Hydrogel Immobilizations for Anammox Enrichment

by
Grant Stein

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented June 7, 2017
Commencement June 2017

AN ABSTRACT OF THE THESIS OF

Grant Stein for the degree of Honors Baccalaureate of Science in Bioengineering presented on June 7, 2017. Title: Hydrogel Immobilizations for Anammox Enrichment.

Abstract approved:_____

Tyler Radniecki

Long delays in reactor startup due to high biomass washout have hindered the development of efficient and cost-effective wastewater treatments based on the anammox process. This can be remedied with efficient biomass retention systems. In the following study, anammox bacteria were successfully enriched in hydrogel immobilizations of polyvinyl alcohol (PVA) and calcium alginate (CA). Despite some biomass washout, a mixed-culture anammox immobilization in 2% CA produced similar activity to a packed-bed sludge reactor and had the highest activity of anammox of the gel immobilizations, achieving a maximum nitrogen removal rate of 1.0 mmol N/hr with 100% efficiency. Due to diffusion limitations in 10% PVA and 10% PVA:1% CA treatments, nitrogen removal achieved a maximum value of 0.4 mmol N/h with 60% efficiency. Modeling of an upflow packed-bed reactor with the modified Stover-Kincannon kinetics model yielded a U_{max} of 0.17 g N/L-day with a K_B of 0.13 g N/L-day.

Key Words: Alginate, Anammox, Hydrogel, Immobilization, Nitrogen Cycle, PVA

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Grant Stein, Author

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Acronyms

AOB……. Ammonia Oxidizing Bacteria

Anammox…… Anaerobic Ammonium Oxidation

CA…… Calcium Alginate

CMC……. Carboxymethyl Cellulose

HRT……. Hydraulic Retention Time

NARR……. Nitrite-Ammonium Removal Rate

NNR…… Nitrite-Nitrate Ratio

NOB……. Nitrite-Oxidizing Bacteria

NRE……. Nitrogen Removal Efficiency

NRR……. Nitrogen Removal Ratio

PVA……. Polyvinyl Alcohol

SBR……. Sequencing Batch Reactor

UFC…….. Up-Flow Column

Symbols

K_2……. second-order substrate removal constant (day^{-1})

K_B……. saturation value constant (mg/L-day)

K_s……. half-saturation constant (mg/L)

Q……. flow rate (L/day)

S_e……. effluent substrate concentration (mg/L)

S_i……. influent substrate concentration (mg/L)

θ_H……. hydraulic retention time (day)

U_{max}…….. maximum substrate utilization rate (day^{-1})

V……. volume of media for all biomass (L)

X……. biomass concentration (mg/L)

Part 1. Background

1.1 Biological Removal of Nitrogen from Wastewater

Nitrogen removal from wastewater is of growing concern worldwide, as ammonia and nitrite can cause eutrophication and create a toxic environment for aquatic organisms (Tchobanoglous *et al*, 2003). Physico-chemical processes have been designed for nitrogen removal from wastewater, but these techniques are less cost-effective than biological processes (Metcalf and Eddy, 2003). Numerous microbial methods have been established for this purpose, but limitations remain due to increasingly strict regulations and high operating costs. Conventional nitrogen removal plants use both autotrophic nitrification and heterotrophic denitrification processes (Whitacre, 2008).

1.2 Nitrification

Nitrification follows a two-step process with co-metabolism of ammonia-oxidizing bacteria (AOB), which oxidize ammonium to nitrite, and nitrite-oxidizing bacteria (NOB), which oxidize nitrite to nitrate, as shown in Eq. (1) and (2).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (1)$$

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

The experimentally determined overall metabolic reaction results in Eq. (3), which shows that oxygen must be present in the system. Bicarbonate is necessary to buffer the system since each ammonium molecule produces a free proton.

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow$$
$$0.021C_5H_7NO_2 + 1.041H_2O + 0.98NO_3^- + 1.88H_2CO_3 \quad (3)$$

1.3 Denitrification

Anoxic denitrification, the second conventional stage of nitrogen removal, is the conversion of nitrate to dinitrogen gas using organic carbon as an electron donor, as shown in Eq. (4). Commonly used electron donors include acetate and organics present in the wastewater, though methanol is generally the preferred carbon source (Metcalf and Eddy, 2003). Additional organic substrate is used to create biomass, (Eq. (5)) (Whitacre, 2008).

$$NO_3^- + 0.833CH_3OH \rightarrow 0.8333CO_2 + 0.5N_2 + 1.167H_2O + OH^- \quad (4)$$

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (5)$$

There are several drawbacks to using the nitrification-denitrification system in the biological removal of nitrogen from wastewater. The two-stage system requires two separate process conditions, thus requiring more space and processing time (Lee *et al*., 2001). A large amount of oxygen is required for nitrification, while large quantities of methanol and external carbon sources are needed for denitrification. These requirements set high operating costs for conventional biological nitrogen removal (Jetten *et al*., 2002).

1.4 Discovery of Anammox

In 1977, it was predicted based on thermodynamics and evolution that two groups of lithotrophs had yet to be discovered. One of these “missing lithotrophs” was thought to use

nitrite as an oxidant in place of oxygen and ammonium to act as an electron donor, permitting the denitrification process to proceed anoxically, directly producing dinitrogen gas (Broda 1977). This prediction was confirmed almost two decades later, when researchers operating a pilot-scale fluidized bed reactor unintentionally discovered what would become known as the "anammox," or anaerobic ammonium oxidation, process (Mulder 1995). The role of anammox in the nitrogen cycle is depicted in Figure 1.

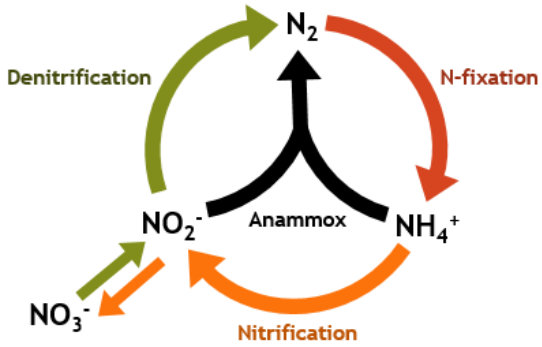

Figure 1. The Role of Anammox in the Nitrogen Cycle.

1.5 Anammox Process

The anammox process is conducted in the anammoxosome organelle of five bacterial genera (*Candidatus Brocadia, Kuenenia, Sclindua, Anammoxoblobus, Jettenia*) in the phylum *Planctomycetes* (Hu *et al*., 2010, Zhang and Lie, 2014). The process uses ammonium as an electron donor and nitrite as an electron acceptor in a three-step reaction. First, nitrite is converted to nitric oxide (Eq. (6)), which permits the anoxic activation of and subsequent conversion of ammonium into hydrazine (Eq. (7)) (Kartal *et al*., 2011). Hydrazine is then

broken down into dinitrogen gas and water (Eq. (8)). The overall catabolic reaction is summarized in Eq. (9).

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (6)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (7)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (8)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (9)$$

Anammox bacteria are autotrophic, using bicarbonate as the carbon source for anabolism Eq. (10) (van Graaf *et al*., 1996). The reduction of bicarbonate is made possible by the generation of electrons in Eq. (8).

$$HCO_3^- + 1.13NO_2^- + 0.066HCO_3^- + 0.053H^+ \rightarrow$$
$$0.99N_2 + 0.066CH_2O_{0.5}N_{0.15} + 0.14NO_3^- + 2.00H_2O \quad (10)$$

When the catabolic and anabolic reactions are combined, the stoichiometry of the overall reaction results in the following theoretical reaction (Eq. (11)):

$$NH_4^+ + 1.13NO_2^- + 0.066HCO_3^- + 0.053H^+ \rightarrow$$
$$0.99N_2 + 0.14NO_3^- + 2.00H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad (11)$$

Strous found that the experimentally-determined stoichiometry, depicted in Eq. (12), is quite similar to the theoretical overall reaction (Strous *et al*., 1998).

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow$$
$$1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \quad (12)$$

1.6 Advantages of the Anammox Process

Since its discovery, a lot of attention has been given to the anammox process as an efficient alternative to traditional nitrification/denitrification methods (Jetten *et al.*, 2002; Whitacre, 2008). When stable operation is achieved with an anammox-based system, it has significant advantages over classical biological nitrogen removal systems. Neither carbon dioxide nor nitric oxide are produced, the process does not require organic carbon or oxygenation, and there is substantially less surplus sludge produced in the anammox process. These factors significantly reduce operational costs of full-scale applications (Jetten *et al*., 2001, Fux *et al*, 2004).

1.7 Challenges of the Anammox Process

The biggest barrier to adoption of the anammox process is the slow growth rate of the bacteria. Under optimal growth conditions, the doubling time of anammox has been reported from 3to 11 days (Strous *et al*., 1998; van der Star, 2007), versus 20 minutes for *E. coli*, a representative heterotroph. Low biomass yield also creates a significant issue for adoption of the process, resulting in a typical reactor startup time of three months (Van de Graaf *et al*., 1996). In 2007, the first full scale-up of a granular anammox process took longer than

anticipated due to poor scale up from low anammox starting concentrations, and process upsets (Abma *et al*, 2007). The granular system was chosen because previously demonstrated biofilm reactors required higher-cost carrier material. However, startup took 3.5 years instead of the anticipated 2 years due to unstable operation, freezing, loss of biomass, poor mixing, and dead zones due to sulphide formation. Biomass retention was also an issue. Anammox enrichment for faster startup has also been hindered by competition with other microbial processes, such as heterotrophic denitrification, which is a faster process than anammox (Ahn, 2006). While there are more than 100 full-scale anammox plants worldwide, (Lackner *et al.*, 2014), these challenges deter further adoption of full-scale anammox systems.

1.8 Immobilization for Biomass Enrichment

Free-cell anammox enrichment is made more difficult by mechanical stress and nutrient shock by high concentrations of nitrite (Isaka *et al*., 2006; Magrí *et al*., 2012). Biomass washout is also an issue due to their often-free-floating nature as plankton, which is intensified by the metabolic production of N_2 gas (Chen *et al.*, 2010). Anammox immobilization has been shown to reduce the effects of these factors, allowing anammox to achieve higher cell densities and superior nitrogen removal rates, as well as permitting simpler reactor operation and maintenance. Immobilization of anammox has been accomplished using fixed-bed and membrane bioreactors with some success (Fux *et al.*, 2004; Trigo *et al.*, 2006).

1.9 Hydrogel Immobilizations for Anammox Enrichment

Immobilizing anammox in gel beads is also a viable option, and has recently been tested with a variety of different gel carriers (Ali *et al*., 2015; Isaka *et al*., 2006; Magrí *et al*., 2012; Zhu *et al.*, 2009). To create an ideal gel matrix, tradeoffs between mechanical stability

and biological activity must be considered. Typically, beads with greater biological activity are less mechanically stable and either dissolve or swell over time (Zhu *et al.*, 2009). Carboxymethyl cellulose (CMC), polyvinyl alcohol (PVA), and calcium alginate (CA) have previously been considered for suitable anammox immobilizing matrices as they are inexpensive, nontoxic, and reversibly crosslinked (Zhu *et al.*, 2009). CMC was shown to be too weak of a gel for long-term enrichment, while CA and PVA are complementary gels that, when blended, exhibit good stability and high biological activity (Zhu *et al.*, 2009).

1.9.1 Calcium Alginate

CA is a water-soluble seaweed derivative used commercially in the food and drug industry and has been commonly used for cell immobilizations since it is nontoxic, inexpensive, and easy to use (Bickerstaff, 1997). Alginate is a polyuronic acid made up of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues which form homopolymeric blocks. These blocks are interspersed with heteropolymeric regions; i.e. MM and GG with MG blocks. Divalent cations covalently bind to alginate, forming a gel. The strength of the binding is directly dependent upon the content of G blocks (Bickerstaff, 1997). Interfacial polymerization occurs instantaneously upon addition to a calcium solution. The interior of the resulting gel continues to polymerize as calcium ions diffuse through the gel. This gel is mechanically stable and highly porous, with pores typically between 5-200 nm, which provides efficient diffusion through the gel. Alginate is affected by the initial gel concentration, the growth of immobilized cells, and hydrodynamic sheer, which may be minimized in packed columns (Bickerstaff, 1997). The matrix is softened by divalent cations such as phosphate and magnesium cations that can exchange with calcium (Zhu *et al.*, 2009). CA also loses its crosslinking calcium ions through passive diffusion.

1.9.2 Polyvinyl Alcohol

PVA is a nontoxic gel commonly used in cell immobilization and is economically feasible for industrial applications (Bickerstaff, 1997). PVA beads are mechanically durable but tend to agglutinate and swell in synthetic wastewater. PVA is typically crosslinked with boric acid to form a bead, which is subsequently solidified with phosphate via esterification. While boric acid is cytotoxic, heterotrophic denitrifiers have been successfully immobilized with good stability over a long time in a continuously stirred tank reactor (Bickerstaff, 1997). Borate can also be replaced with sulfate, which results in reduced toxicity and swelling relative to other crosslinking solutions (Idris *et al*., 2011, Takei *et al*, 2012; Bach & Dinh, 2014). One of the drawbacks to PVA is its dense structure that has poor gas permeability; this can result in buildup of gas bubbles and subsequent floatation of the gel beads (Bickerstaff, 1997). This can be overcome by mixing the gel with CA during polymerization (Bickerstaff, 1997). CA is subsequently washed out by treatment in phosphate solution, creating porous beads. However, the issue of agglutination is still an issue in aqueous environments (Magri *et al.*, 2012).

1.9.3 PVA-Alginate Copolymer

To overcome the limitations of either PVA and CA alone, they can be combined into a homogenous gel matrix that permits good biological activity and mechanical stability. If CA is not removed with phosphate, hydrogen bonding occurs between PVA hydroxyl and CA carboxyl groups (Zhu *et al*., 2009). CA serves to diffuse the electrostatic charge density of the large PVA chains, preventing agglutination of the gel beads. When considering immobilization treatments for biologically removing nitrogen from wastewater, the stability and porosity of the gels are of particular importance (Takei *et al*., 2011). These factors are

largely influenced by the gel to biomass ratio, the ratio between PVA and CA, and the type and duration of crosslinking (Zhu *et al*., 2009).

In a recent study by Ali *et al.*, anammox sludge was successfully enriched in PVA-CA beads in up-flow columns (UFCs). The gel beads permitted substantially better nutrient diffusion than in the granular anammox control, demonstrating the advantage of PVA-CA immobilization (Ali *et al.*, 2015). While promising, several questions remain, such as the effect of chemically crosslinking PVA for long-term stability. In the study, only calcium chloride was used as a crosslinker, so it is unlikely that there was any direct bonding of PVA beyond its interaction with CA. The study was conducted for one month, so it was not determined if the beads could persist with such high microbial activity. It is also not known how anammox will perform in different crosslinking conditions, and it is possible that other crosslinkers may create more stable beads with even greater activity, further optimizing the PVA-CA recipe for anammox enrichment.

1.10 Kinetics

Comparing the relative growth rates of anammox in immobilizations with other systems can elucidate the effectiveness of immobilizations for enrichment. Several models have been used previously to predict reactor effluent substrate concentrations for anammox, with Grau Second-Order (Grau *et al*., 1975) and Stover-Kincannon modified (Stover and Kincannon, 1982) models proving the most reliable (Sultana, 2016). Both models are modifications of the Monod model (Monod, 1949).

The Monod model is commonly used in predicting process kinetics in bioreactors. It represents the kinetics of a single rate-limiting substrate and relating microbial growth in a pure culture suspended in a liquid media at constant temperature and is applicable over a

large range of substrate concentration. The model can be applied to approximate the consumption of complex substrates in a mixed culture, assuming similar kinetics of a single substrate in pure culture (Chen *et al*., 2013). The Monod model relies upon parameters which are difficult to approximate in our system, such as biomass and biomass yield, and supposes that effluent substrate concentration is independent of influent concentrations and flowrate. Yet, substrate effluent concentration is not independent of influent concentration, particularly in mixed cultures (Grau *et al*., 1975). The Monod model must be subsequently modified to fit the needs of these systems. Recently, the Grau Second-Order and Stover-Kincannon modified models have been demonstrated to adequately predict substrate consumption in our mixed anammox cultures (Sultana, 2016).

These models require several assumptions which are common to anaerobic biofilm kinetic models and are applicable to anammox systems (Saravanan and Sreekishnan, 2006). These models assume Monod kinetics under steady-state conditions and that anammox granules are uniform, of spherical size, ideal mass transfer, constant biofilm density, and constant concentration of anammox throughout the biofilm, granule, and reactor.

1.10.1 Grau Second-Order Substrate Removal

The Grau second-order model combines the Monod model and chemical reaction second-order chemical reaction kinetics (Grau *et al*., 1975). The integrated and linearized form of the model is as follows (Eq. (13)).

$$\frac{S_i\theta_H}{S_i - S_e} = \frac{S_i}{K_2 X_i} + \theta_H \qquad (13)$$

Where,

θ_H = hydraulic retention time (day)

S_e = effluent substrate concentration (mg/L)

S_i = influent substrate concentration (mg/L)

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

X = biomass concentration (mg/L)

Eq. (13) can be rewritten with constants *a* and *b* (Eq. (14)), where $a = \frac{S_i}{K_2 X_i}$, and *b* is a dimensionless constant close to 1 that accounts for the reality that substrate concentration will never actually reach zero at any θ_H.

$$\frac{S_i \theta_H}{S_i - S_e} = a + b\theta_H \quad (14)$$

1.10.2 Stover-Kincannon Modified

The Stover-Kincannon model is frequently used to model upflow anaerobic sludge blanket reactors and was first designed to model a rotating biofilm contactor reactor (Stover and Kincannon, 1982). The original model (Eq. (15) predicts substrate removal as a function of substrate loading rate and independent of reaction kinetics under any loading condition.

$$\frac{dS}{dt} = \frac{U_{max}(\frac{QS_i}{A})}{K_B + (\frac{QS_i}{A})} \quad (15)$$

Where, $\frac{dS}{dt} = \frac{Q}{V}(S_i - S_e)$, and

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

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

Q = flow rate (L/day)

S_e = effluent substrate concentration (mg/L)

S_i = influent substrate concentration (mg/L)

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

This model has been modified (Eq. (16)) to account for suspended biomass in the volume of media in an upflow column, wherein most of the biomass exists (Yu *et al.*, 1998). This allows the model to be applied to an upflow column of anammox.

$$\frac{dS}{dt} = \frac{U_{max}(\frac{QS_i}{V})}{K_B+(\frac{QS_i}{V})} \quad (16)$$

Where, V = volume of media for all biomass (L). Since $\theta_H = \frac{V}{Q}$, Eq. (16) can be rewritten as,

$$\frac{dS}{dt} = \frac{U_{max}(\frac{S_i}{\theta_H})}{K_B+(\frac{S_i}{\theta_H})} \quad (17)$$

Where, $\frac{S_i}{\theta_H}$ is the substrate loading rate (Sultana, 2016).

Eq. (17) is like the Monod model but places dependence of the effluent substrate concentration on the influent substrate concentration and the hydraulic retention time. This can also be rewritten to show the saturation constant, analogous to the K_s of Monod, as dependent on the hydraulic retention time (Eq. (18)). Thus, unlike Monod, the modified Stover-Kincannon model places dependence of the effluent substrate concentration on the influent substrate concentration and hydraulic retention time.

$$\frac{dS}{dt} = \frac{U_{max}S_i}{K_B\theta_H+S_i} \quad (18)$$

1.11 Research Plan and Hypothesis

In the following study, a mixed culture containing anammox was immobilized in 10% (w/v) PVA, 10% (w/v) PVA:1% (w/v) CA, 3% (w/v) PVA:1% (w/v) CA, and 2% (w/v) CA. These treatments were compared with non-immobilized sludge in k1 aquarium media. Enrichment and reactor performance were assessed by observing nitrogen removal rates (NRRs), substrate consumption ratios, and nitrogen removal efficiencies (NRE). Grau second-order and Stover-Kincannon kinetics models were applied to the reactors to compare their performance to the literature.

We predict that hydrogel immobilizations will promote better anammox enrichment over non-immobilized anammox by retaining biomass in a stable microenvironment that favors anammox over other species of bacteria in a mixed culture. Immobilizations comprised of alginate, as in the 2% CA treatment, will permit good nutrient diffusion for enrichment but will dissolve over the course of operation, resulting in biomass washout and suboptimal nitrogen removal. The 10% PVA and 10% PVA:1% CA treatment will likely result in swelling and dense crosslinking in the aqueous media due to the high amount of PVA, thus preventing adequate nutrient diffusion and limiting anammox growth. Therefore, a balanced proportion of PVA to CA, as in the 3% PVA:1% CA treatment, will permit good mass transfer while retaining biomass. Subsequent enrichment will be demonstrated by increasing NRRs and NREs during reactor operation, and by comparing substrate consumption ratios to the ideal metabolic ratios of anammox.

Part 2. Materials and Methods

2.1 Inoculum

Anammox was previously grown for 2 years in a sequencing batch reactor (SBR) from sludge originating from the Hampton Roads Sanitation District York River treatment plant, Seaford, Virginia. *Planctomycetes* accounted for 1-3% of the total bacteria population, and this was presumed to be mostly comprised of anammox (Sultana, 2016).

2.2 Growth Conditions

Anammox was grown in synthetic wastewater according to the recipe in Table 2.1. This media was made in 10 L of DI water. The media was purged of oxygen by bubbling with dinitrogen gas for 20 minutes with stirring. Growth medium pH ranged from 7.5-8.5. Each treatment was operated in darkness at 30 °C in 50 mL continuous up-flow columns (UFCs) with hydraulic retention times (HRTs) set by a peristaltic pump from 0.8 to 2.8 hours.

Table 2. Synthetic wastewater media recipe for anammox enrichment

Component	Concentration
$(NH_4)Cl$	8 mM
$NaHCO_3$	12 mM
$NaNO_2$	8 mM
$CaCl_2 \cdot 2H_2O$	4 mM
KH_2PO_4	0.4 mM
$MgSO_4 \cdot 7H_2O$	2 mM
KNO_3	2 mM
$FeSO_4 \cdot 7H_2O$	0.080 mM
EDTA	0.160 mM
Trace Metal Solution*	2 mL/L of media
*Trace Metal Solution Recipe (for 1L volume)	
$ZnSO_4 \cdot 7H_2O$	546 mg
$CoCl_2 \cdot 6H_2O$	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_4	18 mg
EDTA	18.76 g

2.3 Experimentation

Anammox immobilizations were conducted with a range of gel properties to represent the diverse formulations found in literature (Ali *et al.,* 2015; Idris *et al*., 2011; Takei *et al.,* 2012; Zhu *et al.,* 2009). Two primary experiments were run. The original experiment was intended to determine the feasibility of anammox immobilizations and lasted for 116 days. Upon completion, a second experiment was run with the enriched anammox sludge from the previous reactors to determine kinetics between different gel formulations. However, bead degradation occurred before adequate data could be collected for kinetics. Since the second treatments were made with approximately the same biomass for each column, NRRs and NREs were compared to indicate the extent of mass transfer limitations between the treatments.

In the first experiment, sludge was removed from SBR bottles and placed in three gel treatments of 10% PVA:1% CA, 2% CA, and 10% PVA. A fourth UFC was inoculated with sludge in k1 aquarium media. After this experiment was concluded, the sludge was extracted from all reactors, combined, and re-inoculated in four new columns. This time, a 3% PVA:1% CA gel was made instead of a 10% PVA gel with the intent of providing some insight into the success behind the immobilizations of Ali *et al*. (Ali *et al*., 2015). All other treatments were made following the same methods as the original experiment. Triplicates of treatments were not created due to time and space constraints, and triplicate samples were not run. Physical properties, such as the generation of nitrogen gas, reddish color, and granular sludge, are also considered as indicators of anammox enrichment.

2.3.1 Sludge Preparation

For each of the original treatments, sludge was prepared for immobilization by centrifuging 100 mL of SBR sludge at 9,000 rpm for 15 minutes. The supernatant was

decanted and the volume brought to 27 mL with synthetic wastewater. 2 mL was set aside for percent solids and PCR analyses.

Enriched sludge from the first experiment was extracted from the original columns. This sludge was combined and mixed with 8 mM media and equally separated into four falcon tubes. These were centrifuged for 10 minutes at 7,000 rpm. 25 mL supernatant was aliquoted. Approximately 15 mL settled pink sludge remained in 25 mL media in each tube.

2.3.2 10% PVA

25 mL of 20% PVA was mixed 1:1 by volume with anammox sludge. The solution was added dropwise from a height of 10 cm into 500 mL continuously stirred 5% boric acid in media (pH 5). Immediately after being added to the boric acid solution (0-5 minutes), the beads were transferred to 500 mL 1 M sulfate in media (pH 7.5-8.0). After one hour continuously stirred, the beads were transferred to 500 mL 0.5 M phosphate in media (pH 8.6) and allowed to crosslink for one hour with continuous stirring. The 6-10 mm beads were then rinsed three times with DI and placed into a 50 mL column with a packing ratio of 90% (v/v).

2.3.3 10% PVA:1% CA

25 mL of 20% PVA (Spectrum Chemical Mfg Corp, Gardena, California USA), 2% CA (Spectrum Chemical Mfg Corp, Gardena, California USA) was mixed 1:1 by volume with anammox sludge. The solution was added dropwise from a height of 10 cm into 500 mL 5% boric acid (EMD Millipore Corporation, Germany), 2% calcium chloride (Merck KGaA, Germany) in media (pH 5.5). Immediately after being added to the boric acid solution (0-5 minutes), the beads were transferred to 500 mL 1 M sodium sulfate (anhydrous, ACS, USA) in media (pH 7.5-8.0). After one hour continuously stirred, the beads were transferred to 500 mL 0.5 M sodium phosphate (dibasic anhydrous, Amresco, Ohio, USA) in media (pH 8.6)

and allowed to crosslink for one hour with continuous stirring. The 6-8 mm beads were then rinsed three times with DI and placed into a 50 mL column with a packing ratio of 90%.

2.3.4 3% PVA:1% CA

Beads were prepared similarly to Ali *et al*., who demonstrated good anammox enrichment with the following gel ratio and crosslinking method (Ali *et al.*, 2015). 25 mL of 6% PVA, 2% CA was mixed 1:1 by volume with enriched anammox sludge. The solution was added dropwise from a height of 10 cm into 500 mL 4% calcium chloride and crosslinked for 12 hours with continuous stirring. The 4 mm beads were then rinsed three times with DI and placed into a 50 mL column with a packing ratio of 90%.

2.3.5 2% CA

25 mL of 4% CA was mixed 1:1 by volume with anammox sludge. The solution was added dropwise from a height of 10 cm into 500 mL 2% calcium chloride in media (pH 8.0) and allowed to crosslink for two hours with continuous stirring. The 4-6 mm beads were then rinsed three times with DI and placed into a 50 mL column with a packing ratio of 90%.

2.3.6 Control in K1 Aquarium Media

25 mL anammox sludge prepared according to the above protocol was placed in a 50 mL column fully packed to a 90% packing volume with k1 aquarium filtration media.

2.4 HRT and Kinetics

For the original treatments, HRT was maintained at 2.8 h for the first ten days, then was decreased to 2.1 h between 10 and 22 days of operation. HRT was then decreased to 1.0 h between day 22 and 30. The HRT was returned to 2.1 h to minimize biomass washout until the reactors were decommissioned after 116 days of operation.

For the second set of experiments, HRT was maintained at 2.1 h for 15 days of operation to permit the treatments to stabilize. The UFCs were then operated at different flowrates to provide kinetics data for the Grau and Stover-Kincannon models. The 2% CA treatment had already started to dissolve by the time the kinetics tests were initiated. On the 16th day of operation, HRT was decreased to 1.4 h. After 24 hours, the HRT was further reduced to 1 h. The HRT was reduced again to 0.8 h on the 18th day of operation. The HRT was increased to 2.8 h between the 20th and 29th days of operation, at which point the HRT was brought to 1.7 h and maintained for the rest of the experiment.

2.5 Sample Collection and Storage

Influent synthetic wastewater was sampled from the 10 L reservoir, and effluent samples were collected from the UFC effluent lines in 1.5 mL microcentrifuge tubes. Samples were stored in -80 °C freezer before analysis.

2.6 Activity Measurements

Primary catabolic substrates were measured using ion chromatography (NO_2^-, NO_3^-) and spectrophotometry (NO_2^-). Cations were measured using spectrophotometry (NH_4^+). Before analysis, all samples were diluted 1:10 with nitrogen-free synthetic wastewater (no nitrite, nitrate, or ammonium).

2.6.1 Measurements of NO_2^- and NO_3^-

Concentration of anions in synthetic wastewater was determined with a CD20 conductivity meter and a Dionex IonPac AS14 Analytical Column (Dionex, Sunnyvale, CA, USA) at a pump pressure of 1500 psi. The columns used a carbonate eluent comprised of 3.5 mM Na_2CO_3 with 1.0 mM $NaHCO_3$. The test duration was 15 minutes with a flowrate of 1

mL/minute and a 0.6 mL sample volume. Standard curves were prepared from 0.25 and 2 mM of both NO_2^- and NO_3^-.

Nitrite data was also collected colorimetrically in 1 mL volumes at a wavelength of 540 nm on an Orion Aquamate 8000 spectrophotometer (Thermo Scientific). 890 µL sulfanilamide (1% (w/v) in 1 M HCl) and 100 µL N-(1-Naphthyl) Ethylenediamine Dihydrochloride (0.2% (w/v) NED) were added, in order, to 10 µL sample. This solution was mixed by vortexing and allowed to sit at room temperature for 15-60 minutes before reading. Standards were made for the linear range between 0.25 and 2 mM NO_2^-.

2.6.2 Measurements of NH_4^+

Ammonium concentration in synthetic wastewater was determined by colorimetry on a Synergy 2 Biotek Microplate Reader with Gen 5 software. Absorbance of the cation was determined at a wavelength of 660 nm. 275 µL of solution was placed in each microplate well, and was comprised of 25 µL sample, 175 µL citrate (0.014% (w/v) trisodium citrate in 0.1 M HCl, pH 7.0), 50 µL 2-phenylphenol-nitroprusside (3.22% 2-phenylphenol, 0.015% sodium nitroprusside), and 25 µL sodium hypochloride (1% (w/v) in 2 M NaOH, pH 13.0), in that order. The solution sat for 2 minutes after adding citrate. Following addition of all components, the solution was mixed by pipetting and incubated at 37 °C for 15 minutes prior to reading. Standards were made for the linear region between 0.1 and 1.4 mM NH_4^+.

2.7 pH

pH of synthetic wastewater media was determined using an Accumet pH meter and probe (Fisher Scientific). The probe was calibrated weekly using pH 4 and pH 7 buffer solution.

2.8 Calculations

Several parameters are useful in quantifying and comparing anammox productivity. The Nitrogen Removal Rate (NRR) is the amount of ammonium and nitrite removed in the reactor divided by the hydraulic retention time (Eq. (13)) and is an indication of reactor performance and enrichment. Increasing removal rates indicate increased microbial activity.

$$NRR = \frac{([NO_2^-]+[NH_4^+])_{in}-([NO_2^-]+[NH_4^+])_{out}}{\theta_H} \quad (13)$$

Nitrogen Removal Efficiency (NRE) is the total amount of nitrogen removed in the reactor per the amount of nitrogen entering the reactor (Eq. (14)).

$$NRE = \frac{([NO_2^-]+[NH_4^+])_{in}-([NO_2^-]+[NH_4^+])_{out}}{([NO_2^-]+[NH_4^+])_{in}} \times 100\% \quad (14)$$

Nitrite-Ammonium Removal Ratio (NARR) is the amount of nitrite consumed in a reactor per the amount of ammonium consumed (Eq. (15)). This parameter is crucial to determining if substrate depletion is caused by anammox in a mixed culture, and has been widely used to determine the activity, performance, and efficiency of anammox systems. The experimentally-determined metabolic ratio for a pure culture of anammox is 1.32 (Strous *et al*., 1998).

$$NARR = \frac{[NO_2^-]_{in}-[NO_2^-]_{out}}{[NH_4^+]_{in}-[NH_4^+]_{out}} \quad (15)$$

Nitrite-Nitrate Ratio (NNR) is the ratio of nitrite consumed per the amount of nitrate produced (Eq. (16)). About 20% of consumed nitrite is anabolically converted to nitrate, and the experimentally-determined metabolic ratio for anammox is 5.1 (Strous *et al*., 1998).

$$NNR = \frac{[NO_2^-]_{in} - [NO_2^-]_{out}}{[NO_3^-]_{out} - [NO_3^-]_{in}} \quad (16)$$

Part 3. Results and Discussion

3.1 Original Immobilizations

This section shows the results of the first experiment of immobilized sludge in 10% PVA, 10% PVA:1% CA, 2% CA, and k1 media. Effluent nitrogen species, NRRs, NREs, and NARRs are compared to show anammox enrichment. Nitrate data was not collected in the original immobilization treatments.

3.1.1 Effluent Nitrogen Species

This section shows the nitrogen removal achieved in the columns (Figure 3.1) for each treatment and explores the potential reasons for the differences between the treatments. Effluent concentrations of nitrite and ammonium are compared with influent media concentrations to show the raw data upon which further analyses are based.

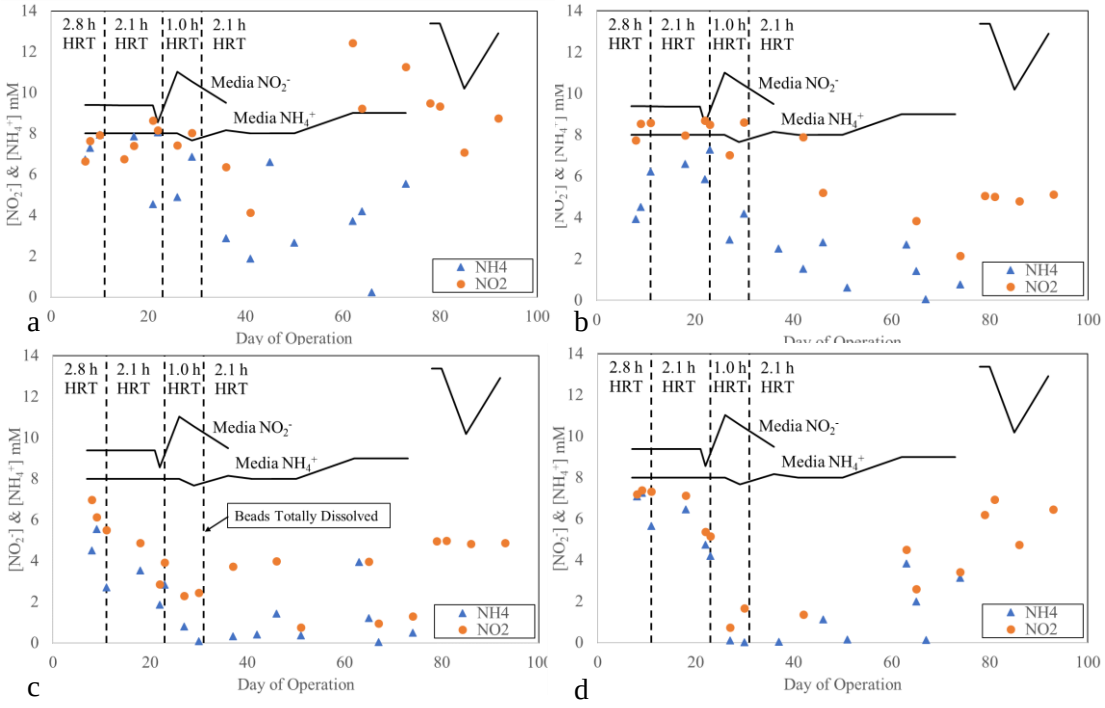

Figure 3.1 Concentrations of influent (media) and effluent nitrogen species in UFCs of original anammox immobilizations of (a) 10% PVA, (b) 10% PVA:1% CA, (c) 2% CA, (d) k1 media.

Removal of both nitrite and ammonium is noticeable throughout operation in all reactors (Figure 3.1). When HRT was decreased to 1.0 h, nitrogen removal increased. This may be due to microbial growth. Another contributing factor to increased nitrogen removal is the reduction in gel matrix, particularly in the 2% CA treatment (Figure 3.1 (c)), resulting in reduced mass transfer resistance. The 2% CA treatment appeared to remove nitrogen as well as, or better than, the k1 media throughout operation (Figure 3.1 (c) and (d)).

Both PVA treatments appeared to remove nitrogen poorly in comparison with the CA treatment and k1 media. Surprisingly, the 10% PVA treatment appears to have formed better than the 10% PVA:1% CA copolymer. The greater nitrogen removal from the 10% PVA :1% CA treatment is surprising, as 1/3 of the initial gel of the copolymer treatment was removed within the second day of operation. Bad swelling resulted in reduced seed biomass and thus,

reduced nitrogen removal. The improved nitrogen removal of the copolymer over the 10% PVA may be due to better mass transfer in the looser structure of the copolymer. Nitrite concentrations in the influent media were not constant during operation, so no influent media concentrations were measured in the gap in influent media nitrite, and no influent media nitrite data was collected between days 40 and 78 of operation. When influent nitrite data was collected again around day 80, nitrite was still being removed in all treatments.

3.1.2 Nitrogen Removal Rates (NRRs)

This section shows the nitrogen removal rates achieved in the columns (Figure 3.2) for each treatment and explores the potential reasons for the similarities and differences between them.

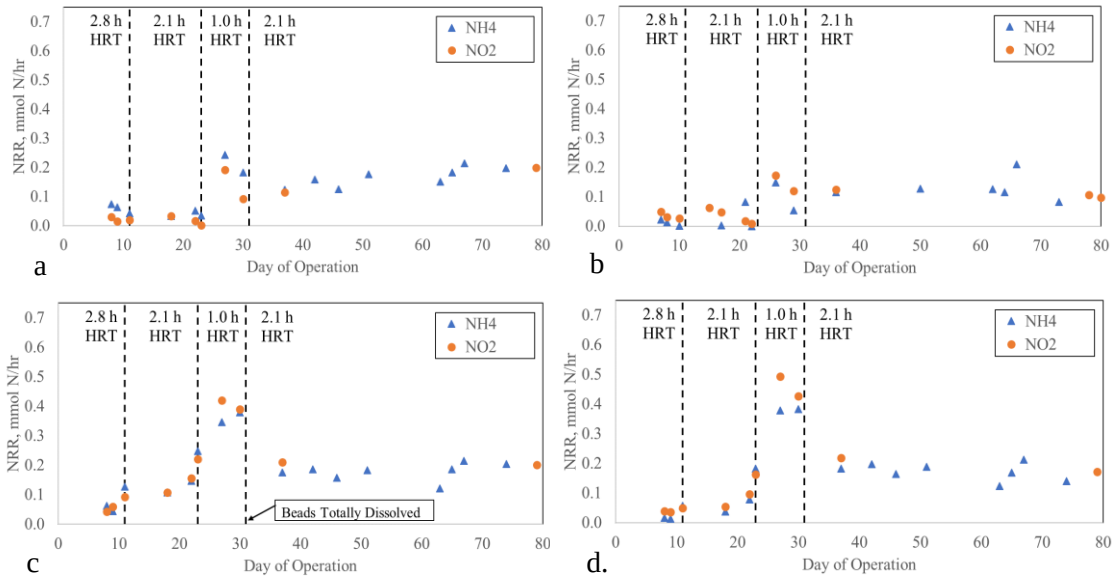

Figure 3.2 Nitrogen removal rates (NRRs) of ammonium and nitrite in UFCs of original anammox immobilizations of (a) 10% PVA, (b) 10% PVA:1% CA, (c) 2% CA, (d) k1 media.

All treatments exhibited similar NRRs at startup (Figure 3.2). NRRs increased for all reactors during operation, suggesting microbial growth. Comparable nitrogen removal rates were found for both PVA treatments (Figure 3.2 (a) and (b)), achieving approximately 0.2

mmol N/h. Both PVA treatments behaved similarly but the NRRs did not increase as much as the other treatments at shortened HRTs. This is likely due to channeling affects within the columns, as the PVA treatments had swollen within the first several days and had to be periodically opened to create space for media flow. The visible growth of pink biofilm, though slow, eventually permitted NRRs comparable to the 2% CA and k1 media treatments.

NRRs were initially higher for the 2% CA treatment (Figure 3.2 (c)) than the k1 media (Figure 3.2 (d)). The k1 media achieved a maximum NRR of 0.5 mmol N/h of nitrite and 0.4 mmol N/h of ammonium, while 2% CA achieved a maximum removal of 0.4 and 0.3 mmol N/h for nitrite and ammonium, respectively. Following complete dissolution of the alginate beads, the 2% CA treatment achieved comparable NRRs to k1 media.

3.1.3 Nitrogen Removal Efficiencies (NREs)

This section shows the nitrogen removal efficiencies achieved in the columns (Figure 3.3) for each treatment and explores the potential reasons for the similarities and differences between the treatments.

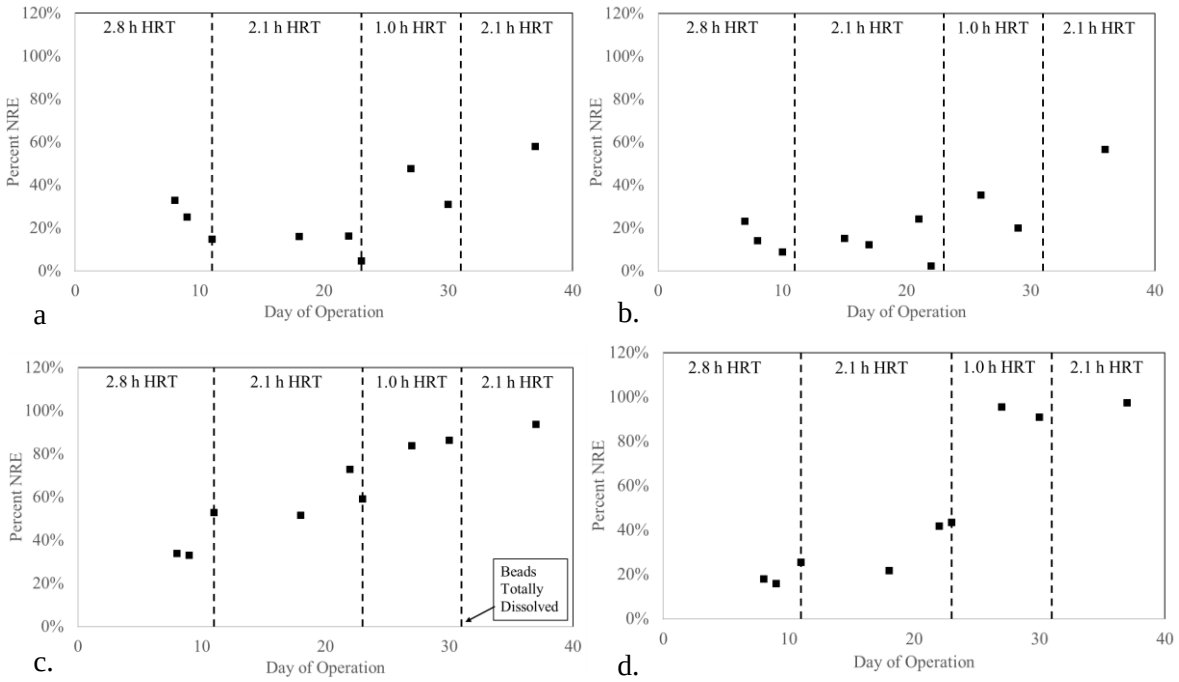

Figure 3.3 Nitrogen removal efficiencies (NREs) of total ammonium and nitrite in UFCs of original anammox immobilizations of (a) 10% PVA, (b) 10% PVA:1% CA, (c) 2% CA, (d) k1 media.

NREs generally increased for the treatments between the start of the reactors and the 37th day of operation (Figure 3.3). The 10% PVA and copolymer treatments decreased in efficiency over the first several weeks of operation (Figure 3.3 (a) and (b)). This is likely due to the continued polymerization of the PVA gels, which was visibly noted by the swelling of the gel matrices over the same time period. Between the 24th and 37th day of operation, NREs increased to 60% efficiency. The increase in NREs for these treatments coincides with increased HRT and NRRs. By this point, the formation of biofilms on the PVA gel matrices may account for the improved nitrogen removal. The formation of biofilms on gel substrate is common among bacteria immobilizations in wastewater treatment (Henze *et al*., 2002).

NREs for both 2% CA and k1 media increased during the first several weeks of operation (Figure 3.3 (b) and (c)). Both treatments reached 100% removal efficiency within

the first 30 days. The k1 media had steadily increasing NREs, but the 2% CA treatment jumped from 40% to 100% efficiency in one week as the beads dissolved. This indicates that nitrogen removal, and corresponding microbial growth, was diffusion-limited in the gel.

3.1.4 Nitrite-Ammonium Removal Ratios (NARRs)

This section shows the NARRs achieved in the columns (Figure 3.4) for each treatment and explores the potential reasons for the similarities and differences between the treatments.

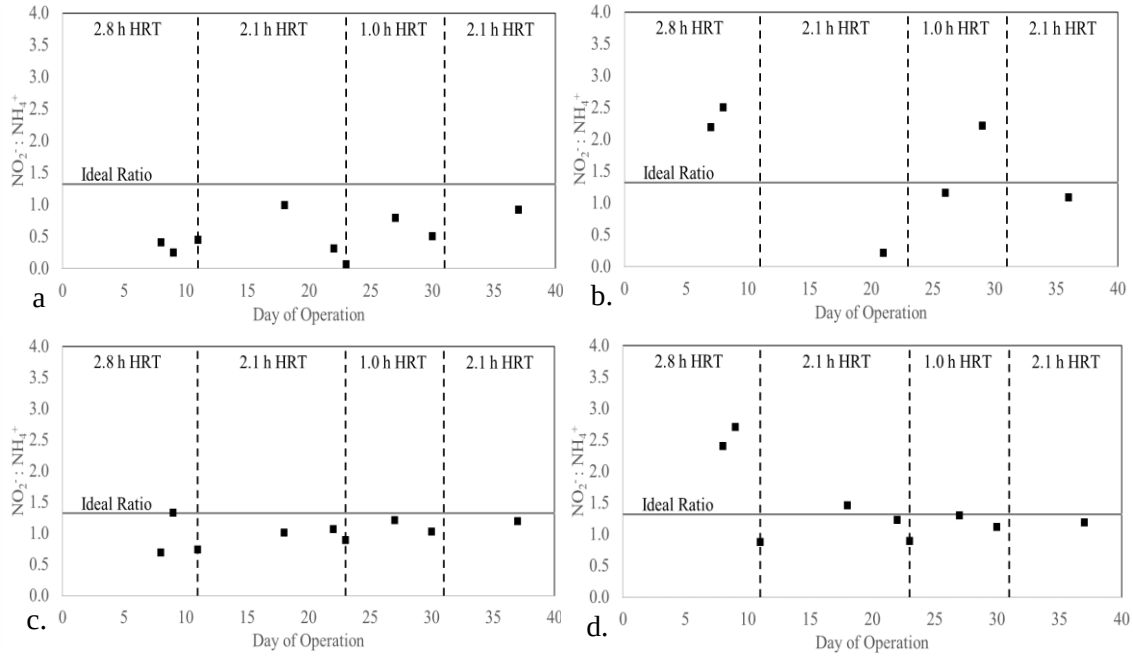

Figure 3.4 NARRs in UFCs of original anammox immobilizations of (a) 10% PVA, (b) 10% PVA:1% CA, (c) 2% CA, (d) k1 media. The ideal metabolic nitrogen removal ratio of anammox is of 1.32.

NARRs for PVA treatments fluctuated more than the 2% CA and k1 media treatments (Figure 3.4), which approached the ideal metabolic ratio of 1.32 within 18 days. Points below the ideal ratio may indicate the presence of NOB activity, while points above the ideal ratio indicate AOB activity. The presence of these bacteria may also account for the

higher NRR of ammonium over nitrite, as seen in the 10% PVA treatment (Figure 3.2 (a)). AOBs and NOBs may be present as the inoculum was a mixed culture and the treatments were inoculated aerobically and could be noticeable in data collected near startup. However, the system was maintained anaerobically, so the existing oxygen in the gels would be consumed quickly and aerobes would be inhibited. Ammonium may be accumulating, as indicated by low values, due to the hydrolysis of proteins that occurs with cell death. It is much more likely that heterotrophic denitrifiers are present, as indicated by high nitrite removal over ammonium.

While anammox activity cannot be discounted in the PVA treatments, substantial NOB activity may have been present in the 10% PVA treatment (Figure 3.4 (a)). Alternatively, low NARRs may indicate low nitrite removal from potential nitrite inhibition (Figure 3.2 (a)). If this were the case, it would be expected to be observed in the other treatments as well. The NARRs approached the ideal ratio several times, and may be within error, though triplicate values were not recorded. The copolymer of 10% PVA:1% CA also approached the ideal ratio, especially on the 26^{th} and 36^{th} day of operation (Figure 3.4 (b)).

PVA did not appear to create as favorable of an environment for anammox as the 2% CA and k1 media treatments. Anammox activity was seen early in the alginate treatment (Figure 3.4 (c)) and remained stable throughout the first 40 days of operation. The improvement of the k1 treatment is impressive considering the high AOB activity in the first week (Figure 3.4 (d)). Within 12 days, the system approached the ideal NARR for anammox.

3.1.5 Summary of Original Immobilizations

Anammox was successfully enriched in the first set of immobilizations, with 2% CA producing the most anammox activity of the gel treatments. NARRs achieved similar metabolic ratios to what has been experimentally determined previously in SBRs with granular anammox (Strous *et al.*, 1998). NRRs and NREs increased for all immobilizations

throughout operation. NRRs stabilized to approximately 0.2 mmol N/h at 2.1 h HRT of both nitrite and ammonium in all columns after 35 days of enrichment. Higher NRRs were achieved by alginate and k1 treatments during faster flowrates, with a maximum NRR of approximately 0.5 mmol N/h. NREs approached 60% for PVA treatments and 100% for the 2% CA and k1 treatments within the first 40 days of operation.

Soon after startup, PVA gel treatments swelled and agglomerated, forming a dense gel matrix which forced channeling of influent media. Gas bubbles, likely of nitrogen generation from anammox activity, became entrapped in PVA gels; this has been predicted in the literature (Bickerstaff, 1997). Reddish biofilms formed on the PVA gels (Appendix A, Figure 1), indicating anammox enrichment despite poor NARRs, an occurrence previously described in these systems (Henze *et al.,* 2002), in addition to a 2 mm layer of reddish biofilm on the base of the column. More PVA gel remained in the 10% PVA treatment than in the 10% PVA:1% CA treatment.

The 2% CA beads provided good mass transfer and dissolved entirely within the first month of operation. Both the sludge in the k1 media and the resultant sludge blanket from the alginate column achieved the reddish color associated with enriched anammox (Appendix A, Table 1). However, biomass washout was noticeable by the formation of reddish granules in the influent and effluent lines (Appendix A, Figure 2). While anammox could be successfully enriched in PVA, this gel was more difficult to work with than CA in UFCs with aqueous media. Smaller ratios of PVA to CA could potentially produce more stable copolymers, but 10% PVA:1% CA was not substantially more effective than simply 10% PVA.

3.2 Second Immobilizations

This section shows the results of the second experiment of immobilized sludge in 10% PVA:1% CA, 3% PVA:1% CA, 2% CA, and k1 media. The new 3% PVA:1% CA treatment was made according to the protocol established by Ali *et al.,* (Ali *et al.,* 2015) due to their reported success. It was intended to compare this formulation with the high PVA or alginate treatments to elucidate the mechanisms behind the success of the 3:1 ratio. Effluent nitrogen species, NRRs, NREs, and metabolic ratios are compared to show anammox enrichment. Nitrate data was collected for only the first 15 days of operation due to analytical equipment failure. 2% Alginate beads dissolved by 80% by volume within 15 days, and were reduced entirely to sludge by the 30th day. 3% PVA:1% CA decreased by 50% volume by the 30th day.

3.2.1 Effluent Nitrogen Species

This section shows the nitrogen removal achieved in the columns (Figures 3.5 (a), (b), (c), (d)) for each treatment and explores the potential reasons for the differences between the treatments. Effluent concentrations of nitrite and ammonium are compared with influent media concentrations to show the raw data upon which further analyses are based.

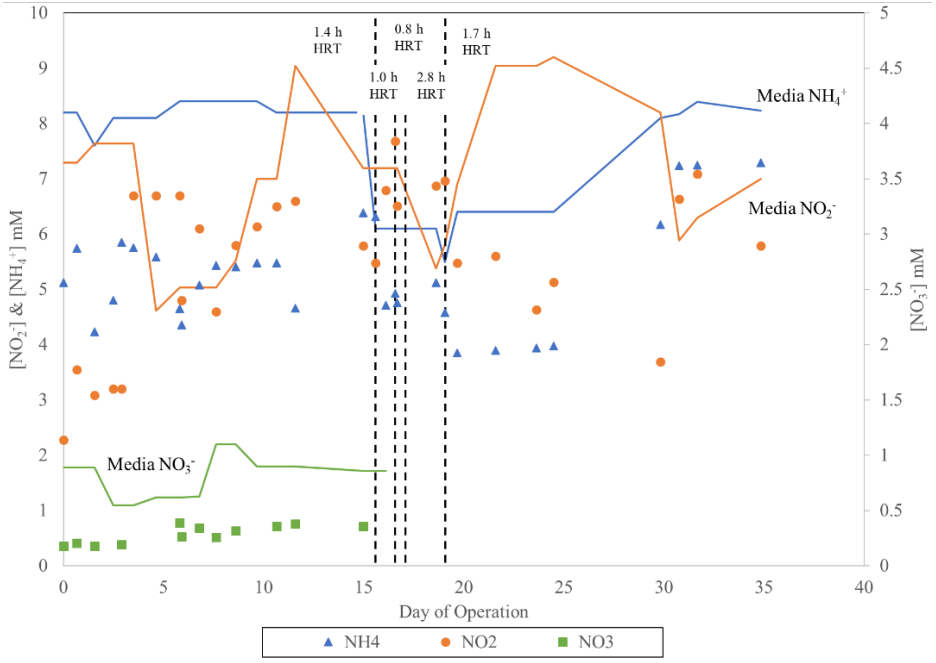

Figure 3.4 (a). Concentrations of influent (media) and effluent nitrogen species in UFCs of anammox treatment of (a) 10% PVA:1% CA. No nitrate data was collected after 15 days of operation.

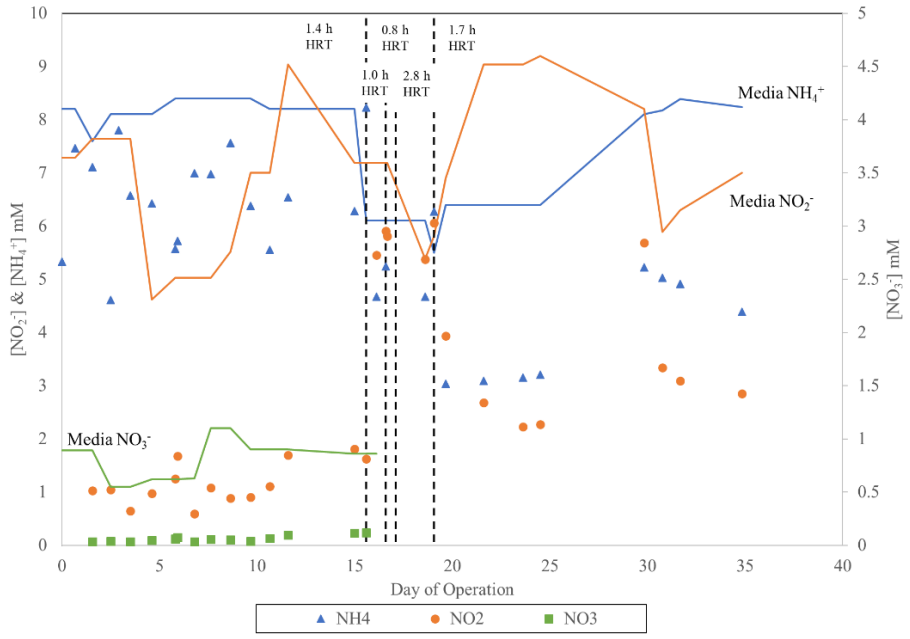

Figure 3.4 (b). Concentrations of influent (media) and effluent nitrogen species in UFCs of original anammox treatment of (b) 3% PVA:1% CA. No nitrate data was collected following 15 days of operation.

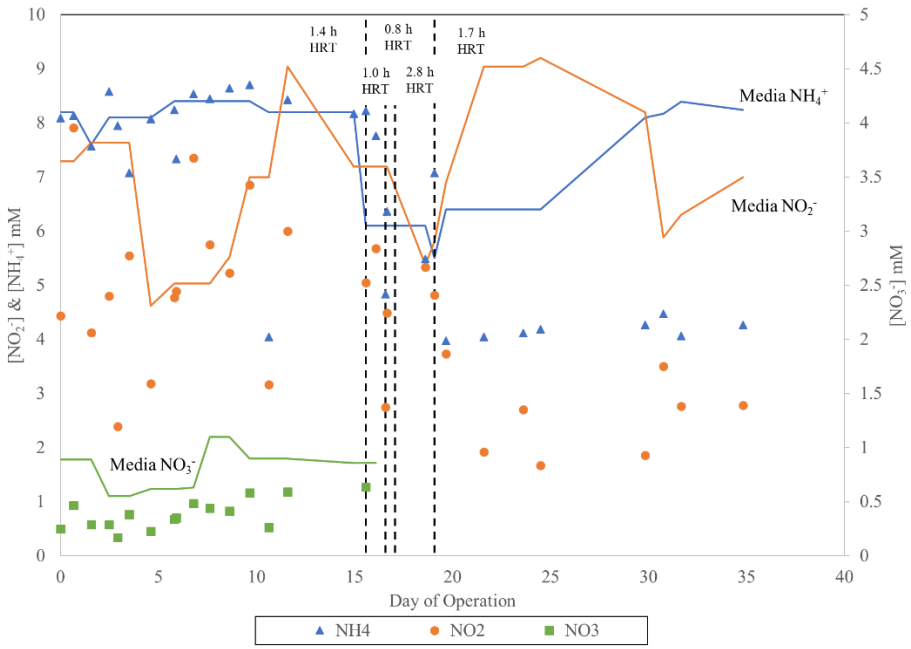

Figure 3.4 (c). Concentrations of influent (media) and effluent nitrogen species in UFCs of anammox treatment of (c) 2% CA. No nitrate data was collected after 15 days of operation.

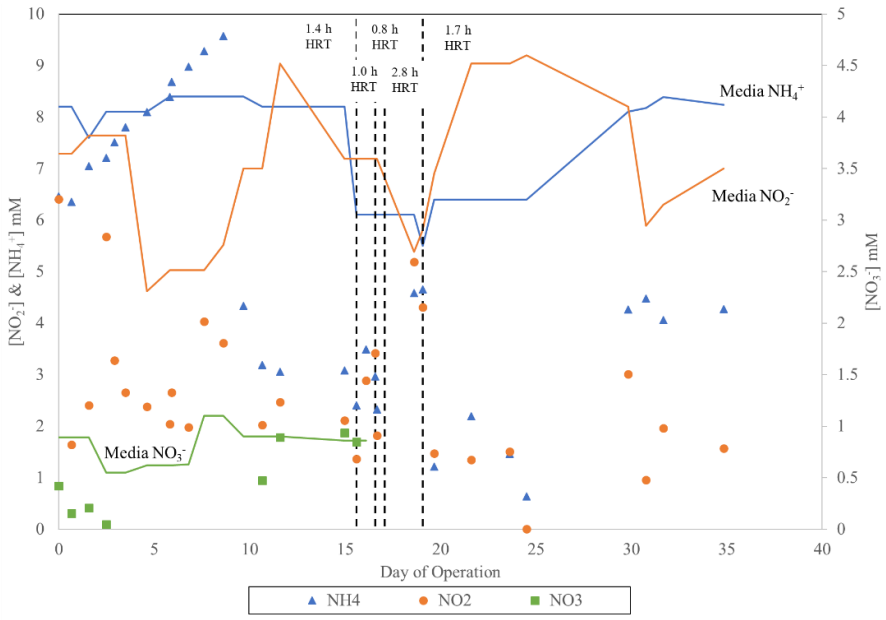

Figure 3.4 (d). Concentrations of influent (media) and effluent nitrogen species in UFCs of original anammox treatment of (d) k1 media. No nitrate data was collected following 15 days of operation.

Influent ammonium and nitrite concentrations were not consistent within the second set of reactors, and nitrate was typically consumed (Figure 3.4). In the 10% PVA:1% CA treatment, effluent nitrogen was initially quite low (Figure 3.4 (a)), indicating good removal, but effluent concentrations climbed within the first several days of operation as the gel swelled and polymerized. Nitrite appears to be produced in the 10% PVA:1% CA and 2% CA reactors, particularly between days 5-10 of operation (Figures 3.4 (a) and (c)). Since the system is anaerobic, nitrite generation may be the result of denitrifying bacteria. Denitrifying bacteria may have been introduced upon reactor startup, or may be due to the death of bacteria that eventually resulted in enough organic carbon to promote the survival of heterotrophic denitrifying bacteria (Sultana, 2016). This appears to be the likely case, as ammonium is noticed to build up in the reactor; ammonia is produced from the hydrolysis of proteins that occurs with cell death. The presence of heterotrophs would also account for the nitrate consumption in the reactors, with particular heterotrophic activity in the k1 media (Figure 3.4 (d)), wherein noticeable ammonium production occurs within a week of operation as nitrite is actively consumed. Since carbon is a more energetically favorable electron donor, heterotrophs have a faster growth rate and can initially outcompete anammox. Eventually, anammox activity outcompetes these heterotrophs as organic carbon is depleted. Anammox activity did stabilize, as seen in the k1 beads; after two weeks of operation, nitrate was being generated and both ammonium and nitrite were being consumed in similar amounts.

Within 35 days of operation, all reactors exhibited removal of nitrite and ammonium, with performance decreasing in the following order: k1 media, 2% CA, 3% PVA:1% CA, 10% PVA:1% CA. This is consistent with expected mass transfer limitations between the treatments. CA is commonly added to PVA treatments to enhance the effective diffusivity of PVA treatments (Bickerstaff, 1997). Furthermore, the 2% alginate beads were noticeably shrinking by the fifteenth day of operation, which corresponds with increased nitrogen removal. This occurred twice as quickly as noted in the original reactors, potentially resulting

from the higher microbial activity of the new reactors (Zhu *et al.*, 2009). The delay in greater ammonium removal (Figure 3.4 (b)) in the 3% PVA:1% CA treatment corresponds with a slower reduction of gel beads that was also noticed; this is likely due to more mechanically stable beads (Appendix 2, Table 3)), a property conferred by the PVA.

3.2.2 Nitrogen Removal Rates (NRRs)

This section shows the nitrogen removal rates achieved in the second columns (Figure 3.5, all) for each treatment and explores the potential reasons for the similarities and differences between them.

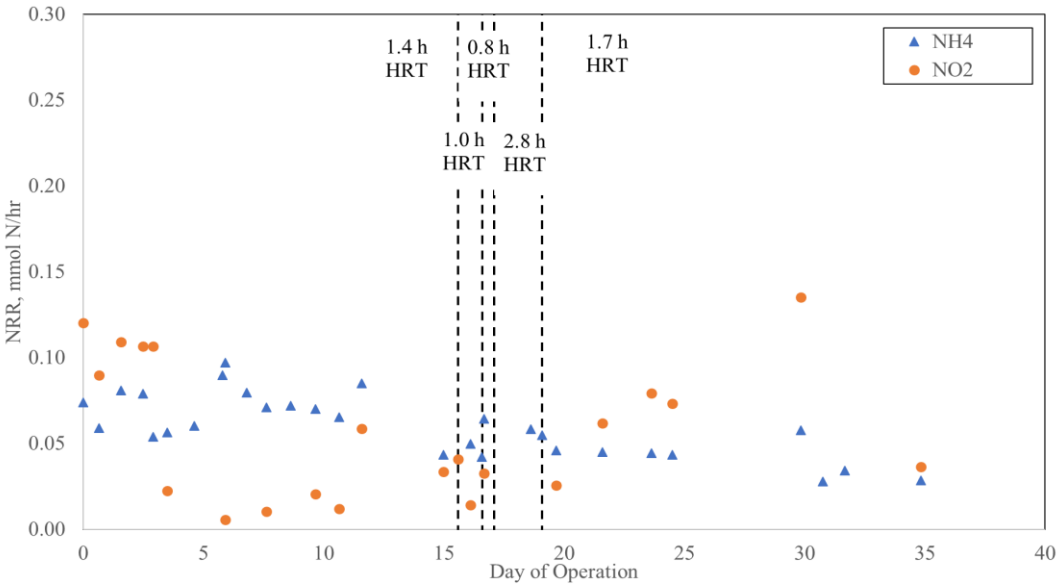

Figure 3.5 (a). NRRs of second anammox immobilization in (a) 10% PVA:1% CA.

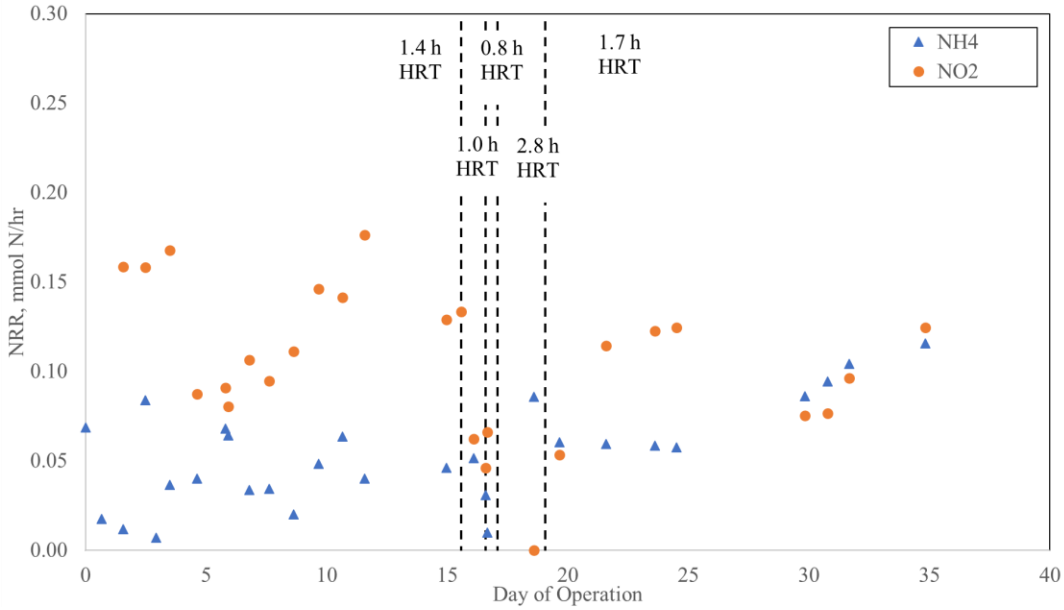

Figure 3.5 (b). NRRs of second anammox immobilization in (b) 3% PVA:1% CA.

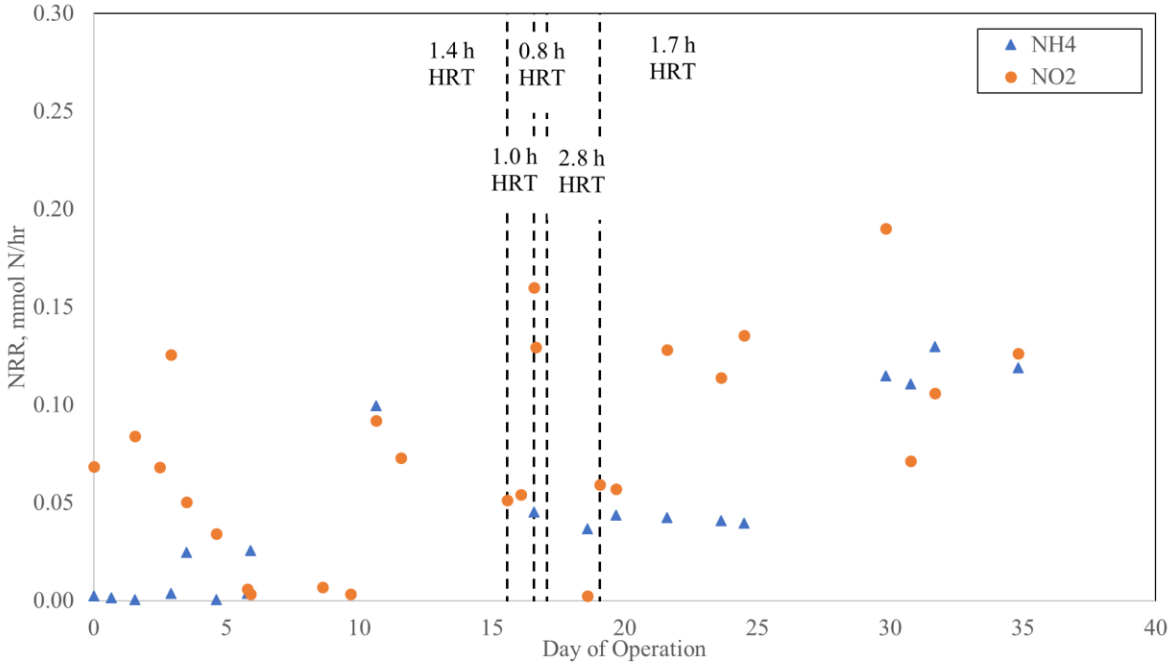

Figure 3.5 (c). NRRs of second anammox immobilization in (c) 2% CA. Beads degraded by approximately 80% by the 15th day.

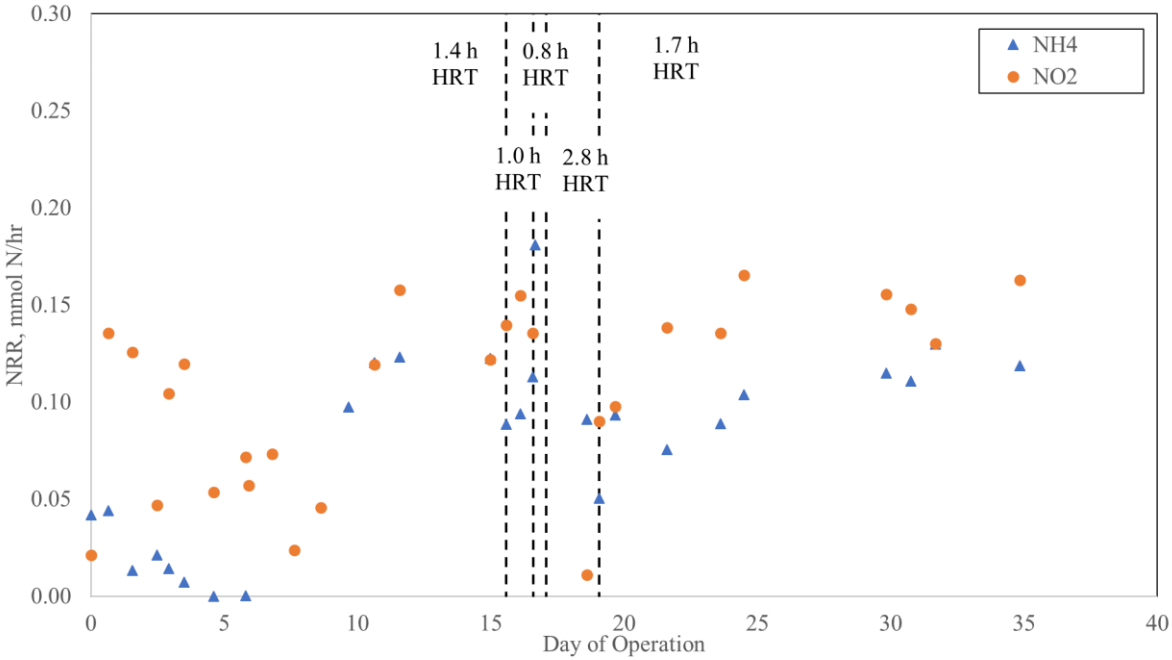

Figure 3.5 (d). NRRs of second anammox immobilization in (d) k1 media.

NRRs (Figure 3.5 (all)) were initially greater for these reactors than in the initial treatments, as expected due to a more enriched starting culture, though a swift decline in activity was surprising and may be due to a changing population in the mixed culture between heterotrophs and anammox. Nitrite removal decreased in the 10% PVA:1% CA treatment (Figure 3.5 (a)) during the first five days and did not increase until much later. This is likely due to the continued polymerization of PVA from natural swelling in synthetic wastewater (Bickerstaff, 1997), resulting in reduced mass transfer and poor microbial activity. It may also be accounted for by the death of NOBs in the reactor, though it is unlikely NOBs were active at all given the anaerobic environment. In the 3% PVA:1% CA treatment, greater NRRs for nitrite over ammonium were maintained, achieving maxima of 0.17 mmol N/h and 0.9 mmol N/h for nitrite and ammonium in the first several weeks, possibly due to heterotrophic denitrifiers. Nitrite removal rates gradually decreased as ammonium removal increased during operation, indicating decreasing heterotrophic activity and increasing anammox activity. In the 2% CA treatment, NRRs for nitrite generally remained higher than ammonium NRRs (Figure 3.5 (c)). This could be due to the presence of heterotrophic denitrifying bacteria, which also appear to have initially contaminated the k1 media (Figure 3.5 (d)); however, the carbon source of these heterotrophs remains uncertain, though the carbon-based polymers may play a role.

3.2.3 Nitrogen Removal Efficiencies (NREs)

This section shows the nitrogen removal efficiencies achieved in the second set of immobilizations (Figure 3.6 (all)) for each treatment and explores the potential reasons for the similarities and differences between the treatments.

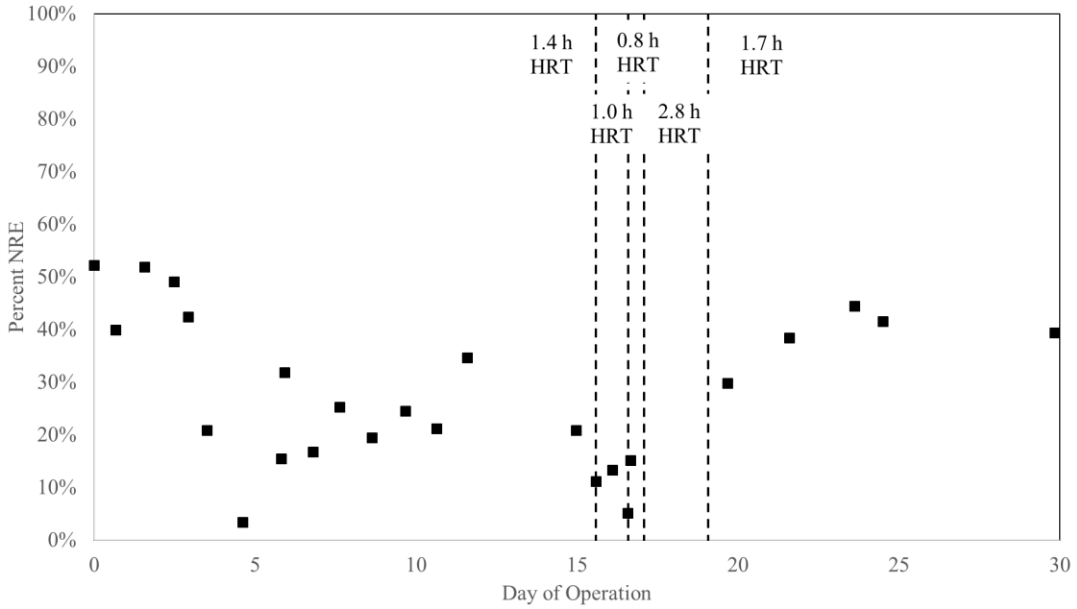

Figure 3.6 (a). NREs of second anammox immobilization in (a) 10% PVA:1% CA.

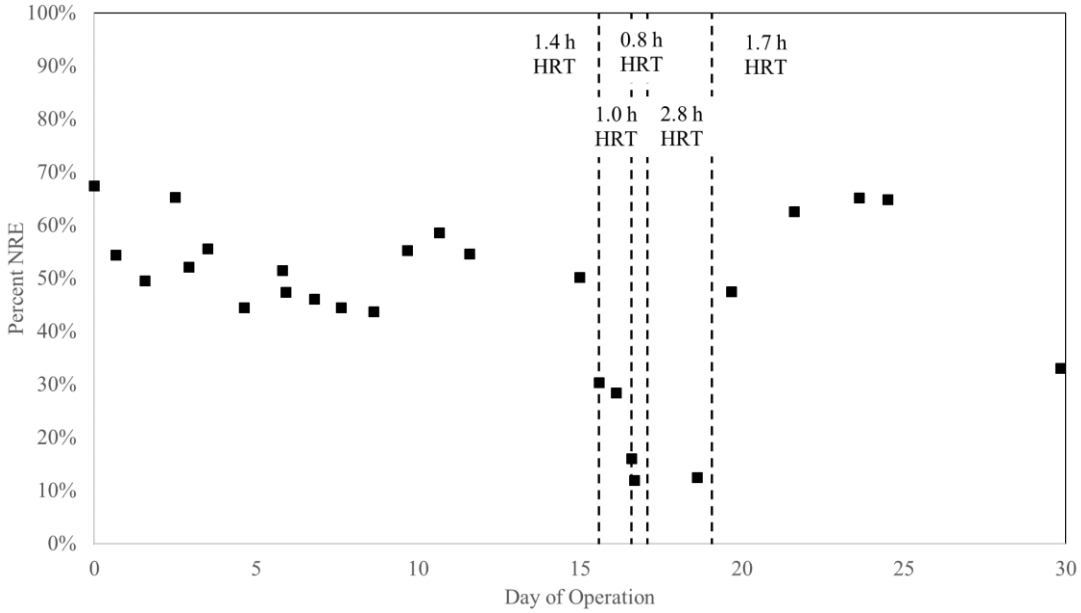

Figure 3.6 (b). NREs of second anammox immobilization in (b) 3% PVA:1% CA.

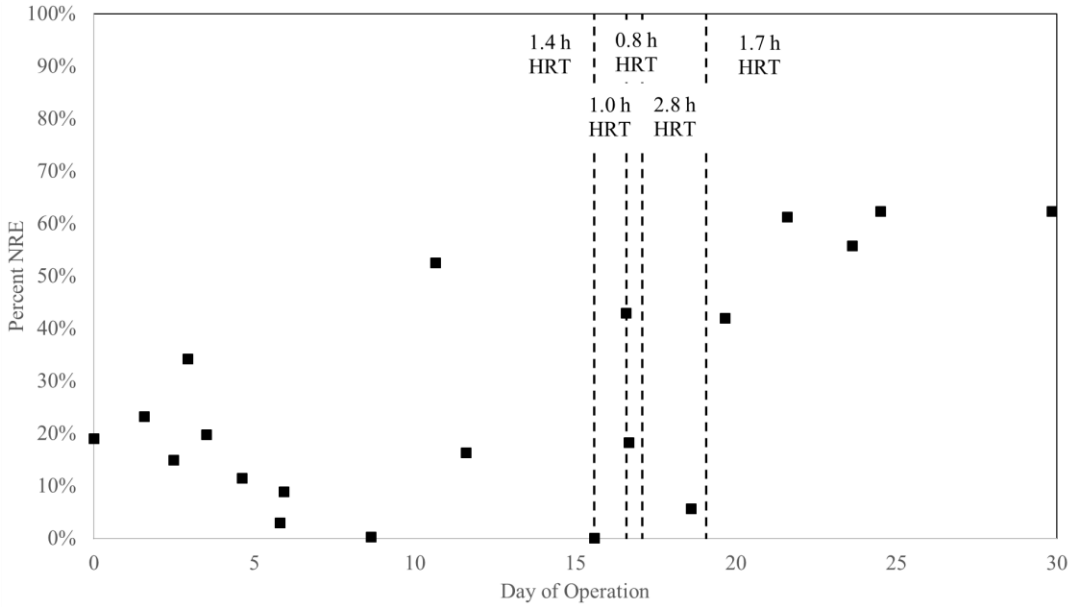

Figure 3.6 (c). NREs of second anammox immobilization in (c) 2% CA. Beads degraded by approximately 80% by the 15th day.

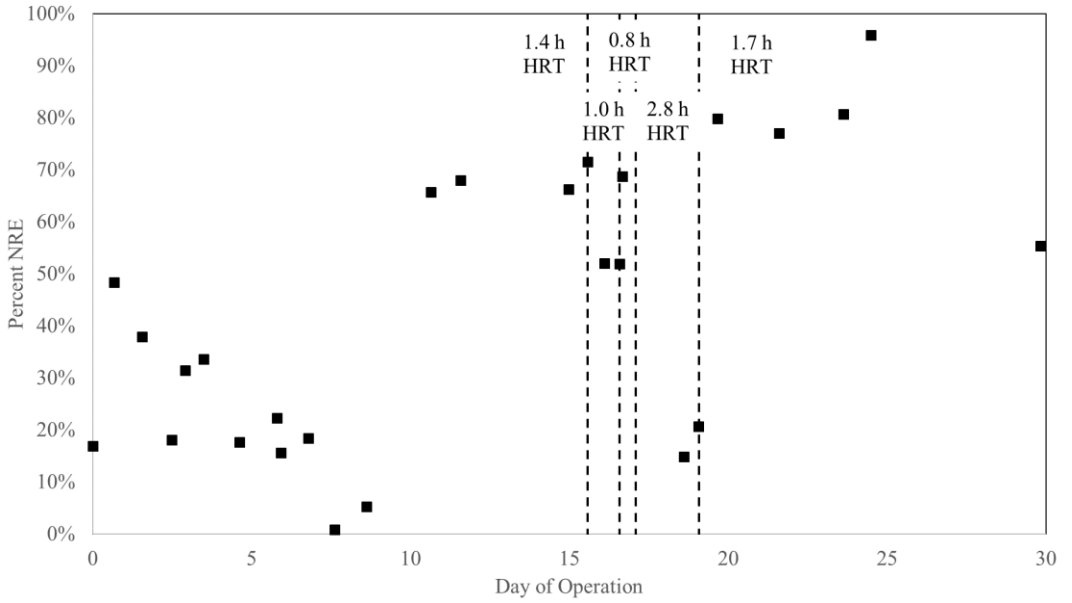

Figure 3.6 (d). NREs of second anammox immobilization in (d) k1 media.

NREs of the second immobilization (Figure 3.6) generally declined over the first ten days of operation for all reactors, especially in the 2% CA and k1 media. This is potentially

due to the death of non-anammox bacteria. Initially active heterotrophic denitrifiers may have exhausted their carbon source, and the gap in nitrogen removal may indicate the slow recovery of anammox. When flowrates were adjusted for the kinetics test, data appeared scattered for all treatments. NREs increased from an initial 20% to 65% within the first 23 days of operation for 2% CA (Figure 3.6 (c)), and increased from 50% to 85% over this period for k1 beads (Figure 3.6 (d)). The results from the 2% CA treatment were surprising, as it was anticipated that NREs would be initially greater in this treatment than the other gel treatments, due to a more porous structure and the previous experiment's results. However, overall improvement of this treatment was greater than the other gel treatments, as expected due to early bead dissolution within 15 days. In the 10% PVA:1% CA treatment, NREs decreased gradually, which is consistent with the expected increase of polymerization and greater resistance to mass transfer as observed in the previous immobilizations (Figure 3.6 (d)). This treatment started with a high NRE of 50%, but ultimately achieved a lower NRE of about 45% by the end of the first month. Increased flowrate resulted in lower NRE, which is consistent with the channeling effects observed previously for this treatment. The most stable treatment was the 3% PVA:1% CA, which maintained an NRE between 50% and 70% throughout operation except at low HRT (Figure 3.6 (b)).

3.2.4 Nitrite-Ammonium Removal Ratios (NARRs)

This section shows the NARRs achieved in the columns (Figure 3.7) for each treatment and explores the potential reasons for the similarities and differences between the treatments. A metabolic ratio of 1.32 is ideal for the anammox process (Strous *et al*., 1998).

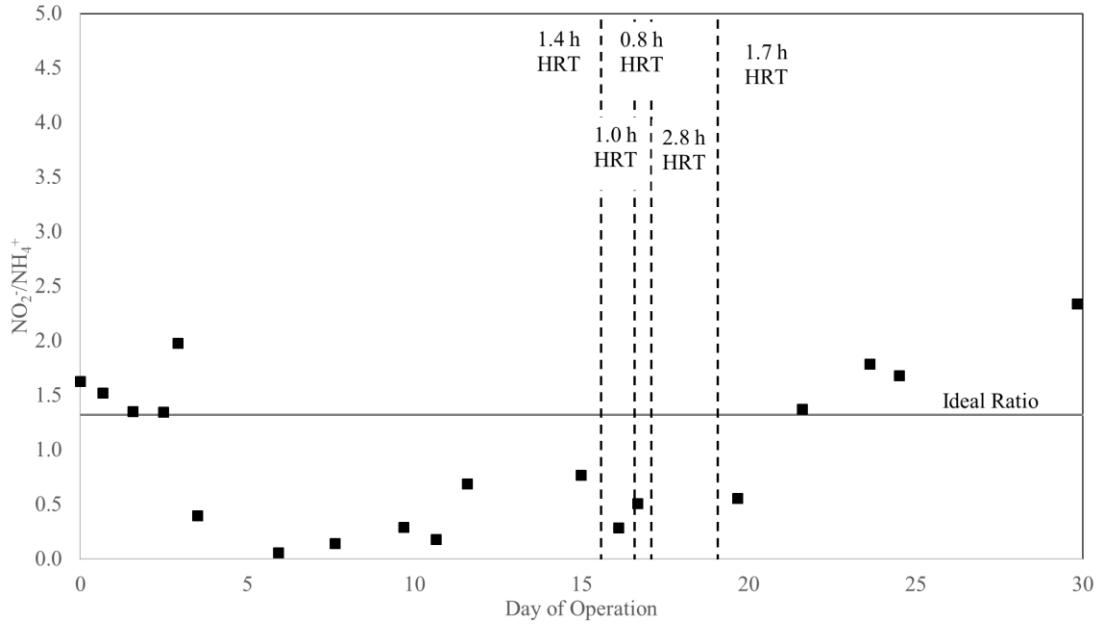

Figure 3.7 (a). NARRs of second anammox immobilization in (a) 10% PVA:1% CA.

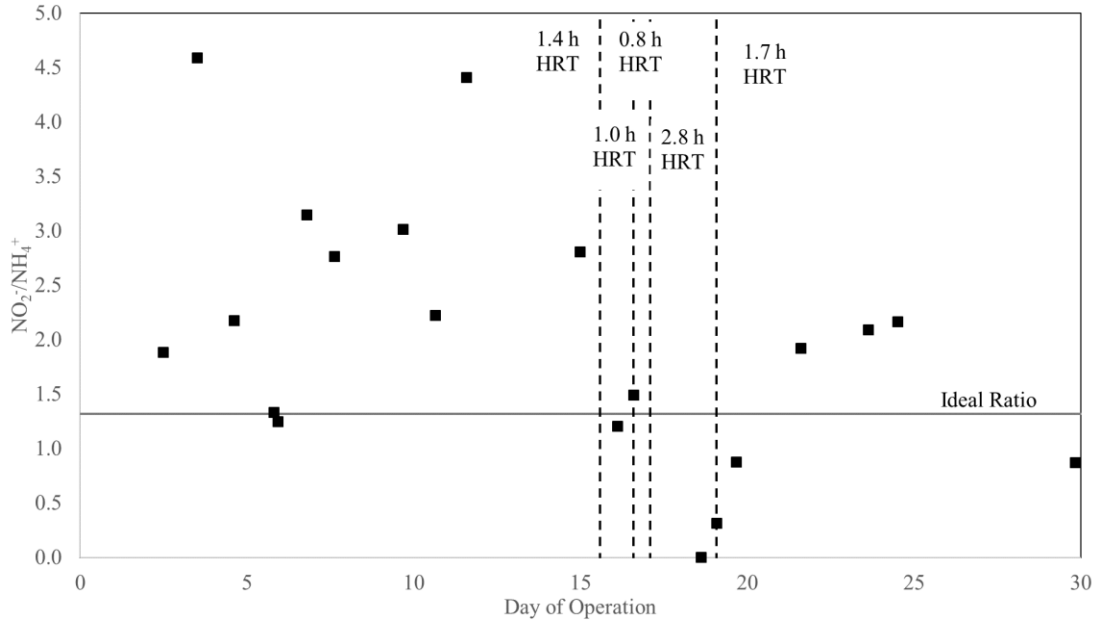

Figure 3.7 (b). NARRs of second anammox immobilization in (b) 3% PVA:1% CA.

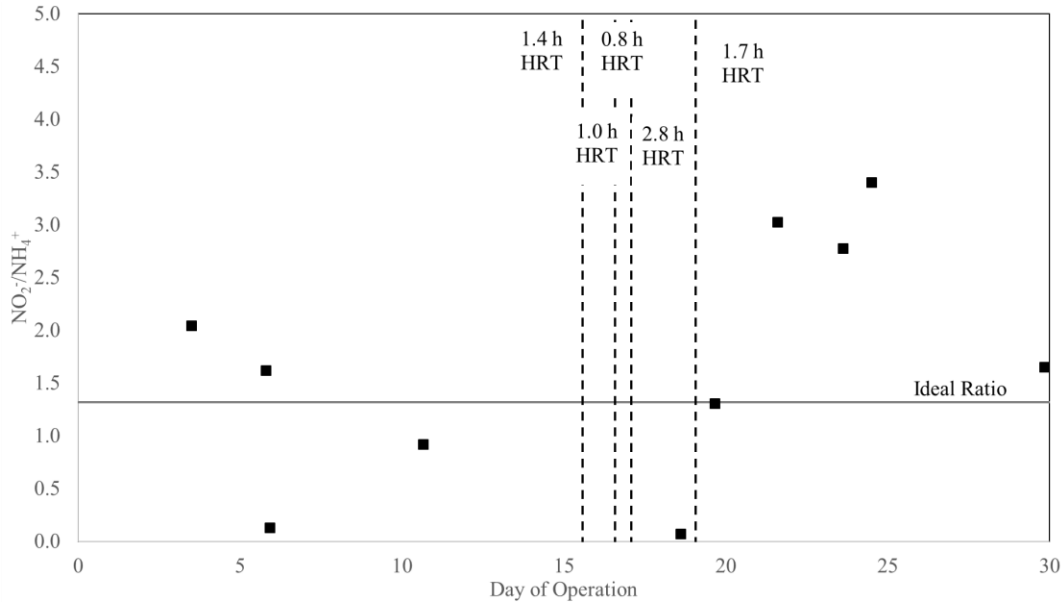

Figure 3.7 (c). NARRs of second anammox immobilization in (c) 2% CA. Beads degraded by approximately 80% by the 15th day.

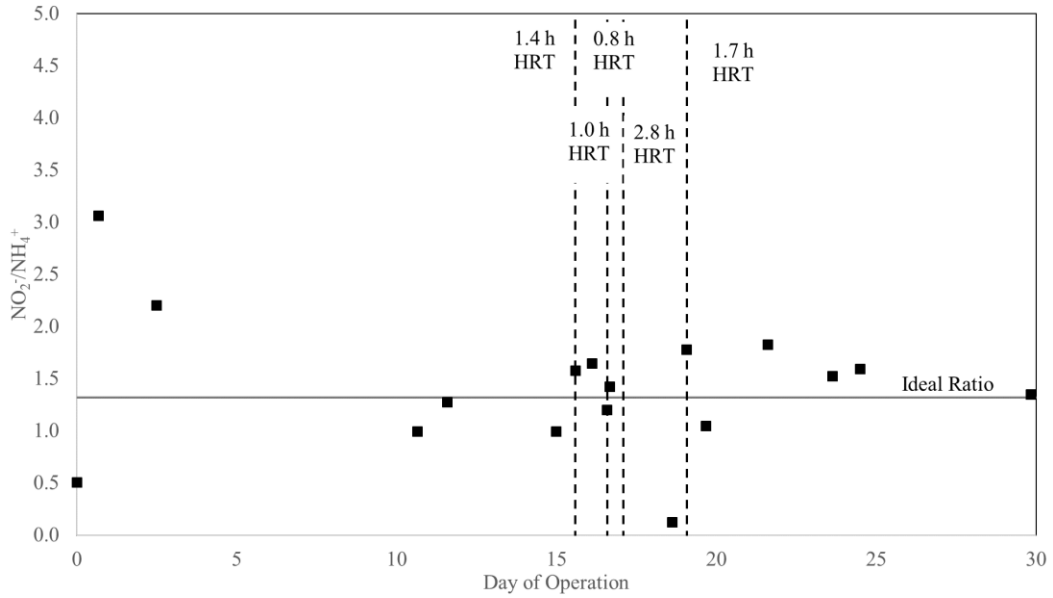

Figure 3.7 (d). NARRs of second anammox immobilization in (d) k1 media.

Initially, the NARRs of the 10% PVA:1% CA treatment were fairly close to the ideal ratio of 1.32 for anammox, with slightly higher nitrite consumption than ammonium (Figure

3.7 (a)), potentially due to heterotrophic activity. Within the first four days, nitrite consumption was substantially less than ammonium consumption, potentially due to the presence of AOBs in the system. These may have been accidentally introduced when this column was opened to remove the gel plug that had formed in the first day of operation. However, this explanation seems unlikely as the UFCs were operated anaerobically, creating an unfavorable environment for AOBs and NOBs. NARRs did not approach the ideal ratio until after 21 days of operation, and then a higher ratio was observed, indicating that NOBs may have been unintentionally enriched instead. For the 3% PVA:1% CA treatment, high NARRs may indicate a substantial amount of heterotrophic denitrification (Figure 3.7 (b)). It is also possible that anammox were additionally converting organic carbon into carbon dioxide using nitrite as an electron acceptor, thus increasing the NARR (Guven *et al.*, 2005). With prolonged operation, the NARRs appear to gradually approach the ideal ratio for this treatment.

The 2% CA treatment fluctuated between nitrifying and denitrifying activity (Figure 3.7 (c)), and rarely approached the ideal anammox ratio. Due to the high variability in this data and lack of triplicate values, this result is inconclusive. In the k1 media treatment, NARRs initially fluctuated but stabilized to near-ideal anammox NARRs within 10 days of operation (Figure 3.7 (d)).

3.2.5 Nitrite/Nitrate Ratios (NNRs)

This section shows the NNRs achieved in the second set of columns (Figure 3.7) for each treatment and explores the potential reasons for the similarities and differences between the treatments. An ideal ratio of 5.1 mole nitrite consumed per mole of nitrate produced corresponds to the ideal metabolic ratio of enriched anammox sludge (Strous *et al.*, 1998).

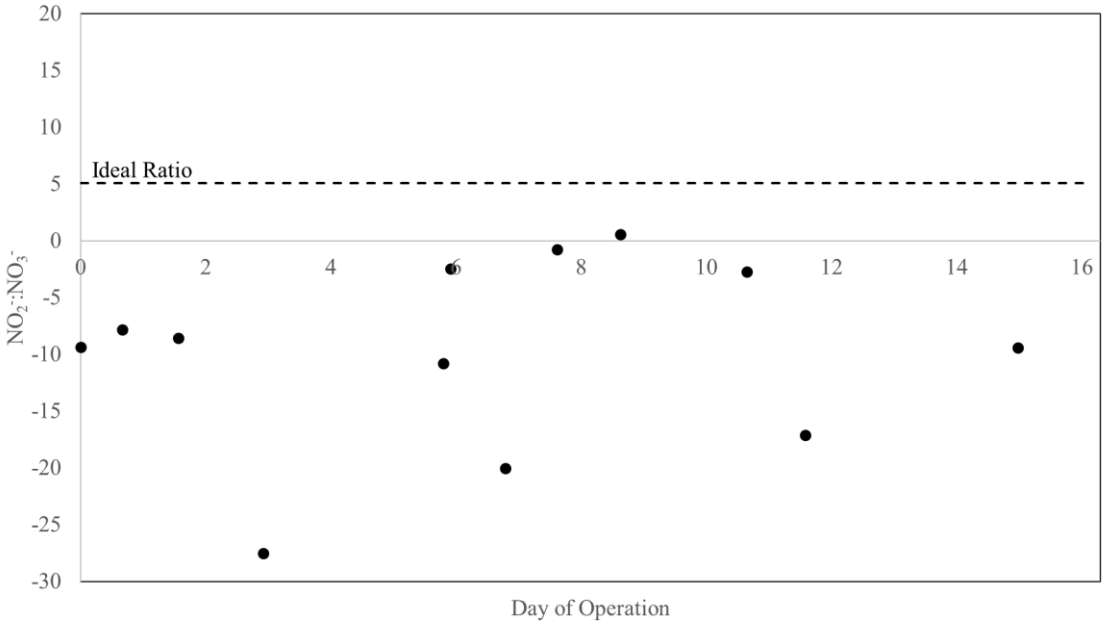

Figure 3.8 (a). NNRs of second anammox immobilization in (a) 10% PVA:1% CA.

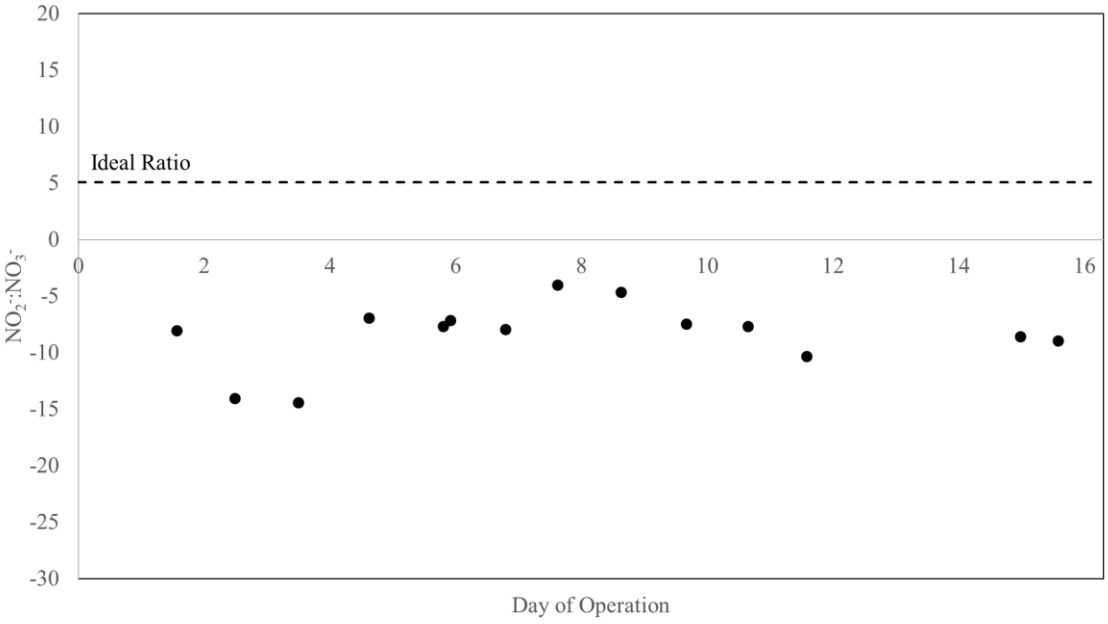

Figure 3.8 (b). NNRs of second anammox immobilization in (b) 3% PVA:1% CA.

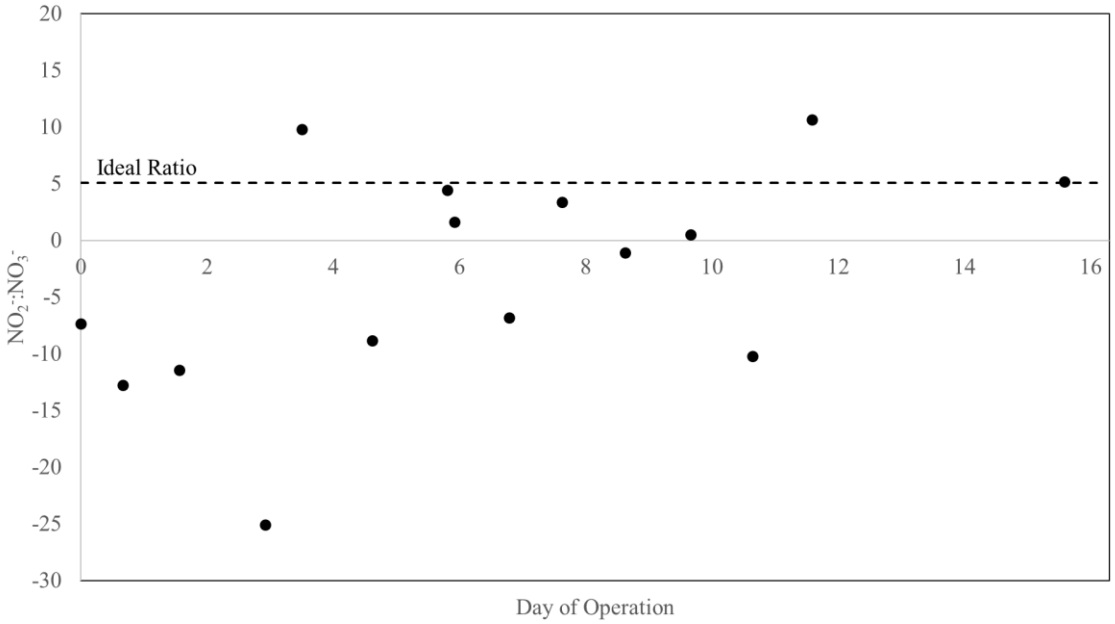

Figure 3.8 (c). NNRs of second anammox immobilization in (c) 2% CA. Beads degraded by approximately 80% by the 15th day.

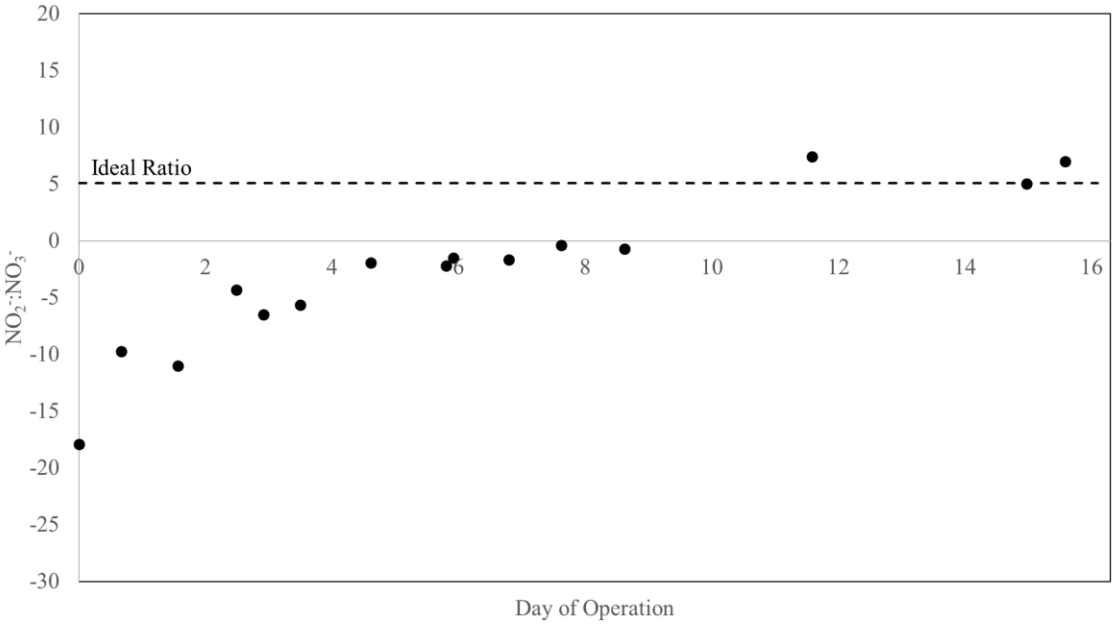

Figure 3.8 (d). NNRs of second anammox immobilization in (d) k1 media.

Negative NNRs correspond with nitrate consumption, and is indicative of heterotrophic denitrification; positive values less than the ideal ratio of 5.1 indicate greater nitrite removal than typically achieved by anammox; and NRRs greater than the ideal ratio of 5.1 could indicate high nitrate generation due to anammox, as these points are still below the theoretical ratio of 8.1 (Eq. 10) (Figure 3.8, (all)). In the 10% PVA:1% CA treatment, NNRs remained very low over the first 15 days of operation, potentially indicating the presence of denitrifiers in the system (Figure 3.8 (a)). Denitrification may have occurred due to the death of bacteria that eventually created enough organic carbon for the growth of denitrifying bacteria (Sultana, 2016). Alginate, and possibly PVA, may even have served as a carbon source for heterotrophs, though this has not been widely reported. Denitrification remained consistent throughout the first 15 days of the 3% PVA:1% CA treatment as well (Figure 3.8 (b)). In the 2% CA treatment, NNRs approached the ideal ratio within the first week, and despite some potentially heterotrophic denitrification, the treatment achieved returned to an ideal ratio within the first 16 days of operation (Figure 3.8 (c)). Potential NOB activity may be due to natural variation of anammox metabolism or is simply due to sampling and detection error. The k1 media treatment achieved the best apparent enrichment over the first 16 days, with nitrate consumption gradually subsiding until it was being generated. By the 12^{th} day of operation, the ideal ratio was achieved (Figure 3.8 (d)).

3.2.6 Summary of Second Immobilizations

The second set of immobilizations were operated for one month and all treatments exhibited nitrogen removal. However, the treatments exhibited more activity by contaminating microbes than seen in the previous immobilizations. It is possible that the beads would stabilize at greater NRRs with continued operation. While it was expected that the second round of immobilizations would be initially more enriched with anammox bacteria than the initial original treatments, contaminating bacteria appeared to have a greater

influence on nitrogen removal than in the previous experiment. There was initially greater nitrogen removal on a basis of sludge volume, but there was a lower amount of sludge added to the second immobilizations, which may account for the perceived decrease in activity in the second immobilizations. This may be due to an aerobic sludge extraction and immobilization procedure; prior to the second immobilization, the enriched sludge from the first set of immobilizations was placed in media for a full day under aerobic conditions. This may have been enough time to inhibit anammox and stimulate the growth of other microbes.

The 2% CA gel was observed to degrade in about half the time (15 days) as the original experiment (Appendix B, Table 3), potentially due to increased microbial activity (Zhu *et al.*, 2009) that was noticed at the beginning of operation. This is supported by the higher NRRs and NREs at initial startup of these reactors (Figures 3.5, 3.6) with respect to the starting NRRs and NREs of the first experiment (Figures 3.2, 3.3). The 3% PVA:1% CA treatment tended to exhibit the most stable operation, but the k1 media permitted the best enrichment over the first month of operation (Figures 3.7, 3.8). More obvious enrichment may continue for all treatments with longer operation.

Since the 3% PVA:1% CA copolymer did not swell as much as gels with high PVA ratios or degrade as quickly as 2% CA gel (Appendix A, Table 4), it is presumed that PVA did not wash out from the beads but formed hydrogen bonds with alginate, as previously described (Zhu *et al.*, 2009). By chemically crosslinking only alginate in the copolymer, the risk of inhibiting the activated sludge with boric acid can be averted. This is likely responsible for the good anammox activity in the immobilizations created by Ali *et al.* (Ali *et al.*, 2015).

While the original experiment was repeated to determine substrate kinetics for the immobilizations, there were substantial fluctuations in nitrogen removal rates and efficiencies in the second experiment, and the gel treatments had already begun to dissolve by the end of the intended stabilization period. Thus, kinetics could not be run on the gel immobilizations

due to unstable operating conditions. Despite the potential presence of heterotrophs, the kinetics models were still applied to the k1 media.

3.3 Kinetics

In this section, kinetic models are presented for the second k1 media treatment. The modified Stover-Kincannon and Grau Second-Order models for predicting effluent substrate were fitted and validated against experimental data over all HRTs of 0.8, 1.0, 1.4, 1.8, and 2.8 hours. Data at the HRT of 2.1 hours was dropped due to high variability.

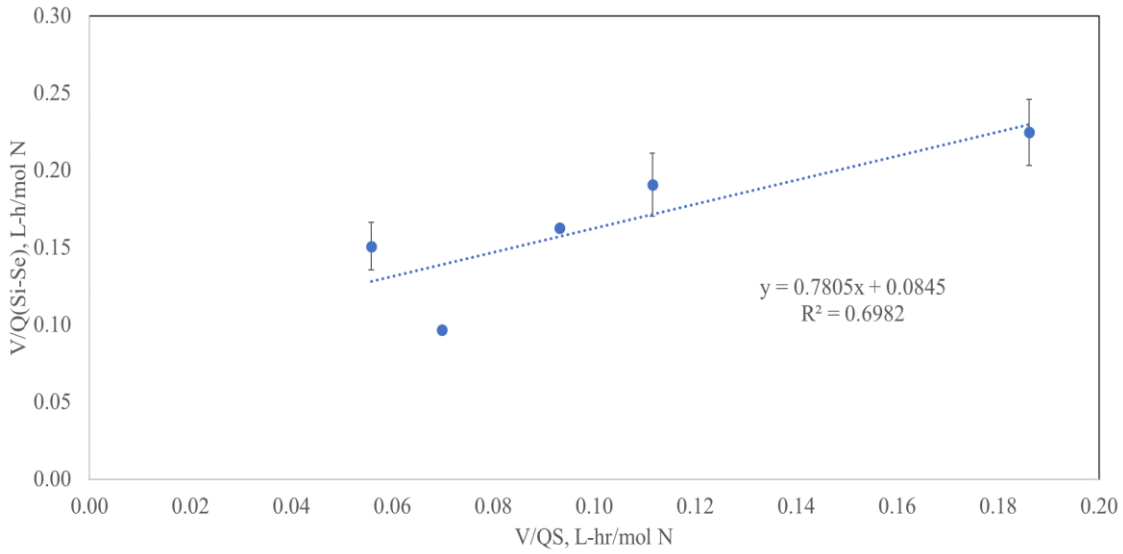

Figure 3.9. Stover-Kincannon modified model for effluent substrate estimation of anammox sludge of second treatment in k1 media. Error bars represent standard deviation of replicates.

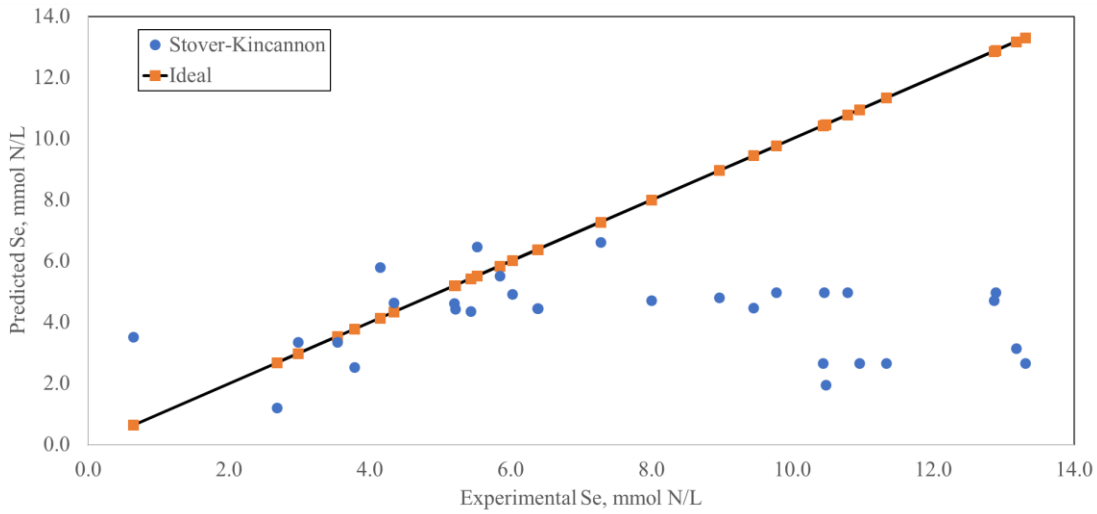

Figure 3.10. Validation of Stover-Kincannon modified model for effluent substrate estimation of anammox sludge of second treatment in k1 media.

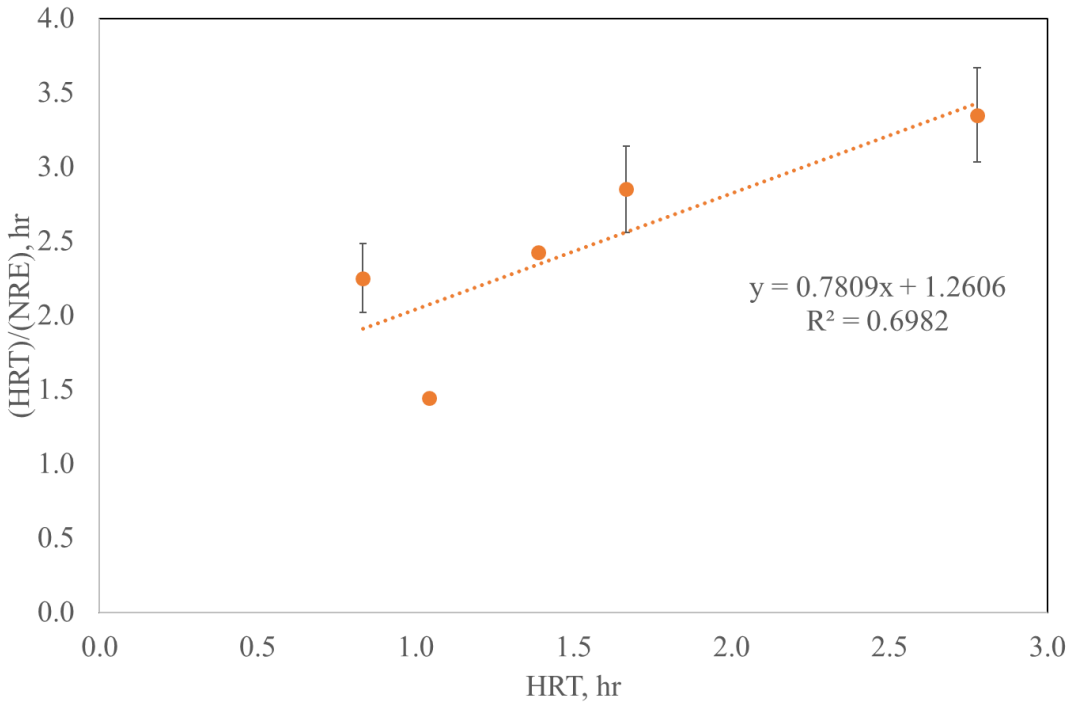

Figure 3.11. Grau second-order model for effluent substrate estimation of anammox sludge of second treatment in k1 media. Error bars represent standard deviation of replicates.

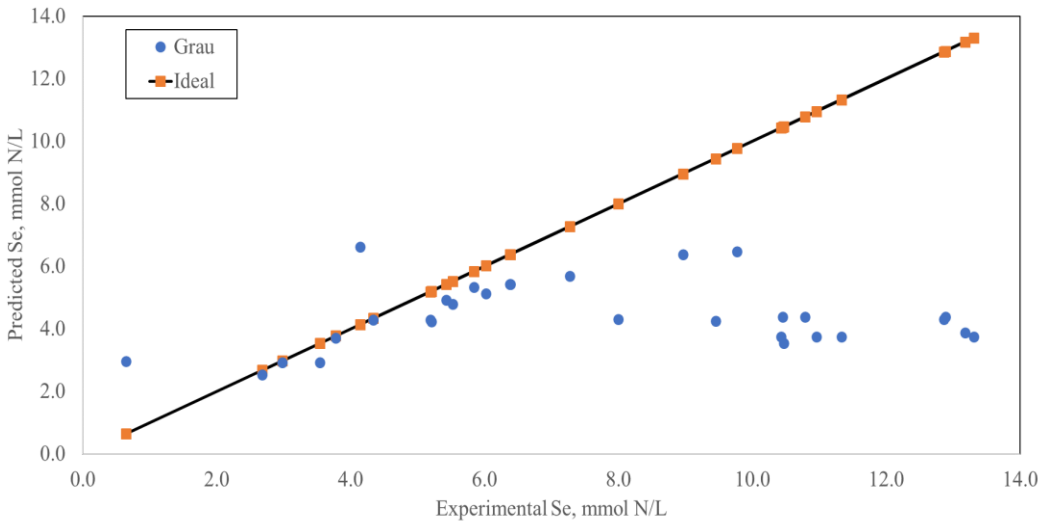

Figure 3.12. Validation of Grau second-order model for effluent substrate estimation of anammox sludge of second treatment in k1 media.

The variability in the nitrogen removal rates and efficiencies of the second k1 media treatment make the system non-ideal for the fitting of kinetics parameters. Nonetheless, the kinetic models were fit to the data. Both the Stover-Kincannon modified model (Figure 3.9) and the Grau Second-Order model (Figure 3.11) fit the data similarly. Thus, either model can be used to approximate the data in place of the other, in keeping with recent findings (Sultana, 2016). When validated against experimental data, neither model fit the data when effluent substrate concentration was less than 2 mM total nitrogen or greater than 7 mM total nitrogen (Figures 3.10 and 3.12). At low effluent concentrations, this may be due to detection limits and natural variability in substrate removal. At higher concentrations, the model breaks down potentially due to the high variation in data associated with limited anammox activity and the potential activity of heterotrophic denitrification.

Table 1. Kinetic model constants for k1 media and literature.

Model	Kinetic Constant	Units	K1 Media	Sultana, SBR	Sultana, UFC	Literature
Grau Second-Order	a	day	1.26	0.12	-	0.964-1.11[1,2]
	b	-	0.78	3.51	-	0.094-1.397[1,2]
Stover-Kincannon	K_B	g N/L-day	0.13	0.17-0.80	15.86	8.97-27.8[3]
	U_{max}	g N/L-day	0.17	0.09-0.27	9.74	7.89-27.4[3]

[1] Jin and Zheng, 2009.
[2] Ni et al., 2010.
[3] Ni et al., 2012.

As seen in Table 1, both Grau second-order and Stover-Kincannon models have kinetic parameters within the range of literature values. These values depend largely upon the extent of anammox enrichment. Sultana determined the above parameters in an unenriched mixed culture containing anammox (Sultana, 2016), while other literature values are reported for highly enriched sludge (Ni *et al.*, 2012). Kinetic parameters for the k1 media indicate a low value relative. This may be due to having normalized these values to the reactor volume instead of sludge volume. Sludge volume was not determined to permit continued operation; however, normalizing to sludge volume would increase these parameters.

Conclusions

Anammox can be successfully enriched in hydrogel immobilizations of polyvinyl alcohol (PVA) and calcium alginate (CA). Despite some biomass washout, 2% CA produced similar activity to a packed-bed sludge reactor and had the highest activity of anammox of the gel immobilizations, achieving a maximum nitrogen removal rate of 0.5 mmol N per hour for both ammonium and nitrite with 100% efficiency. Due to diffusion limitations in 10% PVA and 10% PVA:1% CA treatments, nitrogen removal achieved a maximum value of 0.2 mmol N/h with 60% efficiency. Modeling of an upflow packed-bed reactor with the modified Stover-Kincannon kinetics model showed a U_{max} of 0.17 g N/L-day with a K_B of 0.13 g N/L-day. The Grau second-order kinetics model fit equally well, and these values were similar, though low, to reported literature parameters.

While anammox can be successfully cultured in these PVA-CA formulations, performance is not superior to packed-bed anammox systems for enrichment alone. High concentrations of PVA resulted in poor diffusion properties, preventing dinitrogen gas from escaping as previously reported (Bickerstaff, 1997), making it non-ideal for anammox immobilization. However, PVA can serve as a support matrix for anammox to externally colonize and establish a biofilm, as seen in other systems (Henze *et al*., 2002). Alginate beads were weak and completely dissolved within one month of operation with lower activity sludge, though alginate immobilizations exhibited good mass transfer due to their loose structure. The 3% PVA:1% CA ratio exhibited the best bead stability with limited resistance to diffusion, demonstrating that copolymers of PVA and CA can be optimized to exhibit various desirable properties.

Several questions remain from this study. It remains unclear if the stability of alginate beads were more influenced by sheer stress from increased flowrates, microbial

growth, or by the generation of dinitrogen gas which was observed in the PVA treatments. In the future, controls of uninoculated gel beads should be run in parallel with inoculated gel beads. Additionally, future immobilizations in PVA should utilize sulfate crosslinking, instead of phosphate, to acquire the reduced swelling characteristics reported in literature (Idris *et al*., 2011, Takei *et al*, 2012). Sulfate crosslinking may confer better mass transfer properties for anammox enrichment.

If dissolution of the immobilizing matrix is desired, alginate beads are a good option for future immobilizations, as the sludge recovers as quickly as non-immobilized sludge. This may be useful for the transportation of anammox to new systems. The stability and activity of the 3% PVA:1% CA beads reported above may make this formulation suitable for anammox transportation by providing a stable microenvironment for immobilized cells, minimizing their recovery period when introduced to new environments. The favorability of this microenvironment should also be tested with anammox immobilizations for treatment of landfill leachate, which is known to inhibit non-immobilized anammox (Sultana, 2016).

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Appendix

A. Original Immobilizations

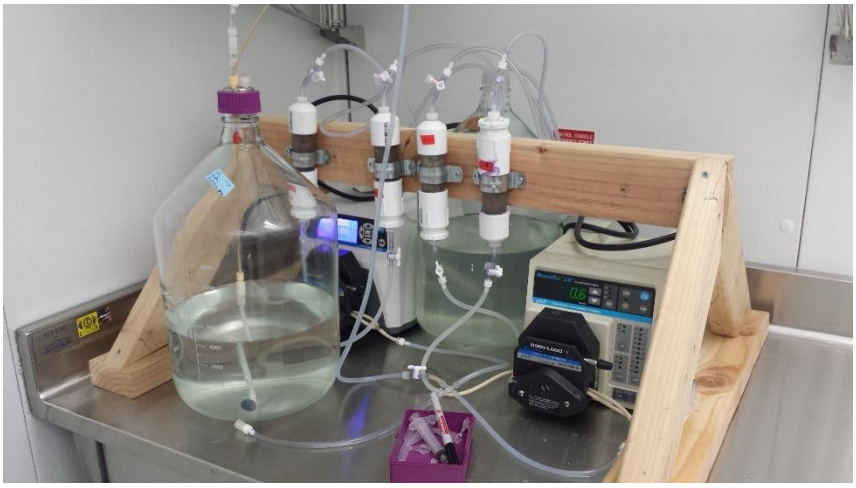


Figure 1. Experimental setup of four UFCs in parallel.

Figure 2. Granular anammox on influent media line of 2% CA UFC.

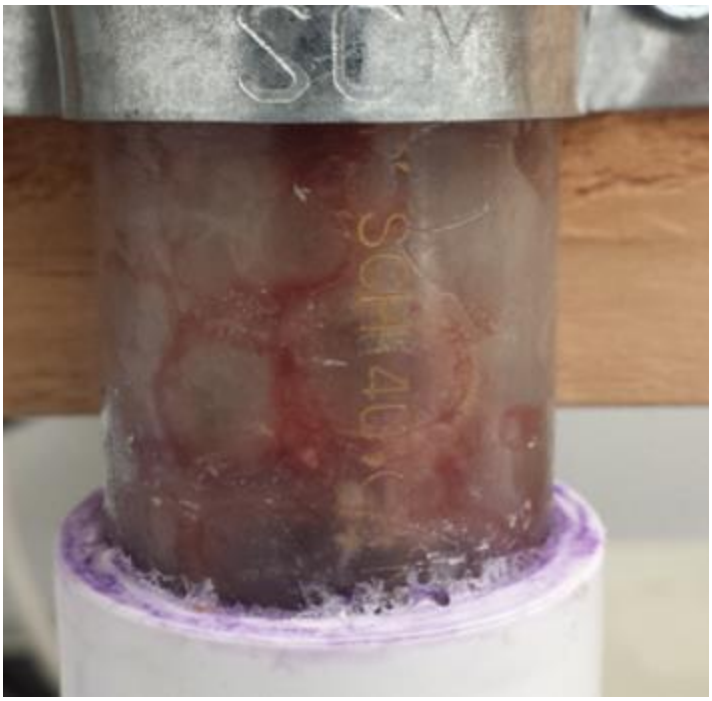
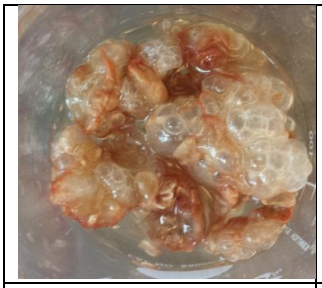
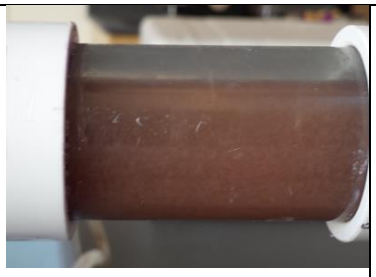
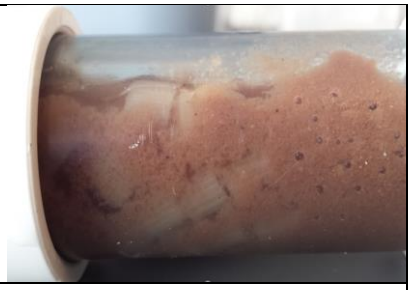


Figure 3. Anammox biofilm on swollen 10% PVA gel in UFC.

Table 1. Results of Original Immobilizations. Both PVA gels were combined in (a).

		
(a) PVA treatments swelled over the first 30 days. Anammox biofilm formed on outside of gel. N_2 gas bubbles from metabolic activity were entrained in the gel matrix.	(b) CA gel was completely dissolved after 30 days, resulting in a sludge blanket at the base of the column. Reddish color indicates anammox enrichment.	(c) K1 beads permitted anammox enrichment, as indicated by reddish color.

B. Second Immobilization Experiment

Table 2. First Day of Operation for Second Immobilization Experiment.

			
(a) 10% PVA, 1% SA, 25 mL sludge, 1:1 gel-sludge. 50 mL upflow column.	(b) 2% SA, 25 mL sludge, 1:1 gel-sludge. 50 mL upflow column.	(c) 3% PVA, 1% CA, 25 mL sludge, 1:1 gel-sludge. 50 mL upflow column.	(d) K1 aquarium filter media, 25 mL sludge.

Table 3. Development of 10% PVA:1% CA anammox immobilization over 30 days of operation.

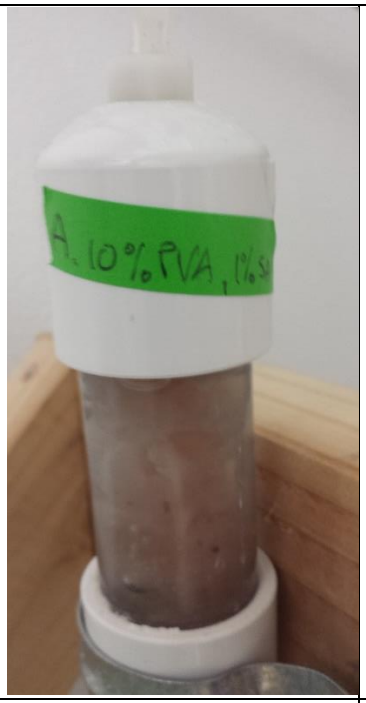
		
4/19/17 (Day 2)	5/1/17 (Day 15)	5/17/17 (Day 30)

Table 4. Development of 2% CA anammox immobilization over 30 days of operation.

		
4/19/17 (Day 2)	5/1/17 (Day 15)	5/17/17 (Day 30)

Table 5. Development of 3% PVA:1% CA anammox immobilization over 30 days of operation

		
4/19/17 (Day 2)	5/1/17 (Day 15)	5/17/17 (Day 30)

Table 6. Development of anammox in k1 media over 30 days of operation

		
4/19/17 (Day 2)	5/1/17 (Day 15)	5/17/17 (Day 30)