國 立 交 通 大 學

環境工程研究所

碩士論文

結合部分硝化、厭氧氨氧化及脫硝作用於單一批次反應槽之發展

Development of simultaneous partial nitrification, anammox and denitrification (SNAD) process in a sequential batch reactor

研 究 生：藍茜茹

指導教授：林志高　教授

中 華 民 國 一 百 年 八 月

結合部分硝化、厭氧氨氧化及脫硝作用於單一批次反應槽之發展

Development of simultaneous partial nitrification, anammox and denitrification (SNAD) process in a sequential batch reactor

研 究 生：藍茜茹　　Student: Chien-Ju Lan

指導教授：林志高　　Advisor: Jih-Gaw Lin

國立交通大學

環境工程研究所

碩士論文

A Thesis

Submitted to the Institute of Environmental Engineering

College of Engineering

National Chiao Tung University

in partial Fulfillment of the Requirements

for the Degree of

Master

in

Environmental Engineering

August, 2011

Hsinchu, Taiwan, Republic of China

中 華 民 國 一 百 年 八 月

中文摘要

本研究旨在探討單一批次反應槽中 (SBR)，結合部分硝化及厭氧氨氮氧化及脫硝技術 (SNAD) 於不同水力停留時間 (HRT) 對於氨氮去除效率與經濟最適化之影響。在 SNAD 技術中先進行部分硝化作用，將氨氮硝化成亞硝酸鹽氮，剩餘氨氮再與亞硝酸鹽氮經由 Anammox 菌作用轉化為氮氣，而同時產生之硝酸鹽氮，與缺氧性脫硝菌進行脫硝作用，消耗水中有機物質。SNAD 技術的優點為能在單一反應槽同時去除水中含氮化合物及有機物質，省去以往需經由兩個反應槽才能達到硝化脫硝之目的。

研究結果顯示當 HRT 從 9 d 降至 3 d 時，氨氮及化學需氧量 (COD) 去除效率則達極限。另外，隨著 pH、曝氣及溫度低於正常操作範圍時，氨氮及 COD 去除效率也隨之降低。在 HRT 9 d 時，氨氮及 COD 其去除效率分別為 96% 及 87%，為本研究之最佳操作水力停留時間。最後，本文中也利用化學計量方程式及模式來推估氨氮去除，在部分硝化、厭氧氨氧化及脫硝之間的比例，結果顯示有 85-87% 的總氮是經由結合部分硝化及厭氧氨氧化作用所去除，而脫硝作用去除比例則占 7-9%。反應槽中菌種鑑定則利用分子生物檢驗法:螢光原味雜交法 (FISH) 及定量聚合酵素鏈鎖反應（qPCR) 分析污泥中菌相。SNAD 系統對基質的負荷反應及操作條件另可藉由敏感性指標 (sensitive index) 來做評估。研究結果能作為提供各污水處理廠未來實廠操作改善或增設水處理除氮設施之參考。

Abstract

To decrease the cost of nitrogen removal process, anaerobic ammonium oxidation (anammox) was developed and coupled with partial nitrification. However, significant quantity of nitrate released from anammox process (10%) is toxic to aquatic environment. Recently, simultaneous partial nitrification, anammox and denitrification (SNAD) process was developed in a sequential batch reactor (SBR) and the influence of hydraulic retention time (HRT) on the SNAD process was investigated in this study. Around 96% NH_4^+-N removal and 87% COD removal were observed at 9 d HRT. Marginal decrease in the removal efficiencies were observed when the HRT was reduced to 3 d or the loading rate was increased by 3 times. On the other hand, a drastic decrease in NH_4^+-N and COD removals were observed when the DO, pH and temperature were dropped shockingly. The response of the SNAD system towards the shock in substrate loading and operating conditions was evaluated by sensitivity index. Finally, the extent of total nitrogen (TN) removal by partial nitrification with anammox and denitrification was modeled using stoichiometric relationship. Modeling results indicated a TN removal of 85-87% by anammox with partial nitrification and 7-9% by denitrification. The bacterial diversity in the reactor was also investigated by fluorescence in situ hybridization (FISH) and quantitative real-time PCR (qPCR) techniques.

誌謝

誠摯感謝我的指導教授林志高老師，不論在生活及研究領域上不厭其煩的諄諄教誨，給予學生最大的幫助、教導及鼓勵。此外本論文的完成亦感謝成功大學鄭幸雄教授，美國愛荷華州立大學宋士武教授，中原大學黃郁慈教授，給予學生寶貴的意見與指導，使得論文整體架構與內容更加嚴謹。

在研究生涯兩年的努力中，特別感謝至誠學長與理安學長不吝指教，總是耐著性子與我討論研究及生活上的難題。碩士班學長學姐紹謙、維芬、依璇、紘瑩、彥均，同窗佩芸、怡君、維倫及學弟信翰與南維，給予我研究上的協助及鼓勵，我的研究生活有你們增添了許多色彩；感謝摯友詩珮、佩昕、欣妤等，在研究遇到瓶頸時分擔壓力。

最後感謝家人長久以來默默的支持與鼓勵，與亡父的庇佑。讓我能全心的完成學業，你們是我完成學業的最大動力，僅以此表達最真誠的謝意。

茜茹 謹致於

交通大學環境工程研究所

2011 年 8 月

Contents

List of Tables

List of Figurs

Chapter 1

Introduction

The release of excessive nitrogen into the aquatic systems leads to acidification and eutrophication problems. At the same time, it can also impair the survival of aquatic plants and other organisms. Thus, the removal of nitrogenous compounds from wastewater systems prior to its disposal is an important issue. Nitrogen removal from wastewaters is usually accomplished through sequential nitrification and denitrification processes, i.e. conventional nitrification-denitrification process. This is recognized as the most suitable process for the treatment of wastewater with high C/N ratio [1]. During the conventional nitrification-denitrification process, NH_4^+ is oxidized to nitrate (NO_3^-) followed by NO_3^- reduction to gaseous nitrogen (N_2). However, several novel nitrogen removal processes have been developed to reduce the energy consumption in the nitrification-denitrification process. These novel processes include single reactor system for high ammonium removal over nitrite (SHARON) [2, 3], completely autotrophic nitrogen removal over nitrite (CANON) [4], oxygen-limited autotrophic nitrification-denitrification (OLAND) [5] and anaerobic ammonium oxidation (Anammox) [6].

Anammox process is gaining lot of importance for nitrogen removal compared to the conventional nitrification-denitrification process. Anammox is an autotrophic oxidation process, which converts NH_4^+ to N_2 using nitrite (NO_2^-) as the electron acceptor. Since, Anammox process is an anaerobic-autotrophic process it eliminates the requirements of aeration and exogenous carbon source [7]. However, the Anammox process depends on the availability of both NH_4^+ and NO_2^- in the system (Eq. (1)); therefore, Anammox process was coupled with partial nitrification in a

single reactor system. The combination of Anammox and partial nitrification decreases the overall cost of the nitrogen removal process; however, a significant quantity of NO_3^- (10%) is released from the Anammox systems. This could be more than the wastewater disposal standards at times. Combined nitrogen removal process such as CANON and OLAND process, both of them are autotrophic nitrogen removal process which operated under oxygen-limited condition, are suitable for the wastewater with relatively high ammonium concentration but without organic consumption. Thus, denitrification was added into the CANON process to solve this problem. On the other hand, combining Anammox and denitrification for complete nitrogen removal has been reported [8]. The overall equation for this process is as follows:

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

Recently, simultaneous partial nitrification, Anammox and denitrification (SNAD) [9] process was developed, which has the potential of treating NH_4^+ and biodegradable organics from wastewaters. The advantages of this process are the complete nitrogen removal and a reduction in the portion of chemical oxygen demand (COD). The granules capable of carrying out the SNAD process were identified in a full-scale landfill-leachate treatment plant in Taiwan [10]. In the SNAD process, majority of nitrogen is removed by the Anammox process. However, developing a SNAD process in the laboratory is highly difficult owing to the requirement of longer start-up time and slow growth rates of Anammox bacteria (the doubling time was reported to be approximately 11 days) [11]. In addition, the reactor carrying out Anammox must be efficient in retaining the biomass [12].

In several studies, the sequential batch reactors (SBRs) have been successfully applied for the enrichment of very slow-growing microbial community [13-15]. Compare to other nitrogen removal configurations, SBR provide efficient biomass retention, leading to a 90% of retention compare to fluidized bed reactor where retention was only 64%. Also the doubling time was reduced from 30 days to 11 days [11, 16] in SBR. However, the optimum conditions of SBR for the enrichment of SNAD organisms (nitrifying, Anammox, denitrifying bacteria) are not well understood. Therefore, the present study was aimed to (1) develop a SNAD process in a laboratory scale SBR using synthetic wastewater, (2) investigate the effect of hydraulic retention time (HRT) on the performance of SNAD and (3) study the bacterial diversity in the SBR.

Chapter 2

Literature Review

2.1 Introduction

Organisms require nitrogen as nutrients to produce a number of complex organic molecules like proteins, enzymes, amino acids, nucleic acids and especially DNA. The nitrogen cycle represents one of the most important nutrient cycles found in ecosystems. The ultimate store of nitrogen is in the atmosphere, where it exists as nitrogen gas (N_2). This store is about one million times larger than the total nitrogen contained in living organisms. Other major stores of nitrogen include organic matter in soil and the oceans (Figure 1).

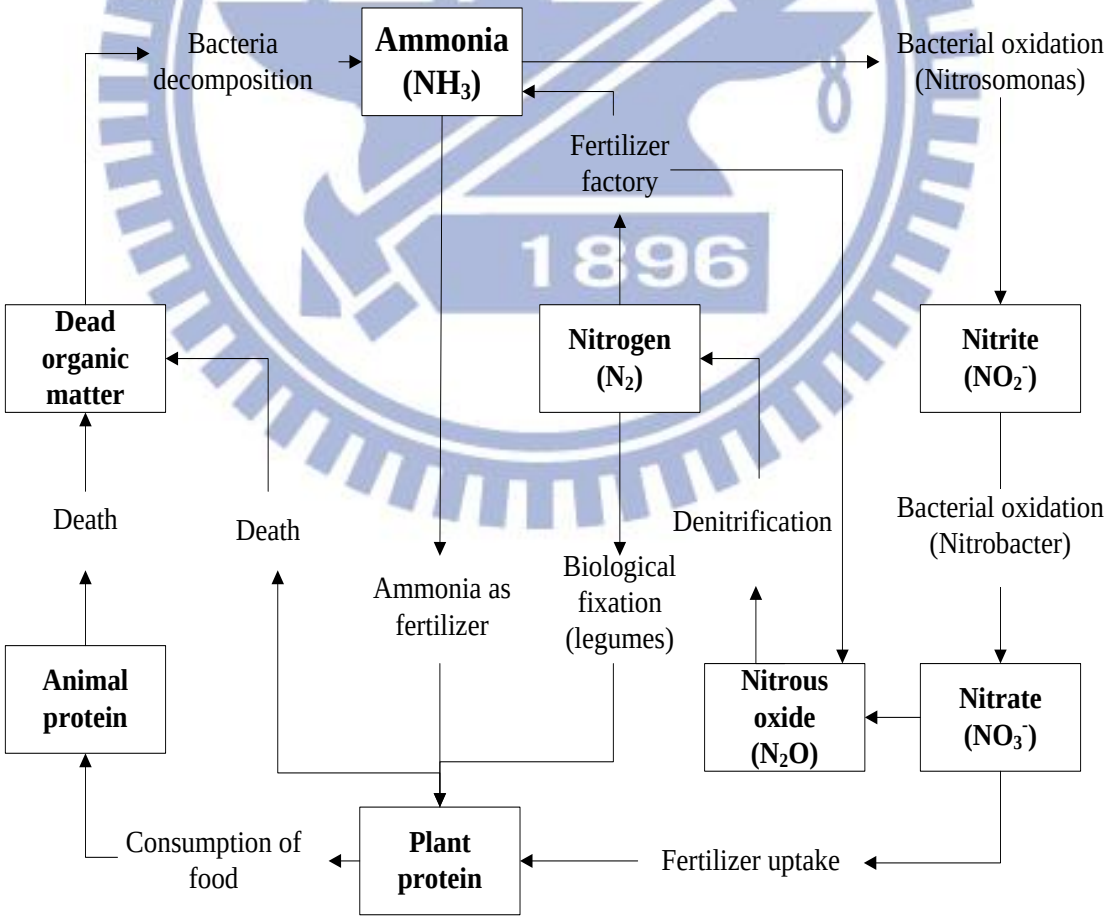


Fig.1. Nitrogen cycle

2.2 Nitrogen pollutants - sources and their impact on environment

Increasing human population have altered nitrogen cycle and accelerated the nitrogen pollutants due to the various human activities, such as providing enough food to the increasing global population. Futhermore, in some parts of the world, nitrogen is responsible for a prevalence of unhealthy diets, while also contributing to a host of environmental problems. Nitrogenous pollutants can enter into the ecosystems from various anthropogenic sources. Some of them are listed in Table 1.

Table 1. Major anthropogenic sources of nitrogenous pollutants [10, 17-20]

Major anthropogenic sources of nitrogenous pollutants
-Alcohol fermentation
- Aquaculture industries (condensates from ferlilizer plants)
- Activities contributing to N mobilization (biomass burning, land clearing and conversion, and wetland drainage)
- Food processing (fish, shrimps, spawns)
-Leather tanning-Industrial wastewater discharges
-Landfill leachate
-Municipal sewage effluent (including effluent from sweage treatment plants without tertiary treatments)
-Overflows of combined storms and sanitary sewers
-Runoff and infliltration from waste disposal sites
-Supernatant from anaerobic sludge digesters
-Wastewaters from livestock farming (cattle, pig, chickens)
- Optoelectronics industrial wastewater
-Semiconductor industrial wastewater

Although, these pollutants can be removed by denitrification process via the formation of nitrous oxide (N_2O), ammonia emissions, and burial of organic matter in sediments [21]. Overall, the impact of nitrogen pollution still remain and has been pointed out three environmental problems: (1) it can drastically decrease the pH of freshwater thereby, impaired the ability of aquatic animals to survive, leading to acidification of water bodies; (2) organic and inorganic nitrogen pollution to waterbodies can induce adverse effects on human health, including malaria, cholera and schistosomiasis [21]; and (3) it can harm ecosystems and contribute to global warming by producing N_2O, a major greenhouse gas which has 310 times higher heat trapping effects than carbon dioxide and even higher than methane (23 times than carbon dioxide) [22].

All these are compelling evidences that human alteration of the nitrogen cycle is negatively affecting human and ecosystem health. Therefore, it is of great importance to determine the most appropriate treatment option as well as the optimal operating conditons to achieve compatibility in combination treatment processes for the maximum removal of nitrogenous pollutant.

2.3 Conventional biological technologies for nitrogen removal

Conventional biological nitrogen removal process has been widely used by a combination of two processes, nitrification and denitrification in separate reactors. Figure 2 shows the schematic representation of conventional nitrification and denitrification process. In nitrification process, ammonium is oxidized first to nitrite and then to nitrate by ammonia-oxidizing bacteria (AOB) with molecular oxygen as electron acceptor. In the subsequent denitrification step, nitrite-oxidizing bacteria (NOB) oxidize nitrite (NO_2^-) to nitrate (NO_3^-) which was further converted to

nitrogen gas by denitrifying bacteria using NO_X^- as electron acceptor and using organic matter as carbon and energy source. Nitrification is an oxygen-requiring process and therefore requires an aerobic environment and most denitrifying bacteria carry out these reactions only under anaerobic conditions.

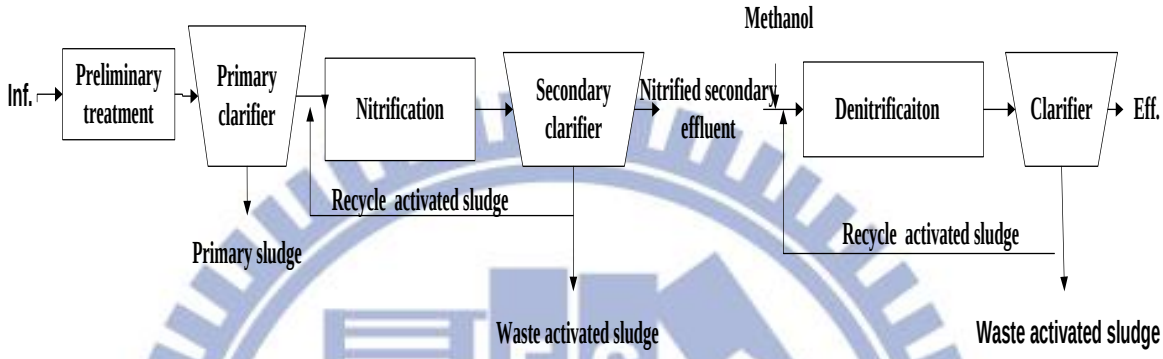


Fig. 2. The schematic of conventional nitrification-denitrification process

However, the limitations of this conventional processes are requirement of high level of oxygen (4.2 g O_2/g NH_4^+-N) for nitrification [23], and sufficient external organic carbon source (2.86 g chemical oxygen demand (COD)/g NO_3^--N) for denitrification [24]. Therefore, external carbon sources like methanol and acetate are normally added to complete the denitrification process when treating wastewaters containing high nitrogen concentration or low C/N ratio, which increases the operational cost. Moreover, nitrification and denitrification process have to be carried out under different oxygen required conditions thus should be designed and operated in two reactors. Consequently, low nitrogen removal efficiency, high oxygen requirement, long retention time, and requirement of an external carbon source are the driving forces for developing new low-cost biological nitrogen treatment processes [25].

2.4 Novel biological processes for nitrogen removal

To overcome the limitations of conventional nitrogen removal process, several novel biological processes are developed. Some of the novel biological nitrogen removal processes are described below:

2.4.1 Anaerobic Ammonium Oxidation (Anammox)

Anammox is a novel and low cost approach to remove nitrogen from wastewater. In 1995, this process was discovered at Gist-Brocades (Delft, The Netherlands) during the effluent treatment from methanogenic reactor in a multistage denitrifying fluidized bed reactor [7, 26, 27]. The researchers found that nitrate and ammonium disappear at the same time in the reactor. The nitrate was first considered as electron acceptor, but it was proved that nitrite was more suitable electron acceptor for Anammox process. In such case, ammonium is oxidized to nitrogen by aerobic AOB with nitrite as the electron acceptor with the production of nitrogen gas and small amount of nitrate. This discovery led to the realization that the enormous of nitrogen losses in the marine environment were due to Anammox process [28].

The discovery of the Anammox bacterium is a revolution in the biological nitrogen cycle. Anammox is a lithoautotrophic biological conversion process, mediated by a group of *Planctomycete* bacteria which named Anammox bacteria. The specific mechanism of Anammox pathway are quite unique, several researchers used ^{15}N-labelled compounds including nitrite, nitrate and hydroxylamine to identify the reaction of Anammox process. Hydrazine and hydroxylamine are both toxic and were found to be the intermediates of the process [6, 16, 29]. Ammonium combined with hydroxylamine to produce hydrazine which subsequently oxidized to nitrogen gas. Schalk et al. (1998) discovered that Anammox bacteria consist of membrane-bound

compartment, anammoxosome, has a large amount of enzyme-liked hydroxylamine oxidoreductase (HAO), which is responsible for oxidation of hydrazine to nitrogen gas [30]. Discovery of hydrazine is exciting as it can be used as rocket fuel and play an important role as electron donor in conversion of nitrite to hydroxylamine. Thus, hydrazine molecule can be used as an energy source by bacteria showing Anammox is a distinct process compare to other nitrogen removal process (Figure 3).

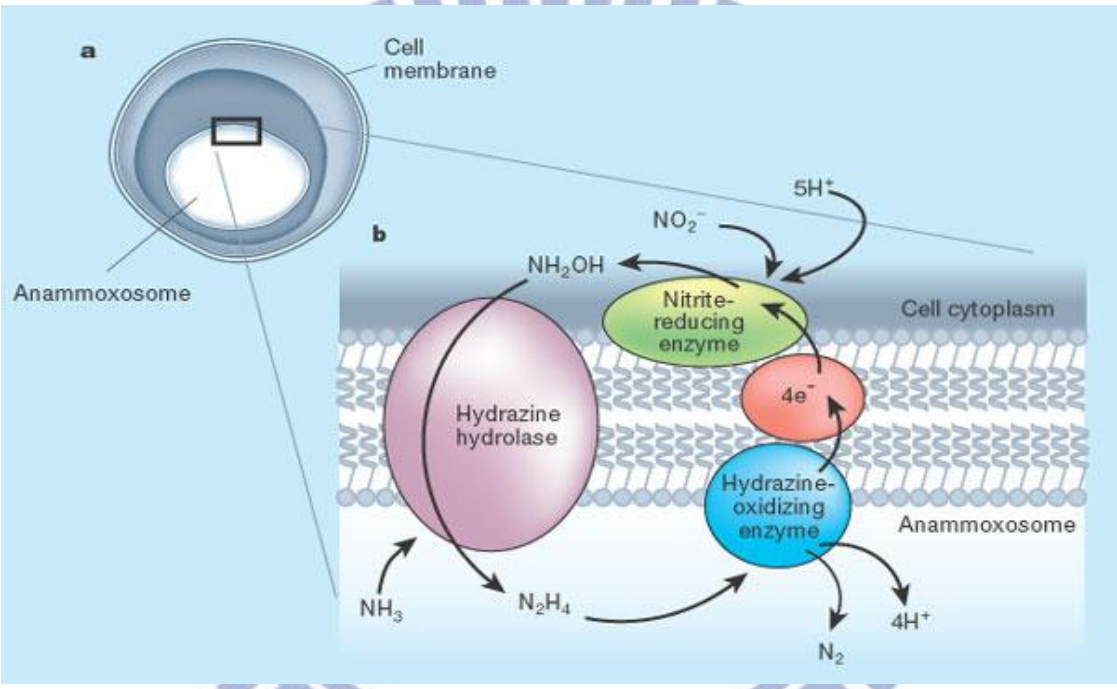


Fig.3. Biochemical pathway of Anammox reaction. a, A simplified depiction of the Anammox microbe, showing the anammoxosome. This is the organelle-like structure in which the energy-generating process involving the combination of ammonia with nitrite takes place. b, The anammoxosome membrane, which consists of the ladderane lipid bilayer, and the anammox reaction pathway. Intermediates in the cycle are hydrazine (N_2H_4) and hydroxylamine (NH_2OH), which are highly toxic [25, 31].

The Anammox process removes about 90% of the incoming nitrogen as ammonium/nitrite and leaves about 10% of nitrogen as nitrate in the effluent (Eq. (1)). External carbon sources are not needed in Anammox because carbon dioxide serves as the main carbon source for aerobic AOB [16].

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

$$\rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O$$

$$\Delta G^0 = -358 \text{ Kj (mol } NH_4^+)^{-1} \quad (1)$$

The first full-scale Anammox reactor was started at the sludge treatment plant in Rotterdam and the reactor treated up to 750 kg-N/d. The Anammox reactor with working volume 70 m^3, fed with partially nitritated sludge liquor from an adjusted nitritation process. However, the application of Anammox process might be limited by slow growth rates of Anammox bacteria (the doubling time of Anammox culture was be reported to be 30 days in a fluidized bed reactor (Table.2)). The critical point of shorten the start-up time of Anammox is having sufficient biomass retention. Thus in order to maintain all the biomass, extending the sludge retention time (SRT) of reactor would help the acclimation of Anammox bacteria. The Anammox processes acclimation have been successfully used in fluidized bed, moving bed biofilm reactor, the rotating biofilm reactor and the anaerobic biological filtrated reactor. But it was very hard to operate in a laboratory-scale reactor owing to its insufficient biomass retention [16].

Table 2. Doubling time of various acclimation reactors

Doubling time (d)	Reactor	Reference
30	FBR[1]	[16]
29	FBR[1]	[6]
21	SBR[2]	[32]
18	MBR[3]	[33]
11	SBR[2]	[11]

*E-coli has doubling time of 0.02 d, [1] fluidized bed reactor, [2] sequential batch reactor, [3] membrane bioreactor

The sequencing batch reactor (SBR) was considered a powerful reactor for studying such slow-growth microorganisms due to the four reasons: (1) efficient biomass retention, leading to a 90% of biomass retention compare to 64% retention in a fluidized bed, also doubling time was reduced to 11 days, (2) a homogeneous distribution of substrates and aggregates, with 1 mm effective aggregate diameter, 50% of the biomass was active, (3) reliable operation for more than one year, and (4) stable conditions for the first time mass balance under defined conditions [11].

Compared with conventional biological nitrogen removal processes, Anammox process has two major advantages. First, Anammox is carried out by autotrophic bacteria under anoxic condition, there is no need for aeration and organic carbon sources, which saves operation costs. Second, the biomass yield during Anammox process is very low (0.11 g VSS/g NH_4^+-N, VSS—volatile suspended solids), which can also save sludge treatment costs [6].

2.4.2 Single reactor High activity Ammonia Removal over Nitrite (SHARON)

In Anammox process, ammonium has to be partly oxidized to nitrite before feeding to the reactor. Thus, the SHARON process was developed in Delft University of Technology for treating recycled water from the sludge digesting unit [2]. In this process, 53% of ammonium was oxidized to nitrite at 1.2 kg N load per m^3 per day, without any need of pH control.

Compare with Anammox process where removal of 1 mole of ammonium consumed 1.32 mole nitrite which needs extra nitrite to complete the reaction, SHARON process offers a good nitrite source without increasing operation cost by purchasing chemical. It's feasible for substantial ammonium reduction in a wastewater with relatively high ammonium content and with an elevated temperature. This process takes advantage of high temperature, enabling high specific growth rates, so that no sludge retention is required and SRT is controlled by HRT. Also, by carefully selecting the HRT, nitrite oxidizers can be washed out, while ammonium oxidizers are retained in the reactor. This process is most suited to treat high ammonium concentration (>500mg-N/L), where the effluent quality is not critical because it can be sensitively influenced by changing the reactor pH between 6.5 and 7.5.

The SHARON process is a partial nitrification process which contains fast growing ammonium oxidizers and this is one of the best suited processes to treat wastewater with a high ammonium concentration. Thus, the SHARON reactor where only 50% ammonium is converted to nitrite (Eq. (2)) can be used to provide the feed for the Anammox process.

$$NH_4^+ + HCO_3^- + 0.75\ O_2 \rightarrow 0.5\ NH_4^+ + 0.5\ NO_2^- + CO_2 + 1.5\ H_2O \quad (2)$$

This stoichiometric reaction shows that no base is needed, since anaerobic digestion will contain enough alkalinity to compensate for the acid production. By using combined SHARON-Anammox process [3], the oxygen requirement for nitrogen removal will be reduced to 60% and no longer require the input of COD. The system can thus be operated independently. The combination of the Anammox process and a partial nitrification (SHARON) process has been tested using sludge digester effluent, successfully (Figure 3). These two new concepts for the removal of nitrogen from wastewater have been developed in which a substantial reduction in the energy and chemical use is achieved.

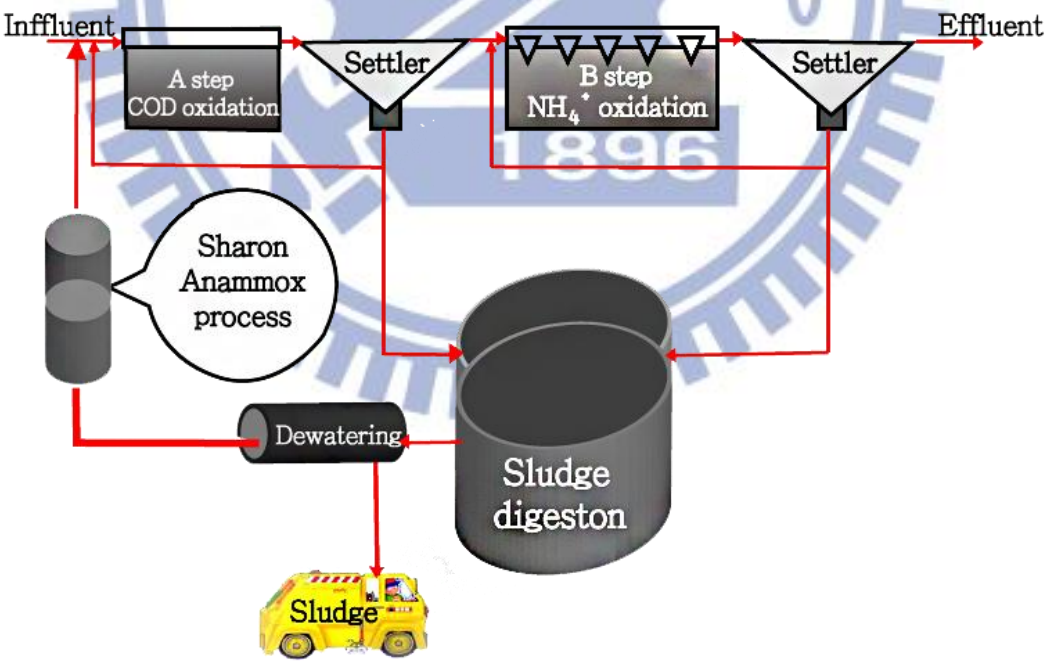


Fig 3. Implementation of the SHARON-Anammox process at the WWTP, Rotterdam-Dokhaven [3].

2.4.4 Completely Autotrophic Nitrogen Removal over Nitrite (CANON)

No matter how Anammox process combined with different novel biological nitrogen removal process, Anammox process has to be operated under anoxic condition. The oxygen-limited condition below 0.5% air saturate provide an adequate environment for Anammox bacteria [12]. Consequently, the CANON process has been discovered where high ammonia and low organics loadings in wastewater treatment plant under oxygen limited condition results in a complete conversion of ammonium to nitrogen gas in a single autotrophic reactor [34, 35]. Subsequently, Sliekers et al. (2002) developed a laboratory-scale reactor to have substantial nitrogen losses with a low dissolved oxygen concentration and with small amounts of COD present in the wastewater [4].

In this process ammonia would be converted partly to nitrite (Eq. (3)) by oxygen-limited AOB (*Nitrosomonas*-like aerobic bacteria) and subsequently, anaerobic ammonium oxidizers (*Planctomycete*-like anaerobic bacteria) would convert ammonia with nitrite to dinitrogen gas (Eq. (4)). The combination of Eq.(3) and (4) results in the following overall nitrogen removal reaction in Eq. (5)[36]:

$$1NH_3+1.5O_2 \rightarrow NO_2^- + H_2O + H^+ \quad (3)$$

$$1NH_3+1.32NO_2^- + H^+ \rightarrow 1.02N_2+0.26NO_3^- +2H_2O \quad (4)$$

$$1NH_3+0.85O_2 \rightarrow 0.11NO_3^- +0.44N_2+0.14H^+ +1.43H_2O \quad (5)$$

There are few key factors for operating CANON process, including ammonia concentration, dissolved oxygen concentration and an AOB population. Especially, oxygen levels (> 0.5 mg/L) has inhibition on anaerobic AOB with extra nitrite production and an ammonia loading of 14 mg/L·hr provide sufficient nitrogen source

in CANON process[37]. The microbial interaction between aerobic AOB and anaerobic AOB affects this process as aerobic AOB utilize ammonia and oxygen as substrates while anaerobic AOB utilize ammonia and nitrite as substrate. In the presence of nitrite oxidizing bacteria (NOB) which utilize oxygen and nitrite as substrates, the CANON process is disrupted because NOB competes with aerobic AOB for oxygen and with anaerobic AOB for nitrite.

However, no extra carbon source is required because it is completely autotrophic. This can be achieved in one single reactor, at oxygen limited conditions, without the production of N_2O or NO. Also, CANON consumes 63% less oxygen than conventional nitrogen removal processes [4]. Altogether, CANON process has a high potential for application in treating low C/N wastewater.

2.4.5 Oxygen-Limited Autotrophic Nitrification–Denitrification (OLAND)

The OLAND process is discovered in a nitrifying rotating contactor treating ammonium-rich leachate without consumption of organic carbon under oxygen-limited condition that can remove the extensive nitrogen by converting NH_4^+ to N_2 [5]. The operative microorganisms were assumed to be autotrophic populations which could denitrify under low dissolved-oxygen (DO) conditions. Therefore, oxygen concentration is critical for OLAND because the population of aerobic AOB drastically decreases at low oxygen concentration. The operative microorganisms were assumed to be autotrophic populations which could denitrify under low dissolved-oxygen conditions. There is no big difference between OLAND and CANON and they differ only on the microbial diversity of these two process, whereas OLAND is achieved by aerobic AOB, while CANON carried out by both aerobic

AOB and anaerobic AOB.OLAND is supposed to take place via two steps (Eq. (6) and (7)). Combining these two steps, it can get an overall reaction in Eq (8): [38]

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (6)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2 \quad (7)$$

$$2NH_4^+ + 1.5O_2 \rightarrow N_2 + 3H_2O + 2H^+ \quad (8)$$

The major advantage of this system is that the inoculums can readily be grown in large quantities which favor the applicability of the OLAND system for practical purposes. Moreover, operation of this system has no requirement for an NO_2^- supply. An ammonium-rich wastewater can be fed directly at a suitable loading rate. Although the process requires limited oxygen conditions, it does not require strictly anaerobic conditions. Therefore, inhibition by trace oxygen exposure is not a serious problem of concern in practice.

2.4.6 Simultaneous partial Nitrification, ANAMMOX and Denitrification (SNAD)

CANON and OLAND process, both of them are autotrophic nitrogen removal process which operated under oxygen-limited condition without organic consumption. These two treatments are suitable for the wastewater with relatively high ammonium concentration but no COD. Thus, a novel non-woven rotating biological contactor reactor was applied for the SNAD process [9]. It allows microorganisms to adhere and colonize throughout the material, making a very well layer of microorganisms. This SNAD process is for the simultaneous nitrogen and COD removal for the high-strength ammonium, with low-carbon wastewater. This is in accord with the principle that partial nitrification requires a certain aerobic condition for oxidation of

ammonia, whereas denitrification and Anammox occurs under anoxic condition in the presence of electron donors [12]. Ammonium is oxidized to nitrite by AOB, subsequently, nitrite can be used by Anammox bacteria which finally convert nitrite to nitrogen gas with small amounts of nitrate under oxygen-limiting conditions. Afterwards, the carbon source may be required since the organic carbon demand for the denitrification reaction is directly consumed from the wastewater COD (as electron donor could deoxidize nitrate to nitrogen gas through denitrifying process (Eq.(9), (10) and (11)), so that the purpose of removing nitrogen and COD can be achieved simultaneously and the total operation cost will be reduces.

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O \quad (9)$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (10)$$

$$NO_3^- + 1.08CH_3OH + 0.24\,H_2CO_3 \rightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68\,H_2O + HCO_3^- \quad (11)$$

The idea of coupling the partial nitrification process with Anammox and denitrification process has been deemed to one of the most economical process and can be used extensively in the ammonium rich wastewater.

2.5 Simultaneous anoxic ammonium removal with sulphidogenesis

As alternatives for oxygen, nitrate and nitrite can be used to control sulfide generation during treatment of S-containing wastewaters. However, sulfate is also likely to be a suitable selection for its strong oxidation capacity. The simultaneous ammonium and sulfate was discovered in an Anammox reactor. The dissimilatory sulfate reducing bacteria (SRB) are usually involved in alternative denitrification routes.

Few researchers postulated that nitrite formation and subsequent Anammox were responsible for following equation:

$$SO_4^{2-} + 2NH_4^+ \rightarrow S + N + H_2O \quad (12)$$

The new postulated anaerobic process of nitrogen and sulfate removal seems to convert into the nitrogen gas and sulfur. The free energy of this reaction is 47 KJ/mol., which make simultaneous anoxic ammonium removal with sulphidogenesis possible.

2.6 Comparison of conventional and novel biological nitrogen removal processes

Table 3 represents the comparative performance of various biological nitrogen removal processes. Combined novel technologies possess advantages in terms of less energy consumption, saving configuration and no need for organic carbon sources. However, each process has its own advantages and potential problems. Many challenges still remain for the optimization and application of Anammox and its combination process either in pilot or full scale treatment plant. The Anammox process can operate under high nitrogen loading and possess distinct advantages of saving aeration costs and carbon source, but the long start-up time for Anammox bacteria still remain as a significant obstacle. On the other hand, CANON and OLAND processes considered using a compact reactor configuration with good biomass retention, nevertheless the enrichment of anaerobic microorganism capable of oxidizing ammonia with nitrite as electron acceptor. The SHARON process is commonly used because it can be operated without any biomass retention, but the high temperature limits its application.

Table 3. Comparative performance in biological nitrogen removal

Process type	**Conventional**	**Anammox**	**SHARON**	**CANON**	**OLAND**	**SNAD**
Common reactor configuration	Activated sludge and biofilm	FBR, SBR, gas lift, fixed bed	Activated sludge and biofilm	Fixed bed, FBR, SBR	Fixed bed, FBR, SBR	Activated sludge, SBR
Operating conditions	Aerobic, anoxic	Anaerobic	Aerobic, anoxic	Oxygen limited	Oxygen limited	Anoxic
Bacteria	AOB: *Nitrosomonas*, *Ntrosococcus*, *Nitrosopira*, *Nitrosovibrio* *Nitrosolobus* NOB: *Nitrospira*, *Nitrospina*, *Nitrococcus*, *Nitrocystis*, *Nitrobacter*	*Candidatus Kuenenia stuttgartiensis*, *Candidatus Scalindua brodae*, *Candidatus Scalindua wagneri*, *Candidatus Anammoxoglobus propionius*, *Candidatus Brocadia*, *Candidatus Jettenia asiatica*	AOB: *Nitrosomonas* NOB: *Nitrobacter*	AOB: *Nitrosomonas*, *Nitrosospira* NOB: *Nitrobacter*, *Nitrospira*	AOB: *Nitrosomonas*, *Nitrosospira* NOB: *Nitrobacter*, *Nitrospira*	AOB: *Nitrosomonas europaea*, *Nitrosomonas oligotropha*

Optimum pH	6.5-8.5	6.7-9.5	7-8	7.8	7-7.2	7.4-8.2
Optimum DO (mg/L)	4-8	<0.2	1-1.5	0.5	<0.1	0.5-0.7
Optimum temperature	12-35	30-40	>25	30-40	30-40	30-40
Oxygen requirement	High	None	Low	Low	Low	Low
COD requirement	Yes	No	No	No	No	No
Substrate	Municipal wastewater	Synthetic wastewater, Anaerobic digester effluent, Piggery waste	Anaerobic digester supernatant/liquor	Synthetic wastewater, Anaerobic liquor	Synthetic wastewater	Synthetic wastewater, Leachate
Sludge production	High	Low	Low	Low	Low	Low
Max N loading (Kg N m^{-3} reactor d^{-1})	2-8	10-20.5	0.5-1.5	2-3	0.1	0.67-0.022
Total nitrogen removal	95%	87%	90%	75%	85%	97%
Reference	[23,24]	[6, 7,12,36]	[2,3]	[4,35]	[5,37]	[9,10,12]

Chapter 3

Material and Methods

3.1 Synthetic wastewater

The SBR was fed with a synthetic wastewater (mineral medium). The composition of the mineral medium used for enrichment of Anammox bacteria is shown in Table 4.

Table 4. Composition of the synthetic wastewater used in this study.

Composition of synthetic wastewater	mg/L	Composition of trace element solution	mg/L
NH_4^+-N (supplied from $(NH_4)_2SO_4$)	200	EDTA	1500
NO_3^--N (supplied from $NaNO_3$)	17	$ZnSO_4$ $7H_2O$	430
$KHCO_3$	2000	$CoCl_2$ $6H_2O$	240
NaH_2PO_4	100	$MnCl_2$ $4H_2O$	990
$CaCl_2 \cdot 2H_2O$	100	$CuSO_4$ $5H_2O$	250
$MgSO_4 \cdot 7H_2O$	58	$NaMoO_4$ $2H_2O$	220
$FeSO_4 \cdot 7H_2O$	18	$NiCl_2$ $2H_2O$	190
EDTA-2Na	20	$NaSeO_4$ $10H_2O$	210
COD* (supplied from glucose)	100	H_3BO_4	14
Trace element (mL/L)	1		

*COD is supplied as glucose ($C_6H_{12}O_6$), and 1 g of glucose produces 1.06 g of COD

3.2 Inoculation sludge

The SNAD seed sludge used for inoculating the synthetic wastewater in SBR was collected from a biological treatment unit (aeration tank) of the full-scale landfill-leachate treatment plant in Taiwan. The operating conditions established the SNAD process in the aeration tank (384 m^3) were: DO ~ 0.3 mg/L, pH ~ 7.4, HRT ~

1.26 d, and the sludge retention time (SRT) ~ 12 to 18 d. The concentrations of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) in the SBR were 1676 and 1140 mg/L, respectively. The fluorescence in-situ hybridization (FISH) and polymerase chain reaction (PCR) techniques were applied to verify the presence of Anammox bacteria in the SNAD seed sludge [10]. In addition to Anammox bacteria the seed sludge also consists of nitrosomonas-like aerobic microorganisms and denitrifiers. The preliminary investigation of the SNAD seed sludge revealed that the seed sludge has very high affinity for NH_4^+ and NO_2^-. The activity of the SNAD sludge in the present study corresponds to a total nitrogen removal of 320 mg/g of VSS, which is several times higher than the activity (a total nitrogen removal of 48 mg/g of VSS) reported by Chen et al. (2009).

3.3 Experimental methods and design

3.3.1 Reactor system and experimental set up

A SBR with a working volume of 18 L was used for the establishment of the SNAD process. The schematic diagram and photograph of the SBR is shown in Figure 4 and 5, respectively.

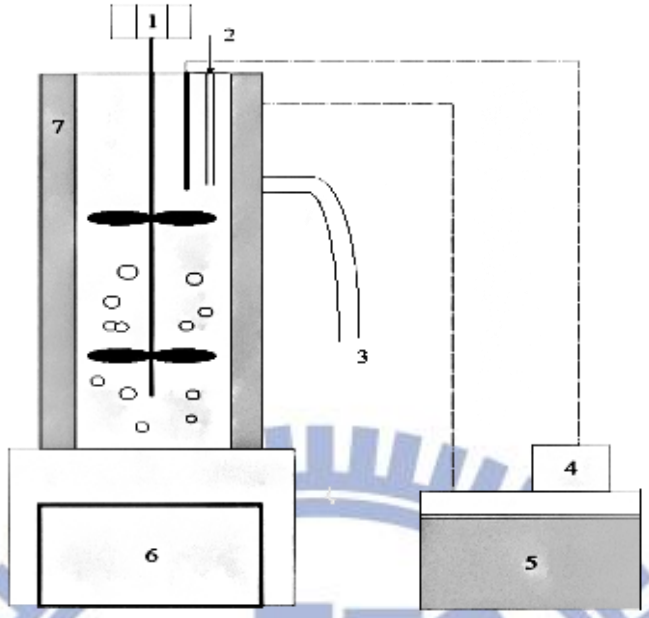


Fig. 4. Schematic of an SBR for SNAD system (1) mechanical stirrer, (2) influent, (3) effluent, (4) DO measurement, (5) thermostat, (6) controller for mechanical stirrer, and (7) thermostatic water jacket

Fig. 5. Photograph of SBR

As a precursor, the acclimation process was started using 2 L of the SNAD seed sludge. The synthetic wastewater was used as a feed; the composition of the feed wastewater is shown in Table 4. The acclimation of the SNAD sludge was started immediately after the inoculation of the seed sludge. The temperature of the SBR was always controlled at 35°C by using a thermostatic water jacket, and the pH was maintained in a range of 7 to 8. The air flow into the reactor was controlled using a pneumatic valve. The dissolved oxygen (DO) concentration in the sample was measured outside the SBR using a DO meter. The DO concentration in the reactor was maintained around 0.3-0.4 mg/L and the alkalinity was maintained in a range of 250-300 mg $CaCO_3$ /L. At the same time, NH_4^+ oxidation to NO_2^- (partial nitrification) was controlled by adjusting the DO concentration in the reactor. At any stage, the NO_2^--N concentration was not allowed to exceed 100 mg NO_2^--N /L beyond which Anammox process could be inhibited [7]. By controlling the NO_2^--N concentration in the SBR, the Anammox reaction was initiated with a proper stoichimetric requirement of NH_4^+ and NO_2^-. A complete mixing inside the SBR was ensured by mixing the reactor contents via a 3-bladed mechanical stirrer at a rate of 100 rpm. After the acclimation process, the performance of the SBR for treating synthetic wastewater with ammonium (200 mg/L) and COD (100 mg/L) was investigated under four different hydraulic retention times (HRTs), i.e. 9 , 4.5, 3 and 6 d. For acclimation as well as studying the effect of HRT, the SBR was operated in cycles of 24 h and each cycle consists of feeding and reaction (23.4 h), settling (0.35 h) and decanting the supernatant (0.25 h).

3.3.2 Analytical methods

The concentrations of nitrogen compounds, suspended solids (SS), volatile suspended solids (VSS), mixed-liquor suspended solids (MLSS), mixed-liquor volatile suspended solids (MLVSS) and alkalinity were measured according to the Standard Methods (APHA, 1998). The NH_4^+–N, NO_2^-–N, NO_3^-–N and SO_4^{2-} concentrations in influent and effluent were determined spectrophotometrically by using standard methods (APHA, 1998), and the organic matter content in the synthetic wastewater was expressed as COD. The pH was determined potentiometrically with a digital pH meter (SUNTEX SP-701, Taiwan) and the DO was monitored outside of the reactor with a digital DO meter (YSI 5100, Taiwan).

3.3.3 Polymerase chain reaction (PCR) and qPCR

The total genomic DNA present in the samples was extracted using the UltraClean Microbial DNA isolation Kit (MO BIO Laboratories, USA). The 16S rDNA sequences were amplified from the genomic DNA by PCR using 11f (5'-GTTTGATCCTGGCTCAG-3') and 1512r (5'-GGYTACCTTGTTACGACTT-3') oligonucleotide primers [39]. The thermal cycling consisted of 10 min at 94 ^oC followed by 35 cycles each of 90 sec at 94^oC, 45 sec at 52^oC, 120 sec at 72^oC and ended by additional 10 min at 72^oC. The nucleotide sequence of PCR products were determined using the BigDye terminator cycle sequencing kit (Applied Biosystems, USA). The resulting sequences were used to do nucleotide-nucleotide blast search through National Center for Biotechnology Information (NCBI). To amplify 16S rDNA of Anammox bacteria, PCR was performed using an oligonucleotide primer pair, 16S-1 (5'-AGTGGCGAAAGGGTGAGTAA-3') and 16S-2 (5'-GGTTACCTTGTTACGACT-3') [40] (referred as primer III) with thermal cycling

of 10 min at 94^{o}C followed by 40 cycles each of 15 sec at 94^{o}C, 2 sec at 50^{o}C, 60 sec at 68^{o}C and ended by additional 10 min at 72^{o}C.

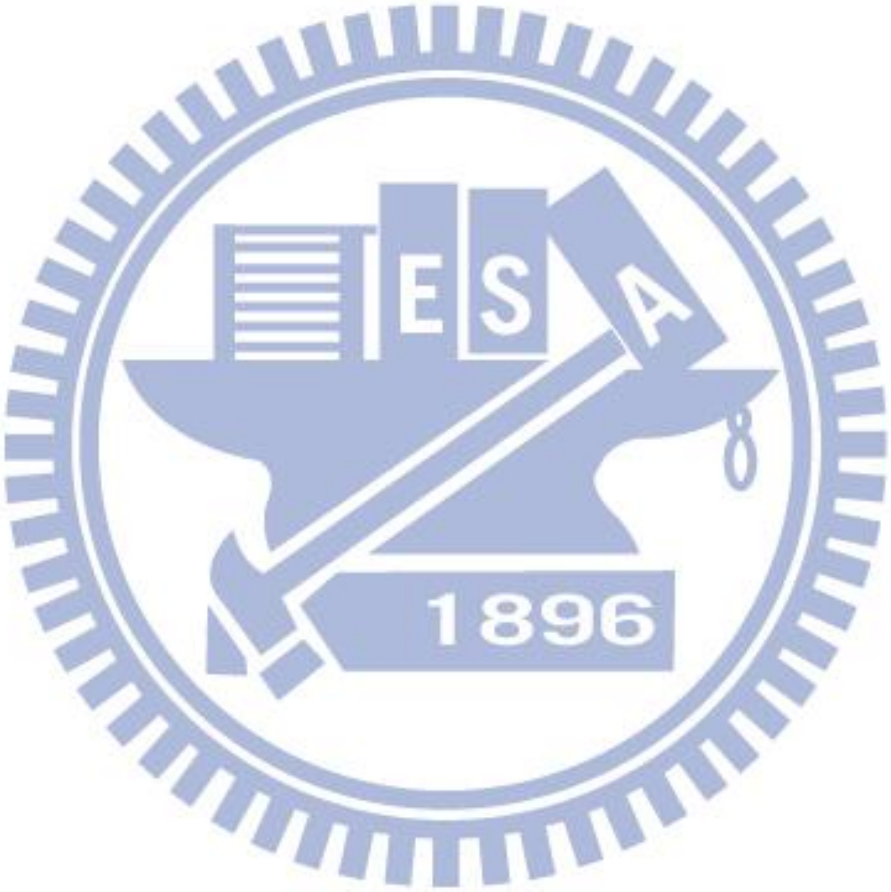
3.3.4 Fluorescence in situ hybridization (FISH)

The 16S rRNA-targeted oligonucleotide probe used in this study was Amx820 [41] for Anammox bacteria. The probe was synthesized and directly labeled with fluorescein isothiocyanate (FITC) at the 5' end. In situ hybridization was performed according to the procedure described by Amann et al. [42]. A100X objective Olympus BX51 microscope (Olympus Optical Co., Japan) fitted with a mercury bulb and blue, green and red filter sets were used for viewing and observing the slides. The photomicrograph was made using an Olympus U-CMAD 3 camera (Olympus Optical Co., Japan) with exposure times of 0.05 s for DAPI and 0.5 s for Amx820.

3.3.5 Terminal Restriction Fragment Length Polymorphism (TRFLP)

TRFLP is based on PCR amplification of a target gene. In the case of TRFLP, the amplification is performed with one or both the primers having their 5' end labeled with a fluorescent molecule. Add 0.5 μl of restriction endonuclease enzyme, *Hhal,* and 2 μl of complimentary buffer into 15μl sample of positive PCR product. The restriction enzyme and complimentary buffer, Buffer C (R003 A), are Catalog No. R6441 System Lot No. 221280 produced by Promega Corporation. The cut sites of the enzyme are 5'GCG^C3' and 3'C^GCG5'. Then put it into thermocycler at 37℃ for two hours. The above procedures are called as digestion reaction. The labeled fragments cuted in digestioin reaction were sent to Nucleic Acid Analysis and

Synthesis Core Laboratory to analyze with ABI PRISM3100 Genetic Analyzer[43,44].



Chapter 4

Result and Discussion

4.1 Profiles of pH and DO

Fig. 4 shows the profiles of pH and DO concentration in the SBR under various HRTs investigated. The pH profile was fairly constant over the HRTs, except the final 6 d of HRT, owing to the malfunction of the aerators on 3 d of HRT. The decrease in pH at any point of time was compensated by the addition of alkalinity to the reactor. If ammonium concentration increased in the effluent, the DO valve was adjusted in such a way that the excess ammonium undergoes partial nitrification. The DO concentration in the reactor was varying a lot in the initial days of operation, i.e. 9 d HRT. The activity of anammox bacteria and denitrifiers in the SNAD system relies on partial nitrification becuase the later supplies NO_2^--N to anammox and denitrification. Moreover, anammox bacteria and denitrifiers prefer anoxic/anaerobic environment. Therefore, there was some difficulty in controlling the air flow rate to the system in the initial days of SBR operation (0-19 d, Fig. 4). After this stage, the airflow was adjusted in such a way to maintain the DO of the reactor at a constant level. To measure the DO concentration precisely in the reactor DO was measured using the BOD bottle at the end of 3 d HRT. The DO concentration at HRT 6 d was close to 0.3-0.4 mg/L.

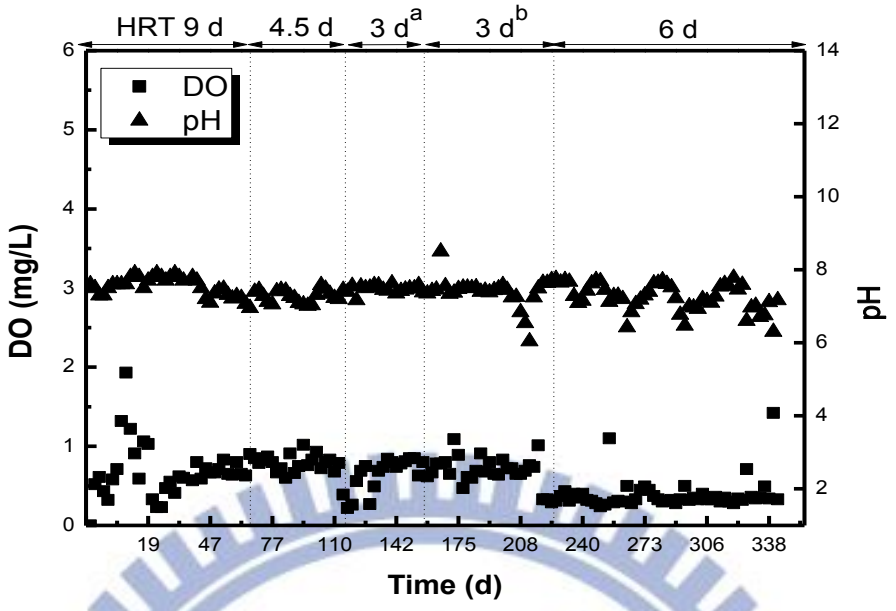


Fig. 6. Time courses of DO and pH during the operation of SNAD process with different HRTs. 3d [a] without aerator and water jacket problems, 3d [b] with aerator and water jacket problems.

4.2 Nitrogen and COD removals under various HRTs

At 9 d HRT, the SBR was operated with influent NH_4^+–N and COD concentrations of 200 mg/L and 100 mg/L, respectively, corresponding to the NLR of 22.2 g/m^3-d and OLR of 11.1 g/m^3-d. Table 6 compare the range of nitrogen loading rates used and total nitrogen removal under different nitrogen removal processes. As shown in Table 6, the loading rates of anammox and OLAND processes are lower than the present study, which evidences that autotrophic nitrogen removal can happen in lower loading rates also. Moreover, this is the first stage of SNAD seed sludge acclimation in the SBR; thus, the reactor was operated in moderate loading conditions to avoid substrate inhibition. The operating conditions of the SBR under various HRTs are shown in Table 6. The organic loading rate (OLR) and nitrogen loading rate (NLR) to the SBR under various HRTs were worked out, and are also shown in Table 5. However, the NH_4^+-N and COD concentrations were kept constant under all HRTs and the ratio of influent COD/TN was maintained at a constant level (0.5).

Table 5. The ranges of loading rate under different nitrogen removal processes

Nitrogen Removal Process	Requirement of O_2/COD	Nitrogen loading (Kg N m^{-3} reactor d^{-1})	Total nitrogen removal (%)	Application status	Common reactor configuration	Reference
Conventional	High/Yes	0.3-9	95	Full-scale	Activated sludge	[23,24]
SND	Low/No	1-3.5	100	Lab-scale	SBR	[43]
ANAMMOX	None/No	0.003-20	87	Full/Lab scale	FBR,SBR	[6,7,12,36]
SHARON	Low/No	0.5-1.5	90	Full-scale	Activated sludge	[2,3]
CANON	Low/No	0.04-3	75	Lab-scale	SBR, UASB	[4,3,5]
OLAND	Low/No	0.001-0.1	85	Lab-scale	SBR, RBC	[5,37]
In this study	Yes/Yes	0.022-0.066	95	Lab-scale	SBR	[9,10,12]

Table 6. Characteristics of synthetic wastewater before and after treatment

HRT (d)	NH_4^+-N (mg/L)		NO_2^--N (mg/L)		NO_3^--N (mg/L)		COD (mg/L)		[c]Inf. COD/TN	OLR (g/m^3-d)	NLR (g/m^3-d)	[c]Eff. TN (mg/L)	Removal (%)		Remarks
	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.	Inf.	Eff.					NH_4^+-N	COD	
9	200	9	0	2.1	17	1	100	14	0.5	11.1	22.2	12.1	96	86	-
4.5	200	10	0	0.4	17	0.1	100	16	0.5	22.2	44.4	10.5	95	84	[d]VFR increased by 2 times
3[a]	200	14	0	2	17	0	100	29	0.5	33.3	66.7	16	93	71	[d]VFR increased by 3 times
3[b]	200	94	0	0.4	17	0.5	100	14	0.5	33.3	66.7	94.9	53	86	Zone of aerator problem (last for 29 days)
6	200	70	0	0.3	17	57	100	13	0.5	16.6	33.3	127.3	65	87	[d]VFR increased by 1.5 times

[a]VFR increased by 3 times, and without aerator and water jacket problems

[b]VFR increased by 3 times, and with aerator and water jacket problems

[c]TN is the sum of NH_4^+-N, NO_2^--N and NO_3^--N;

[d]VFR represents volumetric flow rate and the increases, i.e.1.5, 2 and 3 times, based on 9 d HRT.

The influent and effluent profiles of nitrogenous matter and organics are shown in Fig. 4 and 5, respectively. In the first 40 d of operation, a consistent NH_4^+–N removal (more than 90%) was observed and small quantities of NO_2^--N and NO_3^--N accumulation were found in the SBR. However, SBR displayed a very poor COD removal efficiency (less than 65%) during this period. In the subsequent days (40-65 d), the removal efficiencies increased gradually and have shown a stable NH_4^+–N and COD removal efficiencies of 96% and 87%, respectively.

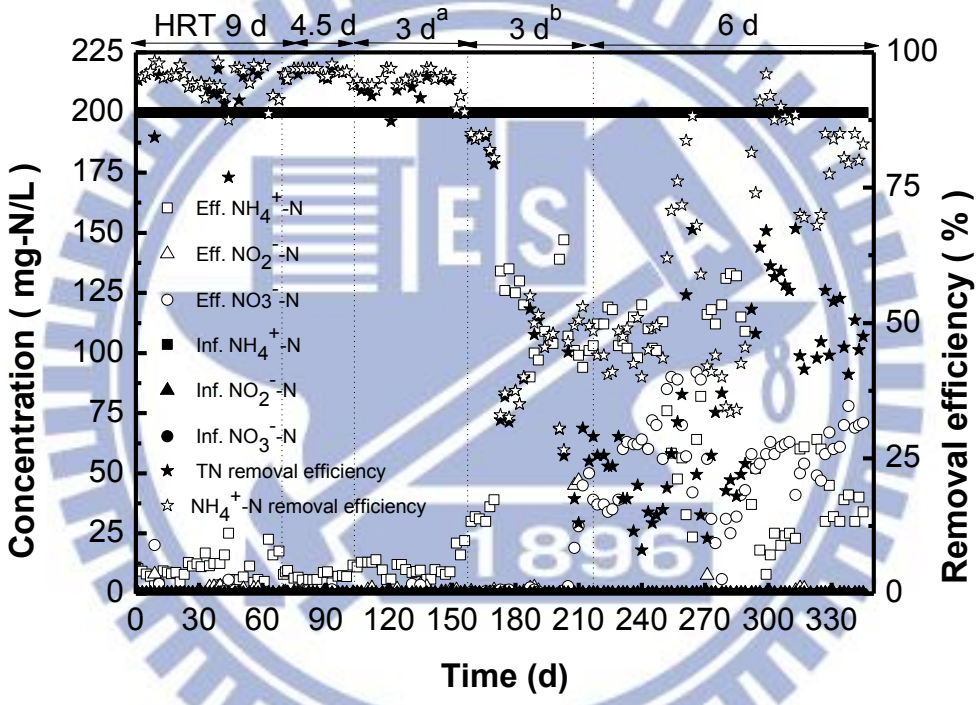


Fig.7. Performance of the concentration of nitrogen compounds and removal efficiency of ammonium and total nitrogen in the SBR at different HRTs. 3d [a] without aerator and water jacket problems, 3d[b] with aerator and water jacket problems.

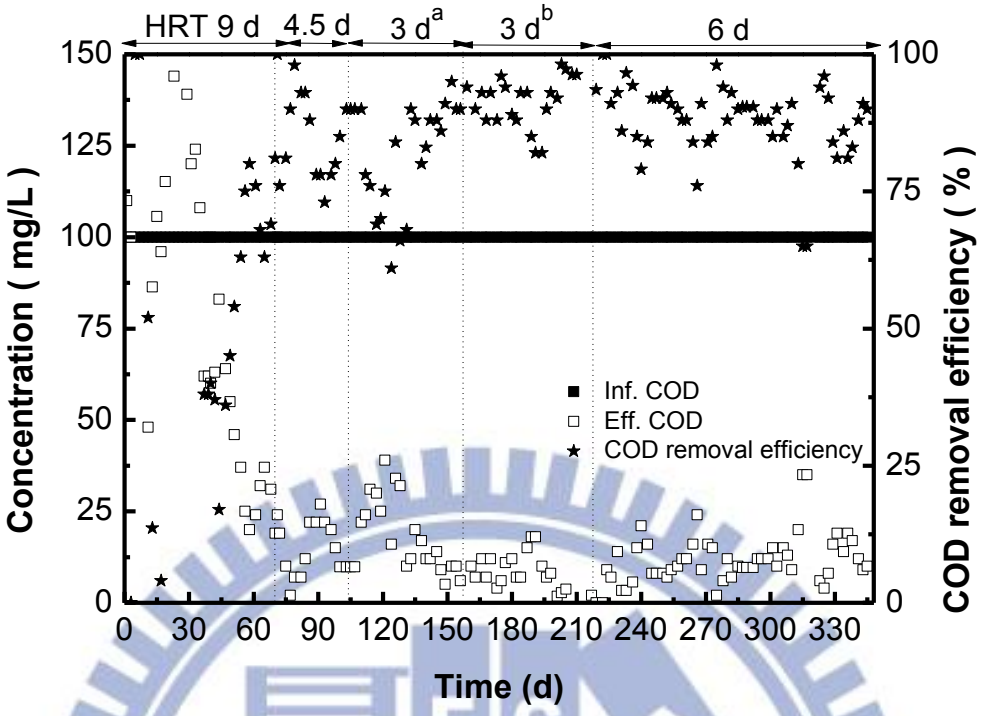


Fig. 8. Performance of the concentration of COD and removal efficiency of COD in the SBR at different HRTs. 3d [a] without aerator and water jacket problems, 3d[b] with aerator and water jacket problems.

In order to find the effect of loading rate on the SNAD process, the NLR and OLR were progressively increased by decreasing the HRT from 9 d to 4.5 d, and operated for 47 d (Table 5). Despite the higher influent NLR and OLR, a stable conversion of NH_4^+–N, without accumulation of NO_2^--N/NO_3^--N was observed in the SBR. The increased NLR (44 g/m^3-d) and OLR (22 g/m^3-d), decreased the COD removal efficiency of the SBR from 87% to 78%, whereas the NH_4^+–N removal efficiency was maintained in the same level, i.e. 95%. This reveals that the increase NLR and OLR have no significant effect on the SNAD system. Table 5 shows the steady-state concentrations of NH_4^+–N, NO_2^--N, NO_3^--N and COD under various HRTs. Following to the steady-state condition at 4.5 d HRT, the reactor NLR and OLR were further increased to 66 g/m^3-d and 33 g/m^3-d, respectively, also the HRT was decreased to 3 d. The decrease in the HRT to 3 d has decreased the NH_4^+–N and COD

removals in the system. An increasing trend in the effluent NH_4^+–N concentration can be noticed in Fig. 5. This indicates that the increases in NLR and OLR (at 3 d HRT) have produced slight inhibition/toxicity to the partial nitrifiers; as a result, insufficient NO_2^--N was produced in the system. Therefore, the performance of Anammox and denitrification were deprived and an overall decrease in the TN and COD removal efficiencies of the system were observed. However, an improvement in the COD removal was observed in the subsequent period (120-150 d) and reached a stable COD removal of 72%.

Unexpectedly, aerator and water jacket were went out-of-order under this recovery stage, which drastically decreased the reactor performance. During this stage, the DO in the SBR has went down to below 0.2 mg/L, pH drop down to less than 6 and the temperature decreased by 5 to 8°C. It can be noticed in Table 5 that only 52% of the NH_4^+–N was removed in the reactor, and interestingly, around 86% of the COD was removed in the reactor. Under this situation, it is hypothesized that Anammox bacteria might be inactive and the NO_2^--N produced as a result of partial nitrification could have been utilized only by denitrifiers. In order not to increase further loading under these circumstances, the reactor NLR and OLR were decreased to 33 g/m^3-d and 16 g/m^3-d. The reactor started to recover when the HRT was increased from 3 to 6 days. The effluent concentration of NH_4^+-N was decreased from 94 mg/L to 25 mg/L, also by slightly adjusting the DO and pH value back to optimal condition, the removal efficiencies of NH_4^+–N and TN has come back to 75% and 67%, respectively. These observations and hypothesis indicate that high DO concentrations (>2 mg/L) could result complete nitrification in the SNAD system, whereas low DO concentration (<0.5 mg/L) could reduce the rate of nitrification and overall performance of the reactor. Moreover, these data reveal that SNAD process is more resistant to substrate

shock loading compared to sudden change in aeration rate and temperature.

Despite of stable conversion of ammonium, nitrate accumulation was detected in effluent from the end of HRT 3 d. Nitrate production was related to a possible response of different electron acceptors such as sulfate which supplied from $(NH_4)_2SO_4$ in the medium instead of nitrite in SNAD process. In many researches, except for nitrite, nitrate and propionate, there might be some other electron acceptors for ammonium oxidation and sulfate is considered to be a suitable selection for its strong oxidization capacity. Polanco et al. (2001) showed the possibility of removing ammonium and sulfate simultaneously. They postulated that the nitrite formation and subsequent Anammox process were responsible for nitrogen removal according to the following equations (Eq. (13), (14), (15) and (16))[44]:

$$3SO_4^{2-}+4NH_4^{+}\rightarrow 3S^{2-}+4NO_2^{-}+4H_2O+8H^{+} \quad (13)$$

$$3S^{2-}+2NO_2^{-}+8H^{+}\rightarrow N_2+3S+4H_2O \quad (14)$$

$$2NO_2^{-}+2NH_4+\rightarrow 2N_2+4H_2O \quad (15)$$

$$SO_4^{2-}+2NH_4^{+}\rightarrow N_2+S+4H_2O \quad (16)$$

After disturbance during HRT 3 d, the reactor was in unsteady state, the end product of combining sulfate with ammonium might also produce nitrate as well. On the other hand, the long period acclimation of SNAD system may result to accumulation of sulfide which can be toxic to microorganisms. The sludge might be covered by sulfur which could limit the sufficient contact among reactants. The Anammox activity might also affect by some middle medium, such as nitrite, H_2S and sulfur causing

nitrate accumulation. With decreasing Anammox activity, further works need to focus on reduction of the released sulfureted hydrogen and collection of sulfur from reaction.

4.3 Model based evaluation of SNAD

The consumption of nitrogen compounds in partial nitrification, Anammox and denitrification are modeled using the stoichiometric equations and the experimental data. Generally, the presence of organic carbon is inhibitory to anammox bacteria. For example, the presence of methanol is found to have irreversible inhibition at concentration as low as 0.5 mM. However, a recent study indicated that anammox bacteria were successful in the oxidation of propionate, and the presence of glucose, acetate, formate and alanine had no effect on the anammox process[45]. The free energy of denitrification using typical organic carbon is shown in Table 7 Moreover, anammox bacteria can be competitive with heterotrophic denitrifiers for the utilization of organic matter, i.e. propionate. But, the rate of propionate utilization by anammox bacteria was 0.6 mM/mg of protein/d, which is far less than the utilization rate by denitrifiers in real-time wastewater systems.

Table 7. Free energy of typical organic carbon with different electron donor in denitrificaiton [46-48].

Denitrification (organic carbon)	**Stoichiometric equation**	**Free energy (kJ/mol)**
Acetate with nitrite	$NO_2^- + 0.375CH_3COO^- + H^+ \rightarrow 0.5N_2 + 0.375CO_2 + 0.375HCO_3^- + 0.875H_2O$	-360

Methanol	$NO_2^-+0.5CH_3OH+H^+ \rightarrow 0.5N_2+0.5CO_2+1.5H_2O$	-388
Glucose	$NO_2^-+0.125C_6H_{12}O_6+H^+ \rightarrow 0.5N_2+0.75CO_2+1.25H_2O$	-402
Acetate with nitrate	$NO_3^-+0.625CH_3COO^-+H^+ \rightarrow 0.5N_2+0.625CO_2+0.625HCO_3^-+1.125H_2O$	-498
Methanol	$NO_3^-+0.83CH_3OH+H^+ \rightarrow 0.5N_2+0.83CO_2+2.17H_2O$	-545
Glucose	$NO_3^-+0.208C_6H_{12}O_6+H^+ \rightarrow 0.5N_2+1.25CO_2+1.75H_2O$	-568

The following stoichiometic relationships are used for modeling: (i) the molar ratio of NH_4^+-N: NO_2^--N in partial nitrification is 1:1, (ii) the stoichiometric consumption (molar ratio) of NH_4^+-N: NO_2^--N in Anammox process is 1:1.32, and produces 0.26 mole of NO_3^--N, subsequently that can be utilized in denitrification, (iii) 1 mg/L of NO_3^--N is used for consuming 1.74 mg/L COD in denitrification. The TN removal in partial nitrification with Anammox and denitrification under all the HRTs based on the stoichiometric modeling are shown in Table 8. Moreover, the detailed modeling concept and the outcomes for 3 d HRT based on the average influent and effluent data are shown in Fig. 9.

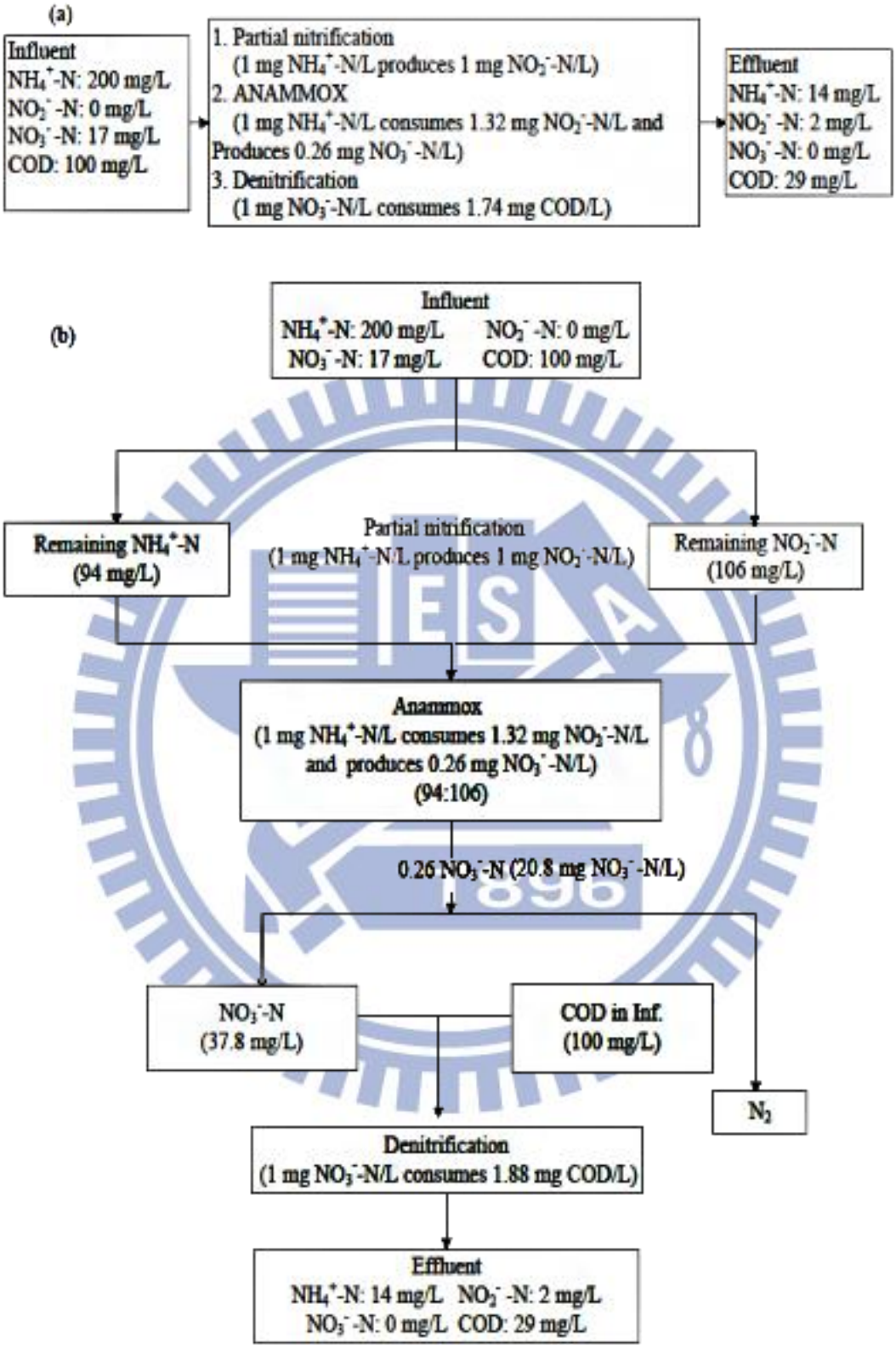


Fig.9. Model based evaluation of the SNAD system.

Table 8 indicates that around 85-87% of the TN removal is by the combination of Anammox and partial nitrification. The NO_3^--N produced in Anammox process is utilized in denitrification along with COD, which is responsible for a TN removal of 7-9%. These observations indicate that under steady-state condition all three processes in the SBR, i.e. partial nitrification, Anammox and denitrification, synchronize each other and establish a firm relationship within the reactor irrespective of the NLR and OLR. However, the shock in the operating DO, pH and temperature of the SNAD system greatly affected the relationship of these processes. This can be evidenced from the poor NH_4^+-N removal efficiency of the system (52%). However, the overall TN removal efficiency of the SNAD system was maintained around 50.7% owing to the consumption of NO_2^--N and/or NO_3^--N in denitrification. The stoichiometric modeling results also indicate that the decrease in the HRT of the system (from 9 to 3 d) could facilitate the increase in the production of Anammox bacteria (from 0.067 to 0.357 g/d). This approach could be useful to enrich the slow growing Anammox bacteria in the real-time conditions. However, a very high volumetric flow rate (VFRs) could wash out the Anammox bacteria from the system.

Table 8. Performance of the SBR under various HRTs

HRT (d)	TN removal (%)		Biomass produced (g/d)	Sensitivity Index (SI)[c*]			
	Partial nitrification + anammox	denitrification		NH_4	NO_2^-	NO_3^-	COD
9	85.7%	8.7%	0.067	- {9}	- {2.1}	- {1}	- {14}
4.5	87.3%	7.8%	0.259	0.4（13）	0.1（2.3）	0.8（1.8）	0.9（27）
3[a]	85.5%	7.3%	0.357	1.3（21）	0.7（3.6）	2.6（3.6）	1.8（39）
3[b]	41.9%	8.7%	0.305	14（135）	0.1（2.2）	0.6（1.6）	2.6（50）
6	32.2%	8.8%	0.197	14（133）	2.5（7.5）	91（92）	1.5（35）

[a]VFR increased by 3 times, and without aerator and water jacket problems

[b]VFR increased by 3 times, and with aerator and water jacket problems

[c]Sensitivity index based on the species concentration at 9 d HRT

* The values within "{}" and "()" indicates average and maximum concentrations in mg/L, respectively

Alternatively, the sensitivity of the SNAD system to the change in VFR was evaluated based on sensitivity index (SI) as shown in Eq. (17) [49].

$$SI = \frac{O_{max} - O_s}{O_s} \quad (17)$$

where, O_{max} is the maximum concentration of substrate in the effluent at 4.5, 3 and 6 d HRTs (mg/L), and O_s is the average concentration of substrate in the effluent at 9 d HRT (mg/L). The values of SI for all nitrogen species and COD are shown in Table 8. The SI values indicate that the SNAD process is not greatly affected by the change in VFR of the system compared to the shock in the operating DO, pH and temperature conditions. Under the shocking DO, pH and temperature conditions, the SI values increased by 14 and 2.6 times for NH_4^+-N and COD, respectively. As indicated before, the Anammox bacteria might be inactive under the shocking condition and the NO_2^--N produced as a result of partial nitrification could have been utilized only by denitrifiers. This reveals that the SNAD system has the capability of acting as shortcut nitrification-denitrification (SND), i.e. NH_4^+-N is oxidized to NO_2^--N in nitritation, and subsequently, the NO_2^--N is reduced to N_2 gas. However, the removal efficiency of the SND system (under shocking condition) is far less than the efficiency observed in the SNAD system.

4.4 Comparison between full-scale SNAD system with lab-scale SNAD system

Landfill is the most common methods of organized waste disposal and remained so in many places around the world. A large number of adverse impacts may occur from landfill operations. One of the impacts is from landfill leachate which contained

organic and inorganic matters characterized by high concentration of nitrogen compounds generated during decomposition of waste in the landfill. Leachate has the specific meaning of having dissolved or entrained environmentally harmful substances which may then enter the environment. In older landfills and those with no membrane between the waste and the underlying, leachate is free to egress the waste directly into the groundwater. The most common method of handling collected leachate is on-site treatment.

The full scale SNAD system is applied in landfill leachate treatment plant [10]. The aeration tank is treating an average leachate flow of 304m^3 d^{-1} with a sludge retention time between 12 and 18 d. Similarly, the full-scale SNAD system was evaluated by the model and sensitivity index describe in previous section. Table 9 shows the result of full-scale SNAD system, it indicated that the nitrogen removal mainly by partial nitrification and Anammox. In 2010, annual precipitation amounts vary from less than 332 mm/month to more than 479 mm/month. This makes the influent concentration varies a lot and the nitrogen removal percentage of partial nitrification and Anammox below than 50%. Moreover, the sensitivity index of ammonium in 2010 has significant effect on the performance of SNAD system.

The comparisons between full-scale and lab-scale SNAD system: (1) Despite of heavy rain in 2010, the full-scale SNAD system demonstrated a stable and high treatment performance for nitrogen removal from actual landfill leachate. Due to the small volume of lab-scale reactor, the buffer capacity of lab-scale SNAD system is way more sensitive than full-scale system, and (2) The operation of lab-scale SNAD system can be more precise on controlling different parameters, such as pH value and DO. pH value in the optimal range to maintain the concentration of free ammonia between 3.5

to 10 mg NH_3-N/L, which made sure the nitrification process stop at ammonium oxidation step and DO concentration in a range of 0.3 to 0.4 mg/L in case nitrite accumulate in the reactor.

Overall, the SNAD process will offer a great future potential for removing nitrogen and organic compounds, it can save energy consumption and cost of adding extra chemical, from wastewater in the industrial application, especially from optoelectronics industrial wastewater in Taiwan.

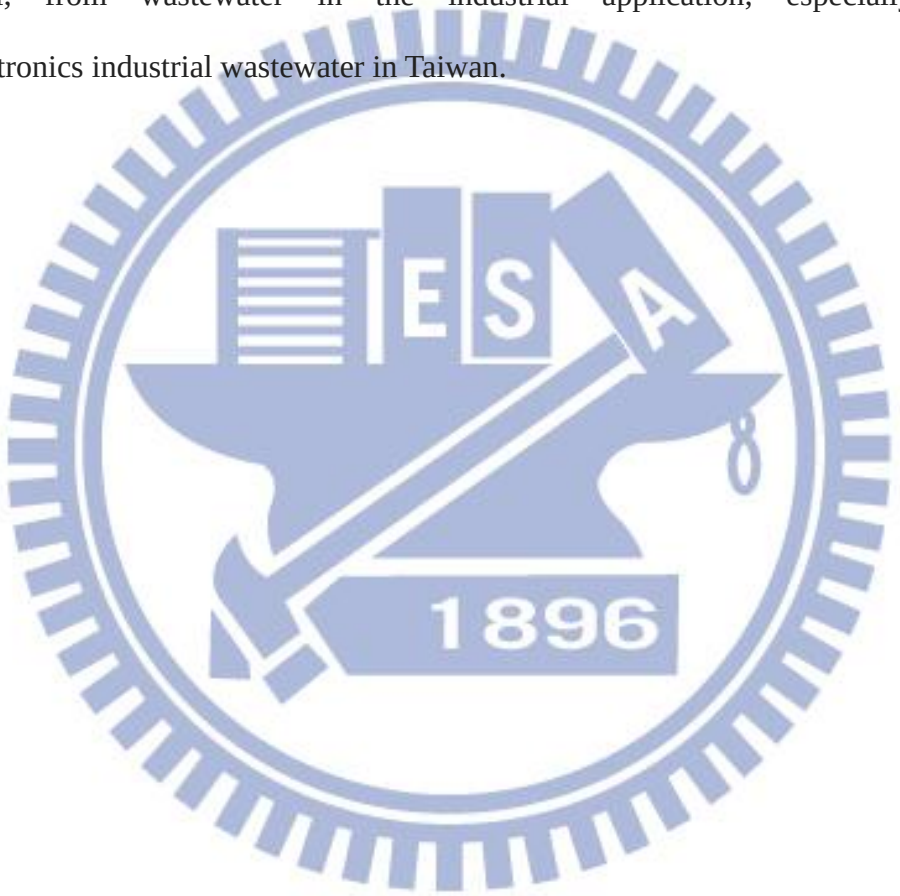


Table 9. Performance of the full-scale SNAD system under different years

Years	TN removal (%)		Sensitivity Index (SI)[b*]			
	Partial nitrification + anammox	denitrification	NH_4	NO_2^-	NO_3^-	COD
2009	71.6%	22.2%	-（224）	-（-）	-（160）	-（492）
2010	47%	23%	2.3（468）	-（-）	1（125）	-0.14（491）
2011[a]	65.2%	13%	1.8（393）	-（-）	1（125）	0.16（319）

[a]The average and maximum concentrations is in 2011/1/1-2011/5/31.

[b]Sensitivity index based on the species concentration in 2009

* The values within "{}" and "()" indicates average and maximum concentrations in mg/L, respectively

4.5 Diversity of the bacterial community in SNAD system

Biotechnological analysis such as FISH and real-time quantitative PCR (qPCR) were conducted to verify the presence of microbial community in SNAD system. Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction, is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, real time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The procedure follows the general principle of PCR; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

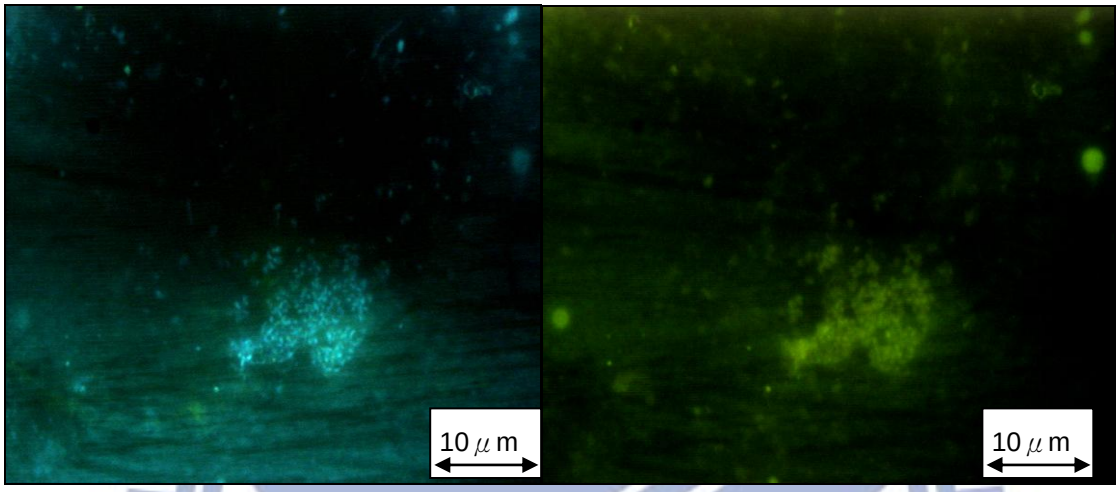
The seed sludge was inoculated from a landfill leachate treatment plant. Figure 10 (a) shows the red granules from aeration tank which found to be typical in Anammox reactor s [10]. Fig. 10 (b) shows the attached growth of Anammox bacteria on the aeration tank wall.

(a)

(b)

Fig. 10 Pictures of red granules from aeration tank (a) Granules in the aeration tank, (b) attached growth of Anammox bacteria on the aeration tank wall.

Red granules taken from landfill leachate treatment plant were analyzed by FISH to confirm the occurrence of Anammox bacteria. Fig.11 (a) shows that all bacterial cells were stained with DAPI；Fig 11 (b) shows that all Anammox bacteria hybridized with probe Amx820.



(a) (b)

Fig. 11. Fluorescence micrographs of bacteria granules collected from the aeration tank (a) DAPI, (b) Amx820

Moreover, 16S rRNA clone analysis revealed that all clones from aeration tank were related to *Kuenenia stuttgartiensis*, *Candidatus* Kuenenia Stuttgartiensis and Anaerobic ammonium oxidizing planctomycete KOLL2a with 99% sequence similarity (Table 9). Similarities with other species are also listed in Table 9. Furthermore, the presence of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) were confirmed by using qPCR and Terminal Restriction Fragment Length Polymorphism (TRFLP or sometimes T-RFLP). TRFLP is a molecular biology technique for profiling of microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. The method is based on digesting a mixture of PCR

amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer. The result is a graph image where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity.

The R^2 value of qPCR is greater than 0.97 for all curves and amplification efficiencies with slopes of -3.44 and -3.17. Two standard curves were constructed using cloned 16S rDNA sequence of eubacteria and Anammox bacteria into pGEM-T (Promega, USA) cloning vector respectively.

Table 10 and 11 provide the detail experimental outcomes of qPCR and the ratio of different bacteria to eubacteria and Anammox bacteria. The relatively quantification percentage of different bacteria to eubacteria are shown in Table 12.

Table 10. Outcomes of Sequence analysis [10].

Species Identified	Similarity (%)	NCBI No.	Reference
Kuenenia stuttgartiensis	99	CT573071.1	[50]
Candidatus Kuenenia Stuttgartiensis	99	AF375995.1	[51]
Anaerobic ammonium oxidizing planctomycete KOLL2a	99	AJ250882.1	[52]
Candidatus Brocadia fulgida	93	EU478693.1	[53]
Planctomycete KSU-1	93	AB057453.1	[40]
Candidatus Brocadia fulgida	93	DQ459989.1	[54, 55]
Candidatus Jettenia asiatica	92	DQ301513.1	[56]

Table 11. Detail outcomes of qPCR.

DNA	Red granule (LWP/fresh) Genomic	Red granule (LWP/fresh) cDNA	Sludge in SNAD system Genomic
Average Ct of eubacteria	18.54	23.20	17.32
Copy number	$2.63*10^6$	$1.17*10^5$	$5.93*10^6$
Average Ct of total Anammox bacteria	16.69	22.42	18.46
Copy number	$7.90*10^5$	$1.24*10^4$	$2.19*10^5$

* Each value was calculated from triplet

Table 12. Relatively quantification of different bacteria to eubacteria

DNA	Red granule (LWP/fresh) Genomic	Red granule (LWP/fresh) cDNA	Sludge in SNAD system Genomic
Average Ct (AOB)	22.27	28.87	19.46
SD (AOB)	0.4755	0.8411	0.2313
AOB /EB	0.075198236	0.019569671	0.227247028
Average Ct (NOB)	22.01	23.90	18.84
SD (NOB)	0.2735	0.1180	0.2828
NOB /EB	0.0896855	0.613658437	0.348210915
Average Ct (KS)	0.0897	0.6137	0.3482
SD (KS)	0.4494	0.0394	0.1800
KS / TA	0.828922332	18.0136671	0.040009267

*AOB: ammonium oxidizing bacteria; EB: eubacteria; NOB: nitrite oxidizing bacteria

Table 13. Relatively quantification of different microbial community in SNAD system

Red granule (LWP/fresh)	KS / EB	AOB / EB	NOB / EB	BA / EB
Ratio (SD)	65.25 (2.78)	7.42 (1.12)	9.71 (2.88)	11.05 (4.1)

*BA: Brocadia anammoxidans

TRFLP with primers amoA-1F and amoA-2R was also carried out to confirm the presence of AOB. The results of TRFLP analyses obtained are shown in the figure 12 which indicated the presence of *Nitrosomonas europaea* and *Nitrosomonas oligotropha*.

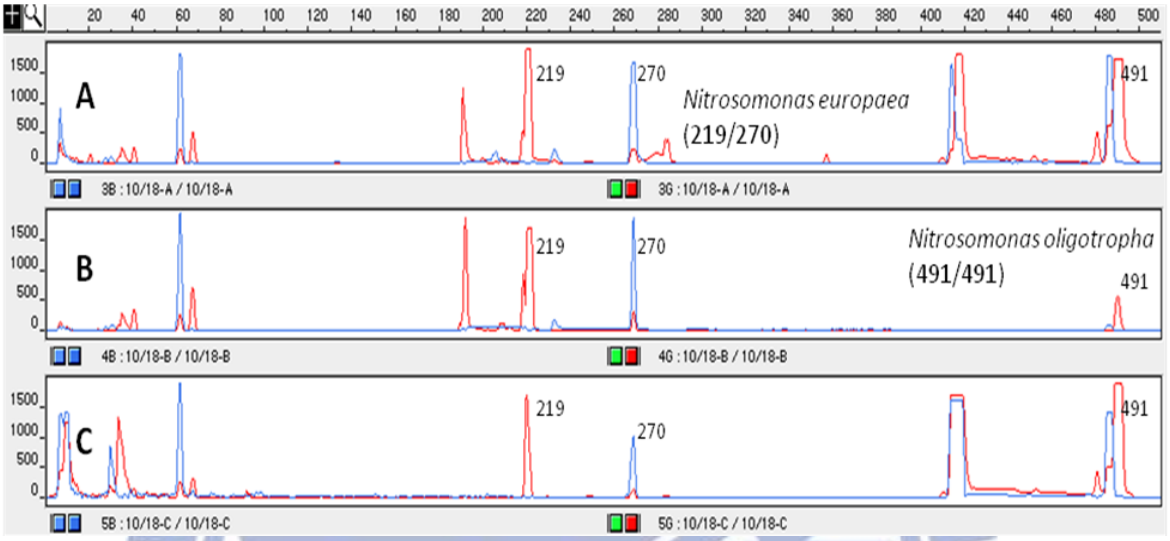


Fig.12. The experimental outcomes of TRFLP analysis.

Chapter 5

Conclusion

The SNAD process was successfully developed in the SBR and the effect of HRTs on the performance of SNAD system were investigated. Around 96% NH_4^+-N removal and 87% COD removal were achieved under the NLR and OLR of 22.2 and 11.1 g/m^3-d, respectively. The increases in NLR and OLR up to 66.7 and 33.3 g/m^3-d have produced little impact on the performance of the reactor; whereas, the sudden reduction/shock in the operating DO, pH and temperature has produced a major drop in the SNAD performance. However, the performance was able to recover from disturbances when NLR and OLR were decreased to 33 g/m^3-d and 16 g/m^3-d, respectively. The removal of nitrogenous compounds in each of the SNAD process was modeled using the stoichiometric relationship. The presence of Anammox bacteria in the SNAD process was confirmed using FISH, qPCR and TRFLP. Overall, SNAD process has potential to successfully remove the nitrogen and carbon compounds from wastewater that can be applied in industrial application for the future.

References

1. Fernandez, I., Vazquez-Padin, J. R., Mosquera-Corral, A., Campos, J. L., and Mendez, R., (2008) Biofilm and granular systems to improve Anammox biomass retention. *Biochemical Engineering Journal*. Vol. 42, No. 3, pp. 308-313.
2. Hellinga, C., Schellen, Aajc, Mulder, J. W., Van Loosdrecht, M. C. M., and Heijnen, J. J., (1998) The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. *Water Science and Technology*. Vol. 37, No. 9, pp. 135-142.
3. Van Dongen, U., Jetten, M. S. M., and Van Loosdrecht, M. C. M., (2001) The SHARON((R))-Anammox((R)) process for treatment of ammonium rich wastewater. *Water Science and Technology*. Vol. 44, No. 1, pp. 153-160.
4. Sliekers, A. O., Derwort, N., Gomez, J. L. C., Strous, M., Kuenen, J. G., and Jetten, M. S. M., (2002) Completely autotrophic nitrogen removal over nitrite in one single reactor. *Water Research*. Vol. 36, No. 10, pp. 2475-2482.
5. Kuai, L. P.andVerstraete, W., (1998) Ammonium removal by the oxygen-limited autotrophic nitrification-denitrification system. *Applied and Environmental Microbiology*. Vol. 64, No. 11, pp. 4500-4506.
6. Jetten, M. S., Strous, M., Van De Pas-Schoonen, K. T., Schalk, J., Van Dongen, U. G., Van De Graaf, A. A., Logemann, S., Muyzer, G., Van Loosdrecht, M. C. And Kuenen, J., (1998) The anaerobic oxidation of ammonium. *FEMS Microbiology Reviews*. Vol. 22, No. 5, pp. 421–437.
7. Strous, M., Kuenen, J. G., and Jetten, M. S. M., (1999) Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology*. Vol. 65, No. 7, pp. 3248-3250.
8. Kumar, M.andLin, J. G., (2010) Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal-Strategies and issues. *Journal of Hazardous Materials*. Vol. 178, No. 1-3, pp. 1-9.
9. Chen, H. H., Liu, S. T., Yang, F. L., Xue, Y., and Wang, T., (2009) The development of simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) process in a single reactor for nitrogen removal. *Bioresource Technology*. Vol. 100, No. 4, pp. 1548-1554.
10. Wang, C. C., Lee, P. H., Kumar, M., Huang, Y. T., Sung, S. W., and Lin, J. G., (2010) Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant. *Journal of Hazardous Materials*. Vol. 175, No. 1-3, pp. 622-628.

11. Strous, M., Heijnen, J. J., Kuenen, J. G., and Jetten, M. S. M., (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology*. Vol. 50, No. 5, pp. 589-596.
12. Strous, M., Vangerven, E., Kuenen, J. G., and Jetten, M., (1997) Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge. *Applied and Environmental Microbiology*. Vol. 63, No. 6, pp. 2446-2448.
13. Dapena-Mora, A., Arrojo, B., Campos, J. L., Mosquera-Corral, A., and Mendez, R., (2004) Improvement of the settling properties of Anammox sludge in an SBR. *Journal of Chemical Technology and Biotechnology*. Vol. 79, No. 12, pp. 1417-1420.
14. Lopez, H., Puig, S., Ganigue, R., Ruscalleda, M., Balaguer, M. D., and Colprim, J., (2008) Start-up and enrichment of a granular anammox SBR to treat high nitrogen load wastewaters. *Journal of Chemical Technology and Biotechnology*. Vol. 83, No. 3, pp. 233-241.
15. Joss, A., Salzgeber, D., Eugster, J., Konig, R., Rottermann, K., Burger, S., Fabijan, P., Leumann, S., Mohn, J., and Siegrist, H., (2009) Full-Scale Nitrogen Removal from Digester Liquid with Partial Nitritation and Anammox in One SBR. *Environmental Science & Technology*. Vol. 43, No. 14, pp. 5301-5306.
16. Vandegraaf, A. A. , Debruijn, P., Robertson, L. A., Jetten, M. S. M., and Kuenen, J. G., (1996) Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology-Uk*. Vol. 142, No. pp. 2187-2196.
17. Camargo, Julio A.andAlonso, Alvaro, (2006) Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. *Environment International*. Vol. 32, No. 6, pp. 831-849.
18. Guo, X., Kim, J. H., Behera, S. K., and Park, H. S., (2008) Influence of dissolved oxygen concentration and aeration time on nitrite accumulation in partial nitrification process. *International Journal of Environmental Science and Technology*. Vol. 5, No. 4, pp. 527-534.
19. Sabumon, P. C., (2008) Development of a novel process for anoxic ammonia removal with sulphidogenesis. *Process Biochemistry*. Vol. 43, No. 9, pp. 984-991.
20. Chen, J. W., Zheng, P., Yu, Y., Tang, C. J., and Mahmood, Q., (2009) Promoting sludge quantity and activity results in high loading rates in Anammox UBF. *Bioresource Technology*. Vol. 101, No. 8, pp. 2700-2705.

21. Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z. C., Freney, J. R., Martinelli, L. A., Seitzinger, S. P., and Sutton, M. A., (2008) Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science*. Vol. 320, No. 5878, pp. 889-892.
22. Duce, R. A., Laroche, J., Altieri, K., Arrigo, K. R., Baker, A. R., Capone, D. G., Cornell, S., Dentener, F., Galloway, J., Ganeshram, R. S., Geider, R. J., Jickells, T., Kuypers, M. M., Langlois, R., Liss, P. S., Liu, S. M., Middelburg, J. J., Moore, C. M., Nickovic, S., Oschlies, A., Pedersen, T., Prospero, J., Schlitzer, R., Seitzinger, S., Sorensen, L. L., Uematsu, M., Ulloa, O., Voss, M., Ward, B., and Zamora, L., (2008) Impacts of atmospheric anthropogenic nitrogen on the open ocean. *Science*. Vol. 320, No. 5878, pp. 893-897.
23. Bruce E.R. And Perry, L.M. , (2001) *Environmental Biotechnology: Principles and Applications*. McGraw-Hill, New York.
24. Gradly, C.P.L and Lim, H., (1980) *Biological Wastewater Treatment*. Dekker, New York.
25. Jetten, M. S. M., Wagner, M., Fuerst, J., Van Loosdrecht, M., Kuenen, G., and Strous, M., (2001) Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. *Current Opinion in Biotechnology*. Vol. 12, No. 3, pp. 283-288.
26. Mulder, A., Vandegraaf, A. A., Robertson, L. A., and Kuenen, J. G., (1995) ANAEROBIC AMMONIUM OXIDATION DISCOVERED IN A DENITRIFYING FLUIDIZED-BED REACTOR. *Fems Microbiology Ecology*. Vol. 16, No. 3, pp. 177-183.
27. Vandegraaf, A. A., Mulder, A., Debruijn, P., Jetten, M. S. M., Robertson, L. A., and Kuenen, J. G., (1995) ANAEROBIC OXIDATION OF AMMONIUM IS A BIOLOGICALLY MEDIATED PROCESS. *Applied and Environmental Microbiology*. Vol. 61, No. 4, pp. 1246-1251.
28. Broda, E. , (1977) Two kinds of lithotrophs missing in nature. *Zeitschrift für allgemeine Mikrobiologie*. Vol. Vol. 17, No. No. 6, pp. pp. 491-493.
29. Kuenen, J. G., (2008) Anammox bacteria: from discovery to application. *Nature Reviews Microbiology*. Vol. 6, No. 4, pp. 320-326.
30. Schalk, J., Oustad, H., Kuenen, J. G., and Jetten, M. S. M., (1998) The anaerobic oxidation of hydrazine: a novel reaction in microbial nitrogen metabolism. *Fems Microbiology Letters*. Vol. 158, No. 1, pp. 61-67.
31. Delong, E.F., (2002) *All in the packaging*. Nature, Vol. 419, pp. 676-677.
32. Vlaeminck, S. E., Cloetens, L. F. F., Carballa, M., Boon, N., and Verstraete, W., (2009) Granular biomass capable of partial nitritation and anammox (vol 58, pg 1113, 2008). *Water Science and Technology*. Vol. 59, No. 3, pp. 609-617.

33. Trigo, C., Campos, J. L., Garrido, J. M., and Mendez, R., (2006) Start-up of the Anammox process in a membrane bioreactor. *Journal of Biotechnology*. Vol. 126, No. 4, pp. 475-487.
34. Helmer, C., Tromm, C., Hippen, A., Rosenwinkel, K. H., Seyfried, C. F., and Kunst, S., (2001) Single stage biological nitrogen removal by nitritation and anaerobic ammonium oxidation in biofilm systems. *Water Science and Technology*. Vol. 43, No. 1, pp. 311-320.
35. Third, K. A., Sliekers, A. O., Kuenen, J. G., and Jetten, M. S. M., (2001) The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: Interaction and competition between three groups of bacteria. *Systematic and Applied Microbiology*. Vol. 24, No. 4, pp. 588-596.
36. Strous, M., (2000) *Microbilogy of anaerobic ammonium oxidation.*Ph D. Kluyver Laboratory. TU Delft University Delft.
37. Sliekers, A. Olav, Third, K. A., Abma, W., Kuenen, J. G., and Jetten, M. S. M., (2003) CANON and Anammox in a gas-lift reactor. *FEMS Microbiology Letters*. Vol. 218, No. 2, pp. 339-344.
38. Poth, M., (1986) DINITROGEN PRODUCTION FROM NITRITE BY A NITROSOMONAS ISOLATE. *Applied and Environmental Microbiology*. Vol. 52, No. 4, pp. 957-959.
39. Amann, R. I., Ludwig, W., and Schleifer, K. H., (1995) PHYLOGENETIC IDENTIFICATION AND IN-SITU DETECTION OF INDIVIDUAL MICROBIAL-CELLS WITHOUT CULTIVATION. *Microbiological Reviews*. Vol. 59, No. 1, pp. 143-169.
40. Fujii, T., Sugino, H., Rouse, J. D., and Furukawa, K., (2002) Characterization of the microbial community in an anaerobic ammonium-oxidizing biofilm cultured on a nonwoven biomass carrier. *Journal of Bioscience and Bioengineering*. Vol. 94, No. 5, pp. 412-418.
41. Schmid, M. C., Maas, B., Dapena, A., De Pas-Schoonen, K. V., De Vossenberg, J. V., Kartal, B., Van Niftrik, L., Schmidt, I., Cirpus, I., Kuenen, J. G., Wagner, M., Damste, J. S. S., Kuypers, M., Revsbech, N. P., Mendez, R., Jetten, M. S. M., and Strous, M., (2005) Biomarkers for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. *Applied and Environmental Microbiology*. Vol. 71, No. 4, pp. 1677-1684.
42. Amann, R. I., Krumholz, L., and Stahl, D. A., (1990) Fluorescent-Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental Studies in Microbiology. *Journal of Bacteriology*. Vol. 172, No. 2,
43. Helmer, ChristineandKunst, Sabine, (1998) Simultaneous

nitrification/denitrification in an aerobic biofilm system. *Water Science and Technology*. Vol. 37, No. 4-5, pp. 183-187.

44. Fdz-Polanco, Fernando, Fdz-Polanco, Maria, Fernandez, Neivy, Urue 鋼, Miguel A., Garcia, Pedro A., and Villaverde, Santiago, (2001) New process for simultaneous removal of nitrogen and sulphur under anaerobic conditions. *Water Research*. Vol. 35, No. 4, pp. 1111-1114.
45. Guven, Didem, Dapena, Ana, Kartal, Boran, Schmid, Markus C., Maas, Bart, Van De Pas-Schoonen, Katinka, Sozen, Seval, Mendez, Ramon, Op Den Camp, Huub J. M., Jetten, Mike S. M., Strous, Marc, and Schmidt, Ingo, (2005) Propionate Oxidation by and Methanol Inhibition of Anaerobic Ammonium-Oxidizing Bacteria. *Applied and Environmental Microbiology*. Vol. 71, No. 2, pp. 1066-1071.
46. Ahn, Y. H., (2006) Sustainable nitrogen elimination biotechnologies: A review. *Process Biochemistry*. Vol. 41, No. 8, pp. 1709-1721.
47. Sabumon, P. C., (2009) Effect of potential electron acceptors on anoxic ammonia oxidation in the presence of organic carbon. *Journal of Hazardous Materials*. Vol. 172, No. 1, pp. 280-288.
48. Sabumon, P. C., (2007) Anaerobic ammonia removal in presence of organic matter: A novel route. *Journal of Hazardous Materials*. Vol. 149, No. 1, pp. 49-59.
49. Jing, C., Ping, Z., and Mahmood, Q., (2009) Simultaneous sulfide and nitrate removal in anaerobic reactor under shock loading. *Bioresource Technology*. Vol. 100, No. 12, pp. 3010-3014.
50. Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M. W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B., Schenowitz-Truong, C., Medigue, C., Collingro, A., Snel, B., Dutilh, B. E., Op Den Camp, H. J. M., Van Der Drift, C., Cirpus, I., Van De Pas-Schoonen, K. T., Harhangi, H. R., Van Niftrik, L., Schmid, M., Keltjens, J., Van De Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M. A., Mewes, H. W., Weissenbach, J., Jetten, M. S. M., Wagner, M., and Le Paslier, D., (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature*. Vol. 440, No. 7085, pp. 790-794.
51. Schmid, M., Schmitz-Esser, S., Jetten, M., and Wagner, M., (2001) 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental Microbiology*. Vol. 3, No. 7, pp. 450-459.
52. Egli, K., Fanger, U., Alvarez, P. J. J., Siegrist, H., Van Der Meer, J. R., and

Zehnder, A. J. B., (2001) Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. *Archives of Microbiology*. Vol. 175, No. 3, pp. 198-207.

53. Woebken, D., Lam, P., Kuypers, M. M. M., Naqvi, S. W. A., Kartal, B., Strous, M., Jetten, M. S. M., Fuchs, B. M., and Amann, R., (2008) A microdiversity study of anammox bacteria reveals a novel Candidatus Scalindua phylotype in marine oxygen minimum zones. *Environmental Microbiology*. Vol. 10, No. 11, pp. 3106-3119.
54. Kartal, B., Van Niftrik, L., Sliekers, O., Schmid, M.C., Schmidt, I., Van De Pas-Schoonen, K., Cirpus, I., Van Der Star, W., Van Loosdrecht, M., Abma, W., Kuenen, J.G., Mulder, J.-W., Jetten, M.S.M., Den Camp, H.O., Strous, M. And Van De Vossenberg, J., (2004) Application, eco-physiology and biodiversity of anaerobic ammonium-oxidizing bacteria. . *Reviews in Environmental Science and Biotechnology*. Vol. 3, No. pp. 255-264.
55. Kartal, Boran, Van Niftrik, Laura, Sliekers, Olav, Schmid, Markus C., Schmidt, Ingo, Van De Pas-Schoonen, Katinka, Cirpus, Irina, Van Der Star, Wouter, Van Loosdrecht, Mark, Abma, Wiebe, Kuenen, J. Gijs, Mulder, Jan-Willem, Jetten, Mike S. M., Den Camp, Huub Op, Strous, Marc, and Van De Vossenberg, Jack, (2004) Application, eco-physiology and biodiversity of anaerobic ammonium-oxidizing bacteria. *Reviews in Environmental Science and Biotechnology*. Vol. 3, No. 3, pp. 255-264.
56. Quan, Z. X., Rhee, S. K., Zuo, J. E., Yang, Y., Bae, J. W., Park, J. R., Lee, S. T., and Park, Y. H., (2008) Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. *Environmental Microbiology*. Vol. 10, No. 11, pp. 3130-3139.