UC Riverside

UC Riverside Electronic Theses and Dissertations

Title

Towards Improved Anaerobic Ammonium Oxidation for Treatment of High Strength Ammonia Wastewater

Permalink

https://escholarship.org/uc/item/0wh7c929

Author

Opot, Stephen Robert Ogola

Publication Date

2018

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, availalbe at https://creativecommons.org/licenses/by/4.0/

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Towards Improved Anaerobic Ammonium Oxidation for Treatment of
High Strength Ammonia Wastewater

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

Stephen Robert Opot

March 2019

Dissertation Committee:
        Dr. Mark R. Matsumoto, Co-Chairperson
        Dr. Jinyong Liu, Co-Chairperson
        Dr. Haizhou Liu

The Dissertation of Stephen Robert Opot is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

ABSTRACT OF THE DISSERTATION

Towards Improved Anaerobic Ammonium Oxidation for Treatment of
High Strength Ammonia Wastewater

by

Stephen Robert Opot

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, March 2019
Dr. Mark Matsumoto, Co-Chairperson
Dr. Jinyong Liu, Co-Chairperson

Excess nutrients, particularly nitrogen (N), from wastewater discharged into waterways lead to eutrophication, hypoxia, and oceanic red tide that threaten public and environmental health. Anaerobic Ammonium Oxidation or Anammox is a relatively new treatment approach for N-removal from wastewater that utilizes less energy and eliminates the need for organic carbon additives. However, the process has encountered pragmatic challenges that impede its widespread adoption as a practical treatment alternative. Foremost among these challenges is the very slow growth rate of the Anammox bacteria, which necessitates long start-up times before high N-removal efficiencies can be obtained on a steady-state basis. As such, it was proposed to amend bioreactors with materials, chabazite, that are capable of concentrating growth factors (e.g., ammonium, NH_4^+), while enabling selective colonization of Anammox bacteria to accelerate the start-up period and to enhance culture development. Nonetheless, bacterial activity in the amended process risk inhibition from cations released during NH_4^+-IX. In relation, the study also sought to

investigate the effect of chabazite type (-Na & -Ca), target cation (NH_4^+), and competing cation (i.e. K^+) on the NH_4^+ oxidation rate.

Anammox culture was developed from atypical thermophilic anaerobic digester (TAD) mixed-sludge. Increased amount of TAD mixed-sludge reduced start-up time, within 59 days, and enhanced Anammox culture development. The Anammox bacteria specie was identified as "*Candidatus* Brocadia sinica."

Chabazite particles had minimal influence on process start-up time but improved N-removal efficiency and Anammox bacteria population. Chabazite-Na reactor was impacted adversely to a greater extent at high feed concentrations than chabazite-Ca reactor with NH_4^+ removal efficiencies decreasing from >95% to 53.5% and >95% to 88%, respectively. The effect of chabazite type on NH_4^+ removal depended on the extent to which NH_4^+-IX impacted the partial nitrification step during the Anammox process. However, chabazite-Na reactor exhibited high Anammox bacteria population due to accelerated bacteria biofilm formation and growth. Upon spiking the feed media with different K^+ concentrations that ranged from 0 to 5.12 meq/L, it was determined that the effect of NH_4^+-IX on partial nitrification was mitigated at increased K^+ concentration in chabazite-Na leading to high NH_4^+ and NO_2^- utilization rates than chabazite-Ca reactor.

Table of Content

List of Figures

List of Tables

Introduction

Nitrogen discharged into open waterways from wastewater treatment plants (WWTPs) has become a widespread problem because most conventional WWTPs are running out of capacity to comply with the decreasing discharge limits (Sushetana *et al*., 2017; Fux *et al*., 2002). Excess nitrogen discharged into waterways lead to eutrophication, hypoxia, and oceanic red tide that threaten public and environmental health (Park *et al.*, 2010). The USEPA recognizes the pragmatic challenges related to site specific nutrient (ammonia) criteria establishment (USEPA, 2013). In efforts to establish more widely acceptable criteria, the USEPA is seeking to develop total ammonia nitrogen (TAN) criteria based on best available science. In 2013, USEPA updated the freshwater ammonia aquatic life ambient water criteria, in accordance with the provisions of section 304(a) of the Clean Water Act (CWA), to revise the Ambient Water Quality Criteria (AWQC) to 17 mg TAN/L and 1.9 mg TAN/L for acute (1-hr average) and chronic (30-day rolling average), respectively, based on pH=7 and temperature = 20°C (USEPA, 2013).

California regulates ammonia (as N) from WWTPs' secondary effluent based on treatment technology but provides a range of 6-18 mg/L for typical domestic wastewater treatment, which became effective in September 2014 (SWRCB, 2014). Even by using performance-based standards in California, several WWTPs are experiencing increasingly high ammonia concentrations averaging 45mg/L (Cruz *et al*., 2018) and reduced flows in the influent (DeZeller and Maier, 1980) due to the increasing population and climate change that has further led to rampant drought conditions and more stringent water conservation measures (Zouboulis and Tolkou, 2015).

Historically, most WWTPs have relied on conventional nitrogen removal (N-removal) processes to meet the compliance demands amidst more stringent regulations. In doing so, conventional N-removal processes use externally applied air and organic carbon to support microbial growth, making the process both energy intensive and anthropogenic source of U.S. greenhouse gas (GHG) emissions (U.S EPA, 2016). With stringent regulations on nutrient (ammonia) and GHG discharge from WWTPs, conventional N-removal process maybe unfavorable to treat high-strength ammonia wastewater.

The use of a combined partial-nitrification and anaerobic ammonium oxidation process (Anammox) is a relatively new alternative for ammonia removal from wastewaters with low carbon to nitrogen ratio (Kim *et al.*, 2008). Compared to the conventional multi-stage nitrification-denitrification nitrogen (N) removal process, the Anammox process utilizes less energy and eliminates the need for organic carbon additives in wastewater treatment plants. The first full-scale reactor was installed in Rotterdam, Netherlands (Van der Star *et al.*, 2007).

Beyond that system, however, to date, less than 100 large-scale processes have been developed worldwide; and less than 10 full-scale processes have been adopted in the U.S (Lackner *et al.*, 2014). Widespread utilization of the process has operational drawbacks that impede its acceptance as a practical treatment alternative. Foremost among these challenges is the very slow growth rate of the Anammox bacteria, which necessitates long start-up times before high nitrogen removal efficiencies can be obtained on a steady-state basis. Attempts to reduce start-up time are challenged by inadequate seed biomass and bacteria washout during operation.

Efforts to improve start-up and long-term process stability have not been successful due to inadequate mass of Anammox bacteria, difficulty to grow the bacteria, and subsequent retention of the bacteria in the treatment reactors (Sliekers *et al*., 2003). Initial seed biomass concentration is critical to the process as it is strongly linked to the abundance and diversity of Anammox bacteria during and after start-up. However, since the bacteria thrive under strict anoxic condition and require an approximate steady concentration ratio of $NH_4^+/NO_2^- = 1.32$ to grow (Lotti *et al*., 2012), it is very difficult to attain the ideal feed substrate ratios in a one-stage process, which makes it more difficult to start the Anammox process (Kanders *et al*., 2014).

Furthermore, due to the low growth rates and potential washouts, it is further challenging to retain the bacteria in the reactor as they remain suspended during culture development in bioreactors (Sliekers *et al*., 2003). This problem is further challenged by inadequate information on the various Anammox bacteria retention methods (Chen *et al*., 2010) and amendment materials (Chen *et al*., 2015) for selective colonization and growth of Anammox bacteria. As such, efforts are needed to promote faster bacterial growth and system retention.

One approach is to amend bioreactors with materials that have beneficial surface and chemical properties that are capable of concentrating growth factors (e.g., ammonium, NH_4^+), while enabling selective colonization of Anammox bacteria on the materials' surfaces. This strategy could reduce start-up time and biomass wasting.

In the first portion of this research (Chapter 2), studies were conducted to evaluate the feasibility of using thermophilic anaerobic digester (TAD) mixed-sludge to enhance

Anammox culture development, to improve performance, and to reduce process start-up time. The objectives of this part of the research were to:

- Assess the potential to use TAD mixed-sludge to improve Anammox activities, process start-up time, and Anammox bacteria population.

- Determine the effect of feed substrates ratio (NH_4^+/ NO_2^-) and alkalinity (HCO_3^-) concentrations on ammonium oxidation rate.

Anammox culture was developed from atypical thermophilic anaerobic digester (TAD) mixed-sludge added proportionately in three bench-scale reactors. All the reactors showed phenotypic Anammox activities at approximately 90% total nitrogen removal with varying start-up times. Start-up time is the time taken to attain maximum specific Anammox activity (SAA^{max}) at maximum and stable nitrogen loading rate (NLR). Reactor 3 which was augmented with the highest proportion of TAD mixed-sludge had the fasted start-up time, 59 days, and attained the highest specific anammox activity (SAA) at highest nitrogen loading rate. Reactor 2 which was augmented with 25% (v/v) of TAD mixed-sludge had the highest Anammox bacteria gene copy numbers after two consecutive doubling times.

The Anammox bacteria species in the reactors was identified as "*Candidatus Brocadia sinica.*" The high abundance of gene copy numbers was attributed to less competition among bacteria during start-up because reactor 2 was augmented with possible optimum proportions of both active Anammox biomass and TAD mixed sludge that mitigated the potential effect of the bacteria mediating the partial nitrification process out-competing the Anammox bacteria in utilizing the available NH_4^+ for metabolic activities

and growth.   To determine the effect of feed characteristics on Anammox rates, highest value of 0.07 g NH_4^+ L^{-1} day^{-1} was achieved at an optimum HCO_3^- concentration value of 541.4 mg/L as $CaCO_3$, which is lower than typical reported value of 1039.4 mg/L as $CaCO_3$ for Anammox systems (Trukhina *et al.,* 2011). The highest specific anammox rate of 0.16 g $NgVSS^{-1}Day^{-1}$ was realized at 3.0 g VSS L^{-1} biomass concentration. The findings in this part of the research imply that thermophilic anaerobic digester (TAD) effluent mixed liquor may be a potential inoculum source with an advantageous application in Anammox process start-up to remove excess ammonium from wastewater.

In chapter 3, chabazite zeolite was used as a strategy to retain bacteria in the treatment reactor to enhance the development of the Anammox culture.   It was hypothesized that Anammox culture development can be enhanced using Chabazite mineral particles as process amendment material. The objectives of this part of the research were to:

- Assess the feasibility of using chabazite to enhance Anammox culture development.

- Assess whether Anammox process start-up time is reduced by using chabazite material to amend the process.

To study the feasibility of using Chabazite particles to enhance Anammox culture development and activities, two 3-L sequencing batch reactors were seeded with fresh return activated sludge (RAS) obtained from Los Angeles – Glendale WWTP owned by the City of Los Angeles.

Chabazite particles obtained from St. Cloud Mining Company (New Mexico, U.S.) was added in one reactor (amended) while the other reactor was operated without chabazite addition and acted as the control.

Chabazite addition in the reactor had minimal influence on the Anammox process start-up time because both the amended (with chabazite) and non-amended reactors exhibited evidence of Anammox activity after 69 days. However, at increased feed variability, the control reactor experienced reduced nitrate production, increased NH_4^+ and NO_2^- effluent concentration, and reduced nitrogen removal efficiency (NRE) from 95% to 86% while the amended reactor's NRE remained constant above 95%.

Chabazite material mitigated the effect of high feed variability resulting in optimum feed concentration needed by the Anammox bacteria. The amended reactor also exhibited quicker recovery than the control reactor at high substrate concentrations, which was attributed to chabazite sorption of NH_4^+ on the surface leading to increased Anammox bacteria population in the amended reactor.

The results in this part reveal that even though there is minimal influence on Anammox process start-up time, addition of chabazite enhanced Anammox culture development at high feed variability.

Based on the second portion of this research, and relying on the tested chabazite form, it was determined that chabazite has minimal influence in Anammox process start-up time. However, chabazite is categorized into four types based on the dominant non-framework cation, *chabazite-Ca, chabazite-K, chabazite-Na*, and *chabazite-Sr* (Coombs *et*

al., 1998; Erdem *et al*., 2004; Sprynskyy *et al*., 2005). Among the mentioned chabazite types, *chabazite-Ca* and *–Na* are the most commonly used (Aponte-Morales *et al*., 2016).

Breck (1974) suggested the likely cation selectivity order and was later modified by Lahav *et al*. (1998). Following both Breck and Lahav cation selectivity orders, it could be speculated that cation prominence on chabazite material may have an impact on ammonium oxidation rate. As revealed by Lahav *et al*. (1998), Na^+ is a less competitive cation than both NH_4^+ and Ca^+ and could easily be exchanged with NH_4^+ faster than Ca^+. In relation, a comparative study was conducted to determine the effective chabazite sub-specie for Anammox process amendment in Chapter 4. The objectives of this part of the research were to:

- Determine whether chabazite-Na has the highest ammonium removal efficiency compared to other chabazite types in the Anammox process.

- Determine how Anammox bacteria population is impacted by the amendment of different chabazite types.

The different chabazite types employed in the study included the Bowie chabazite AZUB (Na/Ca), AZLB-Na, and AZLB-Ca obtained from St. Cloud Mining Company in New Mexico, U.S.

The order of chabazite-amended bioreactor performance in terms of NH_4^+ removal efficiencies was found to be reactors amended with chabazite-Ca > Na/Ca > Na > non-amended (sand control).

Among the chabazite amended bioreactors, the reactor amended with chabazite-Na was the most impacted in terms of nitrogen removal efficiency at high feed concentrations,

while the reactor amended with chabazite-Ca was least impacted depicting a decrease in the ammonium removal efficiencies from $> 95\%$ to 53.5% and $> 95\%$ to 88%, respectively.

High ammonium removal efficiency observed in chabazite-Ca reactor was attributed to high NH_4^+ ion-exchange rate compared to other chabazite types. Effect of chabazite sub-specie on ammonium removal depended on the extent to which NH_4^+ ion-exchange impacted the partial nitrification step of the Anammox process that is mediated by the ammonium oxidizing bacteria. Despite lower reactor performance than chabazite-Ca reactor, the total specific bacteria population was highest in the chabazite-Na reactor among all the reactors.

The high bacteria population in the chabazite-Na reactor resulted from a higher NH_4^+ ion-exchange rate that accelerated bacteria biofilm formation and growth over a prolonged period.

The findings from this part of the research imply that while chabazite-Ca greatly mitigates the effect of high feed variability on Anammox activities compared to other chabazite sub-species, chabazite-Na exhibits high bacteria population attributed to high NH_4^+ ion-exchange rate, which is significant in developing Anammox biomass retention strategies for process utilization.

In Chapter 5, the effect of a competing cation on ammonium oxidation rate was studied. It was hypothesized that the rate of Anammox ammonium oxidation is influenced by the concentrations of a target and competing cation. The concentration of a competing cation, especially in the feed and reactor aqueous solution would affect NH_4^+ ion-exchange which would further impact the Anammox process amended using chabazite material.

Among the possible cations in the feed media, K^+ is the most important competing cation compared to NH_4^+, Ca^+, and Na^+; that is, K^+ could favorably exchange chabazite cations (Ca^+ and Na^+) faster than NH_4^+ as inferred from the cation selectivity order.

The objectives of this part of the research were to:

- Determine the effect of competing cation (K^+) on ammonium oxidation rate.
- Determine the effect of competing cation (K^+) on feed substrates (NH_4^+ & NO_2^-) utilization rates in Anammox processes amended with different chabazite sub-species.

Sequencing batch reactors with active Anammox biomass were amended with appropriate doses of chabazite-Na, chabazite-Ca, and non-amended (control). Upon spiking the feed media with different K^+ concentrations that ranged from 0 to 5.12 meq/L, it was determined that NH_4^+ and NO_2^- utilization rates were higher in chabazite-Na reactor compared to chabazite-Ca reactor at high K^+ concentration. The rates determined in chabazite-Na reactor for NH_4^+ and NO_2 were approximately 1.1 and 1.4 times higher than the rates in chabazite-Ca reactor, respectively.

It was determined that the effect of NH_4^+ ion-exchange on partial nitrification was mitigated at increased K^+ concentration, which led to improved substrates utilization by increased Anammox bacteria population resulting in higher Anammox removal rates in chabazite-Na reactor than chabazite-Ca reactor. The results reveal that chabazite-Na mitigates the effect of competing cations (K^+) during chabazite-amended Anammox process which is significant in developing Anammox biomass retention strategies for enhanced culture development.

From this research, thermophilic anaerobic digester (TAD) effluent mixed-sludge was determined as a potential inoculum source with an advantageous application in Anammox process start-up to remove excess ammonium from wastewater. In employing chabazite to enhance Anammox culture development, it was determined that even though there is minimal influence on Anammox process start-up time, addition of chabazite enhances Anammox culture development at high feed variability. In addition, amending the Anammox process with chabazite-Na could lead to higher development of bacteria population compared to other chabazite sub-species, which was attributed to high NH_4^+ ion-exchange rate that, in effect, accelerates bacteria biofilm formation and growth over a prolonged period. Of the chabazite types, chabazite-Na was found to mitigate the effect of competing cations (K^+) in a chabazite-amended Anammox process leading to improved substrates utilization by the Anammox bacteria.

Chapter 1 : Background

1.1. The Anammox process and benefits

Wastewater treatment plants (WWTPs) often employ conventional nitrogen removal (N-removal) processes. In doing so, conventional N-removal processes rely on externally applied air and organic carbon to support microbial growth making the process energy intensive (Shoener *et al*., 2014). Bolles *et al* (2006) reported that the aeration systems utilized in conventional N-removal process represents 45 to 60% of the treatment plant's energy consumption. In addition, nitrous oxide emitted from conventional N-removal processes account for approximately 5% of the total U.S. greenhouse gas (GHG) emissions (U.S EPA, 2016). With increasing stringent regulation on nutrient (ammonia) and GHG discharge from WWTPs, under the US EPA's National Pollutant Discharge Elimination System (NPDES), conventional N-removal process maybe unfavorable to treat high-strength ammonia wastewater.

The use of a combined partial-nitrification and anaerobic ammonium oxidation process or Anammox is a relatively new alternative for ammonia removal from wastewaters with low carbon to nitrogen ratio (Kim *et al.*, 2008). Anammox activity was first discovered in a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor (Mulder *et al.*, 1995) following an earlier theoretical study in which Broda *et al* (1977) concluded that ammonium (NH_4^+) could be used as an electron (e-) donor and nitrate ($NO_3^{-)}$ as an e- acceptor under strict anoxic environment. Later, Van der Graaf *et al* (1995) reported that nitrite (NO_2^-) was indeed the e-acceptor with hydrazine (Kartal *et al.*, 2011) as an intermediate.

During the Anammox reaction, ammonium and nitrite are converted to N_2 as shown in **Equation 1-1** (Van der Graaf *et al*., 1995). The process utilizes HCO_3^- as the sole carbon source and NO_2^- as the electron acceptor for ammonium oxidation (Mulder *et al*., 1995).

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2$$
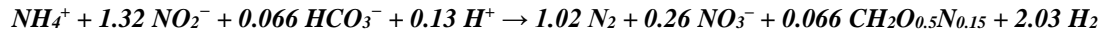
Equation 1-1: Anammox chemical equation.

The bacteria mediating the process are slow growers of the order *Planctomycetales* with approximate doubling times of 10 to 12 days (Strous *et al.*, 1998), which is higher than 0.6 and 4 days associated with activated sludge bacteria and methanogens, respectively (Seviour *et al.*, 2010; Ferry, 1993).

The Anammox biochemical pathway has not been widely investigated, but Kartal *et al* (2011) suggests a likely pathway with hydrazine (N_2H_4) and nitric oxide (NO) as intermediates (Kartal *et al*., 2012).

Due to the low biomass yield (Jetten *et al.*, 1997), reduced O_2 demand, zero addition of external carbon source, and reduced N_2O emissions (Wrage *et al.*, 2001), the process is more favorable to remove nitrogen (as ammonium) from high-strength ammonia wastewater than the conventional process.

1.2. Problems associated with the Anammox process

The first full-scale reactor was installed in Rotterdam, Netherlands (Van der Star *et al*., 2007). Beyond that system, however, to date, less than 100 large-scale processes have been developed worldwide; and less than 10 full-scale processes have been adopted in the U.S (Lackner *et al*., 2014).

Widespread utilization of the process has operational drawbacks that impede its acceptance as a practical treatment alternative including very slow bacteria growth, bacterial washouts during operation, and the risk of process instabilities from inhibition factors such as influent salinity (Abma *et al.*, 2007).

Efforts to improve start-up and long-term process stability have not been successful due to inadequate mass of Anammox bacteria (Van der Star *et al.*, 2007), difficulty to grow the bacteria (Strous *et al.*, 1998), and subsequent retention of the bacteria in the treatment reactors (Sliekers *et al.*, 2003).

Initial seed biomass concentration is critical to the process as it is strongly linked to the abundance and diversity of Anammox bacteria during and after start-up (Park *et al.*, 2010). However, since the bacteria thrive under strict anoxic condition and an approximate steady NH_4^+/NO_2^- ratio of 1.32 to grow (Lotti *et al.*, 2012), it is very difficult to attain the ideal feed substrate ratios in a one-stage process and makes it difficult to start the Anammox process (Kanders *et al.*, 2014).

Furthermore, due to low growth rates and potential washouts, it is challenging to retain the bacteria in the reactor as they typically remain suspended in the culture development bioreactors (Sliekers *et al.*, 2003). This problem is further challenged by inadequate information on the various Anammox bacteria retention methods (Chen *et al.*, 2010) and amendment materials (Chen *et al.*, 2015) for selective colonization and growth of Anammox bacteria. As such, a method or strategy of retaining the bacteria in the treatment reactor is needed to enhance the development of the Anammox culture.

1.3. Improving biological treatment processes

Biological wastewater treatment processes have previously been augmented with desired materials to improve treatment. Materials with unique chemical and physical properties (e.g., the ability to catalyze a process reaction and/or to provide bacteria immobilization surface (Armentano *et al*., 2014)) are often employed in sequencing batch reactors (hybrid systems) to increase bacteria growth rates to enhance systems' performance (Gai and Kim, 2008).

Hybrid systems have previously been used to remove pollutants from wastewater; for instance, Yu *et al.* (2006) reported that by employing Zero-Valent Iron (ZVI) to reduce perchlorate using autotrophic culture, the biomass density was increased and correspondingly led to a 4-fold increase in the rate of perchlorate reduction. Jung *et al.* (2004) also determined that methanogenesis and nitrification rates were enhanced by reducing NH_4^+ concentration levels that caused inhibition when cation exchange materials were added to the bioreactors treating anaerobic wastewater.

1.4. Chabazite characteristics and application to enhance the Anammox Process

Zeolite materials, because of their high cation exchange capacity (CEC) and ammonium ion exchange (NH_4^+ IX) rates, have gained much attention to remove ammonium from wastewater (Booker *et al*., 1996). He *et al.* (2004) used powder zeolite to amend a biological nitrogen removal (BNR) process treating municipal wastewater with a total nitrogen concentration of 54 mg-N L^{-1} and reported that the nitrification rate increased by a factor of two compared to the non-zeolite amended process. Fernandez *et*

14

al. (2008) further reported an improved enrichment of Anammox biomass and reduction of washout to values as low as 3 mg VSS L^{-1}.

Natural zeolites are hydrated aluminosilicates with three-dimensional structure and high adsorption capacity (Erdem *et al.*, 2004). There are approximately 82 variants of zeolite, which are further categorized into different sub-groups (Coombs *et al.*, 1998). Clinoptilolite (zeolite, group 7) is the most abundant and used zeolite species; thus, frequently referred to as the 'natural zeolite' (Sprynskyy *et al.*, 2005).

A zeolite's high CEC is dependent on the presence of either Si^{4+} or Al^{3+} in the mineral lattice. The

Figure 1-1: Anammox bacteria forming a biofilm layer on an ammonium-rich chabazite particle.

substitution of Si^{4+} by Al^{3+} increases the negative charge, which is balanced by the readily available cations (e.g. Na^+) in the aqueous solution. The balanced cations (e.g. Na^+) can subsequently be exchanged with other cations e.g. NH_4^+ (Erdem *et al.*, 2004; Langwaldt, 2008). While focus has been on 'natural zeolite' (clintoptilolite) as an amendment material to enhance biological wastewater treatment, there is recent evidence of higher a CEC zeolite with better NH_4^+ IX rates compared clintoptilolite (Karmen *et al.*, 2013; Langwaldt, 2008). Chabazite (zeolite, group 4) may a better alternative for ammonium removal from wastewater. Chabazite has lower Si^{4+} to Al^{3+} ratio than clinoptilolite (Erdem *et al.*, 2004; Langwaldt, 2008) that could potentially increase the mineral negative charge for cation

substitution leading to increased CEC. In addition, because of its metaphysical and structural properties, chabazite pore cavities could act as anoxic microenvironments for growth of anoxic bacteria (Erdem *et al.*, 2004; Sprynskyy *et al.*, 2005). Moreover, NH_4^+ adsorption and bio-regeneration could be increased because of the negative charge resulting from the high composition of Al^{3+} content within the framework (Lahav *et al.*, 2000). These properties could favor development of anoxic bacteria such as Anammox bacteria. Since Anammox bacteria grow under high ammonia (Hu *et al.*, 2011) and low organic carbon (Dalsgaard and Thamdrup, 2002) conditions; and with relatively higher CEC and NH_4^+ IX capacity than clinoptilolite, chabazite mineral could possibly attain high surface concentrations of ammonium to create a naturally selective environment for proliferation and retention of Anammox bacteria as shown in **Figure 1-1**. It was hypothesized that Anammox culture development could be enhanced by using Chabazite mineral particles as process amendment material.

1.5. Influence of chabazite sub-specie on ammonium oxidation rate

Chabazite is further categorized into four individual sub-species or types, based on the dominant non-framework cation, *chabazite-Ca, chabazite-K, chabazite-Na*, and *chabazite-Sr* (Coombs *et al.*, 1998; Erdem *et al.*, 2004; Sprynskyy *et al.*, 2005). *Chabazite-Ca* and *–Na* are the most commonly used types (Aponte-Morales *et al.*, 2016). Breck (1974) suggested a likely cation selectivity order, later modified by Lahav *et al.* (1998) as shown in **Equation 1-2.**

$$Ti^+ > K^+ > Ag^+ > Rb^+ > NH_4^+ > Pb^{2+} > Na^+ = Ba^{2+} > Sr^{2+} > Ca^{2+} > Li^+ \text{ (Breck, 1974)}$$

$$K^+ > Ca^{2+} > NH_4^+ >> Na^+ > Mg^{2+} \text{ (Lahav et al., 1998)}$$
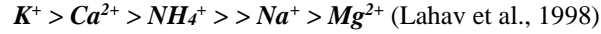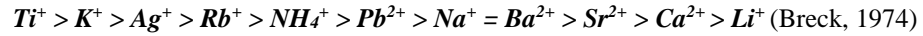
Equation 1-2: Cation selectivity order.

Based on the Breck and Lahav cation selectivity orders, it could be speculated that cation prominence on chabazite material may have an impact on ammonium oxidation rate. As revealed by Lahav *et al*. (1998), Na^+ is a less competitive cation than both NH_4^+ and Ca^+ and could easily be exchanged with NH_4^+ faster than Ca^+. To address whether the type of chabazite affect the ammonium oxidation rate, it was also hypothesized that *chabazite-Na* has a higher ammonium removal rate than *chabazite-Ca* in a one-stage Anammox process (**Hypothesis 3**).

1.6. The effect of competing and target cations on substrate utilization rate

During ion-exchange (IX), non-framework cations are exchanged with the target cation (NH_4^+) and released into the water column. Depending on the concentration of cations in the feed, the released cation could impact the Anammox substrate utilization rate. Sanchez et *al.* (2004) previously reported a decrease in nitrification rate during IX process in the presence of Na^+ at approximately 2000 mg/L.

In addition, the presence of competing cations (e.g. K^+) could further inhibit the process. Referring to the cation selectivity orders, K^+ potentially out-compete NH_4^+, Ca^+, and Na^+ (Langwaldt, 2008) for the exchange sites on chabazite particles. Therefore, NH_4^+ and K^+ concentration in the bulk solution could affect NH_4^+ IX. As such, it was further hypothesized that the rate of substrate utilization is affected by the concentrations of target (NH_4^+) and competing cation (K^+) (**Hypothesis 4**).

1.7. Bibliography

1. Abma W.R.; Schultz C.E., Mulder J.W., et al., *Full-scale granular sludge Anammox process*. Water Science and Technology, 2007. **55**: p. 27–33.
2. Aponte-Morales V.E., Tong S., Ergas S. J., *Nitrogen removal from Anaerobically Digested Swine Waste Centrate using a laboratory-scale chabazite-sequencing batch reactor*. Environmental Engineering Science, 2016. **33**: p. 324-332
3. Armentano I., Arciola C.R., Fortinati E., Mattioli S., Amoroso C.F.; Rizzo J., Kenny J.M., Imbriana M., Visai L., *The interaction of bacteria with engineered nanostructured polymeric materials: a review*. Scientific World, 2014. 410423.
4. Bolles S., *Modeling wastewater aeration systems to discover energy savings opportunities*. Process Energy Services LLC, 2006.
5. Booker N.A., Cooney A.J., Priestley A.J., *Ammonia removal from sewage using natural Australian zeolite.* Water Science & Technology, 1996. **34**: p. 17-24.
6. Breck D.W., *Zeolite Molecular Sieves: Structure, Chemistry, and Use*. Wiley: New York. 1974.
7. Broda, E., *Two kinds of lithotrophs missing in nature*. Basic Microbiology, 1977. **17**: p. 491-493.
8. California (CA) State Water Resource Control Board (CA SWRCQB), General *Wastewater Discharge Requirements for Small Domestic Wastewater Treatment Systems*, 2013. Order WQ 20140-0153-DWQ.
9. Chen G.; Li J., Tabassum S., Zhang Z., *Anaerobic ammonium oxidation (ANAMMOX) sludge immobilized by waterborne polyurethane and its nitrogen removal performance – a lab scale study*. RSC Advances, 2015. **5**: p. 25372-25381.
10. Chen J., Zheng P., Tang Y.Y.C., Mahmood Q., *Promoting sludge quantity and activity results in high loading rates in Anammox UBF*. Bioresource Technology, 2010.**101**: p. 2700-2705.
11. Coombs D.S., Alberto A., Armbruster T., Vezzalini G., *Recommended Nomenclature for Zeolite Minerals: Report of the Subcommittee on Zeolite of the International Mineralogical Association, Commission on New Minerals and Minerals Names*. Mineralogical Magazine, 1998.**62**: p.533-571.
12. Cruz H., Luckman P., Seviour T., Verstraete W., Laycock B., Pikaar I., *Rapid removal of ammonium from domestic wastewater using Polymer hydrogels*. Nature, 2018. **8**: p. 2912.
13. Dalsgaard T., and Thamdrup B., *Production of N2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments*. Applied Environmental Microbiology, 2002. **68**: p. 1312–1318
14. DeZeller J., and Maier W.J., *Effects of Water Conservation on Sanitary Sewers and Wastewater Treatment Plants*. Water Environment Federation, 1980. **52**(1): p.76-88.
15. Erdem E., Karapinar N., Donat R., *The removal of heavy metal cations by natural zeolite*. Colloidal and Interface Science, 2004. **280**: p. 309–314.

16. Fernandez I., Vazquez-Padin, J.R., Mosquera-Corral A., Campos, J.L., Mendez R., *Biofilm and granular systems to improve Anammox biomass retention*. Biochemical Engineering, 2008.**42**: p. 308–313.

17. Ferry, J. G., *Methanogenesis: Ecology, Physiology, Biochemistry & Genetics*. New York: Chapman & Hall, 1993. Print.

18. Fux C., Boehler M., Huber P., Brunner I., Siegrist H., *Biological treatment of ammonium-rich wastewater and subsequent anaerobic ammonium oxidation (anammox) in a pilot plant*. Biotechnology, 2002. **99**(3): p.295-306.

19. Gai X.J., Kim H.S., *The role of powdered activated carbon in enhancing the performance of membrane systems for water treatment*. Desalination, 2008. **225**: p.288-300.

20. He S.b., Xue G., Kong H.N., Li X.*, Improving the performance of sequencing batch reactor (SBR) by the addition of zeolite powder*. Journal of Hazardous Materials, 2007. **142**: p.493-499.

21. Hu B. I., Shen L.D., Xu X.Y., Zeng P., *Anaerobic ammonium oxidation (anammox) in different natural ecosystems*. Biochemical Society Transactions, 2011. **39**: p.1811-1816.

22. Jetten, M. S. M., Horn, S. J., van Loosdrecht, M. C. M., *Towards a more sustainable municipal wastewater treatment system*. Water Science and Technology, 1997. **35**: p. 171-180.

23. Jung J.Y., Chung Y.C., Shin H.S., Son D.H., *Enhanced ammonia nitrogen removal using consistent biological regeneration and ammonium exchange of zeolite in modified SBR process*. Water Research, 2004. **38**: p. 347-354.

24. Kanders L., Areskoug T., Schneider Y., Ling D., Marisa P., Beier M., *Impact of seeding on the start-up of one-stage deammonification MBBRs*. Environmental Technology, 2014. **35**: p. 2767-2773.

25. Karadag D., Koc Y., Turan M., Ozturk M., *A comparative study of linear and non-linear regression analysis for ammonium exchange by clinoptilolite zeolite*. Hazardous Materials, 2007: p. 432-437.

26. Karmen M., Nataša Zabukovec L., Mario S., Anamarija F., *Natural Zeolites in Water Treatment – How Effective is Their Use*. 2013.

27. Kartal B., Niftrik L.V., Keltjens J.T., Op den Camp H.J.M, Jetten M.S.M., *Anammox-Growth Physiology, Cell Biology, and Metabolism*. Advances in Physiology, 2012. **60**: p. 211-262.

28. Kartal, B., Maalcke, W. J., de Almeida, N. M., Cirpus, I., Gloerich, J., Geerts, W., den Camp, H., Harhangi, H. R., Janssen-Megens, E. M., Francoijs, K. J., Stunnenberg, H. G., Keltjens, J. T., Jetten, M. S. M., Strous, M., *Molecular mechanism of anaerobic ammonium oxidation*. Nature, 2011. **479**: p. 127-159.

29. Kim D., Tae-Su K., Yasuhiro T., Ryu H.D., Lee S.I., *Treatment of low carbon-to-nitrogen ratio wastewater using two-stage sequencing batch reactor with independent nitrification*. Process Biochemistry, 2008.**43**: p. 406-413.

30. Lackner S., Gilbert E.M., Vlaeminck S.E., Joss A., Horn H; Loosdrecht., Full-*scale partial nitritation/anammox experiences-An application survey*. Water Research, 2014.**55**: p. 292-303.

31. Lahav O., and Green M., *Ammonium removal using ion-exchange and biological regeneration*. Water Research, 1998. **32**: p. 2019-2028.
32. Langwaldt J., *Ammonium removal from water by eight natural zeolites: A comparative study*. Separation Science and Technology, 2008. **43**: p. 2166-2182.
33. Lotti T., Van der Star W.R.L., Kleerebezem R., Lubello C., Van Loosdrecht M.C.M., *The effect of nitrite inhibition on anammox process*. Water Research, 2012. **46**: p. 2559-2569.
34. Mulder, A., Vandegraaf, A.A., Robertson, L.A.; Kuenen, J.G., *Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor*. FEMS Microbiology Ecology, 1995. **16**: p. 177-183.
35. Park H., Rosenthal A., Jezek R., Ramalingam K., Fillos, Chandran K., *Impact of inocula and growth mode on the molecular microbial ecology of anaerobic ammonia oxidation (anammox) bioreactor communities*. Water Research, 2010. **44**: p. 5005-5013
36. Sanchez O., Aspe E., Marti M.C., Roeckel M., *The effect of sodium chloride on the two-step kinetics of the nitrifying process*. Water environment research, 2004. **76**: p. 73-80.
37. Seviour, R. J., and Per Halkjær. Nielsen, *Microbial Ecology of Activated Sludge*. London: IWA, 2010. Print.
38. Shoener, B. D., Bradley, I. M., Cusick, R. D., Guest, J. S., *Energy positive domestic wastewater treatment: the roles of anaerobic and phototrophic*. Royal Society of Chemistry, 2014. **16**: p. 1204-1222.
39. Sliekers A.O., Third K.A., Abma W., Kuenen J.G., Jetten M.S.M., *CANON and Anammox in a gas-lift reactor*. FEMS Microbiology Letter, 2003. **218**: p. 339-344.
40. Sprynskyy M., Lebedynets M., Terzyk A.P., Kowalczyk P., Namiesnik J., Buszewski B., *Ammonium sorption from aqueous solutions by the natural zeolite Transcarpathian clinoptilolite studied under dynamic conditions*. Colloidal and Interface Science, 2005. **284**: p. 408-415.
41. Strous M., Heijnen J.J., Kuenen J.G., Jetten M.S.M., *The sequencing batch reactors as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms*. Applied Microbiology Biotechnology, 1998. **50**: p. 589-596.
42. Sushetana B., Rajagopalan B., Silverstein J., *Assessment of wastewater treatment facility compliance with decreasing ammonia discharge limits using regression tree model*. Science of the Total Environment. 2017. **598**: p. 249-257.
43. US EPA, Aquatic *Life Ambient Water Quality Criteria for Ammonia – Freshwater Report*. Office of Water. 2013. EPA 822-R-13-001.
44. Van der Star W.R.L, Abma W.R., Blommers D., et al., *Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam*. Water Research, 2007. **41**: p. 4149–4163.
45. Vandegraaf, A. A., Mulder, A., Debruijn, P., Jetten, M. S. M., Robertson, L. A., Kuenen, J. G., *Anaerobic oxidation of ammonium is a biologically mediated process*. Applied and Environmental Microbiology, 1995. **61**: p. 1246-1251.

46. Wrage, N., Velthof, G. L., van Beusichem, M. L., Oenema, O., *Role of nitrifier denitrification in the production of nitrous oxide*. Soil Biology & Biochemistry, 2001. **33**: p. 1723-1732.

47. Yu X., Amrhein C., Deshusses M.A., Matsumoto M.R, *Perchlorate Reduction by Autotrophic Bacteria in the Presence of Zero Valent Iron*. Environmental Science & Technology, 2006.**40**: p. 1328-1334.

48. Zouboulis A., Tolkou A., *Effects of Climate Change in Wastewater Treatment Plants: Reviewing the Problems and Solutions*. Springer Water. 2015. **6**: p. 197-220.

Chapter 2 : Start-up and performance of Anaerobic Ammonium Oxidation process from atypical Thermophilic Digester Effluent

2.1. Abstract

Unique and favorable characteristics coupled with the affirmation of a syntrophic association between anaerobic ammonium oxidizing (Anammox) bacteria and denitrifying anaerobic methane oxidizers (DAMO), offer thermophilic anaerobic digesters (TAD) mixed-sludge a niche to improve Anammox culture development.

This study assessed the feasibility of using TAD mixed-sludge to enhance Anammox culture development to improve performance and to reduce process start-up time. The effect of initial feed substrate ratio, initial biomass, and inorganic carbon concentrations on Anammox activity were also investigated. Anammox cultures seeded with different amounts of TAD mixed-sludge were developed in 3 bench-scale reactors. Reactors 1 (R1) was seeded with active Anammox mixed liquor while Reactor 2 (R2) and Reactor 3 (R3) were augmented with 25% (v/v) and 50% (v/v) of TAD mixed-sludge, respectively.

All three reactors showed phenotypic Anammox activities and attained maximum nitrogen removal efficiencies above 95%. R3 was the fastest to start, 59 days, followed by R2, 85 days, and R1, after 153 days. In addition to having a faster start-up time, R3 also attained the highest maximum Specific Anammox Activity (SAA^{max}) at maximum nitrogen loading rate (NLR). The Anammox bacteria species in all the reactors was identified as "*Candidatus* Brocadia sinica."

Despite having a slower start-up time than R3; R2 attained the highest specific Anammox bacteria population after 44 days to depict approximately 4 generations of the Anammox bacteria assuming each doubling time is 11 days ((Strous *et al.*, 1998). The high Anammox bacteria gene copy numbers determined in R2 was attributed to possible mitigated effect of the bacteria mediating the partial nitrification process out-competing the Anammox bacteria in utilizing the available NH_4^+ for metabolic activities and growth experienced during start-up time.

In assessing the effect of feed characteristics on Anammox rates, the highest rate value of 0.07 g NH_4^+ L^{-1} day^{-1} was attained at 12.5mM HCO_3^- concentration, while the highest specific Anammox rate of 0.16 g $NgVSS^{-1}Day^{-1}$ was determined at 3.0 g VSS L^{-1} biomass concentration. The results imply that TAD mixed-sludge has potential application in Anammox process start-up to remove excess ammonium from wastewater.

2.2. Introduction and Background

Wastewater treatment plants (WWTPs) mostly use multi-stage conventional nitrification and denitrification (NDN) processes to remove nitrogen (as ammonia) from municipal wastewater (Sun *et al*., 2010). The multi-stage nitrification and denitrification processes rely on externally applied air (as O_2) and carbon (as CO_2) to support microbial growth making the process energy intensive (Shoener *et al*., 2014). Bolles *et al* (2006) reported that the biological aeration systems utilized in the conventional NDN process accounts for between 45 and 60% of the total plant's energy consumption. In addition, nitrous oxide emitted from conventional nitrogen removal processes accounts for about 5% of the total U.S. greenhouse gas (GHG) emissions (U.S EPA, 2016). With increasing stringent regulation on nutrient (as ammonia) discharged from WWTPs to waterways, conventional NDN processes has been dimed unfavorable to treat high-strength ammonia wastewater (Third *et al*., 2001).

A two-step process that involves partial-nitrification (step 1) and anaerobic ammonium oxidation (Anammox) (step 2), in a single-stage reactor, has been reported as a better alternative to remove ammonia from wastewater with low carbon to nitrogen (C/N) ratio than the conventional multi-stage NDN process (Kim *et al.*, 2008).

The first step partially oxidizes NH_4^+ to NO_2^- in the presence of the available O_2 (Kalvelage *et al*., 2011). The Anammox process utilizes alkalinity (HCO_3^-) as the sole carbon source and NO_2^- as the electron acceptor for ammonium oxidation producing dinitrogen (N_2) gas (Mulder *et al*., 1995) as shown in **Equation 1-1** (Van der Graaf *et al*., 1995).

The bacteria mediating the process (Anammox bacteria) have characteristically low growth rates because they primarily grow due to inorganic ammonium oxidation coupled to nitrite reduction using HCO_3^- as the carbon source (Kalvelage *et al*., 2011).

Anammox bacteria are of the order *Planctomycetales* with approximately 10- to 12-day doubling times (Strous *et al.*, 1998), which is higher than the 0.6- and 4-day doubling times commonly associated with activated sludge bacteria and mesophilic methanogens, respectively (Seviour *et al.*, 2010; Ferry, 1993).

Kartal *et al* (2011) reported the likely biochemical pathway for the Anammox process and reported hydrazine (N_2H_4) and nitric oxide (NO) as the likely intermediates.

The Anammox process is more suitable in removing nitrogen (as ammonium) from high-strength ammonia wastewater than the conventional process because it produces less sludge, utilizes low O_2, requires zero addition of external carbon source, and have negligible emissions of N_2O (Jetten *et al.*, 1997; Wrage *et al.*, 2001).

Even with the Anammox process advantages over the multi-stage NDN process, the process has not received widespread utilization in mainstream wastewater treatment because of pragmatic operational challenges associated with the Anammox process (Li *et al*., 2018). One of the primary challenges is the slow growth rate of Anammox bacteria, which extends process start-up time (Abma *et al*., 2007).

Further, slow bacteria growth rates result in low biomass yield and long start-up times. To overcome this challenge, recent work has focused on developing strategies to improve biomass yield and retention (Manonmani and Joseph, 2018). However, efforts to improve start-up time and long-term process stability have not been successful due to

inaccessibility of Anammox seed biomass (seed) (Van der Star *et al*., 2007). Seed biomass selection has been shown to be critical in improving biomass yield because the initial seed is strongly linked to the abundance and diversity of Anammox bacteria during and after start-up period (Park *et al.*, 2010).

Nonetheless, selection of seed sources is much difficult as there is inadequate information on the ecological, environmental, and microbial community characteristics on the different types of seed (Tao *et al.*, 2013; Erguder *et al*., 2009). Hu *et al.*, (2011) noted the importance of understanding seed-sludge characteristics and suggested that process start-up and long-term stability could be improved by inoculation if the conditions for competition between the different bacteria were known. As such, selecting a seed source with favorable characteristics is important to develop an Anammox culture (Tao *et al.*, 2013).

With most Anammox processes being seeded and started from conventional sludge sources such as the return activated sludge (RAS) that is characterize with high abundance of nitrifying bacteria (Ye *et al*., 2018; Tomar *et al*., 2016; Tomar *et al*., 2016), competition among other unknown bacteria during acclimatization pose additional challenges in determining optimum operational conditions (Jia *et al.*, 2014).

The Anammox reaction is a tightly coupled cyclic catabolic process, which further makes it vulnerable to disturbances (Kartal *et al.*, 2012).

These challenges, taken together, hinder the enrichment of Anammox culture. Therefore, it is important to explore different strategies to improve the applicability of the Anammox process, which can potentially be achieved through the selection of good seeds

(Li and Yang, 2007; Tomar *et al*., 2016) to reduce process start-up time and improve performance.

Anammox bacteria are ubiquitous in marine and terrestrial environments (Park *et al.*, 2010), and have widely been shown to grow under high ammonia (Hu *et al*., 2011) – low organic carbon (Dalsgaard and Thamdrup, 2002) –psychrophilic (Guo *et al*., 2015) and –mesophilic conditions (Jaeschke *et al*., 2009). Anammox bacteria have also been detected in extremophilic conditions such as thermophilic and halophilic environments (Erguder *et al*., 2009; Li *et al*., 2010; Yoshie *et al*., 2006). The detection of Anammox bacteria in California and Nevada hot-springs (Jaeschke *et al*., 2009) proves that the bacteria can possible grow under thermophilic conditions (Byrne *et al*., 2008). Moreover, recent report on metagenomic studies reveal that Anammox bacteria have a mutualistic coexistence with denitrifying anaerobic methane oxidizing (DAMO) bacteria (Hu *et al., 2015*) and are considered key players in methane production (Zhu *et al*., 2011; Ding et al., 2016).

Similar conditions can be found in thermophilic anaerobic digesters (Desmottes *et al.*, 2015). Thermophilic anaerobic digester mixed sludge (TAD mixed-sludge) being rich in ammonium with proportions of DAMO (Ding *et al*., 2014) could offer a new seeding source for the process. Mixing proportions of active Anammox biomass with TAD mixed-sludge may be ideal to seed and start the process. Mixing or augmenting active Anammox culture and TAD mixed-sludge may also mitigate the effect of increased nonviable bacteria, which are considered a potential source of organic carbon that necessitates the growth of other competitive autotrophs (Hu *et al*., 2010). The presence of proportions of Anammox bacteria and the unique characteristics of TAD mixed sludge (Suvilampi and

Rintala, 2003) may improve Anammox activity and enhance Anammox culture development.

Long start-up time has also been reported to hinder the application of the process (Uyanik *et al.*, 2011). Since TAD mixed-sludge could potentially enhance Anammox culture development, it may also be true that using high proportions of TAD mixed-sludge to augment the mixed inoculum sludge may further reduce the process start-up time leading to better Anammox culture development.

Process instability is another bottleneck towards Anammox process adoption (Jin *et al*., 2008).   The process may experience instability caused by variable feed characteristics (Kanders *et al*., 2014). Understanding the effect of the variability of feed substrates ratio and alkalinity concentrations on the rate of Anammox ammonium oxidation is therefore significant in determining the process stability.

To investigate these hypotheses, this study aimed [1] to assess the potential to use TAD mixed-sludge to improve Anammox activities, process start-up time, and Anammox bacteria population; and, [2] to determine the effect of feed substrates ratio (NH_4^+/ NO_2^-) and alkalinity (HCO_3^-) concentrations on ammonium oxidation rate. If reduced Anammox start-up time and enhanced Anammox culture development is attained, this research contribution will have significant impact towards widespread application of the Anammox process and assist to mitigate ammonium exposure to aquatic environment.

2.3. Materials and Methods

2.3.1. Reactor configuration and operation

Three-liter (3L) Sequencing Batch Reactors (SBRs) were assembled and used to develop the Anammox cultures as shown in **Figure 2-8** in the **Appendix**. The reactors were seeded with thermophilic anaerobic digester mixed liquor from the City of Los Angeles - Hyperion Water Reclamation Plant (HWRP). The seeding mixed liquor characteristics are shown in **Table 2-1**.

Table 2-1: [A] HWRP thermophilic anaerobic digester mixed sludge characteristics (Desmottes et al., 2015). [B] Other volatile compounds found in HWRP thermophilic anaerobic digester mixed sludge

	Parameters	mg/L		Parameters	PPMV
A	pH	> 8	**B**	Carbon Disulfide	0.24
	Alkalinity, as $CaCO_3$	1310-4020		Carbonyl Sulfide	1.43
	TtCOD	859-3130		Dimethyl Disulfide	2.38
	TsCOD	322-2094		Dimethyl Sulfide	0.61
	NH_3-N	367-1120		Methyl Mercaptan	0.25
	NO_2-N	0.2-1		Hydrogen Sulfide	117.04
	NO_3-N	0.2-2		Isopropyl Mercaptan	0.26
	TKN	345-1160		Methane	62.29
	COD/TKN	1.2-5.6		Methyl Mercaptan	1.66
	CO_2	37.10		N-Propyl Mercaptan	0.32
	SO_4^{-2} (Dissolved)	5.94			
	Volatile Acids	291.99			
	Volatile Suspended Particles (VSP)	64.73			
	Total Suspended Particles (TSP)	1.96			

The reactors were stirred using magnetic stirrers set at 60 rpm to achieve complete mixing. The pH in the reactors was controlled between 7.2 and 7.5 by adding 0.5M of HCL to the feed medium. Anoxic conditions in the reactors were maintained by flushing feed media with nitrogen gas. The reactors were fed interchangeably between 1 and 2 days. During feeding, the reactors could settle for 30 min and effluent withdrawn for 10 min.

2.3.2. Feeding composition

The feed medium had the following composition per liter of deionized water: 0.059 –0.177g $(NH_4)_2SO_4$, 0.0121 – 0.0304g $NaNO_2$, 1.25g $KHCO_3$, 0.025g KH_2PO_4, 0.3g $CaCl_2.2H_2O$, 0.2g $MgSO_4.7H_2O$, 0.00625g $FeSO_4$, 0.00625g EDTA, and 1.25mL trace elements solution as prepared by (Zhang *et al.,* 2008). The reactors were fed by carefully increasing the NO_2^- concentrations in the feed media to achieve appropriate NH_4^+/NO_2^- stoichiometric ratio without NO_2^- inhibiting the process because NO_2^- concentration as low as 20 mg/L can upset the process during process start-up. (Strous *et al.*, 1998).

2.3.3. Analytical methods

Ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were analyzed using colorimetric methods according to standard methods of water and wastewater analysis (Albertson, 2000). Biomass concentrations were measured as mixed liquor volatile suspended solids (MLVSS) using standard method. Briefly, to determine MLVSS, dry weight was determined after drying the sample at 105°C for at least 1 hour, and ashed in a furnace (550°C) for 30 min. Dissolved oxygen (DO) concentrations and pH were monitored with selective electrodes. To monitor settling rates, the sludge settling velocity (S.V) and volumetric index (SVI) were occasionally determined following the standard methods.

2.3.4. *Molecular assay*

2.3.4.1.Nucleic acid extraction

Genomic DNA was extracted from samples of enriched biomass using the Qiagen kit. The quality and quantity of extracted DNA was measured by agarose gel electrophoresis and spectrophotometry. The final extracted samples were diluted to between 5 and 10 ng ul^{-1} for use as PCR template.

2.3.4.2.Denaturing gradient gel electrophoresis (DGGE)

All samples were PCR amplified with Anammox bacteria-specific primers (Amx368f/Amx820r) targeting the 16S rRNA gene. The PCR approach for detecting Anammox bacteria followed previously published protocol by Sonthiphand and Neufeld (2013). All PCR amplifications were carried out at initial denaturation temperature of 95°C for 5 min, followed by primer-set-specific thermal cycling conditions with a total of 30 to 35 cycles and a final extension of 72°C for 10 min to complete the reaction (Sonthiphand *et al*., 2013). Final PCR products were verified after amplification via agarose gel electrophoresis to confirm amplicon size using 8% acrylamide gels, and with 30% to 70% denaturing gradients. DGGE gels were also run following the same procedure. Briefly, the DGGE gels were stained with SYBR green and scanned with a Pharos FXTM Plus Molecular Imager. Final representative PCR products were sent to UCR Institute for Integrated Genome Biology for sequencing.

2.3.4.3. Quantitative real-time PCR (qPCR)

The total Anammox bacterial population was investigated following Bipin *et al.* (2007) protocol. The Anammox bacteria standard curves were constructed from Escherichia coli genomic DNA (Sonthiphand *et al.*, 2013). Each constructed *E. coli* PCR product was purified using a MinElute kit (Qiagen, USA) and quantified using Nano-Drop Spectrophotometer. Ten-fold serial dilutions were applied to the standard DNA PCR product template to create qPCR standard curves with efficiencies $\geq 90\%$ and coefficients of determination (R^2) ≥ 0.996 for all standard curves. The agarose gel electrophoresis was employed on all products after each run to confirm the specificity of the qPCR amplification. The qPCR master mix used contained 5 ml of SsoAdvanced SYBR Green Supermix, 0.03 ml of each primer (100 mM stocks), 0.02 ml of bovine serum albumin (10 mg ml^{-1} stock) and 1 ml of genomic DNA template (5–10 ng stock) in a total volume of 10 ml. All qPCR amplifications were performed in duplicate on a CFX96 real-time system (Bio-Rad, USA).

2.3.4.4. Batch assays

Batch assay of known biomass concentrations (gVSS L^{-1}) was employed following the procedure to evaluate specific anammox activity (SAA) as described by Buys *et al* (2000). Briefly, the specific ammonium removal rate was determined from the maximum slope of the curve describing the ammonium removal per time divided by the biomass concentration and the volume of the reactor.

Volumetric nitrogen removal (g-N L^{-1} day^{-1}) and specific Anammox activity (SAA, either mg-N g VSS^{-1} day^{-1} or g-N g VSS^{-1} day^{-1}) rates were employed as basic tools to

assess the effects of substrate ratio (limiting substrate (NO_2^-) to target ion (NH_4^+)) and biomass concentration (cell density, g VSS L^{-1}), and alkalinity on ammonium oxidation rates.

To determine the effect of alkalinity concentration on ammonium oxidation rates, batch experiment with systematic range of $KHCO_3$ concentrations between 0 to 24 mM was conducted. Trukhina *et al* (2011) reported that optimum bicarbonate concentration on a modified deamox process is 24mM. Therefore, significant effect on ammonium oxidation rate would be observed at $KHCO_3$ concentrations between 0 and 24 mM.

To estimate Anammox transient turnover rate, samples were taken from the batch reactors at appropriate time intervals. Synthetic mineral medium with known concentrations of NH_4^+, NO_2^-, and de-oxygenated with N_2 gas was used for this experiment.

2.4. Results and Discussion

2.4.1. *Reactor performances and start-up*

The three bench-scale sequencing batch reactors were monitored for start-up time and performance. Reactor 1 (R1), which was the control reactor, was seeded with active Anammox biomass to accelerate Anammox bacteria growth. The other two reactors were augmented with varying proportions of active Anammox biomass and TAD mixed sludge obtained from Hyperion Wastewater Treatment Plant's (City of Los Angeles) anaerobic digester side-stream. Reactor 2 (R2) and reactor 3 (R3) were augmented with 25% (v/v) and 50% (v/v) of TAD mixed sludge.

The reactors were fed at incremental nitrogen loading rates (NLR) to a maximum value of 35.0 0 mg-N L^{-1} Day $^{-1}$ (**Figure 2-1**) and were simultaneously monitored for

changes in NH_4^+, NO_2^-, and total nitrogen ($NH_4^+ + NO_2^-$) as shown in **Figure 2-7** in the **Appendix**. Alkalinity and biomass (MLVSS) concentrations were also quantified while monitoring changes in temperature, pH, and dissolve oxygen (DO). All the three reactors showed phenotypic Anammox activities and attained maximum nitrogen removal efficiencies greater than 95% at stable state. The proportion of nitrate produced in all the reactors was determined to as 15% of the total nitrogen consumed (result not shown), which is close to 11% that was theoretically determined following **Equation 1-1** by Zhang *et al.* (2008).
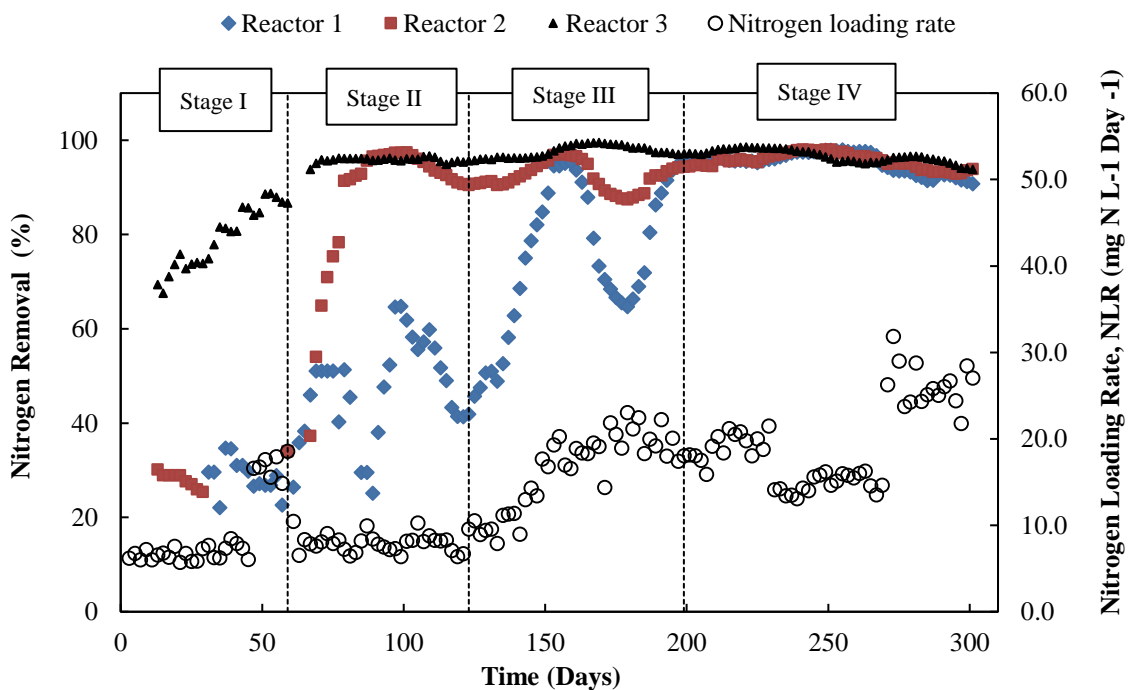
Figure 2-1: Reactors characteristic differences during each Anammox culture development stage

Based on the results depicted in **Figure 2-1**, the nitrogen removal efficiency and NLR profiles could be divided into four distinct stages (Wang *et al.*, 2016; Yu *et al.*, 2013; Tang *et al.*, 2009; Chamchoi *et al.*, 2007). Stage I (approximately 60 days) is the

acclimatization stage that is characterized with average low nitrogen removal efficiencies at 28.6% and 34.0% for R1 and R2, respectively. Conversely, R3's high nitrogen efficiency at 84.4% was attributed to fast Anammox process start -up time. Because of presumed faster start-up time than both R1 and R2, R3 exhibiting phenotypic Anammox activities characterized with high nitrogen removal efficiency at increased nitrogen loading (**Figure 2-2**). Generally, during Stage I, the ammonium effluent concentrations were relatively higher in R1 and R2 (**Figure 2-7,** in the **Appendix**) because the change in environment of the seed sludge might have potentially caused bacteria turnover. That is, the dormant bacteria were killed causing cell lysis and breakdown of organic nitrogen to ammonium leading to increased ammonium effluent concentration even at low nitrogen loading rate (NLR). This assertion can also be supported by the low specific anammox activity depicted as a result of low nitrogen removal and MLVSS concentration in the system (Chamchoi *et al*., 2007). The Specific Anammox Activities (SAA) were not determined at Stage I because it was assumed that most bacteria during this period of development comprised of the nitrifying and denitrifying bacteria that favorably compete for metabolic substrates and grow faster than the Anammox bacteria (Yu *et al*., 2013).
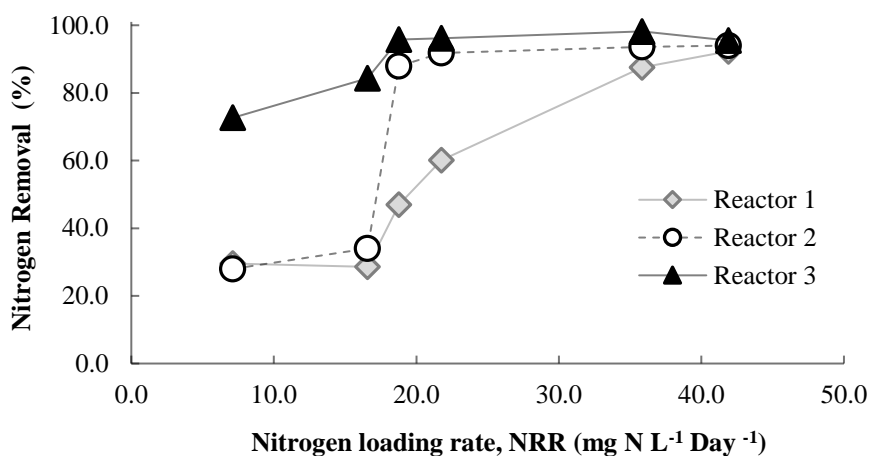
Figure 2-2: Differential nitrogen removal efficiencies with increasing nitrogen loading rates (NLR).

The second stage, Stage II (approximately between 60 -123 days), is the instability stage characterized with near exhaustion of organic substrate from cell lysis and fluctuation of nitrogen removal efficiencies due to coexistence of denitrifying and low populated Anammox bacteria as seen in R1 (Yu *et al*., 2013; Chamchoi *et al*., 2007). However, due to faster start-up time than R1, R2 exhibited increased nitrogen removal efficiency and SAA at 87.9% and 7.29E-02 g-N g-VSS^{-1} Day $^{-1}$, respectively, due to increased Anammox bacteria population (Tang *et al*., 2009).

Stage III (Transition stage) was from approximately 123 to 199 days. During this stage, R3 had already attained a stable nitrogen removal efficiency above 95% and R2's nitrogen removal efficiency was slightly below 95%. However, R1 depicted an increased nitrogen removal efficiency from 47.0% to 87.5%. Despite lower than maximum nitrogen removal efficiencies, Stage III is characterized with improved efficiencies with low accumulation of NH_4^+ and NO_2^-, and generation of NO_3^-. Because R1 start-up time

36

presumably falls within Stage III period, like R2 and R3, the increased nitrogen removal efficiency was as a result of the Anammox activities characterized with high nitrogen removal efficiency. However, due to the sensitivity of Anammox bacteria to exogenous factors such as high nitrite concentration (Jin, *et al.*, 2012) during the transition stage (Yu *et al*., 2013; Tang *et al*., 2009; Chamchoi *et al*., 2007), both R1 and R2 were upset and consequently exhibited decreased nitrogen removal efficiencies from 87.5% to 64.7% and 93.6% to 87.8% for R1 and R2, respectively, when the NLR was drastically increased to approximately 23.0 mg-N L^{-1} Day $^{-1}$. In contrast, R3 nitrogen removal efficiency was not impacted and remained stable above 95% even at high NLR, which is evidence of a fully developed Anammox culture that is often characterized with high resiliency to exogenous and endogenous factors (Egli, *et al.,* 2001; Jin, *et al.*, 2012).

The final stage is the Stable and Effective stage (Stage IV), which is characterized with simultaneous NH_4^+ and NO_2^- removal leading to increased and stable nitrogen removal efficiency (**Figure 2-1**). During this stage, the removal efficiencies were at maximum values for each of the three test reactors indicating that Anammox process became the major pathway responsible for ammonium removal in each of the reactors (Yu *et al*., 2013). Based on the provided conditions, the maximum attained removal efficiencies for all the reactors were above 95%. However, during this stage, there may be deviations of performance in terms of nitrogen removal efficiencies that is dependent on different concentrations of feed substrates and the uniqueness of each developed Anammox culture developed (Chamchoi *et al*., 2007) as summarized in **Table 2-2.**

Table 2-2: Summary of reactors performance and activity during Anammox culture development stages

Reactor	Anammox Performance & Activity					$(SAA^{MAX})^1$ x 10^{-2}	Start-up time (Days)
	Performance & Activity Parameter	*Stage I*	*Stage II*	*Stage III*	*Stage IV*		
R1	Nitrogen removal (%)	28.6	47.0	87.5	97.2	4.2	153
	(SAA^2) x 10^{-2}	-	5.7	4.1	4.2		
R2	Nitrogen removal (%)	34.0	87.9	93.6	97.2	9.6	85
	(SAA^2) x 10^{-2}	-	7.3	9.6	5.2		
R3	Nitrogen removal (%)	72.7	95.8	98.2	96.5	15.7	59
	(SAA^2) x 10^{-2}	-	4.7	15.7	15.7		

At each stage of Anammox culture development, the test reactors in the study simultaneously exhibited varying nitrogen removal efficiencies and SAA (**Table 2-2)**. Reactor 3 attained stable nitrogen removal efficiencies faster than both R1 and R2, while R2 reached stable nitrogen efficiency faster than R1 as shown in **Figure 2-2.** It can be deduced that maximum nitrogen removal efficiency corresponds to the time at which the reactors attained maximum specific anammox activity (SAA ^{max}) that further correlates to the reactors start-up time, which was defined as the time taken to attain SAA^{max} at

---

[1] Maximum Specific Anammox rate at after start-up, SAA^{MAX} (g-N g-VSS $^{-1}$ Day $^{-1}$)

[2] Specific Anammox Activity, SAA (g-N g-VSS $^{-1}$ Day $^{-1}$)

maximum NLR (Dapena-Mora *et al*., 2007). The reactors SAA^{max} were determined as 4.19E-02, 9.55E-02, and 1.57E-01 g-N g-VSS^{-1} Day^{-1}, which corresponded to 153, 85, and 59 days start-up times for R1, R2, and R3, respectively. Based on the improved start-up time and performance exhibited by R2 and R3 under the conditions tested, it can be concluded that TAD mixed-sludge enhanced the Anammox culture development (Zabranska *et al*., 2000; Kumar *et al*., 2010).

2.4.2. *Molecular detection and quantification of Anammox bacteria*

Agarose gel electrophoresis was employed on PCR products to confirm the specificity of the qPCR amplification. The gel image shown in **Figure 2-3** reveals that most bacteria detected were presumably Anammox active bacteria (A+) because the negative controls (NTC, -W-1, and –Ad-1) were not amplified. Using gel 100 bp ladder as a reference, the amplicon size was estimated to be 700-bp, which is similar to Penton *et al* (2006) reported amplicon size of approximately 700-bp 16S rRNA gene that was determined while detecting Anammox bacteria in freshwater and marine sediments. Penton *et al* (2006) designed a new primer set that was 100% specific to recover about 700-bp 16S rRNA gene sequences with greater than 96% homology to the "*Candidatus* Scalindua" group of Anammox bacteria. A search for a potential match on our sequenced data was conducted and the Anammox bacteria specie, *"Candidatus* Brocadia sinica,*"* was found to have the best match at 99% identity and 0 E-value. The identification of the specific Anammox bacteria plus the observed reactor activities strongly confirm successful enrichment and start-up of the Anammox process from TAD mixed-sludge liquor.
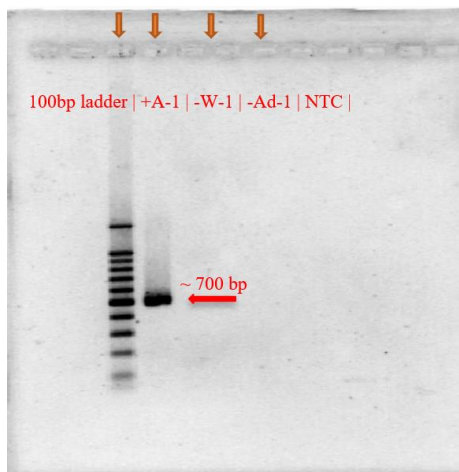
Figure 2-3: Gel image showing bacteria detected
as presumably Anammox

To quantify the bacteria population in the reactors, active Anammox biomass samples were collected after 44 days to depict approximately 4 generations of the Anammox bacteria. **Figure 2-4** shows that the gene copy numbers (GCN) increased more than 2-fold after 44 days in all the reactors, with the highest increase observed in reactor R2. High population of Anammox bacteria in R2 can be attributed to the optimum amount of fresh active Anammox biomass augmented and blended with the right proportion of TAD mixed-sludge that mitigated the effect of competition between the bacteria mediating the two-step process (Kumar *et al*., 2010) in utilizing available NH_4^+ substrate for metabolic activities and growth. The upsurge in Anammox bacteria population over time further confirmed successful growth and enrichment of Anammox bacteria in the bioreactors from TAD mixed-sludge.
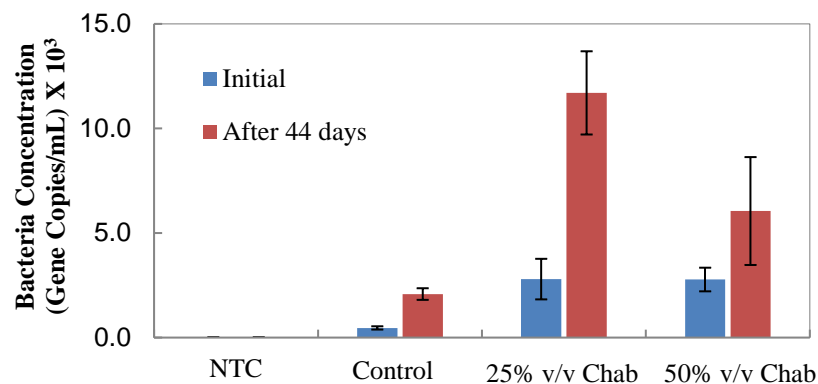
Figure 2-4: Quantified Anammox bacteria in the three reactors**.**
R1 (Control), R2 (25% v/v Chab), and R3 (25% v/v Chab)

2.4.3. *Effect of inorganic carbon (HCO₃⁻) on Anammox rate*

Alkalinity is a significant parameter in the enrichment of Anammox culture. Not only is HCO_3^- reported as the carbon source in the Anammox process, but it is also important in maintaining the suitable pH conditions in the reactor (Karcher *et al.*, 1975) for Anammox culture development. In this study, the maximum Anammox volumetric rate was determined to be 0.07 g NH_4^+ L^{-1} day^{-1} at 12.5mM HCO_3^- concentration, but decreased to 0.06 g NH_4^+ L^{-1} day^{-1} at 20 mM HCO_3^- concentration when free ammonia accumulation increased from 0.03 mg/L to 0.04 mg/L as shown in **Figure 2-5**.
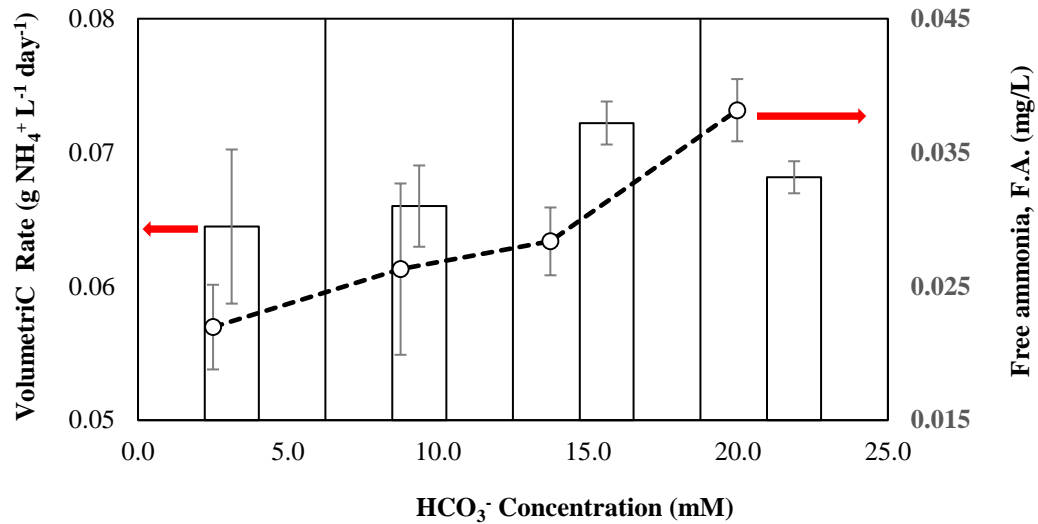
Figure 2-5: Anammox volumetric rate and free ammonia (F.A.) concentration simultaneously increases as HCO_3^- is increased to 20.0 mM. However, sustained accumulation of F.A. > 0.035 mg/L decreases the volumetric rate.

Trukhina *et al.* (2011) reported that the activities of a modified deamox process were negatively impacted at 24mM HCO_3^- concentration which is close to our results. Based on our batch test findings, it can be revealed that the optimum HCO_3^- concentration during the Anammox process was approximately 12.5mM at a total nitrogen removal rate of approximately 0.072 g NH_4^+ L^{-1} Day^{-1}. **Figure 2-5** further shows that the concentration of HCO_3^- below the determined optimum concentration resulted in decreased volumetric ammonium removal rate. To explain the volumetric rate decrease, low influent HCO_3^- concentration led to decreased pH value in the reactors that increased the systems potential to accumulate nitrite and free nitrous acid (FNA), which have both been reported to acutely inhibit the Anammox process (Strous *et al.*, 1998).

At higher HCO_3^- concentration above the determined optimum value, a slight inhibition from HCO_3^- shock potentially occurred that impacted the Anammox rate when

the HCO_3^- concentration was increased to 20mM. The decrease in Anammox rate at high HCO_3^- concentration is most likely attributed to two factors. First, the abrupt increase in HCO_3^- concentration probably and potentially shocked the active Anammox culture that resulted in a slight loss of Anammox activity. Secondly, high HCO_3^- concentrations led to free ammonia (NH_3) accumulation as can be observed in **Figure 2-5** showing a linear correlation between HCO_3^- and free ammonia (FA) concentration. The FA concentration in the batch reactors increased from 0.02 to 0.04 mg/L as the HCO_3^- concentration increased from 2.5 to 20 mM. It had previously been reported that free ammonia (NH_3) inhibits activities of other autotrophs such as *Nitrosomonas* and *Nitrobacter* (Jaroszynski *et al*., 2012), which are key bacteria species mediating the nitrification process. During this study, the free ammonia concentration in the batch reactors was estimated following the expression proposed by Ford *et al*. (1980). In comparison, Belmonte *et al* (2017) similarly reported the effect of alkalinity on partial nitrification process and determined that the decrease of the inlet total alkalinity concentrations increased nitrite accumulation which in-turn contributed to the decrease in pH inside the reactor and further promoted the inhibitory effect of free nitrous acid (FNA) and free ammonia (F.A) on the bacteria mediating the process.

2.4.4. *Effect of substrate ratio on Anammox rate*

Optimum feed substrate ratio (NO_2^-/NH_4^+) is another significant parameter to be considered while enriching the Anammox culture. **Figure 2-6** shows that NO_2^-/NH_4^+ ratio positively correlates with nitrite removal but only slightly impacts the ammonium removal rate. The sharp drop in the rate of NH_4^+ removal is related to inhibition of the partial-

nitrification (P/N) step of the combined two-step process. As earlier noted, during the P/N step, NH_4^+ is partially oxidized to NO_2^- by the Ammonium oxidizing bacteria (AOB) utilizing the available O_2; after which, the remaining NH_4^+ is further oxidized to N_2 by the Anammox bacteria, without O_2, while NO_2^- is reduced and N_2 produced.
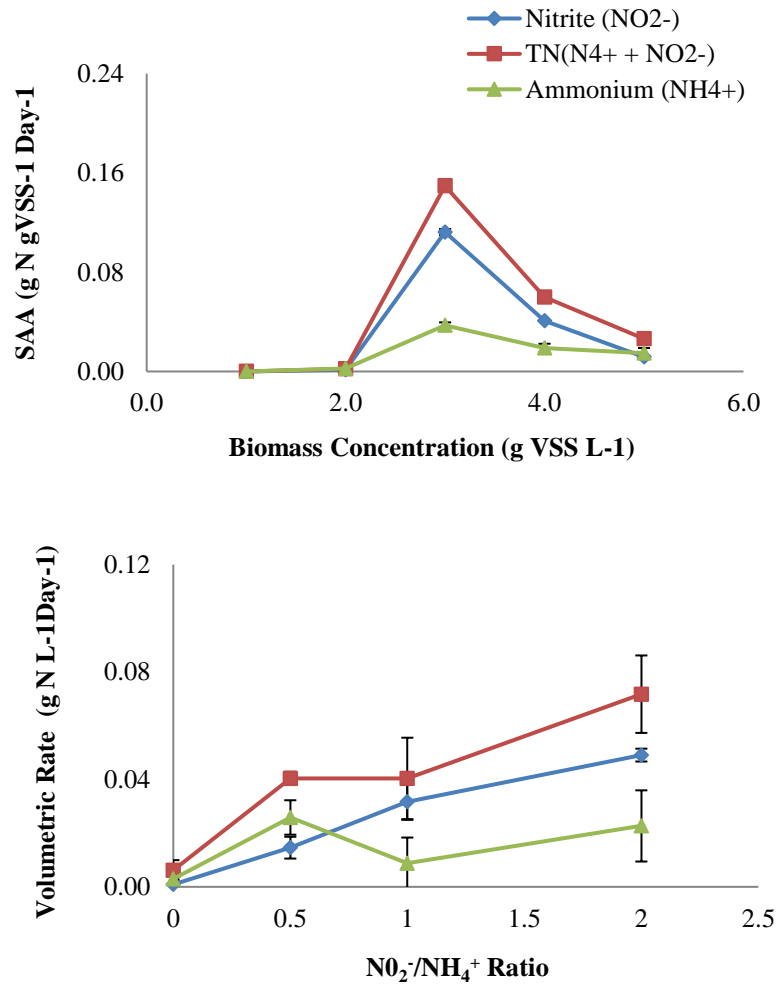
Figure 2-6: (Top) Effect of initial biomass concentrations on specific biomass N-removal rate. (Bottom) Effect of substrate ratio on volumetric N-removal rate

The decreased rate of nitrogen (ammonium) removal at increased biomass concentration is probably the result of the competition among the bacteria mediating the

two-step process. To explain further, at increased initial biomass concentration, the Anammox bacteria are seldom enriched and, as such, the AOB are in abundance compared to the Anammox bacteria. The high AOB population and activities, in-turn, out-competed the Anammox bacteria metabolic activities in utilizing the available NH_4^+ resulting in reduced NH_4^+ removal rate at high initial biomass concentrations.

Previous researchers have also reported similar results (Ying-Cui *et al*, 2014). In their reported study, Ying-Cui *et al* (2014) concluded that high mole ratio of NO_2^-/NH_4^+ negatively influence the Anammox process because it leads to high nitrite residual that promotes the growth of nitrite oxidizing bacteria (NOB) and other denitrifying bacteria that strongly compete with the Anammox bacteria.

2.5. Conclusion

Characterized TAD mixed sludge was obtained from the City of Los Angeles, Hyperion Wastewater Treatment Plant, and its potential to enhance Anammox culture development and to improve process start-up time was investigated. In addition, the effects of inorganic carbon and substrates ration on Anammox rate were studied. Short- and long-term reactor monitoring with and without TAD mixed-sludge augmentation indicated that TAD mixed-sludge in the reactor significantly shortened the Anammox process start-up time and enhanced the Anammox process. Optimum amount of TAD mixed-sludge at 25% (v/v) used to augment the process doubled the Anammox bacteria gene copy numbers after two doubling periods. Increased specific Anammox bacteria population was attributed to negligible competition among the bacteria mediating the two-step partial-nitrification and Anammox processes. High inorganic carbon concentrations and substrates ratio negatively

impacted the Anammox rate. The Anammox volumetric rate reached its highest value of 0.07 g NH_4^+ L^{-1} day^{-1} at 12.5mM HCO_3^- but decreased to about 0.06 g NH_4^+ L^{-1} day^{-1} at 20 mM HCO_3^- due to decreased pH in the bioreactor. Low feed substrates ratio (NH_4^+/ NO_2^-) led to lower Anammox rates but improved at an optimum ratio of approximately 1.5. High and low initial biomass concentrations led to lower specific anammox activities that were also attributed to bacteria competition during start-up time. High specific anammox rate of 0.16 g N $gVSS^{-1}$ day^{-1} was determined at an optimum value of 3.0 g VSS L^{-1} biomass concentration. Reactor performance, Anammox bacteria detection, and bacteria quantification, taken together, confirmed the successful Anammox culture development and enhancement using TAD mixed-sludge. In conclusion, thermophilic anaerobic digester (TAD) effluent mixed liquor has potential applications in Anammox process start-up to remove excess ammonium from wastewater.

2.6. Acknowledgements

2.7.   Bibliography

49. Albertson, O. E., *Changes in the biochemical oxygen demand procedure in the 21st edition of Standard Methods for the Examination of Water and Wastewater*. Water Environment Research, 2007.**79**: p. 453-455.

50. Belmonte M., Hsieh C.F., Campos J. L., Guerrero L., Méndez R., Mosquera-Corral A., Vidal G., *Effect of Free Ammonia, Free Nitrous Acid, and Alkalinity on the Partial Nitrification of Pretreated Pig Slurry, Using an Alternating Oxic/Anoxic SBR*. Bio-Med Research International, 2017.**10**: p. 657-671

51. Bipin K.P., Futaba K., Yasuhiro T., Mori K.; Sumino T., *Quantification of anammox populations enriched in an immobilized microbial consortium with low levels of ammonium nitrogen and at low temperature*. Environmental Biotechnology, 2007.**79**: p.1173-1179.

52. Buys R, Mosquera-Corral A, Sanchez M, Mendez R, *Development and application of a denitrification test based on gas production*. Water Science Technology, 2000. **41**(12): p. 113–20

53. Byrne N., Strous M., Crepeau V *et al.*, *Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents*. The ISME journal, 2008. DOI: 10.1038/ismej.2008.72.

54. Chamchoi N., Nitisoravut S., *Anammox enrichment from different conventional sludges.* Chemosphere, 2007. **66**(11): p. 2225 – 2232.

55. Dapena-Mora, A., I. Fernández, J. L. Campos, A. Mosquera-Corral, R. Méndez, and M. S. M. Jetten., *Evaluation of Activity and Inhibition Effects on Anammox Process by Batch Tests Based on the Nitrogen Gas Production*. Enzyme and Microbial Technology, 2007. **40** (4): p. 859–65.

56. Desmottes C., Saneie S., Chowdhury N., Samar P., Kaldate A., Tashakor N., *Pilot-Scale Experiences of Deammonification of Thermophilic Anaerobic Digester Centrate.* Proceedings from WEFTEC - Water Environmental Federation, 2015: p. 990

57. Ding Z.W., Ding J., Fu L., Zeng R.J., *Simultaneous enrichment of denitrifying methanotrophs and anammox bacteria*. Environmental Biotechnology, 2014. **98**: p.10211-10221

58. Ding Z.W., Lu Y.Z., Fu L., Ding J.; Zeng R.J., *Simultaneous enrichment of denitrifying anaerobic methane –oxidizing microorganisms and anammox bacteria on a hollow-fiber membrane biofilm reactor*. Environmental Biotechnology, 2016. DOI 10.1007/s00253-016-7908-7

59. Erguder T., Boon N., Wittebolle L., Marzorati M., Verstraete W., *Environmental factors shaping the ecological niches of ammonia oxidizing archea*. FEMS Microbiol Review, 2009.**33**: p. 855-869.

60. Guo Q., Xing B.S., Li P., Xu J.L., Yang C.C., Jin R.C., *Anaerobic ammonium oxidation (anammox) under realistic seasonal temperature variations: Characteristics of biogranules and process performance*. Bioresource Technology, 2015.**192**: p. 765-773.

61. Hu B.I., Zheng P., Tang C.J., Chen J.W., Beizen E.V.D., Zhang L., Ni B.J., Jetten M.S.M., Yan J., Yu H.Q., Kartal B., *Identification and quantification of anammox bacteria in eight nitrogen removal reactors*. Water Research. 2010. **44**: p. 5014-5020.

62. Hu B.L., Shen L.D., Xu X.Y., Zheng P., *Anaerobic ammonium oxidation (anammox) in different natural ecosystems*. Biochemical Society Transactions, 2011. **39**: p. 1811-1816.

63. Hu S., Zheng R.J., Haroon M.F., Keller J., Lant P.A., Tyson G.W., Yuan Z., *A laboratory investigation of interactions between denitrifying anaerobic methane oxidation (DAMO) and anammox process in anoxic environments*. Water Microbiology and Microbial Ecology, 2015. **5**: p. 8706. DOI: 10.1038/srep08706

64. Jaeschke A., Camp H.J.M., Harhangi H., Klimiuk A., Hopmans E.C., Jetten M.S.M., Schouten S., Damste J.S.S., *16S rRNA gene and lipid biomarker evidence for anaerobic ammonium-oxidizing bacteria (anammox) in California and Nevada hot springs*. FEMS Microbiol Ecology, 2009. **67**: p. 343-350.

65. Jia F., Peng Y., Yang Q., *Competition and synergism between Anammox bacteria and other bacteria.* Huanjing Kexue Xuebao / Acta Scientiae Circumstantiae, 2014. **34**: p.1351-1361.

66. Jin, R. C., Hu B.I., Zheng, P., Qaisar, M., Hu A.H., Islam, E., *Quantitative comparison of stability of ANAMMOX process in different reactor*. Bioresource, 2008. **99**: p. 1603-1609.

67. Kalvelage T., Jensen M.M., Contreras S., Revsbech N.P., Lam P., Gunter M., LaRoche J., Lavik G., Kuypers M.M.M., *Oxygen Sensitivity of Anammox and Coupled N-Cycle Process in Oxygen Minimum Zones*. PLOS ONE, 2011. **6**(12): p. 292-99

68. Karcher S., Kornmuller A., Jekel M., *Aqueous ammonia equilibrium calculations – effect of pH and Temperature*. Fisheries Research Board of Canada, 1975. **32**(12): p. 2379-2383.

69. Kumar M.; Lin J.G., *Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal – strategies and issues*. Hazardous Materials, 2010. **178**(1-3): p. 1-9.

70. Li H., Chen S., Mu B.Z., Gu J.D., *Molecular detection of Anaerobic Ammonium-Oxidizing (Anammox) bacteria in High-Temperature Petroleum Reservoirs*. Environmental Microbiology, 2010. p. 771-783.

71. Li X., Klaus S., Bott C., He Z., *Status, Challenges, and Perspectives of Mainstream Nitritation-Anammox for Wastewater Treatment*. Water Environment Federation, 2018. **90**(7): p.634-649.

72. Li X.M., and Yang Q., *Enrichment and granulation of Anammox biomass started up with methanogemic granular sludge*. Microbiology and Biotechnology, 2007. **23**: p. 1015-1020.

73. Manonmni U., Joseph K.., *Research advances and challenges in anammox immobilization for autotrophic nitrogen removal*. Chemical Technology & Biotechnology, 2018. **93**(9).

74. Penton C.R., Allan H. D., James M. T., *Molecular Evidence for the Broad Distribution of Anaerobic Ammonium-Oxidizing Bacteria in Freshwater and Marine Sediments*. Applied and Environmental Microbiology, 2006. **71**(10): p. 6829–6832.

75. Sonthiphand P., Neufeld J.D., *Evaluating Primers for Profiling Anaerobic Ammonia Oxidizing Bacteria within Freshwater Environments*. PLoSONE, 2013. **8**: 57242.

76. Sun S. P., Pellicer-Nacher C., Merkey B., Smets B.F., *Effective Biological Nitrogen Removal Treatment Processes for Domestic Wastewaters with Low C/N Ratios: A Review.* Environmental Engineering Science, 2010. **27**(2): p.111-126.

77. Suvilampi J., and Rintala J., *Thermophilic Aerobic Wastewater Treatment, Process Performance, Biomass Characteristics and Effluent Quality.* Review in Environmental Science and Biotechnology, 2003. **2**:35

78. Tang C.J., Zheng P., Mahmood Q., Chen J.W., *Start-up and inhibition analysis of anammox process seeded with anaerobic granular sludge*. Industrial Microbiology Biotechnology, 2009. **36**(8): p.1093 – 1100.

79. Tao Y., Gao D.W., Wang H.Y., Kreuk M., Ren N., *Ecological characteristics of seeding sludge triggering a prompt start-up of anammox.* Bioresource Technology, 2013.**133**: p. 475-481.

80. Third K.A., Sliekers A.O., Kuenen J.G., Jetten M.S.M., *The CANON system (Completely Autotrophic Nitrogen-removal Over Nitrite) and Ammonium Limitation: Interaction and Competition between Three Groups of Bacteria*. System Applied Microbiology, 2001. **24**: p. 588-596.

81. Tomar S., Gupta S.K., *Effects of Seed Culture and Attached Growth System on the Performance of Anammox Hybrid Reactor Treating Nitrogenous Wastewater*. International Journal of Research in Science, 2016. **2**:1.

82. Tomar S., Gupta S.K., Mishra B.K., *Performance evaluation of the anammox hybrid reactor seeded with mixed inoculum sludge*. Environmental Technology, 2016. **37**: p. 1065-1076.

83. Trukhina A.I, Gladchenko M, Kalyuzhnyi S., *Optimization of Sulfide and Organic Modifications of the DEAMOX process.* Applied Biochemistry and Microbiology, 2011. **47**: p. 841-845.

84. US EPA REGULATIONS. *Biosolids Laws and Regulations*. EPA. Environmental Protection Agency, 19 Feb. 1993. Web. 03 Oct. 2016

85. Uyanik S., Bekmezci O., Yurtserver A., *Strategies for Successful ANAMMOX Enrichment at Laboratory Scale.* Clean – Solid, Air, Water, 2011. **39**: p. 653-657.

86. Wang X., Gao D., *In-Situ restoration of one-stage partial nitritation-anammox process deteriorated by nitrate build-up via elevated substrate levels*. Nature, 2016. **6**: p. 37500

87. Ye L., Li D., Zhang J., Zeng H., *Fast start-up of anammox process with mixed activated sludge and settling option*. Environmental Technology, 2018. **39**(23): p. 3088-3095.

88. Ying-Cui Y., Yu T., Da-Wen G., *Effects of HRT and nitrite/ammonia ratio on anammox discovered in a sequencing batch biofilm reactor.* RSC Advances, 2014. **4**: p. 54798.

89. Yoshie S., Ogawa T., Makino H., Hirosawa H., Tsuneda S., Hirata A., *Characteristics of bacteria showing high denitrification activity in saline wastewater*. Applied Microbiology, 2006. **42**: p. 277-283

90. Yu Y.C., Gao D.W., Tao Y., *Anammox Start-up in sequencing batch biofilm reactors using different inoculating sludge*. Applied Microbiology Biotechnology, 2013. **97** (13): p. 6057-6064

91. Zabranska J., Stepova J., Wachti R., Jenicek P., *The activity of anaerobic biomass in thermophilic and mesophilic digesters at different loading rates*. Water Science & Technology, 2000. **42**(9): p. 49-56.

92. Zhang L, Zheng P, Tang C, Jin R., *Anaerobic ammonium oxidation treatment of ammonium-rich wastewater.* Zhejiang University Science B, 2008. **9**(5): p. 416-426

93. Zhu B., Sanchez J., Alen T.A.V., Sanabria J., Jetten M.S.M., Ettwig K.F., Kartal B., *Combined anaerobic ammonium and oxidation for nitrogen and methane removal.* Biochemical Society Transactions, 2011. **39**: p. 1822-1825.

2.8. Appendix
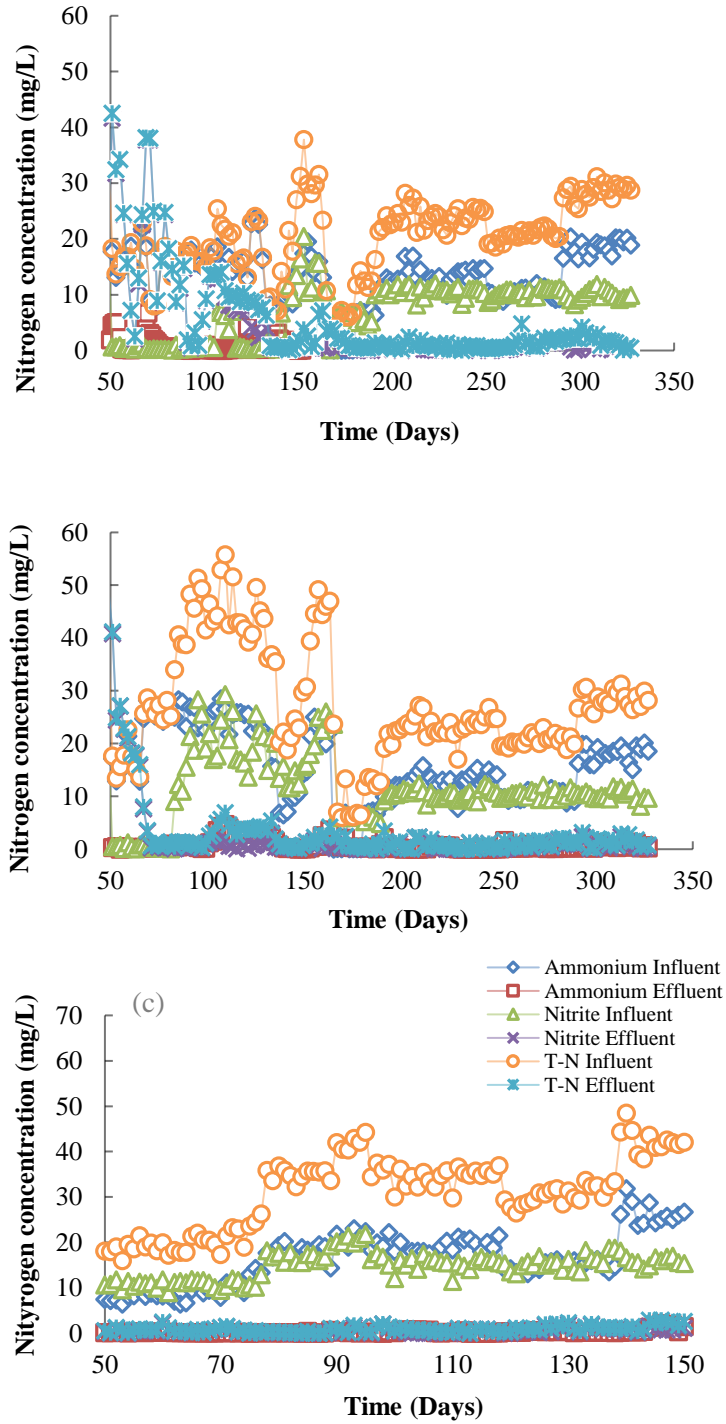
Figure 2-7: Nitrogen in Reactors 1(top), Reactors 2(middle), and Reactors 3(bottom)
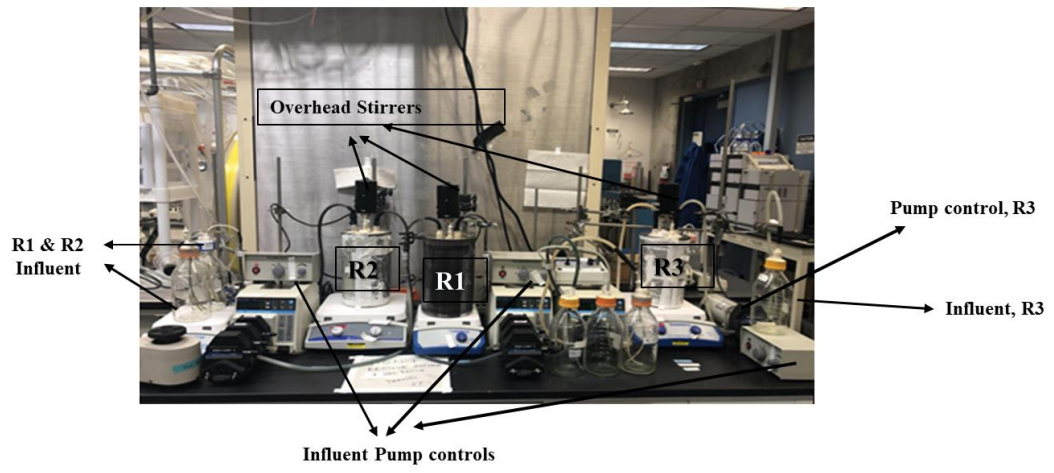
Figure 2-8: Reactors set-up and configuration

Chapter 3 : Chabazite addition enhances Anaerobic Ammonium Oxidation (Anammox) culture development at high feed variability

3.1. Abstract

Anammox activities can be enhanced if materials that have beneficial surface and chemical properties capable of concentrating growth factors, while enabling selective colonization of the Anammox bacteria, were used. With evidence of higher cation exchange capacity and better ammonium ion exchange rate than natural clinoptilolites, chabazite could be the better amendment material contrary to previous reports.

This study aimed to assess whether chabazite addition enhances Anammox culture development and improve process start-up time. Chabazite AZ-UB was obtained from St. Cloud Mining Company in New Mexico, U.S. and pretreated by soaking in deionized water followed by heating at 100°F for 24 hours to remove impurities and to improve kinetics. Batch assays were conducted to determine chabazite cation exchange capacity and isotherm model. Langmuir was the most representative isotherm model with a determined adsorption capacity of 1.18 meq/g-chabazite that is greater than 1.15 meq/g-clinoptilolite.

Two 3-L sequencing batch reactors were started from fresh return activated sludge (RAS) from the City of Los Angeles' joint Los Angeles-Glendale (LAG) water reclamation plant. Appropriate chabazite dose was added in reactor **B** while reactor **A** was operated without chabazite addition (control). Chabazite had minimal influence on Anammox process start-up time because both reactors exhibited evidence of Anammox activity after 69 days. However, at increased substrate concentration, reactor **A** experienced reduced NO_3^- production and increased NH_4^+ and NO_2^- effluent concentrations. Reduced NRR,

from 95% to 86%, was also observed in reactor **A** while reactor **B**'s NRR remained constant at 95%. Chabazite mitigated the effect of feed substrate variability and optimum substrate concentration needed by the Anammox bacteria was determined. At increased substrate concentration, which temporarily inhibited Anammox activities, Reactor **B** recovered quicker than Reactor **A.** Faster recovery was attributed to the presence of chabazite particles that concentrated NH_4^+ on the surface leading to increased Anammox bacteria population from 3.08×10^6 to 5.67×10^7 compared to the population in Reactor A that only increased to 2.17×10^7 copy numbers/g VSS. The results imply that chabazite addition enhances Anammox culture development and process activity during increased substrate concentrations.

3.2. Introduction and Background

The use of combined partial-nitrification and anaerobic ammonium oxidation (Anammox) process is relatively new alternative for ammonia removal from wastewaters with low carbon to nitrogen ratio (Kim *et al.*, 2008). During the Anammox process, ammonium (NH_4^+) and nitrite (NO_2^-) are converted to dinitrogen (N_2) gas as shown in **Equation 1-1** (Van der Graaf *et al.*, 1995). The process utilizes alkalinity (HCO_3^-) as the sole carbon source and NO_2^- as the electron acceptor for ammonium oxidation (Mulder *et al.*, 1995). The bacteria mediating the process are slow growers of the order *Planctomycetales* with approximately 10-12 days doubling times (Strous *et al.*, 1998). In relation, the doubling times are higher than the 0.6- and 4-day doubling times commonly associated with activated sludge process bacteria and mesophilic methanogens, respectively (Seviour *et al.*, 2010; Ferry, 1993).

The biochemical pathway has not been widely investigated, but Kartal *et al* (2011) suggests a likely pathway with hydrazine (N_2H_4) and nitric oxide (NO) as intermediates (Kartal *et al.*, 2012). Due to the low biomass yield (Jetten *et al.*, 1997), reduced O_2 demand, zero addition of external carbon source, and reduced N_2O emissions (Wrage *et al.*, 2001), the process is more favorable to remove nitrogen (as ammonium) from high-strength ammonia wastewater than the conventional process.

Despite Anammox process advantages over the conventional multi-stage nitrification-denitrification nitrogen (N) removal process, its widespread utilization has operational drawbacks that impede its acceptance as a practical treatment alternative. The drawbacks associated with the combined N-removal process include very slow Anammox

bacteria growth, bacteria washouts during operation, and the risk of process instabilities from inhibitory factors such as influent salinity (Abma *et al*., 2007).

Efforts to improve start-up and long-term process stability have not been successful due to inadequate availability of seed Anammox bacteria (Van der Star *et al*., 2007), difficulty to grow the bacteria (Strous *et al*., 1998), and subsequent retention of the bacteria in the treatment reactors (Sliekers *et al*., 2003). Availability of initial seed biomass concentration is critical to the process as it is strongly linked to the abundance and diversity of Anammox bacteria during and after start-up (Park *et al.*, 2010). However, since the bacteria thrive under strict anoxic condition and require an approximate steady NH_4^+/NO_2 ratio 1.32 to grow (Lotti *et al*., 2012), it is very difficult to attain the ideal feed substrate ratios in a one-stage process, which also makes the process very difficult to start (Kanders *et al*., 2014). Furthermore, due to the low growth rates and potential washouts, it is further challenging to retain the bacteria in the reactor as they mostly remain suspended in sequencing batch reactors (SBRs) (Sliekers *et al*., 2003). This problem is further challenged by inadequate information on the various Anammox bacteria retention methods (Chen *et al*., 2010) and amendment materials (Chen *et al*., 2015) for selective colonization and growth of Anammox bacteria. As such, either a method or strategy to retain the bacteria in the bioreactor is needed to enhance the Anammox culture development.

Biological wastewater treatment processes have previously been augmented with various materials to improve treatment. Materials with unique chemical and physical properties (e.g., the ability to catalyze a process reaction to provide bacteria immobilization surface (Armentano *et al*., 2014; Jaroszybski *et al*., 2012) are often employed in sequencing

batch reactors (hybrid systems) to increase bacteria growth rates to enhance performance (Gai and Kim, 2008). Hybrid systems have previously been used to remove pollutants from wastewater; for instance, Yu *et al.* (2006) reported that by employing Zero-Valent Iron (ZVI) to reduce perchlorate using autotrophic culture, the biomass density was increased and correspondingly led to a 4-fold increase in the rate of perchlorate reduction. Jung *et al.* (2004) also determined that methanogenesis and nitrification rates were enhanced by reducing NH_4^+ concentration levels that caused inhibition when cation exchange materials were added to the bioreactors treating anaerobic wastewater. Zeolite materials, because of their high cation exchange capacity (CEC) and ammonium ion exchange (NH_4^+ IX) rates, have gained much attention to remove ammonium from wastewater (Karadag *et al.*, 2007; Booker *et al.*, 1996). He *et al.* (2007) used powder zeolite to amend the biological nitrogen removal (BNR) process treating municipal wastewater with a total nitrogen concentration of 54 mg-N L^{-1} and reported that the nitrification rate was increased by a factor of two compared to the non-zeolite amended process. Fernandez *et al.* (2008) further reported an improved enrichment of Anammox biomass and reduction of washout to values as low as 3 mg VSS L^{-1} when zeolite was used to amend the bioprocess.

As mentioned in **Chapter 1**, natural zeolites are hydrated aluminosilicates with three-dimensional structure and high adsorption capacity (Erdem *et al.*, 2004). Clinoptilolite (zeolite, group 7) is the most abundant and used zeolite specie; thus, frequently referred to as the 'natural zeolite' (Sprynskyy *et al.*, 2005). Zeolite's high CEC is dependent on the presence of either Si^{4+} or Al^{3+} in the mineral lattice. The substitution

of Si^{4+} by Al^{3+} increases the negative charge, which is balanced by the readily available cations (e.g. Na^+) in the aqueous bulk solution. The balanced cations (e.g. Na^+) can subsequently be exchanged with other target cations e.g. NH_4^+ (Erdem *et al*., 2004; Langwaldt, 2008). While focus has been on 'natural zeolite' (clintoptilolite) as an amendment material to enhance biological wastewater treatment, recent evidence of higher CEC and better NH_4^+ IX rate than clintoptilolite (Karmen *et al*., 2013; Langwaldt, 2008) makes Chabazite (zeolite, group 4) a better alternative for ammonium removal from wastewater. Chabazite has lower Si^{4+} to Al^{3+} ratio than clinoptilolite (Erdem *et al*., 2004; Langwaldt, 2008) that could potentially increase the mineral negative charge for cation substitution leading to increased CEC. In addition, because of its metaphysical and structural properties, chabazite pore cavities could act as anoxic microenvironments for growth of anoxic bacteria (Erdem *et al*., 2004; Sprynskyy *et al*., 2005). Moreover, NH_4^+ adsorption and bio-regeneration could be increased because of the negative charge resulting from the high composition of Al^{3+} content within the framework (Lahav *et al*., 2000). These properties can favor the development of anoxic bacteria such as Anammox bacteria. Since Anammox bacteria grow under high ammonia (Hu *et al*., 2011) and low organic carbon (Dalsgaard and Thamdrup, 2002) conditions; and with relatively higher CEC and NH_4^+ IX than clinoptilolite, chabazite mineral can possibly attain high surface concentrations of ammonium to create a natural selective environment for proliferation and retention of Anammox bacteria.

The aim of this study is [1] to assess the feasibility of using chabazite to enhance Anammox culture development and [2] to assess whether Anammox process start-up time is reduced by using chabazite material to amend the process.

3.3. Materials and methods

3.3.1. *Chabazite material preparation*

Chabazite material (Bowie chabazite AZ-UB, 14x40 mesh size) was obtained from St. Cloud Mining Company (New Mexico, U.S). Chabazite AZUB-40 specifications, as outline in the Material Safety and Data Sheet (MSDS), are outlined in **Table 3-1** and **Table 3-2**. The surface dust on the raw material that remains after the grinding process during mining was removed to enhance kinetics (Inglezakis *et al*., 2001). The material was washed and then pretreated by soaking in deionized water (Shcherban and Ilyin, 2016), followed by heating for 24 hours at 100˚C (Ghassemi and Younesi, 2011). Soaking the material in deionized water removes impurities (Jha and Hayashi, 2009), while heating the material either at or above 100˚C removes water molecules and organic materials to increase pore volume and diameter for improved adsorption surface area (Inglezakis *et al*., 1999).

Table 3-1: Typical chabazite (AZ –UB) material properties

Color	Yellowish Tan (dry brightness 43)
Average Chabazite Crystallite	Less than 1 micron
Crystallinity	50 - 60%
Density	1.73 g/cm^3
Total Pore Volume	0.468 cm^3/g
Surface Area	350 m^2/g
Crystal Void Volume	0.47 cm^3/cm^3
Packing Density	Approx. 577 kg/m^3 (36 lbs/ft^3)
SiO$_2$/Al$_2$O$_3$ Ratio	Approx. 4:1
Moisture as Packaged	Less than 12% by Weight
pH of 1% Dispersion	8.5
Stability	pH of 3 through 12
CEC	1.8 meq/g

Table 3-2: Chabazite material chemical analysis (equilibrated at 20 °C and 40% relative humidity)

SiO$_2$	Al$_2$O$_3$	Fe$_2$O$_3$	CaO	MgO	Na$_2$O	K$_2$O	LOI	Dominant Cation
54.6	14.9	2.28	0.22	0.6	6.67	0.9	19.4	Ca/Na

3.3.2. *Chabazite isotherm and kinetic studies*

Isotherm and kinetic studies were conducted by setting up batch experiments using different amounts of chabazite immersed in 200 mL NH$_4^+$ solution ($\sim$ 55 [$\pm$ 5] meq/L, pH adjusted to 7.2) in 250mL Erlenmeyer flasks. The flasks were placed in an incubated orbital shaker maintained at 25˚C and the rotation speed set at 200 rpm. The choice of temperature was consistent with the findings of the study conducted by Lin *et al.* (2013) that showed that the equilibrium adsorption capacity was not distinctively affected by

temperature between 25 to 45˚C range. Samples were taken after 24 hours and analyzed using Dionex Ion-Chromatography instrument (IC DX-120) equipped with an auto sampler to determine the concentration of target cations remaining in the bulk-solution. The results were fit in Langmuir (Yusof *et al.*, 2010) and ion exchange isotherm models (Foo and Hameed, 2010) to determine the best representative adsorption model.

3.3.3. *Chabazite – Anammox hybrid reactor operation*

Two 3-L Sequencing Batch Reactors (SBRs) were seeded with fresh return activated sludge (RAS) obtained from the City of Los Angeles' Los Angeles-Glendale (LAG) water reclamation plant. RAS seed was used instead of TAD to assess the effect of chabazite particles on Anammox process start-up time. Appropriate dose of chabazite material was added in one of the reactors (**B**) while the other was operated without chabazite addition and acted as the control (**A**).

Chabazite dose and contact time needed for effective NH_4^+ ion-exchange was determined using the feed medium. To determine the appropriate dose, batch assays were carried out with varying amounts of chabazite particles in 250mL Erlenmeyer flasks. Samples were taken and analyzed from the flasks at determined time intervals.

All the SBRs were heated using a hot plate set at temperatures between 70 and 75°F. The reactors were stirred using overhead stirrers set at 60 rpm to achieve complete mixing. The pH in the reactors was controlled between 7.2 and 7.5 by adding 0.5M HCL to the feed. Anoxic conditions in the reactors were maintained by flushing the feed media with nitrogen gas. The reactors were fed interchangeably after every 2 days. During feeding, the reactors could settle for 30 min and effluent withdrawn for 10 min.

3.3.4. Feeding composition

The feed medium had the following composition per liter of deionized water: 1.25g
KHCO$_3$, 0.025g KH$_2$PO$_4$, 0.3g CaCl$_2$.2H$_2$O, 0.2g MgSO$_4$.7H$_2$O, 0.00625g FeSO$_4$,
0.00625g EDTA, and 1.25mL trace elements solution as prepared by (Zhang *et al.,* 2008).
The reactors were fed strategically by increasing (NH$_4$)$_2$SO$_4$ (as NH$_4^+$) and NaNO$_2$ (as NO$_2^-$
) concentrations to total nitrogen (NH$_4^+$ + NO$_2^-$) concentration of 400mg/L without abruptly
adding high NO$_2^-$ concentrations that could inhibit the process because NO$_2^-$ concentration
as low as 20 mg/L have been reported to upset the process during start-up period (Strous
et al., 1998).

3.3.5. Analytical methods

Ammonium, nitrite, and nitrate were analyzed using colorimetric methods
according to standard methods of water and wastewater analysis (Albertson, 2000).
Biomass concentrations were measured as volatile suspended solids (VSS). VSS was
determined as dry weight after drying the sample at 105°C for 1 hour then ashed in a
furnace (550°C) for 30 min. Dissolved oxygen (DO) concentrations and pH were
monitored using selective electrodes.

3.3.6. Molecular assay

Nucleic acid (genomic DNA) was extracted from enriched biomass using the
Qiagen kit. The quality and quantity of the extracted DNA samples were measured using
a Nano-Drop spectrophotometer. The final extracted DNA samples were diluted to
between 5 and10 ng ul^{-1} for use as PCR template. Bipin *et al.* (2007) protocol was used to
determine the total Anammox bacterial population against the developed and calibrated

standard curve. The Anammox bacteria standard curves were constructed from Escherichia coli genomic DNA (Sonthiphand *et al*., 2013). Each constructed *E. coli* PCR product was purified using a MinElute kit (Qiagen, USA) and quantified by the Nano-Drop Spectrophotometer. Ten-fold serial dilutions were applied to the standard DNA PCR product template to create the qPCR standard curves with efficiencies $\geq$ 90% and coefficients of determination (R^2) $\geq$0.996 for all the standard curves. The qPCR master mix that was used contained 5 ml of SsoAdvanced SYBR Green Supermix, 0.03 ml of each primer (100 mM stocks), 0.02 ml of bovine serum albumin (10 mg ml^{-1} stock) and 1 ml of genomic DNA template (5–10 ng stock) in a total volume of 10 ml. All qPCR amplifications were performed in duplicate using a CFX96 real-time system (Bio-Rad, USA).

3.4. Results and discussion

3.4.1. *Chabazite characterization*

3.4.1.1. Batch cation exchange capacity (CEC)

Chabazite particles showed good ion-exchange capacity with Na^+ and Ca^{2+} being the main cations exchanged by NH_4^+ as shown in **Figure 3-1** below. As observed, Na^+ equilibrium concentrations were higher than other cations and were inversely correlated to NH_4^+ concentration. It can be inferred that NH_4^+ efficiently exchange Na^+ better than Ca^{2+} and Mg^{2+}. According to chabazite cation selectivity order, NH_4^+ is likely to be exchanged by the dominant cation in the chabazite structure followed by Na^+, Ca^{2+}, and Mg^{2+} respectively. This observation is consistent with Lahav and Green (1998) revelation that the dominant Na^+ cation in chabazite particle is lower in the order of cation selection than

NH_4^+ resulting in high adsorption of NH_4^+ on the particles and, consequently, reduced NH_4^+ concentration in the bulk solution. The four cations were selected for analysis because of their prominence as trace metals in synthetic wastewater make-up and significance in Anammox microbial growth and enrichment (Zhang *et al.,* 2008).
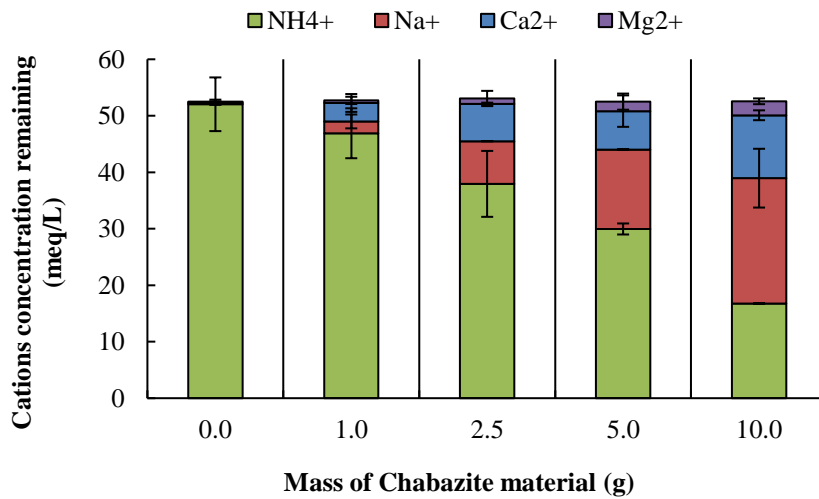
Figure 3-1: Equilibrium cation concentrations during NH_4^+ uptake.

3.4.1.2.Adsorption models

The cation exchange capacity experimental data were fitted in different isotherm adsorption models shown in **Figure 3-2** and **Table 3-3**. As determined, the average R^2 values for all the models were greater than 0.90. In comparison, the Langmuir model was determined to be the best representative model compared to the models that were evaluated in this study. The determined Langmuir parameters include an R^2 value of 0.98, K_L value of 0.0761 (<<1), and q_{max} (adsorption capacity) value of 1.18 meq/g-chabazite. Lin *et al*. (2013) previously reported results closer to the Langmuir kinetic values determined in our study and further concluded that the Langmuir was the best model for ammonium adsorption on zeolite particles. The adsorption model characterization result is also similar

to reports from other past studies such as the study reported by Lin *et al.* (2013). For instance, while characterizing natural chabazite, Stakebake and Fritz (1984) used chabazite materials collected from different geographical locations to measure nitrogen adsorption isotherms and determined that all the evaluated isotherms also obeyed the Langmuir adsorption model.
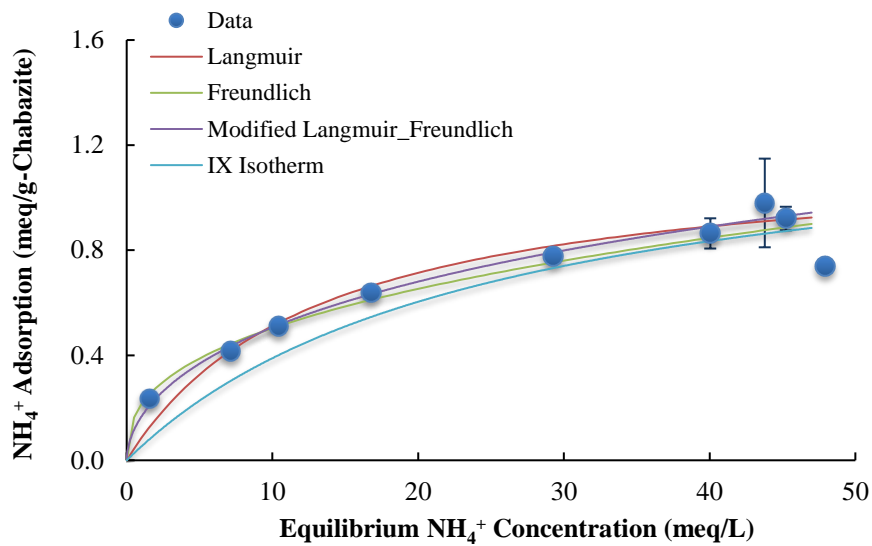
Figure 3-2: Isotherm models for ammonium removal by chabazite material.

The determined adsorption capacity (q_{max}- value) of 1.18 meq/ g-chabazite was higher than the q_{max}- value obtained from other models that were evaluated in this study but was relatively lower than 1.8 meq/ g-chabazite q_{max}- value as indicated on the material safety and data sheet (MSDS) and reported in **Table 3-1**. Lahav and Green (1998) while investigating the effect of biofilm coverage on chabazite kinetic to remove ammonium from wastewater determined a q_{max}- value of 2.6 meq/ g-chabazite which is greater than the q_{max}- value obtained in this study.

Table 3-3: Isotherm exchange coefficients for chabazite AZ-UB

Model	q_{max} (meg/g)	K_L (L/meq)	K_F (L/meq)	K_{IX} (L/meq)	n	R^2
Langmuir	**1.18**	**7.61E-02**	**-**	**-**	**-**	**0.98**
Freundlich	-	-	2.12E-01	-	1/n = 0.38	0.95
Modified Langmuir - Freundlich	2.66	7.15E-03	-	-	n = 0.55	0.90
IX Isotherm Models	0.93	-	-	3.22	-	0.97
MSDS CEC	1.8 meq/g-chabazite					

The significant differences in the values of kinetic parameters obtained in our study and the previously reported values is attributed to the variations on the source of chabazite raw material, pretreatment conditions, and experimental conditions that have been reported to influence chabazite kinetics (Aponte-Morales *et al*., 2016; Hongjian *et al*., 2016). Nonetheless, the chabazite q_{max}- value obtained in this study is higher than the natural clinoptilolite's q_{max}- value of 1.15 meq/ g-clinoptilolite reported by Langwaldt (2008). As characterized, the higher chabazite q_{max} value compared to the clinoptilolite value is evidence that chabazite particles offer better ammonium removal alternative than natural clinoptilolite and can be applied to enhance the Anammox process.

3.4.1.3. Mechanism of ammonium removal using chabazite

Ammonia in solution exists in un-ionized (NH_3) and ionized (NH_4^+) forms that largely depend on the solution pH (Emerson *et al*., 1975). Solution pH affects both the ratio of the two forms of ammonia in solution and the surface charge of the adsorbent (Karcher *et al.*, 2002). In this study, the effect of pH was investigated to attempt to elucidate the mechanism of ammonium removal using chabazite particles because the solution pH has an important

influence on equilibrium ammonia concentration and the ammonia removal efficiency (Gustin and Marinsek, 2011).

Based on **Figure 3-3**, it can be inferred that NH_4^+ removal was mostly observed at pH < 9.3 because NH_4^+ was the most abundant cation in the solution at this pH range. The pK_a value of 9.254 at 298.15K has been reported for NH_4^+ dissociation to H^+ and NH_3 (Maeda et al., 1995), which explains the relatively high concentration and removal of NH_4^+ via cation exchange at pH < 9.3. However, most NH_4^+ removal was observed at pH < 7.2 because of high NH_4^+ concentration and the availability of cation exchange sites on the chabazite particles. At pH > 7.2, it can be observed that NH_3 began to accumulate while NH_4^+ removal decreased. The decrease in NH_4^+ removal at pH > 7.2 can possibly be attributed to saturation of the cation exchange sites that led to decreased NH_4^+ and increased NH_3 removal. In proportion, at pH $<$ 7.2, NH_3 concentration accounted for less than 5% of the total ammonia in the solution and further increased at pH > 7.2.

It was deduced that the ammonia removal mechanism at pH < 7.2 was due to cation ion exchange while at pH 7.2-10.2, the mechanism of ammonia removal was largely attributed to both cation exchange and molecular adsorption. Furthermore, it was observed that the solution mostly contained molecular ammonia (NH_3) at pH 10.2, which is evidence that molecular adsorption could have been the only major contributing mechanism for NH_3 removal using chabazite particles after this pH range. Nonetheless, as the pH was further increased to pH $>$ 10.2, negligible NH_3 was removed from the solution using chabazite material, which could probably be due to the presence of other reactive radicals e.g. OH^- that possibly reacted with the available cations in the solution and the adsorbent material (chabazite) to form salt compounds

and ligands that precipitated and, as a result, occupy the adsorption sites on the chabazite particles leading to decreased NH_3 removal.
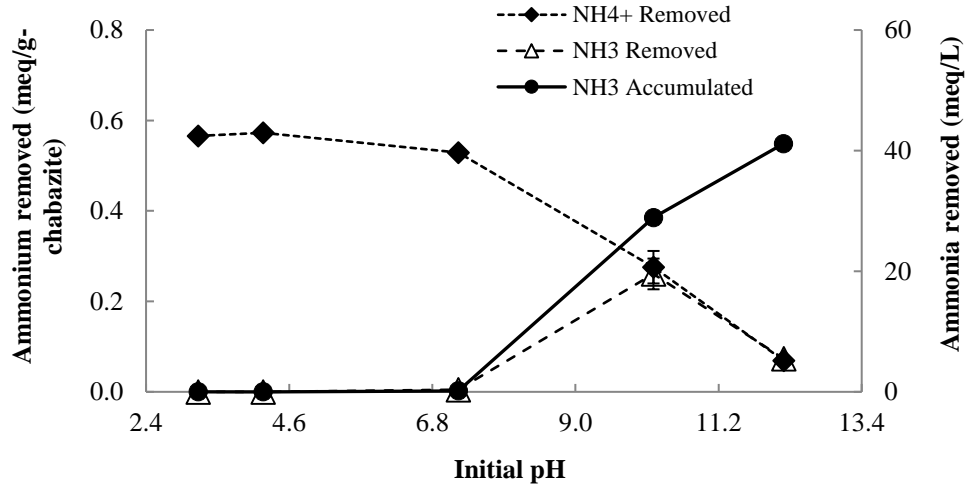
Figure 3-3: Effect of pH on ammonium adsorption on chabazite material showing Ammonium removal using chabazite is a pH- dependent process.

3.4.2. *Reactor start-up and performance*

The reactors showed phenotypic Anammox activities with approximately 95% (removal efficiency) of total nitrogen removal as shown in **Figure 3-4** (A) and (B). In the first 30 days following start-up, the reactors were fed with zero substrates ($NH_4^+ + NO_2^-$) concentration to enable the seed biomass from RAS to acclimatize in the lab reactors. Start-up time was assessed by determining the concentration of nitrate produced as depicted in **Figure 3-4**, which is a product of the Anammox reaction shown in **Equation 1-1**. In both reactors, evidence of nitrate production was observed after 69 days (~ 2 months). After which, the nitrate production increased in all the reactors at influent substrate concentration

Since both reactors exhibited equal start-up time at day 69, it can be deduced that chabazite addition had minimal influence on the Anammox process start-up time.
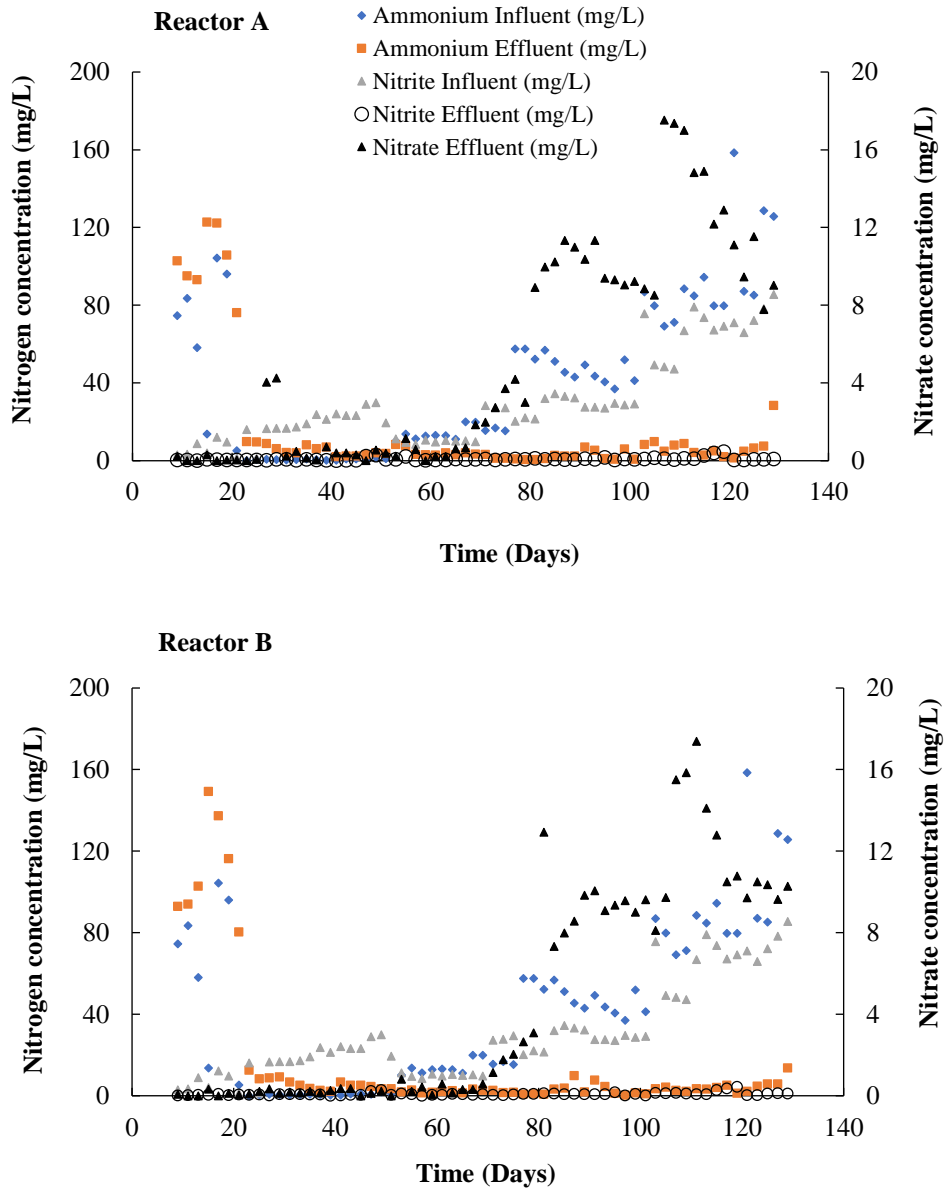
Figure 3-4: Reactors' A (control) and B (with chabazite) performance.

However, reduced nitrate production, increased NH_4^+ and NO_2^- effluent concentration, and reduced NRR with increased NLR in reactor A (control) compared to reactor B (chabazite) after day 90, reveal the resiliency of reactor B at increased influent substrate concentration (variability). Compared to reactor A that solely depended on ammonium removal via the Anammox process, reactor B potentially exhibited two mechanisms for ammonium removal, which are the cation ion-exchange and the ammonium oxidation process via the Anammox bacteria. During ion-exchange, chabazite possibly acted as the 'buffer' material against excess NH_4^+ in the feed. The 'buffer' phenomenon potentially mitigates the effect of high substrate variability in the feed resulting in optimum feed substrate concentration required by the Anammox bacteria.

When the feed substrate concentration was increased to 200mg/L, reactor A further exhibited reduced nitrate production, increased NH_4^+ and NO_2^- effluent concentration, and a drop of the NRR to approximately 86%. In contrast, reactor B's NRR and nitrate production remained relatively constant as shown in **Figure 3-5**. The stable performance of reactor B compared to reduced performance of reactor A is evidence that chabazite addition enhanced Anammox activity at increased feed substrate variability.
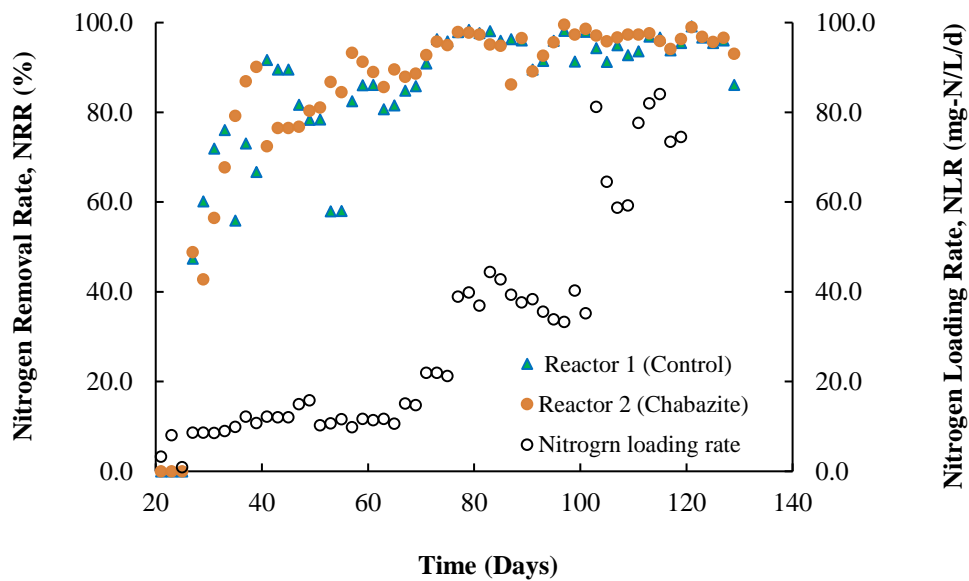
Figure 3-5: Nitrogen removal rate at increased nitrogen loading.

3.4.3. *Anammox bacteria population*

Figure 3-6 shows quantified Anammox bacteria in the reactors. Samples were collected from the reactor every 30 days after start-up until day 120 (4 months). The Anammox gene copy (GC) numbers were determined and normalized with the corresponding total dry weight biomass concentration (g VSS). Specific Anammox bacteria population (copy number/g VSS) between the two reactors were compared to assess the effect of chabazite addition on specific bacteria population. It can be noted that Anammox bacteria population increased steadily after 30 to 60 days when feed substrate concentration was increased in the reactors with reactor A having low bacteria population compared to the population in reactor B. High bacteria population in reactor B can be attributed to the presence of chabazite material that accelerated bacteria biofilm formation and growth.

71

In addition, due to beneficial surface and chemical properties attributed to the chabazite material, concentration of NH_4^+, as growth factor, on the chabazite surface enabled selective colonization of Anammox bacteria on the chabazite surfaces leading to improved bacteria population. Increased feed substrate variability after day 60 led to a drastic drop of the bacteria population in both reactors, which was due to increased substrates concentration that has been reported to reversibly inhibit the Anammox process (Strous *et al.*, 1998). However, chabazite addition in reactor B enabled fast recovery from the inhibition caused by high feed substrate variability leading to even increased bacteria population and improved Anammox process.
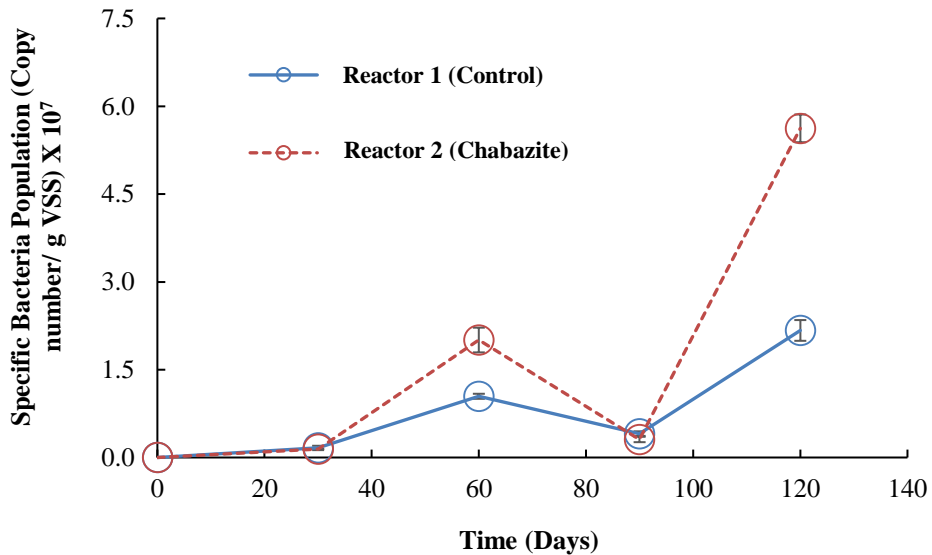
Figure 3-6: Chabazite - zeolite Enhances Anammox Bacteria Population.

3.5. Conclusion

Chabazite material (chabazite) sorption properties, influence on Anammox process start-up time, and nitrogen removal efficiency was investigated in this chapter. In assessing chabazite sorption properties, it was determined that NH_4^+ more readily replaced Na^+ than both Ca^{2+} and Mg^{2+} on chabazite following previously reported cation selectivity orders. The Langmuir model was determined as the most representative among the evaluated isotherm models with an adsorption capacity 1.18 meq/ g-chabazite > 1.15 meq/ g-clinoptilolite. However, due to factors such as differences in the source of raw materials, pretreatment methods, and experimental conditions, 1.18 meq/ g-chabazite < 1.8 meq/ g-chabazite. 1.8 meq/ g-chabazite is the adsorption capacity value reported in the MSDS. Chabazite had minimal influence on Anammox process start-up time because both reactors showed phenotypic evidence of Anammox process after 69 days. However, at increased feed substrates concentration, reactor A (control) experienced reduced nitrate production, increased NH_4^+ and NO_2^- effluent concentration, and reduced nitrogen removal efficiency (NRE) from 95% to 86% while reactor B's NRE remained constant above 95%, which was strongly linked to chabazite presence in reactor B. Chabazite also mitigated the effect of high feed substrate variability and, consequently, an optimum feed substrates concentration needed by the Anammox bacteria for growth was determined. Furthermore, reactor A exhibited low bacteria population at increased feed substrate concentrations compared to reactor B, which was also attributed to the presence of chabazite in reactor B. Anammox bacteria population increased in reactor B because NH_4^+ accumulated (growth factor) on the particles' surface enabling selective colonization and proliferation of Anammox

bacteria. Chabazite further aided in quick recovery from inhibition caused by high feed substrate concentration by quenching excess NH_4^+ in the reactor. Although there was minimal influence on Anammox process start-up time, it can be asserted that chabazite enhanced Anammox culture development at high feed substrate variability. This research contribution will have immense impact towards widespread application of the Anammox process and assist to mitigate ammonium exposure to aquatic environment.

3.6. Acknowledgements

3.7. Bibliography

94. Foo K.Y., Hameed B.H.*, Insights into the modeling of adsorption isotherms systems.* Chemical Engineering, 2010.**156**: p. 2-10.

95. Ghasemi Z., Younesi H., *Preparation and Characterization of Nanozeolite NaA from Rice Husk at Room Temperature without Organic Additives.* Journal of Nanomaterials, 2011.**8**: ID 858961.

96. Gustin S., Marinsek L.R., *Effect of pH, temperature and air flow rate on the continuous ammonia stripping of the anaerobic digestion effluent.* Process Safety and Environmental Protection, 2011, **89**: p. 61-66.

97. Hongjian L., Xiao W., Jun Z., *Kinetics, equilibrium, and thermodynamics of ammonium sorption from swine manure by natural chabazite.* Separation Science and Technology, 2016. **51**(2): p. 202-213

98. Inglezakis V.J., Diamandis N.A., Loizidou M.D., Grigoropoulou H.P.*, Effect of Pore Clogging on Kinetics of Lead Uptake by Clinoptilolite.* Journal of Colloid and Interface Science, 1999.**215**: p. 54-57.

99. Inglezakis V.J., Papadeas C.D., Loizidou M.D., Grigoropoulou H.P., *Effects of pretreatment on physical and ion exchange properties of natural clinoptilolite.* Environmental Technology, 2001. **22**: p. 75-82.

100. Jaroszynski L.W, Cicek N, Sparling R, Oleszkiewicz J.A, *Impact of free ammonia on anammox rate in a moving bed biofilm reactor.* Chemosphere, 2012. **88**: p. 188–195

101. Jha V.K.; Hayashi S., *Modification on natural clinoptilolite zeolite for its NH_4^+ retention capacity.* Journal of Hazardous Materials, 2009. **169**: p. 29-35.

102. Karcher S.; Kornmuller A.; Jekel M., *Anion exchange resins for removal of reactive dyes from textile wastewaters equilibrium, and thermodynamics of ammonium sorption from swine manure by natural chabazite.* Water Research, 2002. **36**(19): p. 4717-4724.

103. Lahav O., Green M., *Ammonium removal using ion-exchange and biological regeneration.* Water Research, 1998. **32**: p. 2019-2028.

104. Lin L., Lei Z., Wang L., Liu X., Zhang Y., Wan C., Lee D.J., Tay J.H., *Adsorption mechanisms of high-levels of ammonium onto natural and NaCl-modified zeolites.* Separation and Purification Technology, 2013. **103**: p. 15-20.

105. Maeda M., Kato K., *Dissociation Constants of Ammonium Ion and Activity Coefficients of Ammonia Nitrate Solutions.* Chemical and Engineering, 1995. **40**: p. 253-256.

106. Shcherban N.D., Ilyin V.G., *Preparation, Physiochemical Properties and Functional Characteristics of Micromesoporous Zeolite Materials.* Theoretical and Experimental Chemistry, 2016. **51**: p. 339-357.

107. Stakebake J.L., and Fritz J., *Characterization of natural chabazite and 5a synthetic zeolites: Part II: Adsorption properties and porosity.* Colloid and Interface Science, 1984. **1**: 100.

108. Waki M., Tokutomi T., Yokoyama H., Tanaka Y., *Nitrogen removal from animal waste treatment water by anammox enrichment.* Bioresource Technology, 2007. **98**: p. 2775-2780.

109. Yusofi A.M., Keat L.K., Ibrahim Z., Majid Z.A., Nizam N.A*., Kinetic and equilibrium studies of the removal of ammonium ions from aqueous solution by rice husk ash-synthesized zeolite Y and powdered and granulated forms of mordenite.* Hazardous Materials, 2010. p. 380-385.

Chapter 4 : Comparative study of the Anaerobic Ammonium Oxidation process amended with different types of chabazite

4.1. Abstract

The study aimed to determine whether chabazite-Na has the highest ammonium removal efficiency compared to other types of chabazite in the Anammox process and, to determine the effect of different types of chabazite on Anammox bacteria population. The chabazite types employed in the study include the Bowie chabazite AZUB (Na/Ca), AZLB-Na, and AZLB-Ca that have reported cation exchange capacities (CEC) of 1.8, 2.50, and 2.50 meq/g, respectively.

Five sequencing batch reactors (SBR) with 0.9L working volume were seeded with active Anammox biomass and amended with appropriate doses of sand [**A**], chabazite-Na (**B**), chabazite-Ca [**C**], chabazite – Na/Ca (**D**), and non-amended (**E**). Among the chabazite amended reactors, B was greatly impacted at high feed substrate concentration while C was least impacted at similar conditions, exhibiting a decrease in the ammonium removal efficiencies from approximately 95% to 53.5% and 95% to 88%, respectively.

The order of ammonium removal performance was found to be, Reactor C > D > B > E = A. High ammonium removal efficiency observed in C was attributed to high NH_4^+ ion-exchange rate compared to other chabazite types. Effect of chabazite type on ammonium removal depended on the extent to which NH_4^+ ion-exchange impacted the initial partial nitrification step of the two-step Anammox process mediated by the ammonium oxidizing (Anammox) bacteria.

Despite lower performance than C, B total specific bacteria population was the highest among all the reactors. The population in B increased to a maximum value of 2.32 x 10^4 copy numbers/mL compared to C that exhibited 1.20 x 10^4 copy numbers/mL and, D at 1.20 x 10^4 copy numbers/mL. High bacteria population in B resulted from higher NH_4^+ ion-exchange rate that accelerated bacteria biofilm formation and growth over a prolonged period. The results imply that while chabazite-Ca greatly mitigates the effect of high feed substrates variability on Anammox activities compared to other chabazite sub-species, chabazite-Na exhibits high bacteria population attributed to high NH_4^+ ion-exchange rate, which is significant in developing Anammox biomass retention strategies for the process.

4.2. Introduction and Background

The anaerobic ammonium oxidation (Anammox) process is a relatively new innovative alternative to remove nitrogen-ammonium from high strength ammonia-wastewater (Strous, *et al*., 1997; Van Dongen, *et al*. 2001; Schmidt, *et al.,* 2003). The bacteria mediating the process consume ammonium (NH_4^+) and nitrite (NO_2^-) as substrates to enhance growth (Dapena-Mora, *et al.,* 2004). Briefly, during the process, alkalinity (HCO_3^-) is utilized as the sole carbon source and NO_2^- as the electron acceptor for NH_4^+ oxidation (Mulder *et al*., 1995). Because the Anammox bacteria utilizes inorganic substrate (NH_4^+ and NO_2^-) for electron transfer and acceptance (He, *et al.*, 2009; Jetten, *et al.*, 2009; Raven, 2009), the bacteria grow slower than commonly known bacteria employed in conventional biological wastewater treatment processes (Schmidt, *et al.*, 2003; Kuenen, 2008; Daims, 2009; Bassin, 2018) leading to low biomass yield and sludge production (Khin, 2004; Ahn, 2006). The Anammox bacteria that mediate the process are of the order *Planctomycetales* with approximately 10 - 12 days doubling times (Strous *et al.*, 1998), which is relatively higher than about 0.6- and 4-days doubling times associated with activated sludge process bacteria and mesophilic methanogens, respectively (Seviour *et al.*, 2010; Ferry, 1993). As a result, the process has significant advantages over the conventional multi-stage nitrification and denitrifcation (NDN) process in removing nitrogen (as ammonium) from high-strength ammonia wastewater that include low sludge production (Jetten *et al.*, 1997), reduced O_2 demand, zero addition of external carbon source, and reduced N_2O emissions (Wrage *et al.*, 2001).

Recent studies on Anammox process have majorly focused on various ways to enhance Anammox biomass yield to improve the process start-up time so as to promote its widespread utilization to replace the more expensive conventional NDN process in mainstream wastewater treatment (van der Star, *et al.,* 2007; Ren, *et al.,* 2015). It is significant to mention that despite the reported Anammox process advantages over the conventional NDN process; Anammox process has not been widely utilized because of significant operational drawbacks that undermine its acceptance as an alternative biological treatment alternative to conventional NDN process (Kartal, *et al.*, 2010; Bassin, 2018). Some of the key drawbacks include very slow Anammox bacteria growth, bacteria washouts during operation, and the risk of process instabilities from inhibition factors such as unfavorable influent substrate ratio (Dapena-Mora, *et al.,* 2007; Van der Star, *et al.,* 2008) and salinity (Abma *et al*., 2007). Slow bacterial growth undermines biomass development to dry-mass concentrations (as a measure of biomass population) capable of withstanding high feed substrates variability (as shock-loads) during municipal and industrial wastewater treatment. In addition, since Anammox bacteria often remain suspended in sequencing batch reactors (Van der Star, *et al.,* 2008), it is likely that enriched Anammox biomass are washed-out at low hydraulic and solids retention times (Strous, *et al.,* 1998). The combined effects of low biomass yield and bacteria wash-out could also lead to process instability (Fernández, *et al*., 2008). Risk of process instability due to bacteria inhibition from exogenous factors can further be compounded with the difficulty to achieve the ideal growth conditions and feed substrate ratio that favors enhanced Anammox bacteria growth (Egli, *et al.,* 2001; Jin, *et al.*, 2012). For instance, in a one-stage

process, the Anammox bacteria thrive under strict anoxic condition and require a steady substrates ratio (NH_4^+/NO_2^-) of approximately 1.32 (Lotti *et al*., 2012). However, attaining these conditions may be very difficult during start-up period because of the competition between the bacteria mediating the conventional NDN and the Anammox processes (Ahn, 2006). Owing to the challenges, enhancing Anammox biomass development is very important in reducing the process start-up time and enriching resilient Anammox bacteria. This study proposed to employ ubiquitous natural amendment materials (Bertino, 2011) with unique properties to adsorb and retain growth factors, such as NH_4^+, to enable the Anammox bacteria to selectively colonize and to proliferate. The proposed potential strategy could enhance Anammox biomass enrichment, retention, and improve the Anammox process widespread utilization in mainstream treatment processes.

Augmenting biological wastewater treatment processes with amendment materials to enhance bacteria biofilm development has been employed in moving bed biofilm reactors (MBBR) to increase biomass concentrations in conventional biological nitrogen process (Ødegaard, 1994; Ødegaard, 2006). For instance, filter bed media (K1 media) has widely been employed as an amendment to augment bioreactors to enhance biomass development in treating ammonium (Bertino, 2011). In addition, unique natural available materials with the ability to catalyze a reaction process and provide the bacteria with immobilization surface (Armentano *et al*., 2014) has previously been used to amend sequencing batch reactors to increase bacteria growth rates and to boost performance (Gai and Kim, 2008). Jung *et al.* (2004) determined that methanogenesis and nitrification rates were enhanced by reducing NH_4^+ concentration levels that caused inhibition when cation

exchange materials were added to the bioreactors treating anaerobic wastewater. Zeolite has also been proposed as possible amendment material to enhance biological nitrogen removal processes because of its potential to concentrate NH_4^+ on the surface (Fernández, et al., 2008). The zeolite material has been reported to enhance biological ammonium and nitrite removal in wastewater, which has been attributed to high cation exchange capacity (CEC), ammonium ion exchange (NH_4^+ IX) rates (Booker et al., 1996; Chen, et al. 2008; Aiyuk, et al. 2006; Fernández, et al., 2008), and the availability of pore-beds that possess finite ammonium adsorption potential (Grismer, Collision, 2017). He et al. (2004) used powder zeolite to amend a biological nitrogen removal (BNR) process treating municipal wastewater with a total nitrogen concentration of 54 mg-N L^{-1} and reported that the nitrification rate was increased by a factor of two compared to the non-zeolite amended process. In addition, enrichment of Anammox biomass was improved and a reduction of bacteria washout to values as low as 3 mg VSS L^{-1} was achieved when zeolite was used as growth media to treat municipal wastewater as reported by Fernández et al. (2008).

When utilizing zeolite to augment biological wastewater treatment processes, much focus has been on using zeolite clintoptilolite specie. However, recent evidence of higher CEC and better NH_4^+ ion-exchange (NH_4^+ IX) rate than clintoptilolite (Karmen et al., 2013; Langwaldt, 2008) makes zeolite chabazite a better alternative for ammonium removal from wastewater. Chabazite has lower Si^{4+} to Al^{3+} ratio than clinoptilolite (Erdem et al., 2004; Langwaldt, 2008) that could potentially increase the mineral negative charge for cation substitution leading to increased CEC. Moreover, NH_4^+ adsorption and bio-regeneration could be increased because of the negative charge resulting from a high composition of

Al^{3+} content within the mineral framework (Lahav, Green, 2000). Since Anammox bacteria grow under high ammonia (Hu *et al*., 2011) and low organic carbon (Dalsgaard and Thamdrup, 2002) conditions; and with relatively higher CEC and NH_4^+ IX than clinoptilolite, chabazite particles could possibly attain high surface concentrations of ammonium to create a naturally selective environment for proliferation and retention of Anammox bacteria. Aponte-Morales *et al,* 2016 showed that the rate of nitrification increased by almost 3-folds when chabazite was added at high NH_4^+ concentrations to a wastewater treatment batch compared to non-amended bioreactor.

Chabazite is further categorized into individual sub-species or types and regarded as a series of four members based on the dominant non-framework cation that include chabazite-Ca, chabazite-K, chabazite-Na, and chabazite-Sr (Coombs *et al*., 1998; Erdem *et al*., 2004; Sprynskyy *et al*., 2005). However, chabazite-Ca and –Na are the most commonly used types of chabazite (Aponte-Morales *et al*., 2016). Breck (1974) suggested a likely cation selectivity order which was later modified by Lahav *et al*. (1998) as shown in **Equation 1-2**. Based on Breck and Lahav cation selectivity orders, it can be speculated that cation prominences on chabazite material have an impact on ammonium oxidation rate. As revealed by Lahav *et al*. (1998), Na^+ is a less competitive cation than both NH_4^+ and Ca^+ and could easily be exchanged with NH_4^+ faster than Ca^+. To determine the effective chabazite sub-specie for Anammox process amendment, this study aimed to determine whether chabazite-Na has the highest ammonium removal efficiency compared to other types of chabazite in an anerorobic ammonium oxidation (Anammox) process and, to determine how Anammox bacteria population is impacted by the amendment of different

chabazite sub-species. If enhanced Anammox culture development and improved Anammox bacteria population is attained, this research contribution will have an immense impact towards widespread application of the Anammox process and assist to mitigate ammonium exposure to aquatic environment.

4.3. Materials and methods

4.3.1. *Chabazite preparation and characterization*

Chabazite types namely the Bowie chabazite AZUB (Na/Ca), AZLB-Na, and AZLB-Ca were obtained from St. Cloud Mining Company (New Mexico, U.S). The materials specifications, as recorded in the material safety and data sheet (MSDS), are included in the **Appendix**. The materials were pretreated to enhance kinetics as follows. Firstly, surface dusts on the raw particles that remain after the grinding process during mining were removed (Inglezakis *et al*., 2001). Secondly, the particles were washed and soaked in deionized water (Shcherban and Ilyin, 2016) then heated for 24 hours at 100°C (Ghassemi and Younesi, 2011). Soaking the materials remove impurities (Jha and Hayashi, 2009) while heating the material at approximately 100°C removes water molecules and organics to increase pore volume and diameter for improved adsorption surface area (Inglezakis *et al*., 1999). Isotherm and kinetic studies were conducted by setting up batch experiments using different masses of chabazite at appropriate intervals immersed in 200 mL NH_4^+ solution (~ 55 [± 5] meq/L, pH adjusted to 7.2) in 250mL Erlenmeyer flasks. The flasks were placed in an incubated orbital shaker maintained at 25°C and the rotation speed set at 200 rpm. Temperature choice was consistent with the findings of the study conducted by Lin *et al.* (2013) that showed that the equilibrium adsorption capacity was not

distinctively affected by any temperature between the 25 to 45°C range. Samples were taken after 24 hours and analyzed using Dionex ion chromatography instrument (IC DX-120) equipped with an auto sampler. Isotherm model determination for chabazite material was report earlier in the corresponding article.

4.3.2. Chabazite – Anammox hybrid reactors operation

Five 1.2-L (900 mL working volume) batch reactors, seeded with active Anammox biomass from an ongoing Anammox culture were started (**Figure 5-4** in the **Chapter 5, Appendix**). The batch reactors included the reactor amended with sand [**A**], which acted as the attached-control reactor, reactors amended with different chabazite types e.g. chabazite-Na (**B**), chabazite-Ca [**C**], chabazite – Na/Ca (**D**), and the non-amended (unattached) control reactor (**E**). Chabazite dose and contact time needed for effective NH_4^+ ion-exchange was determined using the feed medium.

To determine the appropriate dose, batch assays were carried out with varying amounts of chabazite material in 250mL Erlenmeyer flasks. Samples were taken and analyzed from the flasks at determined time intervals. All the SBRs were heated using a hot plate set at temperatures between 70 and 75°F. The reactors were stirred using overhead stirrers (Phipps & Bird Stirrer, Model 7790-400) set at 25 rpm to achieve complete mixing. The pH in the reactors was controlled between 7.2 and 7.5 by adding 0.5M HCl to the feed. Anoxic conditions in the reactors were maintained by flushing feed media with nitrogen gas. The reactors were fed interchangeably every 2 days. During feeding, the reactors were allowed to settle for 20 min and effluent drawn from the reactors for 10 min.

4.3.3. Feeding composition

The feed medium had the following composition per liter of deionized water: 1.25g $KHCO_3$, 0.025g KH_2PO_4, 0.3g $CaCl_2.2H_2O$, 0.2g $MgSO_4.7H_2O$, 0.00625g $FeSO_4$, 0.00625g EDTA, and 1.25mL trace elements solution as prepared by (Zhang *et al.,* 2008). The reactors were fed by steadily increasing the concentrations of $(NH_4)_2SO_4$ (as NH_4^+) and $NaNO_2$ (as NO_2^-) in the feed media every feeding period until the highest total feed nitrogen ($NH_4^+ + NO_2^-$) concentration of 400mg/L spanning over 120 days (4 months) was achieved. This was done to assess the reactors performance at increased feed substrates concentrations.

4.3.4. Analytical methods

Ammonium, nitrite, and nitrate were analyzed using colorimetric methods according to standard methods of water and wastewater analysis (Albertson, 2000). Biomass concentrations were measured as volatile suspended solids (VSS). VSS was determined as dry weight after drying the sample at 105°C for at least 1 hour, and then ashed in a furnace (550°C) for 30 min. D issolved oxygen (DO) concentrations and pH were monitored with selective electrodes.

4.3.5. Molecular assay

Nucleic acid (genomic DNA) was extracted from enriched biomass sampled every 30 days using the Qiagen kit. The quality and quantity of the extracted nucleic acid in the samples were measured using the NanoDrop spectrophotometer. The final extracted samples were diluted to 5–10 ng ul^{-1} for use as PCR template. Total Anammox bacteria population was investigated following Bipin *et al* (2007) protocol. The Anammox bacteria

standard curves were constructed from Escherichia coli genomic DNA (Sonthiphand *et al.*, 2013). Each constructed *E. coli* PCR product was purified using a MinElute kit (Qiagen, USA) and quantified using a NanoDrop spectrophotometer. Ten-fold serial dilutions were applied to the standard DNA PCR product template to create the qPCR standard curves with efficiencies $\geq$ 90% and coefficients of determination (R^2) $\geq$0.996 for all standard curves. The qPCR master mix used contained 5 ml of SsoAdvanced SYBR Green Supermix, 0.03 ml of each primer (100 mM stocks), 0.02 ml of bovine serum albumin (10 mg ml^{-1} stock) and 1 ml of genomic DNA template (5–10 ng stock) in a total volume of 10 ml. All qPCR amplifications were performed in duplicate using a CFX96 real-time system (Bio-Rad, USA).

4.4. Results and discussion

4.4.1. *Chabazite characterization*

Chabazite characterization results had been reported in the preceding chapter. In summary, chabazite exhibited good ion-exchange capacity with Na$^+$ and Ca^{2+} being the main cations exchanged by NH$_4^+$ cation. Na$^+$ equilibrium concentrations were higher than other cations and were increased at similar proportion as the decrease of NH$_4^+$ concentrations in the bulk solution. It was concluded that NH$_4^+$ easily exchange Na$^+$ compared to both Ca^{2+} and Mg^{2+}. The isotherm experimental data were fitted in different isotherm adsorption models as shown in **Table 3-3**. All the models had an average R^2 value greater than 0.90. The Langmuir model was determined to be the best representative isotherm model compared to the other evaluated models. The determined Langmuir model

parameters were as follows: R^2 value of 0.98, K_L value of 0.0761 (<<1), and q_{max} (adsorption capacity) value of 1.18 meq/g-chabazite.

Although the determined Langmuir q_{max} value was higher than the other evaluated models, it was lower than the value reported in the material safety and data sheet (MSDS). The q_{max} value reported in the MSDS is 1.8 meq/g-chabazite. The difference in the MSDS reported q_{max} value and the value determined in this study is strongly attributed to the differences in experimental conditions and pretreatment methods because it has been reported that inconsistent findings of chabazite kinetic parameters in different studies is due to the differences in the source of chabazite raw material, pretreatment conditions, and experimental conditions (Aponte-Morales *et al*., 2016; Hongjian *et al*., 2016). However, despite the variation in kinetic parameters, other studies have reported similar trends with Langmuir model being the best model for ammonium adsorption using zeolite materials such as the study reported by Lin *et al*. (2013). Nonetheless, the chabazite q_{max} value obtained in this study is higher than that of the natural zeolite-clinoptilolite previously reported as 1.15 meq/g-clinoptilolite (Langwaldt, 2008). The result is strong evidence that chabazite particles offer better ammonium removal alternative than natural clinoptilolite and could be applied to enhance Anammox culture development

4.4.2. Reactors performance

All the reactors performed at high removal efficiencies greater than 95% NH_4^+ removal as shown in Error! Reference source not found.. During the first 15 days following s tart-up, the reactors were fed at constant 12mg/L total substrate ($NH_4^+ + NO_2^-$) concentration to enable the Anammox seed biomass to acclimatize.
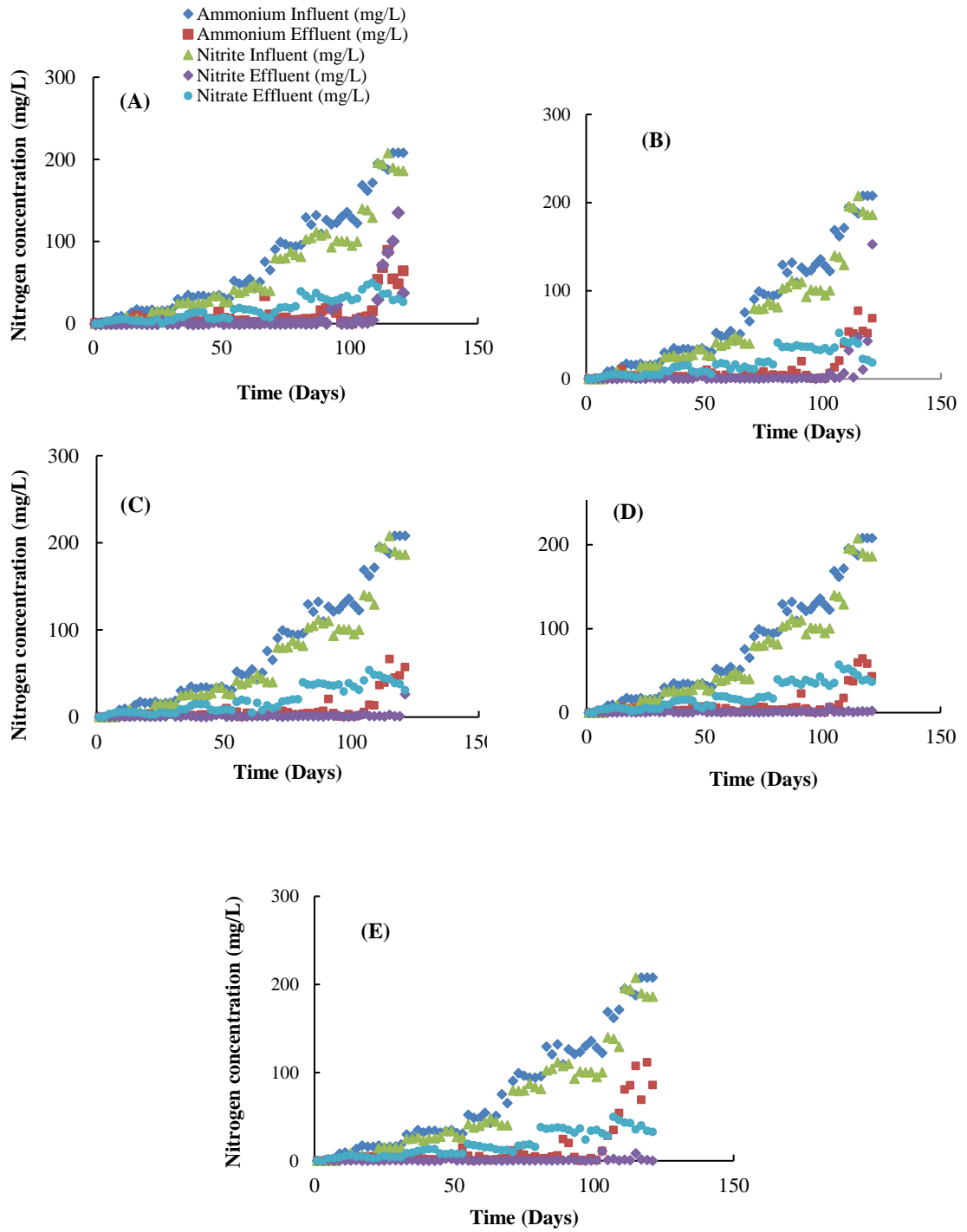
Figure 4-1: (A), (B), (C), (D) & (E) show the reactors performance at increased feed concentrations

Because the reactors were inoculated with active Anammox biomass, evidence of Anammox phenotypic activities was observed in the reactors shortly after the acclimatization stage. For instance, consistent substrates consumption at steady substrates ratio (NH_4^+ /NO_2^-) close to 1.32, coupled with approximate 15% nitrate production was observed in all the reactors as shown in Error! Reference source not found.. The feed s ubstrate concentration was then steadily and strategically increased after the acclimatization stage, at determined time interval, until maximum total nitrogen feed (NH_4^+ + NO_2^-) concentration of 400mg/L was fed on day 115. To mention, all the reactors exhibited similar high ammonium removal efficiency at increased substrate concentration until after 100 days when the total influent substrate concentration was further increased to 260mg/L.

In evaluating the reactors performance as shown in Error! Reference source not f ound., reactor A was mostly impacted at higher feed substrate concentration while reactor C was least impacted showing a drastic drop of the ammonium removal efficiencies from slightly over 95% to 53.5% and $> 95\%$ to 88%, respectively. In the order of ammonium removal efficiency and performance, reactors $C > D > B > E = A$, corresponding to reactors amended with chabazite-Ca, chabazite-Na/Ca, chabazite-Na, and the control reactors that had relatively equal performance efficiencies. Reactor E (non-attached control) had the highest NO_2^- accumulation that inhibited the Anammox activity at high feed substrate concentrations, which is consistent with previous reports that unattached Anammox process is inhibited by NO_2^- concentration as low as 20mg/L in the reactors (Strous *et al*., 1998).
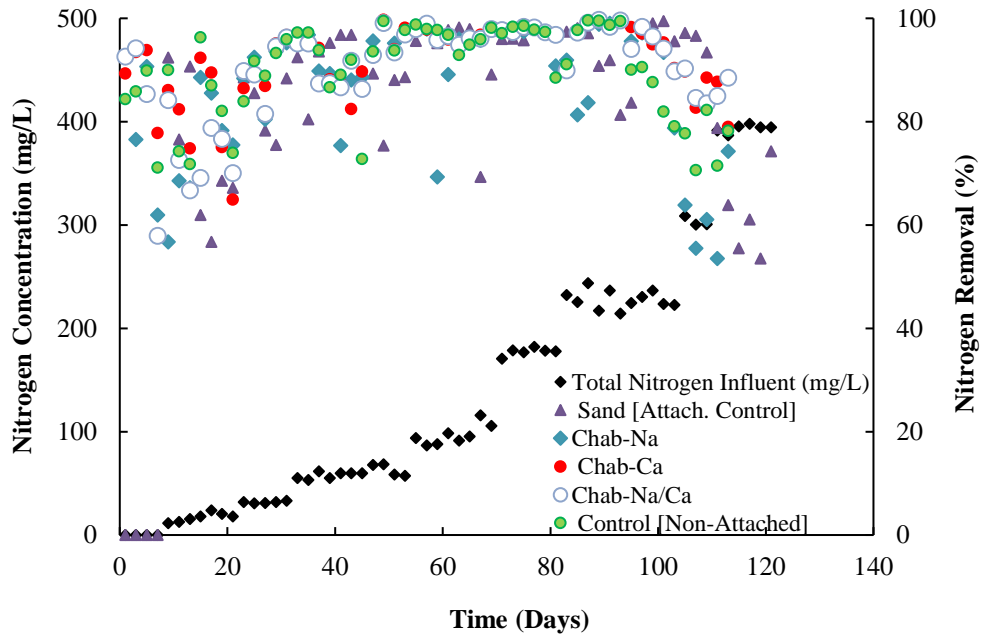
Figure 4-2: Percent nitrogen (ammonium + nitrite) removal for Anammox process augmented with different types of chabazite

The high ammonium removal efficiencies observed in the chabazite amended reactors can probably be attributed to NH_4^+ ion-exchange process that led to accumulation of Anammox bacteria known growth factor (NH_4^+) on chabazite particles. This enabled selective Anammox bacteria colonization and biofilm formation on the particles that further resulted in accelerated Anammox culture development and overall process performance. At high feed substrate concentration, all reactors experienced reduced ammonium removal efficiencies with the lowest efficiency correlating to the highest feed substrates concentration of 400mg/L fed on and after day 115. It had been hypothesized that reactor B (amended by chabazite-Na) would exhibit the highest ammonium oxidation rate and high Anammox activity according to the chabazite cation selective order. However, among the reactors amended using chabazite material, it was determined that

reactor B was the most impacted reactor in removing ammonia at high feed substrates concentration and reactor C (amended using chabazite-Ca) was the least impacted at similar high feed substrates concentration.

To explain the observation, the study looked at the potential role of chabazite particles on each step of the Anammox process. As previously stated, Anammox is a two-step process that, first, involves the partial nitrification process mediated by the aerobic ammonium oxidizing (AOB) bacteria followed closely by the anaerobic ammonium oxidation step mediated by the Anammox bacteria. The high NH_4^+ ion-exchange exhibited by chabazite-Na potentially decreased the total NH_4^+ concentration in the bulk solution available for the partial nitrification step. As a result, this could have impacted the steady substrates ratio (NH_4^+/NO_2^-) of approximately 1.32 (Lotti *et al*., 2012) ideally required in the second step of the Anammox process. With the negative influence of chabazite ion-exchange on partial nitrification process, the overall NH_4^+ oxidation and removal mediated by the Anammox bacteria, in reactor B, was impacted and led to low ammonium removal efficiency. On the other hand, the reactor amended by chabazite-Ca which had been hypothesized to exhibit lower NH_4^+ ion-exchange capacity than the chabazite-Na sub-specie, may have had favorable $NH4^+$ concentration in the bulk solution available for partial nitrification after the ion-exchange process. At favorable NH_4^+ and NO_2^- concentrations after the partial nitrification step, the ammonium oxidation step via the Anammox process would, therefore, successfully proceed and lead to high ammonium removal efficiency. The impact of chabazite sub-specie on Anammox rate could therefore be attributed to the influence of chabazite particles on the partial nitrification step mediated

by the ammonium oxidizing bacteria. It should be inferred and noted that chabazite particles enhance Anammox rate and activity at high feed substrate concentrations because the control reactors (without chabazite) were highly impacted and experienced least ammonium removal efficiencies at high fee substrate condition.

Compared to reactors A and E that solely depended on ammonium removal via the Anammox process, chabazite amended reactors B, C, and D exhibited two mechanisms for ammonium removal which are the cation ion-exchange and ammonium oxidation via the Anammox bacteria. During the ion-exchange process, chabazite particles potentially acted as 'buffer' materials against excess NH_4^+ concentrations in the feed media. This potentially mitigates the effect of high feed substrates variability resulting in optimum feed substrate concentration needed by the Anammox bacteria. When feed substrate concentration was further increased to 400mg/L, reactors A and E further exhibited reduced nitrate production, increased NH_4^+ and NO_2^- effluent concentration, and a drastic drop of the ammonium removal efficiency to 53.5%. The mitigated effect of high feed substrate concentrations on the chabazite particles amended reactors is evidence that chabazite addition enhanced Anammox activity at increased feed substrates concentration with chabazite-Ca amended reactor experiencing the least effect. In relation, it can be concluded that, among the chabazite sub-specie, chabazite-Ca greatly mitigate the effect of high feed substrate variability on Anammox activities.

4.4.3. Anammox bacteria population

Quantified specific Anammox bacteria in the reactors are shown in Error! R eference source not found.. To quantify the specific Anammox bacteria, biomass samples

were collected from the reactors every 30 days until day 120 (4 months) of continuous monitoring. The Anammox gene copy numbers (copy number/mL) were determined and normalized with the corresponding total dry weight biomass concentration (g VSS). Specific Anammox bacteria population (Copy number/ g VSS), as shown in the Supporting Information, among the five reactors were compared to assess the effect of different types of chabazite on specific Anammox bacteria population.
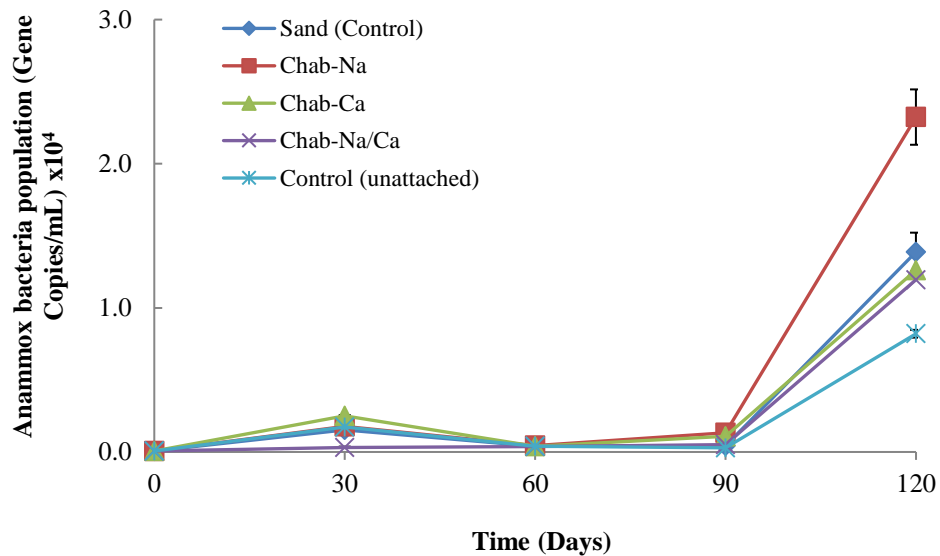
Figure 4-3: Chabazite –Na enhances Anammox bacteria population

It can be observed that Anammox bacteria population increased steadily after start-up time because the seed biomass used to start the processes was obtained from an active Anammox biomass source with phenotypic evidence of the Anammox process. The initial specific Anammox bacteria population increased steadily in all the reactors until day 30 when the total feed substrate concentration was increased to 55 mg/L that consequently led to decreased bacteria population. The decrease in bacteria population was attributed to the

effect of the shock initiated by the abrupt increase in substrates concentration that has been shown to reversibly inhibit the Anammox process. The impact was especially compounded by the effect of potentially high accumulation of NO_2^- that has also been reported to inhibit the Anammox process (Strous *et al*., 1998). However, an increase in bacteria population after the resultant shock effect was observed after about 60 days. In comparison, reactor B had the highest bacteria population among the reactors amended with the chabazite particles. The population rose steadily at continued increase in total feed substrate concentration with reactor B depicting a higher population increase to a maximum value of 2.32 x 10^4 copy numbers/mL compared to Reactor C that exhibited 1.20 x 10^4 copy numbers/mL and, finally, Reactor D depicting a bacteria population of 1.20 x 10^4 copy numbers/mL. Despite lower performance than Reactor C, Reactor B total specific bacteria population was the highest among all the tested reactors. Generally, chabazite addition enabled fast recovery from the inhibition caused by high feed substrate concentration, which led to increased bacteria population because the control reactors showed lower bacteria population at increased feed concentration. High bacteria population in Reactor B can be attributed to higher NH_4^+ ion-exchange rate compared to other chabazite amended reactors that, in effect, accelerated Anammox bacteria biofilm formation and growth over a prolonged period. In addition, due to the beneficial surface and chemical properties associated with the chabazite-Na particles, higher concentration of NH_4^+, as growth factor, was accumulated on the particles' surface compared to the other tested chabazite sub-species, therefore enabling more selective colonization of Anammox bacteria and improved overall bacteria population.

4.5. Conclusion

The different types of chabazites namely the Bowie chabazite AZUB (Na/Ca), AZLB-Na, and AZLB-Ca were obtained from St. Cloud Mining Company (New Mexico, U.S) with cation exchange capacities (CEC) of 1.8 meq/g, 2.50 meq/g, and 2.50 meq/g, respectively. The chabazite materials were pretreated by soaking in deionized water and heated at 100°F for 24 hours. Five sequencing batch reactors (SBR) with 0.9L working volume, seeded with active Anammox biomass, and amended with appropriate doses of sand [A], chabazite-Na (B), chabazite-Ca [C], chabazite – Na/Ca (D), and non-amended (E) were started. Among the chabazite amended reactors, reactor B was greatly impacted at high feed concentrations while C was least impacted depicting a decrease in the ammonium removal efficiencies from > 95% to 53.5% and > 95% to 88%, respectively. In the order of performance, reactor C > D > B > E = A. High ammonium removal efficiency observed in the chabazite amended reactors was attributed to the NH_4^+ ion-exchange process. Since Anammox is a two-step process that involves the partial nitrification step and the anaerobic oxidation step mediated by the Anammox bacteria, high NH_4^+ ion-exchange exhibited by chabazite-Na led to decreased total available NH_4^+ concentration needed for partial nitrification. This impacted the favorable ratio of substrate concentrations needed for the Anammox process and, as result, led to decreased ammonium removal efficiency. Reactor C experienced favorable $NH4^+$ concentration in the bulk solution available for partial nitrification after the ion-exchange process resulting in high ammonium removal efficiency. The impact of chabazite sub-specie on Anammox rate was attributed to the impact on partial nitrification mediated by the ammonium

oxidizing bacteria. Despite lower reactor performance than reactor C, reactor B total specific bacteria population was the highest among all the reactors. The population in reactor B increased to a maximum value of 2.32 x 10^4 copy numbers/mL compared to C that exhibited 1.20 x 10^4 copy numbers/mL and D at 1.20 x 10^4 copy numbers/mL. High bacteria population in reactor B was attributed to higher NH_4^+ ion-exchange rate compared to other chabazite amended reactors that, in effect, accelerated bacteria biofilm formation and growth over a prolonged period.

4.6. Acknowledgements

4.7. Bibliography

110. Ahn, Young-Ho, *Sustainable Nitrogen Elimination Biotechnologies: A Review.* Process Biochemistry, 2006. **41** (8): p. 1709–21.

111. Aiyuk, Sunny, Ilse Forrez, De Kempeneer Lieven, Adrianus van Haandel, and Willy Verstraete, *Anaerobic and Complementary Treatment of Domestic Sewage in Regions with Hot climates—A Review*. Bioresource Technology, 2006. **97** (17): p. 2225–41.

112. Bassin, J. P., Márcia D. G. L., *Advanced Biological Processes for Wastewater Treatment: Emerging, Consolidated Technologies and Introduction to Molecular Techniques*. Springer, 2018. p. 143–203.

113. Bertino, Andrea., *Study on One-Stage Partial Nitritation-Anammox Process in Moving Bed Biofilm Reactors: A Sustainable Nitrogen Removal*. 2011. diva-portal.org. http://www.diva-portal.org/smash/record.jsf?pid=diva2:530273.

114. Chen, Y., Jay J. C., Kurt S. C., *Inhibition of Anaerobic Digestion Process: A Review.* Bioresource Technology. 2008. **99** (10): p. 4044–64.

115. Daims H., Michael W. T., Michael W., *Wastewater Treatment: A Model System for Microbial Ecology*. Trends in Biotechnology, 2006. **24** (11): p. 483–89.

116. Dapena-Mora, A., J. L. Campos, A. Mosquera-Corral, M. S. M. Jetten, and R. Méndez. *Stability of the ANAMMOX Process in a Gas-Lift Reactor and a SBR*. Journal of Biotechnology, 2004. **110**(2): p. 159–70.

117. Egli, K., U. Fanger, P. J. Alvarez, H. Siegrist, J. R. van der Meer, A. J. Zehnder. *Enrichment and Characterization of an Anammox Bacterium from a Rotating Biological Contactor Treating Ammonium-Rich Leachate.* Archives of Microbiology, 2004. **175** (3): p. 198–207.

118. Grismer M., Collison R., *The Zeolite-Anammox Treatment Process for Nitrogen Removal from Wastewater—A Review.* WATER, 2017. **9** (11): p. 901.

119. He S. H., Shi-Yuang H., Bi-Hui J., *The Applications of Zeolite in Water and Wastewater Treatment*. Mining Engineering. *2004.* **1**: p. 7.

120. He Z., Jinjun K., Yanbing W., Yuelong H., Florian M., Kenneth H. N., *Electricity Production Coupled to Ammonium in a Microbial Fuel Cell.* Environmental Science & Technology, 2009. **43** (9): p. 3391–97.

121. Hu B. L., Darci R., Van der Biezen E., Zheng P., Van Mullekom M., Stefan S., Jaap S. S. D., Alfons J. P. S., Jetten M. S. M., Kartal B., *New Anaerobic, Ammonium-Oxidizing Community Enriched from Peat Soil.* Applied and Environmental Microbiology, 2011. **77** (3): p. 966–71.

122. Jetten M. S. M., Laura V. N., Strous M., Kartal B., Keltjens J. T., Huub J. M., Camp O. P., *Biochemistry and Molecular Biology of Anammox Bacteria.* Critical Reviews in Biochemistry and Molecular Biology, 2009. **44** (2-3): p. 65–84.

123. Jin R. C., Yang G. F., Yu J. J., Zheng P., *The Inhibition of the Anammox Process: A Review.* Chemical Engineering, 2012. **197**: p. 67–79.

124. Kartal B., Kuenen J. G., Van Loosdrecht M. C. M., *Engineering. Sewage Treatment with Anammox*. Science, 2010. **328**(5979): p. 702–703.

125. Khin Than and Ajit P. Annachhatre, *Novel Microbial Nitrogen Removal Processes*. Biotechnology Advances, 2004. **22** (7): p. 519–532.

126. Kuenen, J. Gijs., *Anammox Bacteria: From Discovery to Application*. Nature, 2008. **6**(4): p. 320–326.

127. Lahav, Ori, and Michal Green, *Bioregenerated Ion-Exchange Process: The Effect of the Biofilm on Ion-Exchange Capacity and Kinetics*. WATER, 2000. **26**(1): p. 51–57.

128. Langwaldt, Jörg, *Ammonium Removal from Water by Eight Natural Zeolites: A Comparative Study*. Separation Science and Technology, 2008. **43**(8): p. 2166–2182.

129. Ødegaard, H., B. Rusten, T. Westrum, *A New Moving Bed Biofilm Reactor - Applications and Results*. Water Science and Technology, 1994. **29** (10-11): p. 157–165.

130. Ødegaard, Hallvard, *Innovations in Wastewater Treatment: Moving Bed Biofilm Process*. Water Science and Technology, 2006. **53**(9): p. 17–33.

131. Raven J. A., *Contributions of Anoxygenic and Oxygenic Phototrophy and Chemolithotrophy to Carbon and Oxygen Fluxes in Aquatic Environments*. Aquatic Microbial Ecology, 2009. **56**: p. 177–1792.

132. Ren L.F., Shou-Qing N., Cui L., Shuang L., Zhang B., Qiang K., Ning G., 2015. *Effect of Zero-Valent Iron on the Start-up Performance of Anaerobic Ammonium Oxidation (anammox) Process*. Environmental Science and Pollution Research International, 2015. **22** (4): p. 2925–2934.

133. Schmidt I., Sliekers O., Schmid M., Bock E., Fuerst J., Kuenen J. G., Jetten M.S.M., Strous M., *New Concepts of Microbial Treatment Processes for the Nitrogen Removal in Wastewater*. FEMS Microbiology Reviews, 2003. 27(4): 481–492.

134. Strous M., Van Gerven E., Zheng P., Kuenen J. G., Jetten M.SM., *Ammonium Removal from Concentrated Waste Streams with the Anaerobic Ammonium Oxidation (Anammox) Process in Different Reactor Configurations*. Water Research, 1997. **31**(8): p. 1955–1962.

135. Van Der Star, Wouter R. L., Andreea I. M., Van Dongen U. G., Gerard M., Cristian P., Van Loosdrecht M. C. M., *The Membrane Bioreactor: A Novel Tool to Grow Anammox Bacteria as Free Cells*. Biotechnology and Bioengineering, 2008. **101**(2): 286–294.

136. Van D. U., Jetten M.S.M., Van Loosdrecht M. C. M., 2001. *The SHARON®-Anammox® Process for Treatment of Ammonium Rich Wastewater*. Water Science and Technology, 2001. **44**(1): p. 153–160.
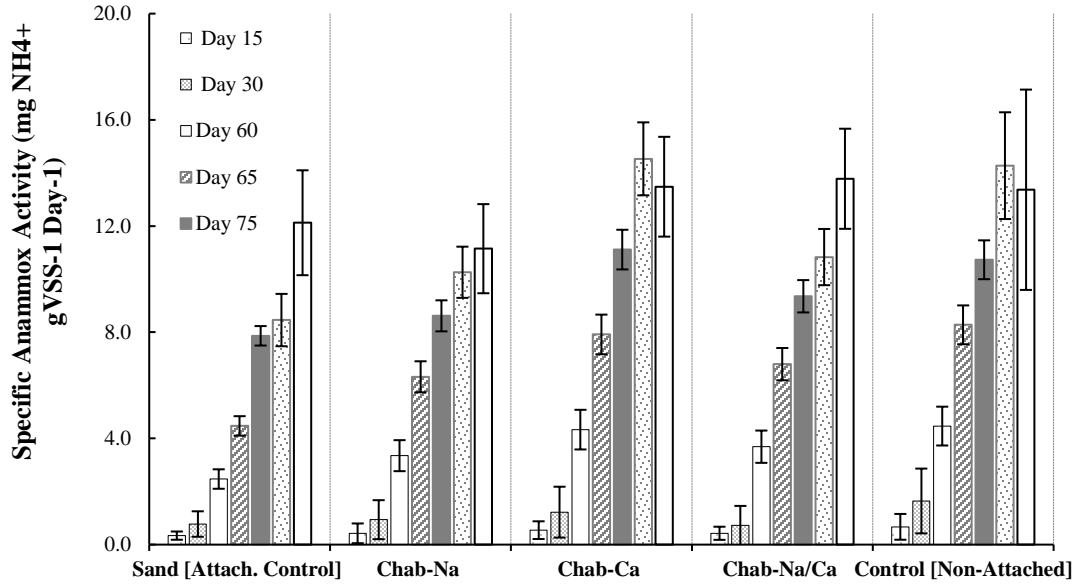
4.8. Appendix

Figure 4-4: Specific Anammox activity for different types of chabazite
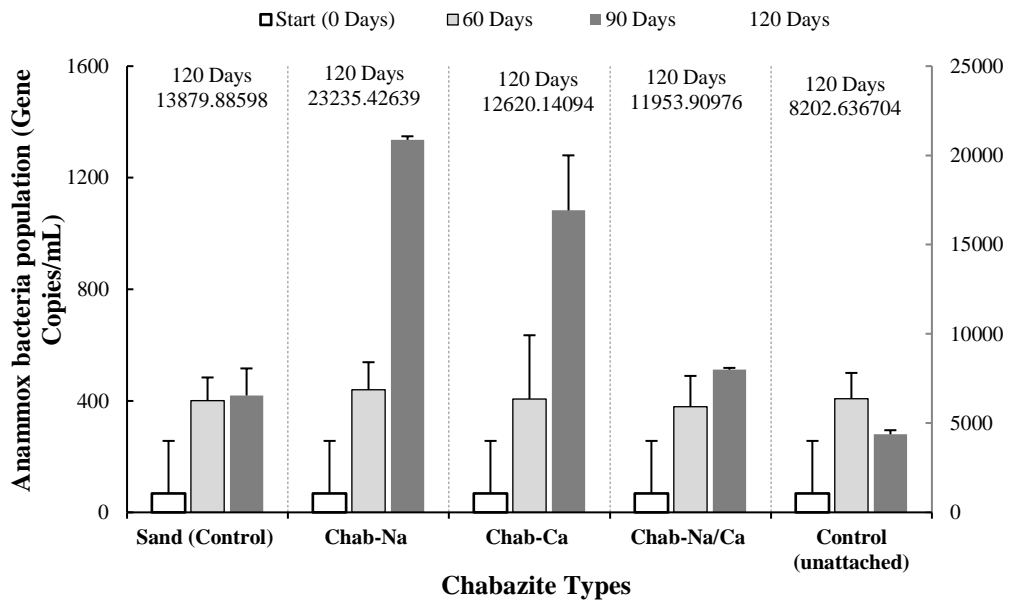
Figure 4-5: Specific Anammox bacteria population in different types of chabazite
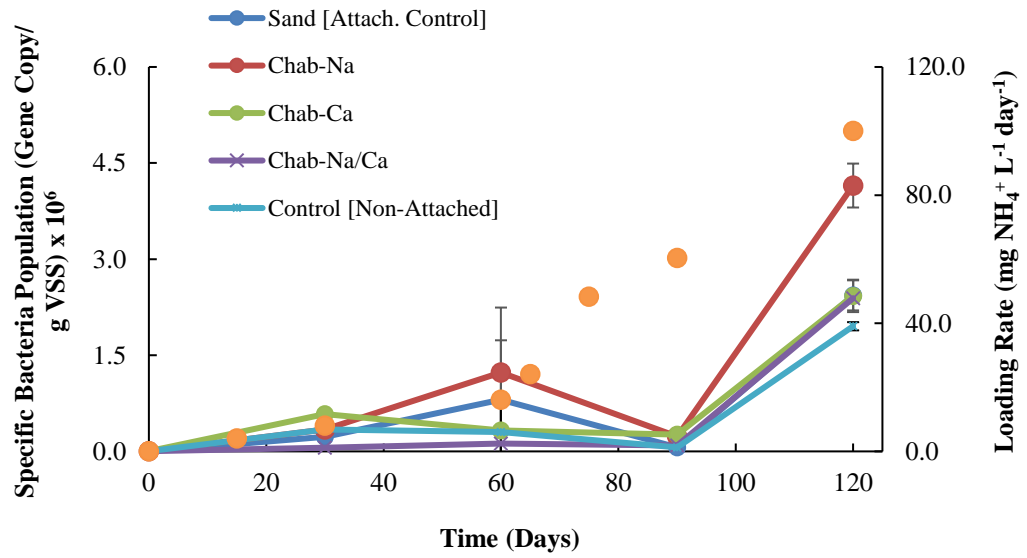
Figure 4-6: Specific Anammox bacteria population increase with increased loading rate

Chapter 5 : Effect of competing cation on ammonium oxidation rate in a hybrid Chabazite-Anammox process

5.1. Abstract

The Anaerobic ammonium oxidation (Anammox) process amended with chabazite particles may experience reduced NH_4^+ removal rates due to competition for the cation-exchange sites. During NH_4^+ion-exchange, the non-framework cations are replaced with NH_4^+ in the bulk solution while the replaced non-framework cations are simultaneously released into the bulk solution causing a cations spike that can potentially impact the Anammox NH_4^+ removal rates. Among the possible cations replaced and released into the bulk solution is K^+ that is the most important competing cation according to the chabazite cation selectivity order (Lahav *et al*.,1998; Breck, 1974).

This study aimed to determine the effect of competing cation on specific ammonium oxidation rate and, to determine the impact of competing cation on feed substrate (NH_4^+) utilization rates during the Anammox process amended with different chabazite sub-species.

The types of chabazite used in the study were the Bowie chabazite AZLB-Na and AZLB-Ca. Three different sequencing batch reactors (SBR) with 0.9L working volume were seeded with active Anammox biomass and amended with appropriate doses of chabazite-Na (**A**), chabazite-Ca [**B**], and non-amended control reactor (**C**). The feed media was spiked with different K^+ concentrations ranging from 0 to 5.12 meq/L. Samples were taken from the reactors at every 4-hour intervals after feeding to determine the feed substrates utilization rates over a 24-hour period. At increased K^+ concentration from 0 to 1.28 meq/L, the NH_4^+ oxidation rate observed in reactor **C** decreased by approximately 2-folds from 1.83 to 1.07 mg NH_4^+ L^{-1}

hr^{-1}. The NH_4^+ and NO_2^- utilization rates were higher in reactor **A** than in reactor **B** at high K^+ concentration. The rates determined in reactor **A** for NH_4^+ and NO_2 were approximately 1.1 and 1.4 times, respectively, higher than the rates in reactor **B**. The effect of NH_4^+ ion-exchange was mitigated at increased K^+ concentration because K^+ out-competed NH_4^+ for the cation exchange sites leading to availability of NH_4^+ needed for the partial-nitrification step of the two-step Anammox process to proceed. Consequently, improved substrates utilization and increased Anammox bacteria population was exhibited that further led to higher Anammox removal rates in reactor **A** than in reactor **B**. The results imply that chabazite-Na mitigates the effect of competing cation (K^+) in chabazite-amended Anammox process. The findings are significant in developing Anammox biomass retention strategies for enhanced culture development.

5.2. Introduction and Background

Nitrogen – which represents 78% of the atmosphere (Fields, 2004) - is an important natural part of the atmosphere and aquatic ecosystems (Rabalais *et al.*, 2002). When either the proportion exceeds 78% in the atmosphere or excessively increases in waterways, nitrogen poses major environmental and economic problems such as eutrophication in natural open waters, groundwater contamination, and atmospheric ozone production (Vitousek *et al.*, 1997). These problems have diverse and far-reaching impacts on public and environmental health (Vitousek *et al.*, 1997). Excess nitrogen (as ammonium) concentrations in aquatic systems and waterways originate from anthropogenic and various industrial activities (Abdelsalam *et al.*, 2018). One significant source of nitrogen (as ammonium) in waterways is effluent discharged from wastewater treatment plants (WWTPs). In composition, approximately 15-30% of nitrogen (as ammonium) contained in WWTPs effluent comes from digester reject-water after the sludge treatment process, while 70-85% comes from residual concentrations after the biological treatment process (Solley, 2000). Other sources of nitrogen (as ammonium) discharged into waterways include agricultural runoff and storm water discharges (Wakida and Lerner, 2005; Line and White, 2007; Li and Davis, 2014).

Ammonia removal in WWTPs is achieved through biological treatment processes. The conventional multi-staged biological nitrogen removal process is the most commonly used process that involve the nitrification and denitrification processes (Peng and Zhu, 2006). However, removal of nitrogen via the conventional multi-stage process requires high supply of oxygen (air), increased carbon demand (alkalinity), and increased supply of

chemicals to augment the process (Wiesmann *et al*., 1994). In addition, the traditional biological denitrification process is only suitable for removal of relatively low ammonia concentration with high carbon to nitrogen (C/N) ratio (López *et al*., 2007; Kim *et al*., 2008). Furthermore, the conventional process faces high demands of energy consumption and production of excess sludge (Wei *et al*., 2003).

To mitigate the problems, the Anaerobic Ammonia Oxidation (Anammox) process has been suggested as an alternative approach to remove nitrogen (ammonia) from high strength ammonia-wastewater at reduced cost and limited resources (Gu *et al.*, 2018; Guerrero *et al*., 2013). In comparison to the conventional process, the Anammox process reduces O_2 demand (energy) by 63%, carbon demand by nearly 100%, and biomass production by 80% (Park *et al*., 2010). The added advantages in adopting the Anammox process can potentially lead to reduced carbon footprint contributed by wastewater treatment plants (Monteith and Hugh, 2005).

Nonetheless, adoption of the Anammox process as a full-scale treatment process has been slow because of the long start-up time associated with the slow growing Anammox bacteria which has a doubling time of approximately 11 days (Jung *et al*., 2007; Star *et al*. 2007; Guerrero *et al*., 2012). Other challenges facing the adoption of the Anammox process include the delicate balance between the oxic and anoxic stages to attain the required population of the different bacteria mediating the Anammox process (Schmidt *et al*., 2003) and the difficult task to maintain the appropriate substrate ratio (NO_2-N/NH_4-N) in the feed media to promote Anammox culture development (Chamchoi and Nitisoravut, 2007; López *et al*., 2007; Tomar and Gupta, 2016).

These challenges result in long start-up time due to Anammox low biomass development and retention (Trigo *et al*., 2006; Fernández *et al*., 2008). To improve the Anammox process start-up, high biomass retention is required (Fux *et al*., 2002; Lackner *et al*., 2014) to minimize wasting. Improving biomass retention would potentially require selection of novel bacteria carrier materials with properties to necessitate bacteria colonization and proliferation (Star *et al*., 2008).

Zeolite has been proposed as a possible material to enhance biological treatment processes because of its potential to concentrate biomass growth factors on the surface (Fernández *et al*., 2008). Specifically, zeolite material has been reported to enhance biological ammonium and nitrite removal in wastewater, which has been attributed to high cation exchange capacity (Chen *et al*., 2008; Aiyuk *et al*., 2006; Fernández *et al*., 2008) and the availability of pore-beds that possess finite ammonium adsorption potential (Grismer and Collision, 2017).

In previous studies, natural zeolite has been applied to improve biological nitrogen removal (BNR) reactor performance (He *et al.*, 2007). He *et al.* (2007) used powder zeolite to amend the biological nitrogen removal (BNR) process treating municipal wastewater with a total nitrogen concentration of 54 mg-N L^{-1} and reported that the nitrification rate was increased by a factor of two compared to the non-zeolite amended process. The improved Anammox reactor performance can also be attributed to sorption of surplus NH_4-N by zeolite particles that act as a preserving reservoir, like a buffer system, generating a stable effluent concentration (Yapsakli *et al*., 2017).

While 'natural' zeolite, clinoptilolite, a member of zeolite group 7, has been reported to enhance biological wastewater treatment, chabazite, a zeolite group 4 member, could be a better alternative for ammonium removal from wastewater (Aponte-Morales *et al.*, 2016) because of its higher cation exchange capacity than clinoptilolite (Ouki and Kavannagh, 1999).

Chabazite high cation exchange capacity could be favorable in the Anammox process as it could adsorb excess ammonium and ease nitrification inhibition. Aponte-Morales *et al.* (2016) study revealed that the rate of nitrification increased almost 3 folds when chabazite was added at high NH_4^+ to a wastewater batch. However, in applying chabazite to augment the nitrification process, challenges with increased competing cations in the bulk solution released during cation exchange could significantly rise (Ouki and Kavannagh, 1999). Similarly, Aponte-Morales observed that Na^+ released from chabazite particles during NH_4^+ ion-exchange plays a role in nitrification inhibition by reducing the ammonium removal rates.

According to the cation selectivity order as reported by Breck (1974) and Lahav *et al.* (1998) shown in **Equation 1-2**, NH_4^+ preferred cations for exchange within the chabazite framework i.e. K^+ and Ca^{2+} could affect the overall ammonium oxidation rate (Lahav *et al.*, 1998). In addition, the presence of competing cations could further inhibit the process as revealed in the selectivity orders. K^+ is the most important competing cation compared to NH_4^+, Ca^+, and Na^+ (Langwaldt, 2008); that is, K^+ could favorably exchange chabazite cations (Ca^+ and Na^+) faster than NH_4^+. It may also be implied that the concentrations of competing cation (K^+), especially in the feed and reactor aqueous solution would affect

NH_4^+ ion-exchange which would further impact the Anammox process amended with chabazite particles.

In relation, this study aimed [1] to determine the effect of two competing cations and corresponding target cation (NH_4^+) on specific ammonium oxidation rate and [2] to determine the effect of competing cation (K^+) on feed substrates (NH_4^+ and NO_2^-) utilization rates in Anammox processes amended with different chabazite sub-species. The objectives would provide new information on the feasibility of using different chabazite material to enhance Anammox culture development and, to improve the process utilization in mainstream biological ammonium removal process.

5.3. Materials and methods

5.3.1. *Chabazite preparation and characterization*

Chabazite types namely the Bowie chabazite AZLB-Na and AZLB-Ca were obtained from St. Cloud Mining Company (New Mexico, U.S). The materials specifications reported on the material safety and data sheet (MSDS) are included in the supporting information.

The surface dusts on the raw materials after the grinding process during mining were removed to enhance kinetics (Inglezakis *et al*., 2001). The material was washed and soaked in deionized water (Shcherban and Ilyin, 2016), then heated for 24 hours at 100˚C (Ghassemi and Younesi, 2011). Soaking the materials has been reported to remove impurities (Jha and Hayashi, 2009) while heating the material at above 100˚C removes water molecules and organics thereby increasing pore volume and diameter (Inglezakis *et al*., 1999).

5.3.2. *Chabazite characterization and dosing*

Cation exchange capacity (CEC) study was conducted by setting up batch experiments using different masses of chabazite at appropriate intervals immersed in 200 mL NH_4^+ solution (~ 55 [± 5] meq/L, pH adjusted to 7.2) in 250mL Erlenmeyer flasks. The flasks were placed in an incubated orbital shaker maintained at 25°C and the rotation speed set at 200 rpm. The choice of temperature was consistent with the findings of the study conducted by Lin *et al.* (2013) that showed that the equilibrium adsorption capacity was not distinctively affected by temperature between 25 to 45°C range. Samples were taken after 24 hours and analyzed using Dionex ion chromatography instrument (IC DX-120) equipped with an auto sampler.

To determine the appropriate dose of the Chabazite - Anammox hybrid reactors operation, batch assays were carried out with varying amounts of chabazite material in 250mL Erlenmeyer flasks. Samples were taken and analyzed from the flasks at determined time intervals. Each SBR was heated using a hot plate set at temperatures between 70 and 75°F. Using overhead stirrers (Phipps & Bird Stirrer, Model 7790-400) set at 25 rpm each flask achieved complete mixing. The pH in the reactors was controlled between 7.2 and 7.5 by adding 0.5M HCl to the feed. To maintain anoxic conditions in the reactors, the feed media was purged with nitrogen gas before feeding. Chabazite-Ca, Chabazite-Na, and Chabazite-Na/Ca were added in separate 1.2-L (0.9-L working volume) Anammox sequencing batch reactors (SBRs). In addition, two similar unattached Anammox SBRs were set-up to act as control reactors with and without K+ addition.

The five reactors were stirred using overhead stirrers (Phipps & Bird Stirrer, Model 7790-400) set at 25 rpm to achieve complete mixing. Two pumps, one for influent, and one for effluent were used to feed and discharge the effluent from the reactors.

The feed was prepared without any supplemental K^+ source and the only source of K^+ was the concentration that was directly spiked into the reactors. Typical synthetic wastewater reagents and solutions was used to make the feed.   The substrates concentrations (ammonium and nitrite) were prepared from $(NH_4)_2SO_4$ (as NH_4^+) and $NaNO_2$ (as NO_2^-).   During feeding, the reactors could settle for 20 min and effluent withdrawn for 10 min. Each was fed interchangeably after every 2 days to allow enough time for ammonium removal.

5.3.3. *Chabazite – Anammox hybrid reactors operation*

Three 1.2-L (0.9 L working volume) batch reactors, seeded with active Anammox biomass from an ongoing Anammox culture were started (**Figure 5-4** in the **Appendix).** The three batch reactors included reactors amended with appropriate doses of chabazite-Na (**A**), chabazite-Ca [**B**], and non-amended control reactor (**C**). Chabazite dose and contact time needed for effective NH_4^+ ion-exchange had previously been determined using the feed medium. To determine the appropriate dose, batch assays were carried out with varying amounts of chabazite material in 250mL Erlenmeyer flasks. Samples were taken and analyzed from the flasks at determined time intervals. All of the SBRs were heated using a hot plate set at temperatures between 70 and 75°F. The reactors were stirred using overhead stirrers (Phipps & Bird Stirrer, Model 7790-400) set at 25 rpm to achieve complete mixing. The pH in the reactors was controlled between 7.2 and 7.5 by adding

0.5M HCl to the feed. Anoxic conditions in the reactors were maintained by flushing feed media with nitrogen gas. The reactors were fed interchangeably after every 2 days. During feeding, the reactors were allowed to settle for 20 min and effluent withdrawn for 10 min.

5.3.4. Feeding composition

The feed medium had the following composition per liter of deionized water: 1.05g $NaHCO_3$, 0.025g NaH_2PO_4, 0.3g $CaCl_2.2H_2O$, 0.2g $MgSO_4.7H_2O$, 0.00625g $FeSO_4$, 0.00625g EDTA, and 1.25mL trace elements solution as prepared by (Zhang *et al.,* 2008). The feed was made without additional K^+ source and the reactors fed at set concentrations of $(NH_4)_2SO_4$ (as NH_4^+) and $NaNO_2$ (as NO_2^-). The feed media was spiked with different concentrations of K^+ (made from KCl) that included 0, 1.28, 2.56, and 5.12 meq/L. Samples were taken from the reactors at every 4-hour interval after feeding to determine the feed substrates (NH_4^+ and NO_2^-) utilization rates over a 24-hour period. The experiments were repeated three times.

5.3.5. Analytical methods

Ammonium, nitrite, and nitrate were analyzed using colorimetric methods according to standard methods of water and wastewater analysis (Albertson, 2000). Ion concentrations during cation-exchange experiments were analyzed using a Dionex ion chromatograph (IC DX-120) equipped with an auto sampler. Dissolved oxygen (DO) concentrations and pH, in the reactors, were monitored with selective electrodes.

5.4. Results and discussion

5.4.1. Chabazite characterization

Chabazite exhibited good ion exchange capacity with Na^+, Ca^{2+}, and K^+ being the main cations replaced by NH_4^+ as shown in **Figure 5-1**. Na^+ equilibrium concentrations were higher than other cations and were increased at approximately similar proportion as the decrease in NH_4^+ concentrations. It can be seen from these results that NH_4^+ more readily replaces Na^+ from chabazite non-framework in comparison to Ca^{2+} and Mg^{2+}.
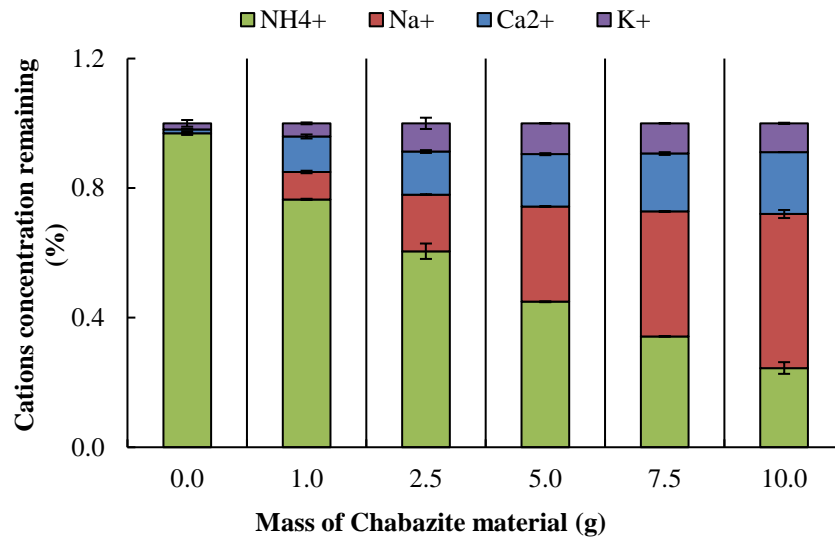
Figure 5-1: Equilibrium cation concentrations during NH_4^+ uptake.

5.4.2. Reactors Performance

The ammonium and nitrite removal rates in the control reactor without chabazite amendment and K^+ addition was determined to be 1.84 mg NH_4^+ L^{-1} hr^{-1} and 1.41 mg NO_2^- L^{-1} hr^{-1}, respectively. It was observed (**Table 5-1)** that high cation concentration (added as K^+) in the feed, decreased the Anammox rates since both ammonium and nitrite utilization

rates decreased from 1.84 mg NH_4^+ L^{-1} hr^{-1} and 1.41 mg NO_2^- L^{-1} hr^{-1} to 1.07 mg NH_4^+ L^{-1} hr^{-1} and 0.91 mg NO_2^- L^{-1} hr^{-1}, respectively. This result is consistent with previously reported findings which stated that high cation concentration decreases ammonium removal rates and efficiencies in biological ammonium removal processes (Green *et al.*, 1996; McVeigh *et al.*, 2008; Ham *et al.*, 2018; Cheng *et al.*, 2017).

Table 5-1: NH_4^+ and NO_2^- removal rates in reactors amended with different types of chabazite

K^+ concentration (meq/L)	Chabazite-Na	Chabazite-Ca	Control No Chabazite & K^+ Addition
0.0	0.65[3] 0.24[4]	0.84 0.53	1.84 1.41
1.28	1.19 0.25	1.07 0.46	1.27 0.39
2.56	1.33 0.62	1.34 0.91	1.07 0.91
5.12	1.48 0.93	1.37 0.68	1.37 0.68

In contrast, an opposite trend was observed in reactors amended with chabazite. Higher K^+ concentrations increased Anammox rates in chabazite amended reactors. Contrary to the control reactor, in the chabazite-Na amended reactor, both ammonium and nitrite removal rates increased from 0.65 mg NH_4^+ L^{-1} hr^{-1} and 0.24 mg NO_2^- L^{-1} hr^-1 to 1.48 mg NH_4^+ L^{-1} hr^{-1} and 0.93 mg NO_2^- L^{-1} hr^-1, respectively, and from 0.84 mg NH_4^+ L^-

---

[3] NH_4^+ rate (mg NH_4^+ L^{-1} hr^{-1})
[4] NO_2^- rate (mg NO_2^- L^{-1} hr^{-1})

^{1} hr^{-1} and 0.53 mg NO$_2^-$ L^{-1} hr$^-$1 to 1.37 mg NH$_4^+$ L^{-1} hr^{-1} and 0.68 mg NO$_2^-$ L^{-1} hr^{-1} in the

chabazite-Ca amended reactor (**Figure 5-2**) and **Table 5-1.**

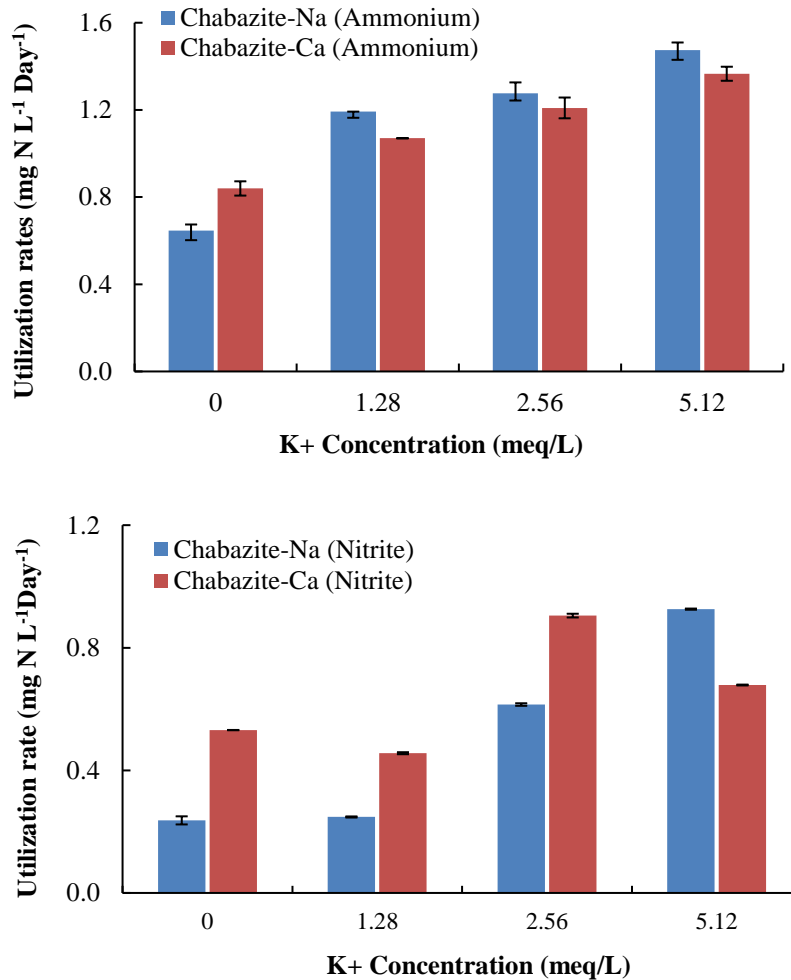**Figure 5-2:** Effect of K$^+$ on ammonium (left) and nitrite (right) removal rate in Anammox bioreactors amended with different types of chabazite.

It was earlier reported (**Chapter 4**) that cation exchange mediated by chabazite addition in the Anammox process results in low ammonium removal rates due to the direct impact of NH$_4^+$ ion-exchange on Anammox's partial nitrification step. From **Figure 5-2**, it

can be inferred that competition between K^+ and NH_4^+ for cation-exchange in the chabazite-amended Anammox process lowers the impact of partial nitrification. To explain, K^+ being higher than NH_4^+ in the cation selectivity order (**Equation 1-2**) readily replaces cations in the chabazite framework compared to NH_4^+, which leads to NH_4^+ availability in the bulk solution that, consequently, allows the partial-nitrification step to proceed. Since the anaerobic ammonium oxidation process is a two-step process i.e. partial-nitrification followed by ammonium oxidation process mediated by Anammox active bacteria, Anammox optimum efficiency is likely exhibited when the two-step proceeds. Therefore, increased K^+ concentration likely mitigates the potential loss of the partial-nitrification process that leads to increased ammonium and nitrite removal rates. Further evidence is depicted by increased nitrite utilization rates at higher K^+ concentrations **(Figure 5-2).** Increased K^+ concentrations lead to low NH_4^+ ion-exchange rates that result in favorable NH_4^+ concentrations required for partial nitrification. At favorable feed substrate concentrations that is ideally 1:1.32 ratios of NH_4^+ and NO_2^-, the anaerobic ammonium oxidation step mediated by the Anammox bacteria would optimally follow leading to increased Anammox removal rates.

Comparing the two chabazite sub-species, Anammox-chabazite-Ca had higher Anammox removal rates than Anammox-chabazite-Na at 0 meq/L of K^+. However, at increased K^+ concentrations, Anammox-chabazite-Na removal rates were higher than the Anammox-chabazite-Ca rates.

To explain this finding, it can be inferred that the effect of NH_4^+ ion-exchange on Anammox partial-nitrification in the Anammox-chabazite-Na process was mitigated at

increased K^+ concentration because K^+ readily replaces Na^+ in the chabazite framework compared to NH_4^+, leading to NH_4^+ availability in the bulk solution that allows the partial-nitrification step of the two-step Anammox process to proceed. The Anammox-chabazite-Na process exhibits two distinct ammonium removal mechanisms, NH_4^+ ion-exchange due to high chabazite-Na CEC, and the ammonium oxidation process mediated by the Anammox bacteria as discussed in **Chapter 4**. In comparison, chabazite-Ca amendment reactors ammonium reduction occurs largely due to oxidation; low NH_4^+ ion-exchange rates are associated with chabazite-Ca.

In addition, chabazite-Na amendment to the Anammox process was determined to enhance Anammox bacteria population. Therefore, at increased K^+ concentration, the effect of NH_4^+ ion-exchange on Anammox process is mitigated in the Anammox-chabazite-Na reactor leading to improved utilization of the available substrate by the Anammox bacteria. Increased substrates utilization at reduced NH_4^+ ion-exchange rates further lead to higher removal rates in the Anammox-chabazite-Na compared to Anammox-chabazite-Ca.

The results of the ammonium and nitrite removal rates across all the reactors amended with different types of chabazite at different K^+ concentrations are shown in **Figure 5-3**. It can be observed that at high K^+ concentration, initial nitrite removal rates are very low because partial nitrification occurs as the initial step of the Anammox process. During partial nitrification, excess ammonium is oxidized by the aerobic ammonium oxidizing bacteria (AOB) using the available oxygen as the electron acceptor resulting in nitrite production (limit reaction). Therefore, nitrite production after partial nitrification

leads to increased nitrite concentration in the reactors, hence the observed overall low nitrite removal rates.
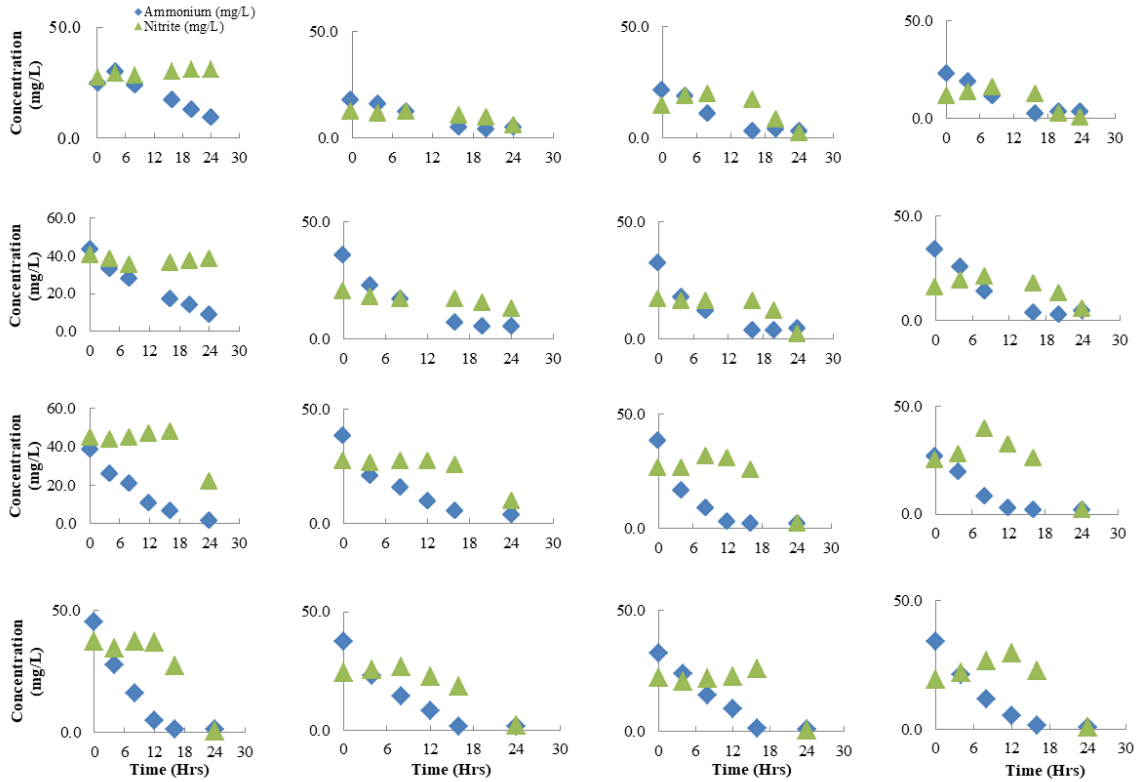
Figure 5-3: Left to right: Control (no chabazite, no addition of K^+), Chabazite-Na, Chabazite-Ca, and Control (No chabazite with K^+ addition). Descending: K^+ concentrations: 0, 1.28, 2.56, and 5.12 meq/L.

Since ammonium is consumed during the partial nitrification step, ammonium removal rates increase at high K^+ concentration as can be observed in **Figure 5-3**. It can further be deduced that at low K^+ concentration, due to the effect of NH_4^+ ion-exchange on partial nitrification, the ammonium removal rates decrease, and the nitrite rates remain constant until after residual favorable substrate concentrations is available that would allow for the Anammox process to proceed.

It can also be observed that both ammonium and nitrite removal rates increased at increased K^+ concentration in the reactor amended with chabazite-Na compared to the reactor amended with chabazite-Ca. **Figure 5-3** further reveals evidence of mitigated impact of NH_4^+ ion-exchange on chabazite-Na at increased K^+ addition. At the high K^+ concentration of 5.12meq/L, nitrite removal rate increases in the reactors amended with chabazite-Na leading to improved ammonium removal rate compared to the reactor amended with chabazite-Ca that had higher removal rates at low K^+ addition.

5.5. Conclusion

Chabazite types namely the Bowie chabazite AZLB-Na and AZLB-Ca were obtained from St. Cloud Mining Company (New Mexico, U.S) each having a cation exchange capacity (CEC) of 2.50 meq/g. The chabazite materials were pretreated by soaking in deionized water and heated at 100°F for 24 hours. Three different sequencing batch reactors (SBR) with 0.9L working volume were seeded with active Anammox biomass and amended with appropriate doses of chabazite-Na (**A**), chabazite-Ca [**B**], and non-amended control reactor (**C**). The NH_4^+ and NO_2^- utilization rates in the control reactor **C** were determined as 1.84 mg NH_4^+ L^{-1} hr^{-1} and 1.41 mg NO_2^- L^{-1} hr^-1, respectively. At increased K^+ concentrations, the rates decreased to 1.07mg NH_4^+ L^{-1} hr^{-1} and 0.91mg NO_2^- L^{-1} hr^-1, respectively, revealing the negative effect of K^+ on Anammox rates. In the chabazite amended reactors, the NH_4^+ and NO_2^-utilization rates increased from 0.65 mg NH_4^+ L^{-1} hr^{-1} and 0.24 mg NO_2^- L^{-1} hr^{-1} to 1.48 mg NH_4^+ L^{-1} hr^{-1} and 0.93 mg NO_2^- L^{-1} hr^-1, respectively, and from 0.84 mg NH_4^+ L^{-1} hr^{-1} and 0.53mg NO_2^- L^{-1} hr^-1 to 1.37 mg NH_4^+ L^{-1} hr^{-1} and 0.68 mg NO_2^- L^{-1} hr^{-1}, respectively, in reactors **A** and **B** respectively. It was

noted that the effect of NH_4^+ ion-exchange on partial nitrification was mitigated at increased K^+ concentration, which led to improved substrates utilization by the Anammox bacteria. Higher substrates utilization due to increased Anammox bacterial population resulted in higher Anammox removal rates in reactor **A** than **B**. The results imply that chabazite-Na mitigates the effect of competing cations (K^+) during chabazite-amended Anammox process.

5.6. Acknowledgements

5.7. Bibliography

137. Abdelsalam Elawwad, *Optimized Biological Nitrogen Removal of High-Strength Ammonium Wastewater by Activated Sludge Modeling*. Watereuse and Desalination, 2017. **8**(3): p. 393-403

138. Cheng Y., Huang T., Shi X., Wen G., Sun Y., *Removal of ammonium ion from water by Na-rich birnessite: performance and mechanisms*. Environmental Sciences, 2017. **57**: p. 402-410.

139. Fields S., *Global Nitrogen: Cycling out of Control*. Environmental Health Perspective, 2004. **112**(2): p. 556 – 563

140. Green M., Mels A., Lahav O., Tarre S., *Biological-ion exchange process for ammonium removal from secondary effluent*. Water Science & Technology, 1996. **34**(1-2): p. 449-458.

141. Guerrero, Lorna, Federico Van Diest, Andrea Barahona, Silvio Montalvo, Rafael Borja, *Influence of the Type and Source of Inoculum on the Start-up of Anammox Sequencing Batch Reactors (SBRs).* Environmental Science and Health, 2013. **48** (10): p. 1301–10.

142. Ham K., Kim B.S., Choi K-Y., *Enhanced ammonium removal efficiency by ion exchange process of synthesized zeolite after Na^+ and heat pretreatment*. Water Science & Technology, 2018. **78**(6): p. 1417-1425.

143. Jung, J. Y., S. H. Kang, Y. C. Chung, D. H. Ahn, *Factors Affecting the Activity of Anammox Bacteria during Start up in the Continuous Culture Reactor*. Water Science and Technology, 2007. **55** (1-2): p. 459–468.

144. Li, Liqing, and Allen P. Davis, *Urban Stormwater Runoff Nitrogen Composition and Fate in Bioretention Systems.* Environmental Science & Technology, 2014. **48** (6): p. 3403–3410.

145. Line, D. E., and N. M. White, *Effects of Development on Runoff and Pollutant Export.* Water Environment Research, 2017. **79**(2): p. 185–190.

146. López, Helio, Sebastia Puig, Ramon Ganigué, Maël Ruscalleda, Maria D. Balaguer, and Jesus Colprim, *Start-up and Enrichment of a Granular Anammox SBR to Treat High Nitrogen Load Wastewaters.* Chemical Technology & Biotechnology, 2008. **83**(3): p. 233–241.

147. McVeigh R.J., Weatherley L.R., *The Effect of Other Cations in Wastewater on the Ion-Exchange Removal of Ammonium Ion*. Chemical Engineering, 2008. **7**(1-2): p. 69-84.

148. Monteith, Hugh D., Halla R. Sahely, Heather L. MacLean, and David M. Bagley. *A Rational Procedure for Estimation of Greenhouse-Gas Emissions from Municipal Wastewater Treatment Plants.* Water Environment Research, 2005.**77**(4): p. 390–403.

149. Ouki, S. K., and M. Kavannagh, *Treatment of Metals-Contaminated Wastewaters by Use of Natural Zeolites.* Water Science and Technology, 1999. 39(10): p. 115–122.

150. Peng, Yongzhen, and Guibing Zhu, *Biological Nitrogen Removal with Nitrification and Denitrification via Nitrite Pathway.* Applied Microbiology and Biotechnology, 2006. **73**(1): p. 15–26.

151. Rabalais, Nancy N, *Nitrogen in Aquatic Ecosystems.* Human Environment, 2002. **31**(2): p. 102–112.

152. Solley, D., *Upgrading of Large Wastewater Treatment Plants for Nutrient Removal.* The Winston Churchill Memorial Trust of Australia: Canberra, Australia. 2000

153. Star, Wouter R. L. van der, Wiebe R. Abma, Dennis Blommers, Jan-Willem Mulder, Takaaki Tokutomi, Marc Strous, Cristian Picioreanu, Mark C. M. van Loosdrecht, *Startup of Reactors for Anoxic Ammonium Oxidation: Experiences from the First Full-Scale Anammox Reactor in Rotterdam.* Water Research, 2007. **41**(18): p. 4149–4163.

154. Tomar, Swati, and Sunil Kumar Gupta, *Investigating the Role of Co-Substrate–substrate Ratio and Filter Media on the Performance of Anammox Hybrid Reactor Treating Nitrogen Rich Wastewater.* Bioscience and Bioengineering, 2016. **121**(3): p. 310–316.

155. Trigo, C., J. L. Campos, J. M. Garrido, R. Méndez, *Start-up of the Anammox Process in a Membrane Bioreactor.* Biotechnology, 2006. **126**(4): p. 475–487.

156. Van Der Star, Wouter R. L., Andreea I. Miclea, Udo Gjm Van Dongen, Gerard Muyzer, Cristian Picioreanu, Mark C. M. Van Loosdrecht, *The Membrane Bioreactor: A Novel Tool to Grow Anammox Bacteria as Free Cells.* Biotechnology and Bioengineering, 2008. **101** (2): p. 286–94.

157. Vitousek, Peter M., John D. Aber, Robert W. Howarth, Gene E. Likens, Pamela A. Matson, David W. Schindler, William H. Schlesinger, David G. Tilman, *Human Alteration of the Global Nitrogen Cycle: Sources and Consequences.* Ecological Applications,1997. **7**(3): p. 737–750.

158. Wakida, Fernando T., and David N. Lerner, *Non-Agricultural Sources of Groundwater Nitrate: A Review and Case Study.* Water Research, 2005. **39** (1): p. 3–16.

159. Wei, Yuansong, Renze T. Van Houten, Arjan R. Borger, Dick H. Eikelboom, Yaobo Fan, *Minimization of Excess Sludge Production for Biological Wastewater Treatment.* Water Research,2003. **37**(18): p. 4453–4467.

160. Wiesmann, U., *Biological Nitrogen Removal from Wastewater.* Advances in Biochemical Engineering & biotechnology, 1994. **51**: p. 113–154.

161. Yapsakli, Kozet, Cigdem Kalkan Aktan, Bulent Mertoglu, *Anammox-Zeolite System Acting as Buffer to Achieve Stable Effluent Nitrogen Values.* Biodegradation, 2017. **28**(1): p. 69–79.

5.8. Appendix

A - sand
B - chabazite-Na
C - chabazite-Ca
D - chabazite-Na/Ca
E - no amendment (Control)
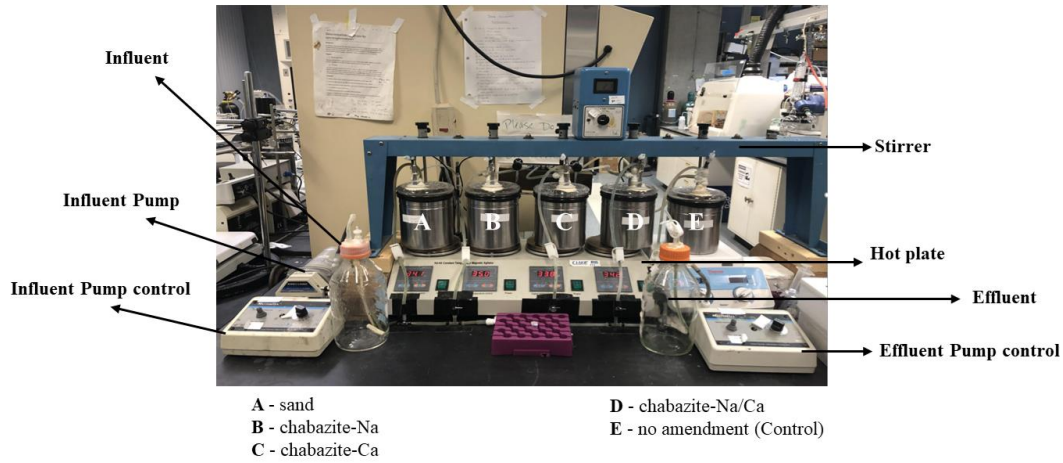
Figure 5-4: Reactors set-up and configuration to investigate the effect of chabazite types and cations on Anammox ammonium removal rate

Table 5-2: Typical chabazite (AZLB-Na) material properties

Color	Reddish/ Tan (dry brightness 40)
Average Chabazite Crystallite	Less than 1 micron
Crystallinity	*90%
Density	1.73 g/cm^3
Total Pore Volume	0.468 cm^3/g
Surface Area	520 m^2/g
Crystal Void Volume	0.47 cm^3/cm^3
Packing Density	Approx. (40-44 lbs/ft^3)
SiO_2/Al_2O_3 Ratio	Approx. 4:1
Moisture as Packaged	Less than 20% by Weight
pH of 1% Dispersion	8.5
Stability	pH of 3 through 12
CEC	2.50 meq/g

Table 5-3: Typical chabazite (AZLB-Na) chemical analysis (Anhydrous Basis)

SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O	Dominant Cation
68.1	18.59	2.84	0.27	0.75	8.32	1.12	Na

*$Tl^+ > Cs^+ > K^+ > Ag^+ > Rb^+ > NH_4^+ > Pb^{2+} > Na^+ = Ba^{2+} > Sr^{2+} > Ca^{2+} > Li^+$

Table 5-4: Typical chabazite (AZLB-Ca) material properties

Color	Reddish/ Tan (dry brightness 40)
Average Chabazite Crystallite	Less than 1 micron
Crystallinity	*90%
Density	1.73 g/cm^3
Total Pore Volume	0.468 cm^3/g
Surface Area	460 m^2/g
Crystal Void Volume	0.47 cm^3/cm^3
Packing Density	Approx. (40-44 lbs/ft^3)
SiO_2/Al_2O_3 Ratio	Approx. 4:1
Moisture as Packaged	Less than 20% by Weight
pH of 1% Dispersion	8.5
Stability	pH of 3 through 12
CEC	2.50 meq/g

Table 5-5: Typical chabazite (AZLB-Ca) chemical analysis (Anhydrous Basis)

SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O	TiO_2	Dominant Cation
69.5	16.6	4.33	4.49	0.89	2.4	1.32	0.47	Ca

*$Tl^+ > Cs^+ > K^+ > Ag^+ > Rb^+ > NH_4^+ > Pb^{2+} > Na^+ = Ba^{2+} > Sr^{2+} > Ca^{2+} > Li^+$

Chapter 6 : Summary

In this research, the feasibility of using atypical thermophilic anaerobic digester (TAD) mixed-sludge to start and enhance the anaerobic ammonium oxidation (Anammox) process was studied. In addition, chabazite was used to enhance the Anammox culture development and improve process performance. Different types of chabazite were also used to determine the most effective chabazite particle to enhance Anammox culture and improve the ammonium oxidation rates. Finally, the effects of target and competing cation concentrations on anaerobic ammonium oxidation rates were investigated.

The initial project was to assess the feasibility of using TAD mixed-sludge to enhance Anammox culture development to improve performance and to reduce process start-up time. The effect of initial feed substrates ratio and biomass concentration, and the concentration of inorganic carbon on Anammox activity were also investigated. Anammox culture was developed from atypical thermophilic anaerobic digester (TAD) mixed-sludge added proportionately in three bench-scale reactors. All the reactors showed phenotypic Anammox activities at approximately 90% total nitrogen removal and had varying start-up times. Reactor 3 which was augmented with the highest proportion of TAD mixed-sludge had the quickest start-up time after 32 days and attained the highest specific anammox activity (SAA) at highest nitrogen loading rate. Reactor 2 augmented with 25% (v/v) of TAD mixed-sludge depicted the highest Anammox bacteria gene copy numbers after two consecutive doubling times. The Anammox bacteria in the reactors were identified as "Candidatus Brocadia sinica." The high gene population was attributed to less competition among bacteria during start-up period.

To determine the effect of feed characteristics on Anammox rates, highest value of 0.07 g NH_4^+ L^{-1} day^{-1} was attained at 12.5mM HCO_3^-, while highest specific anammox rate of 0.16 g $NgVSS^{-1}Day^{-1}$ was realized at 3.0 g VSS L^{-1} biomass concentration. The findings in this part of the research imply that thermophilic anaerobic digester (TAD) effluent mixed liquor may be a potential inoculum source with an advantageous application in Anammox process start-up to remove excess ammonium from wastewater. Future research should focus on understanding the bacteria diversity before and after augmentation using TAD mixed-sludge.

Secondly, chabazite was used as a strategy to retain bacteria in the treatment reactor to enhance the development of the Anammox culture. To accomplish the task, two 3-L sequencing batch reactors were seeded with fresh return activated sludge (RAS) obtained from Los Angeles – Glendale WWTP owned by the City of Los Angeles. Appropriate AZ-UB obtained from St. Cloud Mining Company in New Mexico, U.S was added in one reactor (amended) while another similar reactor was operated without chabazite addition and acted as the control. Chabazite addition in the reactor had minimal influence on the Anammox process start-up time because both the amended (with chabazite) and non-amended reactor exhibited evidence of Anammox activity after 69 days. However, at increased feed variability, the control reactor experienced reduced nitrate production, increased NH_4^+ and NO_2^- effluent concentration, and reduced NRR from 95% to 86% while the amended reactor's NRR remained constant at 95%. It was concluded that chabazite material mitigated the effect of high feed variability resulting in optimum feed concentration needed by the Anammox bacteria. The amended reactor also exhibited

quicker recovery than the control reactor at very high substrate concentration, which was attributed to chabazite property to concentrated NH_4^+ on the surface leading to increased Anammox bacteria population in the amended reactor. The results in this part reveal that even though there is minimal influence on Anammox process start-up time, addition of chabazite enhanced Anammox culture development at high feed variability.

Based on the second portion of the research, and relying on the chabazite sub-specie tested, it was determined that chabazite has minimal influence in Anammox process start-up time. However, chabazite is categorized into individual types or sub-species based on the dominant non-framework cation. Among the mentioned sub-species, *chabazite-Ca* and *–Na* are the most commonly used. As was revealed, Na^+ is a less competitive cation that can easily be replaced with NH_4^+ faster than Ca^+ according to the proposed cation selectivity orders. In relation, a comparative study was conducted to determine the effective chabazite sub-specie for Anammox process amendment. Different types of chabazite employed in the study included the Bowie chabazite AZUB (Na/Ca), AZLB-Na, and AZLB-Ca obtained from St. Cloud Mining Company in New Mexico, U.S. In the order of performance in terms of NH_4^+ percent removal, reactor amended with chabazite-Ca > Na/Ca > Na > non-amended (control) = sand (attached control. To compare the two most commonly used chabazite in the bioreactors, chabazite-Na reactor was greatly impacted at high feed concentrations than the chabazite-Ca reactor with ammonium removal efficiencies decreasing from > 95% to 53.5% and > 95% to 88%, respectively. High ammonium removal efficiency observed in chabazite-Ca reactor was attributed to high NH_4^+ ion-exchange rate compared to other chabazite sub-species. Effect of chabazite

sub-specie on ammonium removal depended on the extent to which NH_4^+ ion-exchange impacted the partial nitrification step of the Anammox process that is mediated by the ammonium oxidizing bacteria. Despite lower reactor performance than chabazite-Ca reactor, chabazite-Na reactor total specific bacteria population was the highest among all the reactors. High bacteria population in chabazite-Na reactor resulted from higher NH_4^+ ion-exchange rate that accelerated bacteria biofilm formation and growth over a prolonged period. The findings from this part of the research imply that while chabazite-Ca greatly mitigates the effect of high feed variability on Anammox activities compared to other chabazite sub-species, chabazite-Na exhibits high bacteria population attributed to high NH_4^+ ion-exchange rate, which is significant in developing Anammox biomass retention strategies for process utilization.

Finally, the effect of competing cation on ammonium oxidation rate was studied. The concentrations of competing cation (K^+), especially in the feed and reactor aqueous solution was hypothesized to affect NH_4^+ ion-exchange that could further impact the Anammox process amended by chabazite material. Among the possible cations in the feed media, K^+ was considered the most important competing cation compared to NH_4^+, Ca^+, and Na^+; that is, K^+ could favorably exchange chabazite cations (Ca^+ and Na^+) faster than NH_4^+ as inferred from the cation selectivity order. Sequencing batch reactors with active Anammox biomass were amended with appropriate doses of chabazite-Na, chabazite-Ca, and non-amended (control). Upon spiking the feed media with different K^+ concentrations that ranged from 0 to 5.12 meq/L, it was determined that NH_4^+ and NO_2^- utilization rates were higher in chabazite-Na reactor compared to chabazite-Ca reactor at high K^+

concentration. The rates determined in chabazite-Na reactor for NH_4^+ and NO_2, which were higher than the rates in chabazite-Ca reactor, were approximately 1.1 and 1.4 times, respectively. It was determined that the effect of NH_4^+ ion-exchange on partial nitrification was mitigated at increased K^+ concentration and led to improved substrates utilization by increased Anammox bacteria population that further resulted to higher Anammox removal rates in chabazite-Na reactor than chabazite-Ca reactor. The results reveal that chabazite-Na mitigates the effect of competing cations (K^+) during chabazite-amended Anammox process which is significant in developing Anammox biomass retention strategies for enhanced culture development.

In conclusion, thermophilic anaerobic digester (TAD) effluent mixed-sludge was determined as a potential inoculum source with an advantageous application in Anammox process start-up to remove excess ammonium from wastewater. In employing chabazite to enhance Anammox culture development, it was determined that even though there is minimal influence on Anammox process start-up time, addition of chabazite enhances Anammox culture development at high feed variability. Amending the Anammox process with chabazite-Na led to higher bacteria population compared to other chabazite sub-species, which was attributed to high NH_4^+ ion-exchange rate that, in effect, accelerates bacteria biofilm formation and growth over a prolonged period. Of the chabazite sub-species, chabazite-Na was found to mitigate the effect of competing cations (K^+) in a chabazite-amended Anammox process leading to improved substrates utilization by the Anammox bacteria.