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THE OPTIMIZATION OF CONDITIONS FOR THE START-UP OF ANAMMOX BACTERIA SEEDED WITH NON-SPECIFIC BIOMASS

Master's thesis

Applied Measurement Science

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LIST OF ABBREVIATIONS

AnAOB – Anaerobic ammonia oxidizing bacteria

AOB – Ammonia oxidizing bacteria

AnMBR – Anaerobic membrane bioreactor

AMO – Ammonia monooxygenase

ATP - Adenosine triphosphate

CANON – Complete autotrophic nitrogen removal over nitrite

COD - Chemical oxygen demand

DEMON – Aerobic/Anaerobic deammonification

DO – Dissolved oxygen

ETC – Electron transport chain

FISH – Fluorescence in-situ hybridization

HAO – hydroxylamine oxidoreductase

HDH – Hydrazine dehydrogenase

HH – Hydrazine hydrolase

HZO – Hydrazine oxidoreductase

HZS – Hydrazine synthase

MLSS – Mixed liquor suspended solid

NOB – Nitrite oxidizing bacteria

NRR – Nitrogen removal rate

NLR – Nitrogen loading rate

NirS/NirK – Nitrite oxidoreductases (k- copper, S- Iron containting)

OLAND – Oxygen limited autotrophic nitrification denitrification

OZM – Oxygen minimal zone

PCR – Polymerase chain reaction

RBC – Rotating biological contractor

rRNA- Ribosomal Ribonucleic acid

SBR – Sequential Batch Reactor

SHARON – Single reactor for high activity ammonium removal over nitrite

SNAP – Single-stage nitrogen removal using anammox and partial nitrification

TN – Total nitrogen

TNRR – Total nitrogen removal rate

UASB – Upflow Anaerobic Sludge Blanket

UBF – Upflow Blanket Filter

WAS – Waste activated sludge

WWTP – Wastewater treatment plant

CHAPTER 1: INTRODUCTION

One of the major advancements in the early 90's is the discovery of anaerobic ammonium oxidation (anammox) as a major part of the world biogeochemical nitrogen cycle. In brief, this process occurs under anaerobic conditions and it is characterized by the autotrophic oxidation of ammonium to dinitrogen gas with an intermediary nitrite serving as an electron acceptor (Mulder et al. 1995; Kuenen 2008).

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

The anammox process since its discovery has been a significant milestone in the biological treatment of nitrogen from wastewater. The aquatic anaerobic bacteria that characteristically mediate this process belongs to the phylum Planctomycetes (Strous et al., 1999).
Anammox bacteria cultivated from anaerobic sludge, which are linked to the anammox process belongs to these genera; *Brocadia, Kuenenia, anammoxoglobus, Jettenia, Scalindua* and others (Kartal et al. 2007; Quan et al. 2008).

The anammox process presents a huge advantage compared to conventional nitrogen removal in wastewater treatment achieved through nitrification and denitrification, the anammox process, which occurs under anaerobic conditions shows significantly higher nitrogen removal efficiency and lower operational cost of about 60%; no requirement for additional organic carbon, reduced oxygen dependency, process can also be carried on a single reactor configuration and lower emission of greenhouse gases (CO_2/N_2O) (Hu et al., 2013). Another important benefit from anammox process is low sludge production; this is majorly due to slow biomass growth rate. Consequently, the slow growth rate (doubling time of about 7 – 20 days) substantially extends the start-up period. There is an incline in the number of full-scale wastewater treatment facilities globally using the anammox process, from study and operations the total removal rates recorded has been significantly noteworthy achieving a nitrogen removal rate, NRR of 9 kg $Nm^{-3}d^{-1}$ for high loading of 750 kg N/L/d (van der Star et al., 2007). The slow growth rate has driven the quest for achieving the best condition suitable for the cultivation and growth associated with start-up of anammox bacteria (Strous et al. 1998; Kartal et al. 2013). Acquisition of the most suitable inoculum is expensive, homegrown biomass is more adapted to local wastewater conditions

(Zekker et al 2014). Therefore, in this thesis anammox cultivation was done from a non-specific inoculum widely available.

The development of anammox in biofilm and suspended biomass for nitrogen removal process have been studied with the speculation of implementation of a hybrid system as being more beneficial and having a potential of increasing stability. Suspended biomass is considerably stable and resilient, with short recovery time (Laureni et al., 2019). The process of enhancing and optimizing the most adequate condition that will facilitate growth of anammox bacteria takes a considerably long period of time, generally more than 200 days (Jetten et al., 2005). Some of the conditions that generally affect anammox growth process include; temperature, hydraulic retention time (HRT), substrate toxicity (ammonia and nitrite), inoculum type and wastewater composition, dissolved oxygen concentration and reactor configuration (Tao et al., 2012).

Salinity optimum at 3 – 10 g NaCl/L promoted the formation of granular sludge and increased the retention of bacteria in the reactor but higher salinity inhibited the anammox process (Jin et al., 2012). Increased salinity results in high osmotic pressure which can lead to dormancy or plasmolysis or possible death of the microorganism. However, it is also pertinent to know that the anammox process is also responsible for 50% nitrogen turnover in marine environments at varying temperatures and high salinity. The adaptability of the anammox bacteria in high salinity has been attributable to acclimatization (Jin et al., 2012; Cho et al., 2019).

The anammox process consists of underlying intermediate reaction and metabolites; Hydrazine, (N_2H_4), Nitric oxide, (NO), Hydroxylamine (NH_2OH), that at optimal concentration promote total nitrogen removal. Hydrazine, enhancing biomass cultivation from zero has not been given much attention. Some works have highlighted the positive effect of hydrazine enhancing and improving recovery of nitrogen removal process, with an optimum concentration of 4 - 5 mg N_2H_4/L, in CANON (complete autotrophic nitrogen removal over nitrite) reactor. Considerably higher concentration is observed to slow down nitrogen removal process, which is possibly attributable to inhibitory effect. (Zekker et al., 2012, Yao et al., 2013).

The efficiency of cultivation of anammox bacteria using different sludge have been reported; an *inoculum* sludge with considerably high settling ability, good resistance and shock load capacity, and high removal efficiency is most suitable (Chamchoi et al., 2007; Quan et al., 2008; Ni et al., 2009). Anaerobic granular sludge has been efficiently used for start-up of anammox bacteria

cultivation due to mentioned properties (Franco et al., 2006). The use of non-specific biomass; aerobic, anaerobic and fermentation biomass has found little or no application in the cultivation of anammox bacteria so far. Seed biomass and *inoculum* from municipal waste providing enriched aerobic and anaerobic bacteria enhancing anammox start-up was successfully started with partial nitritation and denitrification for 315 days yielding a nitrogen removal efficiency of 98.5% (Shalini & Joseph 2013). By putting into consideration, the choice of bioreactor, which should be suited for long-term cultivation, enhancement and analysis. This study undertakes the start-up of anammox bacteria with non-specific *inoculum* as seeding sludge (anaerobic and aerobic sludge) using a single sequential batch reactor (SBR).

Therefore, this study aims to develop anammox process from a mixed culture biomass environment:

1. By accelerating the start-up of anammox bacteria from scratch from monitoring and controlling the bioreactor with the use of synthetic ammonium source and reject water feeding.

2. Using optimum temperature (30 °C) control to cultivate anammox biomass from nitrifying-denitrifying biomass. The process involves the assessment of how optimized growth conditions such as high temperature aid start-up of anammox process from non-specific *inoculum* for the removal of nitrogen from wastewater.

3. Evaluate the effect of optimum salinity; by comparing the impact of controlled salinity on nitrogen removal capacity of anammox bacteria.

4. Effect of Hydrazine in accelerating anammox start up time.

5. The effect of nitrogen loading, nitrite and ammonia rates on start-up process performance upon starting of feeding when nitrite concentration is kept to non-inhibitory level mostly below 1 mgN/L.

CHAPTER 2: LITERATURE REVIEW

2.1 NITROGEN CYCLE

Nitrogen cycle refers to the turnover of nitrogenous compounds in the biosphere. Nitrogen is an abundant element present as molecular nitrogen (N_2) in the atmosphere; it accounts for about 80% of the constituent that makes up air. Nitrogen provides important biological function, as an essential element for life; it is part of hereditary material and an integral framework of components of metabolism. Nitrogen utilization in the anabolic and catabolic reaction process include; ammonium uptake, nitrogen fixation, assimilatory nitrate reduction, anaerobic ammonium oxidation, nitritation, deammonification and dissimilatory nitrate reduction (Dapena-Mora et al., 2007).

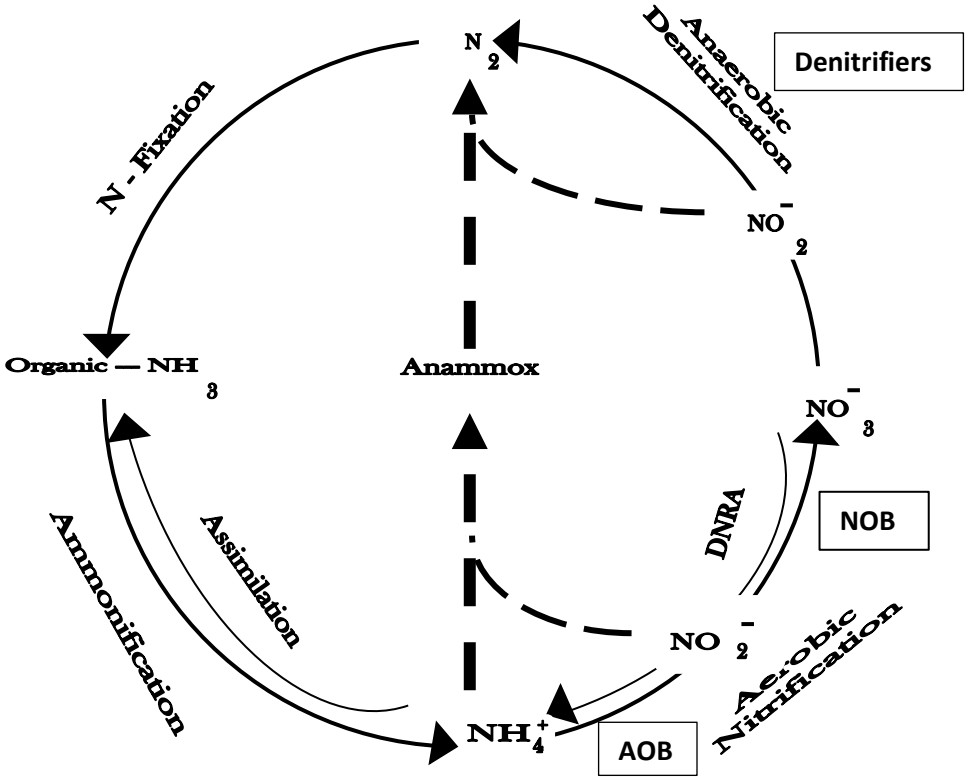


Figure 2.1 A simplified scheme showing the nitrogen cycle. Anammox process is seen at the middle as a short-cut in cycle (Dopena-Mora 2007).

Nitrogen turnover can be briefly described as the transformation of molecular nitrogen to organic nitrogen (nitrogen fixation), autotrophic microorganism mediated conversion of ammonia to nitrite and subsequently to nitrate (nitrification), and assimilation of ammonia in generation of new cells,

heterotrophic microorganism mediated conversion of nitrate or nitrite to nitrogen gas (denitrification) and cellular decomposition/conversion of nitrate to ammonia (ammonification).

The discovery of the microbial group anaerobic ammonium oxidizers (Anammox) that produces nitrogen gas with the use of ammonia and nitrite as substrate during anoxic conditions provided a new understanding to nitrogen turnover, especially in marine environments. The anammox process leads to the production of hydrazine and hydroxylamine intermediates, with the former being highly volatile and toxic (Schalk et al., 1998, Schmid et al. 2003).

2.2 NITROGEN POLLUTION AND WASTEWATER

The presence of nitrogen in wastewater forms a major chemical pollutant. High effluent nitrogen in wastewater leads to undesirable ecological impact and public health concerns. Nitrogen in untreated wastewater is majorly in the form of organic nitrogen and ammonium (NH_4^+). The global concern for environmental protection has given impetus to the implementation of stricter effluent standards and this has also driven the search and development of the most appropriate method to achieving proper effluent quality in terms of nitrogen removal from wastewater. In recent times, activated sludge process has been successfully utilized in the removal of nitrogen and organic carbon compounds from wastewater. Furthermore, the activated sludge process for the efficient removal of nutrients has come under thorough investigation and has seen implementation of functional models in the past decades (Hellinga et al., 1999; Van Loosdrecht & Salem 2006).

Removal of nitrogen from wastewater to acceptable thresholds is particularly important, the aftermath of high concentration of nitrogen in natural water bodies poses huge health and environmental risk. The natural transformation of nitrogen can lead to the production of ammonia, which is an essential plant nutrient and a precursor for nitrification in the generation of nitrates. Nitrate causes methemoglobinemia and in conjunction with high amounts of phosphorus provides nutrients that leads to algal bloom which subsequently leads to eutrophication. High nitrogen effluent causes reduction in dissolved oxygen (DO) concentration in water bodies. The aftereffect of reduced DO concentration and eutrophication can lead to death of life forms in natural waters. Nitrate accumulation in humans is traceable to a host of health problems including cancer (Nourmohammadi et al. 2013; Yamashita & Yamamoto-Ikemoto., 2014).

Nitrogen removal processes from wastewater have undergone immense study and a milieu of different treatment options have been developed over the years, which could be biological and physical/chemical treatment processes (Janus & van der Roest 1997).

2.3 NITROGEN REMOVAL PROCESSES FROM WASTEWATER

In considering the most suitable alternative wastewater treatment option, this is dependent majorly on the cost, effectiveness of the process, chemical requirement, technical operational experience, the energy requirement, reliability of the process and the degree of environmental impact. The choice of technique for nitrogen removal can also be based on the amount of nitrogen represented in wastewater. For wastewater containing higher concentration of ammonium (100 – 5,000 mg N/L), an intensive biological treatment process is required, though some researches have also shown that physico-chemical method can also provide a huge resource recovery approach, but overall process can be relatively expensive (Siegrist, 1996; Janus & van der Roest., 1997; Capodaglio et al., 2015). Some relevant treatment processes are highlighted below to treat high ammonia wastewater:

Physico-chemical techniques

1. Air/Steam stripping process
2. Precipitation of ammonia as struvite
3. Ammonia removal over ion-exchange

Some biological treatment techniques:

1. SHARON process
2. CANON process

2.3.1 PHYSICO-CHEMICAL TECHNIQUES

2.3.1.1 AIR/STEAM STRIPPING

Air stripping is used in the treatment of nitrogen in the form of volatile NH_3 present in wastewater. By regulating the process temperature at 10 – 22 °C and the pH at 10, this facilitates the conversion of NH_4^+ to NH_3, being followed by passing a stream of air through the wastewater. The resulting ammonia is collected in a solution containing sulphuric acid to yield ammonium sulphate,

$(NH_4)_2SO_4$. In steam stripping instead of air, steam is applied in desorption and in collecting free ammonia by condensation (Siegrist, 1996).

2.3.1.2 AMMONIA PRECIPITATION OVER STRUVITE (MAGNESIUM AMMONIUM PHOSPHATE (MAP) PRECIPITATION)

This process characterizes the precipitation of ammonia to yield struvite by the addition of phosphoric acid and magnesium oxide. The overall process is controlled at a pH range 8.5 - 10 to allow for the precipitation of struvite (Siegrist, 1996).

2.3.1.3 ION-EXCHANGE FOR AMMONIA REMOVAL

Naturally occurring zeolite (Aluminosilicate) or synthetic Zeolite serves as an ion-exchange medium. Zeolite has a porous structure and a two-dimensional channel system that allows it to act as a molecular sieve; it can also accommodate a wide variety of cations (Mg^{2+}, Ca^{2+}, Na^{+} and others).

This ion-exchange medium has good structural and temperature stability (700 – 750 °c), catalytic activity, ion-exchange and adsorption capacity, and exchange selectivity. The mechanism of ammonia-nitrogen removal is cationic ion-exchange; by adsorption in the structural pores of the zeolite. The process is pH and temperature dependent (Capodaglio et al., 2015)

2.3.2 BIOLOGICAL PROCESSES

2.3.2.1 SHARON PROCESS

For treatment of wastewater with high ammonia load, Single reactor for high rate ammonium removal over nitrite (SHARON) process offers a compact, cost-effective and sustainable treatment technique. The process compared to biological nitrogen removal over nitrate results in less sludge production (30 %), less carbon requirement (40 %), less oxygen utilization (25 %) and 20 % less CO_2 is produced.

This favours the growth of ammonium oxidizing bacteria (AOB) rather than growth of nitrite oxidizing bacteria (NOB) in a completely mixed reactor. The SHARON process is used to oxidize 50 % of NH_4^+ to NO_2^- in a continuously mixed reactor (HRT, hydraulic retention time 1 day), pH which could be controlled around 6 – 6.7 without sludge retention (Hellinga et al., 1999).

2.3.2.2 CANON PROCESS

This process similar to the SHARON and conventional nitrification/denitrification technique yields the same end result. The first stage involves converting 50% of ammonium to nitrite by AOB under limited oxygen conditions. Anammox bacteria under controlled anoxic condition uses the generated nitrite substrate as electron acceptor for the oxidation of half of the remaining ammonium, producing nitrogen gas (Van loosdrecht & Salem, 2006; Third et al., 2001).

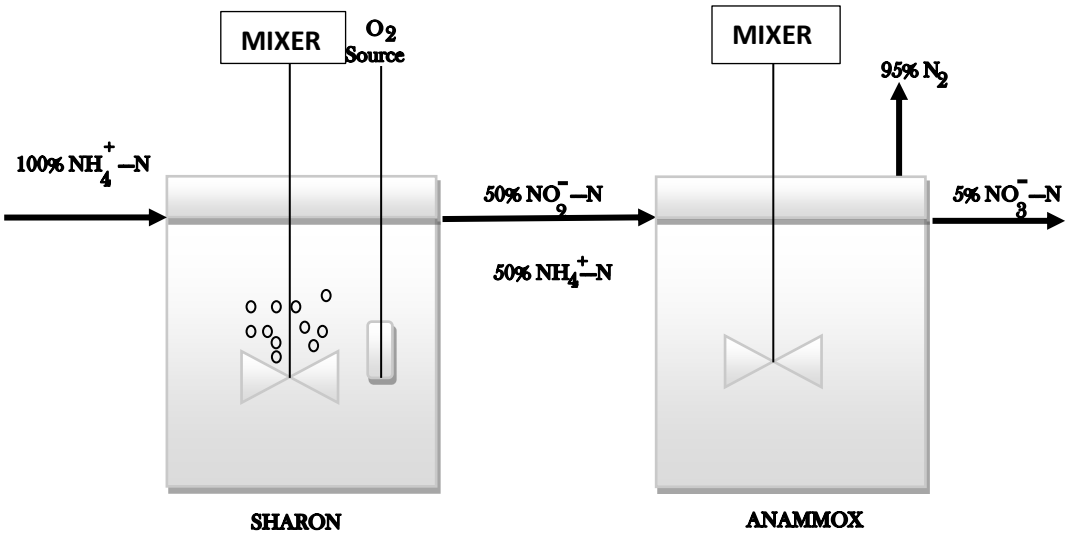


Figure 2.2 Schematic diagram showing combined anammox and SHARON processes (adapted from Jetten et al., 2002)

There are also other useful technologies using a configuration that allows the coexistence of anammox and AOB in a single reactor these are, Oxygen Limited Autotrophic Nitrification and Denitrification (OLAND) process, Single-stage Nitrogen removal using Anammox and Partial Nitritation (SNAP) process and anaerobic/aerobic deammonification (DEMON) process.

2.4 ANAMMOX CULTIVATION IN BIOREACTORS

Anammox bacteria are slow growing bacteria with significantly long doubling time compared with other bacteria; long start-up is required, establishing cultivation and growth can be successfully achieved using bioreactors. Growing a viably active biomass requires a bioreactor that give stability and good retention (van der Star et al., 2008). Studies have established that the type and configuration of a bioreactor affect the start-up and enrichment process of anammox bacteria. A bioreactor with a good surface area that will allow proper circulation of substrate (nitrite) helps to

ensure adequate nitrogen removal process. Increasing the total surface area of the bioreactor to ensure good biomass retention is achieved by the use of carrier materials for the attachment of biofilms (van der Star et al., 2007). Studies have shown that to cultivate bacteria biomass in a granular suspended growth-type, these bioreactor configurations can be implemented: Anaerobic membrane bioreactor (AnMBR), gas-lift bioreactor, moving bed bioreactor (MBR), up-flow anaerobic sludge blanket (UASB), and sequential batch reactor (SBR). However, to effectively develop and cultivate bacteria that is attached to the surface of a carrier material, the following reactor configurations have been used, fluidized bed reactor (FBR), rotating biological contractor RBC and up-flow blanket filter (UBF) (Suneethi et al., 2014).

2.4.1 SEQUENTIAL BATCH REACTOR (SBR)

This process can be carried out in a single tank. This technique for wastewater treatment works using an activated sludge model, which operates on a batch system (filling, reaction and drawing). Characteristically, there are cycles of 5 modes of operation:

a. Feeding
b. React
c. Settle
d. Draw
e. Idle

Some advantages of the SBR include; low-cost, flexibility of operation, compactness and effectiveness in removal of nitrogen (Mace & Mata-Alvarez 2002).

2.5 SLUDGE TYPES

Bacteria growth generally adopts two lifestyles: free-living or growth in a community to form flocs, granules, and biofilms, which have the possibility of attaching to surfaces.

In nitrogen removal technology biofilms and granular sludge types are used, they enhance biomass retention, create diversity and a variation in the environmental conditions across the thickness of the granular and biofilm biomass. The application of an SBR setup adapted with granular biomass is potentially useful in a concerted simultaneous partial nitrification anammox and denitrification process due to the fact that there is a development of a continuous, opposite, sequential macro-

environment that favours the coexistence of different bacteria types. This in a lot of ways help in the reduction of start-up cost of operation due to application of a single reactor, there is also flexibility in operation. Granules describe a more compact and dense aggregation of microbial colonies with an approximately spherical external appearance that do not clump together under hydrodynamic motion and settle significantly faster than sludge flocs (Lemaire et al., 2008). Two major advantages of granular biomass over floccular biomass is that they settle out faster reducing the possibility of biomass wash out. The high density of the granule and the shape leads to high settling velocity of about 18 – 40 m/h (Ni et al., 2009).

Granular biomass offers an efficient method for the separating water phase from biomass and this is particularly important for slow growing bacteria (Anammox) by offering a system that prevent biomass loss during removal of effluent and reduction in idling time of the reactor (Van Hulle et al., 2010). Granulation can also support growth of aerobic and anaerobic bacteria; AOB can be found at the surface of granules where they consume dissolved oxygen. This helps in creating an anoxic zone at the bottom of the system where anaerobic bacteria can effectively grow. This redox stratification creates the co-existence of AOBs and AnAOBs (Vlaeminck et al., 2010).

2.6 METABOLIC PATHWAYS TO BIOLOGICAL NITROGEN REMOVAL PROCESSES IN AMMONIUM-RICH WASTEWATER

Nitrogen present in domestic and industrial wastewater exist majorly in two different forms: ammonia nitrogen (NH_4-N) and organic nitrogen (Dapena-mora et al., 2007). Organic nitrogen and ammonia nitrogen both have a valence state of –3 and are the most reduced forms with similar oxidation state. The pH and temperature of a given solution or medium is particularly important in determining the form at which ammonia nitrogen exists; either as free NH_3 or NH_4^+. Free ammonia is taken up by microorganisms to generate new cells. The following biological processes for nitrogen utilization are considered;

A) Ammonification

B) Nitrification

B) Denitrification

C) Anammox

2.6.1 AMMONIFICATION

Ammonification describes an organic nitrogen transformation process that results in the formation of NH_3/NH_4^+. Animal waste like urea, uric acid, and other organic nitrogen containing compounds are substrates for ammonification (Strock, 2008).

Denitrifying nitrate reduction to ammonium (DNRA) is also an important ammonification reaction. The process proceeds under anoxic conditions and involves the dissimilatory conversion of oxidized nitrogen to NH_4^+ (Canfield and Thamdrup., 2005).

2.6.2 NITRIFICATION

Nitrification is the first stages in the biological nitrogen removal process. It proceeds sequentially by oxidation of ammonium (or other reduced nitrogen compound) to produce nitrite (nitritation)(Eq. 2.2) mediated by ammonia oxidising microorganisms (AOB) and further oxidation produces nitrate (nitratation)(Eq. 2.3), which is mediated by nitrite oxidizers (NOB) (Hellinga et al., 1999). From a biochemical scope, nitrification follows a two-step process (Schmid et al., 2003):

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad \text{Eq. 2.1}$$

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad \text{Eq. 2.2}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad \text{Eq. 2.3}$$

Nitritation which is ammonia oxidation to nitrite offers a more cost-efficient process compared to complete nitrification due to less oxygen demand, less sludge production. Partial oxidation of ammonia to nitrite and residual ammonia not converted can be coupled with the anammox process.

Nitritation is carried out by the chemolithotrophic ammonia oxidizing bacteria that obtain their carbon source from HCO_3^- or CO_2 in the genus *Nitrosomonas* (freshwater), other genera include; *Nitrococcus*, *Nitrospina*, *Nitrosolobus*, *Nitrosovibrio* , which are majorly found in soil (Stahl and del la Torre 2012). Nitratation, which is the second step of the nitrification process is carried out by facultative chemolithotrophic bacteria found in both soil and freshwater: *Nitrobacter*, *Nitrococcus* and *Nitrospira*. Two new NOBs in this group are *Nitrotiga* (cold tolerant) and *Nitrolancea hollandicus* (Sorokin et al., 2012; Lücker et al.,

2015). Nitrification process leading to the synthesis of nitrate following a series of intermediates, it is catalyzed by different enzymes. Ammonia is converted to an intermediate hydroxylamine (NH_2OH) (Eq. 2.4), mediated by the enzyme ammonia monooxygenase (AMO). Subsequently, the enzyme hydroxylamine reductase (HAO) facilitates the production of NO_2^- (Eq. 2.5; 2.6) (Bock &Wagner 2006).

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + 2H_2O \quad \text{Eq. 2.4}$$

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad \text{Eq. 2.5}$$

$$NH_2OH + H_2O + NO \rightarrow 2NO_2^- + 7H^+ + 5e^- \quad \text{Eq. 2.6}$$

The produced nitrite is further oxidized to nitrate by NOB using the enzyme nitrite oxidoreductase (NirS or NirK) (Eq. 2.7):

$$2NO_2^- + 2H_2O \rightarrow 2\,NO_3^- + 4H^+ + 4e^- \quad \text{Eq. 2.7}$$

2.6.3 DENITRIFICATION

Denitrification is a four-step process (Eq. 2.8) that is catalysed by multiple enzymes though not all denitrifiers possess the complete enzymes needed for the process. The enzymes that mediate denitrification process include, nitrate reductases, nitrite reductases, nitric oxide reductases, nitrous oxide reductases (Zumft 1997; Kraft et al., 2011).

Incomplete denitrification caused by a range of factors is the major cause of emission of greenhouse gases (N_2O) (Stein and Klotz 2016). Denitrification is a biochemical process that involves the reduction of oxidized inorganic forms of nitrogen into nitrogen gas (N_2);

$$NO_3^- \rightarrow 2NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad \text{Eq. 2.8}$$

Denitrification is used as an alternative source for oxygen respiration under anoxic or low oxygen concentration. Heterotrophic denitrification takes place in a broad range of bacteria, *Proteobacteria*, *Pseudomonas*, *Thiobacillus*, *Alcaligenes*, *Flavobacterium* and others (Zumft, 1997). Denitrification as a catabolic process in heterotrophic bacteria can be simplified into two step process via nitrate reduced to nitrite and further to N_2 under anoxic condition and the availability of organic carbon sources (acetic acid, methanol, butyric acid and propionic acids) as electron donor and nitrate or nitrite as electron acceptor (Eq. 2.9;2.10). The reaction proceeds thus;

$$8NO_3^- + 5CH_3COOH \rightarrow 4N_2 + 2CO_2 + 6H_2O + 8HCO_3^- \quad \text{Eq. 2.9}$$

$$8NO_2^- + 5CH_3COOH \rightarrow 4N_2 + 8OH^- + 10CO_2 + H_2O \quad \text{Eq. 2.10}$$

2.6.4 ANAMMOX

Anaerobic ammonium oxidation process is a biochemical process that is carried out by lithotrophic organisms in nature with the use of NO_2^- as electron acceptor and NH_4^+ as an electron donor to produce N_2 (Mulder 1995; Eq. 2.12). The anammox process is an alternative means to conventional denitrification process and accounts for a substantial part of nitrogen losses in the marine environment (Kuenen 2008).

The anammox bacteria are neither gram-negative nor gram-positive bacteria. Within their cytoplasm they possess a ribosomal free organelle called the *anammoxosome*, which is the site of anammox catabolism (Kartal et al., 2013).

From the characterization of 16S ribosomal RNA (rRNA) gene sequences 14 species of anammox bacteria have been identified members of phylum planctomycetes; Candidatus *Brocadia anammoxidans*, Candidatus *Brocadia sinica*, Candidatus *Jettenia asiatica*, Candidatus *Anammoxoglobus propionicus*, Candidatus *Brocadia fulgida*, Candidatus *Scalindua brodae*, Candidatus *Scalindua sorokinii*, Candidatus *Kuenenia stuttgartiensis* and others (Strous et al., 1999; Schmid et al., 2003; Kartal et al., 2007; Quan et al., 2008; Oshiki et al., 2011). Classification of these group of microorganisms is majorly based on their habitat and ecological niche; they have been found in Oxygen Minimum Zones (OZMs) at depth of 300 to 1000 meters in oceans, estuarine sediments and marine sediments (Kuenen 2008; Trimmer and Engstrom 2011).

The anammox metabolism follows a complex and unique pathway, leading to the production of N_2 gas, a small amount of the alkalinity and 10 % of nitrogen is converted to NO_3^- (Strous et al., 1998).

$$1.32NO_2^- + 0.066HCO_3^- + NH_4^+ + 0.13H^+ \rightarrow$$

$$\rightarrow 1.02N_3^- + 0.26NO_3^- + 2H_2O + 0.066CH_2O_{0.15}N_{0.15} + 10CO_2 \quad \text{Eq. 2.11}$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad \text{Eq. 2.12}$$

The stoichiometry of the reaction shows nitrite and ammonium conversion ratio of 1.32:1 and the production of nitrate, with nitrate and ammonium ratio as 0.26:1 (Jetten, 2001). Bicarbonate is the only source of carbon for the process; approximately 1 mol of HCO_3^- is utilized in the oxidation of 15 mol of ammonium. The complex biochemical pathway is mediated by the following enzymes, hydroxylamine oxidoreductase (HAO), hydrazine oxidoreductase (HZO) also called hydrazine dehydrogenase (HDH), nitric or nitrous oxide oxidoreductase (copper containing NirK or Iron containing NirS) and hydrazine synthase (HZS). The reaction can be described in 3 steps;

1. The first step catalyzed by nitrite oxidoreductase produces nitric oxide (NO) from the utilization of NO_2^-. The reduction reaction is as follows:

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \qquad \text{Eq. 2.14}$$

2. Hydrazine synthase mediates the synthesis of hydrazine from available substrate NO and NH_4^+:

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \qquad \text{Eq. 2.15}$$

3. The last reaction involves oxidation of N_2H_4 to generate 4 electrons and N_2 in a reaction catalyzed by hydrazine oxidoreductase:

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \qquad \text{Eq. 2.16}$$

It has also been proposed that the group of anammox bacteria (Candidatus *Brocadia anammoxidans*) lacking the nitrite oxidoreductase enzyme reduces NO_2^- via hydroxylamine with the concomitant reaction of hydroxylamine with NH_4^+ to generate hydrazine (Oshiki et al., 2011). The reaction is summarized thus:

$$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O \qquad \text{Eq. 2.17}$$

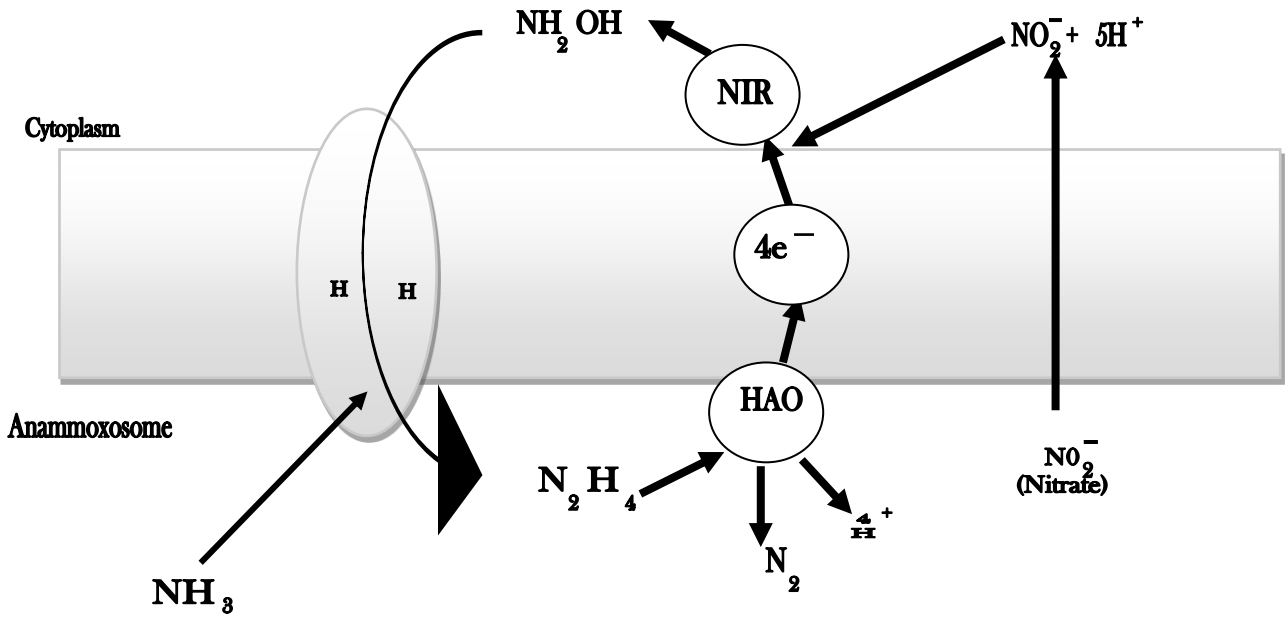


Figure 2.4 A schematic diagram showing anaerobic ammonia oxidation production of N2 catalyzed by HAO from N_2H_4 with the concomitant production of $4e^-$. The $4e^-$ is used by NIR to produce NH_2OH from NO_2^-. The enzyme HH produces N_2H_4 from NH_4^+ and NH_2OH (Adapted from Jetten et al., 2002).

2.7 FACTORS AFFECTING ANAMMOX KINETICS

2.7.1 TEMPERATURE

Several researches have shown the optimum temperature range favourable for the anammox process at 30 – 40 °C (Strous et al., 1999, Dosta et al., 2008). Some researches done at temperature range of -1.7 – 20 °C have proved to be a challenging process (Rysgaard et al., 2004, Dostal et al., 2008).

In the natural habitat, anammox bacteria can adapt to temperature range form -1.4 – 80 °C (Tao et al., 2012). Studies by Dosta et al. (2008) on short-term and long-term effects of temperature on anammox activities showed maximum activity at range of 35 – 40 °C, for none of adapted biomass at higher temperature of about 40 °C led to loss of activity by plasmolysis. Anammox bacteria represents a unique group of bacteria that can adapt to low temperature by acclimatization. Temperature is an important kinetic parameter that can be effectively controlled in improving anammox process.

2.7.2 SALINITY

Anammox bacteria can be found in both freshwater and marine habitats with significantly high salt concentration. Although the anammox process offers a favourable means of treatment for wastewater containing high salt content; high salt concentration can lead to inhibition of the synthesis of many enzymes, decrease in the cell metabolism and increased osmotic pressure within the cell that causes loss of biomass activity by plasmolysis of the bacteria. The tolerance and activity of the anammox bacteria in high salt concentration is largely dependent on the anammox species type and acclimatization. The species of bacteria within the genus *Scalindua* have been detected in halophilic habitat (Schmid et al., 2003). Studies have shown that the Candidatus *Kuenenia stuttgartiensis* favourably acclimated to high salt concentration of 90 % NaCl and 10 % KCl (Kartal et al., 2006). The inhibitory effect of high salt concentration on the activity of anammox biomass is reversible. Most of the other anammox bacteria thrive in freshwater environments. Studies have reported that salinity at concentration within the range of 3 – 15 g NaCl L^{-1} facilitated retention of biomass and formation of anammox granular sludge (Dapena-mora et al., 2010).

Further studies have also indicated that the type of salt determines the effect that will be exerted on anammox activity. Salt concentration of 6 – 10 g NaCl L^{-1} also showed stimulatory effect on anammox activity while at higher salinity a decrease in anammox performance (Windey et al., 2005; Dapena-mora et al., 2007; Oshiki et al., 2011).

2.7.3 pH

Effective regulation of the anammox process requires proper control of the pH. Strous et al., (1997) highlighted the optimum pH range for anammox activity at 6.7 – 8.3 with an optimum pH at 8. However, if the anammox was enriched at an operational condition within the alkaline/acidic pH range, the biomass can adjust to these conditions.

2.7.4 ORGANIC MATTER

Anammox bacteria are chemolithoautotrophic, which means they can utilize inorganic carbon sources HCO_3^- and CO_2. Prolonged exposure of cultivated anammox bacteria to biodegradable organic carbon sources inhibits the bacteria; this can also result in the growth of heterotrophic denitrifying bacteria which can inadvertently compete with anammox bacteria for available nitrite (Chamchoi et al., 2008; Molinuevo et al., 2009). Organic carbon induced

inhibition of the anammox process is observed in influents containing C/N ratio that is higher than 2 (Kumar and Lin 2010).

Studies have also shown that biomass containing certain species of anammox bacteria (Candidatus *Brocadia propionicus* and Candidatus *Brocadia fulgida*) can oxidize particular organic compounds such as propionate, acetate and formate (Kartal et al., 2008). In contrast to earlier result enrichment media containing Candidatus *Brocadia sinica* lost 85 % of activity upon exposure to 32 mg L^{-1} of methanol (Oshiki et al., 2011).

Inhibition by certain organic compounds like aldehyde, alcohol (ethanol), phenol and antibiotics can lead to complete or irreversible loss of microbial activity by enzyme inactivation or microbial poisoning (Jin et al., 2012).

2.7.5 FREE AMMONIA/AMMONIUM

A study to show the effect of ammonia nitrogen was carried out using a concentration of 1000 mg N L^{-1} for one week in an SBR reactor, but did not lead to loss of anammox activity (Strous et al., 2000). While a 50 % loss of activity was observed at concentration of 770 g NL^{-1} (Dapena-mora et al., 2007).

Some studies have also indicated that high concentration free ammonia rather than ammonium results in decline of anammox activity (Aktan et al., 2012; Fernández et al., 2012). The strong inhibitory effect by free ammonia may be attributed to set-up, reactor condition, type of microbial population and sludge type. Biofilm and granular sludge type are seen to be more useful compared to single cell in enabling tolerance inhibitory effect by free ammonia (Aktan et al., 2012).

2.7.6 NITRITE AND HYDRAZINE

Nitrite plays an important role as a substrate in the anammox process, although studies have also demonstrated that this compound can lead to loss of anammox activity at concentrations of 100 mg NO_2^--N L^{-1} (Strous et al., 1999). The exerted inhibitory effect by NO_2^- has been shown to be reversible, though the observed reversibility also depends on the time frame of exposure and concentration of nitrite (Lotti et al., 2012).

Externally added hydrazine is utilized in the production of extra energy, this consequently helps in the acceleration of growth during the process of startup. It is also reported that external addition limits the accumulation of NO_3^- and N_2O production (Ganesan and Vadivelu, 2019).

Hydrazine is produced as an intermediate in the anammox metabolism of nitrite and ammonium: the anammox bacteria is able to uniquely metabolize and use this compound as an energy source (Schalk et al., 1998). The conversion of hydrazine to N_2 results in the release of four electrons that is subsequently utilized in the production of ATP (Kartal et al., 2011). Hydrazine has also been reported to show latent toxicity on bacteria activity: it is known to inhibit other bacteria (AOB) that are involved in biological treatment of wastewater excluding the anammox bacteria. (Qiao et al., 2016). Ganesan and Vadivelu (2019) also highlighted that the external addition of hydrazine at 10 mg L^{-1} helped in the reduction of start-up time by 50 % to achieve maximum removal of nitrite and ammonium after 49 days compared to sludge types which used non-anammox inoculum for start-up.

2.8 COEXISTING MICROORGANISMS FOR WASTEWATER TREATMENT

Wastewater management nowadays is geared towards sustainability and conservation of resources; this has increased attention in selecting the most effective process that will conserve energy and resources. The discovery of the anammox has since opened up a new wave of possibilities to the removal of nitrogen from wastewater. A combination of several microbial processes and pathways can lead to advancement of already existing technologies and the development of newer technologies.

Researches have highlighted the coexistence of aerobic and anaerobic bacteria, which can be particularly important in treating high loads of carbon and nitrogen containing wastewaters (Third et al., 2001; Kumar and Lin, 2010).

2.8.1 ANAMMOX AND DENITRIFIERS

The synchronisation of anammox and denitrifying bacteria in the natural environment and in the laboratory have been studied (Kumar and Lin 2010). Developing technique allowing the coexistence of anammox and denitrifiers in WWTPs will result in solving the problem of possible nitrate accumulation during anammox process, this reduction will improve NRR (Ni et al., 2009). Denitrifiers convert NO_3^- to N_2 through a sequential process with NO_2^- as an intermediate under anoxic conditions; the produced nitrite is used by anammox bacteria in the oxidation of ammonia (van Hulle et al., 2010). The possible challenge that can result in coexistence is the problem where denitrifiers outcompetes anammox in the reduction of nitrite to N_2 (Ahn et al., 2004). However, this challenge can be mitigated by reducing the organic C/N ratio to below 2,

which will help in slowing down the denitrification process (Kumar and Lin, 2010). More research is needed to understand the complexity of the harmonization between denitrifiers and anammox in wastewater treatment technology.

CHAPTER 3: MATERIALS AND METHOD

The successful implementation of the anammox process will require methods to control the conditions that allow for growth and to assess biomass activity by carrying out batch testing: batch testing helps to measure the influence of certain kinetic parameters and the effect of possible toxic compounds. By monitoring the concentration of key substrates like NH_4^+-N, NO_3^--N, and NO_2^--N as they are consumed within the possible space of time. It is also necessary to monitor and assess how certain compounds influence and contribute to the activity of the anammox bacteria.

The start-up of the anammox bacteria activity using non-specific biomass was attempted under favourable conditions in this study. The conditions were optimized to favour the growth and increase the activity of anammox biomass. Application of PCR and FISH analytical method offers the possibility of determining the group and range of bacteria that makes up the biomass.

3.1 EXPERIMENTAL SETUP

This work was designed to successfully start-up cultivation of anammox bacteria using a Sequential Batch Reactor. An SBR reactor that can take a liquid volume of 20 L with a height of 52 cm and diameter of 25 cm. Schematic of the setup is seen in figure 2.3. The bioreactor was connected to a regulated stirrer from the top and an inlet for pH measurement. The lid of the reactor also has an inlet for feeding liquid and an outlet for draining out effluent liquid. First stage of the cultivation process required the reactor to be operated at moderate stirring rates of 200 ± 5 rotations per minute (RPM), this was later regulated intermittently using a time relay interval of 15 mins to allow for continuous mixing of biomass and ensuring homogeneity of feed into the reactor for analysis. Before removal of effluent liquid, the bioreactor mixing is stopped for 30 mins to 1 hour to allow for biomass settling before draining.

3.2 INOCULUM AND CONDITIONS IN BIOREACTOR

A mixed sludge was applied for this start-up. The sludge was obtained from the nitrification-denitrification process and anaerobic digestion stage of Tartu municipal wastewater treatment plant (WWTP), Estonia. The plant operates a conventional nitrogen removal process and attempts to go on a full-scale deammonification bioreactor for treatment of rejected water. 5 L each of activated and anaerobic sludge types were diluted with tap water for the start-up. Initial *inoculum* MLSS was 10.8 ± 0.25 g/L and MLSS was 9.0 ± 0.05 g/L after inoculation. The hydraulic retention time (HRT) was 2 – 3 days with a water exchange of 10 L done once per week. The bioreactor

was initially opened on top to allow for minimal aeration to favour simultaneous activity of oxic and anaerobic bacteria. The bioreactor was later covered by plastic cover to avoid nitrite oxidation to nitrate. Reject water was applied as a nitrogen source, which contains appropriate composition of trace elements and minerals.

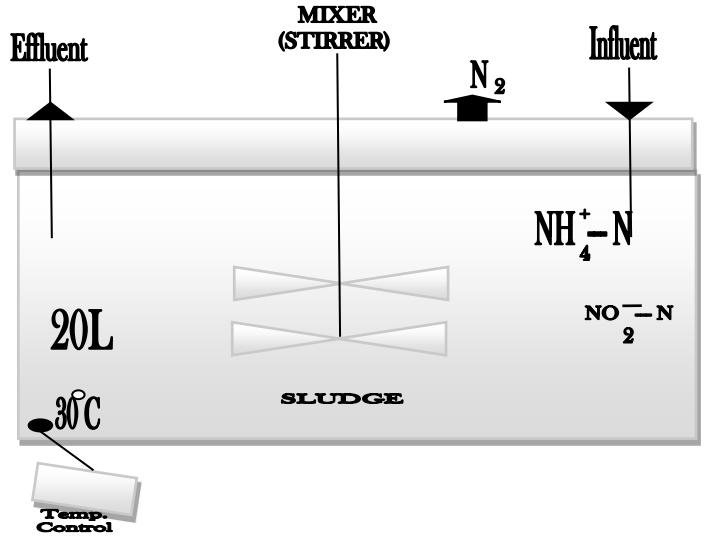


Figure 3.1 Schematic diagram showing a lab-scale SBR reactor setup for anammox process.

3.3 SAMPLE COLLECTION

Samples were collected after every 2 – 3 days. Nitrogen parameters were analysed in both influent and effluent sample mixtures after centrifuging at 4500 rpm. Sample for microbial analysis was taken at take-off of the cultivation process and subsequently at different stages of cultivation process.

3.4 BATCH TESTING

Batch testing was conducted to assess the effect of spiking with different concentrations of N_2H_4 (hydrazine) 2, 2.5, 5, 7.5, 10 and 12.5 mg/L on total nitrogen removal rate. Batch testing also covered the study of the effect of addition of synthetic and reject water on the nitrogen removal efficiency. Tests were done at room temperature in a 20 L reactor measuring nitrogen compounds after every 2h during the 10h period.

Batch assay to study the combined effect of spiking with acetate and optimum amount of hydrazine on both anammox and denitrifiers was carried out.

The following nitrogen species NO_2^--N and NH_4^+-N were added synthetically and using reject water that contains high ammonia loads (1400 mg/L) as well as containing moderate amounts of micronutrients. The amount of biomass in the reactor was determined as mixed liquor suspended solid (MLSS) from the start of the cultivation process and subsequently as the start-up process continued. Before start-up, the mixed sludge was diluted using tap water to achieve a NH_4^+-N concentration of 272 mg/L. Periodic dilution was carried out in the reactor to reduce the effect of high salt concentration and other substrate in the bioreactor. Batch testing was carried out at low, medium and high salinity (0.09 g Cl^-/L, 18 g Cl^-/L and 0.4 g Cl^-/L respectively; Appendix 1).

3.5 SAMPLE ANALYSIS

Total nitrogen in effluent and influent mixture was analyzed for in mg N/L concentration of NO_3^--N, NO_2^--N and NH_4^+-N from the bioreactor 3 times every week, pH, temperature was monitored daily. Total nitrogen was determined using ion chromatography and spectrophotometric methods performed at the department of environmental technology, Institute of Chemistry, University of Tartu, Estonia.

3.5.1 NITRITE DETERMINATION

Samples were taken with the range of 0.1-10 ml and diluted in a 25 ml volumetric flask with deionized water. 0.5 ml volume of NO_2-N sulfanylamide and 0.5 ml of diamine were consecutively using a pipette. The mixture is shaken vigorously and allowed to stand for 10 minutes for reaction to occur, a gradual change in the color is seen either a pink or purple depending on the concentration. Duplicate measurement is taken using a 10 mm cuvette on a spectrophotometer (Hach Lange DR 2800 Spectrophotometer). The readout concentration multiplied by dilution factor to obtain the actual concentration.

3.5 2 ION CHROMATOGRAPHY

Ion chromatography Metrohm was used for nitrite/nitrate and salinity measurements as well. The instrument uses a cationic exchange stationary phase in the elution of only anions. The anion elution buffer is 1.0 mM $NaHCO_3$ and 3.2 mM Na_2CO_3. Column temperature 30°C and flow rate 0.7 mL/min. The instrument works with a conductivity detector.

3.5.3 NITRATE DETERMINATION

Sample is taken with the range of 1-2 ml depending on the concentration into the clayware crucible followed by the addition of 1 ml of sodium salicylate. The resulting solution is shaken to allow for

proper mixing and then allowed to evaporate in a water bath. Evaporation is followed by cooling. After cooling, 1 mL of H_2SO_4 is added and allowed to stand for 10 minutes. 8 ml of deionized water is then added followed by the addition of 10 ml of NaOH + EDTA solution. The sample is transferred to a volumetric flask (flask chosen for dilution depending on the observed coloration, the darker the yellow, the more dilution) and filled with deionized water to the mark. The final mix was measured using a custom (wider) cuvette with a Hach Lange DR 2800 spectrophotometer. The spectrophotometer displays final concentration of nitrate in mg NO_3^--N /L, this is multiplied by the dilution factor.

3.5.4 AMMONIUM DETERMINATION

Ammonium determination is carried out using the Nessler's colorimetric method. 0.1-10 ml of the sample is pipetted into a 25 ml volumetric flask and the flask was filled to about 1/3 its volume with deionized water. This was followed by the addition of 3 drops mineral stabilizer reagent solution with the consecutive addition of 2 drops of polyvinyl alcohol dispersing reagent. The flask is filled to the mark with deionized water and shaken to allow for proper mixing. The final step involves the addition of 1 ml of Nessler reagent followed by mixing. The solution is allowed to stand for 2 minutes after the addition of Nessler reagent. Ammonium concentration was measured using a cuvette from a Hach Lange DR 2800 spectrophotometer. Spectrophotometer will give ammonium concentration in mg NH_4^+-N/ L which is then multiplied by dilution factor.

3.5.5 PH

For testing anammox activity, it is necessary to stabilize pH within the optimum range 7.5 - 8.3, pH was measured before and after each sampling process. The Evikon E6115 pH meter was used for this purpose, which was calibrated daily using a 2-point calibration system with standard buffer solutions of pH 4 and pH 7 before the start of the experiment.

3.5.6 CHEMICAL OXYGEN DEMAND

A chemical oxygen demand (COD) is required to find the total organic matter content. COD reveals the decomposition of organic matter in wastewater by the action of strong oxidants, which can be used to assess the level of water pollution. The higher the COD reading, the more organic matter that is oxidized in the wastewater. The oxidizing organic substance consumes dissolved oxygen in the test. The organic matter present in the solution is converted into carbon dioxide and water by the action of a strong oxidant in an acidic medium. The amount of chemical oxygen

demand is calculated from the amount of reacted strong oxidant which is measured using a cuvette prepared according to the standard EVS-ISO 15705:2004.

COD cuvette tests contains:

I. Silver sulfate in sulfuric acid
II. Mercury (II) sulfate
III. Potassium dichromate

Cuvettes can be used to determine the chemical oxygen demand between 100 and 2000 mg/L, and the oxidizer is potassium dichromate ($Cr_2O_7^{2-}$). After shaking the primary tube, 2 ml of the sample is added and shaken again. The cuvette is placed in a Nanocolor Vario 3 machine and heated at 148 °C for 2 hours. It is then shaken again and allowed to cool in cold water. The chemical oxygen demand is determined using a Hach Lange DR 2800 spectrophotometer, which measured the chromium Cr^{3+} at an absorbance of 605 nm. The final concentration displayed by the spectrophotometer is in mg O_2/L, this can be multiplied by the dilution factor if dilution was carried out.

3.5.7 DETERMINATION OF MLSS (MIXED LIQUOR SUSPENDED SOLID)

The MLSS determination was carried out from a well-mixed sample. Parallel sample using two 50 mL centrifuge tubes was weighed to obtain the mass of the empty tube. 20 mL of sample is pipetted into each tube. Sample is centrifuged for 10 minutes. The supernatant is carefully decanted. The tube is then placed in a drying cabinet at 105 °C for 24 hours. After drying the tubes, the tubes are cooled in a desiccator. The weight of the tube containing dried sample is measured to obtain actual dry mass of sample.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 BIOREACTOR PERFORMANCE AND DIVISION INTO THREE PHASES

The bioreactor was started with physical parameters; temperature set at 30 ± 0.5 °C and pH maintained at range of 7.5 – 8.3 to ensure proper operating condition for anammox bacteria from an influent of non-specific nitrification/denitrification sludge having ammonia concentration at 270 mg/L, followed by a subsequent addition of NH_4Cl and reject water as alternate ammonium source during start-up period.

The bioreactor achieved an efficiency of approximately 90 % of total nitrogen removal after 100 days of start-up (Fig. 4.1).

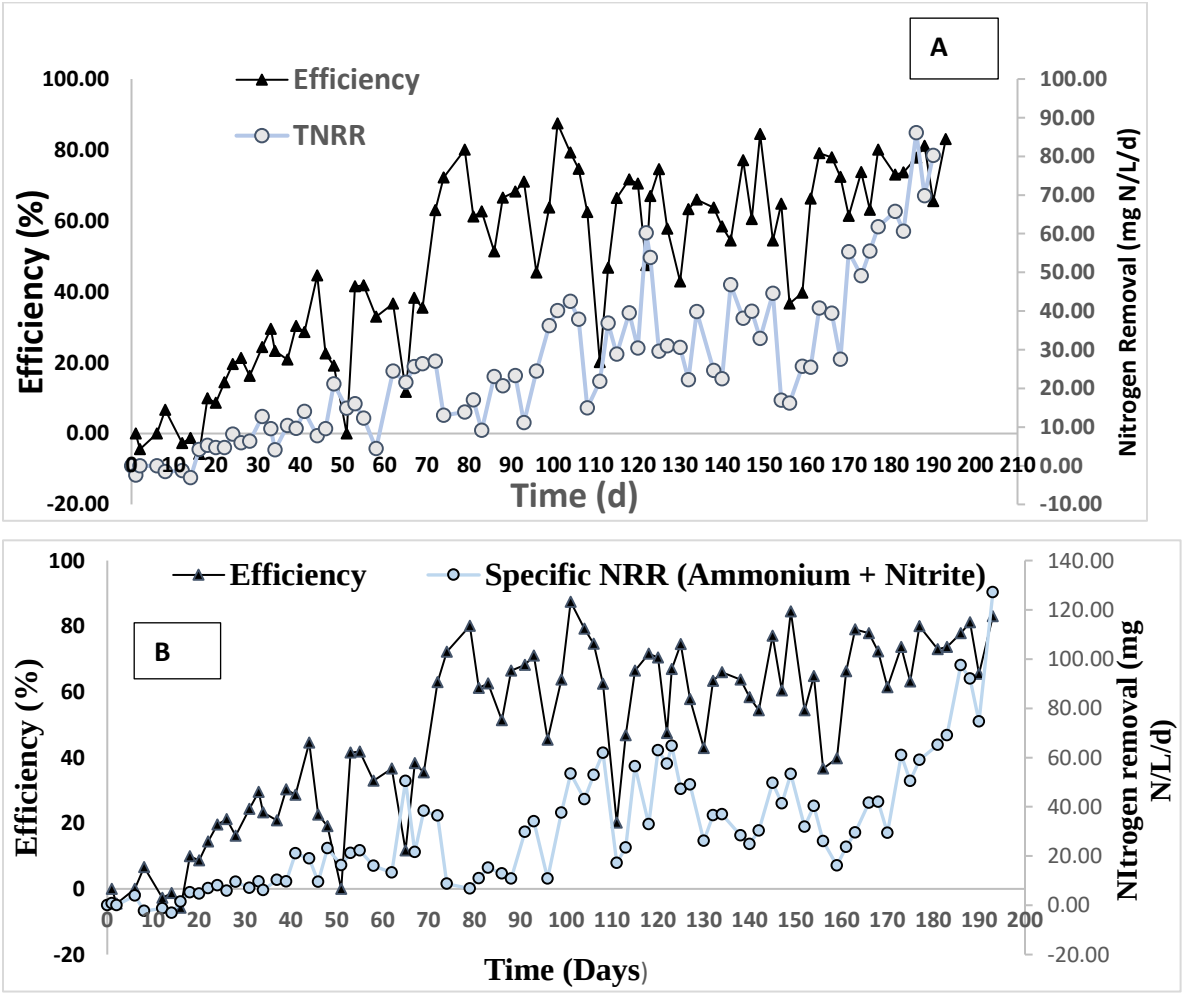


Figure 4.1. Overall performance of reactor in nitrogen TNRR, specific NRR and nitrogen removal efficiency within 194 days of start-up.

The analysis of the biomass and anammox activity in the overall start-up process is divided into 3 phases with characteristic changing periods. The first period was microbial acclimatization/activation phase with only nitrite feeding in the form $NaNO_2$ as nitrogen source as ammonium was still present from inoculation sludge. This phase was characteristically low salinity phase. The second phase was the high salinity phase, which followed the addition of NH_4Cl as ammonium source. The third phase followed the addition of reject water (from anaerobic digester sludge), which is characteristic of optimum salinity phase.

The total nitrogen loading rate was started at 0.08 kg N/m^3 /d and was increased 0.1 kg N/m^3 /d depending on the performance of the reactor.

The seeding sludge was taken from Tartu municipal wastewater treatment plant. The *inoculum* consisted of anaerobic and activated sludge. There was no addition of external organic matter into the bioreactor to benefit autotrophic process start-up.

4.2 ADAPTATION/ ACTIVATION PHASE

In the first phase of operation biomass concentration measured in MLSS decreased from 9.0 g/L to 7.1 g/L (Appendix 1). This decrease is connected to adaptation incapacity occasioned by changes in environmental condition after inoculation (Wang et al. 2009).

This phase is considered as low salinity phase; NO_2^--N was the only external substrate added to the reactor at 10 mg/L depending on the NO_2^--N removal. The comparison of influent and effluent NH_4^+-N concentration indicated an increase in effluent NH_4^+-N in the bioreactor, while, influent NO_2^--N decreased considerably well compared to effluent. The increase in NH_4^+-N was caused by cell autolysis and the decay of dead cells anaerobically broken to organic substances (Gutwiński et al., 2006). The organic material produced within this period served as substrate for denitrifiers for denitrification process with the concomitant consumption of 100 % NO_2^--N and NO_3^--N. Batch testing result revealed the production of NO_3^--N, but daily effluent result showed the absence of nitrate NO_3^--N within this phase, which evidently reveals the presence and activity of denitrifiers. The stagnation phase lasted for 65 days with no observable decrease in ammonia to indicate anammox activity.

The total nitrogen removal efficiency within this period was within the range 20 % to 40 % (Fig. 4.1). The estimation of total nitrogen concentration removed in the stagnation phase was

contributed by NO_2^--N and NO_3^--N (Fig. 4.2B&C), while, the decrease in the ammonia concentration was not noticed, contributed majorly by assimilation of nitrogen from decayed bacteria (Fig. 4.2A). NH_4^+-N removal was considerably low compared to the total NO_3^--N and NO_2^--N removal (Fig. 4.2).

4.3 HIGH SALINITY PHASE

In this phase anammox activity was seen with concomitant decrease in NO_2^--N and NH_4^+-N. In this phase synthetic NH_4^+-N was used as ammonium source in the form of NH_4Cl, while NO_2^--N was introduced in the form of $NaNO_2$. This phase lasted for 65 – 100 days of start-up (Fig. 4.2). The simultaneous addition of NH_4Cl and $NaNO_2$ lead to an increase in the salinity of the bioreactor (Appendix 1). The salinity was 10 – 18 g Cl^-/L. The ratio of NH_4^+-N/NO_2^--N in the bioreactor was higher than 1:1.3, at an average of 1:2.5 (Appendix 1). This was indicative of the concerted activity of denitrification and the anammox process. The utilization of nitrite for anammox activity was considerably lower compared to overall nitrite utilization. The effluent nitrate produced within this phase was consumed and this also demonstrated the activity of denitrifiers. The activity of anammox bacteria is largely dependent on the cell density of biomass; an increase in the activity is achieved when the concentration of cells is within the range of 10^{10} - 10^{11} cell/mL (Wu et al., 2018).

This phase demonstrated that at high salinity there exists a synergy of both denitrifying and anammox bacteria in the granular biomass. Applied SBR, which is particularly useful for its high biomass retention capacity, supported growth and function of different bacteria groups within the community of bacteria in the biomass.

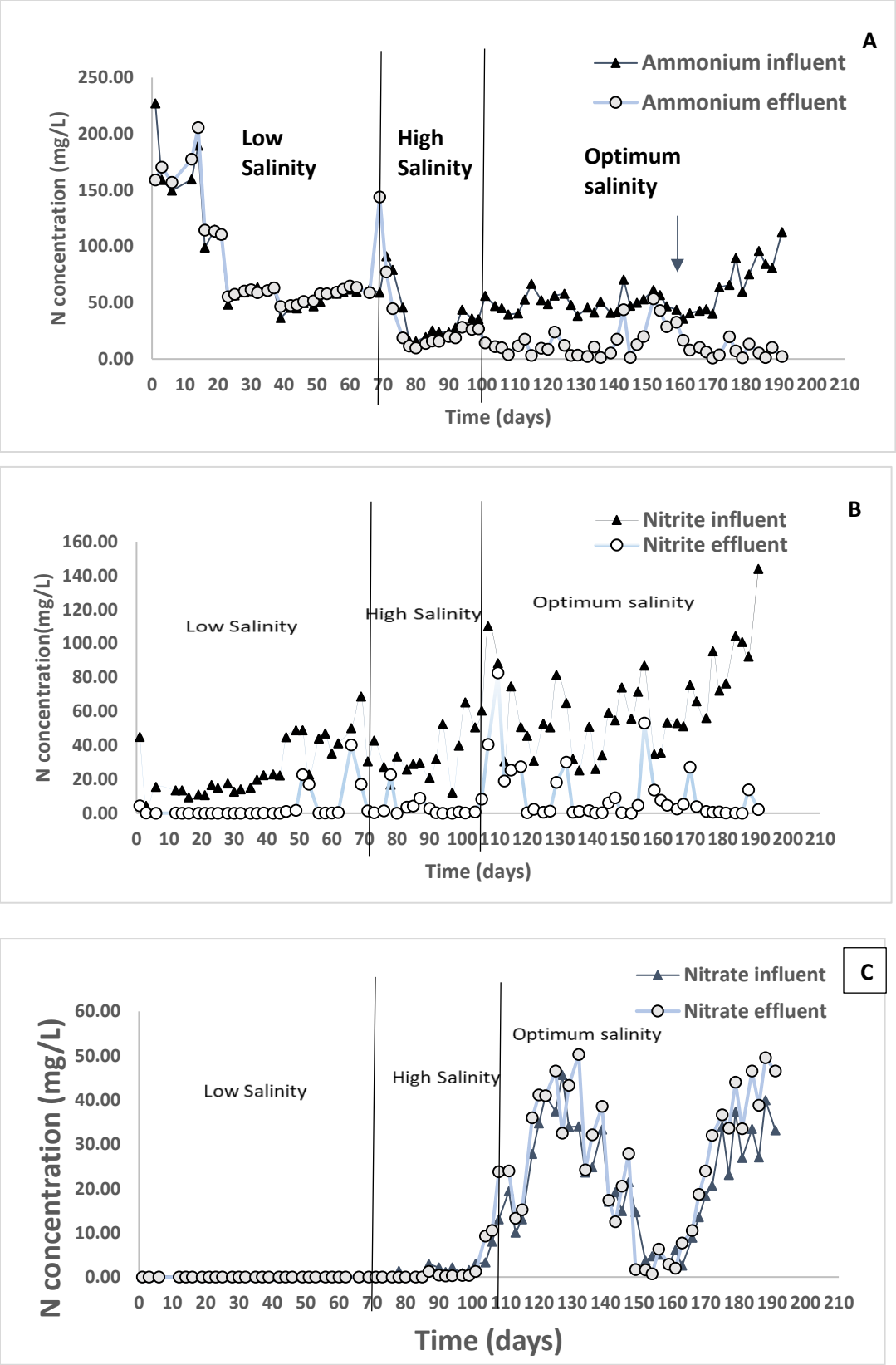


Figure 4.2. The flux of NH_4^+-N, NO_2^--N and NO_3^--N influent and effluent within start-up period.

4.4 OPTIMUM SALINITY PHASE

In this phase it was evident that there was complete depletion of organic carbon from cell autolysis that supported the activity of denitrifiers. The synergy between denitrifiers and anammox and possible competition of both groups of microorganisms for nitrite noticeable in the high salinity phase ended with anammox activity emerging as dominant from 101 days onwards (Fig 4.2). The salinity was below 0.5 g Cl^-/L in this phase which is a drop from 18.2 g Cl^-/L from the previous stage. The performance of the bioreactor in this phase of optimum salinity was achieved by the discontinuance of NH_4Cl addition as ammonium source to the use of reject water. The influent concentration of NO_2^--N and NH_4^+-N added were increased according to the performance of the bioreactor. The ratio of NH_4^+-N/ NO_2^--N was at an average of 1/1.33 ± 0.06 which was indicative that the bioreactor achieved full anammox function according to the (Eq. 2.12) (Strous et al., 1998) (Appendix 1). Total nitrogen removal efficiency of 87 % was achieved with the reactor stabilized at temperature 30 ^{0}C and pH 7.5 – 8.3 (Fig. 4.1).

Previous studies have highlighted the successful start-up using different *inocula*. Start-up have been achieved at low temperature using UASB, CAMBR at low temperature (Zekker et al., 2014, Wu et al., 2018). Although, more time is required to achieve start-up of anammox activity at low temperature. Using conventional activated sludge in SBR start-up was achieved for the anammox process at 101 days at 30 ^{0}C (Wang et al., 2012). Using two different sludge types at 30 ^{0}C faster start-up was achieved at 65 days using an SBR in this study.

In the anammox reaction (Eq. 2.12) the utilization of 1 mole of NH_4^+-N requires 1.32 moles of NO_2^--N with the concomitant release of 0.26 mole of NO_3^--N. In this phase the effluent nitrate increased in direct relationship to the amount of NO_2^--N that was consumed. This phenomenon leads to a higher amount of NO_3^--N observed in the effluent compared to influent NO_3^--N (Fig. 4.2C). A build-up of nitrate in the reactor is indicative of complete anammox process.

The obtained result also showed instances, where the removed NH_4-N/NO_2^--N ratio was 1:1.32 - 1:1. Previous studies have highlighted the prevalence of partial denitrification occurring simultaneously with anammox, which can lead to nitrate reduction. The use of organic carbon by anammox bacteria as electron donor to favour organotrophic anammox process rather than autotrophic anammox process have also been mentioned for certain groups of anammox bacteria (Wu et al., 2018). Anammox bacteria has also been linked to mediating

dissimilatory nitrate reduction to ammonia (DNRA); this process accounts for partial loss of nitrate under anaerobic condition by anammox bacteria (Castro-Barros et al., 2017). The possible out-competition for the utilization of organic carbon at low C/N ratio (<0.2) released from DNRA process by Candidatus *Brocadia sinica* over denitrifiers have also been reported in previous work (Shu et al., 2016). Accounting for some of the NO_3^--N losses observed in this phase this can be traceable to partial DNRA activity by anammox bacteria and partial denitrification by denitrifying bacteria.

In this study, by inoculating two different sludge types it is evident that rapid start-up was achieved, with stable nitrogen removal during experimental period of 193 days sustained. The total NLR rose by approximately 100% within this period of optimum salinity compared to other phases.

4.5 BATCH CYCLE ANALYSIS

Three different cycles of batch analysis were carried out in reactor at a 30-day interval during the first 90 days of startup without the addition of hydrazine (Fig. 4.3). The first 90 days represents the adaptation/stagnation phase of the anammox bacteria. During this period reduced anammox activity were noticed for the first 60 days of anammox bacteria start-up with specific anammox activity (NH_4^++NO_2^-) at 0.16 mg N/g MLSS/h to 0.08 mg N/g MLSS/h for a 30-day interval in the batch cycle assay. The last 30 days batch assay revealed an increase in specific anammox activity with total nitrogen removal rate of 0.62 mg N/g MLSS/h (Fig. 4.3).

The activity of anammox bacteria was also monitored in cycles, in a total duration of 10h spiking with different concentrations of anammox intermediate hydrazine; this was done to assess the effect of different concentration on the activity of anammox biomass.

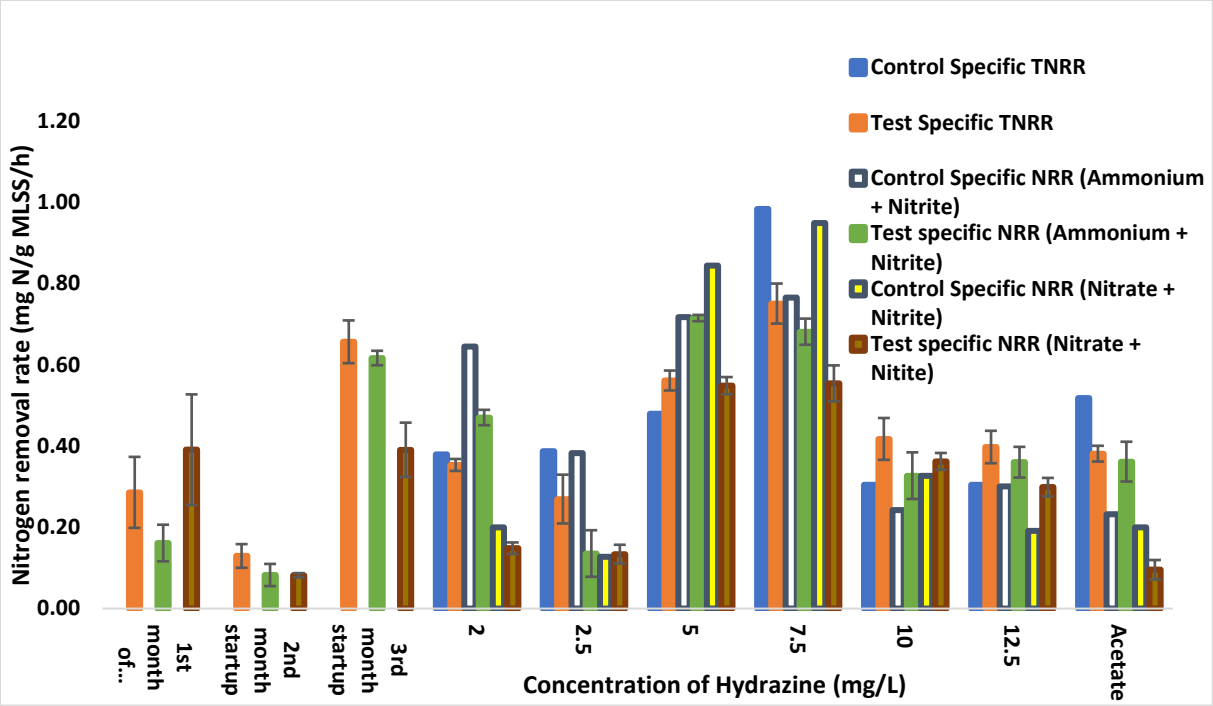


Figure 4.3: Batch assay for the 90 days without addition of Hydrazine, spiking with hydrazine and also spike with hydrazine and acetate.

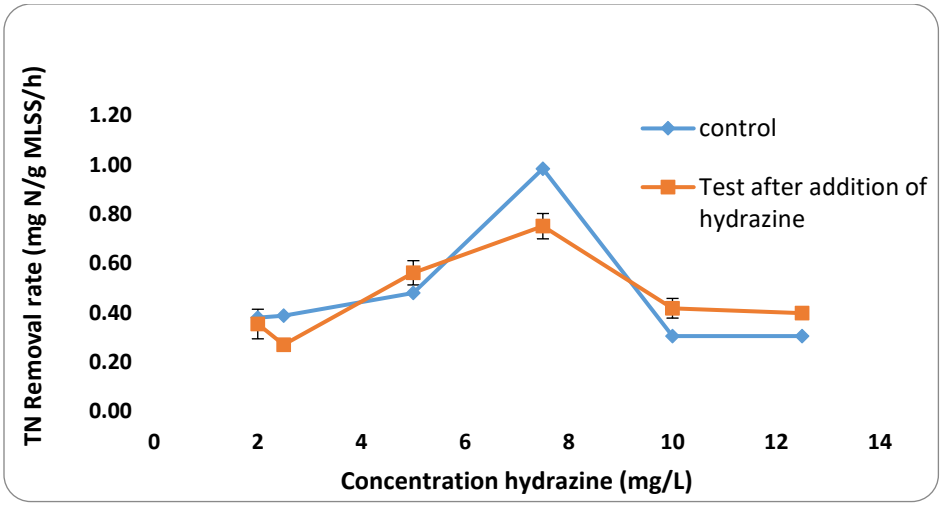


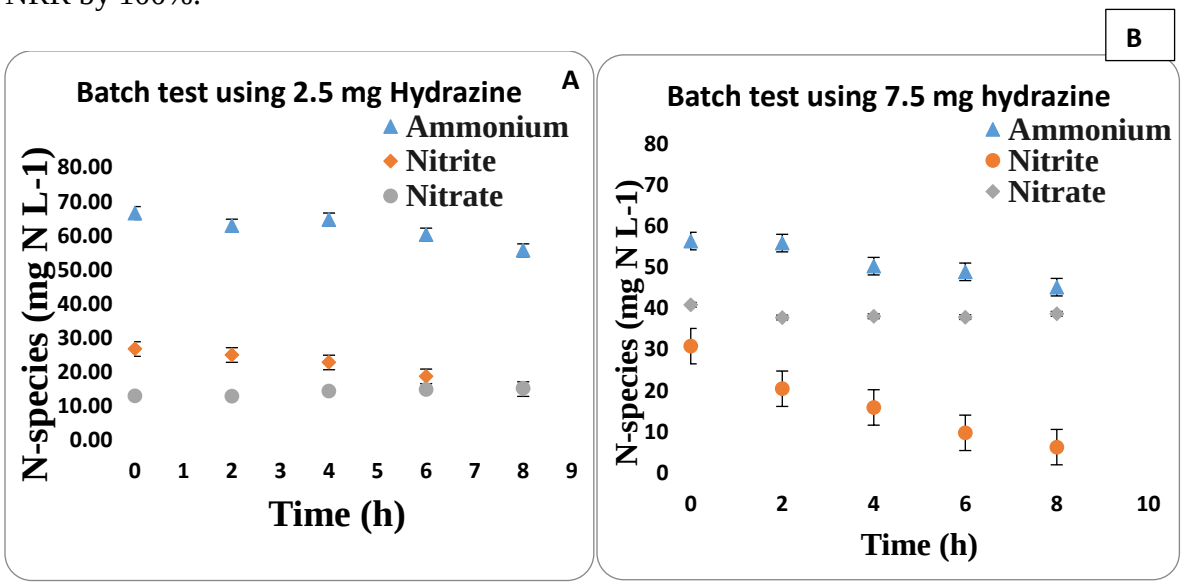
Figure 4.4: Effect of spiking with different concentration of hydrazine on specific TN removal rate.

The first 2h of the batch cycle analyses was the control test without addition of hydrazine, immediately after substrate addition. The following hydrazine concentrations were used as spiking: 2 mg/L, 2.5 mg/L, 5 mg/L, 7.5 mg/L, 10 mg/L, and 12.5 mg/L (Fig. 4.3; 4.4). The test

using 5mg/L N_2H_4 resulted in specific nitrogen removal rate (NH_4^++NO_2^-) of 0.72 ± 0.01 mg N/g MLSS/h, while that of the control rates are similar at 0.68 ± 0.06 mg N/g MLSS/h (Fig. 4.3;4.4C). Spiking with 10 mg/L and 12.5mg/L N_2H_4 showed a specific removal rate for test analysis with an identical trend to results obtained for 5mg/L (Fig. 4.3) with specific removal rates of 0.33 ± 0.1 mg N/g MLSS/h and 0.36 ± 0.07 mg N/g MLSS/h respectively.

It can therefore be deduced that spiking with different concentrations of N_2H_4 did not always significantly increase nitrogen removal rate from the test result. However, 5 mg/L N_2H_4 improved anammox activity when compared to another test rate. Previous studies carried out using two different reactors, a control and a test bioreactor with hydrazine. Results showed that hydrazine addition did not significantly increase nitrogen removal rate but rather helped to achieve a shorter start-up time of 7 weeks compared to that of the control that took 11 weeks (Ganesan & Vadivelu, 2019).

Batch test spiking with 7.5 mg/L of N_2H_4 as test and addition of acetate to increase C/N ratio 3:1 was carried out (Fig. 4.3; Appendix 2). This test was done to see if hydrazine will enhance anammox activity in conditions of high C/N ratio (Fig. 4.3). High concentration of C/N ratio led to the inhibition of anammox activity with the concomitant increase activity of denitrifiers. The observed 90% reduction in anammox activity was restored after addition of 5 mg/L N_2H_4 for 7 days. This result shows that external addition of N_2H_4 can help in achieving stable anammox activity and recovery from inhibition by substrate. Hydrazine addition also enhanced NLR and NRR by 100%.



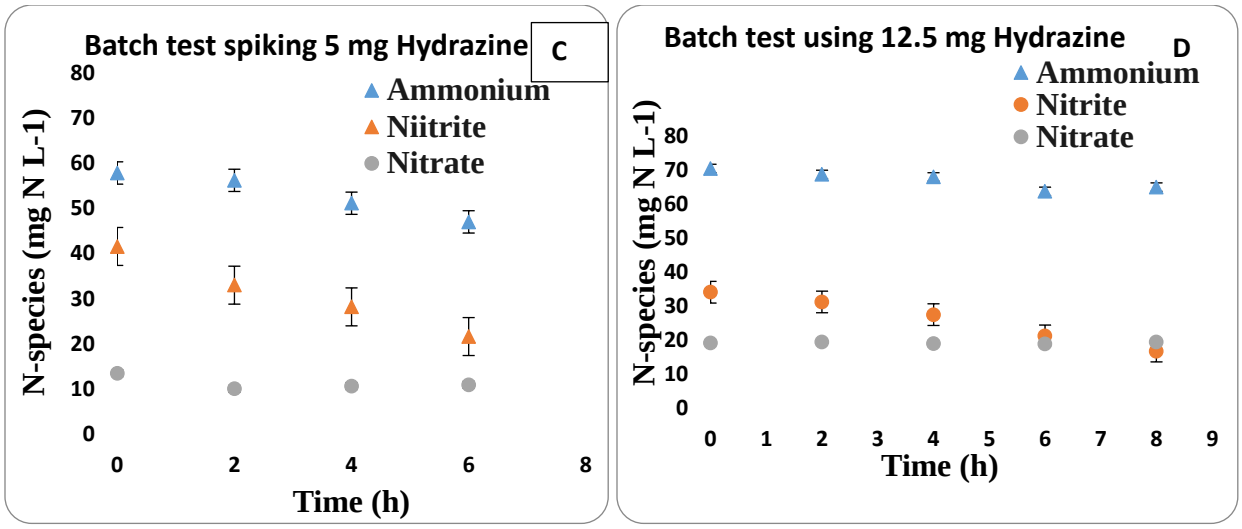


Figure 4.5 Time course of nitrogen species transformation during batch testing after addition of different concentration of hydrazine.

4.6 EFFECT OF HYDRAZINE ON ANAMMOX ACTIVITY

The effect of hydrazine as an anammox intermediate nitrogen compound was studied to enhance anammox process efficiency by spiking at a short-term. Optimum hydrazine concentration to reach the highest nitrogen removal rate was searched. Also, nitrite and nitrate reduction by denitrifying processes was aimed to diminish by hydrazine addition. The activity of anammox bacteria with hydrazine was studied using batch cycles of 2 h for a total period of 10h on a weekly basis depending on the performance of the bioreactor.

Previous studies have highlighted that long-term addition of N_2H_4 in a test reactor increased NLR by 190% compared to the reference reactor. The overall nitrogen removal rate was 0.432 g N/L/d (Midonski et al., 2019). Hydrazine effect in increasing NRR and NLR was observed in this study after inhibition by high C/N ratio. There have also been earlier reports of the positive effect of N_2H_4 in accelerating NRR and also overcoming inhibition as a result of substrate (Bettazzi et al., 2010, Zekker et al., 2012). Short-term addition of N_2H_4 for 7 days significantly led to a boost in anammox utilization of substrate with over 100% increase in NLR and NRR at 0.1 g N/L/d and 0.127 g N/L/d respectively (Fig. 4.1B).

The stoichiometry of nitrate produced during anammox process is 0.26 moles per mole of ammonia oxidized (equation 2.12). It was observed that during the batch cycle test, spiking with N_2H_4, led to the occurrence of a stepwise nitrate reduction before the end of the cycle tests indicating a

possible deactivation of partial DNRA activity and increase by anammox bacteria (Castro-barros et al., 2017; Fig 4.5). This trend can be indicative of hydrazine ability to destimulate pathways that will result in the utilization of nitrate for other metabolic purposes. Xiao et al. (2015) highlighted that long-term addition of trace hydrazine improved the performance of nitrogen removal and the strong inhibitory effect on NOB in a CANON bioreactor with NO_3^--N/NH_4^+-N ratio lowered more than that of theoretical value.

External N_2H_4 provides an easy substrate to metabolize by the anammox bacteria. The effect of N_2H_4 in accelerating nitrogen removal rate and start-up can be explained in two ways. Firstly, the metabolism of externally added N_2H_4 through a short reaction step by the anammox bacteria in their anammoxosome provides means of an additional energy source that can be used to carry out other processes. Externally added N_2H_4 is directly oxidized to N_2 through a one step process that leads to the generation of 4 electrons that is channeled into the electron transport chain (ETC) for the production of ATP (Yao et al., 2016). The availability of the extra source's energy drives growth, repair, and storage of nutrient within the cell; the stored nutrient within the cell in the form of glycogen is deployed during stress and starvation to generate energy and as carbon sources (van Niffrik et al., 2008). Lastly, N_2H_4 has been reported to inhibit the activity of other bacteria except the anammox bacteria; NOB is inhibited by N_2H_4 reducing the bacteria capacity to utilize nitrite in the formation of nitrate, making nitrite, which is an electron acceptor in the anammox process, readily available for the anammox bacteria (Xiao et al., 2015).

It has been established that anammox bacteria is inhibited by certain substrates such as nitrite. Nitrate accumulation resulting from high activity of NOB causes increased concentration of nitrate which can inhibit anammox activity. An even higher concentration of nitrite above 100mg/L can inhibit anammox activity, but this inhibitory effect is reversible (Strous et al., 1999). This phenomenon was observed in this study when nitrite concentration reached 110 mg/L in the bioreactor. High concentration of N_2H_4 has also been linked to inhibit anammox activity at concentrations greater than 10 mg/L (Carvajal-Arroyo et al., 2013).

Table (4.1) shows the comparison of nitrogen removal rate achieved during the start-up of anammox process for different reactors available in the literature and one from current study. These reactors were operated under different conditions; temperature, type of sludge and reactor configuration. The time taken to achieve start-up of anammox process, efficiency and nitrogen

removal rate differs in relation to a lot of contributing factors. Start-up was achieved at low temperature (10 – 20 °C) using an SBR/MBR bioreactor to reach a nitrogen removal rate of 0.027 g N/L/d, while that of a gas-lift reactor was 0.29 g N/L/d (Hendrickx et al, 2012; 2014). Hu et al, (2013) achieved start-up using a partial nitritation anammox process in an SBR at 30 °C and reached a nitrogen removal rate of 0.028 g N/L/d. In this study, startup of anammox process was achieved using a simplified SBR setup and 0.08 g N/L/d was achieved for total nitrogen removal rate, while NH_4^+-N and NO_2^--N was 0.127 g N/L/d.

Table 4.1: comparison of anammox removal rates by different sludge and reactor types.

Bioreactor type	Temp. (°C)	Start-up time (days)	Volume of reactor (L)	NRR (g N/L/d)	Include nitritation	Sludge type	Author
SBR/MBR	10	727	4.2	0.027	-	Mixed activated sludge	Hendrickx et al., 2014.
SBR	30	345	5	0.028	+	Start-up	Hu et al., 2013.
RBC	29	300	2.5	0.60	+	Start-up	De Clippelier (2013)
Gas-lift reactor	20	253	4.5	0.29	-	Anammox sludge	Hendrickx et al., 2012.
MBR	35	110	4.8	0.159	+	Conventional activated sludge	Wang et al., 2012.
SBR	30	193	20	0.127	-	Start-up with activated and anaerobic sludge	Current study

CHAPTER 5: CONCLUSION

In this study a simplified SBR configuration setup was used to achieve nitrogen removal by anammox bacteria seeded with non-specific inoculum sludge. The bioreactor was operated for an entire period of 193 days with start-up of anammox bacteria achieved after 65 days of cultivation of the biomass. The treatment system design and operation in this study was successful, achieving a nitrogen removal efficiency of 87 % and specific NRR 0.127 g N/L/d. The cultivated anammox biomass showed a huge capacity for nitrogen removal and nitrogen removal rate was further enhanced by more than 100% after the application of hydrazine for a short period of time. The use of synthetic ammonium substrate as NH_4Cl and reject water was applied; the yield of anammox activity with a concomitant increase NLR and NRR achieved during the use of reject water was higher compared to that of synthetic substrate. The process of controlling and optimization of conditions, pH 7.5 – 8.3, temperature at 30 °C, programmed circulation and mixing, closed anaerobic reactor system and monitoring of substrate dynamics, a stable anammox bacteria biomass was achieved. The goal of achieving a stable start-up of anammox process presented a lot of challenges especially starting with a mixed bacteria type (AOB, NOB and denitrifiers) present at the beginning of the process and the subsequent high salinity conditions during the second phase. The monitoring and control of the concentrations of ammonium and nitrite was an important factor in achieving start-up of anammox process. Implementing an HRT of 2 - 3 days was important in reducing toxicity and inhibition from substrate on anammox activity, this can also help in proper biomass retention that can support growth of anammox bacteria in an SBR system.

The overall result shows that the use of SBR, with non-specific inoculum and application of optimum concentration of hydrazine can effectively improve start-up of anammox process and scale-up total nitrogen removal process. The treatment system design and operation at steady optimum conditions in this study was successful using suspended granular biomass, achieving a stable nitrogen removal efficiency and increase in specific NRR.

RECOMMENDATIONS

- Evaluation of the impact of low temperature on anammox biomass; development of possible adaptation of microorganisms for the treatment of wastewater at significantly reduced temperature present in mainstream wastewater flows constituting as major wastewater flow.
- Adaptation of conditions with the use of a high C/N ratio to drive simultaneous activity of anammox and denitrifiers to prevent the possible accumulation of nitrite that can result in inhibitions of anammox bacteria.
- Investigation and implementation of full-scale application of this technique in partial nitrification anammox process with the use of a combination of non-specific sludge.
- Automation of bioreactor configuration and setup for applicability in full-scale.
- Microbial analysis at different stages of the start-up to validate more reliable process development based on anammox bacteria quantities.
- Enrichment of anammox bacteria and development of conditions that will enhance the growth and dominance and emergence of specific anammox bacteria by the aid of hydrazine addition.

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APPENDIX 1

MLSS is calculated using this formula;

$$\text{MLSS (g/L)} = \frac{dry\ mass\ (g) - mass\ of\ empty\ tube(g)}{Sample\ volume\ (mL)} * 1000$$

Total nitrogen removal rate was calculated using this formula;

$$\text{TNRR} = \frac{Influent - Effluent}{Vol * d}$$

Total nitrogen removal efficiency was calculated using this formula;

$$Efficiency = \frac{Influent - Effluent}{Influent} * 10$$

Table showing salinity level at different period of start-up.

days	Concentration (g Cl^- /L)
10	0.072
17	0.094
23	0.075
31	0.148
46	0.730
76	2.047
88	10.562
91	18.186
113	0.35
186	0.206
191	0.100

Table showing conversion ratio based on mass balance NO_2/ NH_4 within start-up period

Start-up period (days)	NO_2/ NH_4
66-99	2.52 (± 0.51)/1
101-125	1.03 (± 0.16)/1
126-145	1.12 (± 0.12)/1
146-194	1.33 (± 0.06)/1

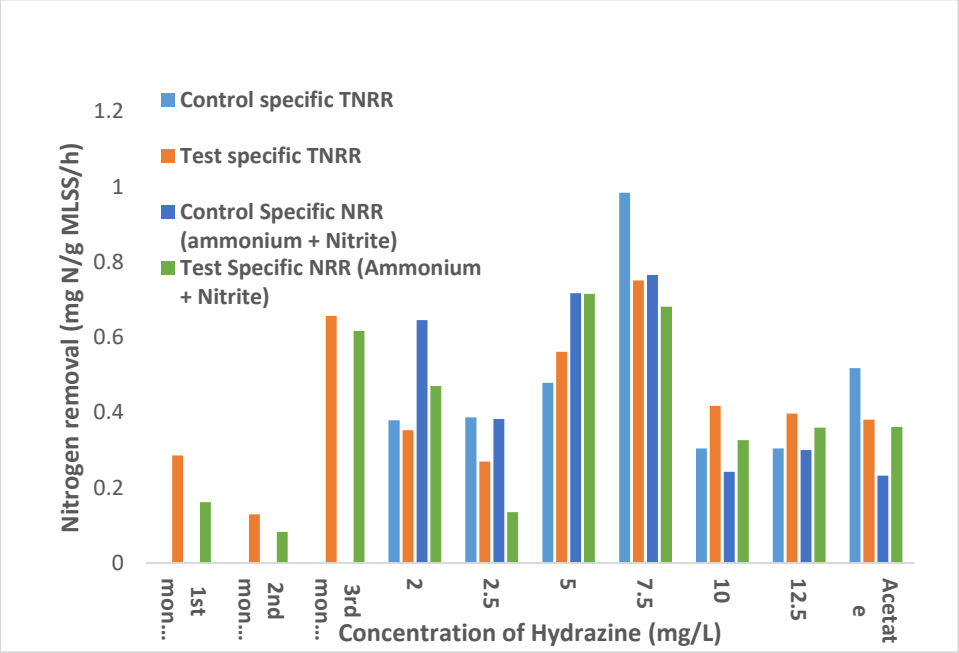
Table showing MLSS concentration within start-up period

Start-up period (days)	MLSS (g/L)
1	10.8 ± 0.25
10	9.00 ± 0.05
31	8.10 ± 0.35
71	7.10 ± 0.82
144	6.73 ± 0.23

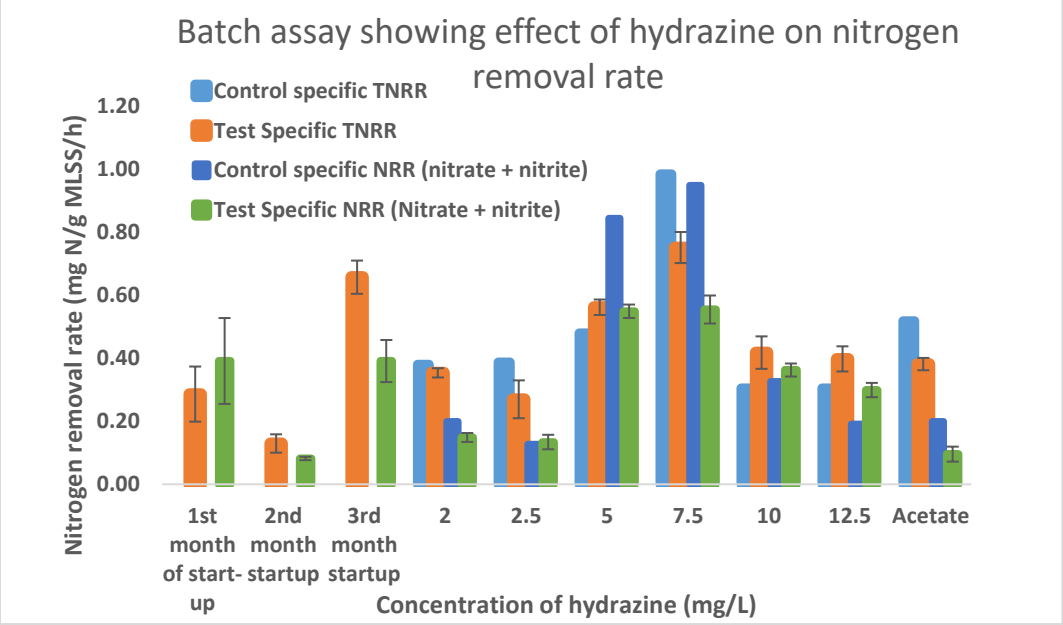
Table COD concentration for 10h at 2h interval during batch test with organic carbon addition

Time (2h)	COD (mg/L)
2	1007.30
4	966.04
6	947.64
8	933.91

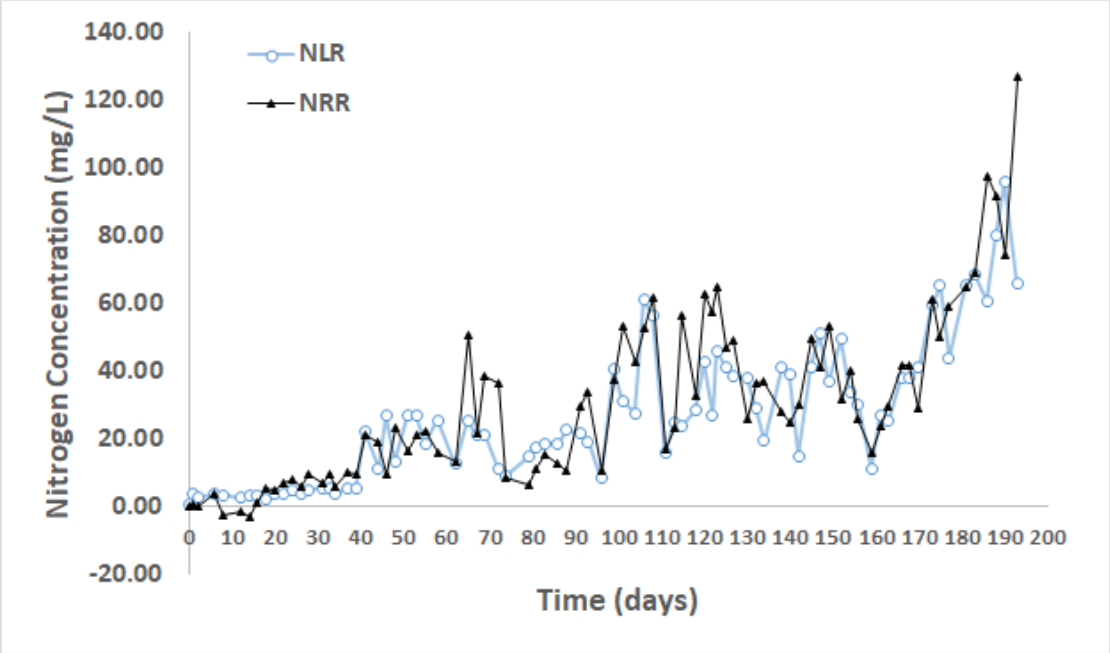
APPENDIX 2



This figure shows batch testing result for specific nitrogen removal rate (NH_4^+-N+NO_2^--N) first 90 days, spiking with different concentration of hydrazine and spiking with hydrazine and acetate.



This figure shows batch testing result for specific nitrogen removal rate (NO_3^--N+NO_2^--N) first 90 days spiking with different concentration of hydrazine and spiking with hydrazine and acetate.



This figure shows nitrogen removal rate, NRR (NH_4^+-N+NO_2^--N) and nitrogen loading rate, NLR for 193 days of start-up.

OPTIMISEERITUD TINGIMUSED ANAMMOX PROTSESSI KÄIVITAMISEKS MITTESPETSIIFILISE BIOMASSIGA

Märksõnad: Anaeroobne ammooniumi oksüdatsioon (anammox), anammox bakterid, soolsuse mõju, suspendeeritud biomass, granuleeritud biomassiga, anammox aktiivsus, lämmastiku ärastamine.

Anaeroobsete ammooniumit oksüdeerivate (anammox) bakterite poolt vahendatud lämmastiku ärastamise protsessi käivitamist sünteetilise ja vädu (settevee) reovee korral uuriti annuspuhasti reaktoris (SBR), mis oli inokuleeritud mittespetsiifiliste seemnemudaga (aeroobse ja anaeroobse muda seguga). Samuti testiti anammox vaheühendi hüdrasiini toimet lämmastikuärastusele. Rakendati lihtsustatud SBR-seadistust, reguleerides pH ja temperatuuri vastavalt 7,5 - 8,3 ja 30 ± 0,5 ° C juures. Need tingimused kohandati anammox bakterite kasvatamise soodustamiseks.

Süsteemi käivitati ammooniumi lahusega ja sellele lisati sünteetilise ja vädu reoveega kontsentratsioonidel 10 mg N L^{-1} kuni 110 mg N L^{-1} , hüdraulilise viibeajaga (HRT) 2–3 päeva kogu algusperioodi vältel. Sünteetilise ammooniumi ja nitriti kasutamine lämmastikuallikana moodustasid reaktori opereerimisel kolm erineva soolsuse perioodi: madala, kõrge ja optimaalse soolsuse periood. Settevesi andis optimaalse soolsuse perioodil soolsuse kuni 0,5 g L^{-1} . Lämmastiku eemaldamise efektiivsus oli 87%.

Viidi läbi hüdrasiini erineva kontsentratsiooniga lisamine reaktorisse annuskatsetel. 5 mg N_2H_4 L^{-1} näitas optimaalset kontsentratsiooni, mis suurendas anammox protsessi aktiivsust , saavutades lämmastiku eraldumise biomassi spetsiifiliseks kiiruseks 0,72 mg N / g MLSS / päevas. Lühiajalise hüdrasiini lisamine suutis suurendada lämmastiku eemaldamise kiirust umbes 100% pärast protsessi pärssimist kõrge C / N suhtega.

Lämmastiku reostuskoormus kasvas kogu 193-päevase käivitusperioodi jooksul 0,08 kg N m^{-3} L^{-1} kuni 0,1 kg N $m^{-3}L^{-1}$. Lämmastiku (NH_4^+ + NO_2^-) ärastuse kiirus oli 0,127 g N $L^{-1}d^{-1}$. Anammox bakterite käivitamine suspendeeritud biomassiga oli edukas pärast 65-päevast operatsiooni hüdrasiini lisamisel, kiirendades anammox protsessi aktiivsust märkimisväärselt.

THE OPTIMIZATION OF CONDITIONS FOR THE START-UP OF ANAMMOX BACTERIA SEEDED WITH NON-SPECIFIC BIOMASS

Keywords: anammox, reject water, salinity, suspended biomass, granular biomass, anammox activity, nitrogen removal.

Start-up of anammox bacteria mediated nitrogen removal from synthetic and reject water sources was investigated using a sequential batch reactor (SBR) inoculated with non-specific seed sludge (aerobic and anaerobic sludge types). The effect of anammox reaction intermediate hydrazine was also tested. A simplified SBR setup was implemented controlling pH and temperature at 7.5 – 8.3 and 30 ± 0.5 °C respectively. This condition was stabilized and adapted to favour the cultivation of anammox bacteria from scratch.

The system was started with an initial ammonium concentration from inoculum sludge and was supplemented with synthetic and reject water at 10 mg L^{-1} to 110 mg L^{-1} with a hydraulic retention time (HRT) of 2 – 3 days during the entire period of start-up. The use synthetic ammonium and nitrite as nitrogen source as well as reject water created three distinct period of salinity: low, high and optimum salinity period. Reject water gave the optimum salinity period of less than 0.5 g L^{-1} with significantly higher nitrogen removal rate observed. Nitrogen removal efficiency of 87 % was achieved.

Spiking with different concentration of hydrazine was carried out. 5 mg N2H4 L^{-1} indicated optimum concentration that enhanced anammox activity during batch testing, achieving specific nitrogen removal rate of 0.72 mg N/ g MLSS/d. Addition of trace hydrazine for short-term was able to enhance nitrogen removal rate (NRR) and nitrogen loading rate (NLR) by approximate 100 % after inhibition by high C/N ratio.

Nitrogen loading rate increased during the entire start-up period of 193 days from 0.08 kg N $m^{-3}L^{-1}$ to 0.1 kg N $m^{-3}L^{-1}$. Specific nitrogen (NH_4^+ + NO_2^-) removal rate of 0.127 g N L^{-1} d^{-1} was achieved. The start-up of anammox bacteria in suspended granular biomass was successful after 65 days of operation, with hydrazine significantly accelerating anammox activity.

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