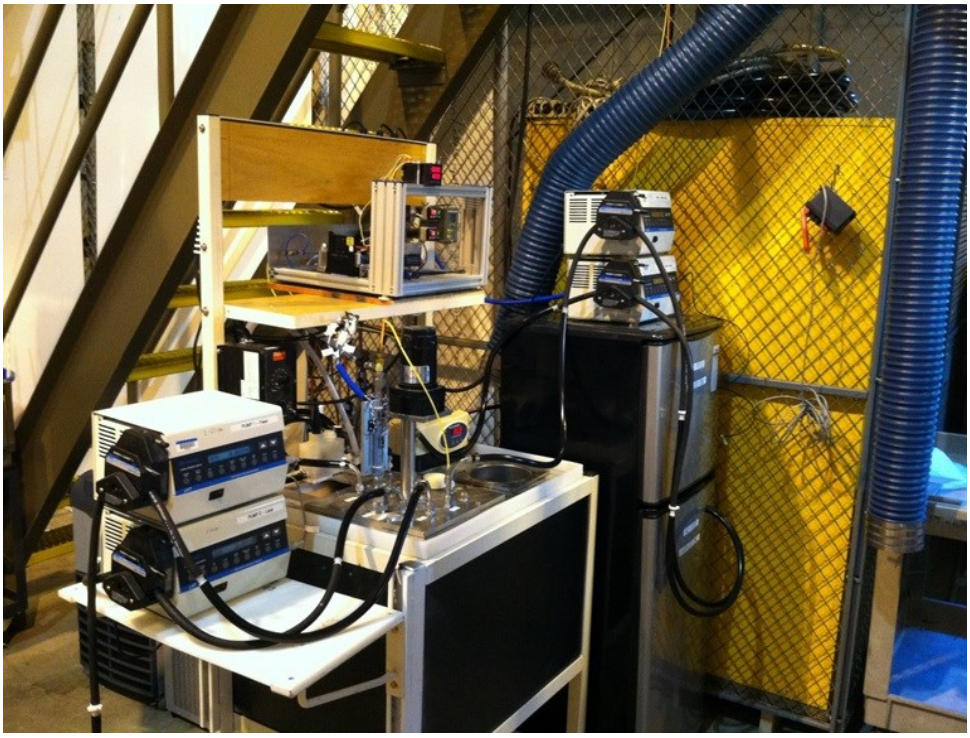

Figure A.3: The laboratory SBR for nitritation/anammox treatment of anaerobically digested swine waste.

Figure A.4: Anammox biofilm growing on reactor baffles. A similar biofilm was observed on reactor walls (photo not available).

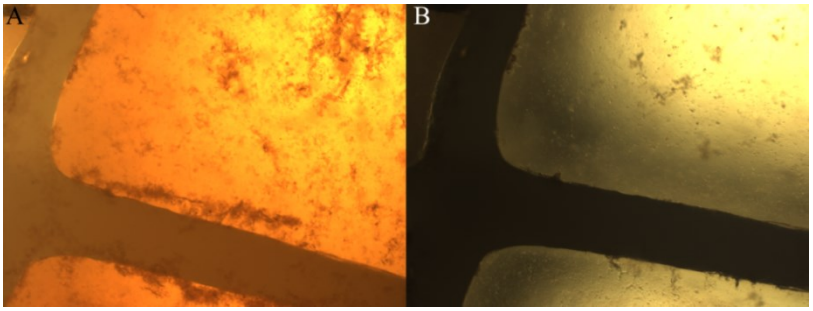

Figure A.5: Representative photo of the LAB associated with the plastic media. (A) unwashed; (B) washed.

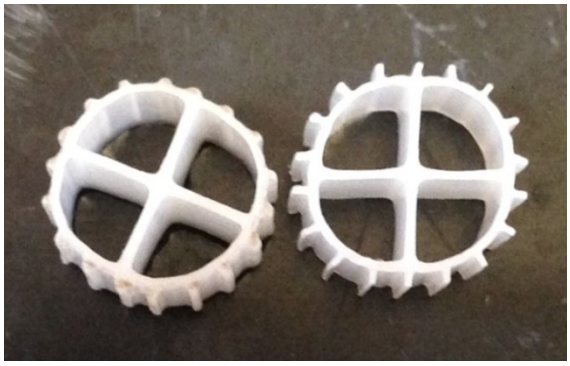
Figure A.6: Used (left) and new (right) K1 media.

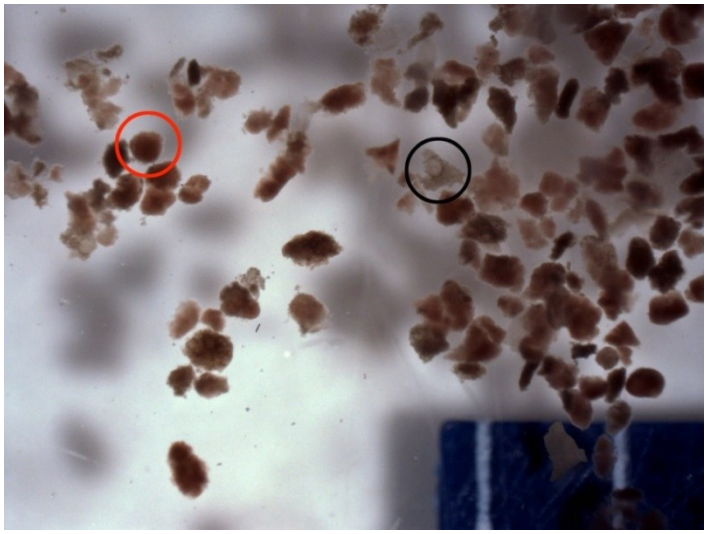
Figure A.7: Granules from the nitritation/anammox SBR. A typical red granule is highlighted by the red circle and a typical brown granule by the black circle. Distance between white marks in lower right =1 mm.

Figure A.8 Biofilm growing on impeller shaft in the SBR above the liquid surface.

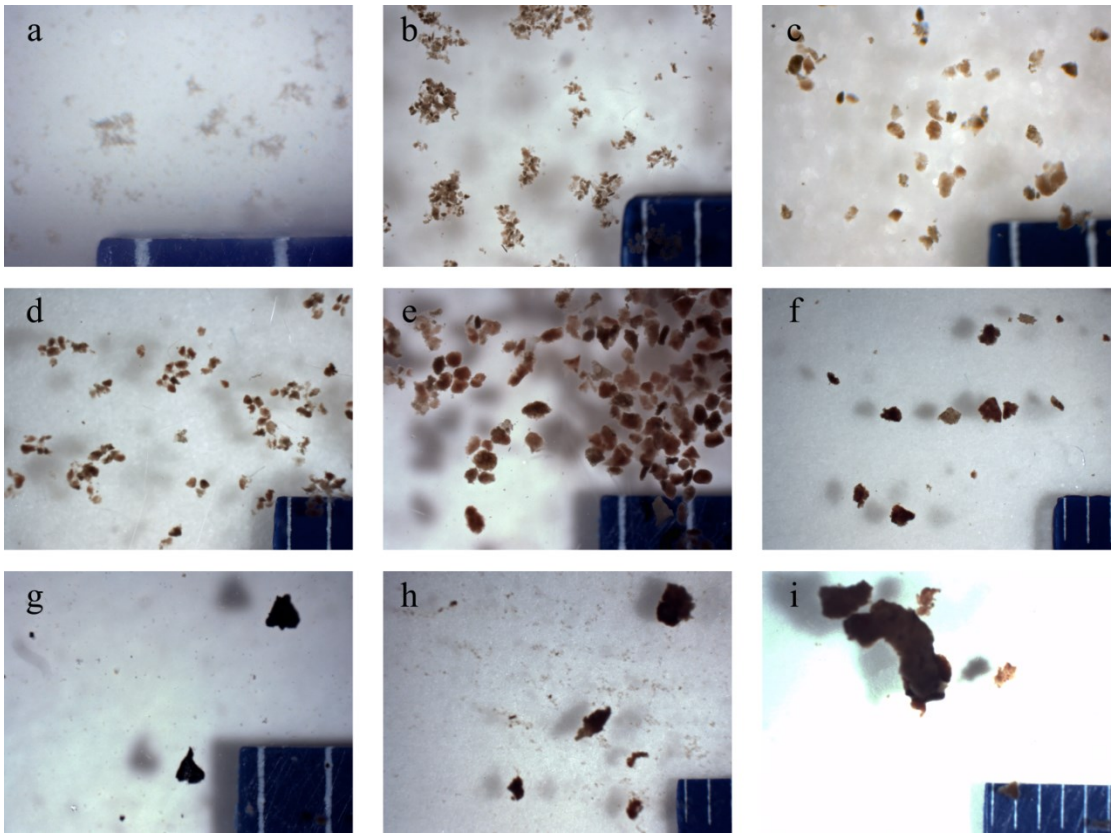

Figure A.9: Representative photos of size fractionated biomass from the nitritation/anammox SBR. (a) <75 µm, (b) 75-125 µm, (c) 125-177 µm, (d) 177-250 µm, (e) 250-500 µm, (f) 500-850 µm, (g) 850 µm – 1.00 mm, (h) 1.00-2.00 mm, (i) 2.00-3.50 mm

APPENDIX B: DETAILS OF SBR PHYSICAL DESIGN AND OPERATION

B.1 Physical Design

The laboratory-scale nitritation/anammox reactor is described in the text (Section 5.2.3). Tubing for influent, effluent and the recirculation loop for pH and DO monitoring was 0.95-cm ID SS connected above the reactor headplate to food-grade PVC, which in turn was connected to Norprene® tubing near the pump head for each pump. The vertical placements of the tubing attached to the reactor headplate are summarized in Table B.1. The indicated placement is the discharge or intake point of the indicated tube.

Table B.1: Placement of SBR tubing components.

Tube	Placement from bottom of headplate (cm)
Influent	0 cm
pH/DO loop draw	27.3 cm
pH/DO loop return	0 cm
Thermistor well	23.5 cm
Effluent	14.0 cm
O_2 diffuser	28.0 cm

The mixer for the reactor comprised two impellers located at different depths. The lower impeller consisted of two blades made of 0.8 mm-thick SS, 1.9 cm wide, 12.2 cm long at a pitch ~30° CCW off horizontal. The top of the impeller was located 30.5 cm from the bottom of the reactor headplate (~10 cm from the bottom of the reactor vessel). Impeller rotation was counterclockwise at ~135 rpm. The upper impeller consisted of two blades made of 0.8 mm-thick SS, 2.5 cm wide, 6.4 cm long, with a total length of 15.2 cm; the blades were oriented perpendicular to the liquid surface. The upper impeller had 9 holes (28 mm diameter each) arranged in a 3x3 parallelogram, 1.3 cm between centers and aligned 40° off vertical.

B.2 Operation

Cumulative mass of ammonium-N loaded to and discharged from the laboratory SBR for the duration of reactor life from start-up to autopsy is shown in Figure B.1. On day -390 biofilm (Figure A.4) was physically removed from the reactor walls resulting in the rapid accumulation of effluent ammonium (Figure B.2). To promote AOB activity, on day -379 the air flow was adjusted to 0.167 min min^{-1} (5 min air flow; 25 min no flow) resulting in rapid accumulation of nitrite. Anaerobic incubation begun (day -376) to consume excess nitrite. After compete consumption of reactor nitrite, aerobic activity was resumed (day -363) and a rapid increase in effluent nitrite observed.

Air flow was reduced to 0.01 (15 seconds of flow; 25 min no flow) on day -358 to promote anammox activity and reduce the relative contribution of AOB but due to the continued high concentration of nitrite in the reactor the air pulse was eventually terminated and the reactor incubated anaerobically (day -349). The reactor operated under high ammonium, high nitrite conditions for 25 days with little to no ammonium consumption; fresh granular anammox sludge was added and aerobic pulsing resumed on day -324. Ammonium decreased and feeding was resumed on day -308.

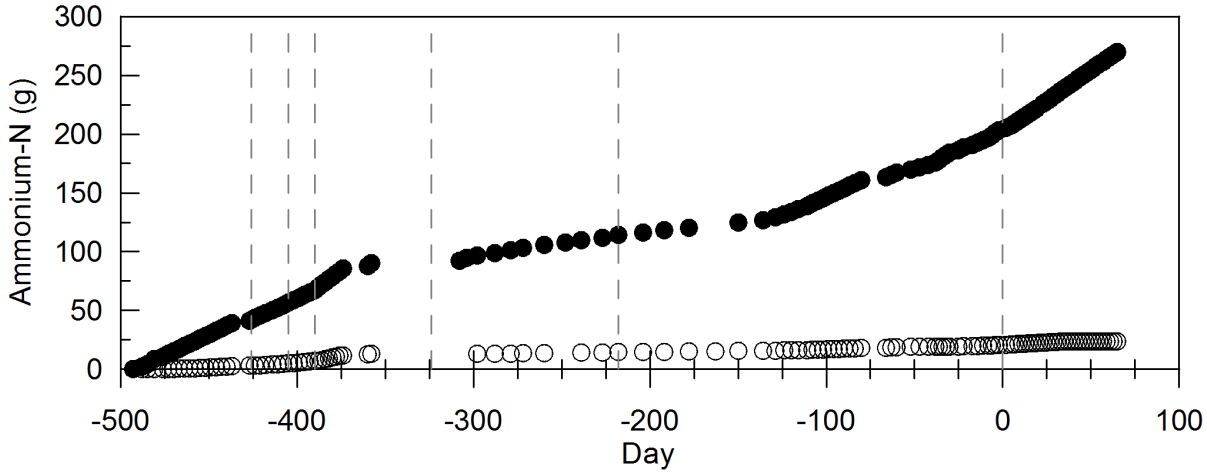

Figure B.1: Cumulative mass loading and discharge for the entire duration of SBR life. Important events are denoted by dashed grey lines. Data is cumulative mass loaded (●) or discharged (○).

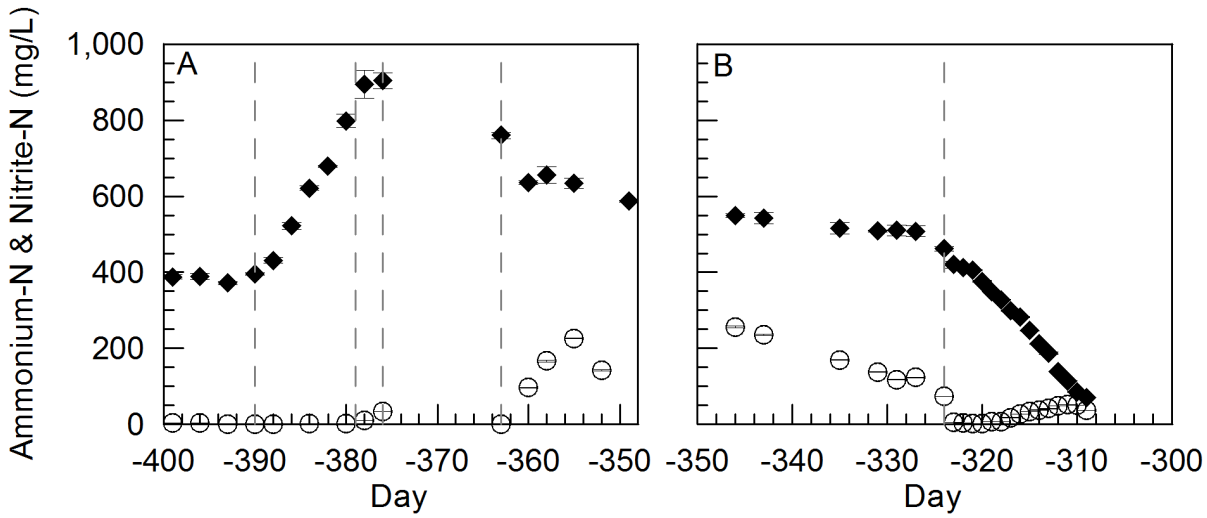

Figure B.2: Concentration profile of effluent (A) and internal (B) ammonium-N (◆) and nitrite-N (●) for select operating conditions. Dashed lines denote important events and are referred to by day number in the text (Appendix B.2). Data are means and standard deviations of triplicates; some error bars are smaller than the symbol size.

APPENDIX C: SBR CHARACTERIZATION

C.1 Tracer Study

A tracer study using nitrate was employed to confirm complete mixing of the liquid phase after feeding. Because the influent to the reactor was normally delivered at 4°C and the internal recirculation loop recirculated the reactor contents at 35°C, nitrate solutions at both 4°C and 35°C were used. Stock solution (1 L; 1.6 g KNO_3 L^{-1}) was pumped through the feed line from a pre-rinsed 2-L graduated cylinder. Samples were collected after 0, 10, 20, 30, and 45 min of mixing. Results are provided in Figure C.1. Complete mixing of the tracer simulating the feed temperature was virtually instantaneous, and complete mixing of the tracer simulating internal recirculation occurred within 10 min.

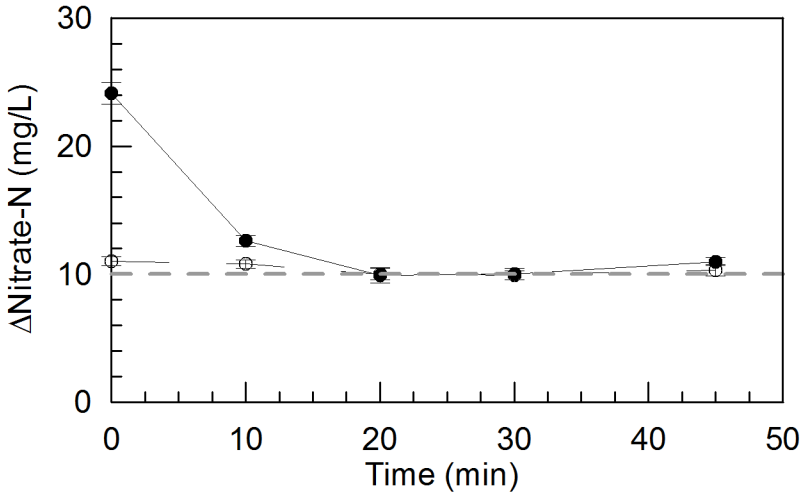

Figure C.1: Concentrations of nitrate above background levels as a function of time in tracer studies. Warm (35 °C) tracer is shown by filled circles and cold (4 °C) tracer by open circles. The dashed grey line represents the expected ΔNO_3^--N concentration resulting from instantaneous complete mixing. Data is concentration of nitrate above background levels and error bars are the standard deviation.

C.2 Oxygen Transfer

Due to the rapid nature of oxygen pulsing, less oxygen was provided per pulse than predicted by the duration of each pulse (6 s) and flow rate (1.0 L min^{-1}). Oxygen supply per pulse was estimated by integrating the manually recorded flow rate over time (Figure C.2).

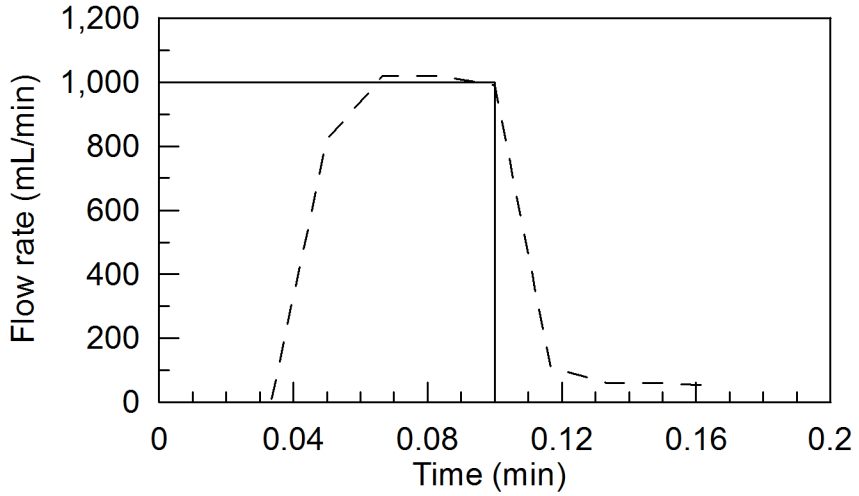

Figure C.2: Programmed (solid line) and measured (dashed line) oxygen flow. Integration of programed flow gives oxygen addition of 100 mL pulse^{-1}; actual flow ~69 mL pulse^{-1}.

Oxygen transfer efficiency of the diffuser was assessed by continuously bubbling air (24.7% O2) at 2.5 L min^{-1} and following DO in the reactor. The plug for the sampling port in the reactor headplate was opened to vent excess gas and maintain atmospheric pressure. After accumulation of DO, the headplate plug was replaced and air supply stopped. The DO was recorded manually and converted to total mass in the reactor (Figure C.3). Oxygen transfer efficiency was determined assuming gas mixing behaved ideally. The rate of DO consumption after the air flow was stopped was added to the rate of DO accumulation to obtain a total rate of oxygen transfer to the reactor during the period of air supply. The transfer efficiency was calculated as the mass rate of oxygen transferred to the reactor contents divided by the mass rate at which it was delivered.

From the data in Figure C.3, oxygen accumulated in the liquid phase at a rate of 2.45 mg O_2 min^{-1} and was consumed at a rate of 1.00 mg O_2 min^{-1}. From these results, the estimated oxygen transfer efficiency of the diffuser was 0.46 %.

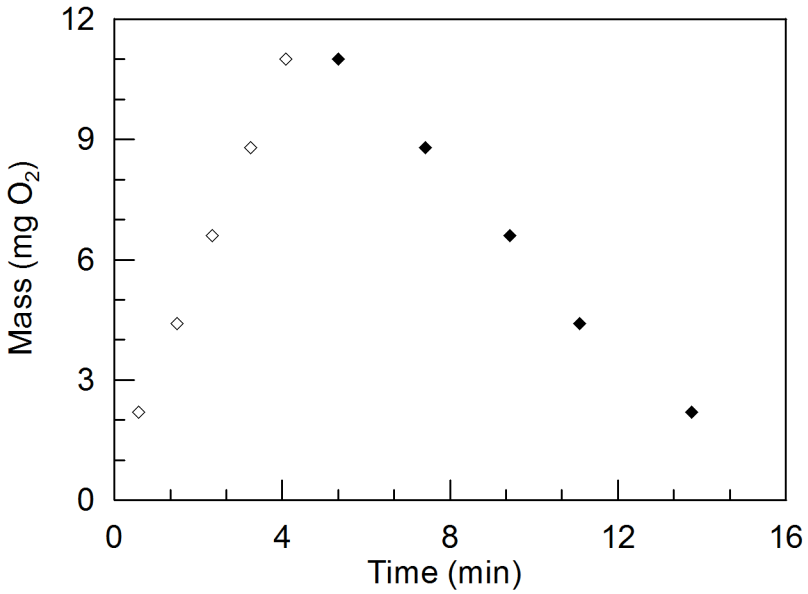

Figure C.3: Mass of DO in the reactor liquid phase during the period in which air (24.7% oxygen) was supplied (◇) at 2.5 L min^{-1} (794 mg O_2 min^{-1}) and after the air flow was stopped (◆).

Total oxygen loading was determined by the known volume of oxygen addition per pulse and the number of pulses per day. Total oxygen discharge was determined by the measured average composition and flowrate (Section 5.2.7 Offgas composition and flow). Net oxygen consumption was determined as the difference in loading and discharge.

C.3 Effect of Mixing Intensity on N Removal

To evaluate the effect of mixing speed on oxygen transfer and, therefore, aerobic ammonium oxidation, an order-of-magnitude estimate of the relative contributions of anammox and nitritation to ammonium removal was determined assuming a 1:1 stoichiometry of both reactions:

$$r_{NH_4^+} = r_{NH_4^+,anammox} + r_{NH_4^+,AOB} \quad \text{(C.1)}$$

$$r_{NO_2^-} = r_{NO_2^- AOB} + r_{NO_2^-,anammox} \quad \text{(C.2)}$$

$$r_{NH_4^+,anammox} = r_{NO_2^-,anammox} \quad \text{(C.3)}$$

$$r_{NH_4^+,AOB} = -r_{NO_2^- AOB} \quad \text{(C.4)}$$

Where r is the rate of consumption and subscripts denote the species and reaction. Solving this system yields:

$$r_{NO_2^-,anammox} = \frac{r_{NH_4^+} + r_{NO_2^-}}{2} \quad \text{(C.5)}$$

$$r_{NH_4^+,AOB} = \frac{r_{NH_4^+} - r_{NO_2^-}}{2} \quad \text{(C.6)}$$

The net rates of ammonium (r_{NH4+}) and nitrite (r_{NO2-}) consumption were measured and substituted into Equations C.5 and C.6 to estimate the rates of anammox and nitritation, respectively. Results of the analysis are shown in Figure C.4, which clearly indicates a reduced rate of nitritation when the mixing intensity in the reactor was decreased.

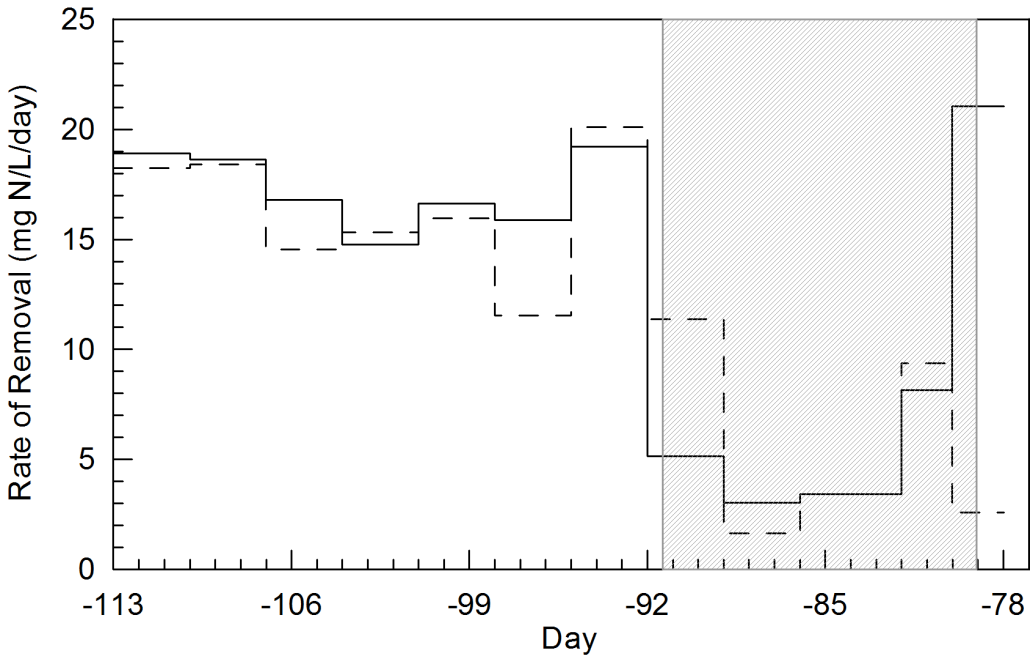

Figure C.4: Effect of mixing speed on rates of nitritation (solid line) and anammox (dashed line). The period of lower mixing speed (80 rpm, reduced from 135 rpm) is shown in the shaded region.

C.4 Anammox Stoichiometry

Lower and upper bounds for the stoichiometry of oxygen consumption to nitrogen removal in a combined nitritation/anammox system (shown below; equation C.9) were determined by the mass rate of oxygen consumption (Appendix C.2) divided by the mass rate

of nitrogen removal (upper bound) or subtracting COD removal from oxygen consumption and dividing by nitrogen removal (lower bound).

Net oxygen consumption can be used to estimate the anammox stoichiometry by combining equations C.7 and C.8 to produce C.9.

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad \text{(C.7)}$$

$$\underline{NH_4^+ + xNO_2^- \rightarrow yN_2 + zH_2O + aNO_3^-} \quad \text{(C.8)}$$

$$(1+x)NH_4^+ + 1.5xO_2 \rightarrow yN_2 + 2xH^+ + (x+z)H_2O + aNO_3^- \quad \text{(C.9)}$$

From the measured oxygen to nitrogen stoichiometry the anammox stoichiometry can be estimated.

C.5 Mixed Liquor Activity Shortly After Addition of Granules

A sub-sample of reactor mixed liquor was collected from the SBR recirculation line in triplicate 60-mL amber-glass serum bottles, plugged with butyl-rubber stoppers and sealed with aluminum crimp caps. Vacuum and nitrogen were alternatingly applied to the headspace three times, and the bottles were incubated anaerobically at 35°C and shaken at 250 rpm. Subsamples (1.5 mL) were collected at selected time points and filtered through GF/B glass fiber filters. After complete consumption of nitrite, additional nitrite (0.1 mL; 123 mg $NaNO_2$ mL^{-1}) was added with a syringe though the stopper. Results are shown in Figure C.5. Reactor data from this time period indicated anammox was happening in the reactor but could not be replicated *ex situ*. This result implies the reactor biofilm was important for N removal and biofilm carriers were added to increase biomass retention.

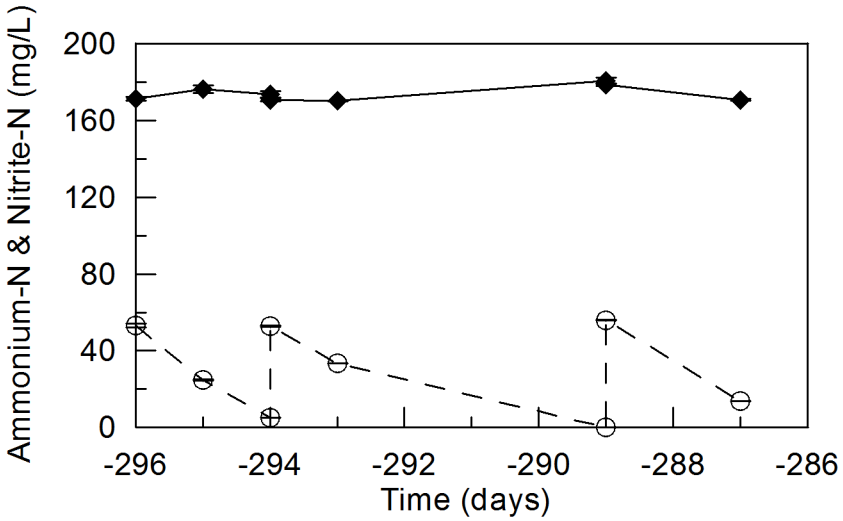

Figure C.5: *Ex situ* anammox activity assay performed in anaerobic incubations by following ammonium-N (◆) and nitrite-N (○). Increases in nitrite-N represent spiking of additional nitrite. Data are means and standard deviations of triplicates; some error bars are smaller than the symbol size.

C.6 *Ex situ* Anammox Kinetics

To determine a half-saturation constant for nitrite removal, the activity of the mixed liquor was tested in batch anaerobic incubations as described in Appendix C.5. The integrated Monod kinetic model was fitted, assuming constant biomass concentration. The estimated half-saturation coefficient for NO_2^--N was 2.7 mg L^{-1} (Figure C.6).

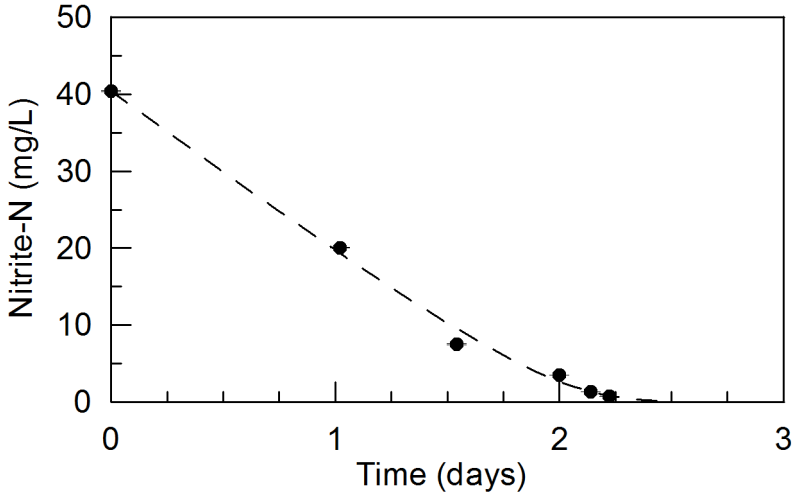

Figure C.6: Time course of the concentration of nitrite-N in a batch anaerobic incubation used to estimate the half-saturation coefficient. The fit of the integrated Monod model with constant biomass is shown as the dashed line. Data is measured concentration of nitrite-N; error bars are smaller than the symbols.

C.7 Effect of Increased COD Load

On day -10 (Figure C.7, grey line), a fresh batch of waste was fed with a higher COD content and effluent ammonium-N increased. On day 0 (Figure C.7, grey dashed line) the sparge gas was switched from air to pure oxygen resulting in stabilized and subsequent decrease effluent ammonium (Figure C.8; Appendix C.7).

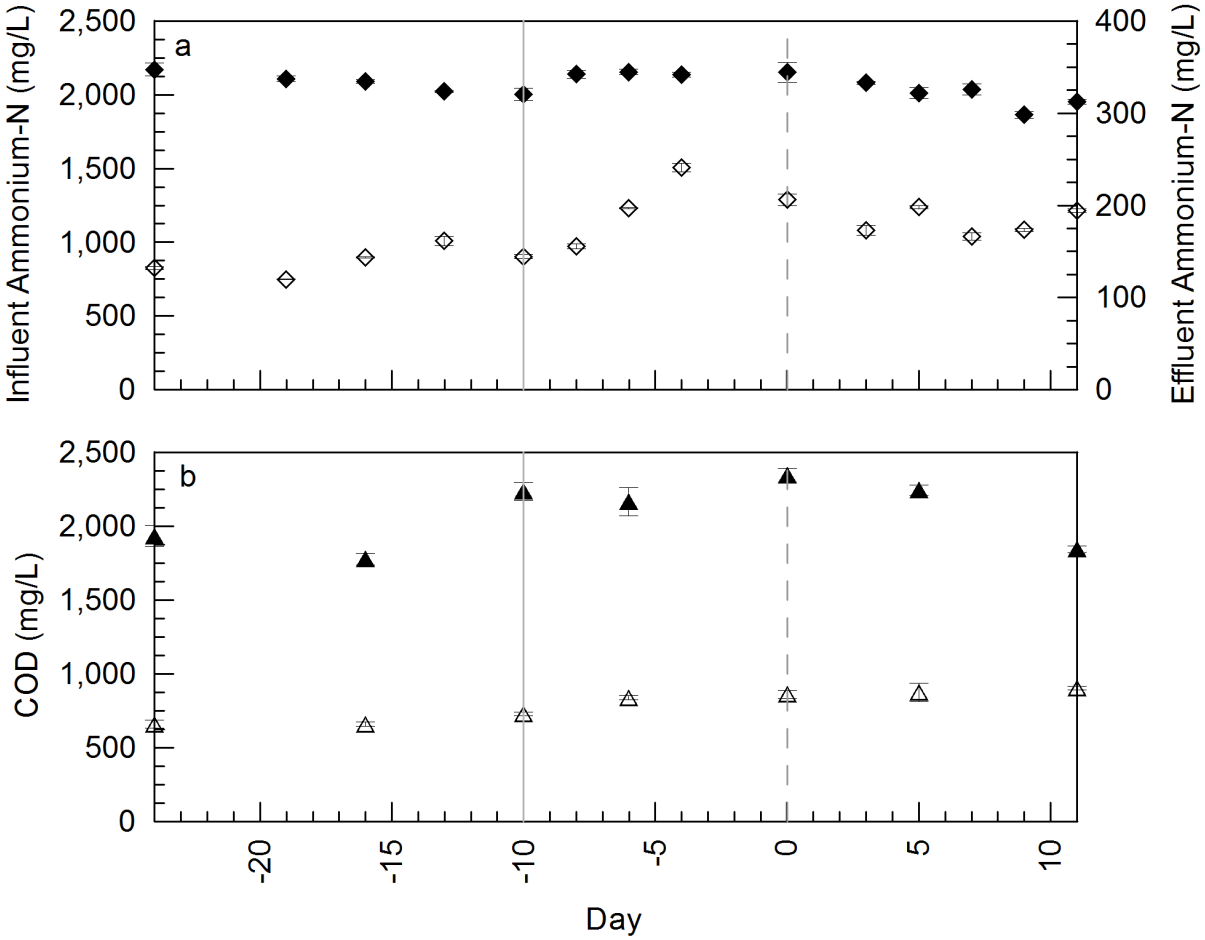

Figure C.7: Effect of increased COD load on effluent quality. Legend: diamonds – ammonium-N, triangles – COD, solid symbols – influent, hollow symbols – effluent. Note different y-axis for influent and effluent ammonium-N. Data are means and standard deviations of triplicates; some error bars are smaller than the symbol size.

C.7 Trends in Effluent Concentrations of Nitrogen Species

Concentrations of the inorganic N species and TN in the reactor effluent are shown in Figure C.8. Removal of total-M improved through the end of the study. Data for the final 25 days of operation are summarized in Tables C.1 and C.2.

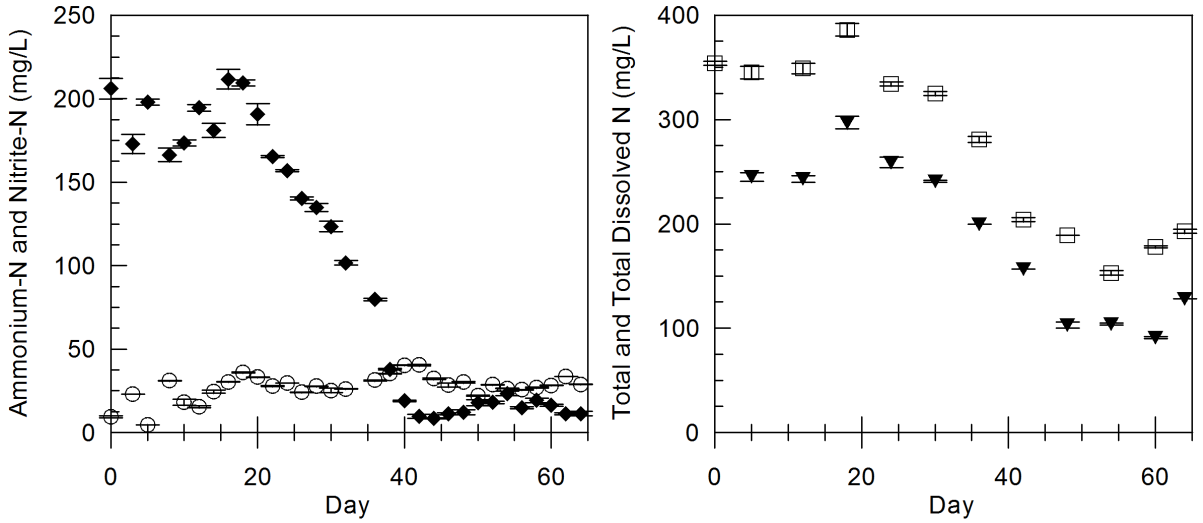

Figure C.8: Effluent concentration of ammonium-N (◆), nitrite-N (○), total-N (□), and total dissolved-N (▼). Data are measured concentration, error bars the standard deviation. Some error bars are smaller than the symbols.

Table C.1: Effluent quality from the SBR over the final 25 days of operation.

Parameter	Effluent [a]	
Nitrogen		
Ammonium-N	15.1 ± 4.6	(12)
Nitrite-N	30.3 ± 5.6	(12)
Nitrate-N	27.3 ± 4.5	(12)
Total Inorganic-N	72.7 ± 6.6	(12)
TDN	113 ± 26	(4)
TN	181 ± 19	(4)
COD		
Total	2,800 ± 140	(7)
Soluble	1,060 ± 30	(7)
Alkalinity		
Bicarbonate	1,710 ± 70	(12)
Total	2,210 ± 60	(12)

[a] Data represent means ± standard deviation. The number of samples analyzed is in parentheses. Units are mg L^{-1} except units for alkalinity (mg $CaCO_3$ L^{-1}).

Table C.2: Summary of loading and discharge rates over the final 25 days of operation.

	Loading [a]		Discharge		% Removal
Ammonium-N	52.1 ± 0.2	(1.00)	0.407 ± 0.013	(0.989)	99.2 ± 3.2
TDN	56.1 ± 1.4	(0.999)	2.42 ± 0.17	(0.985)	95.7 ± 7.2
TN	58.6 ± 1.6	(0.999)	3.93 ± 0.25	(0.988)	93.3 ± 6.4
tCOD [a]	122 ± 0	(1.00)	65.0 ± 0.9	(0.999)	46.9 ± 0.7
sCOD	66.7 ± 0.7	(0.999)	26.7 ± 0.2	(1.00)	59.9 ± 0.8
Alkalinity	246 ± 0	(1.00)	55.5 ± 0.2	(1.00)	77.4 ± 0.3

[a] Notes as in Table 5.3 of the text.

C.9 Offgas Ammonia

Reactor offgas was discharged through a commercially available aquarium airstone submerged in 0.2% boric acid. Captured ammonia from the gas phase was measured as liquid phase ammonium. Standards for analysis were prepared in 0.2% boric acid.

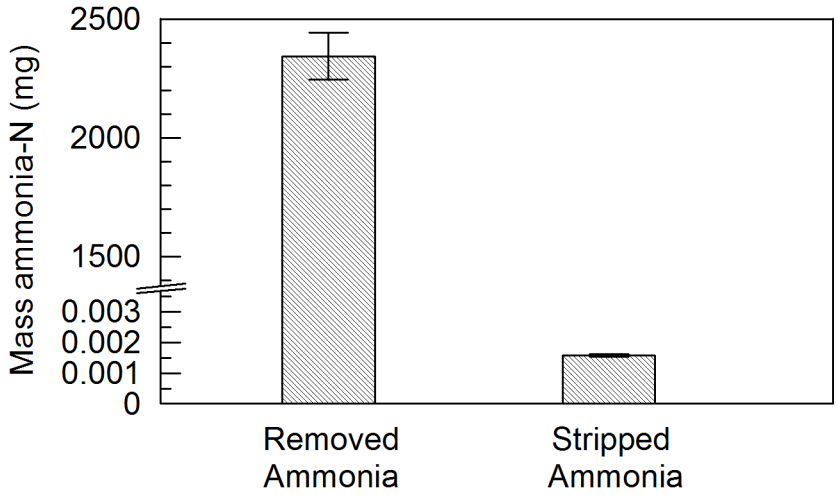

Figure C.9: Mass of ammonia captured in reactor offgas with 0.2% boric acid. Note break in the y-axis.

C.10 Biomass Features

Biomass in the reactor was present in three forms: flocculent, granular, and loosely associated biofilm (LAB) on the K1 media. The LAB could be separated easily from the K1 media by gently dipping the carriers in deionized water (Figure A.5); there was no other biofilm growth associated with the media after 284 days of operation (Figure A.6). The floc was a brown color and the granules were both brown and red (Figure A.7). The size of the granules shown in Figure A.7 is 250 – 500 μm.

The lack of biofilm growth on the K1 media is likely due to excessive mixing energy (110 kW m^{-3}, assuming an impeller Newton Number of 4). Mixing energy > 0.1 kW m^{-3} has previously been found to adversely impact anammox granules (*110*) and likely impacted biofilm development as well. Because oxygen transfer occurred primarily from the headspace

rather than from the diffuser, high mixing intensity was necessary to maximize oxygen transfer. Reduced mixing resulted in reduced N removal as a result of diminished nitritation due to poor oxygen transfer (Appendix C.3).

C.11 Estimating Effluent tCOD

Due to the presence of abraded plastic in the effluent from the nitritation/anammox SBR, the concentration of tCOD was over-estimated. In an effort to correct for the contribution of plastic COD to the measured value, the ratio of sCOD/tCOD consumption was used (Equation C.10). The values substituted into Equation C.10 are shown in Table C.3.

$$\frac{sCOD_{in}-sCOD_{out}}{tCOD_{in}-tCOD_{out}} = \frac{sCOD}{tCOD}_{consumed} \approx 0.7 \qquad \text{(C.10)}$$

Where:

$sCOD_{in}$ = Concentration of influent sCOD

$sCOD_{out}$ = Concentration of effluent sCOD

$tCOD_{in}$ = Concentration of influent tCOD

$tCOD_{out}$ = Concentration of effluent tCOD

Table C.3: Parameters for estimating $sCOD/tCOD_{consumed}$

Parameter	Mean	n
$sCOD_{in}$	2,440	17
$sCOD_{out}$	974	18
$tCOD_{in}$	4,610	17
$tCOD_{out}$ [a]	2,480	1

[a] Measured on sample that had been passed through a 75 μm screen

C.12 Concentration of sCOD During Intracycle Sampling

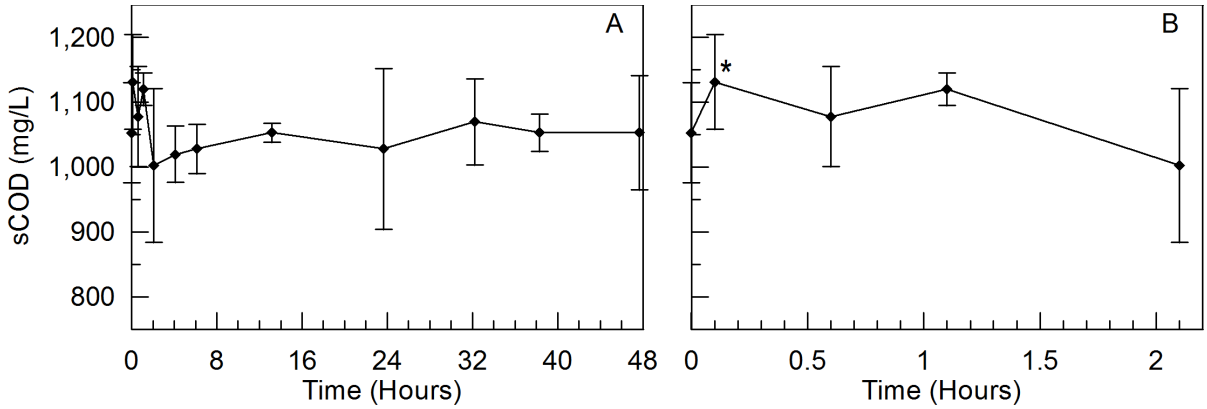

Figure C.10: Concentration profile of sCOD during intracycle analysis (Section 5.2.8). Caption as in Figure 5.3.

APPENDIX D: SOLIDS AND MICROBIAL COMMUNITY CHARACTERIZATION

D.1 Phylogeny of Abundant OTUs

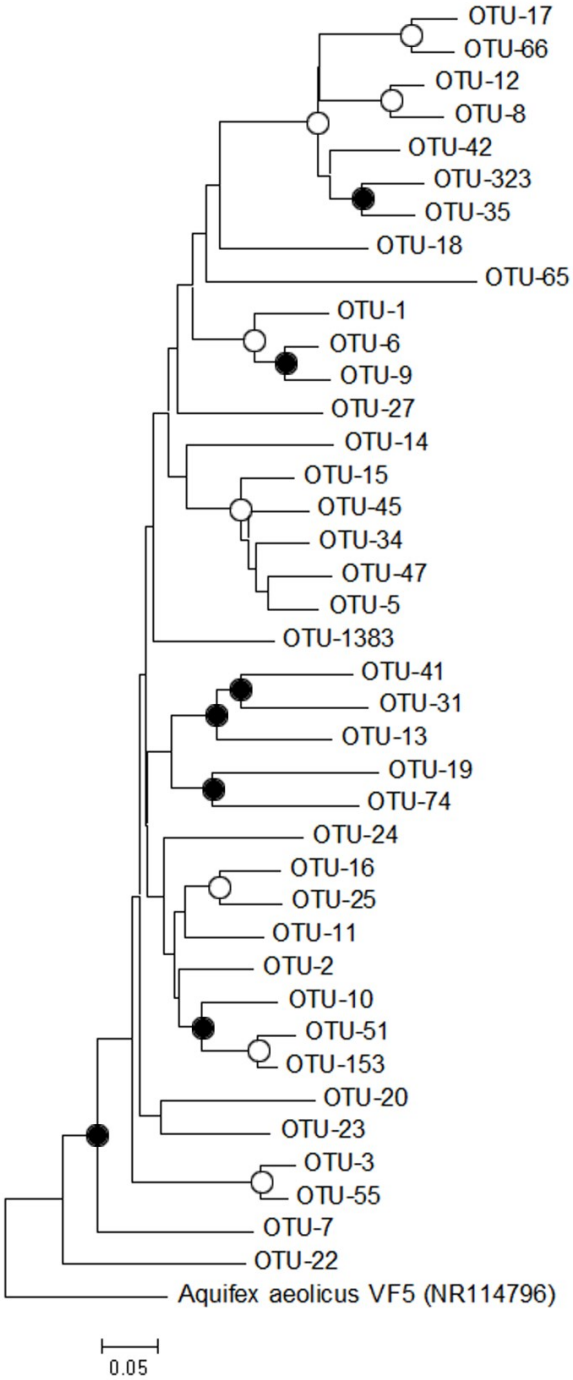

Figure D.1: Neighbor-joining tree showing relationship of abundant (≥2%) OTUs from the barcoded amplicon sequencing libraries. *Closed* and *open* circles on nodes indicate ≥ 50%

and ≥ 95% bootstrap values, respectively. GenBank accession numbers are in parentheses. The tree used *Aquifex aeolicus*, the deepest branching known bacterium, as an outgroup.

D.2 Solids Recovered from the Nitration/Anammox SBR

Table D.1: Mesh sizes used for separation of the reactor biomass.

Screen Opening Size	Tyler Standard Mesh Number
75 µm	200
125 µm	115
177 µm	80
250 µm	60
500 µm	32
850 µm	20
1.00 mm	18
2.00 mm	9
3.50 mm	6

Table D.2: Characterization of solids retained by each sieve.

Sample Name	TSS (g)[a]	VSS (g)	VSS/TSS	% of total[b]	DNA Yield (ng (mg VSS)$^{-1}$)
Mixed Liquor [c]	44.7 ± 0.915	31.4 ± 0.6	0.703 ± 0.005	N/A	443 ± 6
<75 µm	30.1 ± 1.442	19.4 ± 0.5	0.645 ± 0.036	96.4 ± 6.4	445 ± 0
75-125 µm	0.306 ± 0.002	0.274 ± 0.001	0.896 ± 0.003	0.981 ± 0.046	69.2 ± 0.0
125-177 µm	0.171 ± 0.001	0.142 ± 0.002	0.831 ± 0.018	0.549 ± 0.026	59.6 ± 0.2
177-250 µm	0.243 ± 0.031	0.194 ± 0.030	0.799 ± 0.025	0.778 ± 0.106	54.1 ± 4.4
250-500 µm	0.218 ± 0.015	0.181 ± 0.014	0.830 ± 0.015	0.700 ± 0.058	89.0 ± 1.4
500-850 µm	0.084 ± 0.009	0.059 ± 0.005	0.694 ± 0.025	0.271 ± 0.030	97.8 ± 0.3
850 µm-1.00 mm	0.032 ± 0.001	0.010 ± 0.001	0.327 ± 0.042	0.103 ± 0.005	35.6 ± 0.7
1.00-2.00 mm	0.035 ± 0.009	0.018 ± 0.008	0.493 ± 0.087	0.113 ± 0.029	130 ± 40
2.00-3.50 mm	0.031 ± 0.001	0.015 ± 0.140	0.491 ± 0.021	0.099 ± 0.006	402 ± 9
Sum[d]	31.2 ± 1.443	20.3 ± 0.5	0.650 ± 0.035	69.8 ± 3.5[e]	N/A

[a] Data represent mean ± standard deviation of triplicate measurements. N/A, not applicable
[b] Percent of TSS recovered.
[c] Sample collected before sieving.
[d] Sum of solids in all size fractions.
[e] Percent of mixed liquor TSS recovered.

D.3 qPCR Product Melting Temperature

The melting temperature of the qPCR products were accessed by differentiating the fluorescence signal (measured in relative fluorescence units; RFU) with respect to temperature. The temperature corresponding to the maximum rate of change of the fluorescence signal corresponds to the melting temperature of the product. In the course of reactor operation the melting temperature of the *hzsA* qPCR product shifted from ~84°C to ~80°C (Figure D.2).

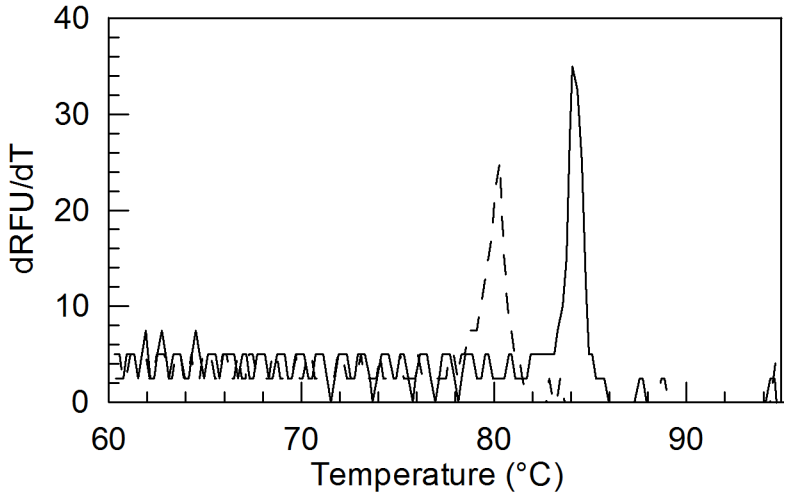

Figure D.2: Derivative melting curve for samples from the HRSD seed culture (solid line) and reactor biomass (dashed line).

D.4 Abundant Sequences Recovered from the SBR

Table D.3: Percent relative abundance of well-represented (>5%) phyla in the MiSeq libraries.

Phylum	Swine Waste	Mixed Liquor A	Mixed Liquor B	<75µm	Granules [a]	LAB [b]	Biofilm	HRSD [c]
Proteobacteria	7.10	50.3	26.4	25.4	32.7 ± 3.0	29.6	30.7	35.3
unclassified	7.38	27.6	39.6	40.1	5.14 ± 0.81	35.0	5.16	4.70
Firmicutes	30.3	3.55	4.35	5.01	3.41 ± 1.24	6.34	5.45	1.10
Bacteroidetes	23.2	7.52	12.6	11.9	12.8 ± 2.8	10.0	27.5	24.4
Chlorobi	0.66	1.19	1.69	1.74	12.6 ± 2.4	1.38	2.46	8.12
Chloroflexi	0.68	0.88	1.23	1.39	8.36 ± 2.84	1.76	3.34	3.27
Synergistetes	10.7	2.01	3.63	4.79	0.72 ± 0.18	4.12	1.58	0.32
Planctomycetes	0.46	0.59	0.93	0.81	8.98 ± 1.17	0.91	1.41	7.66
Verrucomicrobia	2.09	1.38	2.17	2.20	3.70 ± 0.75	2.18	6.73	3.38
Actinobacteria	0.81	1.26	1.73	1.58	2.39 ± 0.51	2.92	6.85	0.62
Acidobacteria	0.42	0.71	1.24	1.08	4.77 ± 0.63	1.12	3.59	2.93
Other	16.1	2.98	4.46	4.03	4.40 ± 0.40	4.74	5.30	8.220

[a] Mean ± Standard Deviation of combined fractions > 75 µm.
[b] LAB – Loosely Associated Biofilm
[c] HRSD – Hampton Roads Sanitation District

Table D.4: Percent relative abundance of well-represented (>2%) sequences in the MiSeq libraries [a]. Values ≥ 2% are in bold.

OTU	Taxonomy	Swine Waste	Mixed Liquor A	Mixed Liquor B	<75µm	Granules	LAB	Biofilm	HRSD
OTU-1	*Bacteria*	0.29	**25.8**	**37.5**	**37.8**	**2.57 ± 0.63**	**32.7**	**3.45**	0.24
OTU-2	*Alteromonadales*	0.14	**3.14**	**6.75**	**3.91**	**4.93 ± 0.90**	**6.59**	**7.28**	0.83
OTU-3	*Candidatus Kuenenia*	0.06	0.08	0.10	0.08	**5.17 ± 1.24**	0.07	0.07	0.31
OTU-5	*Betaproteobacteria*	0.11	1.25	1.88	**2.72**	**3.89 ± 1.19**	**3.62**	**3.02**	0.99
OTU-6	*Ignavibacteriaceae*	0.27	0.38	0.43	0.41	**4.28 ± 0.83**	0.37	0.54	**6.31**
OTU-7	*Verrucomicrobia*	0.13	0.82	1.43	1.12	**3.11 ± 0.64**	1.49	**5.91**	1.77
OTU-8	*Chitinophagaceae*	0.19	1.10	1.71	1.41	**2.20 ± 0.51**	1.44	**5.99**	0.26
OTU-9	*Ignavibacteriaceae*	0.11	0.36	0.52	0.45	**3.29 ± 0.45**	0.45	0.82	0.97
OTU-10	*Sinobacteraceae*	0.25	0.42	0.53	0.49	1.00 ± 0.11	0.81	1.03	**12.8**
OTU-11	*Dokdonella*	0.11	0.24	0.28	0.31	**2.03 ± 0.64**	0.33	0.43	1.40
OTU-12	*Chitinophagaceae*	0.13	0.82	1.26	0.95	1.50 ± 0.37	1.12	**5.80**	0.19
OTU-14	*Chlorobi*	0.10	0.18	0.31	0.39	**2.20 ± 0.54**	0.21	0.85	0.42
OTU-15	*Rhodocyclaceae*	0.04	0.97	1.44	**2.38**	1.05 ± 0.21	1.84	0.54	0.17
OTU-16	*Syntrophobacteraceae*	0.15	**4.21**	**7.09**	**7.50**	1.67 ± 0.39	**6.82**	1.00	0.15
OTU-17	*Bacteroidetes*	**8.16**	1.10	**2.05**	**2.38**	0.15 ± 0.04	1.67	0.29	0.33
OTU-18	*Chlorobi*	0.11	0.13	0.19	0.23	**2.72 ± 1.76**	0.15	0.19	0.13
OTU-19	*Dethiosulfovibrionaceae*	**4.20**	0.86	1.80	**2.29**	0.12 ± 0.04	1.84	0.16	0.03
OTU-20	*Acidobacteria*	0.08	0.29	0.66	0.46	1.33 ± 0.47	0.46	**2.49**	0.68
OTU-22	*Phycisphaerales*	0.03	0.14	0.26	0.18	**2.16 ± 0.43**	0.19	0.62	0.04

Table D.4: (continued)

OTU	Taxonomy	Swine Waste	Mixed Liquor A	Mixed Liquor B	<75µm	Granules	LAB	Biofilm	HRSD
OTU-25	*Syntrophobacteraceae*	0.12	0.28	0.25	0.19	**2.12 ± 0.79**	0.27	1.10	0.10
OTU-27	*Clostridium*	**2.89**	0.14	0.14	0.14	0.52 ± 0.53	0.39	0.59	0.05
OTU-34	*Nitrosomonas*	0.06	0.41	0.36	0.24	0.61 ± 0.17	0.45	**3.64**	0.84
OTU-35	*Bacteroidetes*	0.17	0.07	0.10	0.08	0.09 ± 0.02	0.07	0.13	**8.61**
OTU-42	*Bacteroidetes*	0.37	0.15	0.16	0.14	0.09 ± 0.02	0.12	0.15	**8.13**
OTU-45	*Comamonadaceae*	0.04	0.07	0.08	0.06	0.12 ± 0.03	0.08	0.09	**3.11**
OTU-47	*Methylotenera*	0.03	**12.7**	0.06	0.06	0.07 ± 0.02	0.06	0.07	0.07
OTU-51	*Methylococcaceae*	0.05	**17.1**	0.04	0.05	0.06 ± 0.02	0.05	0.06	0.07
OTU-55	*Candidatus Brocadia*	0.04	0.01	0.02	0.02	0.12 ± 0.03	0.01	0.01	**2.66**
OTU-66	*Bacteroidetes*	**2.00**	0.13	0.27	0.36	0.02 ± 0.01	0.18	0.05	0.12
OTU-74	*Synergistaceae*	**2.00**	0.10	0.17	0.27	0.03 ± 0.01	0.22	0.05	0.04
OTU-153	*Crenothrix*	-	**2.21**	0.01	0.01	0.01 ± 0.01	-	0.01	0.01
OTU-323	*Flavobacteriales*	0.26	1.56	**2.78**	**2.07**	**5.96 ± 1.45**	1.95	**9.75**	0.61

[a] Footnotes as in Table D.3.

D.5 PCoA Analysis of Size-Separated Fractions

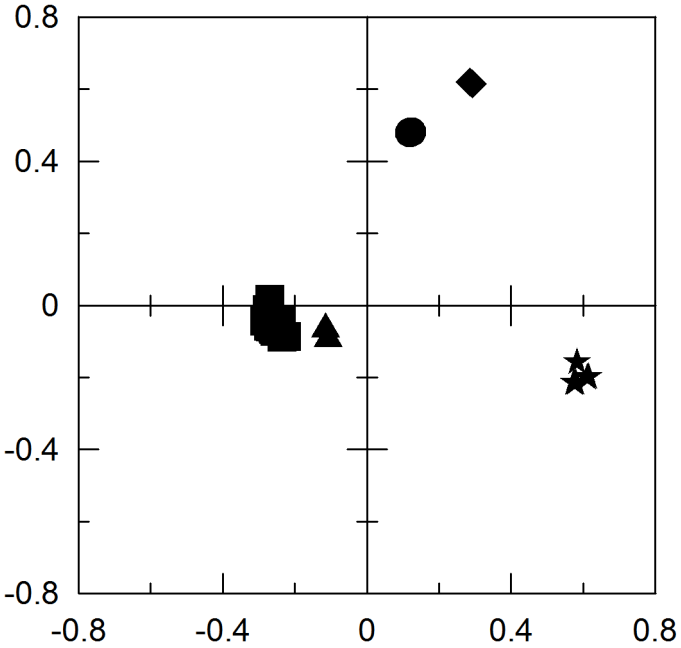

Figure D.3: Principal coordinate analysis (PCoA) of sequences libraries. All libraries were randomly reduced to the size of the smallest libraries (24,450 sequences). Symbol shapes are indicative of statistical distinct groups. Legend: granules (■), impeller shaft biofilm (▲), HRSD seed (●), digested swine waste (◆), and biomass <75 µm (★).

D.6 Heavy Metals Analysis

Metals content (Table D.5) was determined by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). Samples (100 µL) were oven-dried at 90°C overnight in polypropylene tubes and subsequently digested with 100 µL Optima grade nitric acid (Fisher Scientific, Pittsburgh, PA, USA) overnight at 90°C while covered. Samples were cooled to room temperature and 100 µL 30% Optima grade hydrogen peroxide was added prior to heating to 90°C for two hours. Samples were stored for 1 week at 4°C prior to analysis on an Agilent 7500cx ICP-MS with Agilent 1260 Infinity Bioinert LC (Agilent technologies). Calcium and iron were analyzed in hydrogen mode and other metals analyzed in helium mode.

Table D.5: Concentration (mg L^{-1} except where noted) of heavy metals in the anaerobically digested swine waste and their respective IC50 values (*173*, *241*, *242*). Metals that could be inhibitory are shown in bold.

Element	Total	Soluble	IC50
Ca	142 ± 1	100 ± 3	N/R [a]
Mg	38.0 ± 0.2	33.4 ± 0.3	N/R
Fe	**7.61 ± 0.05**	**0.75 ± 0.02**	**1.1**
Ni	0.16 ± 0.00	0.14 ± 0.00	48.6
Zn	**5.21 ± 0.03**	**2.15 ± 0.01**	**3.9-7.6**
Cu	**3.17 ± 0.02**	**0.19 ± 0.00**	**1.9-5.0**
As[b]	10.3 ± 0.6	9.86 ± 0.72	N/R

[a] N/R – not reported

[b] Concentration in μg L^{-1}

APPENDIX E: COLOUR SEGREGATED SEQUENCING

E.1 Summary of Sampling

Mixed liquor was collected from the reactor and aggregates collected by difference in density. Separated granules were manually screened based on colour ("Red" and "Brown"; Figure A.7). DNA was extracted from colour-separated granules, non-granular reactor mixed liquor ("Floc"), reactor effluent ("Effluent"), reactor influent ("Swine Waste"), and the initial anammox seed culture from HRSD ("HRSD") using MoBio Powersoil Powerlyzer DNA extraction kits. DNA extracts were sent to MRDNA (www.mrdnalab.com, Shallowater, TX, USA) for sequencing on the Illumina MiSeq platform. All sequencing was done in duplicate (designated as "A" and "B" for a given sample).

E.2 Summary of Sequencing

A total of 1,594,130 sequences were obtained from the Illumina MiSeq sequencing run and processed as described in the text (Section 6.2.4 Illumina MiSeq sequencing and analysis). In the cleaned data, 63,566 unique sequences were analyzed for chimeras, 31,367 potential chimeras were detected and removed from the dataset. A total of 232,558 sequences were retained for downstream analysis. The smallest library contained 10,965 sequences while the largest contained 27,588.

Table E.1: Summary of samples and library sequence data from the colour separated sequencing run

Sample	No. of Sequences	No. of OTUs[a]	Inv. Simpson[a,b]
Swine Waste A	22,171	939	37.2
Swine Waste B	23,577	1135	34.7
HRSD A	21,651	1268	23.6
HRSD B	27,588	1485	22.8
Red A	19,167	1320	31.3
Red B	22,933	1196	32.9
Brown A	16,793	1200	31.5
Brown B	10,965	1007	28.4
Floc A	15,953	1692	64.4
Floc B	18,989	1506	61.0
Effluent A	18,342	952	25.3
Effluent B	14,429	960	24.7

[a] Values based on a random subsample of 10,965 sequences per library.
[b] Inverse Simpson Index, a dimensionless number ranging from 0 to ∞ with higher numbers indicating more intra-sample diversity.

E.3 Community Analysis

The relationship among the replicates is shown below in a phylogenetic tree (Figure D.1). Reactor samples (influent swine waste, effluent, granules, and floc) clustered into a clade showing difference from the initial seed culture. The two types of granule (red, brown) had high similarity, which was expected due to imperfect separation. Floc and Effluent were most similar to each other and also were the most similar to the Swine Waste. Phylum level community analysis is shown in Figure D.2.

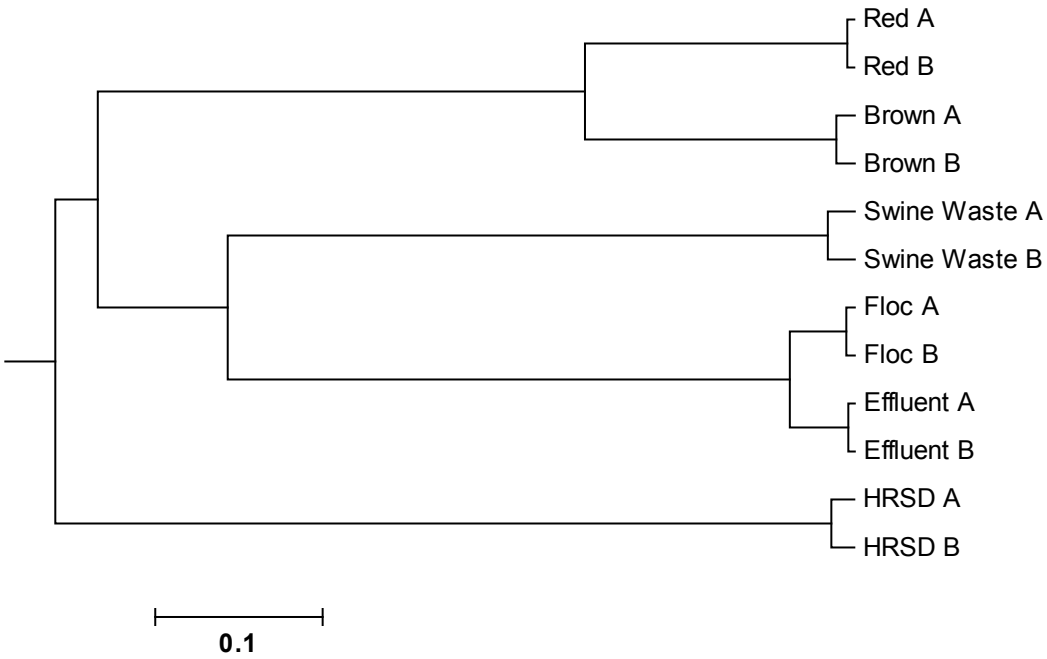

Figure E.1: Phylogeny of the sequenced microbial communities.

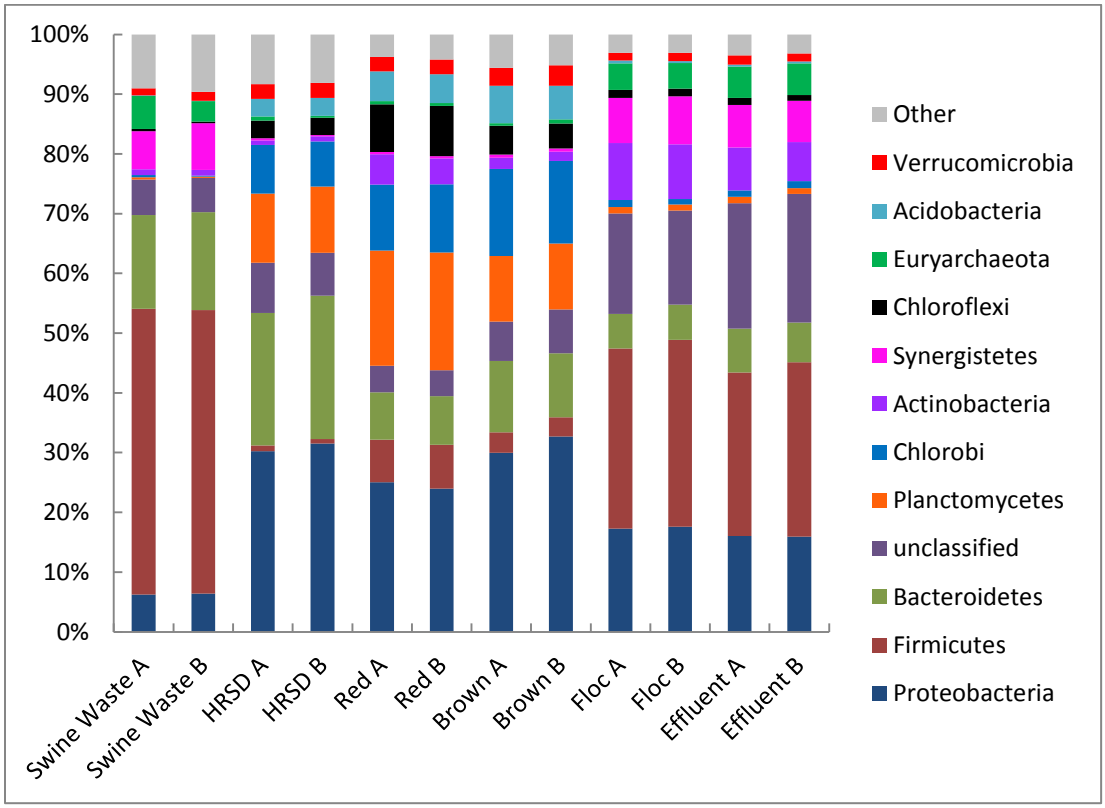

Figure E.2: Phylum level classification of sequences from Illumina MiSeq sequencing. "Other" represents combined phyla that each account for <1% of the total microbial community. Anammox bacteria are well established as members of *Planctomycetes*.

Bacteria known to be associated with relevant N transformations are shown in Table D.2. Note enrichment of anammox bacteria in the red reactor granules, with a shift in the community from *Candidatus Brocadia* in the seed culture to *Candidatus Kuenenia* in the reactor (both known anammox genera). Nitrite oxidizing bacteria (*Nitrospira* and *Nitrobacter*) were successfully repressed in the system and ammonium oxidizing bacteria (*Nitrosomonas*) were primarily associated with the floc. Other known anammox genera (*Candidatus Jettenia, Candidatus Anammoxoglobus,* and *Candidatus Scalindua*) were not detected.

Table E.2: Percent relative abundance of sequences of interest in libraries. Values ≥1% are in bold. Dashes indicated <0.01%.

Sample	*Candidatus Brocadia OTU-14*	*Candidatus Kuenenia OTU-4*	*Nitrosomonas OTU-25*	*Nitrospira OTU-90*	*Nitrobacter OTU-332*
Swine Waste A	0.07	0.12	0.03	0.01	-
Swine Waste B	0.02	0.04	0.02	-	-
HRSD A	**7.28**	0.22	0.85	**1.01**	0.01
HRSD B	**6.60**	0.08	0.88	**1.04**	0.01
Red A	0.07	**16.5**	0.13	0.01	0.06
Red B	0.06	**16.8**	0.15	0.01	0.04
Brown A	0.10	**7.79**	0.43	0.01	0.14
Brown B	0.17	**7.34**	0.38	0.02	0.10
Floc A	0.07	0.17	**1.89**	0.01	-
Floc B	0.05	0.17	**2.00**	0.01	0.02
Effluent A	0.05	0.15	**1.68**	0.01	0.02
Effluent B	0.03	0.18	**1.57**	0.02	0.01

Tables E.3: Percent relative abundance of significant (>2%) members of the microbial community in each library which are not known to be associated with N metabolism. These OTUs are classified according to their highest known taxonomic rank (e.g. unclassified families are sorted by order). Averages of the duplicate sequencing runs are reported. Values ≥1% are in bold.

OTU	Highest Known Taxonomic Rank		Swine Waste	HRSD	Red	Brown	Floc	Effluent
OTU-1	Bacteria	(Domain)	0.20	0.23	**1.21**	**3.49**	**8.04**	**17.95**
OTU-15	Bacteroidetes	(Phylum)	0.07	**9.34**	0.07	0.11	0.05	0.08
OTU-8	Clostridiaceae	(Family)	0.06	**9.30**	0.53	0.54	0.23	0.28
OTU-2	Ignavibacteriaceae	(Family)	**8.83**	0.10	**2.30**	0.36	**5.23**	**5.09**
OTU-3	Alteromonadales	(Order)	0.04	0.62	**3.96**	**8.31**	**3.20**	**3.14**
OTU-6	Sinobacteraceae	(Family)	0.10	**6.01**	**2.78**	**3.71**	0.14	0.17
OTU-17	Bacteroidetes	(Phylum)	0.06	0.14	**4.00**	**5.89**	0.38	0.42
OTU-21	Bacteroidetes	(Phylum)	0.02	**5.67**	0.05	0.06	0.04	0.01
OTU-7	Ignavibacteriaceae	(Family)	0.07	0.96	**4.98**	**5.33**	0.28	0.32
OTU-53	Sulfurimonas	(Genus)	**3.47**	0.07	0.12	0.18	**5.06**	**4.22**
OTU-12	Actinobacteria	(Phylum)	0.05	0.03	**1.05**	0.55	**4.49**	**3.09**
OTU-16	Bacteroidetes	(Phylum)	**4.42**	0.09	0.11	0.14	**1.21**	**2.00**
OTU-22	Chlorobi	(Phylum)	0.03	0.30	**2.69**	**2.90**	0.28	0.24
OTU-11	Clostridiaceae	(Family)	**2.84**	0.03	0.52	0.08	**2.07**	**2.55**
OTU-36	Bacteria	(Domain)	0.01	**2.77**	0.04	0.05	0.03	0.03
OTU-53	Syntrophobacteraceae	(Family)	0.02	0.03	**1.84**	**2.70**	0.06	0.09
OTU-42	Gammaproteobacteria	(Class)	0.02	0.03	0.49	**2.70**	0.05	0.03
OTU-10	Turicibacter	(Genus)	**2.29**	0.05	0.51	0.15	**2.56**	**2.65**
OTU-20	Betaproteobacteria	(Class)	0.03	0.45	**2.54**	**1.87**	1.00	0.80
OTU-18	Verrucomicrobia	(Phylum)	0.02	**1.02**	**2.12**	**2.50**	0.58	0.64
OTU-9	Clostridiaceae	(Family)	**1.37**	0.04	0.90	0.12	**2.40**	**2.18**
OTU-28	Comamonadaceae	(Family)	0.02	**2.38**	0.05	0.08	0.02	0.02
OTU-23	Methanospirillum	(Genus)	**1.58**	0.07	0.16	0.13	**2.10**	**2.36**
OTU-54	Bdellovibrio	(Genus)	0.02	**2.36**	0.01	0.03	0.02	0.01

Tables E.3: (continued)

OTU	Highest Known Taxonomic Rank		Swine Waste	HRSD	Red	Brown	Floc	Effluent
OTU-36	Syntrophobacteraceae	(Family)	0.03	0.05	0.99	**1.58**	**2.21**	**2.11**
OTU-29	Chloroflexi	(Phylum)	0.01	0.11	**2.15**	**1.37**	0.05	0.03
OTU-24	Acidimicrobiales	(Order)	0.03	0.03	**1.61**	0.63	**2.09**	**1.39**
OTU-13	Clostridiaceae	(Family)	2.23	0.04	**0.35**	0.08	**1.80**	**1.64**
OTU-44	Chlorobi	(Phylum)	0.03	0.04	0.61	**1.99**	0.14	0.16

Table E.4: Description of BLAST results of dominant bacteria from Table D.3.

OTU	Classification	BLAST Results
OTU-1	Bacteria	Uncultured Bacteria from wastewater and activated sludge systems; suspected denitrifier
OTU-38	Bacteria	Uncultured Chloroflexi from dry/arid soils; seen associated with Fe-Mn nodules in China
OTU-15	Bacteroidetes	Uncultured bacteria from anammox granular systems
OTU-16	Bacteroidetes	Uncultured bacteria from anaerobic systems associated with volatile fatty acid oxidation
OTU-21	Bacteroidetes	Uncultured bacteria from wastewater treatment systems
OTU-17	Bacteroidetes	Uncultured bacteria associated with wastewater systems including an anammox SBR
OTU-44	Chlorobi	Uncultured bacteria from anammox reactors
OTU-22	Chlorobi	Uncultured bacteria from wastewater systems
OTU-18	Verrucomicrobia	Misc sources (anammox granules, anammox biofilm, human skin, Australian Vertisol)
OTU-29	Chloroflexi	Uncultured bacteria from subsurface/groundwater
OTU-12	Actinobacteria	Uncultured bacteria from soil
OTU-20	Betaproteobacteria	Uncultured bacteria from activated sludge and anammox systems
OTU-42	Gammaproteobacteria	Misc sources (hydrocarbon contaminated soil, wastewater of oil refinery, chironomid eggs)
OTU-3	Alteromonadales	Uncultured bacteria from activated sludge
OTU-24	Acidimicrobiales	Uncultured bacteria from wastewater systems including anammox biofilm
OTU-2	Ignavibacteriaceae	Uncultured bacteria from anaerobic digester associated with acetate metabolism.
OTU-7	Ignavibacteriaceae	Uncultured bacteria treating antibiotic wastewater
OTU-9	Clostridiaceae	Uncultured bacteria associated with acetate degradation from anaerobic digester supernatant
OTU-11	Clostridiaceae	Uncultured bacteria associated with acetate degradation from anaerobic digester supernatant
OTU-13	Clostridiaceae	Uncultured bacteria associated with acetate degradation from anaerobic digester supernatant
OTU-8	Clostridiaceae	Uncultured bacteria from wastewater systems treating livestock (including swine) waste
OTU	Classification	BLAST Results
OTU-53	Syntrophobacteraceae	Misc Sources (rice paddy, tadpole gut, volcano mud, chlorohydrocarbon river bed)
OTU-6	Sinobacteraceae	Uncultured bacteria from mainstream anammox and mangrove soil

Table E.4: (Continued)

OTU-36	Syntrophobacteraceae	Uncultured bacteria from soil samples
OTU-10	Turicibacter	Uncultured bacteria associated with acetate degradation from anaerobic digester supernatant
OTU-23	Methanospirillum	Uncultured archaeon associated with propionate degradation from anaerobic digester supernatant
OTU-5	Sulfurimonas	Uncultured bacteria associated with anaerobic digester treating swine waste
OTU-28	Comamonadaceae	Misc sources (potato risosphere, wastewater, freshwater wetland soils)
OTU-54	Bdellovibrio	Misc sources (marine hydrothermal, redhead grass risosphere, hydrocarbon contaminated soil)

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