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FMH606 Master's Thesis 2021

Master of Science, Energy and Environmental Technology

Study on the Start-up of Anammox Process in Lab-Scale Moving Bed Biofilm Reactor (MBBR)

Sabin Pathak

Faculty of Technology, Natural sciences and Maritime Sciences

Campus Porsgrunn

University of South-Eastern Norway

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Course: FMH606 Master's Thesis, 2021

Title: Study on the Start-up of Anammox Process in Lab-Scale Moving Bed Biofilm Reactor (MBBR)

Number of pages: 60

Keywords: Nitrogen compounds transformation, biofilm, partial-nitritation, anammox, moving bed biofilm reactor, OUR

Student:	Sabin Pathak
Supervisor:	Eshetu Janka Wakjera, Carlos Dinamarca, Hildegunn Hegna Haugen
External partner:	Shuai Wang; Bio water Technology AS
Availability	Open

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Summary:

The discharge of wastewater containing nitrogen compounds is harmful to marine life and human health, hence, many biological nitrogen removal methods are in use in many treatment plants in recent years. Among the several alternatives, the deammonification method is considered the best because it is both energy and cost-effective. However, due to the difficulty of suppressing NOB bacteria for partial-nitritation and the slow growth rate of anammox bacteria, implementation is difficult and needs further study. Therefore, this research aimed to gain a better understanding of the partial-nitritation anammox process and to accomplish it without the use of any specific anammox sludge. However, the carriers from KRA`s nitrification-denitrification reactor were used to provide sufficient AOB biofilm.

A moving bed biofilm reactor was set up at USN laboratory for this experiment and was fed with synthetic wastewater. The influent ammonium concentration was maintained at around 140 mg/L, and the alkalinity concentration was adjusted to compensate for the hydrogen ion produced by partial-nitritation. The temperature was maintained around 30^{o}C throughout the whole period, while other operating parameters such as SALR, HRT, and DO were modified over time based on observed data. The transformation of ammonium to other nitrogen compounds by different groups of bacteria was measured by laboratory analysis of nitrogen compounds. In addition, the bacteria growth and its composition in the biofilm were detected by dry weight measurement and the oxygen uptake rate (OUR) test, respectively.

The reactor was operated with different SALR under continuous aeration to achieve partial-nitritation by suppressing NOB. Despite the DO/TAN ratio was low enough and free ammonia levels were above the inhibition range to suppress NOB, the NPR was always higher than NAR. The failure to achieve partial-nitritation was caused by either *Nitrospira apps* growth or a thin biofilm, but to support this conclusion, additional microbial analysis should be performed. The aeration strategy was therefore changed to intermittent, and two different intermittent aeration cycle was applied i.e., IAC-2 and IAC-4. The close condition of partial-nitritation was achieved with the average value of 35%, 49%, and 74% of NPR, ARE, and NAR, respectively when the IAC-2 was stabilized at IAC-3. Another intermittent aeration cycle (IAC-4) results in a slight decrement in ARE and NPR, while maintaining the same NAR as in IAC-2.

The OUR test shows that the washout of NOB bacteria from the carrier is time-consuming, and most of the time was utilized to remove it. This led to the long and slow start-up of the anammox process. However, implementation of shorter length in the aerobic phase of intermittent aeration cycle and seeding of sludge from anaerobic digestor or denitrifying basin after completion of partial nitritation may result in quick and successful start-up of anammox process.

Preface

This research was carried out as a master thesis, which is a prerequisite for graduating from the University of South-Eastern Norway with a degree in Energy and Environmental Technology. This was a continuation of the previous master's project to develop an anammox method, but the reactor scale was modified from pilot to lab-scale due to the failure of pilot-scale reactor research to develop this condition for various reasons. For this one moving bed biofilm reactor (MBBRs) was set up at the USN laboratory.

This study was organized by USN in the collaboration with Bio-water Technology AS.

I would like to express my heartfelt gratitude to Researcher Eshetu Janka Wakjera and Assoc. prof Carlos Dinamarca for their technical advice, feedback, and much-needed assistance in bringing this project to a successful conclusion. Also, I would like to express my gratitude to senior engineer Hildegunn Hegna Haugen for her laboratory-related suggestions and guidance, which made my project much easier. In addition, I am grateful to Shuai Wang from bio-water technology for his technical advice and assistance with the experimental planning for this project's completion.

Porsgrunn, 16.05.2021

Sabin Pathak

Contents

Nomenclature

AF	Anaerobic biological Filter reactor
Anammox	Anaerobic Ammonium Oxidation
AOB	Ammonia Oxidizing Bacteria
ASP	Activated Sludge Process
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
FA	Free Ammonia
FISH	Fluorescence in situ hybridization
KRA	Knarrdalstrand Wastewater Treatment Plant
MBBR	Moving Bed Biofilm Reactor
NOB	Nitrite Oxidizing Bacteria
NOB	Nitrite Oxidizing Bacteria
OUR	Oxygen Uptake Rate
PNA	Partial Nitritation Anammox
qPCR	quantitative Polymerase Chain Reaction
RBC	Rotating Biological Contactors
SCRB	Suspended Carrier Biofilm Reactor
SHARON	Single reactor High Activity Ammonia Removal Over Nitrite
TAN	Total NH_3-N+NH_4-N concentration
UASB	Upward Flow Anaerobic Sludge Blanket
WWWTPs	Wastewater Treatment Plant

1 Introduction

Nitrogen compounds (NH_4^+, NO_3^-, NO_2^-) present in wastewater negatively affect the environment and human health. Hence, environmental laws and regulations regarding the discharge of nitrogen compounds into natural water bodies are strict. Free ammonia at a concentration of over 1.7 mg/L has a toxic effect on fish [1]. The high concentration of ammonium reduces the oxygen concentration in the water body because nitrifying bacteria consume dissolve oxygen (DO) to oxidize ammonia to nitrite (NO_2^-) and nitrate (NO_3^-,) [1]. Moreover, a nitrate concentration greater than ten ppm has dismissive health effects on infants and pregnant women [2]. It should therefore be removed from wastewater before discharging to natural ecosystems. Biological processes have been extensively used to remove nitrogen pollutants, converting ammonium presence in wastewater to nitrogen gas by naturally occurring bacteria. Several biological processes remove nitrogen from wastewater, such as traditional nitrification-denitrification, one stage PNA (Partial Nitritation Anammox), two-stage PNA, etc.

Nowadays the partial nitritation combined with anammox is considered the most reliable biological process for nitrogen removal from wastewater. This process is widely used to treat the ammonium reach wastewater from the supernatant of anaerobically digested sludge (i.e., Reject water) in most wastewater treatment plants (WWTPs). The one-stage partial nitritation anammox process removes the nitrogen in a shortcut way (i.e., without producing nitrate) by autotrophic bacteria, resulting in less aeration requirement [3], less sludge production and lower carbon footprint than other biological nitrogen removal processes [4]. Moreover, using this process result in less space requirement and high volumetric nitrogen removal rate than the two-stage partial nitritation anammox process.

The nitritation process begins in the last century, whereas first discovery of anammox bacteria was on denitrifying reactor at baker's yeast factory Gist-Brocades in Delft, The Netherlands in 1985 [5], where they found that oxidation of ammonia in anoxic condition. Since then, many scientist and researcher invest their time to find activity and suitable conditions of anammox processes. As a result, many Wastewater Treatment Plants (WWTPs) such as, Hattingen,

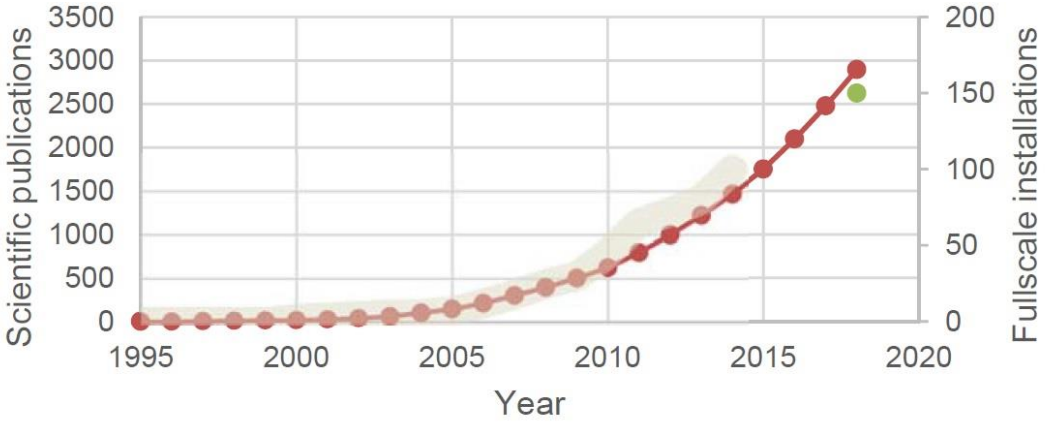

Figure 1.1: Scientific research and WWTPs operating with anammox process from 1995 to 2020 where, pink line shows cumulative publications, green point shows total full-scale plant in 2018, white dark line shows cumulative full-scale installation worldwide.

Germany [6], Strass, Austria [7], Zurich, Switzerland [4], etc., are operated with ammonia removal by deammonification process (i.e. PNA). Regarding the PNA process, in the last three decades there are over 3000 scientific research paper published and over 150 full scale installations worldwide using anammox process (Figure 1.1) [8]. Due to the slow growth rate of anammox bacteria and have a long startup-time for full scale development, which range from one to two-and-a-half years [6], [7], most of the recently operated WWTPs have been seeded with anammox inoculum from another anammox plant [8].

In this thesis, removing nitrogen from one stage PNA using moving bed biofilm reactor (MBBR) reactor was studied. One stage PNA consists of two steps in one reactor: first oxidation of half concentration of ammonium presence in wastewater to nitrite by ammonia oxidizing bacteria (AOB) in aerobic environment which is known as partial nitritation, then half of the remaining ammonium is oxidized by anammox bacteria with consumption of previously formed nitrite in an anaerobic environment. It is possible to achieve simultaneous partial nitritation and anammox condition in MBBR reactor because AOB, responsible for partial nitritation, is attached in outer layer biofilm. In contrast, Anammox grows in the inner anoxic layer (Figure 1.2) [1]. The stable partial nitritation biofilm is required to develop anammox condition because it provides a suitable ammonium to nitrite ratio to anammox bacteria. However, the development of partial nitritation is a time-consuming process, so around 60% of total carrier used in the lab reactor was from nitrification-denitrification reactor of Knarrdalstrand Wastewater Treatment Plant (KRA) of Porsgrunn, Norway to ensure sufficient nitritation biofilm.

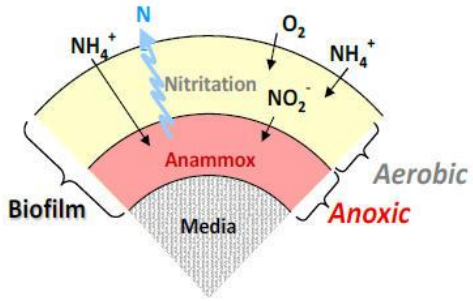

Figure 1.2: Bacteria location and nitrogen compounds transformation within the biofilm.

1.1 Problem description

Although the study of anammox bacteria has been running since its discovery, there are still many questions regarding the anammox behavior and suitable condition to cultivate anammox bacteria. Moreover, stable partial nitritation, which is considered a prerequisite of anammox condition is also hard to achieve because of same autotrophic and aerobic nature of AOB and NOB. Therefore, there is need for more research on both anammox condition and stable partial nitritation to remove nitrogen efficiently by PNA process.

1.2 Aims and Objectives

This thesis is a continuation of the previous project to develop a suitable environment for the anammox process. However, the lab-scale reactor is used instead of a pilot reactor situated in KRA WWTPs, and synthetic wastewater is used rather than reject wastewater. The use of synthetic wastewater and lab reactor enables to control the desired condition for stable ammonia concentration and careful monitoring the process. Hence, this thesis's main aim is to obtain a suitable condition for the start-up of anammox process in the lab scale reactor using synthetic wastewater. To promote anammox condition and cultivation of anammox biomass, the following subtask was applied:

1. Generate experimental data which is suitable for anammox condition using synthetic wastewater.
2. Apply different aeration to achieve different dissolve oxygen concentration, and different hydraulic loading rate to optimize the reactor.
3. Analyse the generated data and perform mass balance, compare generated data with other works (i.e., literature) of Anammox process.

The detailed task description is annexed in Appendix A.

1.3 Structure of report

This report contains a total of 7 subchapters. Chapter 1 briefly introduces the needs for nitrogen compounds removal, partial nitritation anammox process, and project objectives. The theory and literature review regarding nutrient removal technique with focus on anammox process is detailly explained in chapter 2. Chapter 3 interprets the detailed material and methods used to achieve the thesis objectives. Finally, chapter 4, chapter 5, chapter 6, and chapter 7 explain the result from the experiment carried out for thesis objective, discussion of the result by comparing it with different works of literature, the conclusion from the discussion, and future work needs for anammox start-up, respectively.

2 Theory and literature review

This subchapter explains the different process used in wastewater treatment with brief explanations of MBBR, the different methods can be used to remove nitrogen compounds from the wastewater with focus on anammox process, and complete autotrophic nitrogen removal techniques. In addition, the different strategy and factor for the nitrite oxidizing bacteria (NOB) suppression and theory related to oxygen uptake rate (OUR) to find bacteria composition in biofilm is also described.

2.1 Biological process for wastewater Treatment

There are three methods for removing unwanted concentration of contaminants in wastewater such as physical, chemical, and biological processes. Among them, the biological process is an economical and efficient technique. The biological process consists of naturally occurring bacteria, responsible for the oxidation of soluble and colloidal materials from wastewater. The biological process can be broadly categorized into two parts: the attached growth process and the suspended growth process.

2.1.1 Attach growth process

The attached growth process is a biological wastewater treatment process in which active sludge responsible for wastewater treatment is attached in the reactor bio-carrier [9]. The groups of microorganisms also called active sludge, which can attach to the surface, are called biofilm [10]. The waste flows over the carrier and gets in contact with active sludge to remove undesirable pollutant concentrations in wastewater. Various natural and artificial materials such as glass, peat, rock, natural zeolite, and expanded clay fibrous carrier has been tested as carriers in the attached growth treatment reactors in recent years. However, Le et al.[9] reported that well-known carriers used in attach growth process are made from polyethylene and polyurethane. It is crucial to consider material selection for carriers because appropriate material enhances the large surface area per unit volume, resulting in a high amount and variety of biomass concentration [11]. Moreover, the percentage of void and porosity of carrier material decide which will be the dominant microorganisms in the treatment process [12].

The attached growth system can be categorized into two classes based on carriers' movement: i) Fixed biofilm system, ii) Moving biofilm system. The biofilm is developed on a fixed position carrier such as rock, plastic profile, etc., in a fixed biofilm system. Furthermore, the wastewater and nutrition for the bacteria move through fixed bed pores, and unwanted material removal occurs. Typical examples of fixed biofilms systems are trickling filters, biological disks, anaerobic up-flow filters, etc. The fixed film bioreactors have several advantages, such as high removal efficiency in higher loading rate due to the long retention time of active biomass in the reaction zone, perform well to organic shock load and toxic inputs [13]. However, one main disadvantage is clogging of media pores due to active biomass growth, resulting in more need for backwashing for efficient operation [14]. On the other hand, in a moving biofilm system such as aerated biofilter, suspended carrier biofilm reactor (SCRB), biological fluidized bed, etc. biofilm is attached to the continuously moving carrier through an aerator or mechanical stirrer. Moving of biofilm in the reactors is suitable for retaining the slowly growing active biomass, such as nitrifiers, in the reactor [14]. Overall, This attach

growth process's main advantage is high biomass concentration, simultaneous nitrification-denitrification, and resistance to shock loading [9]. Moreover, it requires less space and no need of secondary clarifier (due to absence of return sludge line) than conventional activated sludge process.

2.1.1.1 **Moving bed biofilm reactor (MBBR)**

Since fixed film attach growth has the main problem of clogging media, which hindered the efficient operation and require frequent backwashing. In recent years, suspended carrier biofilm reactors (SCRB) such as moving bed biofilm reactors (MBBR) have been considered a promising solution to mitigate this problem. Moving bed biofilm reactor is first invented by professor. Hallvard Ødegaard at Norwegian University of Science and Technology (NTNU) in late 1980 [15]. This reactor is suitable for nitrification because of having a higher sludge retention time and simultaneous nitrification-denitrification because of low oxygen diffusion through the biofilm and can maintain an anoxic and aerobic environment inside and outside of biofilm. Moreover, Mazioti et al.[16] reported that to achieve more active sludge inside the reactor, hybrid MBBR consisting of attached and suspended biomass is a promising technology.

The efficiency of MBBR reactors depends on the carrier material used inside the reactors. The Figure 2.1 depicts some of the widely used bio carriers in MBBRs reactors [17]. A higher specific surface area carrier enhances a higher concentration of biofilm in the carrier, resulting in more efficient treatment. The percentage of carrier filling should be appropriate according to the treatment objective and type of wastewater [18]. Moreover, Adequate flow and mixing are the crucial parameter to maintain appropriate turbulence, which maintains the suitable thickness of biofilm [18]. The thickness of biofilm less than 100 μM is suitable for full substrate penetration. However, the efficient thickness of the biofilm can vary depending upon condition such as fully aerobic, simultaneous anoxic and aerobic condition. High turbulence causes more detachment of the biofilm from the carrier, and low turbulence results in slower movement of the carrier and higher thickness of microorganisms in biofilm.

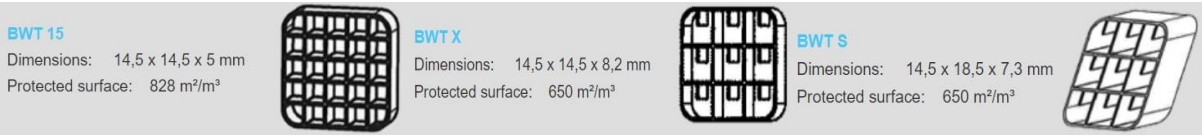

Figure 2.1: Bio-carriers with their properties used in MBBR reactors.

2.1.2 Suspended growth process

The suspended growth process is a biological treatment process that is used to treat the wastewater by micro-organisms, which are float and/or suspended in the wastewater to be treated. The wastewater flow through and around the suspended organisms, and the removal of unwanted concentration take place. Based on oxygen, suspended growth can be categorized into two classes: i) the aerobic suspended growth process and ii) the anaerobic suspended growth process. The detailed classification of the suspended growth process is shown in Figure 2.2 [19]. Aerobic suspended growth processes are mainly used to treat municipal and low

strength industrial wastewater whereas, the anaerobic suspended growth process treats high strength industrial wastewater [19].

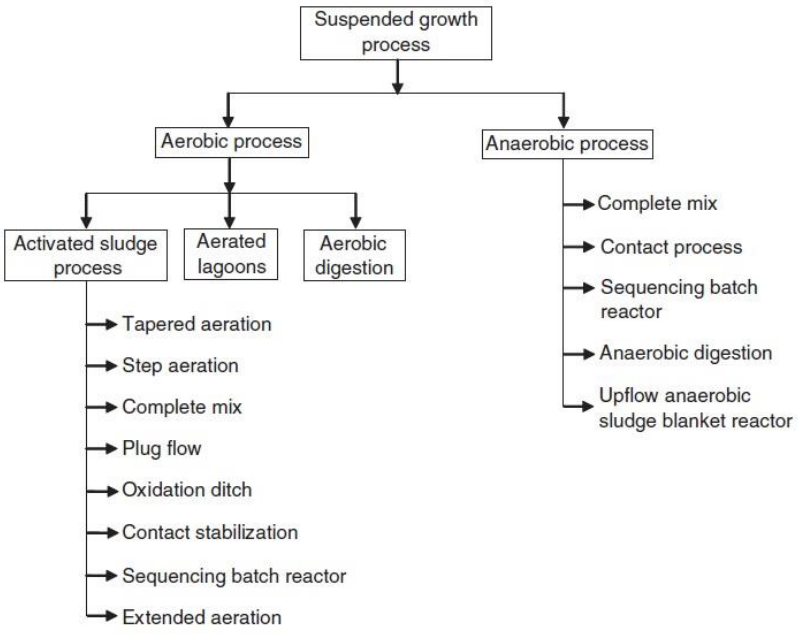

Figure 2.2: Classification of suspended growth process.

The most common use suspended growth treatment method in the municipal wastewater treatment system is the activated sludge process (ASP). The activated sludge process is the biological treatment process in which activated sludge is continuously circulated to come in contact with wastewater to oxidize carbon and nutrient present in it. The activated sludge is a biological floc consist of a mixture of microorganisms, non-living organic matter, and inorganic matter [20]. This process consists mainly of two components, i) aeration tank or anoxic tank and ii) clarifier. In an aeration/anoxic tank, microorganisms degrade the soluble and the colloidal material while, the settlement of microorganisms in the form of biological flocs occurs in the clarifier. An appropriate flow of settled sludge is recycled back into an aeration/anoxic tank to remove a high concentration of soluble and colloidal material. Two scientists Clark and Gage first developed this process in 1913 at Lawrence experiment station in Massachusetts, USA [19]. To obtain effluent with minimum organic and nutrient, two main parameters are important for ASP: maximum removal of organics and nutrient in a shorter possible time and producing good settling biological flocs [20]. However, both conditions cannot be fulfilled because biological floc at high speed has a higher capacity of removal and have poor settling characteristics and vice versa. Hence, the design engineer should know the incompatibility of two parameters to design efficient activated sludge process.

2.2 Biological Nitrogen removal from wastewater

Nitrogen can be removed from wastewater by different techniques such as chemical, physical, and biological processes. The biological process is the most promising technology because of its low operational cost, less use of chemicals, and the lower complexity of plant and

management than a physical and chemical process [21]. This chapter deal with different types of biological nitrogen removal process along with their advantages and drawbacks.

2.2.1 Conventional nitrification-denitrification

Nitrification combined with denitrification is the most common method of biological nitrogen removal from wastewater. The nitrogen presence in wastewater in the form of ammonia is oxidized into nitrogen gas by two sequential steps: i) nitrification and ii) denitrification.

2.2.1.1 **Nitrification**

Nitrification is the biological process in which ammonium (NH_4^+-N) presence in wastewater oxidized into nitrate (NO_3^--N) in an aerobic environment. This process generally occurs in two-step: first, ammonia is converted into nitrite (NO_2^--N) by ammonia oxidizing bacteria (AOB) known as nitritation (Equation 2.1), then nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate called nitratation (Equation 2.2). Ammonium and nitrite act as electron donors for nitritation and nitratation, respectively, while oxygen acts as an electron acceptor for both steps. Both AOB and NOB are autotrophic organisms because they utilize inorganic carbon (i.e., CO_2) as their carbon source. The bacteria community that capable of oxidizing ammonium are *Nitrosomonas, Nitrosococcus*, *Nitrosopira*, *Nitrosovi-brio*, and *Nitrosolobus* [22]. On the other hand, several genera such as *Nitrobacter, Nitrospira*, *Nitrospina, Nitrococcus*, and *Nitrocystis* oxidize nitrite to nitrate [22]. Among different genera of bacteria for nitrification, the most common bacteria are *Nitrosomonas* and *Nitrobacter* for nitritation and nitratation, respectively,

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \quad (2.1)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2.2)$$

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2\ H^+ \quad (2.3)$$

which have the nitrifying capacity of 1000 to 10000 times higher than other genera [23].

In the nitrification reaction, only nitritation process is the hydrogen ion (H^+) producing step, hence sufficient alkalinity as the buffer is necessary to avoid pH drop and to maintain suitable pH range of 7-8 for AOB and NOB growth [21]. The pH value below 6.0 stops the nitrification steps [24]. The stoichiometric equivalent is for 1 mole of NH_4^+-N oxidation, 2 moles of alkalinity as HCO_3^- require consuming hydrogen ion produced in the nitritation process (Equation 2.4), which is similar with 7.14 g alkalinity as $CaCO_3$ required for 1 g of NH_4-N oxidation.

$$NH_4^+ + 2\ HCO_3^- + 2\ O_2 \rightarrow NO_3^- + 2\ CO_2 + 3\ H_2O \quad (2.4)$$

As the nitrification process occurs in the aerobic environment, sufficient oxygen is necessary for complete nitrification. According to the stoichiometry, for 1 g of NH_4-N oxidation 4.57g oxygen is required (Equation 2.3). Therefore, dissolved oxygen (DO) concentration is the essential parameter for nitrification. Bertino et.al [23] reported that a DO concentration above 2-3 mg/l is essential for complete nitrification. On the other hand, A DO range between (0.5-2.5) mg/l may inhibit the nitrification kinetics for both attach and suspended growth treatment

process [21]. The actual value of critical DO for nitrification kinetics depends on the type of growth process (i.e., suspended or attached growth), the degree of diffusion of oxygen from bulk liquid to active biomass, Ammonia loading rate, etc.

2.2.1.2 **Denitrification**

Denitrification is a biological process where facultative heterotrophic bacteria reduce nitrate to nitrogen gas (N_2) via series of intermediate products (Equation 2.5). Heterotrophic bacteria consume organic carbon as their carbon source. Facultative bacteria can get their oxygen either from dissolved oxygen of bulk liquid or from nitrate molecule. It is necessary to maintain anaerobic or anoxic conditions for denitrification because the facultative bacteria first take DO from bulk, resulting in a lower nitrate reduction [24]. This process is also known as the dissimilatory nitrate reduction process. The most common bacteria responsible for denitrification are *Achromobater, Pseudomonas, Micrococcus, Bacillus,* and *Alcaligens* [22]. In the denitrification reaction, nitrate (NO_3^-) acts as an electron acceptor while organic carbon donates electrons.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2.5)$$

The denitrification process is the alkalinity production process. Stoichiometrically, one mole of alkalinity as hydroxide (OH^-) is produce for each mole of nitrate reduction (Equation 2.6). This is equivalent to 3.57g of alkalinity as $CaCO_3$ production from 1 g of nitrate reduction [21]. In consequence, about one-half of the alkalinity consume under nitrification can be recovered. The pH generally increased due to the production of alkalinity by the denitrification reaction. The suitable range of pH for efficient denitrification is 7-8, while if pH value less than 7, affects the denitrification rate [25].

$$5\ CH_3OH + 6\ NO_3^- \rightarrow 3\ N_2 + 5\ CO_2 + 7\ H_2O + 6\ OH^- \qquad (2.6)$$

Since the denitrification process requires organic carbon, it is unsuitable for wastewaters having a high concentration of ammonia and low COD, due to the additional cost of supplying organic carbon. The addition of external organic carbon, especially ethanol or acetic acid, enhances the growth rate of denitrifying bacteria [24]. If the denitrification is carried out on the raw wastewater, it results in a slightly lower growth rate of denitrifying bacteria and the lowest growth rate if the microorganisms rely on carbon source from endogenous decay [24].

2.2.2 Nitritation-Denitritation

The Nitritation-Denitritation process, commonly known as the single reactor system for high activity ammonium removal over nitrite (SHARON) process, is a sustainable alternative to traditional nitrification-denitrification for nutrient removal from wastewater [26]. This method was first developed at TU Delf by Hellinga in 1990 [27]. In this process, ammonia present in wastewater is converted into nitrogen gas by two sequential steps, nitritation and denitritation. The nitritation process includes ammonia oxidation into nitrite by AOB under aerobic condition (Equation 2.1), while heterotrophic bacteria under anaerobic condition reduce nitrite into nitrogen gas, called denitritation (Equation 2.7).

$$NO_2^- + 0.5\ CH_3OH \rightarrow 0.5\ N_2 + 0.5\ CO_2 + O.5\ H_2O + OH^- \quad (2.7)$$

This process utilizes a shortcut path to remove nutrient removal from wastewater than traditional nitrification-denitrification (Figure 2.3) [23]. As a result, this process reduces the oxygen and organic carbon requirement by 25% and 40%, respectively. In addition, the production of sludge is also lower than the traditional nitrification-denitrification process.

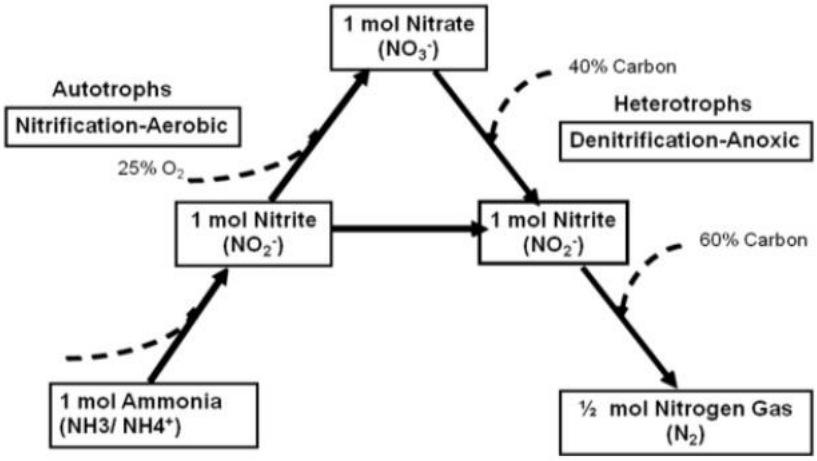

Figure 2.3: Nitritation-Denitritation and Traditional nitrification-denitrification pathways.

2.2.3 Anammox process

The anammox process is the novel and unique method to oxidize ammonia present in wastewater to nitrogen gas under anaerobic conditions by anammox bacteria. The anammox reaction proceeds with the consumption of nitrite and ammonium in the stoichiometric ratio (i.e. NO_2^-/ NH_4^+) of 1.32 to yield nitrogen gas (Equation 2.8) [26], where ammonium and nitrite act as an electron donor and electron acceptor, respectively. However, Lotti et.al [28] reported that the stoichiometry of the nitrite to ammonium ratio is 1.146 rather than 1.32. Therefore, the anammox process needs > 50% nitrite content for efficient operation. Along with nitrogen gas, the anammox process also yields nitrate at a rate of 11% as the by-product. Hence, Complete removal of ammonia to nitrogen gas is not possible via the anammox process.

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O \quad (2.8)$$

The mass balance analysis carried out by Strous et.al [29] on the anammox enrichment culture shows that anammox bacteria use CO_2 as their carbon source to yield the biomass ($CH_2O_{0.5}N_{0.15}$). Therefore, the requirement of organic carbon for denitrification is completely omitted by an anammox process. The oxidation of ammonia along with nitrite into nitrogen gas proceeds through series of intermediate steps: firstly, nitrite is reduced partially to

hydroxylamine (NH_2OH), and then ammonia reacts with hydroxylamine to form hydrazine (N_2H_4), which further oxidize to nitrogen gas (N_2) (Figure 2.4) [22].

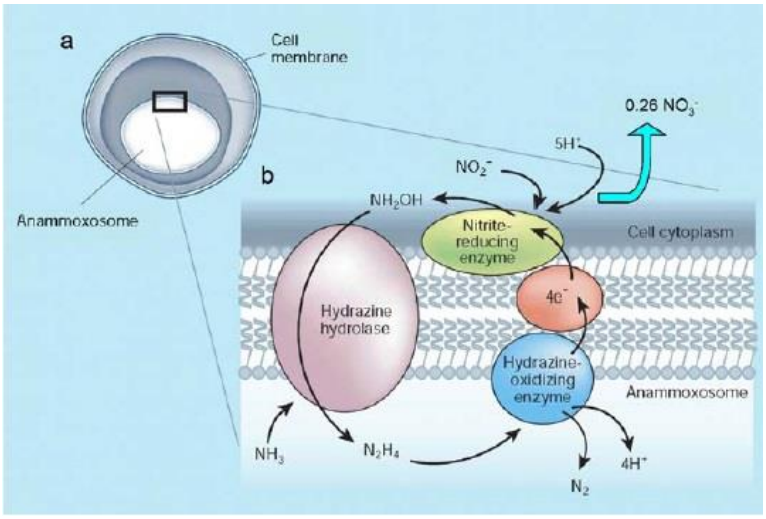

Figure 2.4: Biochemical Pathway of anammox process. a) Simple figure of anammox bacteria. b) Different intermediate reactions locations with in anammox cell, and their products.

Anammox activity has been documented in a variety of wastewater treatment facilities since its discovery [30]. Moreover, anammox bacteria are present in the natural environment such as in sea and river, contributing to the world nitrogen cycle by producing 70% of nitrogen gas in marine sediments [31]. The bacteria responsible for the anammox process are *chemolithoautotrophic* bacteria of the *Planctomycetes* order. Despite many bacteria species that can perform the anammox process (Table 2.1), rarely different anammox species can occur in the same treatment facilities because of their own environmental conditions [32]. However, Furukawa et.al [33] reported that the presence of two different anammox species in the lab-scale partial-nitritation anammox reactor.

Table 2.1: Microbial Species of Anammox bacteria [26].

Genus	Species	Sources
Brocadia	*Candidatus Brocadia anammoxidans*	Wastewater
	Candidatus Brocadia fulgida	Wastewater
Kuenenia	*Candidatus Kuenenia stuttgartiensis*	Wastewater
Scalindua	*Candidatus Scalindua brodae*	Wastewater
	Candidatus Scalindua wagneri	Wastewater
	Candidatus Scalindua sorokinii	Seawater
	Candidatus Scalindua arabica	Seawater

Jettenia	*Candidatus Jettenia asiatica*	Not reported
Anammoxoglobus	*Candidatus Anammoxoglobus propionicus*	Wastewater

In late 1990, when the anammox bacteria was first discovered, it was considered as the extremely slow-growing bacteria, which have 11-30 days doubling time [29], [34]. However, a recent study of anammox bacteria on synthetic medium claims that the anammox bacteria can be double in the population (i.e., doubling time) within 2-4 days [35]. Several studies on anammox bacteria in the lab-scale reactor using synthetic wastewater reported the different doubling times of anammox bacteria as shown in the Figure 2.5 [8]. The reason behind the difference in doubling time of anammox bacteria could be the use of different process and reactors such as MBBR, batch reactors, etc. [35]. Moreover, the operating temperatures and the type of anammox bacteria can also result in different doubling times [36]. However, Van Hulle et.al [26] reported the main reason for variations in doubling time is due to different methods used to determine the growth rate of anammox bacteria, such as direct counts of anammox bacteria, growth rate based on biomass yield, and nitrogen removal rate.

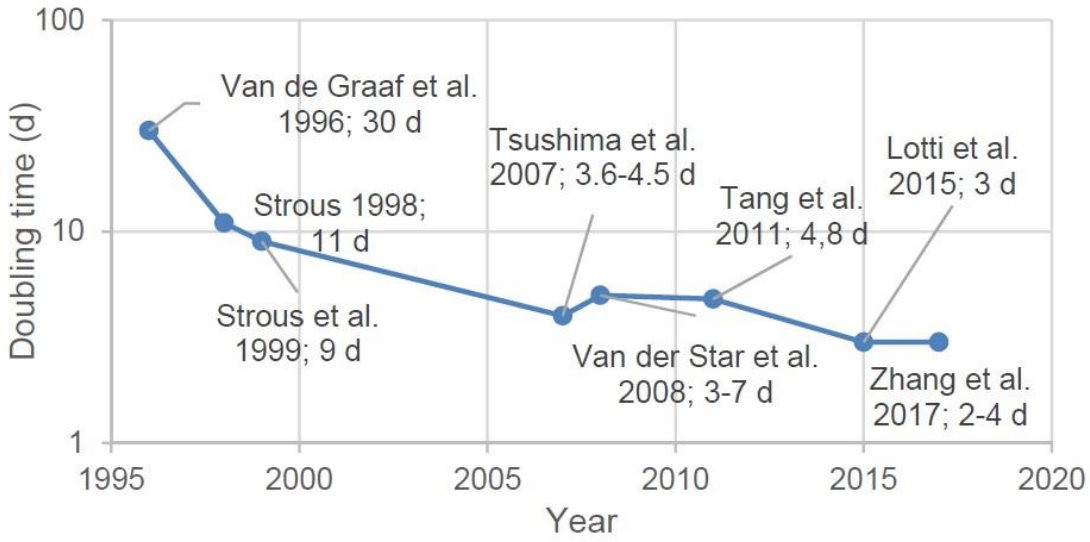

Figure 2.5: Anammox doubling time according to different study. Note that y-axis is in logarithmic scale.

2.2.3.1 **Factor influencing the Anammox Process**

The anammox process is affected by several factor and are described below.

2.2.3.1.1 Inhibition of substrate and products

Although nitrite is the substrate for the anammox bacteria, high concentration exposure of it to anammox bacteria inhibits the anammox process. However, no specific threshold value has been reported up to now. Mora et.al [37] found that the nitrite concentration higher than 350 mg-N/L led to inhibit anammox process by 50% whereas, long-term exposure of 40 mg-N/L of nitrite results in irreversible inhibition of the anammox process, as suggested by Christian Fux [38]. Even after long-term exposure to high concentrations of nitrite, this reduced activity

due to nitrite inhibition can be restored by adding trace amounts of the anammox intermediates hydroxylamine, and hydrazine [38].

Moreover, a concentration of 1 g-N/L, ammonium and nitrate do not affect the anammox process [38]. However, Mora et.al [37] found that the ammonium and nitrate concentrations of 770 mg-N/L and 630 mg-N/L, respectively have 50% inhibition in the anammox process.

The influent bicarbonate concentration also influences the anammox activity because these bacteria are chemolithoautotrophic, which consume inorganic carbon CO_2 as a carbon source. Low CO_2 production results from the lower bicarbonate to ammonia ratio below 2.3 in the influent suppresses anammox activity [39]. However, a high ratio of bicarbonate to ammonium (i.e., 4.7) also inhibits the anammox process. The inhibition is due to high free ammonia concentration result from an increase in pH by the high bicarbonate concentration [26].

2.2.3.1.2 Inhibition by DO

Anammox bacteria are completely anaerobic. Hence, even with low concentrations of DO can inhibit the anammox process. However, depending upon the DO concentration, the inhibition in the anammox process by DO is either reversible or irreversible. The low DO concentration (i.e., air saturation in between 0.25-2%) has a reversible effect on anammox bacteria [40] whereas, a higher concentration (i.e., air saturation > 18%) inhibits the anammox process irreversibly [41].

2.2.3.1.3 Effect of organic carbon

There is still conflict between different research articles on the anammox process inhibition by organic carbon. Van Hulle et.al [26] reported that even if the digested wastewater has high COD content, it is still considered the best wastewater for the anammox process. In this wastewater, the readily nonbiodegradable organic matter gives a high contribution to COD content because the readily biodegradable organic matter was feed by the bacteria to produce biogas in anaerobic digestion. As a result, heterotrophic denitrifiers' growth is lower in the reactors, so they are unable to outcompete the anammox bacteria for denitrification. Furthermore, even though the wastewater contains a high proportion of easily biodegradable organic matter, heterotrophic denitrifiers mature at a slower rate. This is maybe due to the consumption of fast degradable organic matter in the proceeding partial nitritation steps [42].

Meanwhile, many other studies reported the negative effect of organic carbon on the anammox process [43]. Even in the low concentration of organic carbon, anammox bacteria cannot compete with heterotrophic bacteria for denitrification. This is because anammox bacteria have a slower growth rate than heterotrophic denitrifiers [44], and the heterotrophic denitrification reaction is thermodynamically more favorable due to the high Gibbs free energy [43]. The COD to nitrogen compound ratio at which heterotrophic denitrifiers outcompete the anammox process differs between different research papers. According to Güven et.al [45], anammox bacteria cannot compete with heterotrophic denitrifiers when the COD/N ratio is greater than 1, while Chamchoi et.al [46] found that when the COD/N ratio is greater than 2 in upward flow anaerobic sludge blanket (UASB) reactor feed with fat milk as organic carbon, anammox bacteria are fully inhibited.

Moreover, anammox process cannot completely remove the nitrogen present in wastewater although the process has 100% efficiency. This due to the production of nitrate as a byproduct in the anammox reaction. Hence, For the complete nitrogen removal, the anammox process can be coupled with denitrifiers in one reactor. The denitrifiers can feed the nitrate produce by anammox to yield nitrite or nitrogen gas in anaerobic conditions. [47]

2.2.3.1.4 Temperature and pH

Anammox bacteria have high activity at the temperature between 30ºC- 40ºC. The batch test carried out by Dosta et al. [48] to find the temperature dependence of anammox bacteria shows that the anammox bacteria have the highest activity in the temperature range of 35ºC -40ºC, while a higher temperature (i.e., >45ºC) results in an irreversible decrease of anammox activity due to biomass lysis (i.e., disintegration of cell). The optimal temperature for the highest activity of anammox bacteria also depends upon the type of anammox bacteria in the system.

However, the successful operation of the anammox process was achieved in the lower temperature of 20ºC in rotating biological contactors (RBC) [49] and anaerobic biological filtered reactor (AF)[50]. At the lower temperature, anammox bacteria have low activity, resulting in slow adaptation of anammox sludge. This could be the key factor for the operation in low temperature because an abrupt change in operational condition can lead to destabilization of the process. Therefore, a suitable startup strategy of anammox sludge is needed before to operate at a lower temperature. Firstly, the anammox sludge should be developed in a different reactor with the temperature at which the anammox bacteria have the highest activity. Then the anammox sludge is adapt to the lower temperature by decreasing the temperature. Lastly, the adapted anammox sludge can be inoculated in the low-temperature reactor for the operation [51].

The anammox process has a pH range of 6.7 to 8.3 with an optimal pH of 8.0 [52]. The low pH results in a high concentration of free nitrous acid result in suppression of anammox activity. On the other hand, high pH has high free ammonia, which also inhibits anammox bacteria.[26]

2.2.3.1.5 Biomass concentration

The anammox activity directly depends on the quantity of anammox biomass. Enough cells should be present for the anammox activity because the lower quantity of cells results in poor intercellular communication for the activity [27]. Strous et al. [52]found that the anammox is active only when the cell concentration is higher than $10^{10} – 10^{11}$ cells/ml in the purified culture.

2.2.3.1.6 Effect of light

The anammox activity is sensitive to visible light. Van de Graaf et al. [34] reported that anammox activity decrease by 30 to 50% due to visible light. Hence, the anammox reactor should be cover with black plastic or paper to eliminate the radiation of light to anammox bacteria.

2.3 Biological Autotrophic Nitrogen removal

Since the discovery of the anammox bacteria, the path of removing nutrients (i.e., ammonia) from wastewater has been changed to a fully autotrophic process. The partial nitritation combined with the anammox process, also known as the partial-nitritation anammox process (PNA), utilizes AOB for partial-nitritation and anammox for denitrification, result in complete autotrophic nitrogen removal, making no need for organic carbon for the nutrients removal. In the PNA process, firstly, AOB oxidizes half of the ammonium to nitrite without producing nitrate (Equation 2.9) [22], and then the remaining ammonium along with nitrite is utilized by the anammox process to yield nitrogen gas (Equation 2.8). This process can be implemented in a single reactor (one reactor system), and by using two reactors (two-reactor system) [26].

$$NH_4^+ + 0.75\ O_2 + HC0_3^- \rightarrow 0.5\ NH_4^+ + 0.5\ NO_2^- + CO_2 + 1.5\ H_20 \qquad (2.9)$$

The path of ammonia oxidation to nitrogen gas for traditional nitrification-denitrification and PNA has shown in the Figure 2.6 [53]. The PNA process only requires oxidation of half of the ammonium to nitrite without accumulation of nitrate in an aerobic environment (i.e., partial nitritation). Hence, this process decreases the aeration requirement by 63% than the traditional nitrification-denitrification process [53]. Moreover, the sludge yields lower by 80% than the traditional nitrification-denitrification process due to the low synthetic yield value of autotrophic bacteria [54], making it easy to handle excess sludge and reduce the transportation cost of sludge for WWTPs. Moreover, the carbon dioxide (CO_2) produced by AOB in the partial nitritation step is consumed by anammox bacteria, resulting in the lower carbon footprint than traditional nitrification-denitrification process [55].

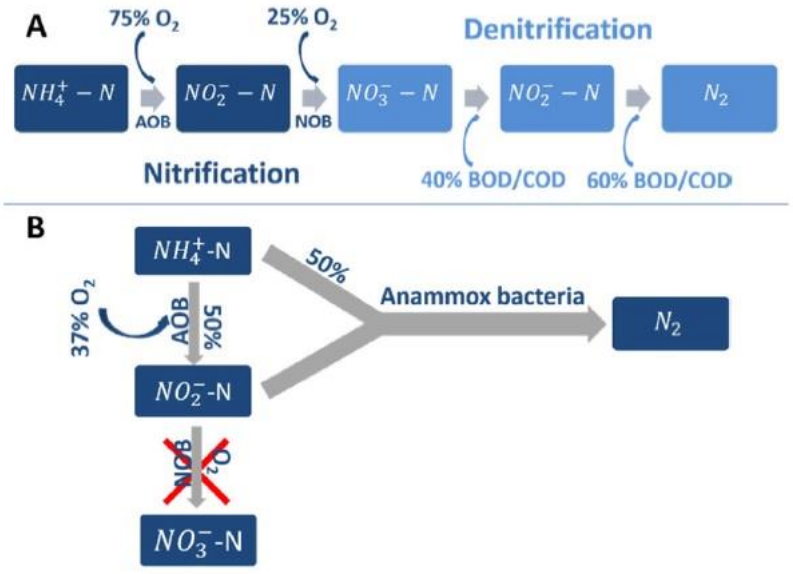

Figure 2.6: Simplified path of traditional nitrification-denitrification process (A), and PNA process (B).

Meanwhile, the practical implementation of the PNA process is quite challenging due to the slower growth rate of AOB and anammox bacteria [29]. This problem can be mitigated by

using an attached growth process such as moving bed biofilm reactor (MBBR) in which active biomass can retain for longer time [56]. Moreover, the NOB should be suppressed for efficient nitrogen removal from the PNA process because at the time of substrate consumption (i.e., nitrite), NOB outcompetes the anammox bacteria if they are present in significant amount, resulting in nitrate accumulation.

2.3.1 Partial nitritation anammox in one reactor (one reactor system)

One reactor system for the nutrient (i.e., ammonia) removal from wastewater is a well-known technology in which nitrifiers (i.e., AOB) for partial nitritation and anammox for autotrophic denitrification coexist in the same reactor [57]. This process has known by several names, such as the OLAND process (Oxygen Limited Autotrophic Nitrification and Denitrification) [58], aerobic /anoxic deammonification or DEMON [59], SNAP process (Single-stage Nitrogen removal using Anammox and Partial nitritation) [33], and the CANON process (Completely Autotrophic Nitrogen removal Over Nitrite) [60]. Initially, it was assumed that the nitrifiers under low DO concentration are responsible for the anaerobic ammonium oxidation to nitrogen gas in the OLAND and DEMON process. However, in the CANON process, it was believed that the anammox bacteria perform a key role in anaerobic ammonium oxidation. This conflict is neutralized by the Pynaert et al. [61] and Helmer -Madhok et al. [62] study in all three reactors using fluorescence in situ hybridization (FISH) analysis for bacteria composition, showing that anammox is responsible for anaerobic ammonium oxidation. The operating conditions in the reactor should be maintained to favor both partial nitritation and anammox simultaneously for the efficient nitrogen removal from one stage PNA.

Because of AOB's aerobic nature and anammox bacteria's anoxic nature, oxygen-limited conditions (i.e., low DO concentration) should be used to avoid oxygen inhibition in anammox bacteria and to provide enough oxygen for partial nitritation in AOB [23]. The simultaneous aerobic/ anoxic conditions can be achieved by using biofilm or granules reactors. In these reactors, the AOB present in the outside layer consumes the oxygen and produces sufficient nitrite, resulting in an anoxic condition and sufficient substrate for the anammox bacteria which grow in the inner layer [26]. The optimum value of DO for the one-stage PNA process is depends upon the reactor configuration and the influent ammonia concentration [23]. In our case (i.e., MBBR reactor) optimum DO depends on biofilm thickness and density, boundary layer thickness, and temperature [26].

Since both nitritation and anammox process occurs in the same reactor, one stage PNA required less space than two-stage PNA, where two separate reactors are used for partial nitritation and anammox. Moreover, Wyffels et al. [63] reported that this process has a generally higher volumetric removal rate than two-stage PNA. However, maintaining optimum DO in this process for a long time is quite challenging, especially when the ammonia loading rate is varying in the influent [64].

2.3.2 Partial nitritation anammox in two reactors (Two reactor system)

Two-stage PNA is the complete autotrophic nitrogen removal process in which two separate reactors in series are used for the nutrient removal from the wastewater. In the first reactor, AOB converts about half of the influent ammonia to nitrite under aerobic conditions to produce

anammox-friendly effluent i.e., ammonium to nitrite molar ratio of 1:1, while the second reactor is responsible for anaerobic oxidation of ammonia and nitrite to yield nitrogen gas by anammox bacteria [22]. This process is also known by several names, such as the combined SHARON-Anammox process, and autotrophic nitrogen removal process [23]. The schematic diagram of the two-stage PNA process has depicted in the Figure 2.7 [23].

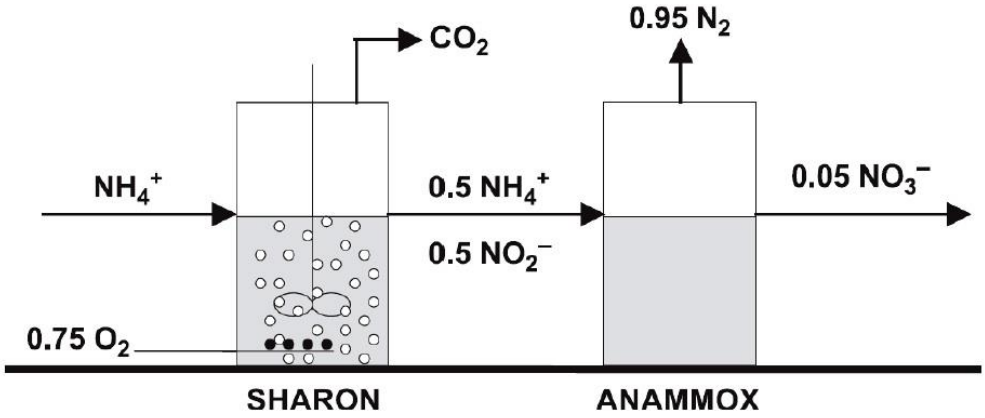

Figure 2.7: Two stage partial nitritation anammox process.

This process allows more flexible operating conditions and stable process performance than one stage PNA since two-step (i.e., partial nitritation and anammox) in two separate reactors can be controlled separately [63]. Moreover, nitrogen removal with two-stage PNA yields less amount of N_2O than one-stage PNA [65]. This process is more appropriate to treat the wastewater having a high content of toxic or biodegradable compound because this compound will be degraded in the partial nitritation reactor, resulting in the non-toxic influent for the anammox reactor [66].

2.4 Factors for NOB suppression in MBBR reactor

To achieve nitrogen removal either from nitritation-denitration or from PNA, the NOB bacteria should be suppressed. This is also called the partial nitrification process. Different factors as described below can be considered to achieve partial nitrification.

2.4.1 Low dissolved oxygen

Low DO is the widely used strategy by different researcher to suppress NOB bacteria. This method is based on the fact that the AOB bacteria have higher DO affinity than NOB bacteria due to different DO half-saturation constant (0.032-0.48 for AOB and 0.7-5.3 for NOB), resulting in NOB suppression under low DO concentration [53]. In other words, under low DO concentration, AOB has a higher growth rate than NOB due to a lower DO half-saturation constant of AOB. However, a recent study concludes that only low DO oxygen concentration cannot suppress NOB for long time operation [67]. This is due to the growth of *Nitrospira* NOB, which has less DO half-saturation constant value than AOB (i.e., 0.33), resulting in higher growth rate even in low DO concentration [68].

2.4.2 Free Ammonia inhibition

The nitrite-oxidizing bacteria are more sensitive to the high free ammonia concentration than ammonia oxidizing bacteria. Anthonisen et al.[69] reported that free ammonia concentration higher than 8-120 mg/l inhibits AOB, while the free ammonia in low concentrations (i.e., 0.08-0.82 mg/l) can inhibit NOB. Hence, the suppression of NOB without affecting AOB can be done by applying free ammonia concentration higher than the inhibition range of NOB and lower than the AOB inhibition range. The free ammonia concentration depends upon the temperature, pH, and total ammonium concentration (Equations 2.10 -2.11)[21]. At higher pH and temperature, a higher amount of TAN (NH_3-N+ NH_4-N) shifts to NH_3-N.

$$NH_3\text{-}N = \frac{TAN\,(10^{pH})}{\left(\frac{1}{k_a}\right) + 10^{pH}} \tag{2.10}$$

$$\frac{1}{K_a} = \exp\left(\frac{6334}{273+T}\right) \tag{2.11}$$

Where, TAN = total NH_3-N + NH_4-N concentration

T = temperature, ℃

K_a = Ionization constant for ammonium

2.4.3 Intermittent aeration cycle

To suppress NOB, oxygen can be applied through intermittent aeration. Intermittent aeration is the aeration strategy in which alternating aerobic, and the anaerobic condition is applied into the reactor. This strategy can inhibit the NOB growth since the NOB has lower starvation recovery dynamics than AOB, resulting in the AOB recovery earlier than AOB when the reactor shift from an anoxic phase to an anaerobic phase [70]. Due to the high recovery dynamics of the AOB bacteria, it transfers the ammonium to nitrite as soon as the reactor shift from the anoxic phase to the aerobic phase. However, NOB takes a longer time to convert nitrite to nitrate due to low recovery dynamics. In the meantime, nitrite can accumulate in the system and whenever NOB starts to oxidize the nitrite, aeration should be turn off to shift the reactor to an anoxic phase. Moreover, AOB can exert hydroxylamine when the aeration is suddenly turn off [71]. Also, AOB produces nitric oxide (NO) by performing denitrification under anaerobic conditions [72]. Both of these compounds exerted by AOB under intermittent aeration are inhibitory for NOB, resulting in suppression of NOB [73]. The intermittent aeration cycle is also useful to develop anammox condition due to the presence of an anaerobic phase.

Meanwhile, the application of intermittent aeration enhances the production of nitrous oxide (N_2O). The nitrous oxide can be formed in PNA by the oxidation of nitric oxide (NO) and hydroxylamine (NH_2OH) [74]. Also, it is hard to maintain the length of aerated and non-aerated phases due to a lack of knowledge of the actual lag time of NOB.

2.4.4 Other influencing factor

The addition of different acids such as formic, acetic, propionic, and n-butyric acid inhibits the NOB bacteria, while it does not have any inhibition effect on AOB [75]. Moreover, A study carried out by Peng et al.[76] on dozens of inhibitory compounds for nitrification shows that chlorate, cyanide, azide, and hydrazine have more inhibition on NOB than AOB. Hence, the additions of these compounds in appropriate amounts also help to achieve partial nitrification.

2.5 Oxygen uptake rate test to quantify bacterial composition.

Oxygen uptake rate is a measure of oxygen consumption of two nitrifiers (i.e., AOB, NOB) and heterotrophs by using inhibitors. It is a simple, robust, and qualitative method to quantify bacterial activity in suspended liquid or in the Biofilm. This method can be applicable for suspended liquid with Biofilm [77] and for suspended liquid only [78]. It is relied on the successive addition of two chemicals: sodium chlorate ($NaClO_3$) and allylthiourea ($C_4H_8N_2S$), inhibitors of NOB and AOB. A typical oxygen utilization curve from the OUR test, given in Figure 2.8 [78], clarifies this test concept. The oxygen uptake rate is calculated as from the slope of the linear regression of the measured DO profile. The sample's oxygen utilization from

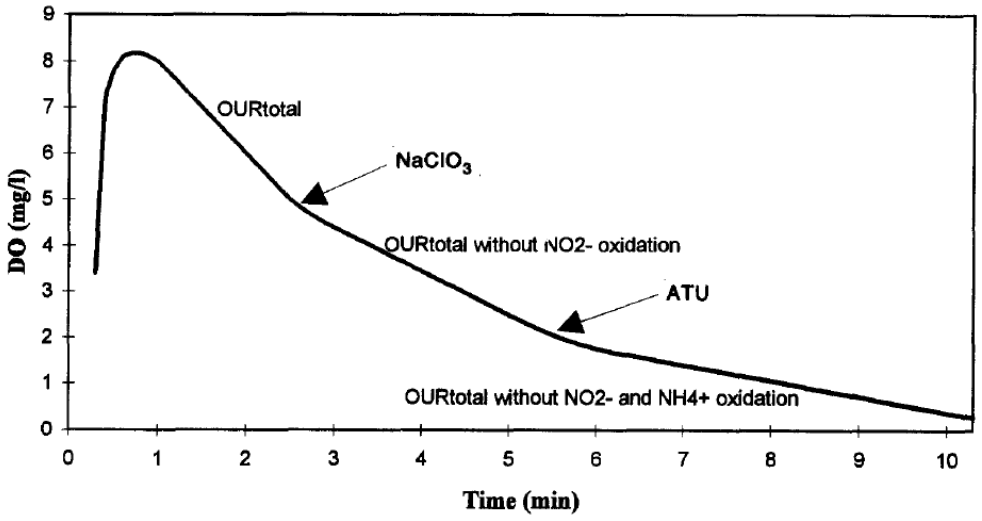

Figure 2.8: Oxygen utilization curve obtained from OUR test after linear regression for suspended liquid.

high DO concentration (7-8 mg/l) in the appropriate time (different for suspended and Biofilm sample) is considered total oxygen uptake rate, consisting of oxygen utilized by all three microorganisms (i.e., AOB, NOB, and heterotrophic bacteria). The difference between total OUR and the OUR after the addition of the $NaClO_3$ is considered the oxygen uptake rate of NOB. Similarly, the difference between NOB oxygen uptake rate and the oxygen uptake rate after adding two inhibitors (i.e., $NaClO_3$ and allylthiourea) considered as AOB uptake rate. Lastly, the DO profile slope after the addition of two inhibitors is the Heterotrophic oxygen uptake rate. From this experiment, it is impossible to distinguish whether the endogenous decay or the substrate consumption takes oxygen.

3 Material and Methods

A moving bed biofilm reactor (MBBR) was set-up at the University of South-Eastern Norway (USN) laboratory to achieve the anammox conditions. The reactor was feed with synthetic wastewater (Section 3.4) to ensure the desired substrate for the fast anammox process start-up.

Samples were taken out almost daily (sometime in one day gap) for NH_4-N, NO_2-N, NO_3-N, and alkalinity analysis, whereas onsite measurements such as DO, and temperature were measured as part of the daily routine. Furthermore, the dry weight of the biofilm was measured one time per week.

This sub-chapter presents the details about the reactor set-up, biochemical analysis procedure for wastewater quality, synthetic wastewater constituent, biofilm weight measurement, etc. Furthermore, the material and methods used to carry out oxygen uptake rate to quantify the bacterial composition is also described.

3.1 Laboratory set-up of Moving Bed Biofilm reactor (MBBR)

The experimental set-up of the PNA MBBR reactor is shown in Figure 3.1. The reactor was continuously fed with synthetic wastewater using a peristaltic pump from 20L influent bottle. The mechanical stirrer was inserted into the reactor to ensure biofilm movement, and the rotation speed was set to 140 rpm. The reactor was wrapped with a heater belt, and the temperature was constant at 30±1℃. Moreover, A black foam was used to insulate the reactor, to protect the active biofilm from the sunlight. The detailed design parameter and the operating condition of the reactor are presented in Table 3.1 and Table 3.2, respectively.

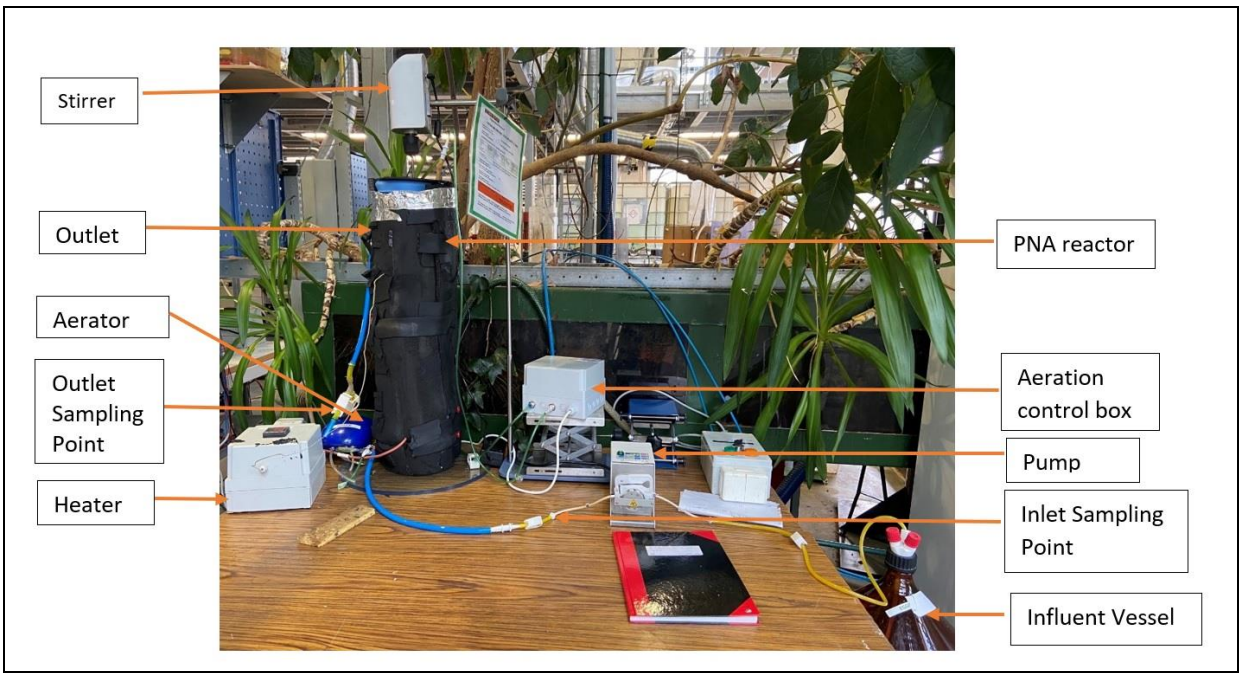

Figure 3.1: Experimental reactor set-up at USN.

3.1.1 The biofilm carrier in the reactor

The type of carriers used was BTWS which has dimension of **14.5×18.5×7.3mm** and a protected surface area of **650 m^2/m^3** (Figure 3.2) [17]. Further details of the carriers, such as filling ratio, and total protected surface area, are presented in Table 3.1.

Figure 3.2: Carrier used in PNA reactor.

3.1.2 Aeration in reactor

Aeration was supplied through a horizontal round circular bottom pipe, which has many small holes to distribute the same amount of air throughout the reactor. The aeration strategy (i.e., continuous, and intermittent) used and the duration of aeration is presented in the Table 3.2.

Table 3.1: Design parameter of PNA MBBR reactor.

Items	Units	Value
Working Volume	L	10.5
Water depth	m	0.61
Cross sectional area	Sq.m	0.148
Volume of old carrier from KRA	L	3
Volume of new carrier	L	2
Percentage filling of carrier	%	47.6
Effective volume of water inside reactor	L	8
Total surface area of carrier	Sq.m	3.25

Table 3.2: Operating conditions of reactor.

Items	Unit	value	Duration (Days)
Feed flow rate	L/d	3.3 6.5	1 - 21 21 - 90
HRT	d	2.42 1.23	1 -21 21 - 90
Surface ammonia loading rate (SALR)	g-N/m^2 d	0.15 0.28	1 -21 21 - 90
Aeration Strategy	Continuous Intermittent		1 - 58 58 - 90

3.2 Wastewater quality analysis

3.2.1 Dissolve Oxygen and Temperature.

The dissolved oxygen concentration and the temperature of the reactor were measured daily by using WTW Oxi 3310 (Weilheim, Germany).

3.2.2 pH

The pH of samples was measured by using Beckman 390 pH-meter. The samples were homogeneously mixed at room temperature, and the pH meter was calibrated with two buffer solutions of pH 4.0 and 7.0 before measuring pH.

3.2.3 Nitrogen compounds and Alkalinity concentration analysis

The samples from the reactor were first filtered through the 0.45µm GxF multi-layered acrodisc PSF filters. Then the filtered samples were diluted with the distilled water by the dilution factor 5. The concentration of ammonium-nitrogen (NH_4-N), nitrite-nitrogen (NO_2-N), and nitrate-nitrogen (NO_3-N), Alkalinity as $CaCO_3$, were then determined by using the US standard methods of number 114559, 100609, 114563, 101758, respectively [79].

3.3 Biofilm measurement

The biomass on carriers was measured one time a week. Five old carriers and five new carriers were taken out from the reactor. The carrier was placed into an aluminum plate and dried at 105^{o}C for 24 hours. The dried carriers along with the aluminum plate were placed into a desiccator for 10 minutes to maintain the room temperature. The cooled carriers and aluminum

plate were weighted in analytical balance and noted as W1. After that, carriers were soaked into Hypochlorous acid (HOCl(aq)) for 2 hours, and biomass was washed away by using the brush and tape water. Again, the cleaned biomass was dried, cooled, and weighted the same as above, and noted as W2. The weight of biomass per carrier was calculated by using Equation 3.1.

$$\text{Biomass per carrier (W)} = \frac{W_1 - W_2}{\text{No of carrier}} \quad (3.1)$$

3.4 Synthetic wastewater

The synthetic wastewater was prepared in the 20L glass vessel in a two-day gap by using tape water. To obtain the desired concentration of the ammonium-nitrogen (≈140 mg/L), and buffer (500-1100 mg/L as $CaCO_3$) for the nitrification, the appropriate amount (calculation is annexed in Appendix B) of ammonium chloride (NH_4Cl) and sodium hydrogen carbonate ($NaHCO_3$) respectively was used. Also, the Vitamins (1ml/L), and Minerals (1ml/L) solution were added to synthetic medium as micro and macro nutrients. After the addition of whole components, the glass botttle was placed on the magnetic stirrer for the proper mixing and to ensure the homogeneity of solution. The compounds and their proportion used to make vitamins and mineral is shown in the Table 3.3.

Table 3.3: Constituents of vitamins and minerals solution [80].

Vitamins (g/L)	Minerals (g/L)
Thioctic acid: 0.05	$CuSO_4.5H_2O$: 0.055
p-aminobenzoic acid: 0.05	$MnSO_4.H_20$: 0.04
Pantothentic acid: 0.05	$FeSO_4.7H_2O$: 2.7
Vitamin B12: 0.001	$NiCl_2.6H_2O$: 0.1
Thiamine: 0.05	$ZnSO4.7H_20$: 0.088
Nicotinic acid: 0.05	$CoCl_2.6H_2O$:0.05
Riboflavin: 0.05	H_3BO_3: 0.05
Pyridoxine hydrochloride: 0.1	
Folic acid: 0.02	
Biotin: 0.02	

3.5 Oxygen uptake rate procedure (OUR)

The methods for experimenting with suspended liquid and suspended liquid along with biofilm are different as suggested by Gosaka et.al [78] and Gutt et.al [77], respectively.

3.5.1 OUR in suspended liquid

The experimental set-up of oxygen uptake rate test for the suspended liquid is depicted in the Figure 3.3-A. The suspended liquid of volume 100ml was taken out from the PNA reactor and placed into the round bottom flask. The vessel and the sample were placed on a magnetic stirrer and inside heater for the whole experiment period for perfect mixing and to maintain the same temperature as in the reactor. Also, the round bottom flask was closed tightly to avoid oxygen diffusion from the surrounding. The DO meter (WTW Oxi 3310) was dipped inside the round bottom flask to measure DO concentration digitally. An aerator was used to aerate the sample until the DO concentration reached 7-8 mg/L. Once the DO concentration was reached 7-8 mg/L, the aeration was turn off, the DO concentration of the sample starts to decrease, which signifies the beginning of the experiment. When the DO concentration of the sample became 5mg/l, $NaClO_3$ was added (final concentration of 2.13 g/L). Finally, After the DO concentration became 3 mg/L, allylthiourea (final concentration is 5mg/L) was added and the DO concentration was recorded for next 2-3 minutes.

3.5.2 OUR in suspended liquid along with biofilm

Figure 3.3-B shows the experimental setup of the OUR test for a mixture of suspended liquid and biofilm. The suspended liquid of volume 300ml was taken out from the reactor and placed into a 600ml working volume round bottom flask. Another procedure such as mixing, recording, and experimental condition like temperature is the same as in the suspended liquid test. After the suspended liquid was aerated for 3 hours, biofilm (volume of 300ml) was added. After 1.5-2 minutes, 5 ml of Naclo3 (final concentration of 17mM/L) was applied. Again after 3-4 minutes, 5 ml of allylthiourea (final concentration of 43µM/L) was added, and DO concentration was recorded for the next 2-3 minutes.

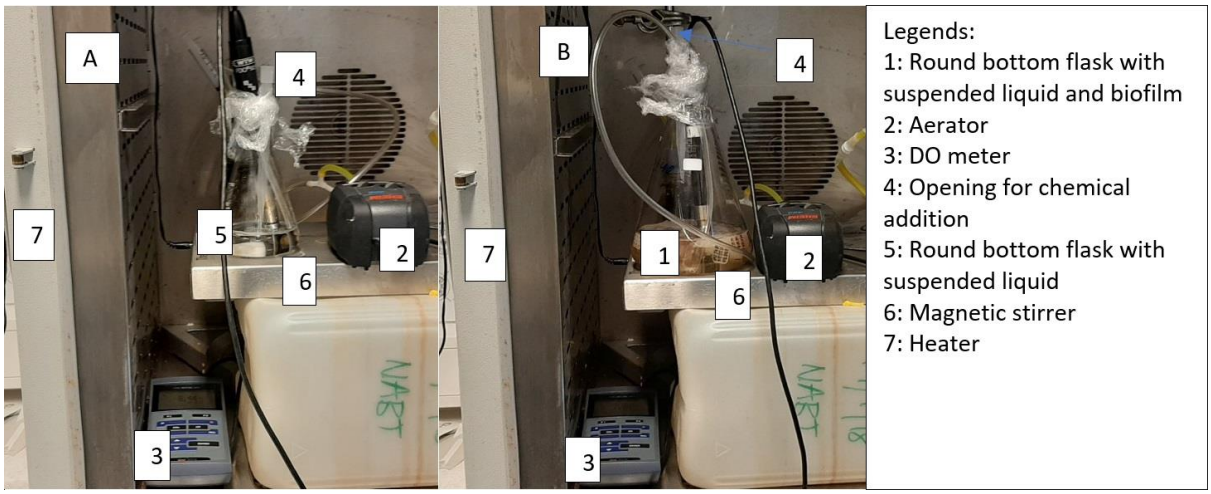

Figure 3.3: Experimental set-up of OUR test. A) For suspended liquid, B) For suspended liquid along with biofilm.

4 Result and Experimental Planning

This section discusses the results of nitrogen compound transformations under various operating conditions, the different mode of aeration and its effect on ammonium conversion to other nitrogen compounds, bacterial quantification on biofilms, and the relationship of DO with pH and alkalinity in the PNA process. All the relevant data from laboratory analysis were calculated and plotted in MS excel.

The concentration of nitrogen compounds is presented in terms of surface ammonia loading rate (SALR), surface ammonia removal rate (SARR), nitrate production rate (NPR), nitrite accumulation rate (NAR), ammonium removal efficiency (ARE), were respectively calculated by using Equations (4.1)- (4.5).

$$\text{SALR } [\frac{\text{gN}}{m^2.\text{d}}] = \frac{C_{NH4\text{-}N,in} \times Q}{A_{carrier} \times 1000} \quad (4.1)$$

$$\text{SARR } [\frac{\text{gN}}{m^2.\text{d}}] = \frac{(C_{NH4\text{-}N,in} - C_{NH4\text{-}N,out}) \times Q}{A_{carrier} \times 1000} \quad (4.2)$$

$$\text{NPR } [\%] = \frac{(C_{NO3\text{-}N,out} - C_{NO3\text{-}N,in})}{(C_{NH4\text{-}N,in} - C_{NH4\text{-}N,out})} \times 100 \quad (4.3)$$

$$\text{ARE } [\%] = \frac{(C_{NH4\text{-}N,in} - C_{NH4\text{-}N,out})}{C_{NH4\text{-}N,in}} \times 100 \quad (4.4)$$

$$NAR\ [\%] = \frac{(C_{NO2-N,out} - C_{NO2-N,in})}{(C_{NH4-N,in} - C_{NH4-N,out})} \times 100 \quad (4.5)$$

Where, $C_{NH4\text{-}N,\ in}$ = Inlet ammonium concentration [mg/L]

$C_{NH4\text{-}N,\ out}$ = Outlet ammonia concentration [mg/L]

$A_{carrier}$ = Total surface area of carrier [m^2]

$C_{NO3\text{-}N,\ in}$ = Inlet nitrate concentration [mg/L]

$C_{NO3\text{-}N,\ out}$ = Outlet nitrate concentration [mg/L]

$C_{NO2\text{-}N,\ in}$ = Inlet nitrite concentration [mg/L]

$C_{NO2\text{-}N,\ out}$ = Outlet nitrite concentration [mg/L]

Q = Water flow rate [L/d]

4.1 Nitrogen compounds transformation under continuous aeration

The reactor is operated with different DO concentration, which was provided through continuous aeration from day one to day 58, to achieve partial nitritation. The operating

conditions were applied based on two requirements for partial nitritation: i) around 50% ammonium removal efficiency (ARE), and ii) almost 0% nitrate production rate (NPR).

In this period, the reactor was subjected to two different SALR to maintain different HRT by applying different influent flow rate (Table 3.2). In this section, the result of the conversion of influent ammonium to another nitrogen compound (NO_2-N, and NO_3-N) in different SALR under continuous aeration is described.

4.1.1 Nitrogen compound transformation under low SALR

The transformation of influent ammonium to nitrite and nitrate along with their accumulation rates under different DO/TAN and in the same SALR (0.14±0.1 gN/m^2. d) is depicted in Figure 4.1. In the beginning, when DO/TAN was higher than 0.025, around 90% of the influent ammonium was converted into other nitrogen compounds, resulting in around 10 mg/L ammonium in the outlet (Figure 4.1-A). In this period, the NAR was first increased and started to decline from day 7, but the NPR was low initially and was continuously increased over time. This indicates that the operating condition could not meet the requirements of partial nitritation. Hence, the dissolved oxygen was decreased significantly.

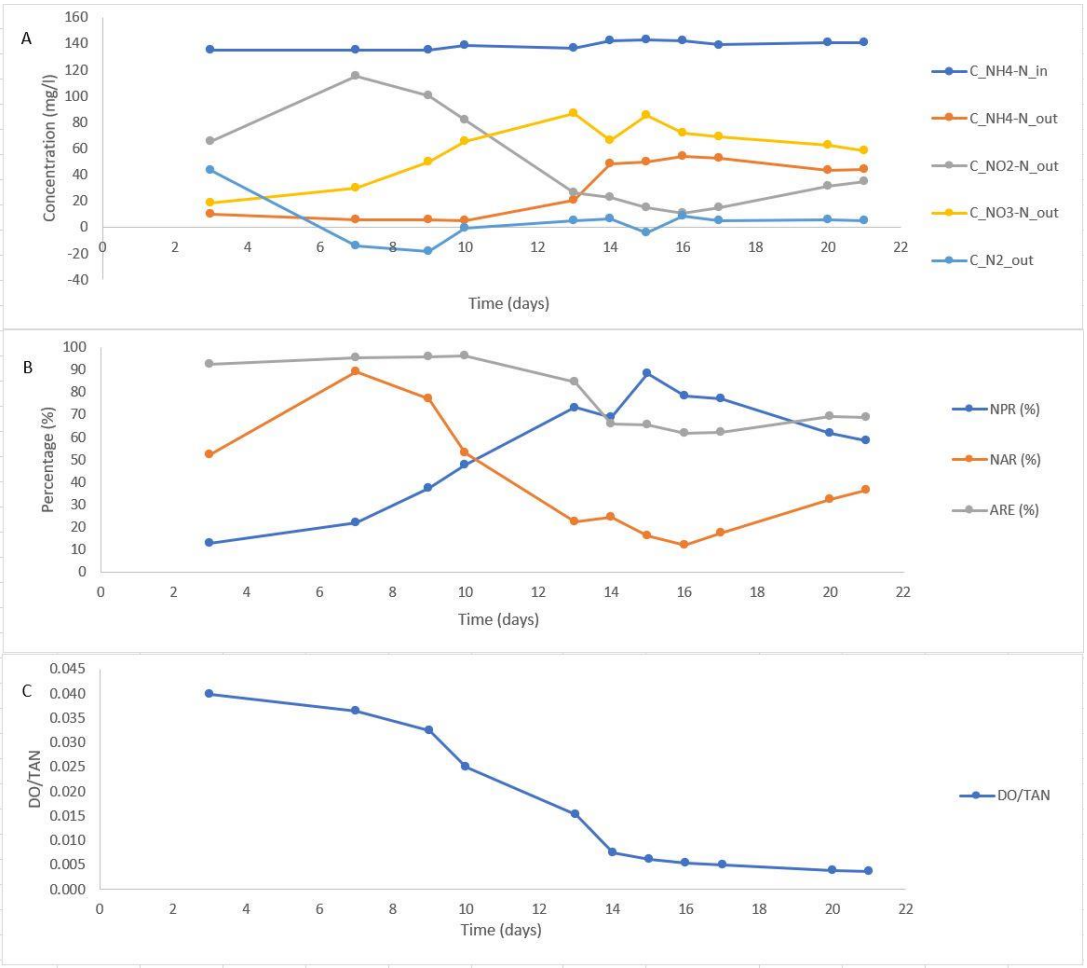

Figure 4.1: Nitrogen compounds transformation with different DO/TAN in low SALR. A) Nitrogen compounds variation. B) Production rate of nitrogen compounds and ammonium removal rate. C) DO/TAN ratio as operating conditions of the reactor.

As the ratio of DO/TAN started to fall below 0.025 from day 10 to day 14, ARE was also decreased gradually due to an increase in outlet ammonium concentration ($C_{NH4\text{-}N,\ out}$). In this operating condition, as time goes on, the large proportion of consumed ammonium was transformed to nitrate, which resulted in an increment in outlet NO_3-N concentration, and a decline in NO_2-N concentration in the outlet.

From day 14 to day 22, the DO/TAN was maintained stable at 0.005. This results in stable ARE with a value of around 62%. The NPR was fluctuating in the beginning, and gradually decreased, and was reached 60%. Moreover, throughout the whole period, the NPR was always higher than NAR, which means the higher fraction of consumed ammonium was transferred to nitrate. Although this operating condition met the first requirement of partial nitritation, the NPR value was higher than the desired value. Therefore, SALR was decided to increase by increasing the influent flow rate.

Moreover, the nitrogen compounds mass balance (Appendix C) reveals that there was a higher volume of nitrogen gas exhausting from the reactor in the beginning, but it disappeared entirely by day 6 (Figure 4.1-A).

4.1.2 Nitrogen compounds transformation under high SALR

The influent ammonium transformation to nitrate and nitrite, when the reactor is subjected to high SALR (i.e., 0.27±0.005 $gN/m^2.d$) and under different DO/TAN is presented in the Figure 4.2. For the first 8 days, the reactor was operated with stable DO/TAN of value around 0.04, which result in stable ARE with a value of around 20% (Figure 4.2-B). In this condition, the NPR nearly equal to NAR, signifying half of consumed ammonium was accumulated as $N0_3$-N with a value around 20 mg/L (Figure 4.2-A). Since this operating condition could not result in the first requirement of partial-nitritation, the DO/TAN was decided to increase.

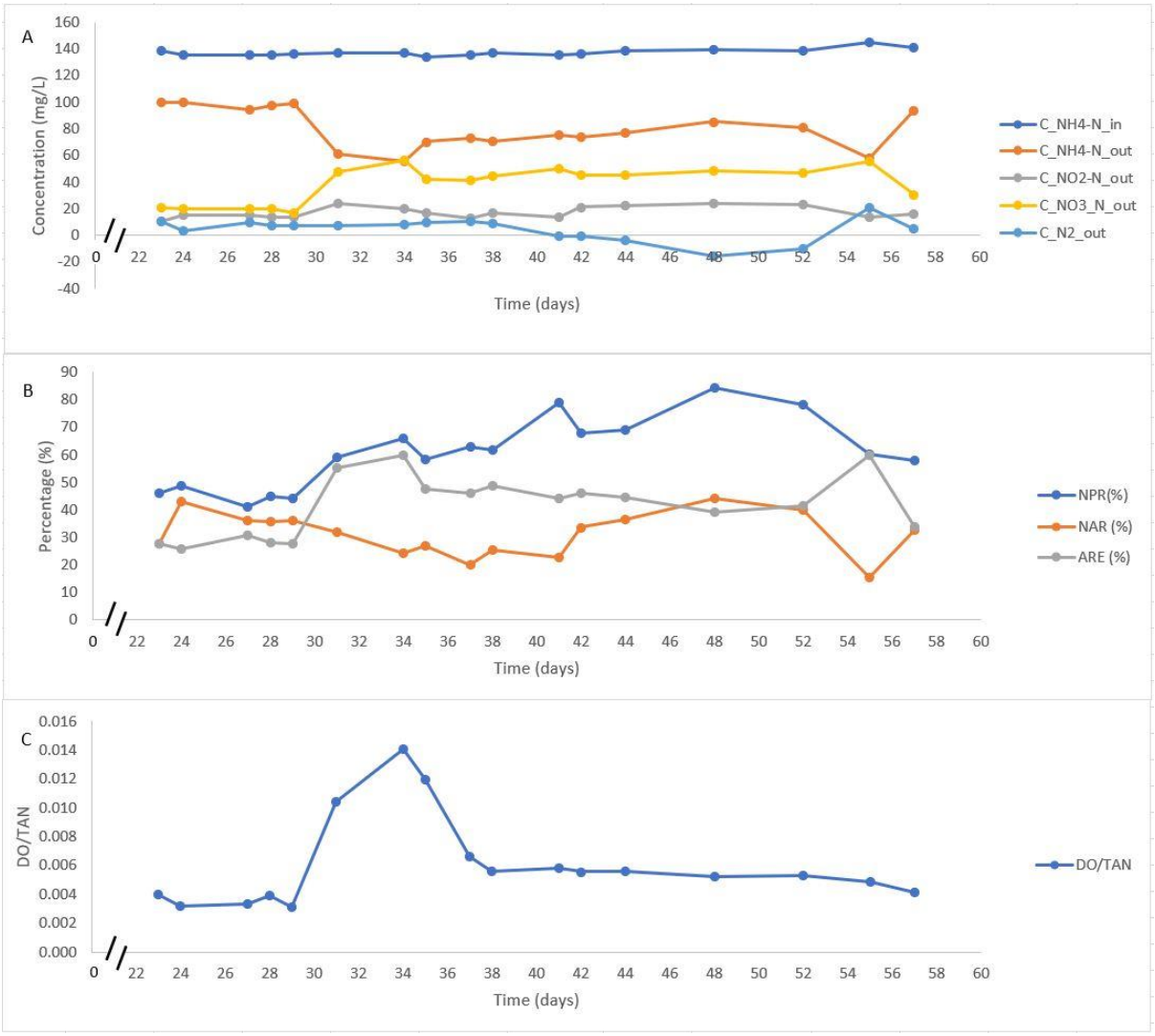

Figure 4.2: Nitrogen compound transformation with different DO/TAN in high SALR. A) Nitrogen compound variations. B) Production rate of nitrogen compounds and ammonium removal efficiency. C) DO/TAN as operating conditions.

As the DO/TAN start to rise above from day 29, ARE was also increased and was reached 60%, resulting in around 60 mg/L NH_4-N in the outlet (Figure 4.2-A and B). This condition enhances the accumulation of NO_3-N in the outlet, as the NPR was started to be increased and it crossed over 60% on day 31. Although the DO/TAN gave a promising result in terms of the first requirement of partial-nitritation, the second requirement was far from the desired value.

The DO/TAN was therefore decreased continuously from day 34 and maintained stable around 0.55 after day 37 (Figure 4.2-C). ARE followed the same trend of DO/TAN and was relatively stabilized at 40 % at stable DO/TAN. However, NPR was gradually increased over the time and reached above 80% on day 48 (Figure 4.2-B). Afterwards, the NPR started to fall, but never reached to the desired value of second condition of the partial nitritation.

4.1.3 Effect of the SALR on nitrogen compounds transformation

The effect of different surface ammonia loading rates (SALR) on the transformation of influent ammonium to other nitrogen compounds (i.e., nitrite and nitrate) under low DO/TAN (i.e., 0.0053±0.0010) is presented in the Figure 4.3. On the same value of DO/TAN, when the reactor was subjected to high SALR by decreasing HRT, low ARE was observed. Therefore, DO/TAN and SALR should be increased simultaneously to maintain the same ARE. Moreover, ARE was closer to the first requirement of partial nitritation condition in high SALR (Figure 4.3). However, although the NAR was in an increasing trend and, NPR was in decreasing trend, the NPR and NAR value was higher than the second requirement, suggesting no sign of partial nitritation has occurred. As seen from trends of NPR and NAR, increasing SALR can give partial nitritation. However, in our reactor, the SALR was not increased above 0.27±0.005 $gN/m^2.d$ (the explanation is in section 5.3). Therefore, the aeration strategy was changed to an intermittent aeration cycle from day 62.

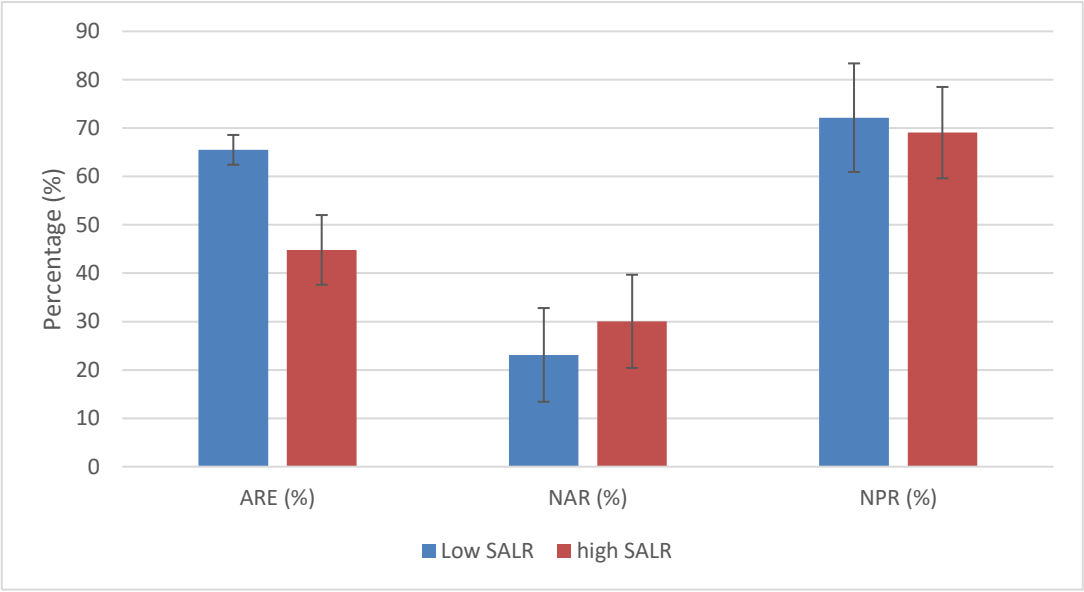

Figure 4.3: Effect of SALR on nitrogen compound transformation under low DO/TAN.

4.2 Intermittent aeration cycle and its effect on nitrogen compound transformation

The dissolved oxygen for active biofilm was provided by means of intermittent aeration from day 59 to day 90 under same HRT, and SALR (Table 3.2). This section describes the different DO profile of different intermittent aeration cycle along with their cycle length, and effect of it on the influent ammonium transformation.

4.2.1 Intermittent aeration cycle trial

The reactor had 45% ARE under DO/TAN value of 0.0053 (Figure 4.2), which was equivalent to 0.72 mg/L DO concentration. Hence, the reactor was subjected to different aeration rate and with different aeration cycle lengths to achieve DO concentration in the range of 0.8-1 mg/L aerated phase and around 0.1 mg/L in the non-aerated phase. After several test, nearly closer DO profile was found in intermittent aeration cycle of 20 min ON / 30 min OFF cycle length with around 1.2 mg/L DO in aerated phase and around 0.25 mg/L DO in non-aerated phase. This result was under certain rate even though aeration rate was not recorded due to lack of flow meter (IAC-1) (Figure 4.4-A).

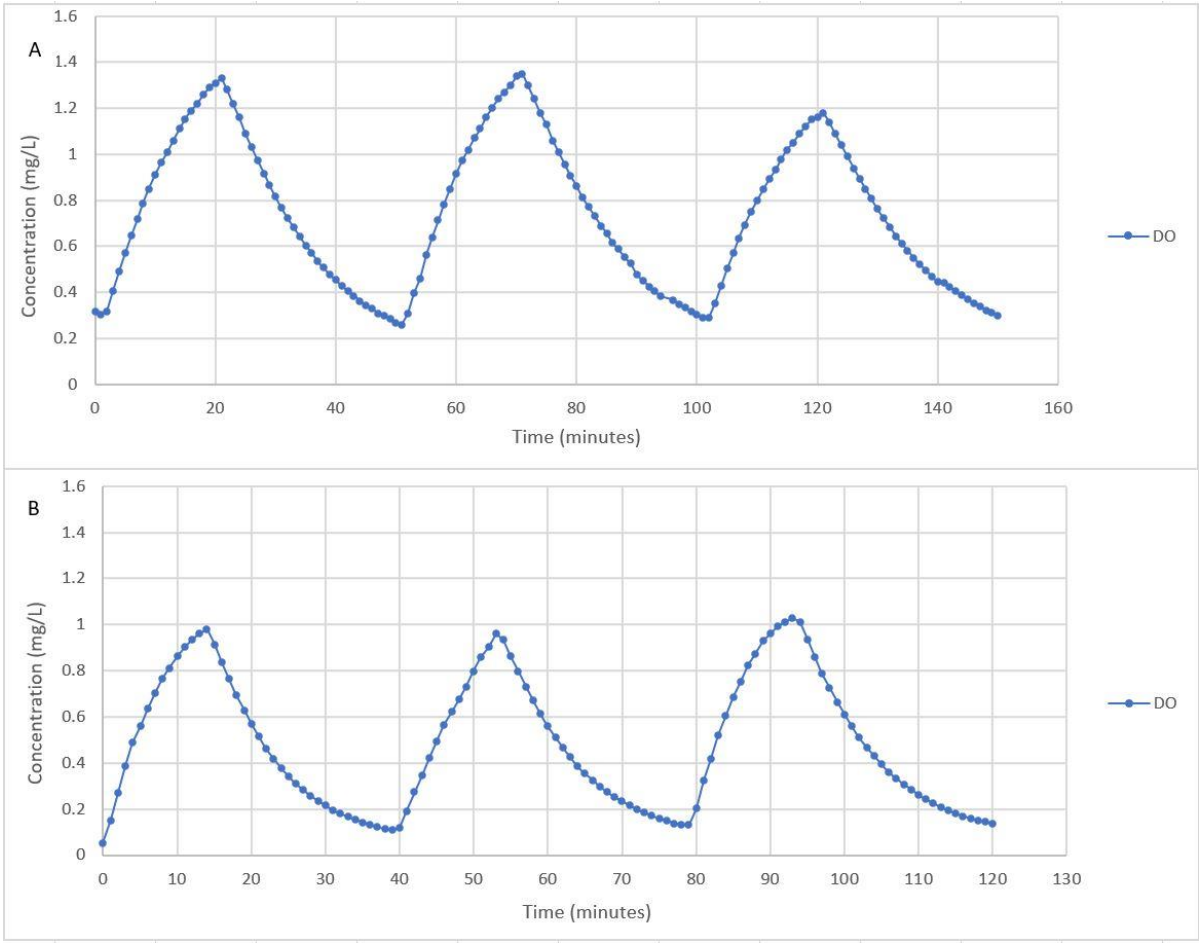

Figure 4.4: DO profile of intermittent aeration cycle. A) Intermittent aeration cycle with cycle length 20 min on/30 min off (IAC-1). B) Intermittent aeration cycle with cycle length 15 min on/25 min off (IAC-2).

In this cycle, as soon as the aeration was ON, the DO start to rise sharply and reached 1 mg/L after 15 min. Therefore, to achieve the desired DO profile, the length of aeration was shorted by 5 min in both aerated phase and non-aerated phase, respectively making the cycle length of 15 min ON / 25 min OFF (IAC-2), as shown in (Figure 4.4-B), which was applied in the reactor from day 59.

However, afterwards when the reactor was subjected to this cycle, a gradual decrement of DO concentration was observed, and reached to 0.5 mg/L and 0.075 mg/L in aerated phase and non-aerated phase (IAC-3), respectively at day 79 (Figure 4.5).

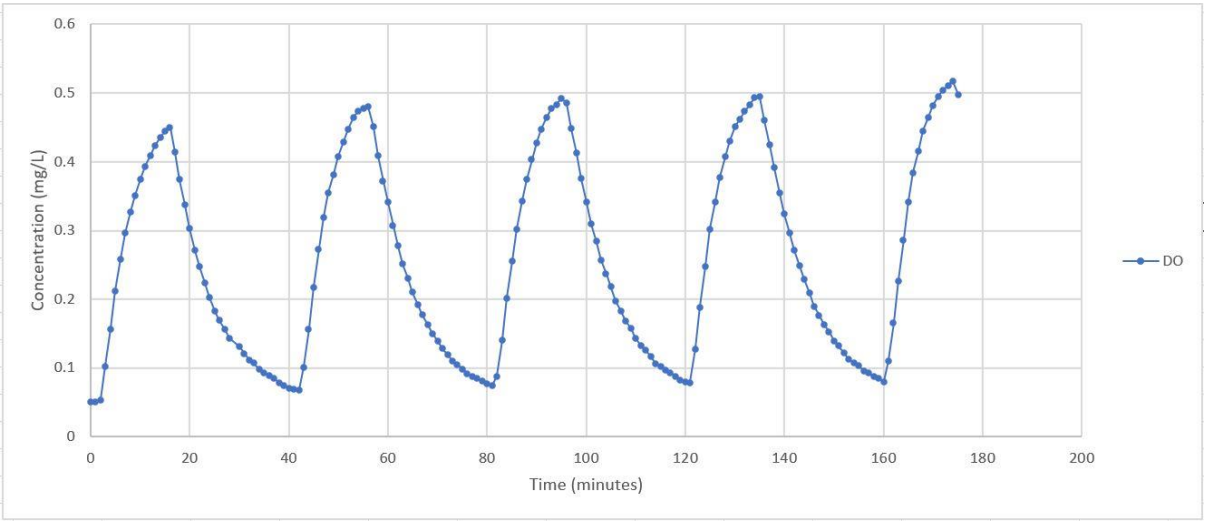

Figure 4.5: DO profile of intermittent aeration cycle with cycle length 15 min ON/ 25 min OFF measured on day 79 (IAC-3).

Based on the observed data of ammonium transformation (Section 4.2.2), from day 85 the cycle length in the non-aerated phase of the IAC-3 was increased by 5min, making cycle length 15 min ON/ 30 min OFF with aerated phase DO same as IAC-3 and non-aerated phase DO around 0.05 mg/L (IAC-4), as shown in the Figure 4.6.

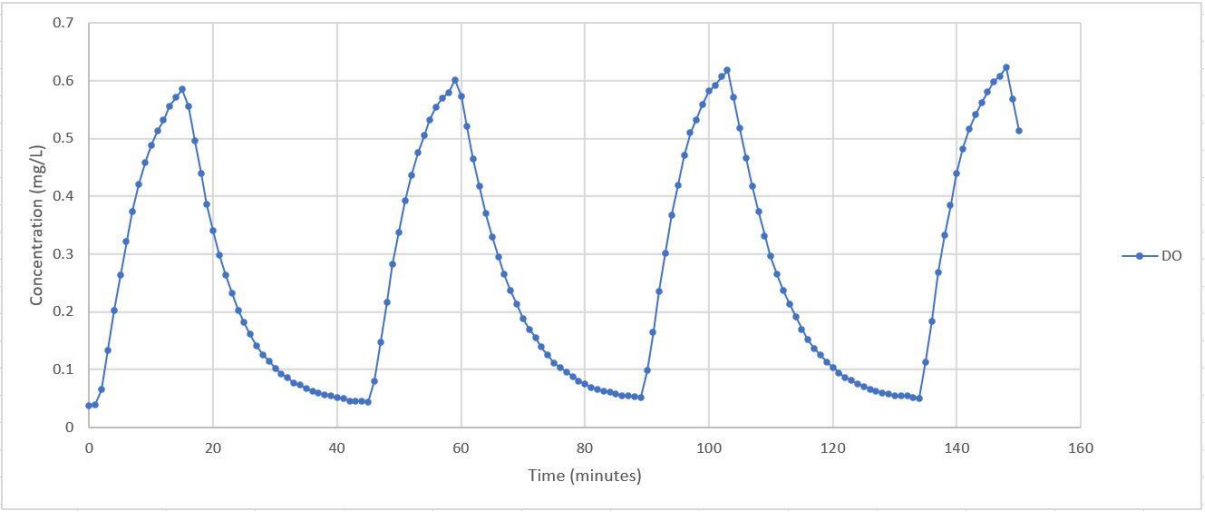

Figure 4.6: DO profile of intermittent aeration cycle with cycle length 15 min ON/30 min OFF (IAC-4).

4.2.2 Effect of intermittent aeration on the nitrogen compounds transformation

The transformation of influent ammonia to other nitrogen compounds when the intermittent oxygen is provided in the reactor is shown in the Figure 4.7. After the application of IAC-2, ARE started to increase gradually over time and became stable from day 76 with at a value of around 45%, resulting the decrement of the outlet NH_4-N concentration. The NAR was low at

the beginning than NPR, but it increased sharply starting from day 64 and became stable with value of around 75% from day 78 (Figure 4.7-B). The NO_3-N in the outlet was stable in the beginning, signifying the consumption of high ammonia with time was accumulated as NO_2-N. However, it started to decrease slowly from day 70 and increased this intensity from day 79. As a result, NPR reached around 29% on day 83. This result in favorable condition in ARE for partial nitritation. However, NPR was still high. Hence the aeration cycle length was decided to change.

The extended intermittent aeration (IAC-4) results in a slight decrement of ARE, and NPR, causing stable NAR (Figure 4.7-B).

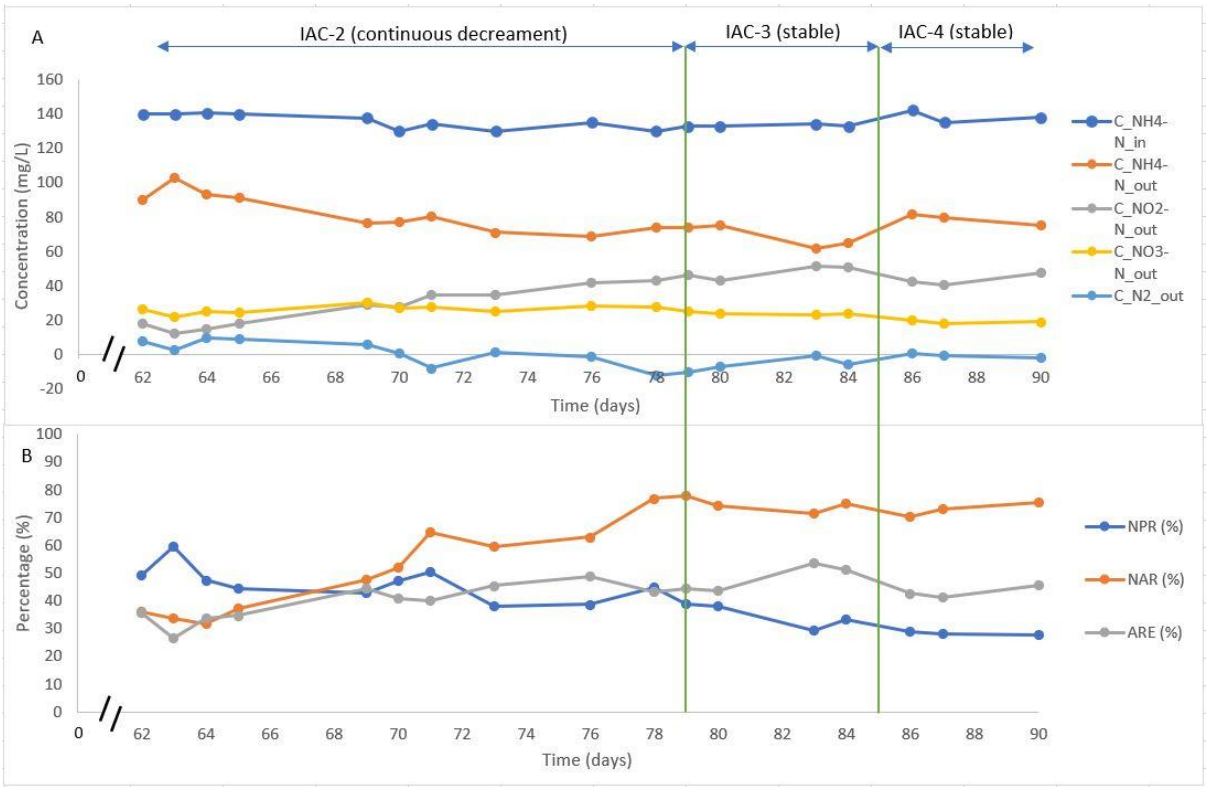

Figure 4.7: Effect of intermittent aeration cycle on nitrogen compound transformation. A) Nitrogen compounds variation in different aeration cycle. B) Nitrogen compounds production rate and ammonium removal efficiency.

4.3 Biofilm quantification

The biomass in the biocarrier was quantified by measuring dry weight, and the composition of the bacteria in the biofilm was measured in terms of OUR.

4.3.1 Biomass weight on carrier

Figure 4.8 depicts the cumulative weight of biomass per carrier over the experimental period. The accumulated biomass weight on the new carriers was significantly low when compared

with the old carriers. Since the cumulative value was nearly constant in both the old and new carriers, almost no measurable biomass was accumulated over time.

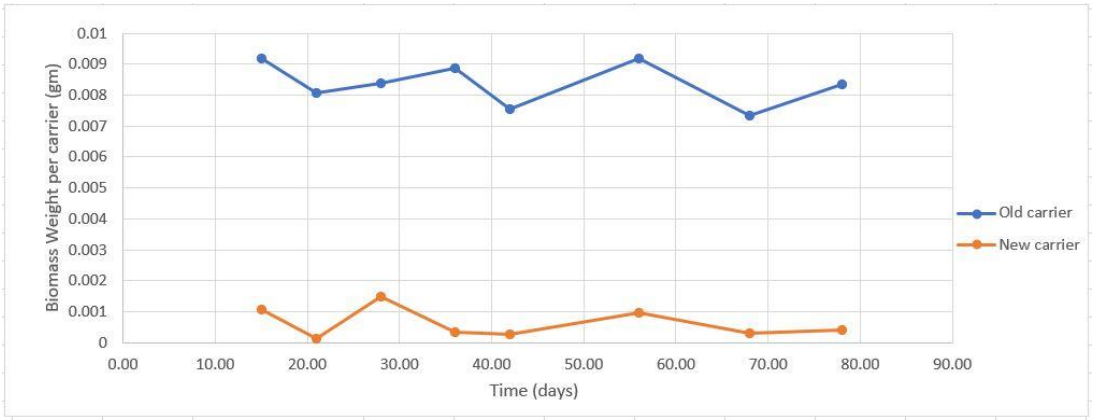

Figure 4.8: The cumulative accumulated of biomass in old and new carriers.

4.3.2 Bacterial composition in the biofilms

The consumption of oxygen by different bacteria groups in the suspended liquid and mixed suspended liquid and biofilm is shown in the Figure 4.9. For simplicity and to compare with each other, the oxygen taken by suspended liquid and suspended liquid along with the biofilm

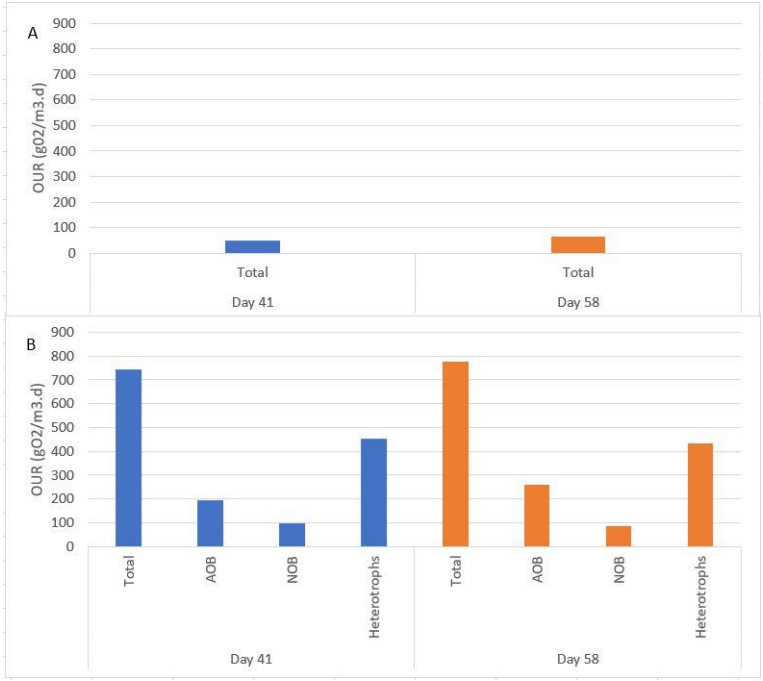

Figure 4.9: Oxygen consumption by different bacteria groups. A) In suspended liquid. B) In combined suspended liquid and biofilm.

was calculated in the same unit (i.e., $gO_2/ (m^3.d)$). As seen from the bar graphs, there were negligible bacteria groups in the suspended liquid on both test days. The oxygen consumption

by AOB, NOB, and heterotrophs was taken around zero since the oxygen concentration never falls below 7 mg/L when OUR experiment was conducted.

There was a significant numbers of bacteria in biofilm as oxygen uptake rate was higher than 700 $g0_2/m^3$.d, and this was in slightly increasing trend over time. The composition of bacterial groups i.e., NOB, AOB, and heterotrophs was in ascending order. Moreover, the composition of AOB was in slightly increasing trends whereas, the NOB was in a very weak decreasing trend over time. Meanwhile, the composition of heterotrophs had stayed consistent and stable on both experimental days.

4.4 pH and Alkalinity variations

The concentration variation of alkalinity as $CaCO_3$ in the inlet and outlet over the experimental period is shown in the Figure 4.10. The inlet concentration was higher than outlet for all time. For the first 21 days, the influent alkalinity concentration was 978± 75 mg/L, then it was maintained stable around 490±57 mg/L for the rest of the days. The effluent had low alkalinity for the first 13 days, which was around 78±27 mg/L. Then this concentration sharply increased to around 400 mg/L on day 14. Afterwards, this concentration was decreased gradually over time and was stable starting from day 28 with an average value of 118± 41 mg/L. However, between days 57-65 and 71-79, high and low alkalinity concentrations values, respectively were observed.

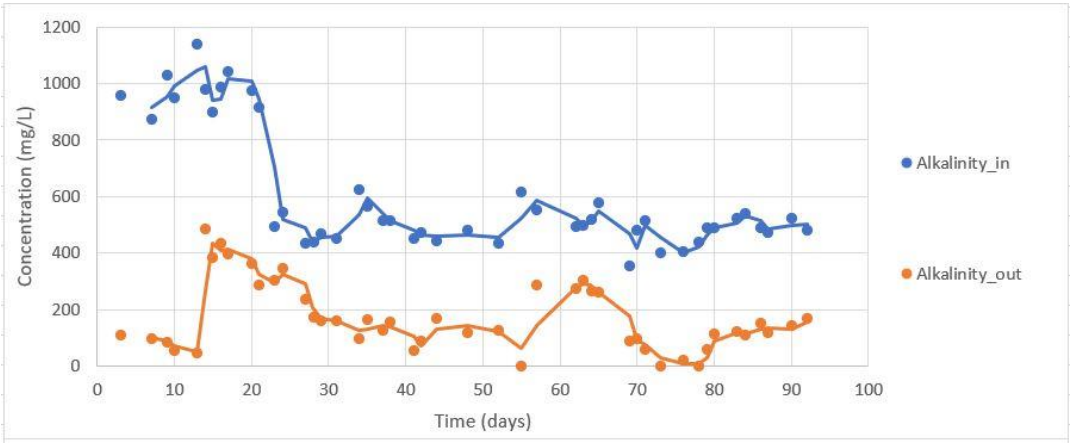

Figure 4.10: Alkalinity variations in inlet and outlet.

Figure 4.11 depicts the variations in pH over time in the inlet and outlet of the reactor. The influent had stable pH throughout the experimental period with an average value of 8.1±0.12. The influent pH was slightly higher than the effluent pH, which had an average value of 7.8±0.25.

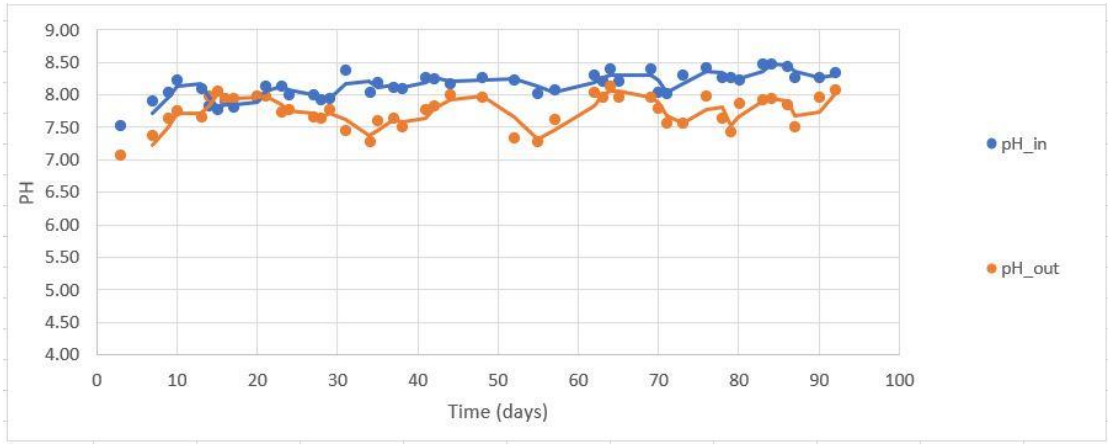

Figure 4.11: pH variations in inlet and outlet.

5 Discussion

In this section, the results are further explained and compared with the literature to identify key findings of the process progress and to come up with some conclusion.

5.1 Synthetic medium concentration and operating conditions

The influent ammonia concentration was decided to maintain around 140 mg/L throughout the experimental period. For the anammox process start-up, this concentration was chosen based on two factors: i) To encourage anammox and AOB while suppressing NOB, free ammonia (FA) levels should be between 1 and 10 mg/L [81] and ii) the nitrite concentration should be less than 60 mg/L; otherwise, the anammox process will be inhibited [82]. The selected ammonium concentration resulted in FA below 10 mg/l (Equation 2.10) with pH and temperature of 7.8 and 30^{o}C which were the favorable operating parameter for the anammox process (Section 2.2.3.1.4), respectively. However, during the experimental sometimes pH crossed 8 and resulted in high free ammonia. Furthermore, partial nitritation of this ammonium concentration produces the above-mentioned nitrite concentration as well as a sufficient stoichiometric ratio of substrates for the anammox process (Section 2.2.3).

The inlet alkalinity concentration was first maintained around 978±75 mg/L, which is a necessary alkalinity requirement for the full consumption of influent ammonium by AOB (complete nitritation) to develop nitritation biofilms in the new carriers (Section 2.2.1.1). Since the development of the nitritation biofilms is a time-consuming process due to lower cellular growth yield of value between 0.04-0.45 g biomass/gN [83]. Hence, the reactor was operated to achieve partial nitritation and the alkalinity concentration was decreased to 490±57 mg/L from day 33.

5.2 Partial nitritation under different SALR with continuous aeration

There was a considerable amount of nitrogen gas (N_2) generated by the reactor at the beginning (Figure 4.1). This was due to the heterotrophic-denitrifier biofilms since the seeded bio-carrier was from KRA's nitrification-denitrification reactor. However, due to a lack of COD source in the reactor, this production completely ceases on day 8 and thereafter. Again, up until day 13, the NPR was lower than the NAR, indicating that AOB was more involved than NOB. Since the bacterial culture was starved of the substrate while being transferred from the KRA to the lab reactor, the AOB recovered faster than the NOB, with recovery times of 4 days [84] and one week to one month [85], respectively.

After day 13, NPR was always higher than NAR in different SALR under continuous aeration (Figure 4.1- 4.2). The reactor was operated with different DO/TAN ratio ranging from 0.005-0.04, a far lower value than 0.25 for NOB suppression according to Bartrolí et.al [86]. Also, the FA was higher than 1 mg/L throughout this period (Appendix D), enough for the NOB suppression (Section 2.4.2). Despite maintaining both conditions, the NOB activity was higher in the reactor. In the same way, Schopf et al. [87] could not achieve partial nitritation in MBBR reactor with 0.11 DO/TAN below SALR of 6.5 gN/m^2.d, this was due to the relatively smaller

thickness of biofilm, resulting in the sufficient diffusion of substrates in the inner part of biofilm, where the NOB lies. Similarly, Choi et al. [88] observed partial nitritation above 2.16 kg-N/m^3.d SALR. The qPCR (quantitative polymerase chain reaction) analysis showed that there was a high amount of Nitrospira spp was present in the biofilm, causing unsuccessfulness in getting partial nitritation under the lower SALR. The Nitrospira spp are the k-strategists (Lower substrate concentration with high affinity) type NOB bacteria that are predominant in low-strength ammonia wastewater having DO below 2 mg/l [89], [90]. Furthermore, suppression of NOB by higher FA than inhibition range is not promising solution since the NOB becomes more resilient to FA inhibition over time [91].

Based on the above findings, it is clear that the higher ammonia surface loading rate gives stable partial nitritation under continuous aeration, as in our case, 0.27 g-N/m^2.d SALR was a bit closer to partial nitritation than 0.15 g-N/m^2.d SALR in same DO/TAN (Figure 4.3). However, the actual cause of failure in partial nitritation up to 0.27gN/m^2.d SALR was either by the growth of Nitrospira spp or the by limited thickness of biofilms, and should be confirmed by qPCR analysis.

5.3 Achieving partial nitritation via intermittent aeration

A higher SALR than 0.27 gN/m2.d was not applied to remove NOB. This is because increasing the SALR necessitates either increasing the flow rate or increasing the inlet ammonia concentration. Both approaches are ineffective for anammox growth since a higher flow rate results in a far lower HRT than the Klaus et al. [92] recorded for a successful startup of 33 hours, whereas a higher ammonia concentration results in a higher free ammonia concentration than 10 mg/L. Therefore, the aeration strategy was changed from continuous to intermittent from day 59.

After the IAC-2 was first introduced, ARE decreased from an average of 44% to 36% on day 62 and an even lower value on day 58. This was due to the biofilm's inability to adapt to its new surroundings [93]. Until day 79, the biofilms gradually adapted to its new environment by consuming DO from the bulk liquid and completely adapted after that because DO in the intermittent aeration cycle remained constant at IAC-3. Even though ARE remained stable after day 76, the NAR increased while the NPR decreased over time, indicating the sign of partial-nitritation. The suppression of the NOB by intermittent aeration is due to the different aspect as described in Section 2.4.3.

Since IAC-4 gives the reactor more anoxic time than IAC-3, the reactor's ARE had decreased slightly. In this condition, the NAR remained stable while the NPR steadily decreased, indicating that NOB was suppressed more than IAC-3. This is due the decay rate of NOB was higher than AOB in anoxic phase [94].

5.4 Active biofilm on biocarriers

Since both carriers have around constant accumulated dry weight throughout the experiment, almost no net significant biomass was growing over time. This was due to the low cellular yield of autotrophic bacteria, low strength ammonium wastewater, and the no biomass in the synthetic medium. The new carrier had almost zero biomass weight throughout the experiment, which suggests that the fast development of biofilm needs some source of biomass from

influent. If the reject water is used as the influent, it could result in a significant increase in dry weight on both carriers because the reject water contains a significant amount of bacteria [8].

The OUR test shows a negligible number of active biofilms in the suspended liquid compared to the biofilms, suggesting the complete attached growth process. Since the reactor was subjected to favourable conditions of AOB for a long time, the composition of the AOB grew over time. However, the NOB composition was very weakly decreasing over time despite the unfavourable conditions to grow. This is because the KRA's biofilm contains a considerable amount of NOB during seeding, and it is difficult to wash out after it has been attached to the carrier [95]. In addition, the heterotrophic composition was higher on both days, which was impossible due to a lack of COD source and no denitrification because N_2 gas production was near zero after day 8. The higher composition of these bacteria was resulted from imperfect inhibitor mixing with liquid during the OUR experiment.

5.5 pH and Alkalinity variations

Since nitritation produces H^+ ions (Equation 2.1), some of the inlet alkalinity was used to neutralize them, resulting in an outlet alkalinity concentration that was always lower than the inlet. Moreover, in our case, the high concentration of $HCO3^-$ gives high alkalinity concentration which result in high pH, and vice versa. Therefore, most of the time the outlet pH was lower than the inlet. Furthermore, the alkalinity in the outlet was higher between days 57 and 65 due to low H^+ ion production, as ARE was low during those time.

5.6 Anammox start-up

The anammox process was not seen as no N_2 was observed throughout the experimental period except beginning. The suppression of the NOB bacteria was necessary for the partial-nitritation, which provides sufficient substrate for the anammox start-up. Different techniques were used to achieve partial nitritation, and intermittent aeration was eventually effective in bringing the reactor close to partial nitritation. Because each of these techniques had to be maintained for a certain period in order to see how they affected the operation. Furthermore, the washout of pre-establish NOB bacteria from bio carrier is a lengthy procedure (Refer to section 5.4). The limited-time availability in the thesis and the above-mentioned reasons led to an unsuccessful anammox start-up.

However, from this condition over time it possible that anammox process can be achieved. The intermittent aeration of cycle length 15 min ON/ 30 min OFF also could not achieve complete partial- nitritation because some NOB activity is still there. Therefore, the cycle length in the aerated phase should be decreased to make the aeration cycle more alternating aerobic/anoxic than before because this is the most effective factor in intermittent aeration for NOB suppression [96] . Once the complete nitritation is achieved, appropriate seeding should be added for the fast start-up of the anammox process. The seeding can be done either using specific anammox sludge, or sludge from the anaerobic digester [97], or sludge from the denitrifying basin [98].

6 Conclusion

The anammox process was not achieved during the experimental period allocated due to the bulk of the time was spent suppressing the NOB bacteria for partial-nitritation since the attached NOB were very challenging to wash out. Various methods were used, including different loading rates with continuous aeration and intermittent aeration cycles of varying lengths of aeration cycles to achieve the partial-nitritation. The laboratory analysis data under these operating conditions are compared to various literature to conclude the reactor's overall performance, which is listed below.

- The influent ammonium concentration and operating conditions should be chosen to encourage anammox and AOB growth while suppressing NOB growth for the start-up of PNA process.
- The ratio of DO/TAN is a critical operating condition in the PNA process.
- It is impossible to achieve partial nitritation in relatively low SALR with continuous aeration.
- The higher free ammonia solely cannot suppress NOB due to its resilient characteristics.
- The intermittent aeration cycle is a promising strategy to achieve the partial-nitritation by suppressing NOB under low SALR.
- The autotrophic biofilm takes a long time to develop, so the reject water should be used as a feed to speed up the process.
- It is difficult to remove the biofilms from the bio carrier once it is attached.
- The nitritation process, alkalinity concentrations, and pH concentrations are closely related with each other in the PNA process.
- Due to a limited of time in the thesis, the quick anammox start-up could not be completed.

7 Future works

- To further suppress NOB, the aerated duration in the intermittent aeration cycle should be reduced to allow for further alternating of aerobic and anoxic conditions.
- To gain a better understanding of the bacterial composition on the biofilm, microbial analysis should be performed.
- A close monitor of the reactor and frequent laboratory analysis should be done to keep the reactor on track.
- Seeding of either anaerobic digestor sludge or sludge from the denitrifying basin should be done for the quick anammox start-up after achieving perfect partial-nitritation.

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Appendices

Appendix A **Master thesis task description**

Appendix B **Quantity calculation of NH_4Cl and $NaHCO_3$**

Appendix C **Nitrogen Mass Balance**

Appendix D **Measured data and safety forms**

Appendix A Master thesis task description

University of South-Eastern Norway

Faculty of Technology, Natural Sciences and Maritime Sciences, Campus Porsgrunn

FMH606 Master's Thesis

Title: Study on the Start-Up of Anammox Process in Lab-Scale Moving Bed Biofilm Reactor (MBBR)

USN supervisor: Eshetu Janka Wakjera, Hildegunn H. Haugen and Carlos Dinamarca

External partner: Shuai Wang, Biowater Technology http://www.biowatertechnology.com

Task background:
The general purpose is to study the important parameters to establish anammox process in Moving bed biofilm reactors (MBBR). Anammox is a biological process for the treatment of ammonium rich wastewater such as reject wastewater treatment. However, fast start-up of anammox process is challenging due to low cellular yield and long generation time. Previous experiments conducted to establish anammox process in MBBR reactors at Knarrdalstrand wastewater treatment plants (KRA) was not successful due to various biological, chemical and physical reasons. Hence, this study is a continuation of previous projects to establish anammox process in laboratory scale reactor with a mixture of activated sludge as inoculum and ammonium rich synthetic wastewater.

Task description:
Main task:- Obtain right conditions that promote annamox process in the MBBR system.
Sub tasks:

- Generate relevant experimental data for fast start-up of anammox process in MBBR with synthetic wastewater, low aeration, oxygen concentration, mixing and various hydraulic loading rates (i.e. ammonium concentration).
- Analysis generated data, nitrogen mass balance and conduct literature reviews for comparing with traditional annamox systems.
- Modify and apply standard activated sludge models to model, simulate and interpret the experiment data at experimental conditions (e.g. biofilm, anammox process).

Student category: EET or PT students
Practical arrangements: Experimental work, analysis and theoretical work at USN.
Supervision:- As a general rule, the student is entitled to 15-20 hours of supervision. This includes necessary time for the supervisor to prepare for supervision meetings (reading material to be discussed, etc).

Signatures:

Supervisor (date and signature): 21/1/21

Student (write clearly in all capitalized letters): SABIN PATHAK

Student (date and signature): 20-01-2021

Address: Kjølnes ring 56, NO-3918 Porsgrunn, Norway. **Phone:** 35 57 50 00. **Fax:** 35 55 75 47.

Appendix B Quantity calculation of NH_4Cl and $NaHCO_3$

The amount of NH_4Cl and $NaHCO_3$ used to achieve the desire concentration of NH_4-N and Alkalinity as $CaCO_3$ in the synthetic medium is based on the following calculation.

1. Conversion from NH_4CL to NH_4-N:

When NH_4Cl is dissolve in water, it dissociates to give following product as in the Equation B.1.

$$NH_4Cl \rightarrow NH_4^+ + Cl^- \qquad (B.1)$$

Hence, 1 mol of $NH_4Cl \rightarrow$1 mol of NH_4^+

Molar mass of NH_4Cl and NH_4^+ are:

MW_{NH4CL} = 14+4+35.5 g/mol

MW_{NH4+}= 18 g/mol

Therefore, 53.5 g $NH_4CL \rightarrow$18 g of NH_4^+

53.5 g $NH_4CL \rightarrow$18*(14/18) g of NH_4^+-N

1 g NH_4-N = 3.8214 g NH_4Cl

Therefore, we can conclude that 1 mg/l NH_4-N is equivalent to 3.8214 mg/l NH_4Cl.

2. Conversion from $NaHCO_3$ to $CaCO_3$:

Assume, for the calculation, the concentration of $NaHCO_3$ is 1 g/L.

When $NaHCO_3$ is dissolved in water, it dissociates to give following product as in the Equation B.2.

$$NaHCO_3 \rightarrow Na^+ + HCO_3^- \qquad (B.2)$$

Hence, 1 mol of $NaHCO_3 \rightarrow$ 1 mol of HCO_3^-

Molar mass of $NaHCO_3$ and HCO_3^- are:

MW_{NaHCO3}= 84.01 g/mol

MW_{CaCO3} = 100 g/mol

Therefore, 84.01 g $NaHCO_3 \rightarrow$ 61 g HCO_3^-

1 g $NaHCO_3 \rightarrow$ 0.72 g HCO_3^-

In the neutral liquid, presence of carbonate ions in the form of either HCO_3^- or $CaCO_3^-$ or combination of both mainly gives alkalinity. In our case, since we are adding $NaHCO_3$, HCO_3^- is responsible for alkalinity of synthetic medium.

Equivalent weights:

Eq.wt of HCO_3^- = 61/ 1(charge) = 61 g/Eq

Eq.wt of $CaCO_3$ = 100/2 (oxidation state) = 50 g/Eq

No.of equivalent of HCO_3^- = 0.72 (g/L) / 61 g = 0.0118 eq/L

Alkalinity as $CaCO_3$ = No.of equivalent of HCO_3^- * Eq.wt of $CaCO_3$

= 0.118*50 = 0.59 g/L

Therefore, we can conclude that 1 mg/L $NaHCO_3$ is equivalent to 0.59 mg/L as $CaCO_3$.

Appendix C Nitrogen Mass Balance

The Nitrogen gas (N_2) gas production from the reactor was calculated by using nitrogen mass balance equation as shown in Equation C.1. In this mass balance, no production of nitric oxide (NO) and nitrous oxide (N_2O) was considered.

$$C_{NH4\text{-}N,in} + C_{NO2\text{-}N,in} + C_{NO3\text{-}N,in} = C_{NH4\text{-}N,out} + C_{NO2\text{-}N,out} + C_{NO3\text{-}N,out} + C_{N2} \quad (C.1)$$

Where, $C_{NH4\text{-}N,\ in}$ = Inlet ammonium concentration [mg/L]

$C_{NH4\text{-}N,\ out}$ = Outlet ammonia concentration [mg/L]

$C_{NO3\text{-}N,\ in}$ = Inlet nitrate concentration [mg/L]

$C_{NO3\text{-}N,\ out}$ = Outlet nitrate concentration [mg/L]

$C_{NO2\text{-}N,\ in}$ = Inlet nitrite concentration [mg/L]

$C_{NO2\text{-}N,\ out}$ = Outlet nitrite concentration [mg/L]

C_{N2} = Nitrogen gas concentration [mg/L]

Appendix D Measured data and safety forms

The data obtained from laboratory analysis and safety forms are available in Microsoft teams.

The address of Microsoft teams is given below:

https://teams.microsoft.com/_#/school/files/General?threadId=19%3A08265a44cdf942319a92a6e184b04ec3%40thread.tacv2&ctx=channel&context=Anammox%2520Reactor%2520Process%2520hall&rootfolder=%252Fsites%252FKRAProject%252FShared%2520Documents%252FGeneral%252FAnammox%2520Reactor%2520Process%2520hall

Appendices