Anammox Enrichment and Kinetics

by
Rahasudha Kannan

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
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degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

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Rahasudha Kannan for the degree of Honors Baccalaureate of Science in Bioengineering and Honors Baccalaureate of Arts in International Studies presented on June 2, 2017. Title: Anammox Enrichment and Kinetics.

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Tyler Radniecki

Anammox (Anaerobic Ammonium Oxidation) is a significant biogeochemical process carried out by certain bacteria, generally referred to as anammox bacteria. These bacteria have the ability to anaerobically oxidize ammonium (NH_4^+) with nitrite (NO_2^-) to form nitrogen gas (N_2) without an organic carbon source. While anammox bacteria are found in a variety of environments, their metabolism can be especially useful for ammonium removal in wastewater treatment plants. When compared to conventional nitrogen removal processes, the greatly decreased aeration and methanol requirements significantly reduce cost and greenhouse gas production. This study focused on the enrichment of anammox bacteria from three different sources (wastewater treatment plants in Rotterdam, Netherlands; Virginia, USA; and Oregon, USA) using sequencing batch reactors and up-flow column reactors as enrichment techniques. Anammox activity, monitored by measuring nitrogen species in influent and effluent samples over time, indicated that up-flow column reactors were more suitable for anammox enrichment, and that treatment history of the sludge source affected enrichment at the lab-scale. The first-order substrate removal model, Grau second-order substrate removal model, and modified Stover-Kincannon models were applied to the three anammox up-flow column reactors to describe and predict anammox growth kinetics.

Key Words: anammox, enrichment, kinetic models

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Honors Baccalaureate of Science in Bioengineering and Honors Baccalaureate of Arts in International Studies project of Rahasudha Kannan presented on June 2, 2017.

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Rahasudha Kannan, Author

Introduction

Anammox (Anaerobic Ammonium Oxidation) is a microbial process carried out by certain bacteria, generally referred to as anammox bacteria. These bacteria were recently discovered (1994) in the Netherlands and have since become a topic of international research. During the early stages of this study, the author of this thesis was able to meet one of the researchers who pioneered this research, Dr. Mike Jetten, and his research team at Radboud University in the Netherlands.

These bacteria have the ability to anaerobically oxidize ammonium (NH_4^+) with nitrite (NO_2^-) to form nitrogen gas (N_2). While anammox bacteria are found in a variety of environments, the process they carry out can be especially beneficial for ammonium removal in wastewater treatment plants as it would significantly reduce cost and help lower greenhouse gas production due to decreased aeration and methanol requirements compared to traditional nitrogen removal processes.

However, the extremely slow growth rate of anammox bacteria combined with a limited understanding of how common wastewater contaminants influence the anammox process has slowed its implementation in full-scale wastewater treatment plants. Understanding how to best enrich anammox bacteria and how they interact with common wastewater components would be beneficial in designing robust anammox based nitrogen removal treatment processes. Thus, the purpose of this particular research project was to study the enrichment of anammox to improve culturing methods. Anammox from three different sources, each with different treatment process background, was cultured using two enrichment techniques: a sequencing batch reactor (SBR) and an up-flow column reactor. A secondary goal of this project was to apply different mathematical models to anammox growth kinetics and use these to verify anammox enrichment.

Background Information

Importance of Nitrogen and Nitrogen Removal

Nitrogen, which is abundant on Earth and makes up 78% of Earth's atmosphere, is essential for living organisms to function.[1] However, in excess, nitrogen can be detrimental to human health and the environment. Reactive nitrogen from animal-raising facilities, manufactured fertilizer run-off, septic systems, and other sources can enter bodies of water and lead to eutrophication.[1] Increased levels of nitrate provide the necessary nutrients for algae blooms, which often deplete the dissolved oxygen in the water and prevent the body of water from sustaining other aquatic life.[1] Thus, nitrogen removal is an essential part of wastewater treatment. Biological conversion of ammonia (NH_3) to nitrogen gas (N_2) is typically carried out in two steps: the oxidation of ammonia to nitrate (NO_3^-) (Equation 1 and 2) and the reduction of nitrate to nitrogen gas (Equation 3).[2] The first process, referred to as nitrification, is an aerobic process carried out by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB).[2] Denitrification, the second process, is an anaerobic process carried out by a few different groups of bacteria that require organic carbon as a food source.[2]

AOB: $NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+$ (1)

NOB: $NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ (2)

Heterotrophic denitrifying bacteria:

$NO_3^- + 0.83\ CH_3OH \rightarrow 0.5\ N_2 + 0.83\ CO_2 + 1.17\ H_2O + OH^-$ (3)

Anammox Process and Anammox Bacteria

In the 1970s, Austrian physiochemist Broda predicted the existence of two groups of autotrophs, anammox being one of them, based on evolutionary and thermodynamics grounds.[3] However it wasn't until 1994 that anammox bacteria were accidentally discovered by researchers in the Netherlands.[3]

During the early stages of this study, he author of this thesis had the opportunity to meet with Dr. Mike Jetten, who was part of the team that discovered anammox, Dr. Boran Kartal, and other members of their anammox research laboratory at Radboud University in Nijmegen, Netherlands. Jetten and Kartal are among those who paved the way for anammox research and have continued to make important contributions to this area of research. Since then, anammox has become a major topic of international interest (discussed further in the "International Anammox Research" section below) because they play a very important role in the nitrogen cycle. In fact, they are thought to be responsible for ~50% of nitrogen gas released into the atmosphere.[3]

Anammox bacteria are able to anaerobically oxidize ammonia, using nitrite (NO_2^-) as an electron acceptor, to produce nitrogen gas without an organic carbon source (Figure 1). The anammox process has many advantages over the traditional denitrification process including low biomass yield, no need for aeration, and no addition of organic carbon sources, all of which make it more cost-effective and sustainable.[4] Reduced energy consumption, greenhouse gas emission, and sludge production all contribute to the cost-effectiveness, sustainability, and understandably the popularity of anammox research.[5]

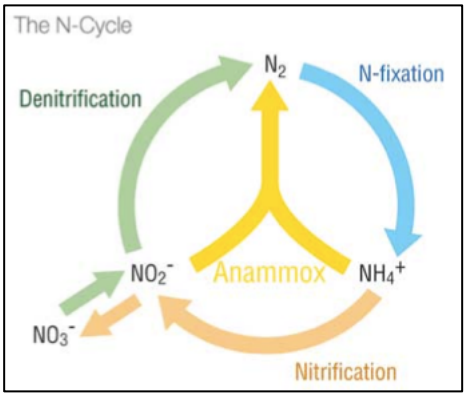

Figure 1. Traditional nitrogen cycle (green, blue, and orange arrows) with the anammox process (yellow arrow).[6]

Anammox bacteria are a characteristic red-orange color (Figure 2) when highly enriched and are difficult to culture due to their extremely slow growth rates, with a doubling time of 7-22 days under favorable growth conditions.[3]

Figure 2. Enriched anammox bacteria from an anammox lab at Radboud University in the Netherlands

The reaction shown in Figure 3 describes the overall metabolism and growth of anammox cells.[3] The stoichiometric ratio of nitrite consumed to ammonium consumed (1.32 to 1) can be used as an indicator of anammox activity.

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

Figure 3. Reaction describing anammox metabolism[3]

To understand how anammox is able to carry out this process researchers have investigated the anammox enzyme pathway and proposed the existence of an organelle called an anammoxosome, which contains three key enzymes (Figure 4).[3] The first, nitrite reductase (Nir), reduces nitrite to nitric oxide (NO). Nitric oxide is combined with ammonium in hydrazine synthase (HZS) to produce hydroxylamine (NH_2OH) and hydrazine (N_2H_4). The hydroxylamine is reduced back to nitric oxide, while the hydrazine is oxidized into nitrogen gas by the third enzyme, hydrazine dehydrogenase (HDH). Hydrazine is one of the major components of rocket fuel and is one of the most reduced compounds on earth, making it very reactive.[7]

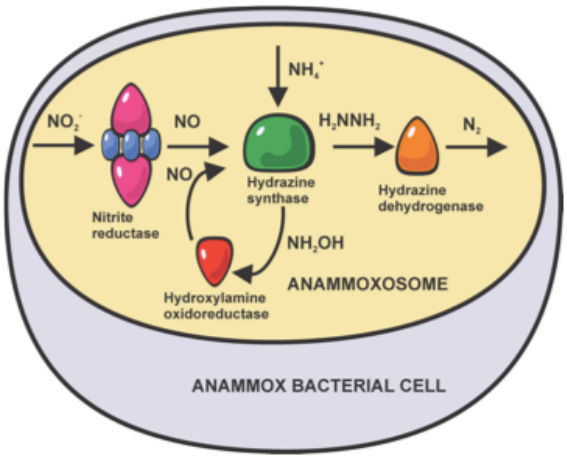

Figure 4. Summary of anammox bacteria enzyme pathway[8]

Research and Implementation of Anammox

International Anammox Research

A bibliometric analysis of the trends of anammox research identified that the number of publications about anammox has increased rapidly since 2000, with 968 publications between 1995 and 2012.[9] Although the Netherlands was the origin of anammox discovery, research groups in China have produced the most publications on anammox (27.71% of publications).[9] Figure 5 reveals the top-ten most productive countries in terms of anammox research.[9]

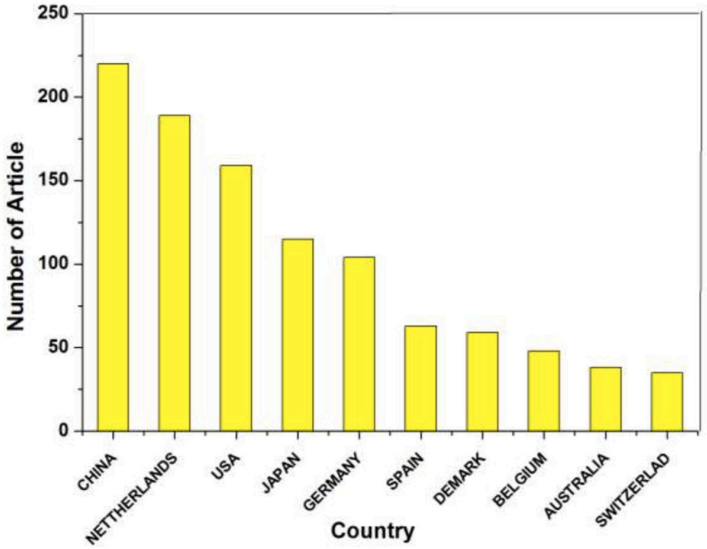

Figure 5. Top-ten most productive countries in terms of anammox research related publications. China accounts for 27.71% of publications, followed the Netherlands (23.8%), and the USA (20.03%).[9]

Of the articles investigated in this bibliometric analysis, nearly 30% of the articles were internationally collaborative articles.[9] Of the different focuses in anammox research, the anammox process receives the most attention (62% of publications).[9] Anammox inhibition and diversity studies receive some attention (13% and 12%), but anammox physiology research is not growing as rapidly as these other subtopics.[9]

Anammox in Wastewater Treatment Systems

As of 2014, there were more than 100 full-scale partial-nitritation/anammox installations worldwide.[10] Of these plants more than 50% are operated as sequencing batch reactors (SBRs), 88% are operated as single-stage systems, and 75% are being used for side stream treatment of municipal wastewater.[10] Different locations are implementing the anammox process differently, so various anammox treatment systems have emerged. These include CANON (Completely Autotrophic Nitrogen removal Over Nitrite); OLAND (Oxygen-Limited Autotrophic Nitrification/Denitrification); DEMON (Deammonification); SHARON (Single reactor system for High-rate Ammonium Removal Over Nitrite); and SNAD (Simultaneous Nitrification, Anammox, and Denitrification).[9] However, the anammox treatment systems relevant to this study are the SHARON and DEMON processes.

The SHARON process uses an aerated, well-mixed, continuous flow reactor to convert ammonium exclusively to nitrite by controlling process parameters such as pH, temperature, and aeration rate.[5] Maintaining the listed process parameters in certain ranges allows AOBs to thrive and NOB to be washed out of the reactor. In this system, granules with AOB on the outside and anammox on the inside are formed.[5] Since this is a continuous flow reactor, sludge retention time is the same as the hydraulic retention time.

The DEMON process (deammonification) also takes place in a single reactor, but with an SBR instead of a continuous flow reactor.[5] This allows the sludge retention time to be much higher than the hydraulic retention time. The DEMON process takes place in two steps: the partial nitritation of ammonia (an aerobic process), followed

by the anaerobic ammonium oxidation by anammox.[5] Partial nitritation is the process of converting ammonium into nitrite.

Kinetic Models

Kinetic models are valuable tools for the description and prediction of reactor performance.[5] Popular kinetic models for anaerobic treatment systems include the Monod model, the first-order substrate removal model, the Grau second-order model, and a modified Stover-Kincannon model.[5] While the Monod model can be used to study both pure cultures and mixed bacterial culturing, assuming growth kinetics are similar for the two culture systems, it was not suitable for modeling the anammox sludge system since certain parameters required for the Monod model were difficult to obtain for the anammox reactors used in this study. The Monod equation is shown below:

$$\mu = \frac{\mu_{max} * S_e}{K_S + S_e} \quad (4)$$

Where,

μ = specific growth rate (day^{-1})

μ_{max} = maximum specific growth rate (day^{-1})

K_S = half saturation constant (mg/L)

S_e = total nitrogen concentration in effluent (mg N/L)

<u>First-Order Substrate Removal Model</u>

The first-order substrate removal model assumes that first-order kinetics are dominant in the reactor and that reactor contents, including substrate concentration, are well-mixed.[5] Equation 5 presents the general first-order kinetics equation.

$$-\frac{dS}{dt} = \frac{QS_i}{V} - \frac{QS_e}{V} - K_1 S \quad (5)$$

Where,

$\frac{dS}{dt}$ = Substrate removal rate (mg N/L-day)

$\frac{QS_i}{V}$ = Rate of substrate entering the reactor or loading rate (mg N/L-day)

$\frac{QS_e}{V}$ = Rate of substrate exiting the reactor (mg N/L-day)

K_1S = Rate of substrate conversation due to the first-order reaction (mg N/L-day)

Q = Flow rate (L/day)

V = Volume of reactor (L)

S_i = Influent substrate concentration (mg N/L)

S_e = Effluent substrate concentration (mg N/L)

K_1 = First-order substrate removal rate constant (day^{-1})

The substrate removal rate becomes negligible $\left(\frac{dS}{dt} = 0\right)$ under pseudo steady-state conditions, allowing the equation to be modified as shown below.

$$\frac{QS_i}{V} - \frac{QS_e}{V} = K_1S_e \quad \text{or} \quad \frac{S_i - S_e}{\theta_H} = K_1S_e \tag{6}$$

Where, $\theta_H = \frac{V}{Q}$ = Hydraulic retention time (day)

Grau Second-Order Substrate Removal Model

The Grau second-order was originally developed for multicomponent substrate removal by activated sludge.[5] This model combines second order kinetics (Equation 7) with the Monod model. The integrated and linearized form is shown in Equation 8).

$$-\frac{dS}{dt} = K_2X\left(\frac{S_e}{S_i}\right)^2 \tag{7}$$

$$\frac{S_i\theta_H}{S_i - S_e} = \theta_H + \frac{S_i}{K_2X_i} \tag{8}$$

Where,

$\frac{dS}{dt}$ = Substrate removal rate (mg N/L-day)

K_2 = second order substrate removal rate constant (day^{-1})

X = biomass concentration (mg/L)

S_i = Influent substrate concentration (mg N/L)

S_e = Effluent substrate concentration (mg N/L)

$\theta_H = \frac{V}{Q}$ = Hydraulic retention time (day)

Modified Stover-Kincannon Model

Originally developed to model biofilm systems on rotating biofilm contactor (RBC) reactors, the Stover-Kincannon model is its capable of predicting the substrate removal rate independent of reaction kinetics at any substrate loading condition.[5] Substrate removal rate, considered a function of substrate loading rate, is given by:

$$\frac{dS}{dt} = \frac{U_{max}\left(\frac{QS_i}{A}\right)}{K_B + \left(\frac{QS_i}{A}\right)} \quad (9)$$

Where,

$\frac{dS}{dt} = \frac{Q}{V_r}(S_i - S_e)$ = Substrate removal rate (mg N/L-day)

Q = Flow rate (L/day)

V_r = Volume of reactor (L)

S_i = Influent substrate concentration (mg N/L)

S_e = Effluent substrate concentration (mg N/L)

U_{max} = maximum substrate utilization rate (mg N/L-day)

K_B = saturation value constant (mg N/L-day)

A = disc surface area that affects bacterial growth on the disk in an RBC

While original model assumes that suspended biomass is negligible compared to the immobilized biomass (i.e. the biofilm), this did not hold true for the anammox UFRs in this study since all the biomass was suspended in media. The model was modified to use the volume of media instead of the disc surface area:

$$\frac{dS}{dt} = \frac{U_{max}\left(\frac{QS_i}{V}\right)}{K_B + \left(\frac{QS_i}{V}\right)} \quad (10)$$

Where, V = volume of media for all biomass (L)

Linearizing Equation 10 yields the following equation:

$$\left(\frac{dS}{dt}\right)^{-1} = \frac{V_r}{Q(S_i - S_e)} = \frac{K_B}{U_{max}}\frac{V_r}{QS_i} + \frac{1}{U_{max}} \quad (11)$$

Where,

$\frac{V_r}{Q(S_i - S_e)}$ = inverse of the substrate removal rate (L-day/mg N)

$\frac{V_r}{QS_i}$ = inverse of substrate loading rate (L-day/mg N)

Q = Flow rate (L/day)

V_r = Volume of reactor (L)

S_i = Influent substrate concentration (mg N/L)

S_e = Effluent substrate concentration (mg N/L)

U_{max} = maximum substrate utilization rate (mg N/L-day)

K_B = saturation value constant (mg N/L-day)

Rewriting Equation 11 yields the following equation:

$$\frac{dS}{dt} = \frac{U_{max}S_i}{K_B\theta_H + S_i} \quad (12)$$

Where, $\theta_H = \frac{V_r}{Q}$ = Hydraulic retention time (day)

Equation 12 resembles the Monod model. Unlike the Monod model, in the modified Stover-Kincannon model the substrate removal rate (dS/dt) is dependent on the substrate loading rate (S_i/θ_H).

Materials and Methods

Sources of Anammox Reactor Biomass

Anammox bacteria used in this study were acquired from three different sources in three separate locations. The first source, an anammox bacteria containing sludge, was obtained from the Hampton Roads Sanitation District (HRSD) York River wastewater treatment plant in Virginia, USA. This full-scale anammox treatment plant uses the DEMON process to treat their wastewater.[6] The second source came from the Dokhaven wastewater treatment plant in Rotterdam, Netherlands. This plant was the first full-scale anammox treatment plant in the world and operates using the

SHARON-anammox process.[11] The third source was a highly enriched granular anammox culture from a pilot-scale anammox treatment plant in Clackamas, Oregon, USA, which used the SHARON process as well.

Anammox Media Preparation

Anammox media was prepared in a glass bottle by adding the components shown in Table 1 and brining the final volume to 10 L using DI water Figure 6.

Figure 6. Anammox media with an N_2 gas bag attached to prevent a vacuum from forming as media is pumped out of the bottle.

Once the components were added, the media was mixed, and purged with N_2 for 20-40 minutes using a sparging stone. While the media recipe in Table 1 was used for most of the study, the recipe underwent changes during the course of this study. The anammox media recipe used ammonium sulfate ($(NH_4)_2SO_4$) as its ammonium source instead of ammonium chloride (NH_4Cl) until December 2016. Up-flow reactors with the Netherlands anammox (NL_UFR) and Virginia anammox (VA_UFR) were fed media with final nitrite, ammonium, and sodium bicarbonate concentrations of 4 mM, 4 mM, and 12 mM, respectively, until 11/16/2016.

Table 1. Anammox media recipe used for up-flow column reactors

Component	Final Concentration in Media
Dry Additions	
NH_4Cl	6 mM
$NaHCO_3$	9 mM
$NaNO_2$	6 mM
Liquid Additions	
$CaCl_2 \cdot H_2O$	2 mM
KH_2PO_4	0.2 mM
$MgSO_4 \cdot 7H_2O$	1 mM
KNO_3	1 mM
$FeSO_4 \cdot 7\ H_2O$	0.040 mM
EDTA (hydrous)	0.080 mM
Trace Metals Solution*	1 mL/L of media
*Trace Metals Solution Recipe (for 1 L of solution)	
$ZnSO_4 \cdot 7H_2O$	546mg
$CoCl_2 \cdot 6H_2O$	309 mg
$MnSO_4 \cdot H_2O$	1.065 g
$CuCl_2 \cdot 2H_2O$	222 mg
$Na_2MoO_4 \cdot 2H_2O$	266 mg
$NiSO_4 \cdot 6H_2O$	368 mg
K_2SeO_4	155 mg
H_3BO_3	14.3 mg
EDTA (hydrous)	18.75 g

Sequencing Batch Reactor Set Up and Operation

All sequencing batch reactors (SBRs) in this study were set up in 250 mL glass bottles and were stored in a 25-30° C shaking incubator (Figure 7). The very first SBR (SBR A), set up on 4/15/2015, was filled with 100 mL of anammox media and 50 mL of anammox sludge from the DEMON process WWTP in D.C. Approximately 75 mL of the anammox media was withdrawn and replaced with fresh media every day, after which the SBR was purged with N_2 for 20 minutes. This SBR was later split into three different SBRs (SBR 1, SBR 2, and SBR 3) and operated from 10/14/15 until 12/4/15, after which they were recombined into one SBR referred to as SBR 6. SBR 6 contained ~60 mL of anammox sludge.

SBR 4 contained granular anammox pellets from the anammox treatment plant in the Netherlands while SBR 5 contained a white powder, which had originally been anammox sludge. SBRs 1, 2, 3, 4, and 5 contained 120 mL of media that were

replaced three times a week until 11/30/2015. From 11/30/2015 until 5/11/2016, only 40 mL of the media were replaced once every three days.

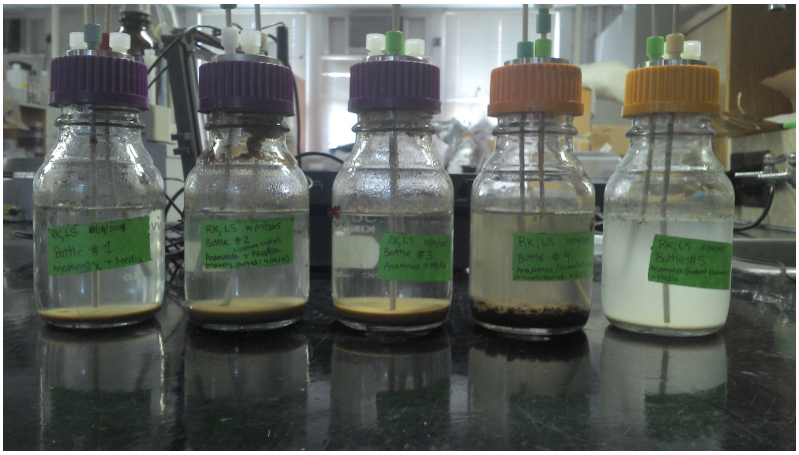

Figure 7. The sequencing batch reactors used in this study: SBRs 1, 2, 3, 4, and 5 (left to right). SBRs 1-3, 5 contained Virginia anammox and SBR 4 contained Netherlands anammox.

Up-flow Column Reactor Set Up and Operation

Two up-flow column reactors were set up in 140 mL clear PVC tubes (H = 26 cm, D = 2.5 cm) on 5/11/2016 using the anammox sludge from SBRs 4, 5, and 6. The contents of SBR 4 (~45 mL of anammox sludge from the Netherlands) were transferred into up-flow column reactor 1 (UFR1) while the contents of SBR 5 (white powdered anammox) and SBR 6 (~ 29 mL of anammox sludge from D.C.) were transferred into up-flow column reactor 2 (UFR 2). Both reactors were continuously fed with anammox media using a peristaltic pump (Figure 8).

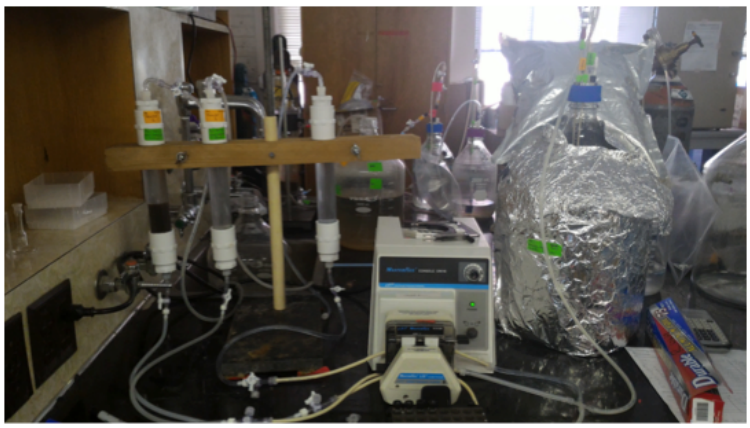

Figure 8. Up-flow column reactor set up. Media bottle (far right) was wrapped in aluminum foil. Influent is pumped from media bottle through the bottom of each UFR (far left) and out of the top of each UFR.

The anammox media concentration was altered from 4 mM NO_2^-, 4 mM NH_4^+, 1 mM NO_3^-, 12 mM $NaHCO_3$ to 6 mM NO_2^-, 6 mM NH_4^+, 1 mM NO_3^-, 9 mM $NaHCO_3$ on 11/16/2016. The Clackamas anammox (~9 mL of granules) was set up in a 15 mL up-flow column reactor (H = 13.5 cm, D = 1.1-1.9 cm) on 5/15/16. All reactors were operated at room temperature (with no temperature regulation) until 1/9/2017 when all up-flow reactors were moved into a temperature controlled 30°C room.

Sample Collection and Storage

The SBR samples were extracted from the reactor using a large plastic syringe. For the up-flow column reactors influent samples were collected directly from the media bottle using a plastic syringe, while effluent samples were allowed to drip from the effluent tube directly into their storage containers. All influent and effluent samples were collected in 1.5 mL microcentrifuge tubes and stored at -80° C.

Nitrogen Species Measurements

The Dionex ICS-5000 (ion chromatography system) was used to measure was used to measure the concentration of nitrogen species (NO_2^-, NH_4^+, and NO_3^-) in some influent and effluent samples. A Dionex IonPacTM AS18 column with 15 mM NaOH was used for anions, and a Dionex IonPacTM CS16 column with 30 mM Methanesulfonic Acid (MSA) as eluent was used for cations. The pump pressure was

maintained at 1600 with an 8-minute test duration for anions, while the pump pressure was maintained at 2000 psi with a 15-minute test duration for cations. Standard curves for NH_4^+ and NO_2^- ranged from 2-8 mM, and the standard curve for NO_3^- ranged from 1-3 mM. Standard solutions for NO_2^-, NH_4^+, and NO_3^- were created using NH_4Cl, $NaNO_2$, and KNO_3, respectively, in nitrogen-free anammox media.

Colorimetric assays were used to measure nitrogen species concentrations in all remaining samples. Standard curves for all nitrogen species measurements were prepared in nitrogen-free anammox media. Anammox media without any N was used as a blank for all assays. Samples were diluted 1:4 or 1:5 for the colorimetric nitrite and ammonium assays, and were diluted 1:10 for the colorimetric nitrate assay. Sample absorbances were measured with an Orion™ AquaMate 8000 UV-Vis Spectrophotometer or a Biotek Synergy 2 Multi-mode Microplate Reader.

Colorimetric nitrite assay

This colorimetric assay measures low levels of nitrite (NO_2^-), between 0 and 3 mM. The following components were added to a 1.5 mL microcentrifuge tube in the order listed: 890 µL of 1% (w/v) Sulphanilamide in 1 M HCl, 10 µL of the sample, and 100 µL of 0.2% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride (NED). The solution was mixed using a Vortex machine and absorbance of the solution was measured at 540 nm after 10 minutes (or longer, but less than 8 hours). Samples containing nitrite will turn the solution a shade of pink (Figure 9). Absorbance was measured using a spectrophotometer. A standard curve for NO_2^- ranging from 0.5-8 mM (diluted 1:4) was created using $NaNO_2$ in nitrogen-free media for nitrite assay samples measured using the spectrophotometer. Both reagents were stored in amber bottles or in aluminum-foil covered containers. The NED reagent was replaced if it was not clear in appearance.

This assay was also scaled down to total reaction volume of 200 µL (178 µL of 1% (w/v) Sulphanilamide in 1 M HCl, 2 µL of the sample, and 20 µL of 0.2% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride). The solution was mixed by pipetting the

solution in and out several times, and the absorbance was measured using a plate reader. A standard curve for NO_2^- ranging from 0.5-10 mM (diluted 1:5) was created using $NaNO_2$ in nitrogen-free media for nitrite assay samples measured using the plate reader.

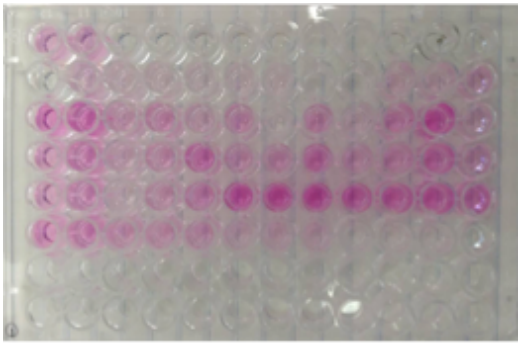

Figure 9. Colorimetric nitrite assay in a 96-well plate

Colorimetric ammonium assay

This colorimetric assay measures low levels of ammonium (NH_4^+), between 0-25 mg N/L (0-1.78 mM). The assay was prepared in a 96-well plate by adding the following components in the order listed. First, 25 µL of the sample and 175 µL Citrate reagent were added to a well and were allowed to sit for 1 minute. Then, 50 µL of 2-Phenylphenol-Nitroprusside (2-PP) reagent and 25 µL of buffered hypochlorite reagent were added to the well and mixed by pipetting the solution in and out several times. Samples containing ammonium will turn the solution a shade of green (Figure 10). The reaction was incubated at 37° C for 15 minutes before measuring the solution absorbance at 660 nm using a plate reader. A standard curve for NH_4^+ ranging from 0.178-1.78 mM was created using NH_4Cl in nitrogen-free media.

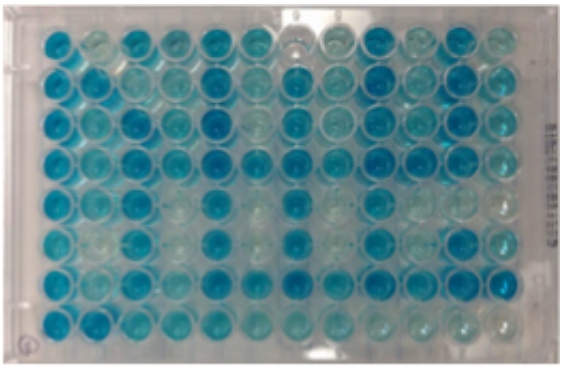

Figure 10. Colorimetric ammonium assay in a 96-well plate

The Citrate reagent was prepared by dissolving 5 g trisodium citrate in 100 mL DI water, adjusting the pH to 7.0 using 0.1 M HCl, and adding 250 mL DI water. The 2-Phenylphenol-Nitroprusside reagent was prepared by dissolving 3.22 g of 2-phenylphenol and 0.015 g sodium nitroprusside in 100 mL DI water. The buffered hypochlorite reagent was prepared by adding 1 g Na_3PO_4 to 80 mL DI water and 10 mL sodium hypochlorite solution, adjusting the pH to 13.0 using 2 M NaOH, and adding 10 mL of DI water. All reagents were stored at 4° C.

Colorimetric nitrate assay

This colorimetric assay measures low levels of nitrate (NO_2^-), between 0-22 mg NO_3^- /L (0-0.323 mM NO_3^-). The assay was prepared inside a laminar flow hood using a 96-well plate. The following components were added to a well in the order listed: 20 µL of the sample and 200 µL of the NAS reagent. After mixing the solution with a transfer pipette, the reaction was allowed to sit for 20-30 minutes. Samples containing nitrate will turn the solution a shade of purple (Figure 11). Solution absorbance was measured at 570 nm using a plate reader. A standard curve for NO_3^- ranging from 0.0357-0.357 mM was created using KNO_3 in nitrogen-free media.

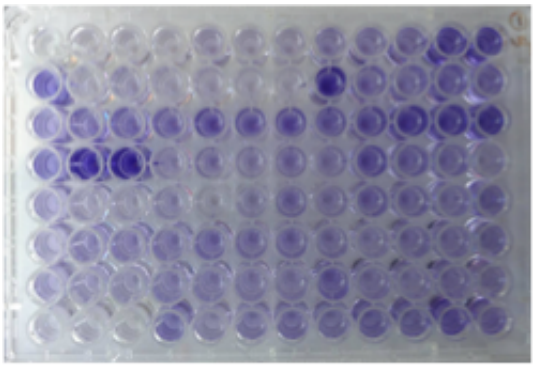

Figure 11. Colorimetric nitrate assay in a 96-well plate

The NAS reagent was prepared by mixing equal volumes of analytical grade concentrated phosphoric acid and concentrated sulfuric acid, and allowing the mixture to stand for one week in a laminar flow hood. The lid was left slightly ajar during this equilibration period. After one week, solid Polysciences NAS reagent was

added to the acid mixture for a final concentration of 5 g/L. The bottle was tightly capped and shaken until the NAS reagent was solubilized. This colorless reagent should be stored in a laminar flow hood.

Applying Mathematical Models to Anammox Growth Kinetics

Kinetic Experiments

The flow rate of media into each reactor was changed in order to alter the nitrogen loading rate and sludge contact time. The reactors were allowed to operate at a given flow rate for 2-3 weeks, during which influent and effluent samples were collected. The NE_UFR was operated at flow rates between 0.24-0.53 L/day, the VA_UFR at flow rates between 0.22-0.55 L/day, and the CL_UFR at flow rates between 0.17-0.45 L/day. A detailed summary of the flow rates, nitrogen loading rates, and duration kinetic experiments for each reactor is given in Table 3. The total nitrogen in the media was 126 mg N/L for the NL_UFR and VA_UFR until 11/16/2016, after which the total nitrogen in the media was increased to 182 mg N/L. The media for the CL_UFR contained 182 mg N/L during the entirety of its operation.

Table 3. Summary of flow rates and N loading rates into up-flow reactors

Dates of Operation	NL_UFR		VA_UFR		CL_UFR	
	Flow Rate (L/day)	N Loading Rate (mg N/L-day)	Flow Rate (L/day)	N Loading Rate (mg N/L-day)	Flow Rate (L/day)	N Loading Rate (mg N/L-day)
5/27/2016 - 6/27/2016	0.53	800 - 1100	0.50	1400 - 1800		
6/28/2016-10/15/2016	0.24	500 - 800	0.22	760 - 1100		
10/17/2016 - 11/15/2016	0.26	700 - 1500	0.26	1200 - 2400	0.24	4400 - 5500
11/16/2016 - 1/30/2017	0.26	900 - 1100	0.26	1400 - 1800	0.24	4300 - 5500
1/31/2017 - 2/13/2017			0.40	2200 - 2900	0.32	5700 - 7400
2/14/2017 - 2/28/2017			0.24	940 - 1800	0.17	2100 - 4100
2/29/2017 - 3/21/2017			0.55	3300 - 5800	0.45	8700 - 15000
3/22/2017 - 4/5/2017			0.36	2100 - 2700	0.29	5500 - 7000

The three mathematical models were applied only to the VA_UFR and CL_UFR because the NL_UFR was discontinued before sufficient kinetic experiments could be conducted. The volume of sludge was used in place of the volume of reactor in all models because the volume of anammox sludge in the reactor did not equal the

volume of the reactor. This modification allowed the models to more accurately reflect anammox sludge contact time with substrates.

First-Order Substrate Removal Model

Using the first-order substrate removal model under pseudo steady-state conditions (Equation 6), the first-order substrate removal rate constant, K_1, was determined by plotting $\frac{S_i - S_e}{\theta_H}$ against S_e and obtaining the slope of the plot.

Grau Second-Order Substrate Removal Model

The integrated and linearized form of the Grau second-order substrate removal model (Equation 8) can be used to extract kinetic parameters. Since the substrate removal efficiency, E, can be written as $E = \frac{S_i - S_e}{S_i}$ the term on the far left in Equation 8 was rewritten as $\frac{\theta_H}{E}$. Plotting $\frac{\theta_H}{E}$ against θ_H and applying a linear trend line yielded an equation with the form:

$$\frac{\theta_H}{E} = a + b\theta_H \quad (13)$$

Where,

$\theta_H = \frac{V}{Q}$ = Hydraulic retention time (day)

Q = Flow rate (L/day)

V = Volume of reactor (L)

E = Substrate removal efficiency

$a = \frac{S_i}{K_2 X_i}$, the second-order multicomponent substrate removal constant (day^{-1}) was the y-intercept

b = dimensionless kinetic constant was the slope

If X_i, biomass concentration, is known then K_2 can be calculated from the "a" term, but K_2 was not obtained for this study since biomass concentrations in the anammox reactors were not measured.

Modified Stover-Kincannon Model

The linearized form of the Stover-Kincannon model, modified for suspended biomass (Equation 11) was used to obtain kinetic parameters. Note that $\frac{V}{Q(S_i - S_e)} = \frac{\theta_H}{(S_i - S_e)}$ and $\frac{V}{QS_i} = \frac{\theta_H}{S_i}$ in Equation 11. Kinetic constants U_{max} and K_B were obtained by plotting $\frac{\theta_H}{(S_i - S_e)}$ against $\frac{\theta_H}{S_i}$ and applying a linear trend line, where the slope was K_B/U_{max} and the y-intercept was $1/U_{max}$.

Results and Discussion

Anammox Enrichment in Sequencing Batch Reactors

The change in concentration of each nitrogen species (NO_2^-, NH_4^+, and NO_3^-) over time for SBR A (Netherlands sludge) is shown in Figure 12. Consumption of nitrite and ammonium (in a 1.32 : 1 ratio) with nitrate production would be indicative of anammox activity[12], but the plot below does not demonstrate these trends. Removal of ammonium with minimal removal of nitrite and nitrate bacteria indicates that the system maybe have been partially aerobic since AOB and NOB require oxygen to convert ammonium into nitrite, and nitrite into nitrate, respectively. Any heterotrophic denitrifying bacteria that survived the partially aerobic system could have converted the nitrate being produced by NOB into nitrogen gas.

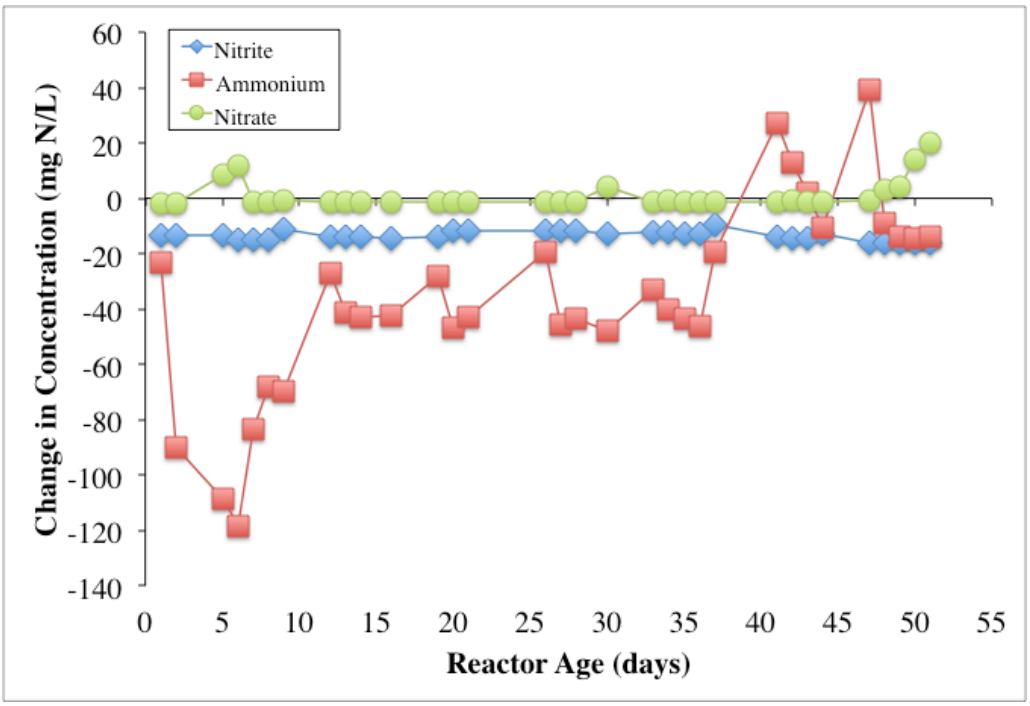

Figure 12. Change in nitrogen species over time in SBR A (Netherlands anammox). The trends in consumption and production of the three N species are not characteristic of anammox activity. Ammonium removal with little nitrite removal indicated that the system might have been aerobic in the early stages.

Similarly, a plot of the ratio of change in nitrite concentration to change in ammonium concentration ($\Delta[NO_2^-]$: $\Delta[NH_4^+]$) over time revealed that the ratios for SBR A did not align with the expected ratio of 1.32 to 1 (Figure 13). See Appendix A for change in N-species over time plots and $\Delta[NO_2^-]$: $\Delta[NH_4^+]$ over time plots for each of the other SBRs.

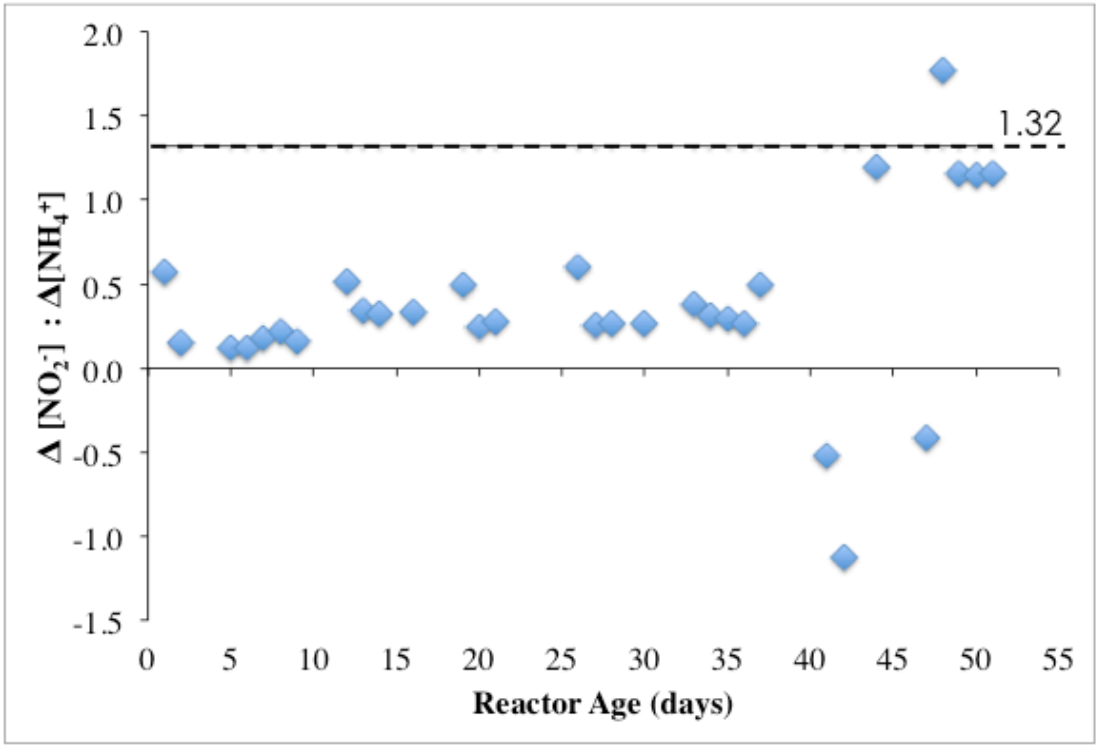

Figure 13. Ratio of change in nitrite to change in ammonium concentration over time for SBR A (Netherlands anammox)

Poor enrichment in the SBRs was unexpected since several other studies have successfully enriched anammox using sequencing batch reactors.[12,13] SBRs have many advantages including efficient biomass retention, and homogeneous distribution of substrates, products, and biomass in the reactor.[12] The stoichiometric equation shown in Figure 3 was developed by Strous *et al.* by culturing anammox in a sequencing batch reactor with a mixed culture system.[12] Another study using an SBR to enrich anammox detected anammox activity after 2 months of operation and achieved 82% nitrogen removal efficiency with the reactor.[13]
Furthermore, SBRs were identified as the most popular reactor type in anammox research by a bibliometric analysis of anammox research trends.[9]

Minimal enrichment in the SBRs in this study may have been because of improper operating conditions and operating procedures, such as a lack of a completely anaerobic environment, lack of temperature control, and unintentional biomass

removal during media replacements. Another reason for the lack of noticeable anammox activity may have been the ineffective removal of organic carbon from the reactors. Heterotrophic denitrifying bacteria that may have been present in the sludge require organic carbon to use as an electron donor[5], so they would have been able to survive in the reactors until the organic carbon in the reactor was depleted.

Anammox Enrichment in Up-flow Column Reactors

The anammox sludges from SBRs discussed earlier were transferred to up-flow column reactors (UFRs) upon seeing the difficulties with operation, cultivation, and biomass retention. Although the SBRs provided a longer sludge contact time with substrates, the UFRs allowed for easier operation & maintenance as well as more control of and consistency with nitrogen loading rates. Upflow anaerobic sludge blanket reactors (UASBRs) offer many advantages over other reactor systems, including high-space loading, low footprint, and resistance to shock and toxins.[14] Jin *et al*. compared the stability of the anammox process in different reactor configurations and found that the UASB reactor was the most stable configuration for substrate concentration compared to an upflow stationary fixed film (USFF) reactor, and an anaerobic SBR.[15] Sultana compared anammox enrichment in SBRs to that in a membrane upflow anaerobic sludge blanket (MUASB) system and observed more effective enrichment in the MUASB.[5] That study proposed the MUASB had better anammox enrichment than the SBRs because of the former's ability to capture planktonic anammox cells and to enhance the anammox granulation process.[5]

In this study, the Netherlands anammox from SBR 4 was transferred to a UFR that will be referred to as NL_UFR; the Virginia anammox from SBRs 5 and 6 was transferred to a UFR that will be referred to as VA_UFR; lastly, the Clackamas anammox was set up in a UFR that will be referred to as CL_UFR. Enrichment of anammox in these three UFRs, each with a different anammox sludge source, will be discussed and compared in this section. Change in nitrogen species concentration, nitrite consumption to ammonium consumption ratio ($\Delta[NO_2^-] : \Delta[NH_4^+]$), total

nitrogen removal, and total nitrogen removal efficiency were used to quantify anammox enrichment.

Anammox Activity in the Netherlands Anammox (NL_UFR)

The plot of change in N-species over time for NL_UFR revealed that anammox activity was not apparent until day 160 (~5 months) of operation (Figure 14). During the first 5 months, nitrite and nitrate were being consumed while the ammonium concentration remained relatively constant, which was indicative of heterotrophic denitrifying bacteria activity. Since the Netherlands anammox arrived in a mixed sludge form this behavior is not surprising. However, the length of this heterotrophic denitrifying bacteria activity may have been reduced under more optimal operating conditions, including a temperature regulated environment, optimal nitrogen loading rate, and optimal hydraulic retention time.

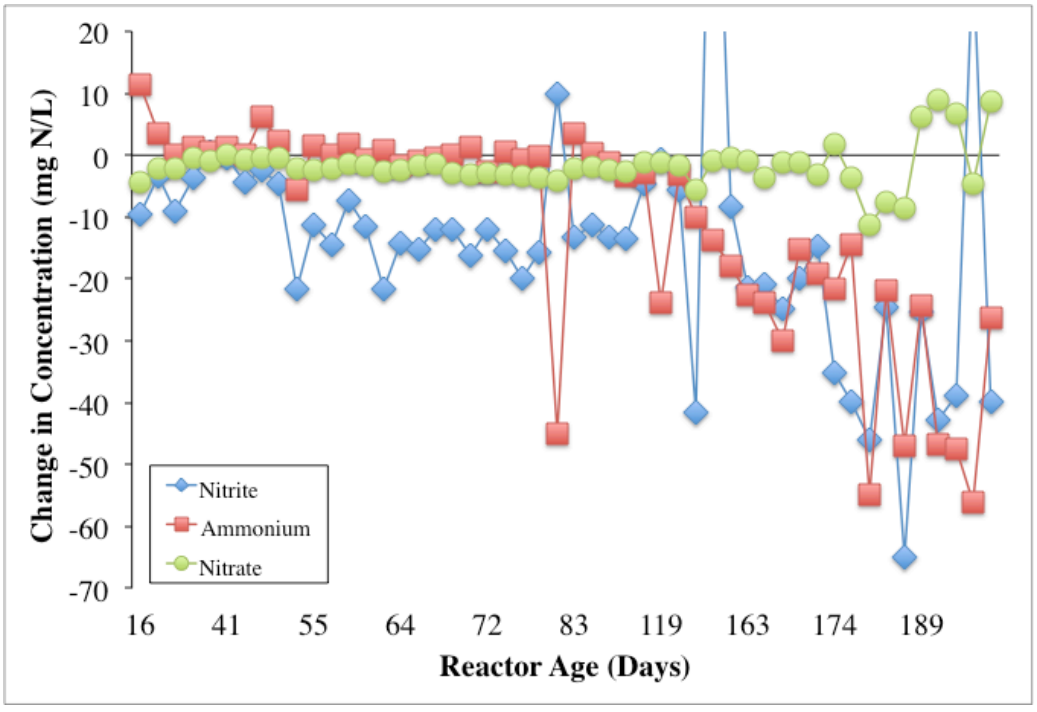

Figure 14. Change in nitrogen species over time in NL_UFR

The same trends are demonstrated by the nitrite consumption to ammonium consumption ratios (Figure 15). The ratios begin aligning around 1.32 after day 160 just as anammox activity became apparent in the previous plot.

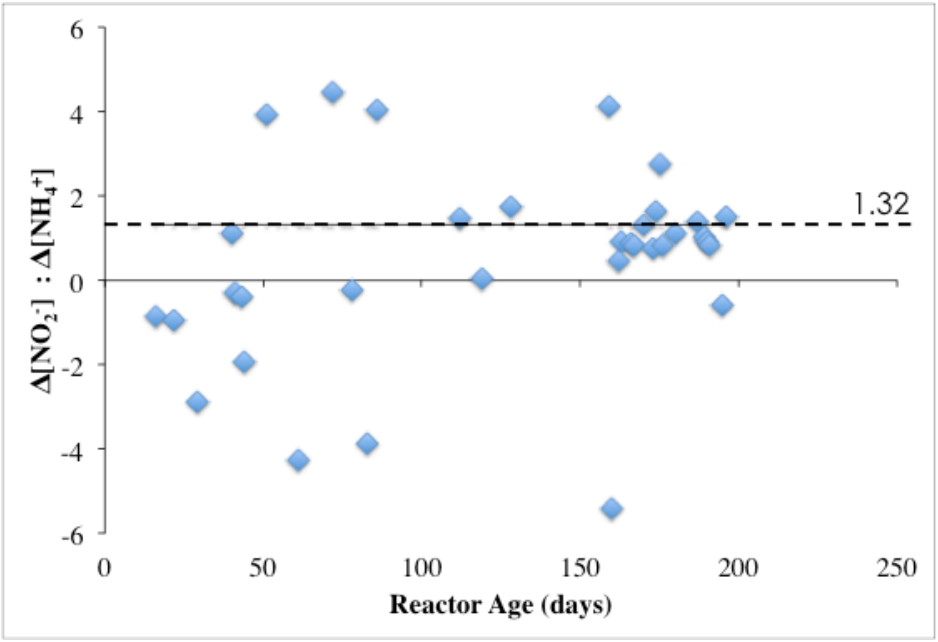

Figure 15. Ratio of change in nitrite to change in ammonium concentration over time for NL_UFR

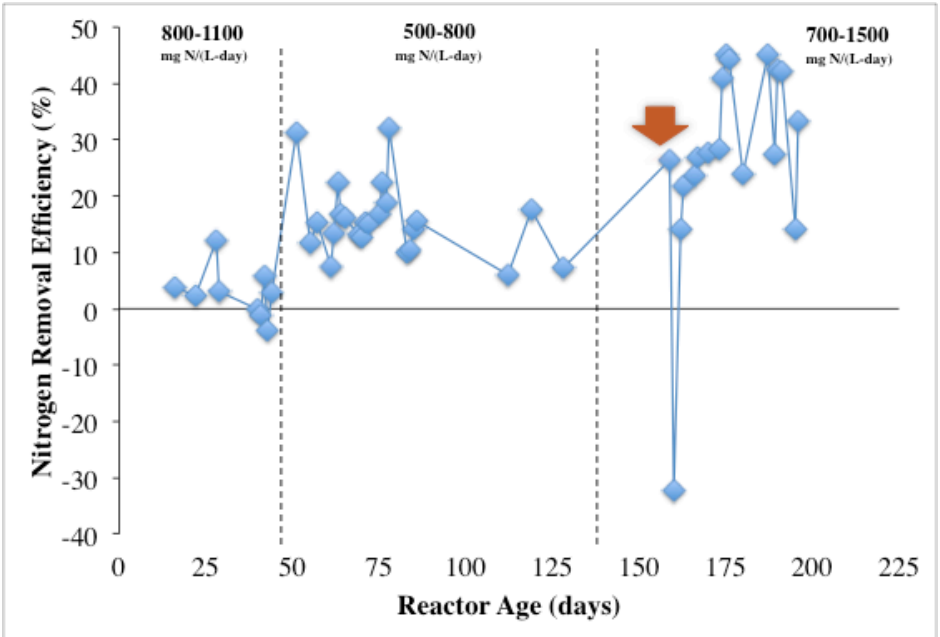

Figure 16. Total nitrogen removal efficiency in NL_UFR. The red arrow indicates beginning of noticeable anammox activity

Total nitrogen removal efficiency in NL_UFR increased over time, with a maximum removal efficiency of 45% (Figure 16). Additionally, total nitrogen removal rates (Figure 17) increased significantly around the same period that anammox activity becomes noticeable (indicated by the red arrow). This trend is reasonable since ammonium, which was not being removed earlier, started being consumed in addition to nitrite.

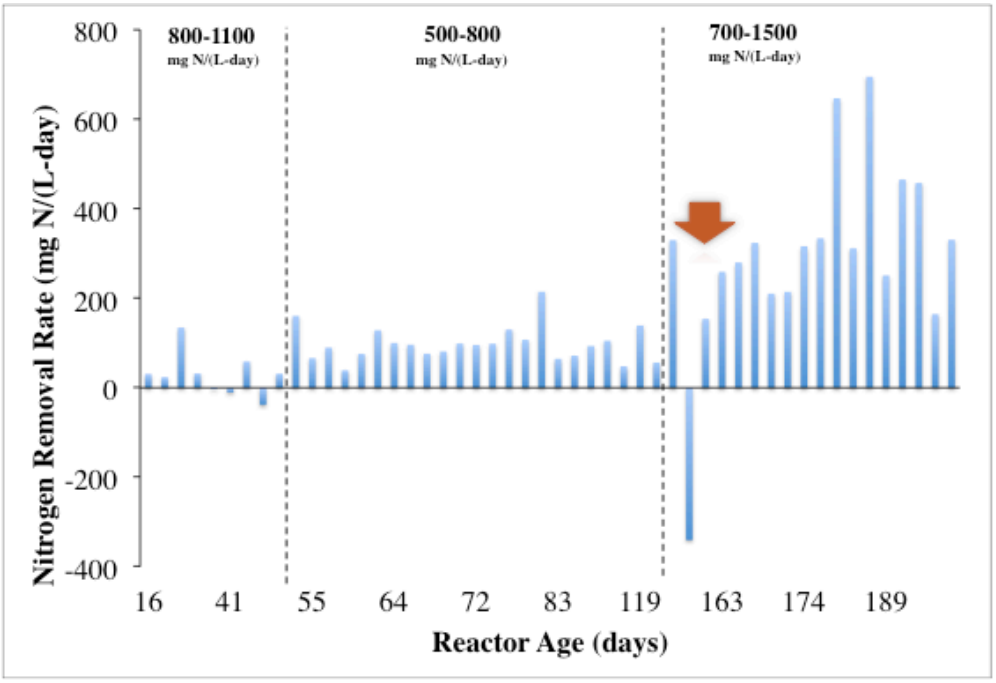

Figure 17. Total nitrogen removal rates in NL_UFR. The red arrow indicates beginning of noticeable anammox activity. Change in nitrogen loading rate is indicated by the vertical dotted black lines.

Anammox Activity in the Virginia Anammox (VA_UFR)

The plot of change in N-species over time for VA_UFR revealed that anammox activity was not apparent until day 112 (nearly 4 months) of operation (Figure 18). During the first 4 months, nitrite and nitrate were being consumed with no to minimal ammonium consumption, which was indicative that heterotrophic denitrifying bacteria activity was dominant. Similar to the NL_UFR, this behavior was not surprising since the Virginia anammox also arrived in a mixed sludge form. However, the anammox activity becomes apparent and eventually dominant in this reactor. Both the Netherlands anammox and Virginia anammox began exhibiting anammox activity ~5 months of enrichment in the UFR.

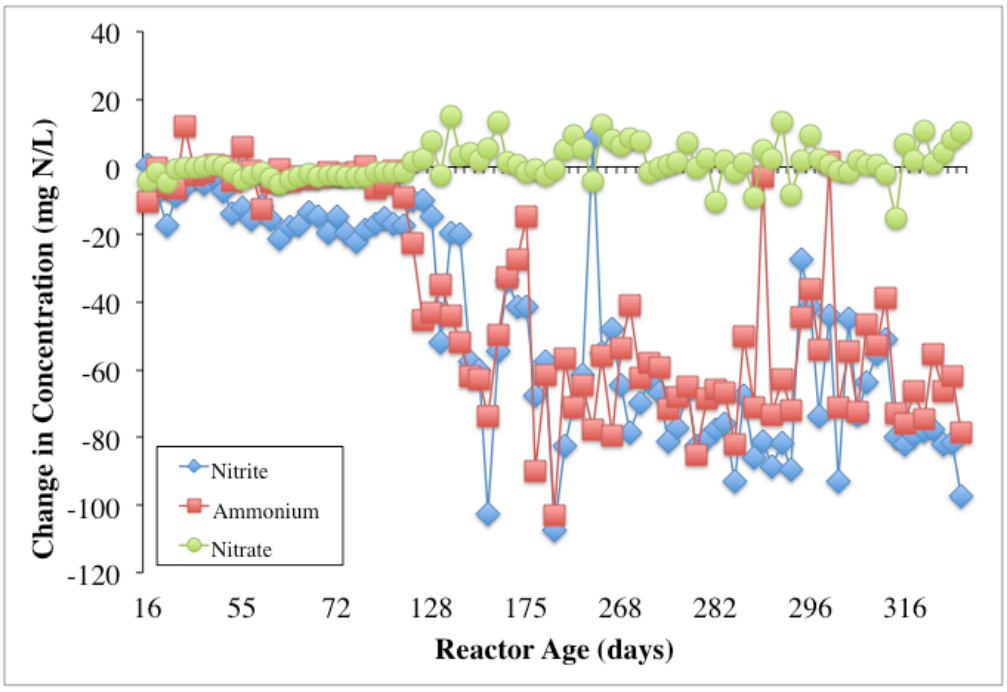

Figure 18. Change in nitrogen species over time in VA_UFR

The ratios of nitrite consumption to ammonium consumption in the VA_UFR were more consistently around 1.32, especially after the first 4 months of operation (Figure 19).

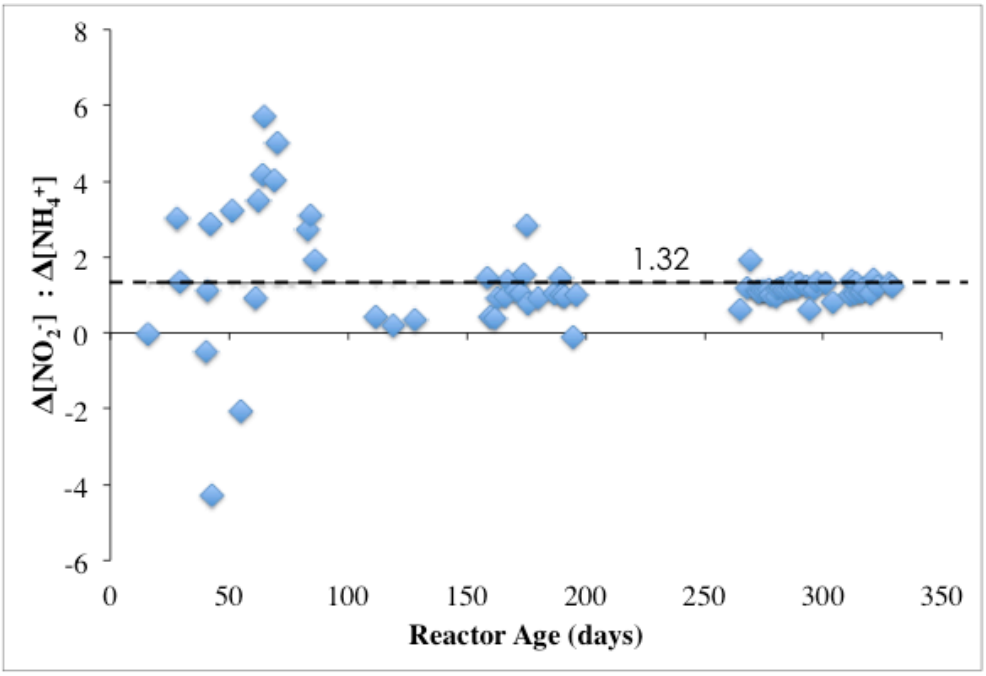

Figure 19. Ratio of change in nitrite to change in ammonium concentration over time for VA_UFR

Total nitrogen removal efficiency in the VA_UFR also increased over time, but achieved a maximum removal efficiency of 90%, nearly double that of the NL_UFR (Figure 20). Note that removal efficiencies were highest during nitrogen loading rates of 0.9-2.4 mg N/(L-day).

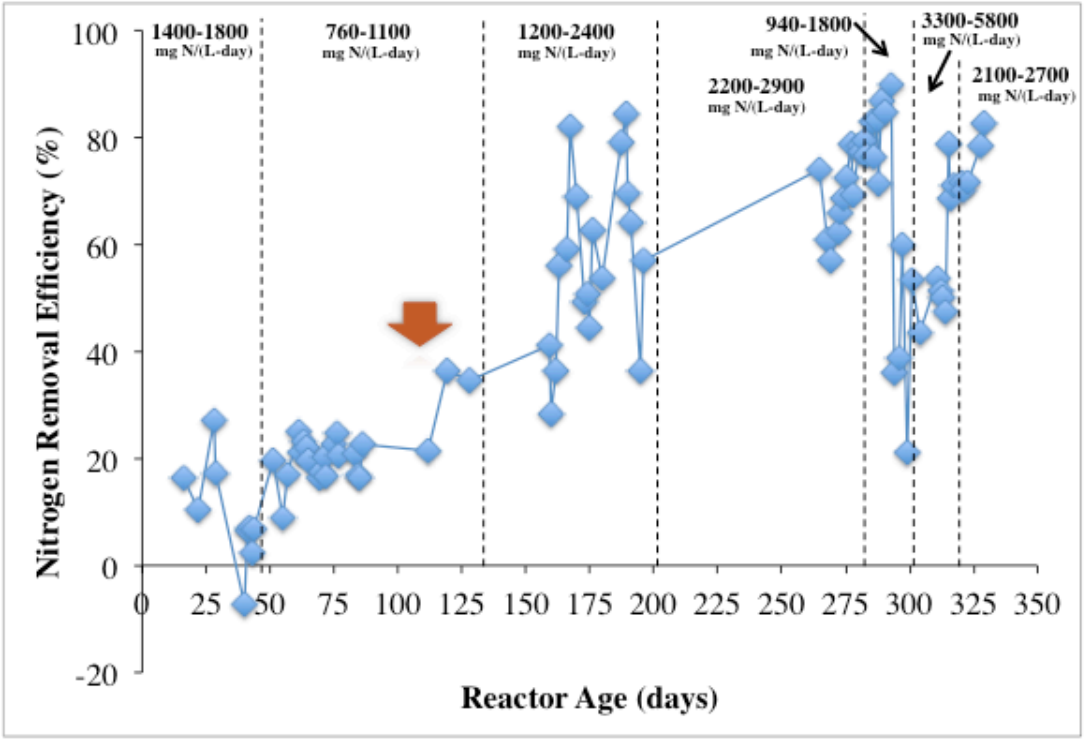

Figure 20. Total nitrogen removal efficiency in the VA_UFR. The red arrow indicates beginning of noticeable anammox activity

Similar to the trend in the NL_UFR, the total nitrogen removal rates (normalized to sludge volume) over time in VA_UFR also increase significantly once anammox activity becomes noticeable (Figure 21). In the VA_UFR the effect of changing the total nitrogen loading rate can be clearly see in the figure below as well. As the total nitrogen loading rate increased the total nitrogen removal rates also increased.

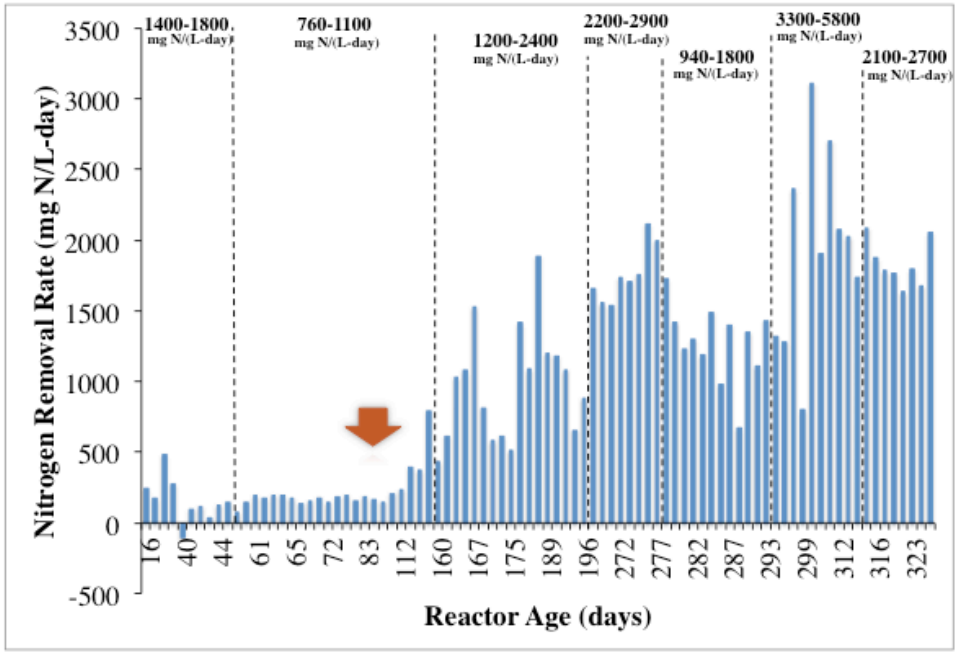

Figure 21. Total nitrogen removal rates in VA_UFR. The red arrow indicates beginning of noticeable anammox activity. Change in nitrogen loading rate is indicated by the vertical dotted black lines.

UFR 4: Clackamas Anammox

Unlike the other two reactors, the CL_UFR did not have a period during which heterotrophic denitrifying bacteria activity dominated (Figure 22). The plot of change in nitrogen species demonstrates anammox activity since the beginning of operation.

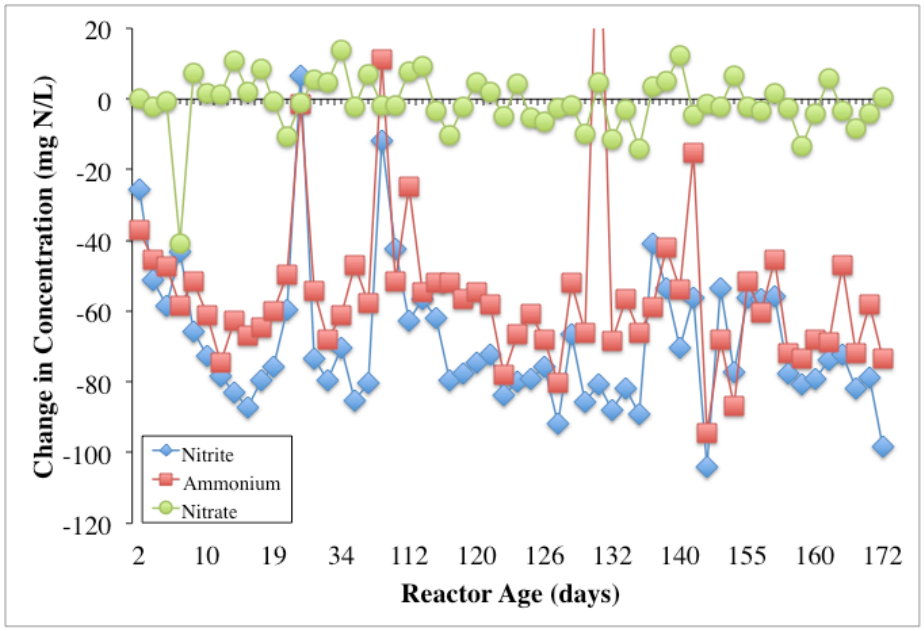

Figure 22. Change in nitrogen species over time in the CL_UFR

The ratios of nitrite consumption to ammonium consumption remain clustered around 1.32 throughout the entire operation time (Figure 23).

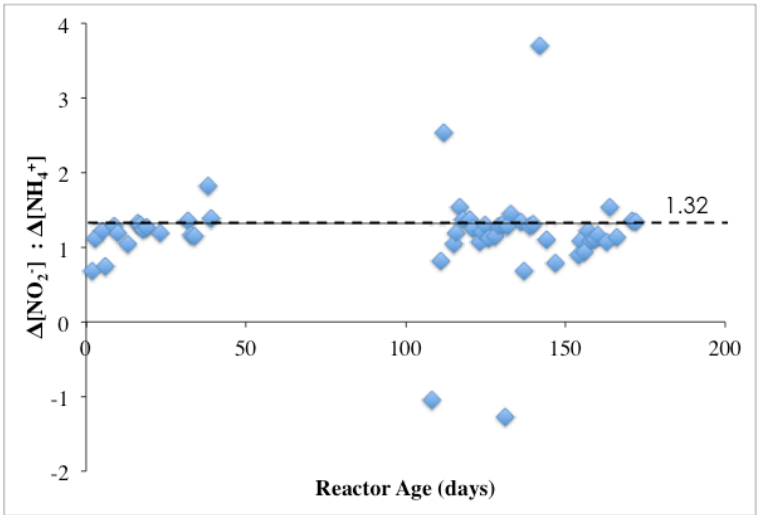

Figure 23. Ratio of change in nitrite to change in ammonium concentration over time for the CL_UFR

Total nitrogen removal efficiency in the CL_UFR started out much higher than the efficiency in the other two reactors, but achieved a maximum removal efficiency of 90%, similar to that of the VA_UFR (Figure 24). Day 30 and day 108 had the two lowest removal efficiencies likely because there were pump and fluid flow issues on those days. Note that removal efficiencies were highest during nitrogen loading rates of 2-6 mg N/(L-day).

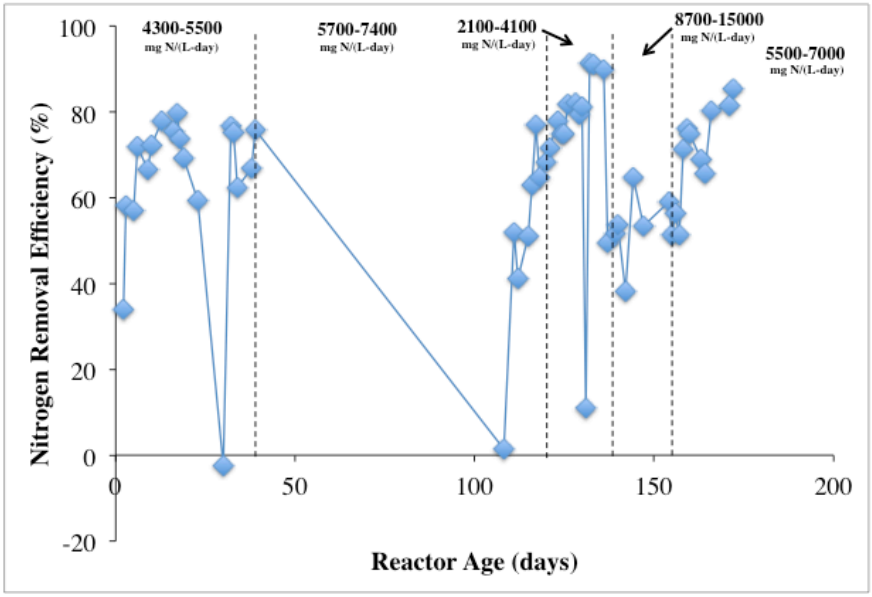

Figure 24. Total nitrogen removal efficiency in the CL_UFR

Similar to the trend seen in the VA_UFR the total nitrogen removal rates also increased as the total nitrogen loading rates were increased in the CL_UFR (Figure 25).

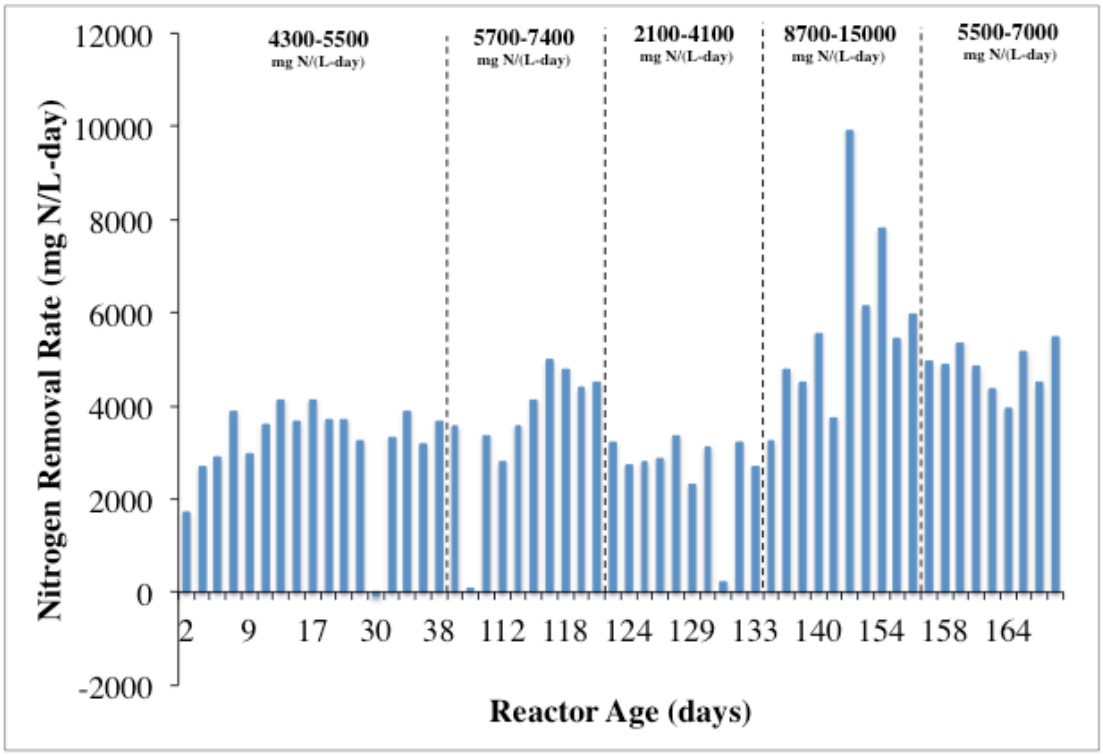

Figure 25. Total nitrogen removal rates in CL_UFR. Change in nitrogen loading rate is indicated by the vertical dotted black lines.

Comparing Anammox Activity in NL_UFR, VA_UFR, and CL_UFR

Based on the anammox activity data above, it was apparent that all UFRs exhibited anammox activity. However, the NL_UFR and VA_UFR both experienced a significant period of time (~5 months) without detectable anammox activity. The lack of noticeable anammox activity can be attributed to the long start-up period of anammox reactors, likely due to the slow growth rate of anammox. Dapena-Mora *et al*. found that it took 60 days before they observed the stable stoichiometric ratio of nitrite to ammonium consumption.[13] A study on the set-up and operation of the first full-scale anammox treatment plant in the Netherlands observed similar trends to those seen in the NL_UFR and VA_UFR during the start-up period. The anammox treatment plant Rotterdam had periods of growth, but no detectable anammox activity for the first 800 days of operation.[16] The researchers observed low nitrite conversion

as the predominant activity during the first 800 days and proposed that it was likely due to denitrification consuming nitrite with excess COD in the reactor since there was no ammonium removal or observable nitrate production.[16]

Comparing the maximum total nitrogen removal rates (normalized to anammox sludge volume) of the three reactors illustrated that the Clackamas anammox had the highest removal rates, followed by the Virginia anammox, and lastly the Netherlands (Figure 26). The CL_UFR maximum nitrogen removal rate was 14X higher than that of the NL_UFR and 4X higher than that of the VA_UFR. However, this cannot be attributed to higher anammox activity in one reactor since the highest removal rate occurred during the highest corresponding nitrogen loading rate range for each reactor, and these ranges do not overlap with one another. The nitrogen loading rate corresponding to the maximum nitrogen removal rate for the CL_UFR was 10-12 times that of the loading rate for the VA_UFR and ~3 times that of the NL_UFR. Thus, the maximum nitrogen removal rates of each reactor cannot be directly compared with each other.

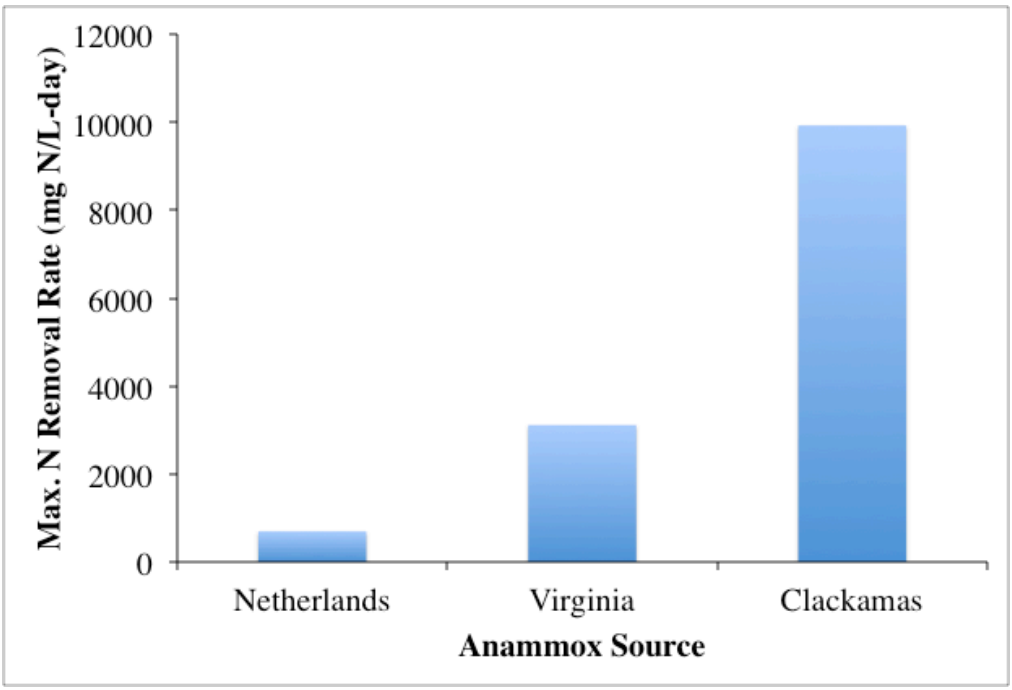

Figure 26. Comparison of the maximum nitrogen removal rates in the NL_UFR, VA_UFR, and CL_UFR

Sultana, who cultivated anammox bacteria from the same source in the Netherlands and Virginia in separate SBRs, observed much lower total nitrogen removal rates than the ones observed in this study. Note that total nitrogen was defined as N from NH_4^+ and NO_2^- only in that study. Sultana observed a maximum removal rate of 0.092 g N/L-day (0.092 kg N/m^3-day) in the VA_SBR while this study achieved a maximum removal rate of 3.1 g N/L-day (3.1 kg N/m^3-day) in the VA_UFR.[5] The observed maximum removal rate for the NL_UFR (0.70 g N/L-day or 0.70 kg/m^3-day) was also higher than the observed maximum removal rate for the NL_SBR (0.1 g N/L-day or 0.1 kg/m^3-day) in Sultana's study.[5] However, both studies consistently showed poorer anammox activity and enrichment in the Netherlands anammox from the SHARON process than the Virginia anammox from the DEMON process.

Differences in reactor type, loading rates, sludge volume, and the duration of anammox enrichment may explain the difference in removal rates (for the same anammox source) between the two studies. The CL_UFR observed the highest removal rates at 9.9 kg/m^3-day. The significant difference in loading rates could likely account for the large difference in maximum loading rates between the UFRs in this study. Additionally, studies have shown that reactors inoculated with highly enrich anammox granules perform better and tend to be more robust in response to sudden shocks of higher substrate concentration than reactors inoculated with sludge.[12, 15]

Also note that the maximum removal rates in this study were calculated per volume of anammox sludge since the sludge did not occupy the entire volume of the reactor. Adjusted to the reactor volume the maximum removal rates for the VA_UFR, NL_UFR, CL_UFR were 0.22 kg/$m^3_{reactor}$-day, 0.64 kg/$m^3_{reactor}$-day, and 6.0 kg/$m^3_{reactor}$-day.

Compared to the maximum removal rate of 1.5 kg N/m^3-day achieved by Strous *et al* in a fluidized-bed anammox reactor fed with sludge digestion effluent from a wastewater treatment plant, Sultana's values are much lower.[5] López *et al*. showed a

similar maximum nitrogen removal rate of 1.6 g N/L-day after a year of start-up in an SBR with activated sludge.[17] The NL_UFR in this study appeared to perform poorly relative to literature values, but the other two UFRs appeared to have significantly higher removal rates than those seen in literature. Differences in reactor type and culturing methods may explain the difference in max removal rates seen in literature and this study.

A comparison of the maximum nitrogen removal efficiencies in the reactors was more revealing about the level of anammox activity in the reactors. Both the VA_UFR and CL_UFR had similar maximum removal rates (90% and 91%, respectively), double that of the NL_UFR (45%) (Figure 27). The VA_UFR began with a much lower removal efficiency (~20%) than the one CL_UFR (~80%) began with, suggesting that the Virginia sludge was significantly enriched over time.

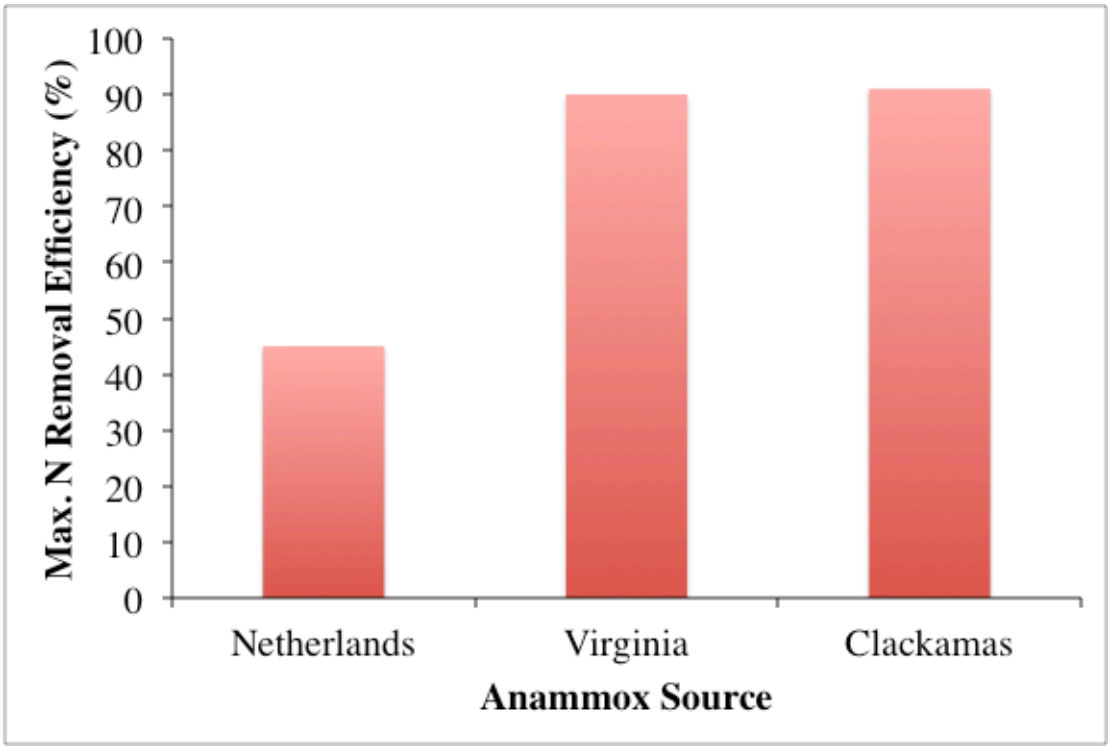

Figure 27. Comparison of the maximum nitrogen removal efficiency in the NL_UFR, VA_UFR, and CL_UFR

The difference in activity between the three reactors can also be contrasted using the anammox color (Figure 28). The NL_UFR was a dark brown, the VE_UFR a light

brown, and the CL_UFR was the characteristic red-orange color. To a certain extent, these colors correspond to the level of activity seen in the three reactors.

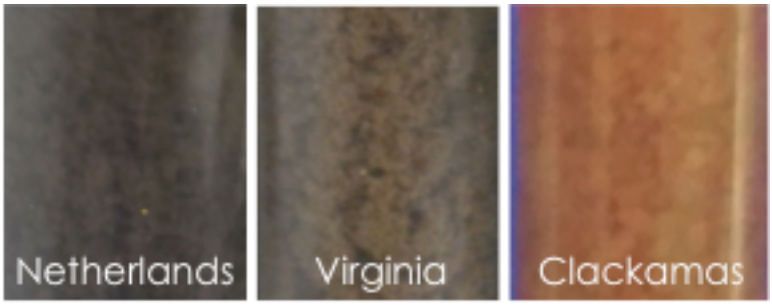

Figure 28. Comparison of the color of anammox in NL_UFR, VA_UFR, and CL_UFR

Mathematical Models for Anammox Growth Kinetics

First-Order Substrate Removal Model

The fit of the first-order substrate removal model is extremely poor with $R^2 < 0.1$ for both the VA_UFR and the CL_UFR (Figure 29a and 29b). The poor fit of this model was not surprising since the first-order model assumes that one of the substrates is limiting in a multi-component system. This assumption did not hold true for the anammox reactors since the nitrite consumption to ammonium consumption ratio was expected to be 1.32. Since the fit of the model was so poor, the first-order rate constants extracted from the model (0.34 for VA_UFR and -3.1 for CL_UFR) were not reasonable. In contrast, Sultana's study, which enriched the same Virginia anammox in SBRs, achieved a much better fit for the VA_SBR using the first-order substrate removal model ($R^2 = 0.80$).[5]

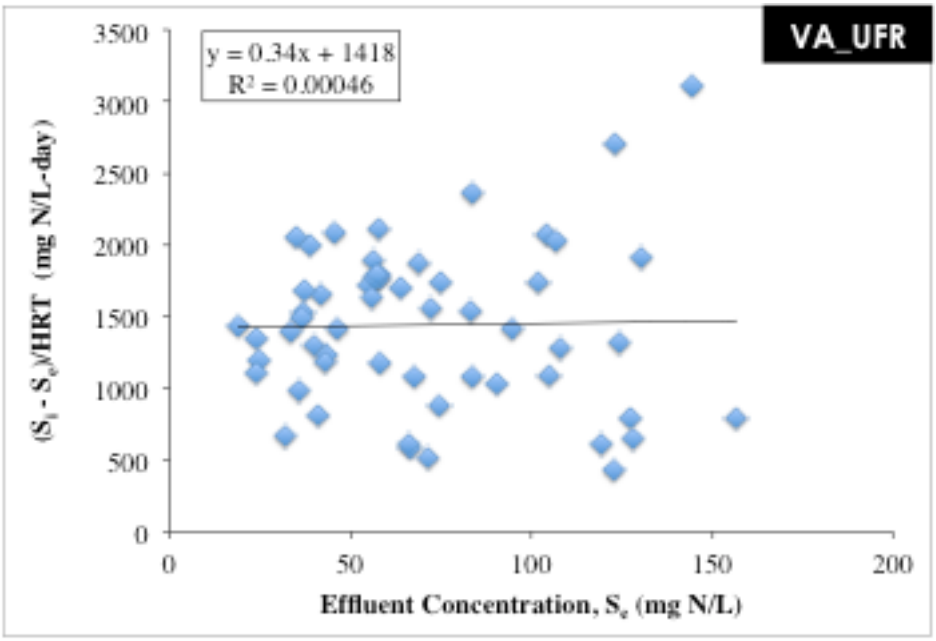

Figure 29a. First-order substrate removal model applied to the VA_UFR

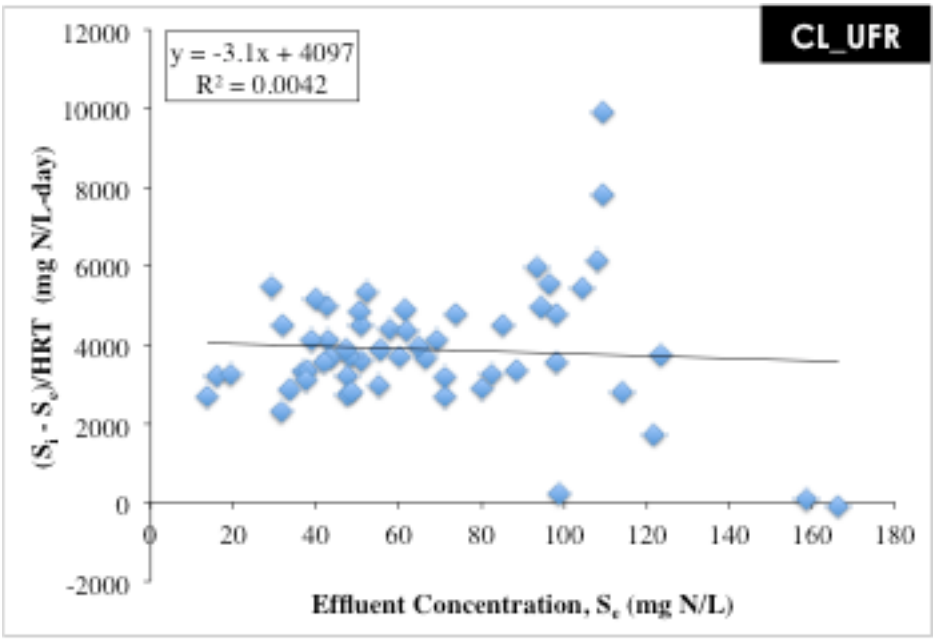

Figure 29b. First-order substrate removal model applied to the CL_UFR

Grau Second-Order Substrate Removal Model

The Grau second-order substrate removal model appeared to fit the data better than the first-order model, but the fit was still poor with $R^2 = 0.25$ and $R^2 = 0.40$ for the VA_UFR and the CL_UFR, respectively (Figure 30a and 30b). The improved fit was

likely because this model considers multi-component substrate systems. Sultana's study also saw an improvement in fit with the Grau model, but once again achieved a much better fit for the VA_SBR ($R^2 = 0.88$) than that achieved for the VA_UFR in this study.[5]

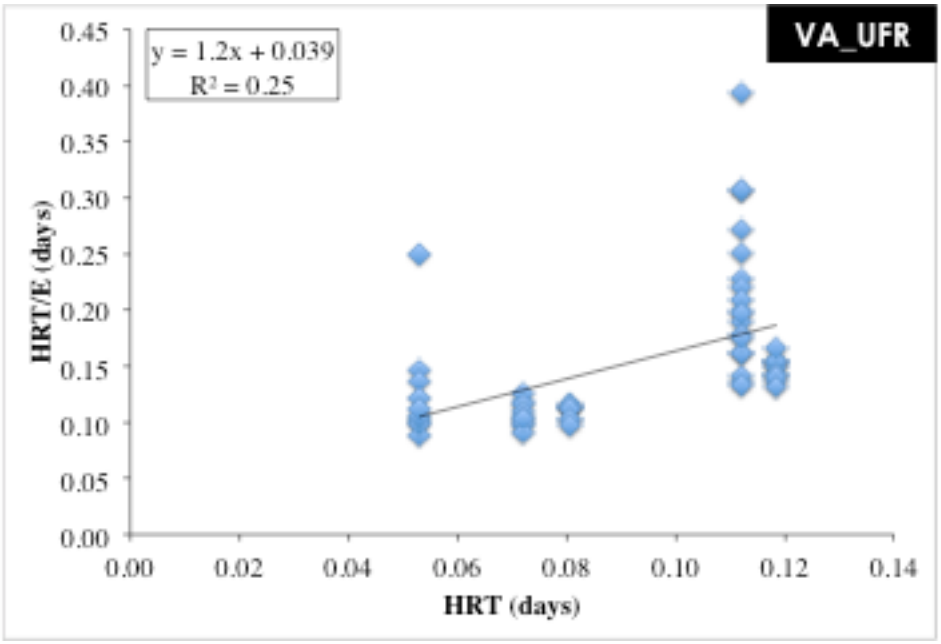

Figure 30a. Grau second-order substrate removal model applied to the VA_UFR

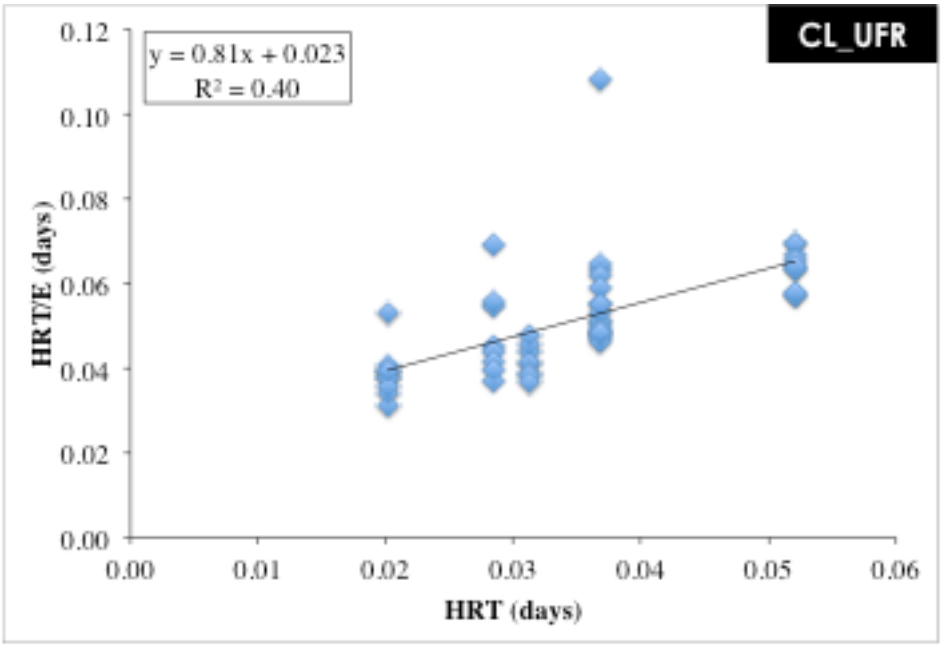

Figure 30b. Grau second-order substrate removal model applied to the CL_UFR

Modified Stover-Kincannon Model

The modified Stover-Kincannon model appeared to fit the data better than the other two models, but the overall fit was still poor with $R^2 = 0.48$ and $R^2 = 0.53$ for the VA_UFR and the CL_UFR, respectively (Figure 31a and 31b). As with the other two models, Sultana's study achieved a much better fit than this study ($R^2 = 0.89$) for the VA_SBR.

The maximum substrate utilization constant, U_{max}, and saturation constant, K_B, were determined from the slope and y-intercept using the method described in the Materials and Methods section (Table 4).

Table 4. Kinetic parameters from the modified Stover-Kincannon model for VA_UFR and CL_UFR

Anammox Source	**U_{max} (g N/L-day)**	**K_B (g N/L-day)**
Virginia	13	19
Clackamas	10	9

The U_{max} of the VA_UFR appears to be higher than that of the CL_UFR (13 g N/L-day compared to 10 g N/L-day). However, error of the values themselves (due to poor model fit) may explain the difference. Since the VA_UFR and CL_UFR also appeared to have achieved similar removal rates at the end of this study, it would not be surprising if the U_{max} values were also similar.

Sultana found the U_{max} and K_B for the VA_SBR to be 0.27 g N/L-day and 0.8 g N/L-day.[5] While the difference in quality of fit could partially explain the difference in kinetic parameters between this study and Sultana's, it is more likely that Sultana's reactors weren't as highly enriched with anammox as the reactors in this study. This pattern was evident in the lower nitrogen removal rates and removal efficiencies seen earlier as well.

The U_{max} and K_B for both UFRs in this study were comparable to literature values, although the values in this study may have a large error due to the relatively poor fit

of the model. Ni *et al*. observed a U_{max} and K_B of 11.4 and 12.4 g N/L-day, respectively, for a UASB reactor with granular anammox.[18] Ni *et al*. used highly enriched (93.7%) anammox granules in the study.[18] This may also explain the difference in kinetic parameters obtained by Sultana and this study, since the anammox in this study appeared to be more enriched than the anammox in Sultana's study.

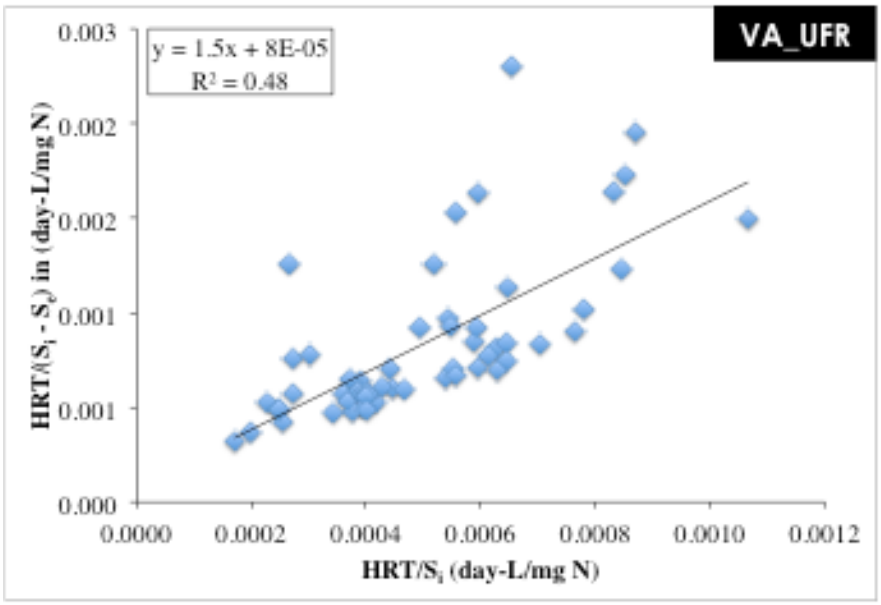

Figure 31a. Modified Stover-Kincannon substrate removal model applied to the VA_UFR

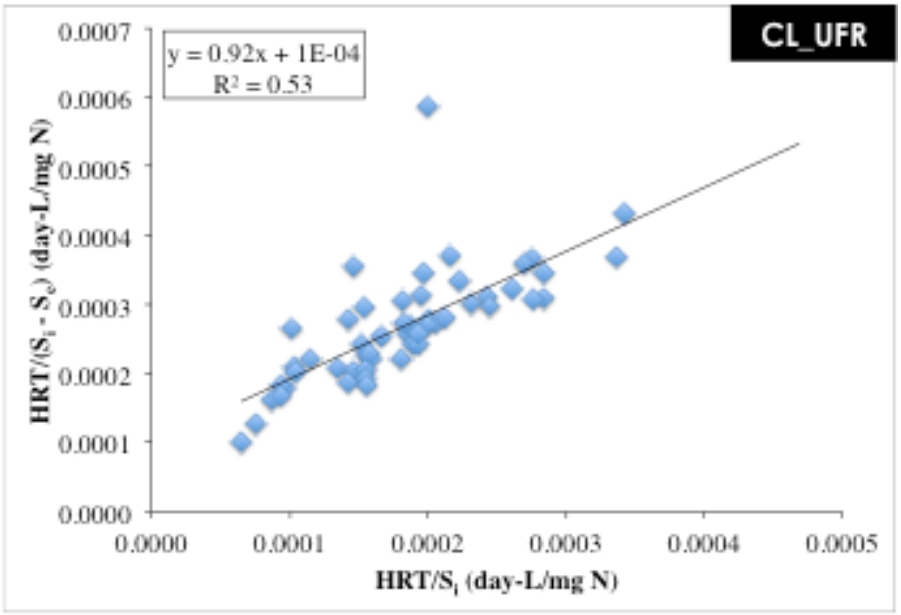

Figure 31b. Modified Stover-Kincannon substrate removal model applied to the CL_UFR

Differences in fit of the three models between Sultana's study and this study can likely be explained by the lack of adjustment time provided after changing loading rates in the anammox reactors in this study. The reactors were not allowed to reach steady state before taking samples used to create the mathematical models, while Sultana gave the SBRs in her study a 1-2 week adjustment period before performing kinetic experiments.[5] This likely contributed to the poor fit of the models as well as errors in kinetic parameters calculated in this study.

Conclusion

Research into the anammox has been rapidly growing since its discovery ~23 years ago. Both application of anammox technology and research are being conducted at the international level with China, Netherlands, the USA, Japan, and Germany currently leading in anammox-related publications.[9] While anammox can help reduce cost and greenhouse gas production if successfully implemented in WWTPs, anammox is difficult to culture due to its slow growth rate. This study focused on anammox improving enrichment using two different reactor configurations and applied mathematical models to describe anammox growth kinetics.

Anammox sludge from the Dokhaven Treatment plant in the Netherlands, HRSD York River Treatment plant in Virginia, and the pilot-scale anammox treatment plant in Clackamas, Oregon were enriched in this study. The Netherlands and Virginia anammox were initially cultured in SBRs (NL_SBR and VA_SBR), but were transferred to up-flow column reactors due to poor anammox activity in the SBRs. Up-flow column reactors appear to be more suitable for anammox enrichment than the small-scale sequencing batch-reactors used in this study, likely due to superior biomass retention and organic carbon removal in the UFRs. The nitrogen species consumption over time as well as the stoichiometric ratios of nitrite to ammonium consumption over time indicated that all UFRs had anammox activity by the end of this study. The maximum nitrogen removal efficiencies achieved were 45% (NL_UFR), 90% (VA_UFR), and 91% (CL_UFR).

Comparison of the three UFRs also suggested that the type of anammox treatment process used at a WWTP facility might affect the length of time required to enrich anammox. Particularly, the anammox fraction of the biomass may affect the ability of anammox to compete with heterotrophic denitrifying bacteria in the reactor. Lastly, the modified Stover-Kincannon model appeared to be the most suitable for modeling the kinetics of the anammox reactors in this study.

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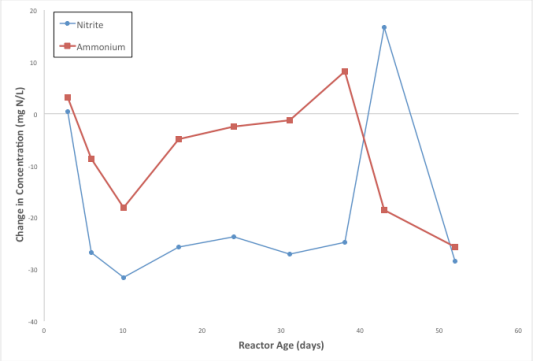
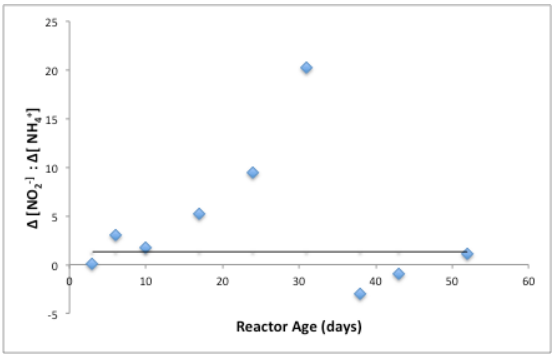
Appendices

Appendix A: Sequencing Batch Reactor Data

SBR 1 (Virginia Anammox)





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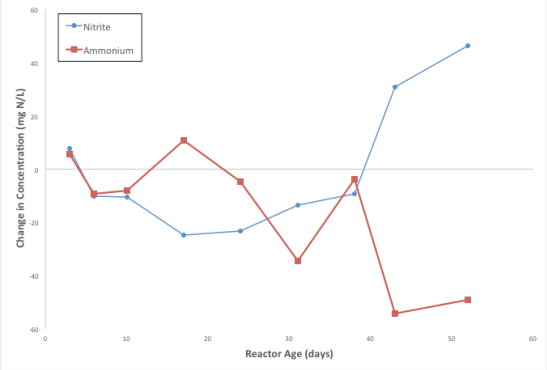
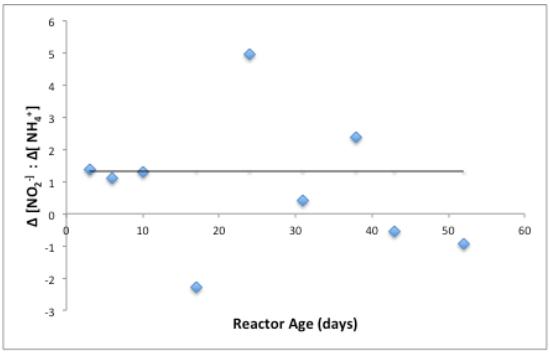
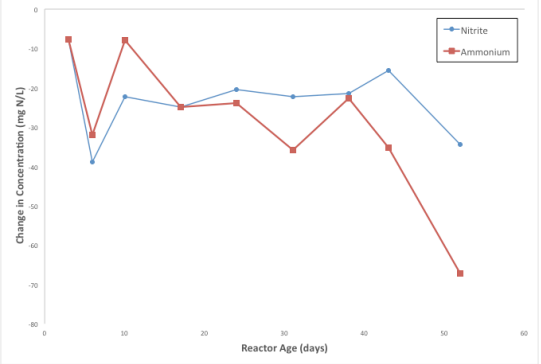
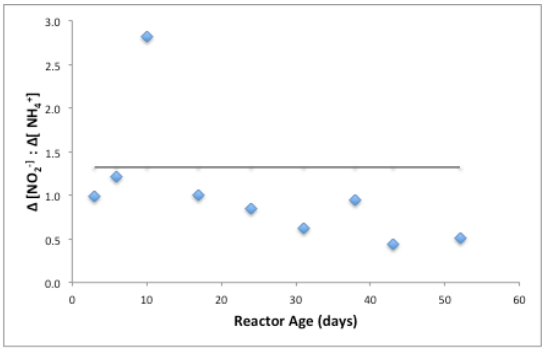


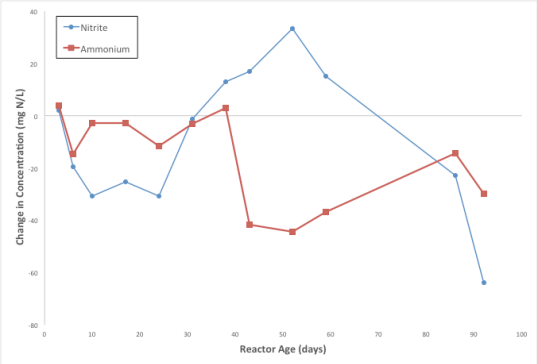


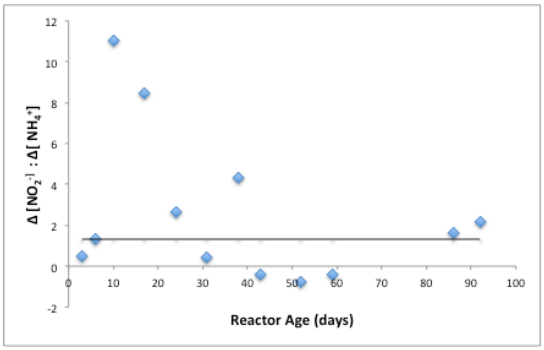
SBR 3 (Virginia Anammox)



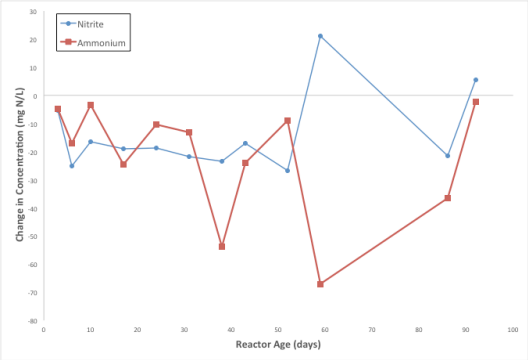


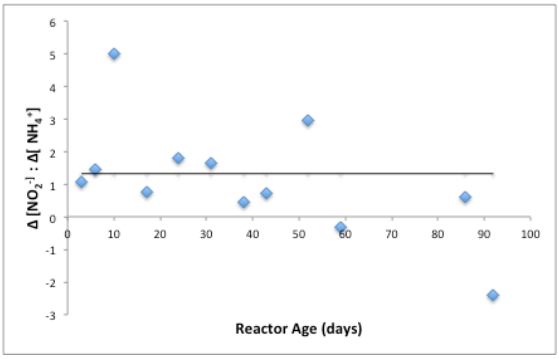
SBR 4 (Netherlands Anammox)



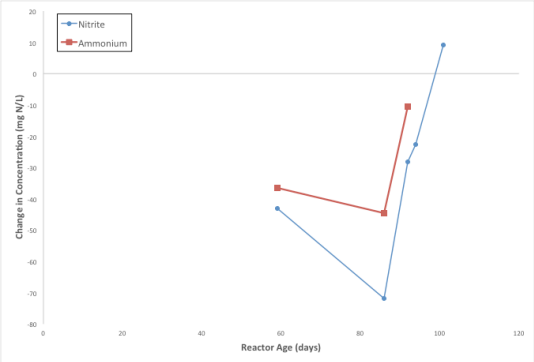


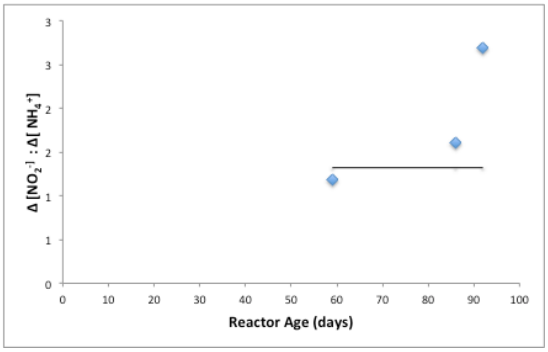
SBR 5 (Virginia Anammox)





SBR 6 (Virginia Anammox)

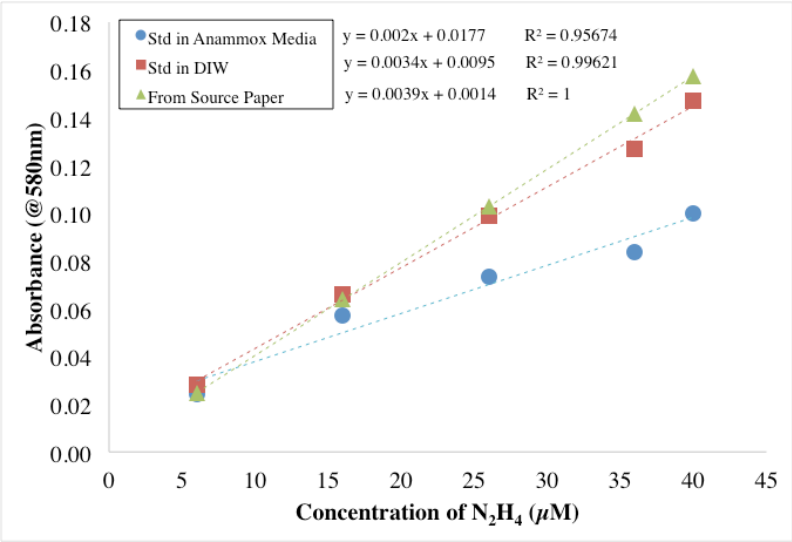




Appendix B: Hydrazine Standard Curve and Experiment

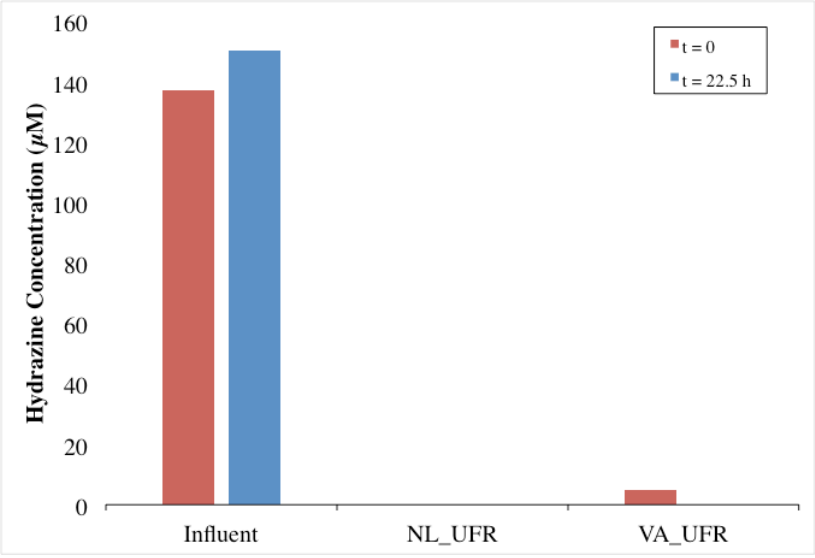
The methodology for the hydrazine assay was adapted from a paper by Zargar and Hatamie.[19]

Hydrazine Standard Curve

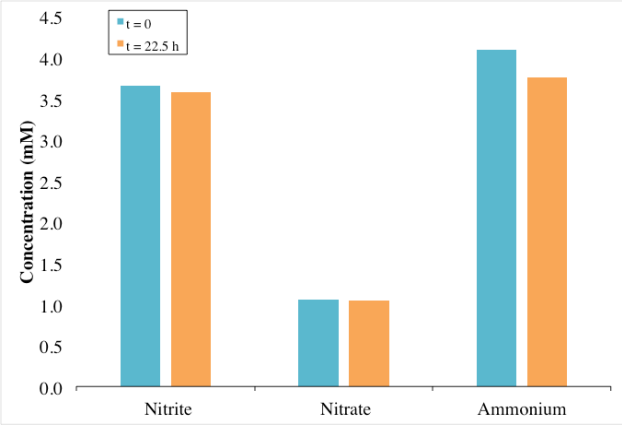


For the hydrazine experiment 4.86 mg of N_2H_4 per L (0.15 mM) was added to regular anammox media. Hydrazine, NO_2^-, NO_3^-, and NH_4^+ were measured at t = 0 and t = 22.5 hours.

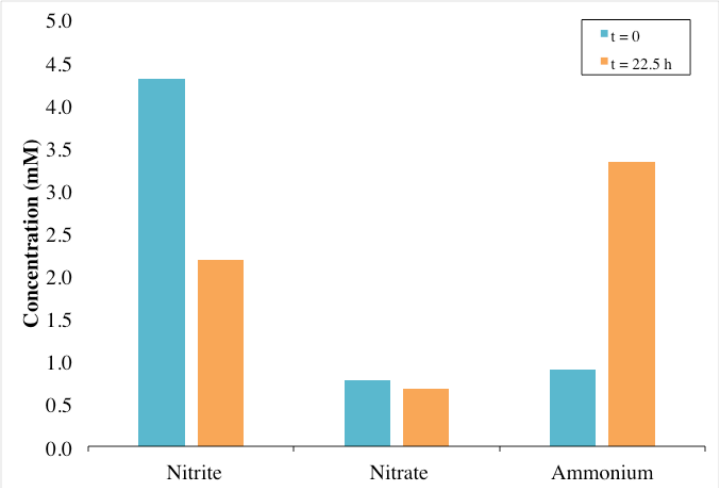
Hydrazine Concentration in NL_UFR and VA_UFR



Nitrogen Species Concentrations in Influent



Nitrogen Species Concentrations in Effluent of NL_UFR



Nitrogen Species Concentrations in Effluent of VA_UFR