EFFECTS OF STARVATION ON ANAMMOX PROCESS AND BACTERIA

AIDIL FAIZIE BIN SHADAN

UNIVERSITI SAINS MALAYSIA

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EFFECTS OF STARVATION ON ANAMMOX PROCESS AND BACTERIA

by

AIDIL FAIZIE BIN SHADAN

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LIST OF ABBREVIATIONS

AMO	Membrane-bound ammonia monooxygenase
Anammox	Anaerobic Ammonia Oxidation
AOB	Ammonia Oxidizing Bacteria
CANON	Completely Autotrophic Nitrogen Removal Over Nitrite
EPS	Extra polymeric substances
FNA	Free nitrous acid
HAO	Hydroxylamine oxidoreductase
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
NLR	Nitrogen Loading Rate
NOB	Nitrite-oxidizing bacteria.
TN	Total Nitrogen
SHARON	Single reactor system for High activity Ammonium Removal Over Nitrite
WWTPs	Wastewater treatment plants

KESAN KEBULURAN TERHADAP PROSES ANAMMOX DAN BAKTERIA

ABSTRAK

Sistem Anammox mengandungi ammonium dan nitrit, sebagai satu-satunya penderma elektron dan penerima elektron, manakala karbonat merupakan satu-satunya sumber karbon yang disediakan. Kehadiran nitrit dan ketiadaan penderma elektron organik adalah penting untuk aktiviti Anammox. Kajian kebuluran telah dijalankan kerana kebuluran bakteria adalah fenomena yang biasa di alam dan dalam proses rawatan air buangan, terutama di bawah keadaan persekitaran yang bervariasi dan rumit dalam biofilem. Tiga jenis kebuluran diperhatikan semasa kajian ini, iaitu kelaparan nitrit, kelaparan amonia dan kelaparan kedua-dua nitrit dan amonia dengan penambahan hidrazin. Jenis kebuluran ini disiasat dengan tempoh masa kebuluran yang sama selama 15 hari pada suhu operasi 35 ˚C. Semasa tempoh kebuluran, kepekatan komponen nitrogen (ammonia dan nitrit) di dalam air sisa diperhatikan untuk mengkaji kesan kebuluran pada proses Anammox. Sementara, Cecair Bercampur Campur Pepejal (MLSS), Cecair Becampur Campir Pepejal Meruap (MLVSS) dan bahan polimer tambahan (EPS) bakteria anammox diperhatikan semasa tempoh kebuluran untuk mengkaji kesan kebuluran bakteria. Telah didapati bahawa, kebuluran nitrit dalam proses Anammox memberikan sedikit penyingkiran kepekatan ammonia dalam air sisa yang dari 50 mg/L hingga 33.8 mg/L selepas tempoh kebuluran. Seterusnya, kebuluran ammonia dan kebuluran kesuluruhan dengan penambahan hidrazin masing-masing menghasilkan penghapusan nitrit dan hidrazin. Jumlah kelaparan dengan penambahan hydrazine memberikan kepekatan tertinggi MLSS dan MLSS, iaitu 250 mg / L dan 170 mg / L masing-masing.

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ABSTRACT

Anammox system contained ammonium and nitrite, as the only electron donor and electron acceptor, respectively, while carbonate was the only carbon source provided. The presence of nitrite and the absence of organic electron donors are essential for Anammox activity. Starvation studies have been carried out because bacterial starvation is a common phenomenon in nature and in wastewater treatment process, especially under conditions of varying influent and complicated environment in the biofilm. Three types of starvation were observed during this study, which is starvation of nitrite, starvation of ammonia and starvation of both nitrite and ammonia with addition of hydrazine. These types of starvation were investigated with same period of starvation time of 15 days at operating temperature of 35 ˚C. During the starvation period, the concentration of nitrogen component (ammonia and nitrite) in the wastewater were observed to study the effects of starvation to the Anammox process. While, Mixed Liquor Suspended Solid (MLSS), Mixed Liquor Volatile Suspended Solid (MLVSS) and extra polymeric substances (EPS) of the anammox bacteria was observed during the starvation period to study the effects of starvation to the bacteria. It have been found that, starvation of nitrite in the Anammox process gives small removal of ammonia concentration in the wastewater which is from 50 mg/L to 33.8 mg/L after the starvation period. While, starvation of ammonia and total starvation with addition of hydrazine result in total removal of nitrite and hydrazine respectively. Total starvation with addition of hydrazine give the highest concentration of MLSS and MLSS, which is 250 mg/L and 170 mg/L respectively.

CHAPTER ONE

INTRODUCTION

1.1 Biological Wastewater Treatment

Industrial activities release large quantity of polluted wastewater. The contamination of water bodies with such wastewater results in aquatic ecological disability and causes various diseases, such as kidney failure, bone deformation, skin disease, and cardiovascular abnormalities. Industrial wastewater can be treated using physical, chemical and biological methods. Physical method involved in the beginning of wastewater treatment to separate large solid from the liquid. Chemical and biological treatment involved when wastewater cannot be treated by using physical methods. Biological treatment is an important and integral part of any wastewater treatment plant that treats wastewater from either municipality or industry having soluble organic impurities or a mix of the two types of wastewater sources. The obvious economic advantage, both in terms of capital investment and operating costs, of biological treatment over other treatment processes like chemical oxidation and thermal oxidation has cemented its place in any integrated wastewater treatment plant (Mittal, 2011). The major application of this treatment is for removal of soluble organic matter. This occurs as the microorganisms use it as a food source, converting a portion of the carbon into new biomass. Other than that, biological treatment convert soluble inorganic matter. Since the discovery during the 1960s of the effects of eutrophication cause by nitrogen and phosphorus, engineers have been concerned about the removal of inorganic nutrient from wastewater (Grady Jr et al., 2011).

1.2 Nitrogen Removal from Wastewater

Biological wastewater treatment also known as the secondary treatment in wastewater treatment plant. The focus in this treatment is to remove waste that cannot be removed during pre-treatment and primary treatment using activated sludge. In this biological wastewater treatment, nitrogenous components in the wastewater will removed from the wastewater and released to atmosphere in the form nitrogen gas. The biological removal involves two processes, namely, nitrification and denitrification.

Nitrification is the process by which ammonia is first converted to nitrite by ammonia-oxidizing bacteria (AOB) oxidize ammonium (NH_4^+) to nitrite (NO_2^-) via hydroxylamine (NH_2OH) as shown in Equation 1 and 2 below. Membrane-bound ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) are involved in these two reactions. In the second step, nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate (NO_3^-) with the involvement of membrane-bound nitrite oxidoreductase (NOR) (reaction 3) (Zhu et al., 2008).

$$NH_3 + O_2 + 2[H] \xrightarrow{AMO} NH_2OH + H_2O \quad (1)$$

$$HN_2OH + 0.5O_2 \xrightarrow{HAO} NHO_2 + 2H^+ + 2e^- \quad (2)$$

$$NO_2^- + 0.5O_2 \xrightarrow{NOR} NO_3^- \quad (3)$$

In anoxic denitrification, NO_3^- and NO_2^- are reduced to gaseous nitrogen with a variety of electron donors, such as methanol, acetate, and organic substances in wastewater (reactions 4 and 5) (Zhu et al., 2008). Gaseous nitrogen is produced from nitrite and nitrate during denitrification (Cheremisinoff, 1997).

$$2NO_3^- + 10H^+ + 10e^- \rightarrow N_2 + 2OH^- + 4H_2O \quad (4)$$

$$2NO_2^- + 6H^+ + 6e^- \rightarrow N_2 + 2OH^- + 2H_2O \quad (5)$$

1.2.1 Forms of Nitrogen

Nitrogen has the ability to exist in seven oxidation states, ranging from minus 3 to plus 5, and therefore, is found in many compounds. In wastewaters, nitrogen may be found in four forms: organic nitrogen, ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen (Cheremisinoff, 1997).

Ammonia nitrogen may exist in aqueous solution as either ammonium ion or unionized ammonia. The relationship between the two forms is pH dependent and may be expressed in accordance with the following equation:

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^-$$

Nitrite nitrogen is unstable and easily oxidized to nitrate compare to nitrate nitrogen. It exists as an intermediate compound during the oxidation of ammonia nitrogen to nitrate nitrogen. If exist in wastewater, the concentration is usually less than 1.0 mg/l (Cheremisinoff, 1997).

1.3 Importance of Removing Nitrogen from Wastewater

Nitrogenous compounds can cause a significant depletion of dissolved oxygen in receiving waters, exhibit toxicity towards fish, and therefore, decrease the productivity of streams and lakes, and present a public health hazard (Cheremisinoff, 1997). Nitrogen in all form can be available as a nutrient and therefore contributes to

eutrophication in aquatic system. Thus, this will harm the environment and removing nitrogen from wastewater is an important process in wastewater treatment.

1.4 Problem Statement

There are several biological methods for removing nitrogenous component from wastewater. The examples of methods are nitrification, denitrification, Anammox (Anaerobic Ammonia Oxidation) and SHARON (Single reactor system for High activity Ammonium Removal Over Nitrite). Anammox and SHARON system contained ammonium and nitrite, as the only electron donor and electron acceptor, respectively, while carbonate was the only carbon source provided. The presence of nitrite and the absence of organic electron donors were essential for Anammox activity. Adequate nitrite and ammonia are needed in the process. Over the years, many researches carry out studies about Anammox process as this process is still not commercialise in industrial scale. Many researchers study about what if the process have inadequate feed. Thus, effects of different types of starvation on Anammox and bacteria has been carry out in this study.

1.5 Research Objectives

1. To study the effect of nitrite and ammonia starvation on Anammox process and bacteria.
2. To study the effects of hydrazine addition during starvation on Anammox process bacteria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Anaerobic Ammonia Oxidation

Anammox process was discovered in the early nineties and has great potential for the removal of ammonia nitrogen from wastewater. The responsible bacteria transform ammonium (NH_4^+) and nitrogen dioxide (NO_2) into nitrogen gas (N_2) and water (H_2O). This saves costs as less energy for aeration and no organic carbon sources (e.g. methanol or recirculated sludge) are required. During the last 20 years, many researches were conducted on the Anammox process. In 2007, the first large-scale Anammox reactor was built in Rotterdam. It displays the vast possibilities of this new process (Kuenen, 2008).

Anammox is a biologically mediated reaction in which ammonium (NH_4-N) is oxidized to nitrogen gas using nitrite (NO_2-N) as the electron acceptor. There are many important benefits for nitrogen removal from wastewater using Anammox compared to conventional process. The benefits of Anammox compared to conventional process are less oxygen demand, external carbon supply and excess sludge production (Park et al., 2017).

2.1.1 The Anammox Process

Anaerobic Ammonium Oxidation, (Anammox) is an innovative technological advancement in the removal of ammonia and nitrogen in wastewater. This new process oxidize ammonia and use nitrite as electron donor and form nitrogen gas (Shivaraman,

2003) in one single process instead of passing through a two stage process of aerobic nitrification and anaerobic denitrification. It was discovered about 15 years ago and has resulted in new opportunities for research and development of sustainable nitrogen removal systems. Compared to conventional nitrification/denitrification in activated sludge systems, Anammox eliminates the necessity of an organic carbon source for nitrification, reduces energy demand for aeration and has a smaller production of excess sludge and lower CO_2 emissions. The Anammox reaction can be represented as below:

$$NH_4- + NO_2- = N_2 + 2H_2O$$

This reaction is carried out by Anammox bacteria (Candidatus Brocadia anammoxidans) belonging to the group of planctomycete (Shivaraman, 2003).

2.1.2 The Activated Sludge

Anammox bacteria, which were discovered in wastewater sludge in the early 1990s, have the unique metabolic ability to combine ammonium and nitrite or nitrate to form nitrogen gas. This discovery led to the realization that a substantial part of the enormous nitrogen losses that are observed in the marine environment up to 50% of the total nitrogen turnover were due to the activity of these bacteria (Kuenen, 2008).

Anammox bacteria have two microbial reactions that feature ammonium as an electron donor for denitrification with nitrite (NO_2^-) (equation 1) and photosynthesis (equation 2)

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

$$4NH_4^+ + 3HCO_3^- \rightarrow 2N_2 + 3CH_2O + H^+ + 6H_2O \quad (2)$$

Observation by Arnold Mulder from Gist Brocades Fermentation Company in their denitrifying pilot plant that is the disappearance of ammonium at the expense of nitrate (NO_3^-) and a clear increase in nitrogen production. 'Anammox' is the given name of the process based on metabolism that involved, which is anaerobic ammonium oxidation.

Other than Equation 1 and 2, there is an intermediate product in the reaction before converting ammonium into nitrogen. The intermediate product is hydrazine as shown in Equation 3.

$$NH_2OH + NH_4^+ \rightarrow N_2H_4 + H_2O + H^+ \qquad (3)$$

Gijs Kuenen and Mike Jetten have done an anammox reaction but nitrogen production ceased within 24 to 48 hours. However, their reaction contained ammonium and nitrite rather than nitrate. Eventually, after several studies they detect hydrazine in the enrichment culture (Kuenen, 2008). Hydrazine is an energy-rich compound that used as a rocket fuel. Indeed, hydrazine is a powerful, yet potentially toxic, molecule that used as an energy source by bacteria.

Candidatus K. stuttgartiensis is a member of Planctomycetes. It contains subcellular compartments, including the anammoxosome, where energy conservation takes place. The sample was high-pressure frozen, freeze substituted and Epon embedded. The riboplasm is the equivalent of the ribosome-containing cytoplasm in most other bacteria. Figure 2.1 below shows transmission electron micrograph of *Candidatus* K. stuttgartiensis. The Photograph courtesy of L. van Niftrik, Radboud University, Nijmegen, The Netherlands (Kuenen, 2008).

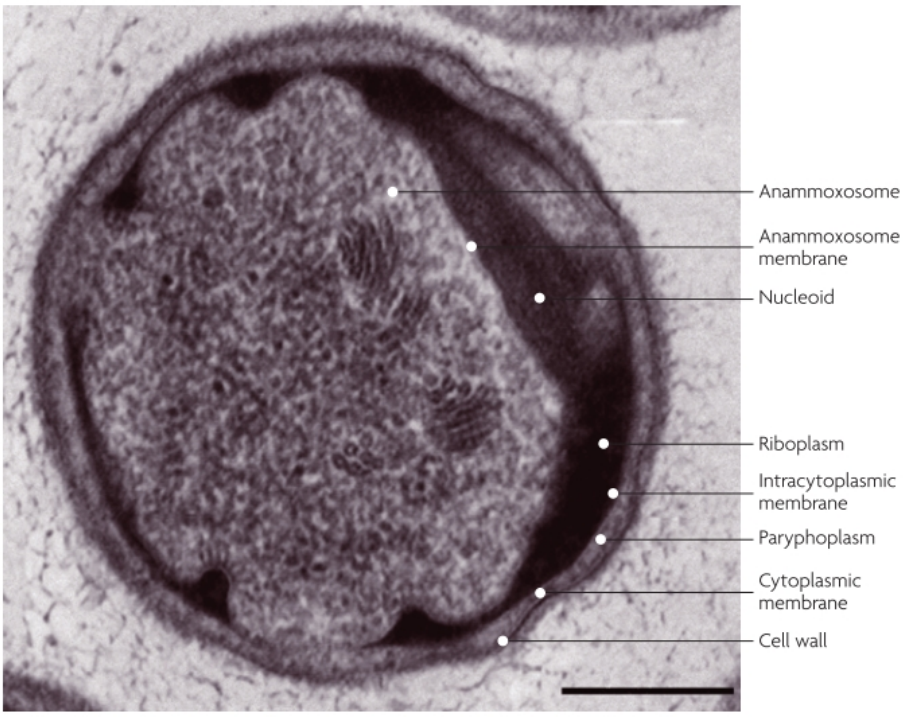

Figure 2.1: Transmission electron micrograph of a Candidatus Kuenenia stuttgartiensis cell (Kuenen, 2008). The scale bar represents 200 nm

2.2 Inhibition of Anammox Process

Anammox process is inhibited by many factors, which prevent improvements on the process as well as the application of the Anammox process. A variety of inhibitory substances, such as substrates (ammonia and nitrite), organic matter (nontoxic organic matter and toxic organic matter), salts, heavy metals, phosphate and sulfide, are commonly present in the practical applications (Jin et al., 2012). The application and industrialization of the Anammox process have been restricted by the growth characteristics of the Anammox bacteria and the widespread inhibition factors existing in nitrogen-rich wastewater. Anammox bacteria have a slow growth rate (their doubling time at 30–40 ˚C is approximately 10–14 day (Van der Star and Strous, 2007).

2.2.1 Substrate Inhibition

In most wastewater treatment plants (WWTPs), ammonium is one of the main forms of nitrogen (S.W.H. Van Hulle, 2010). Nitrogen-containing wastewater, such as sludge digestion liquid, landfill leachate, monosodium glutamate wastewater, nitrogen fertilizer production wastewater, coking wastewater and other industrial wastewater, is often rich in ammonium (Van Hulle, 2010). For nitrite contain, it has been reported that the presence of a high-concentration of nitrite severely inhibited a wide range of microorganisms (Zhou, 2011).

Many researchers have studied the substrate inhibition on Anammox process and the Total Nitrogen Removal when the process is inhibited. Zhang et al. (2016) have done a study about inhibition process caused by high substrate concentration. From their study, it can be concluded that the stability of the anammox reaction was easily disrupted by various factors. Inhibitions were caused by the synergistic effect of high pH, excessive substrate and a lack of K^+. Among the inhibitors, FNA was considered the most toxic factor (Zhang et al., 2016).

Ma et al. have also done a study about substrate inhibition in Anammox process (Ma et al., 2017). One of their study objective is effect of substrate concentration at a constant Nitrogen Loading Rate (NLR). The experiment have phase I until VIII in 442 days period. In phase IV, the substrate concentration was set at 350 mg/L, which resulted in the TN removal efficiency recovering to 82.5% on the 5th day and reached an average efficiency of 84.1 ± 3.9% after continuous operation for 33 (Ma et al., 2017) days as shown in Figure 2.2 below.

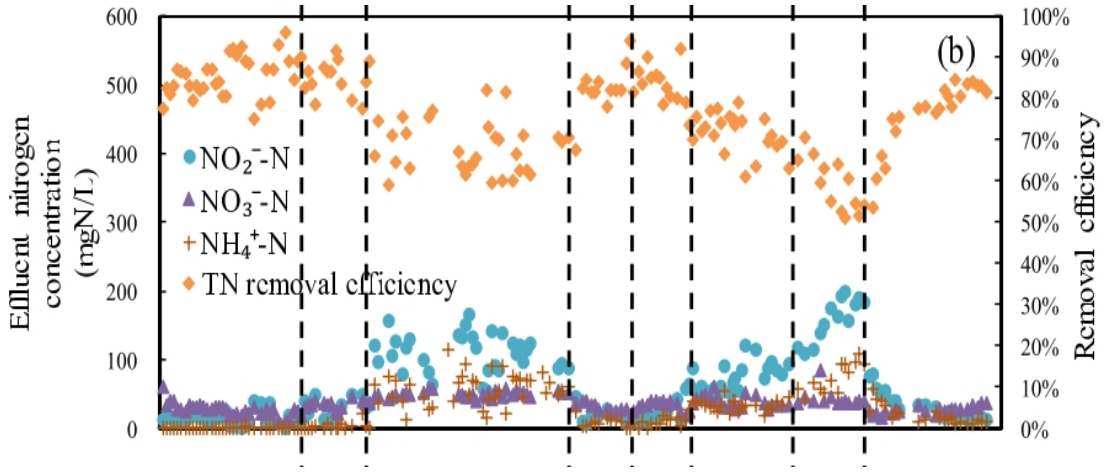

Figure 2.2: Variation of effluent nitrogen compounds and TN removal ratio (Ma et al., 2017)

2.3 Starvation of Anammox Process and Bacteria

Bacterial starvation is a common phenomenon in nature and in wastewater treatment process, especially under conditions of varying influent and complicated environment in the biofilm. One of the most critical aspects for anammox reaction stability is the concentration of substrates, namely ammonium and nitrite (Wu et al., 2015). From a study, it have stated that no matter in the actual industrial wastewater or the natural environment, the fluctuation of substrate concentrations usually occurred (Tang, 2010).

2.3.1 Feed Starvation

Wu et. al. have carry out a study about different starvation stress in order to determine the tolerance of anammox bacteria at 35 ˚C (Wu et al., 2015). From their study, it has been determined that period of starvation tolerance was 4 weeks in the absence of nitrite, 5 weeks in the absence of ammonium, 7 weeks for the absence of these two substrates at 36 ˚C under pH 7 - 8 and anaerobic conditions. Wu et. al. analyse the

bacteria from the starvation process by bacteria settling characterization and extra polymeric substances (EPS) composition after starvation process. Their results well indicated that the starvation stress induced by different substrates depletions resulted in different bacteria settling capacities (Wu et al., 2015). The bacteria aggregation and settling are actually determined by EPS (Song et al., 2017).

Reeve et al. have done a study to validate the laboratory scale results of Wu. et al (Wu et al., 2015) which demonstrated Anammox bacteria cultures starved in synthetic media were able to tolerate four weeks in the absence of nitrite (Reeve et al., 2016). The study by Reeve et al. to investigate the impact of feed starvation on the ability of an industrial scale SBR to regain nitritation and Anmmox acitivity. Characterisation of the microbial abundances Anammox bacteria indicated the starvation period did not have adverse effects on the microbial community. The results indicate that Anammox activity is quiet robust and there is no need for constant feed supply as the bacteria can survive for 60 days as the microbial community can restore within a day or two. This study also indicates that there is no need for additional capital investment to build a large centrate feed storage tank and that Anammox Sequencing Batch Reactor (SBR) can be operated successfully with intermittent dewatering systems without impacting the nitrogen removal rates (Reeve et al., 2016).

2.3.2 Hydrazine Addition

Hydrazine is an important intermediate for Anammox, in which ammonium and nitrite are converted to nitrogen gas. Hydrazine addition is known to improve the nitrogen removal capacity in Anammox-based processes (Ma et al., 2018). Ma et al. have carry out a study to investigate the hydrazine addition on conversion performance for normal

Anammox substrates. The result obtained from this study indicates that hydrazine could increase the Specific Anammox Activity (SAA) in an Anammox system as shown in Figure 2.3 below.

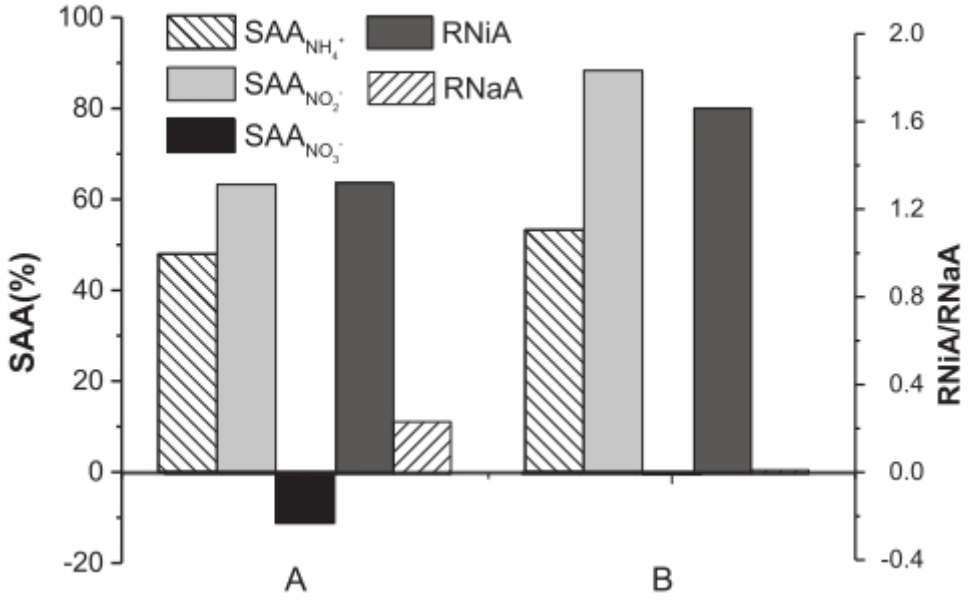

Figure 2.3 : Changes in conversation characteristics of anammox substrates without (A) or with (B) hydrazine addition (Ma et al., 2018)

This result also corroborates the findings by Yao et al. and Xiao et al. (Xiao et al., 2015) which is their study about the effects of hydrazine addition in Completely Autotrophic Nitrogen Removal Over Nitrite (CANON) process (Yao et al., 2013). However, there are variation in their reports on the increase in total nitrogen removal by adding hydrazine. 83.3% reported by Yao et al. (2013) and 200% increases in CANON by Xiao et al. (2015).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Equipment and Chemicals

The list of the equipments and its general use is tabulate in Table 3.1 below.

Table 3.1: List of equipment use in Anammox starvation experiment

Equipment	Purpose
Hot plate magnetic stirrer	Stir the system with constant rate and temperature
Oven	Evaporate water in sample.
Furnace	Burning volatile substance in sample
UV VIS Spectrophotometer	Characterize the sample using wavelength.
Centrifuge	Separate solid from liquid by using microtube.
Sonicator	To agitate the particles in the sample.

The list of chemical used throughout the experiments are list in Table 3.2.

Table 3.2 : List of chemical used in starvation of anammox

Chemical	Range / Purity
Ammonia Salicyclate	0.4 – 50 ppm
Ammonia Cyanurate	0.4 – 50 ppm
Nitrite Reagent	0.2 – 250 ppm
Hydrazine Reagent	0 – 600 µg/L
BCA solution	
Cupric sulfate	4%

Phenol	4%
Sulphuric acid	95 – 97%

3.2 Research Flow Chart

The overall experimental activities was carried out as presented below.

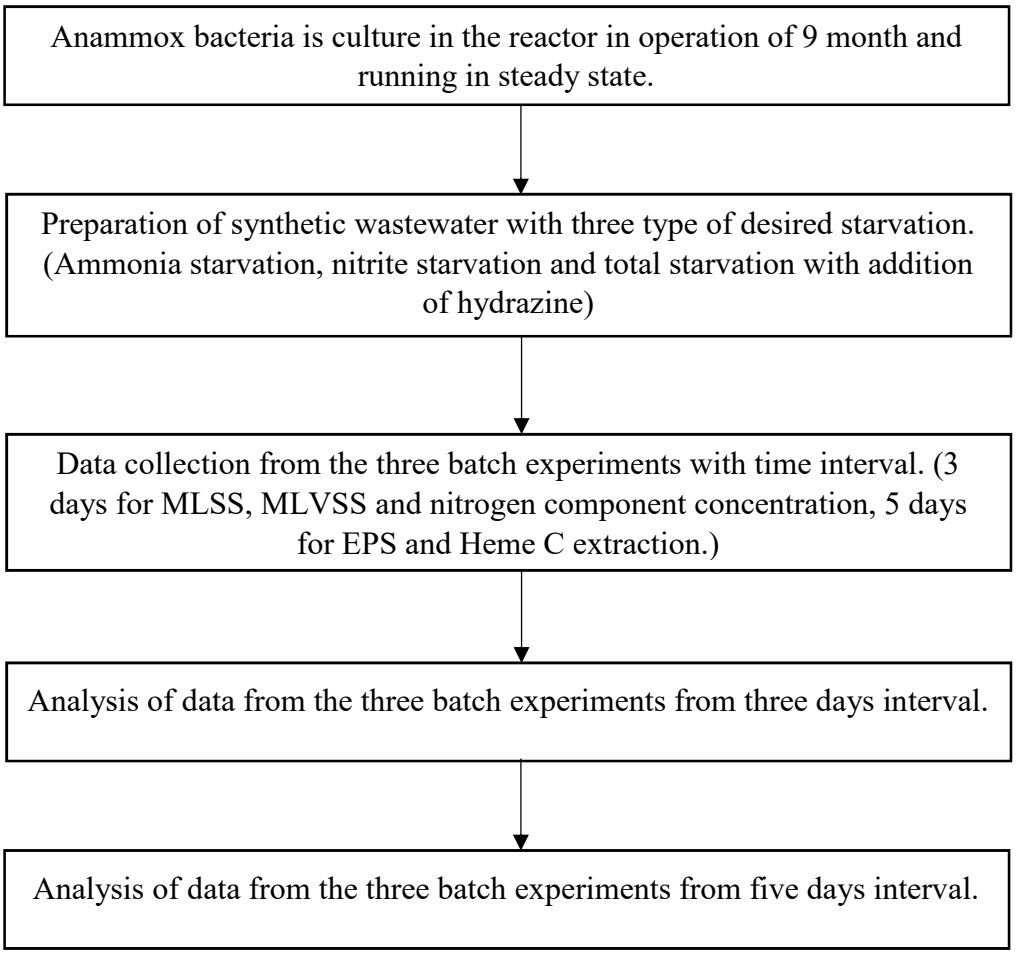

Figure 3.1: Schematic flow diagram of experimental activities

3.3 Experimental Procedure

3.3.1 Anammox Bacteria

In this study, Anammox bacteria used from the main bacteria reactor in the Environmental Laboratory, School of Chemical Engineering. The bacteria reactor have been operate for more than 9 months and running in steady state.

3.3.2 Preparation of Synthetic Wastewater

The composition of synthetic wastewater was (g/L): KH_2PO_4 0.0272, $MgSO_4.7H_2O$ 0.18, $CaCl_2.2H_2O$ 0.12. This composition was fixed. Addition of NH_4Cl 0.3178 and $NaNO_2$ 0.07498 will give different type of starvation. 1 ml of trace elements solutions I and II were added into synthetic wastewater. The trace elements solution I contained (g/L): EDTA 5 and $FeSO_4$ 5 while trace solution II contained EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $NaMoO_4.2H_2O$ 0.22, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21 and H_3BO_4 0.0014. The synthetic wastewater was deoxygenated by flushing with nitrogen gas before place in the 500 ml bottle.

3.3.3 Preparation of Batch Experiments with Different Type of Starvation

In this study, three batch of experiments with 500 ml working volume with different type of starvation were set up. The first batch of experiment was the Anammox system with starvation of nitrite. 0.3147 g of ammonium chloride (NH_4CL) was added into Batch 1 as the ammonia feed. Second batch of experiments was starvation of ammonia in the system. 0.07498 g of sodium nitrite ($NaNO_2$) was added into Batch 2 as the nitrite feed. Finally, last batch of experiments was total starvation with no ammonium

and nitrite but with addition of hydrazine as the feed. 20 ml of hydrazine (N_2H_2) stock solution was added into the Anammox system as Batch 3.

Summary of preparation of batch experiments is tabulated in Table 3.3 below.

Table 3.3: Summary of experimental preparation

Experiment	Type of Starvation	Nitrogen Feed	Concentration (ppm)
Batch 1	Nitrite	Ammonia	50
Batch 2	Ammonia	Nitrite	50
Batch 3	Total starvation	Hydrazine	5

3.3.4 Preparation for Mixed Liquor Suspended Solid (MLSS) and Mixed Liquor Volatile Suspended Solid (MLVSS)

A filter paper was weight before filter the sample. For Mixed Liquor Suspended Solid (MLSS), 10 ml of sample was filtered by using vacuum pump and put in an oven with 100 ˚C for one day. After dry in the oven, the weight filter paper with sample was measured. For Mixed Liquor Volatile Suspended Solid (MLVSS), the previous filter paper with dried sample in the oven was put in a furnace with 550 ˚C. After it cool down, the weight of sample from the furnace was measured.

3.3.5 Preparation for ammonia, nitrite and hydrazine solutions

The ammonia solution was prepared in between 0.4 – 50 mg/L by adding 5 ml of ammonia reagent and 0.1 ml sample from batch experiments. After adding these two in a vial, ammonia salicyclate and ammonia cyanurate were added respectively. Shake the vial well and the reaction will occur about 20 minutes. Nitrite solution was

prepared in between 0.2 – 250 mg/L. 10 ml of sample from batch experiments was added with nitrite reagent in a screw cap test tube. Shake the screw cap test tube well and the reaction will occur about 5 minutes. Hydrazine solution was prepared in between 0 – 600 µg/L. 10 ml of sample from batch experiments range added with 0.5 ml hydrazine reagent in a screw cap test tube. Slowly tilt the screw cap test tube and the reaction will occur about 10 minutes.

3.3.6 Extra Polymeric Substance (EPS) Extraction

The two main substances that were analysed were carbohydrate and protein. When carbohydrate and protein concentrations were total up it equals to EPS. 1.5 ml of sludge was transferred into a 1.5 ml microtube. Than the microtube containing sludge was centrifudged at 12500 rpm for 20 minutes. The supernatants were discard and deionise water is filled into the microtube until finf the volume of 1.5 ml. The sedimentation was stirred by using a sterile needle so that a uniform centrifugation obtained. The microtubes were further centrifudged at the same speed and time. The tubes was taken out, the supernatants were discarded and the sediments were stirred again after filling the tubes with deionised water. This was continued by sonicating the microtubes for 10 minutes under 30˚C. Finally the microtubes are again centrifuged for 30 minutes at 12500 rpm. After centrifugation the supernatants are the extra polymeric substance (EPS) sample.

To determine the protein concentration, test tube assay test was carried out. 50 µL of sample was pipette from the microtubes into screw caped test tubes. Then, 20 µL of 4% Cupric Sulphate was mixed with 1 ml of BCA solution to produce a BCA working reagent. 1 ml of BCA working reagent was transferred into the screw caped test tube

which contain the sample and it was mixed by gentle vortexing. After that, it was incubated in a water bath for 30 minutes at 37 ˚C to allow the reaction to occur. The mixture was cooled to room temperature.

For carbohydrate concentration determination, Phenol-Sulphuric Acid Assay was carried out. 500 µL of EPS sample from the centrifuged and sonicated supernatants were transferred into screw caped test tubes and then added with 500 µL of 4% phenol and 2.5 ml of sulphuric acid.

3.3.7 Heme C Extraction

Covalently bound c-type heme was removed using 2-nitrophenylsulfenyl chloride (Sigma-Aldrich, St. Louis, MO) according to the method of Fontana et al. (1973). SDS-PAGE with a SH- reducing reagent was used to determine the molecular mass of the protein whose heme had been removed.

3.4 Analysis

3.4.1 Nitrogen Component Concentration

For ammonia concentration testing, ammonia salicyclate and ammonia cyanurate were use as the reagent. Add 0.5 ml of sample into the reagent vial. After the addition of these reagents, shake the vial to ensure the sample and reagents well mixed. The vial left for 20 minutes for the reaction to take place. A green colour solution will form indicate the high concentration of ammonia in the sample while a yellow colour solution indicate low concentration of ammonia in the sample.

For nitrite concentration testing, nitrite reagent was use. Add 10 ml of sample into empty screw cap test tube and add nitrite reagent. After the addition of nitrite reagent, shake the vial to ensure the reagent and the sample well mixed. The test tube left for 5 minutes for the reaction to take place.

Then for hydrazine concentration testing, hydrazine reagent was use. Add 10 ml of sample into empty screw cap test tube and add 0.5 ml of hydrazine reagent. Gently tilt the screw cap test tube to mix the solution and avoid bubbles from formed. The test tube left for 10 minutes for the reaction to take place.

The concentration of ammonia, nitrite and hydrazine were read by UV-Vis HACH sprectrophotometer. The result from UV-Vis HACH was in concentration (mg/L) unit.

3.4.2 Protein and Carbohydrate Concentration from Extra Polymeric Substances Extraction

Extra polymeric substances responding data require two test, which are protein and carbohydrate composition testing. BCA solution and cupric sulphate were add into the sample for protein test. While for carbohydrate test, phenol and sulphuric acid were add into the sample. The concentration of protein and carbohydrate were then determine from the calibration curve. From the calibration curve, linear regression (R^2) was plotted and from the straight line equation, the concentration of carbohydrate and protein can be calculated.

3.4.3 Heme C Concentration

Protein concentrations were determined with the BCA protein assay kit (PIERCE, Rockford, IL), using bovine serum albumin as the standard (Smith et al., 1985).

CHAPTER FOUR

RESULTS AND DISCUSSION

This chapter depicts the experimental results obtained and its related discussion which consist of three main sections. The first part shows the substrate removal, which is the nitrogen component at different type of Anammox bacteria starvation. Second part shows the relationship of MLVSS and Heme C in the bacteria in the Anammox system on different type of anammox bacteria starvation. Finally, the relationship of extra polymeric substance and MLVSS on different type of anammox bacteria starvation.

4.1 Effects of Starvations to the Anammox Process

Figure 4.1 shows the substrate concentration profile during the starvation period of Batch 1. Batch 1 contain ammonia substrate and the bacteria was starved from nitrite.

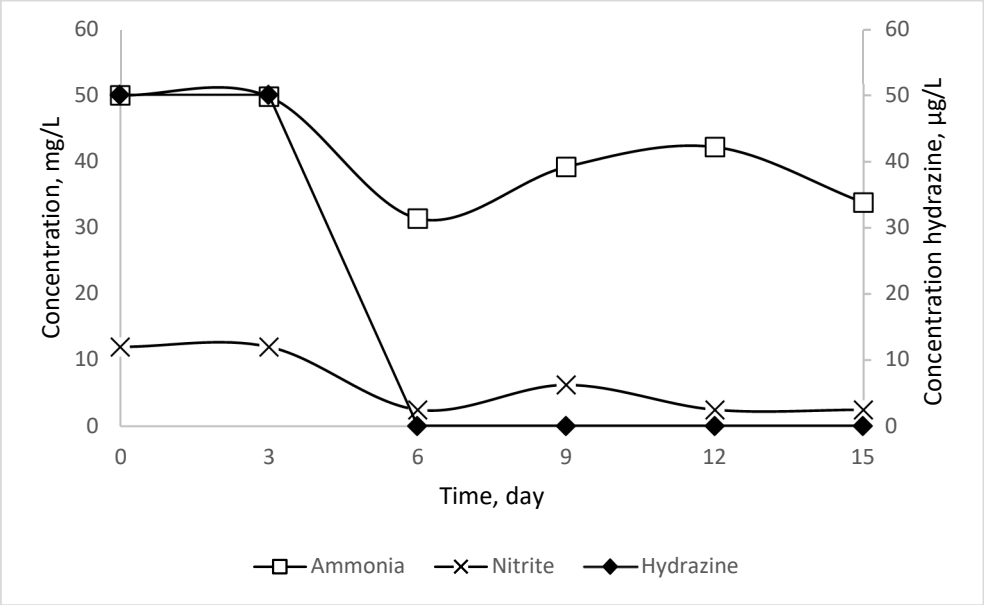

Figure 4.1: Substrate concentration profile during the study of nitrite starvation for Batch 1

Ammonia concentration profile in Batch 1 show fluctuated trend throughout the starvation period. From day 0 – 6, concentration of ammonia show decreasing trend. This ammonia removal was caused by ammonium oxidation under anaerobic conditions using nitrite as the electron acceptor with molecular dinitrogen as the final product. Even though the system was starved with nitrite, the Anammox bacteria may carried nitrite from the main reactor before experiment started. As the nitrite is totally consumed in day 0 – 6, ammonia removal from day 6 – 15 does not happen since there are no electron acceptor to undergo oxidation process.

Figure 4.2 shows the substrate concentration profile during the starvation period of Batch 2. Batch 2 contain nitrite substrate and the bacteria was starved from ammonia.

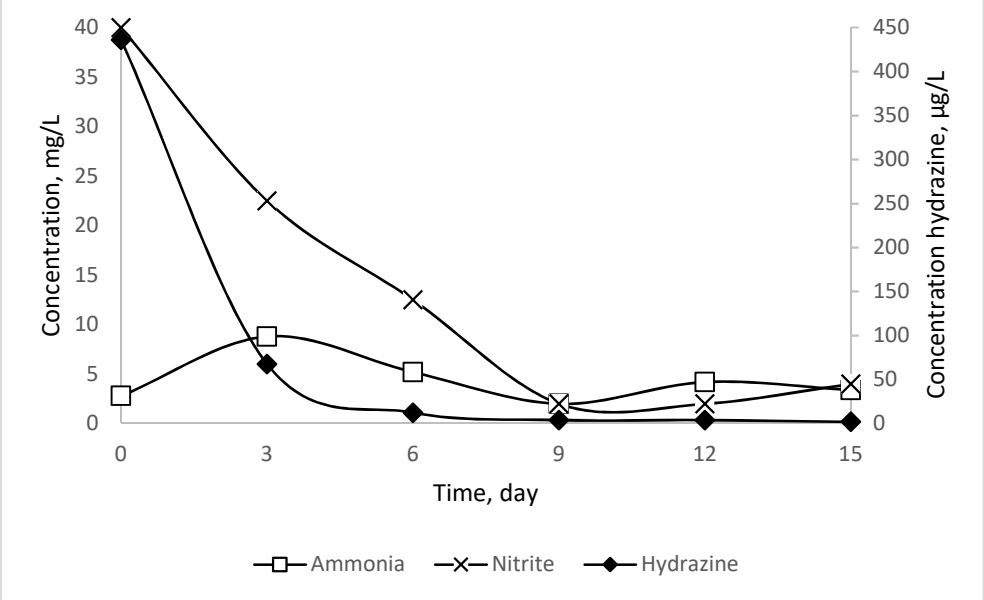

Figure 4.2: Substrate concentration profile during the study of ammonia starvation for Batch 2

Nitrite concentration in Batch 2 shows drastic decreasing trend from day 0 – 9 and constant from day 9 – 12. Nitrite is used as electron acceptor from ammonia to be oxidize to nitrogen gas. Then, the existing 2.8 mg/L ammonia carried by Anammox

bacteria from the main reactor before the experiment started. However, the concentration of ammonia started to increased back at day 9 due to bacteria decay. Hydrazine concentration also show decreasing trend because the molecule in Anammox bacteria used it as energy source for sustainability as survivability.

Figure 4.3 shows the substrate concentration profile during the starvation period of Batch 3. Batch 3 starve from ammonia and nitrite but hydrazine is added into the Anammox system.

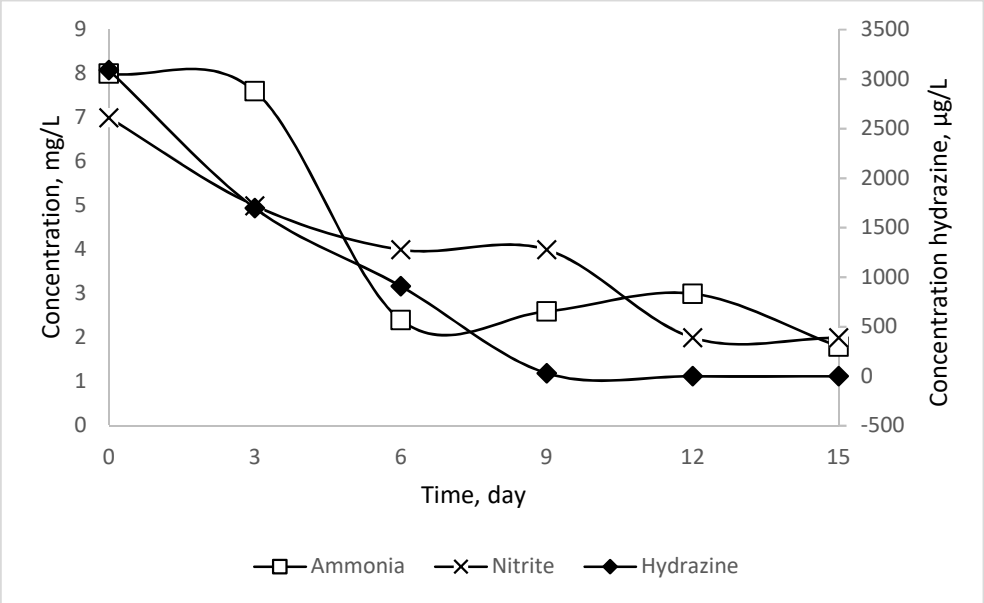

Figure 4.3: Substrate concentration profile during the study of nitrite and ammonia starvation with addition of hydrazine for Batch 3

Hydrazine concentration in Batch 3 shows decreasing trend. Hydrazine is consumed completely by the bacteria in order to survive and do maintain since there are no addition of substrate ammonia or nitrite. Since the anammox bacteria able to sustain, the little amount of ammonia and nitrite that exist in the system can be remove.

Figure 4.4 shows the summary of substrate nitrogen removal profile for 3 types of starvation during the starvation period.

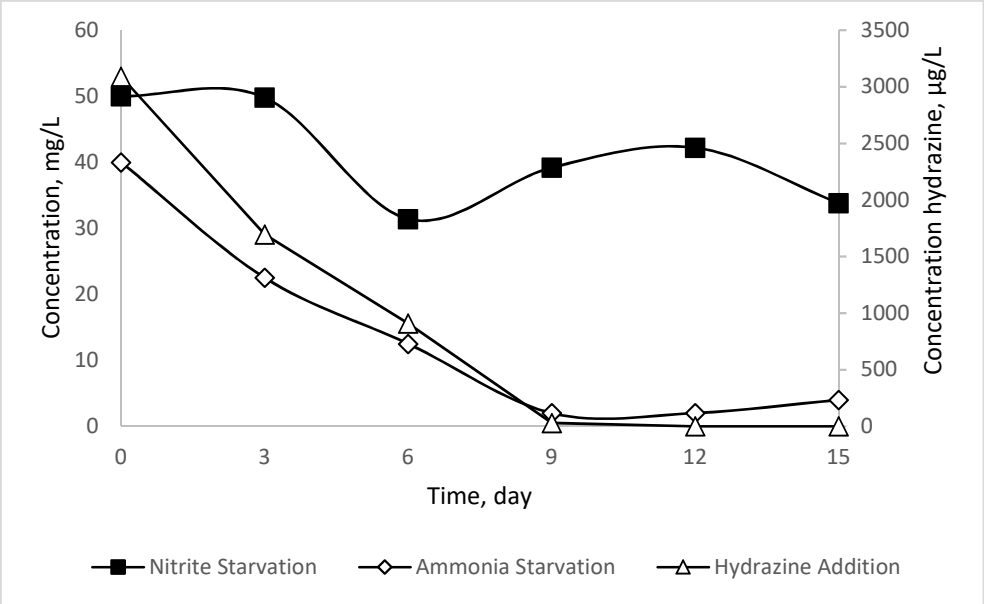

Figure 4.4: Substrate nitrogen removal for 3 different type of starvation during starvation period

From the summary Figure 4.4 above, the most obvious thing is nitrite starvation (Batch 1) does not completely remove ammonia substrate in the Anammox system since there are no electron acceptor to oxidize the ammonia. As a result, after starvation period, the concentration of ammonia substrate in the Batch 1 was still high which is 33 mg/L. Only 17 mg/L of ammonia substrate was removed by the Annamox process from 50 mg/L at the beginning of Batch 1 experiment.

While, ammonia starvation (Batch 2) remove 36 mg/L of the nitrite feed substrate from 40 mg/L to 4 mg/L in the Anammox system. Nitrite in Batch 2 is used as electron acceptor in order to remove existing ammonia in the system. At the end of the starvation period, the concentration of both nitrite and ammonia in Batch 2 are 4 mg/L.