國立交通大學

環境工程研究所

博士論文

建立同時部分硝化、厭氧氨氧化及脫硝系統

Development of Simultaneous Partial Nitrification, Anammox and Denitrification (SNAD) process

研究生：王至誠

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中文摘要

以生物方法去除水中氨氮，傳統上都以硝化脫硝兩個步驟來進行。在硝化時需要大量的曝氣動力提供氧氣作為電子接受者，使氨氮轉換為硝酸鹽而進行脫硝步驟。脫硝需要加入大量的有機物作為電子提供者，此方法不僅使得操作費用提升，更有可能導致處理效率低落時，有機物隨著放流水排出，成為另一種污染源。再者，在脫硝不完全時將會產生一氧化二氮此種溫室氣體，而加劇溫室效應。本研究將建立一種新穎脫硝方法，“同時部分硝化、厭氧氨氧化及脫硝技術”，將約莫二分之一的氨氮硝化成亞硝酸鹽後，利用氨氮作為電子提供者，亞硝酸鹽為電子接受者，直接進行脫硝，不需要添加任何有機物，也可節省一半以上的曝氣費用。再者，若污水中含有少量之有機物，亦可進行脫硝，去除有機物。同時部分硝化、厭氧氨氧化及脫硝技術比較傳統方法可節省60%以上的費用。

本文中將找尋適當污泥，並針對所取得之污泥進行16S-rRNA分析，比對基因庫中相關文獻的菌種相似度，以確認取得污泥中有所需要之厭氧氨氧化菌。同時進行質能平衡之計算，探討氮在本系統中的流佈。在實驗室中建立一組穩定操作之反應槽，以垃圾掩埋場滲出水作為進流，探討在不同的氮負荷及有機負荷條件下部分硝化、厭氧氨氧化及脫硝程序之氨氮去除效能。結果發現，氮負荷率的提升會影響處理效率，經處理後亞硝酸鹽氮濃度幾乎趨近於零，而硝酸鹽氮濃度則不超過36 mg/L，氨氮去除效率最高可達94%。經模式計算後，滲出水總氮的去除在此程序中有69-88%是藉由部分硝化及厭氧氨氮氧化所完成，而化學需氧量去除率則只有21-45%。最後分析污泥中菌相的分佈，利用qPCR進行分析，包括好氧氨氧化菌，好氧亞硝酸鹽氧化菌，厭氧氨氧化菌以及與總菌數之間的比例。本研究成功地利用同時部分硝化、厭氧氨氧化及脫硝程序去除污水中含有氮及有機污染物，並證實所馴養的微生物包含厭氧氨氧化菌。

Abstract

The Simultaneous partial nitrification, anaerobic ammonium oxidation (Anammox) and denitrification (SNAD) process is an innovative biotechnology for the replacement of the traditional nitrification followed by denitrification. The advantage of the SNAD process include less than 60% of operation cost from aeration over the traditional nitrification followed by denitrification, because only half of ammonium stream is required to converting intonitrite and consequently couples the other half of ammonium stream to nitrogen gas. Moreover, the other merit of the SNAD process could remove organic matter by denitrification in the same reactor, which is not able to accomplish by the other autotrophic denitrification processes. The SNAD process was successfully operated in a continuous stirred tank reactor (CSTR) landfill-leachate treatment plant and in a lab-scale sequence batch reactor (SBR). To reveal the SNAD microbial community in the landfill-leachate treatment plant, the 16S rRNA of the sludge from it was analyzed by the molecular tools, which are DNA extraction and Polymerase Chain Reaction (PCR). The result of the 16S rRNA analysis identified that Anammox bacteria were dominant in the SNAD process. On the other hand, we confirm that the Anammox activity contribute most of nitrogen removal by a nitrogen mass balance approach . The result from it indicated the total nitrogen (TN) removal from the combined partial nitrification and Anammox route accounted for 75.5%, while the heterotrophic denitrification contributed to TN removal of 7.7% and COD removal of 23.2%.

For the lab-scale study, the lab-scale SBR SNAD process was initially inoculated the biomass from the full-scale landfill-leachate treatment plant. After adaptation of the biomass, four stages (I to IV) with varying nitrogen loading rate (NLR)was examined for the process performance. The increase of the NLR reduced the ammonium removal proportionately. The nitrite concentration was close to zero and the nitrate concentration was less than 36 mg/L in all the stages during the operation period. The total nitrogen removal in the SBR resulted mainly from partial nitrification and Anammox (69-88%) that was evaluated by a stoichiometric model. Overall, the SNAD process offers validated performance on simultaneous nitrogen and chemical oxygen demand (COD) removal economic-friendly.

誌　謝

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至誠　謹致於

交通大學環境工程研究所

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Chapter 1 Introduction

Nitrogen removal from wastewater accomplished commonly by biological nitrification followed by denitrification has raised a lot of attention in the past 100 years. Nitrification repairs abundant air for oxidation of ammonium to nitrate. On the other hand, huge quantity of organic carbon source requires for complete denitrification. The requirement of aeration and organic source enhances the overall operation cost of a wastewater treatment plant. However, the conventional nitrification followed by denitrification process is not suitable for treating a wastewater containing high ammonium with low organic carbon in it.

Generally, livestock waste contains high ammonium concentration especially the manure. Besides, the effluent from anaerobic digesters treating animal carcasses also contains high ammonium concentration. When the animal carcass is decomposed in the anaerobic digestion, protein converts to ammonium, accumulates in the reactor and inhibits the activity of microorganism. This causes the failure of the anaerobic digestion system. Several researchers attempted to develop a suitable process for treating this high ammonium containing wastewater. In the last decade, a novel autotrophic nitrogen removal process called "anaerobic ammonium oxidation" (Anammox) was developed. Moreover, Anammox is recognized as the process responsible for the completion of nitrogen cycle in the marine/deep-ocean environments. However, the presence of nitrogen along with organic carbon is toxic to Anammox species. Therefore, the major focus of this study was to develop an Anammox based system for nitrogen and organic removal. The objectives of this study were as follows: (i) Enrichment of the Anammox bacteria. (ii) Analysis of the DNA sequence of Anammox bacteria and comparison with the DNA sequence in the gene data base and (iii) Development of a stable simultaneous partial nitrification, Anammox and denitrification process in the laboratory.

Chapter 2 Literature review

Ammonium disappearing from the ocean has been extensively surveyed in several research labs [1]. Ammonium been considered an inner compound in the environment, microorganism is difficult to harvest energy from ammonium degradation. Therefore, ammonium missing in the ocean was a legend in past 20 years. Some researchers asserted that there must contribute from a lithotrophic bacterium, which converts ammonium to nitrogen gas directly using proper electron acceptor. They also calculated the stoichiometric equation to predict the ammonium oxidation using nitrite as electron acceptor in the anaerobic condition. The stoichiometric equation of the Anammox reaction illustrated shown in the eq. (1). The free energy of Anammox was -358 kJ/mole. However, the researcher could not identify the microorganism that proceeds anaerobic ammonium oxidation in water environments. The first identification of the Anammox process was in the fluidized bed reactor treating fermentation effluent in The Netherlands and it evidenced the possibility of nitrogen cycle in the marine/deep-sea environments [2]. Consequently, the Anammox process was developed for treating high ammonium and low or without carbon content wastewater in the recent years.

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

The various commonly adopted ammonium treatment techniques including nitrification/denitrification, Anammox, SHARON, CANON and SNAD are introduced in Fig. 1 [3].

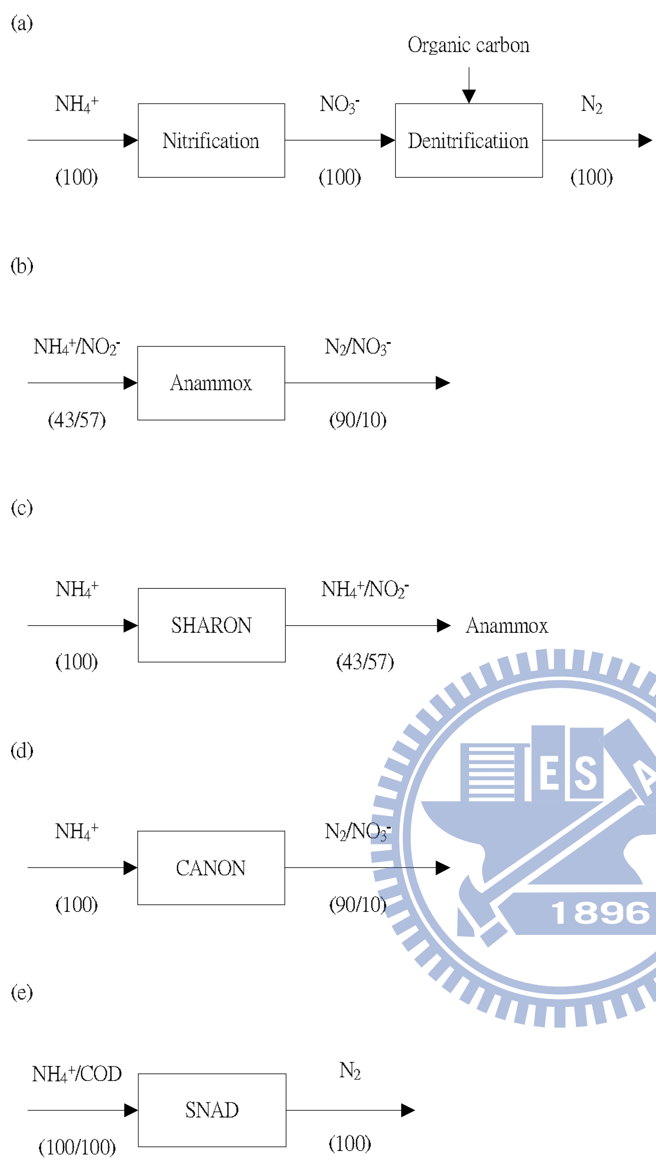


Fig. 1 Flux diagrams of the nitrification-denitrification (a); Anammox (b); SHARON (c); CANON (d); and SNAD (e).

2.1 Nitrification/denitrification

Conventionally, biological nitrogen removal is achieved by the nitrification followed by denitrification process [4], i.e. (i) aerobic nitrification of ammonium by chemolithoautotrophic bacteria to nitrite and further to nitrate with oxygen as the electron acceptor, and (ii) anoxic denitrification of nitrate to nitrite to gaseous nitrogen by heterotrophic denitrifying bacteria using organic matter as carbon and energy source.

The complete nitrification reaction is shown in eq. (2). The nitrification includes two steps which are nitritation and nitratation. Nitritation is transformation of ammonium to nitrite, and nitratation is transformation of nitrite to nitrate. Nitritation and nitratation are listed in eq. (3) and eq. (4), respectively. Ammonium is converted to nitrite through ammonium monooxygenase (AMO) [5] and hydroxylamine oxidoreductase (HAO). The AMO and HAO are the two kinds of enzymes binding in the bacterial membrane. Hydroxylamine is an intermediate in these two steps. The relative reaction equation of hydroxylamine production is given in eq. 5, 6 and 7. Adding up of these equations (eq. (5), (6) and (7)) gives the eq. (3).)

$$NH_3+2O_2 \rightarrow NO_3^-+H^++H_2O \quad (2)$$

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

$$NO_2^-+0.5O_2 \rightarrow NO_3^- \quad (4)$$

$$NH_3+2H^++2e^-+O_2 \rightarrow NH_2OH+H_2O \quad (5)$$

$$NH_2OH+H_2O \rightarrow HNO_2+4H^++4e^- \quad (6)$$

$$2H^++0.5O_2+2e^- \rightarrow H_2O \quad (7)$$

The genes of ammonium oxidizing bacteria (AOB) involving in nitritation are *Nitrosomonas*, *Ntrosococcus*, *Nitrosopira*, *Nitrosovibrio* and *Nitrosolobus*. The second stage of the nitrification reaction is nitratation which oxidizes of nitrite to nitrate. The complete oxidation of one mole of ammonium to nitrate consumes two moles of oxygen. The well-known nitrite oxidizing bacteria (NOB) are *Nitrospira*, *Nitrospina*, *Nitrococcus* and *Nitrocystis*. In most wastewater treatment processes, *Nitrospira* is often identified as the dominant AOB.

The typical reactor used for nitrification shown in Fig. 2 [6]. Most of the nitrification processes combine BOD removal by combining   in sequence. Sometime, an extended aeration is necessary to proceed nitrification (Fig. 2). While nitrification reaction takes place, it consumes the alkalinity of wastewater. Therefore, the addition of bicarbonate is the most common operation strategy for neutralization of produced proton of nitrification from providing necessary alkalinity. However, the following denitrification reaction could only compensate half of the alkalinity consumed in nitrification.

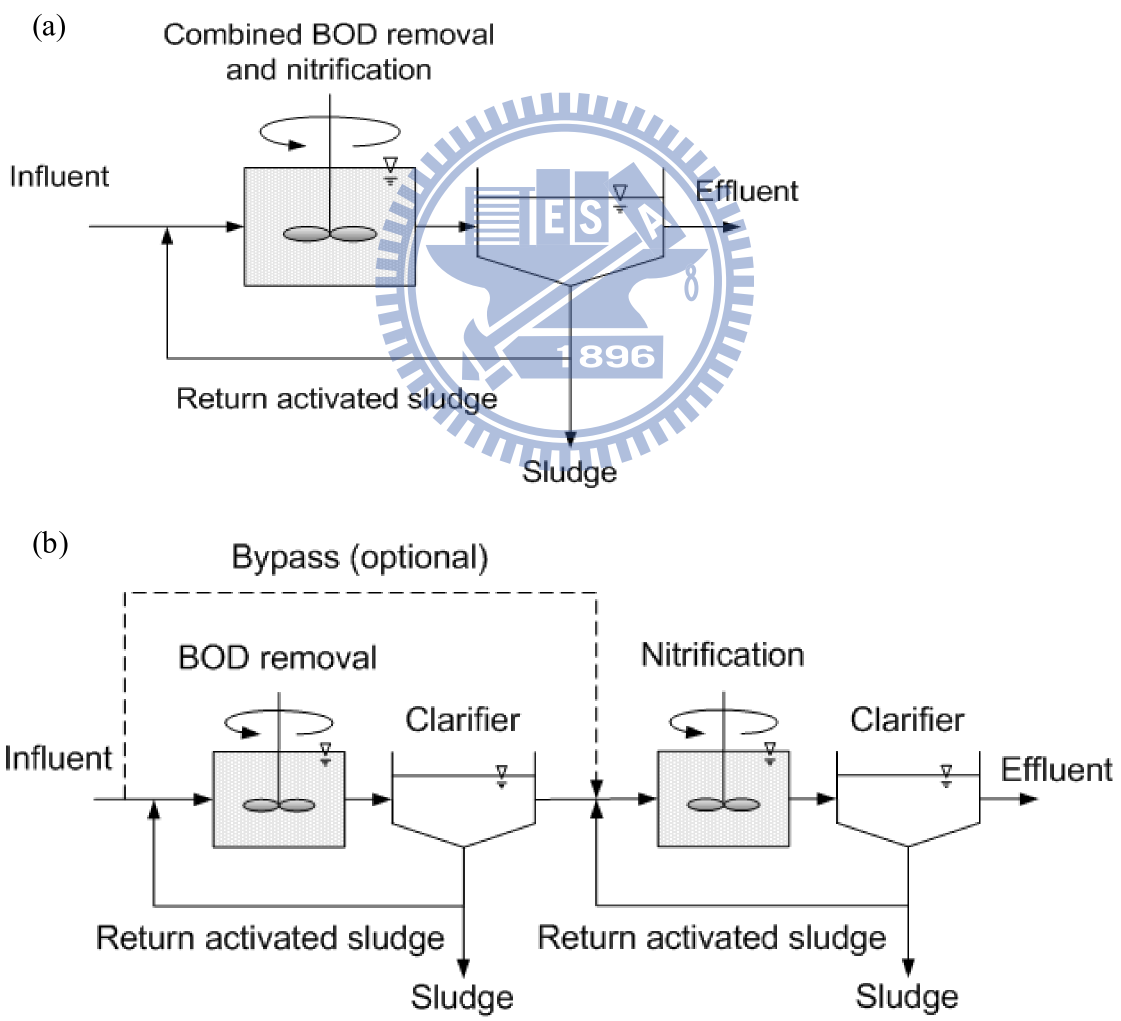


Fig. 2 Reactor setup for nitrification process (a) in a single aeration tank (b) extended aeration [6]

Nitrification efficiency can be affected by change in pH, temperature, ammonium concentration and dissolved oxygen (DO) concentration. The optimum pH for nitrifying bacteria growth is between 7.5 and 8.0. The change in pH value can affect the ammonium and free ammonia (FA) concentrations. The FA could inhibit nitrifying bacteria activity when the concentration exceeds 150 mg/L. The relationship between ammonium and FA is given in eq. (8).

$$NH_4^{+}+OH^{-} \leftrightarrow NH_3+H_2O \quad (8)$$

This equation demonstrates that the decrease in pH could drop-down the FA concentration. When pH value dropped down to 6.4, all FA will convert to ammonium; when pH value increased up to 9.4, all ammonium converted to FA. Besides, nitrifying bacteria have high affinity to oxygen. In the DO level between 0.2-0.5 mg/L, nitrifying bacteria (especially AOB) stop forming nitrate. This shows another possibility of nitrite removal short-cut process. Therefore, nitrite produced from partial nitrification is able to complete the denitrification. This short-cut process is applied in simultaneous nitrification-denitrification (SND)[7].

Denitrification reaction reduces the oxidized form of nitrogen, i.e. nitrite or nitrate converted to nitrogen gas. Organic carbon is used as the electron donor in denitrification. Oxidation of the organic carbon provides electron for reducing nitrite or nitrate. The end product of denitrification is nitrogen gas or nitric oxide (NO) or nitrous oxide (N_2O).

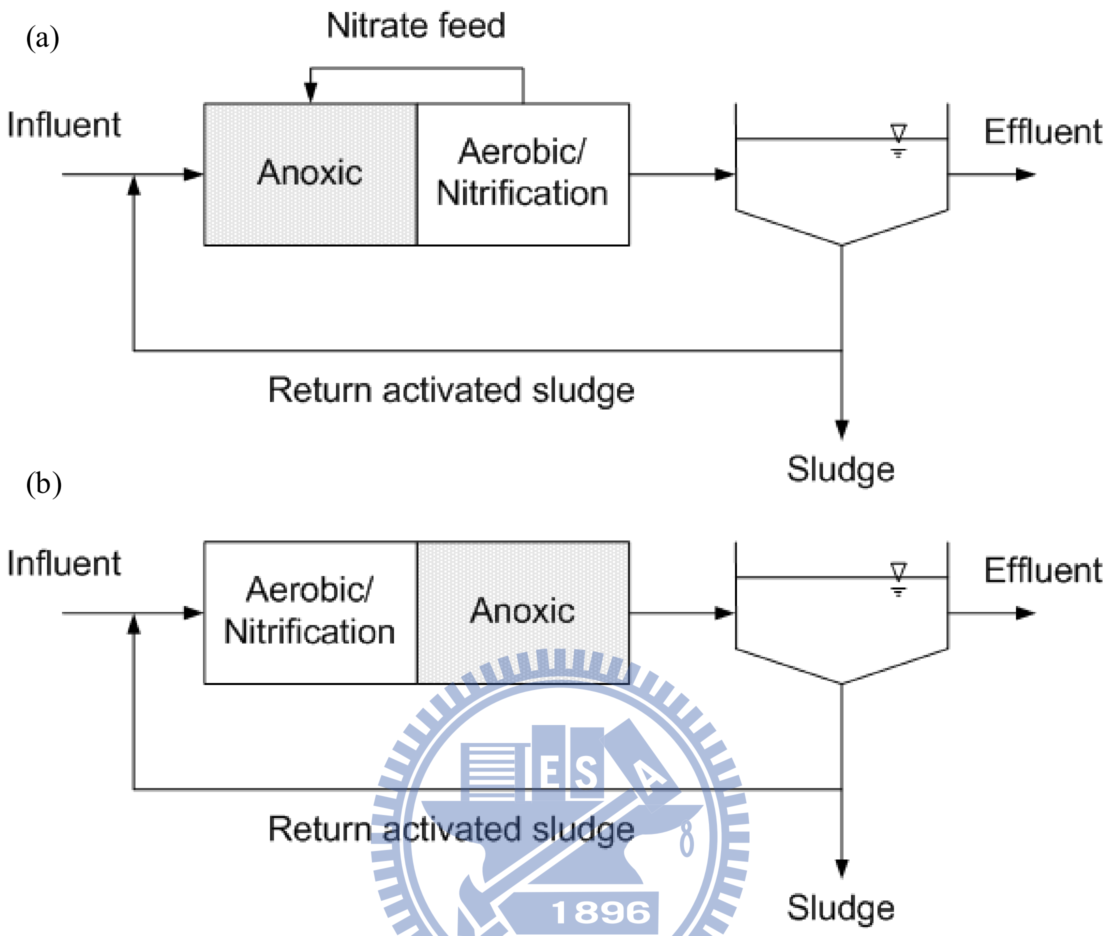


Fig. 3 Typical reactors arrangement in nitrification-denitrification process (a) in Anoxic-aerobic (AO) (b) in aerobic-anoxic (OA)[6]

The typical denitrification process is always required nitrification process occurred in advanced shown in Fig. 3 [6]. Two ways could be adopted for developing nitrification followed by denitrification, i.e. anoxic condition followed by aerobic condition with nitrate recycling back or aerobic condition followed by anoxic condition.

Nitrous oxide has long atmospheric lifetime approximately 120 years and heat trapping effects 310 times than carbon dioxide on a per molecular basis even higher than methane (23 times than carbon dioxide). Nitrous oxide is an important greenhouse gas and is produced from various biological sources. When wastewater was decanted into water bodies, nitrous oxide will be produce due to low DO content.

Denitrifying bacteria could be found in a wide variety of bacteria groups and also widely spread in water environments. The optimal growth temperature of the denitrifying bacteria is between 30 and 35^{o}C and the pH of range 7.0-8.2 [8]. Denitrifying bacteria are able to consume organic or inorganic carbon for their growth and respiratory process. On the other hand, autotrophic denitrifying bacteria oxidize hydrogen or reduced sulfur compounds to gain energy and use CO_2 as the carbon source. The heterotrophic denitrifying bacteria has ability to utilize organic compounds such as glycerol, glucose, volatile fatty acids, polyvinyl alcohol and even aromatic petroleum products as the carbon source [8].

2.2 Anaerobic ammonium oxidation (Anammox)

Denitrification and nitrification processes were found in 1882 and 1890, respectively (Fig. 4)[4]. Over 100 years after its identification of the denitrification process, the nitrogen cycle was generally believed to be completed.

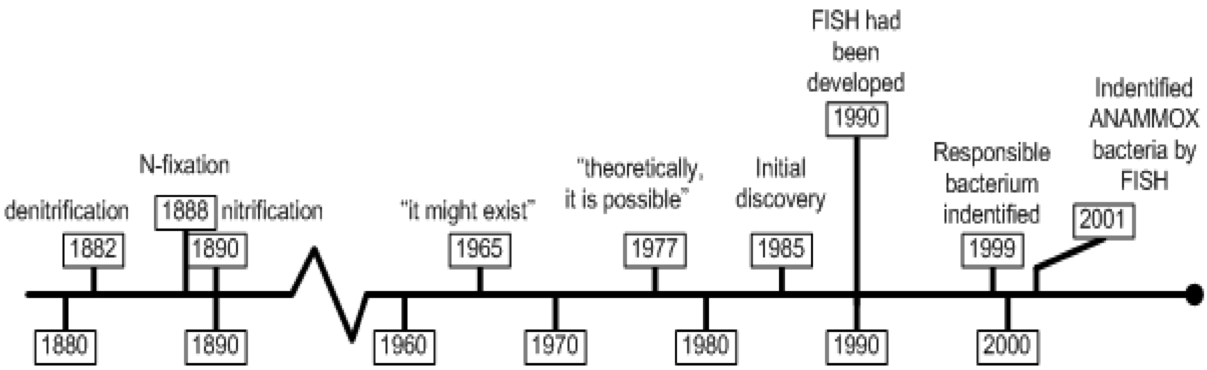


Fig. 4 Timelines of discoveries in the fields of ammonium and Anammox [4].

In 1995, Mulder et al. start up a 23-L capacity fluidized bed reactor for treating bakery yeast wastewater effluent in The Netherland [2]. Mulder et al. found that the nitrate and ammonium disappear at the same time in the reactor. The nitrification and denitrification could not remove ammonium only in an anoxic condition except assimilation. Excluding the assimilation, ammonium disappeared in the reactor, which raised interest by the researchers to advance survey on it. They recalled an article published in 1977 by Broda et al. which predicted that there are two lithotroph missing in nature [9]. One lithotroph is as reported to utilize ammonium as electron donor and nitrite as electron acceptor to form nitrogen gas. The difference between Mulder et al. and Broda et al. is the electron donor form of utilized in the reaction. Eq. 9 represents the finding of Mulder et al.

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \quad (9)$$

In eq. (9), the researcher considered that nitrate is used as the electron acceptor instead of nitrite. Later, Graaf et al. demonstrated the utilization of nitrite as the electron acceptor by tracer experiment [10]. They designed a fluidized bed reactor introducing $^{15}NH_4^+$ and $^{14}NO_2^-$ as tracers. The end product of the reaction was nitrogen gas composed by $^{14\text{-}15}N_2$. Two nitrogen atoms came from $^{15}NH_4^+$ and $^{14}NO_2^-$. Graaf et al. successfully demonstrated the anaerobic ammonium oxidation using nitrite and not by nitrate. In the Mulder et al. study [2], nitrate reduced to nitrite as the first step and combined ammonium and nitrite to proceed Anammox. Based on the amazing discovery, the nitrogen cycle in biological treatment was revised as shown in Fig. 5.

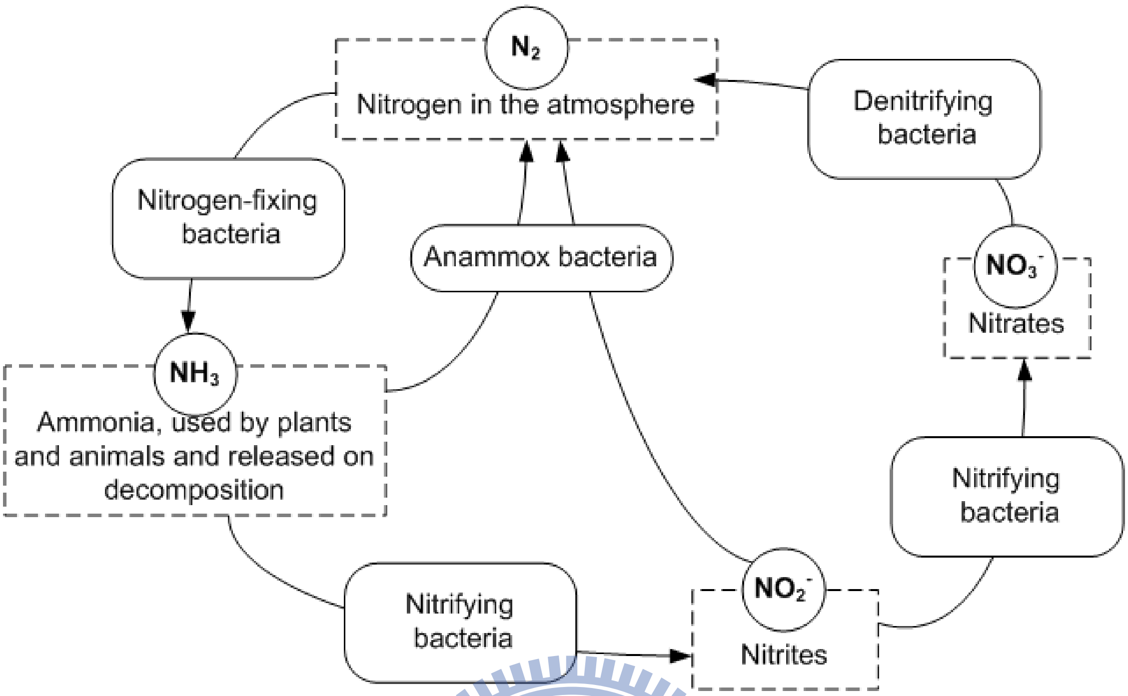


Fig. 5 Biological nitrogen transformation cycle and Anammox shortcut.

Subsequently, Stous et al. formulated a complete metabolic equation for Anammox reaction based on the mass balance [11]. The protein content and elemental composition of the biomass were found to be $CH_2O_{0.5}N_{0.15}$. According the biomass composition and mass balance, the stoichiometry of Anammox is illustrated in eq. (10).

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

Several interesting details can be noticed from the stoichiometry of Anammox. First, the biomass yield is extremely low, one mole of ammonium only yield 0.066 mole of the biomass. The extremely low yield of the biomass means the cultivation should spend a long time and also less sludge production saves sludge treatment cost. Second, Inorganic carbon is carbon source in the

Anammox reaction. It is a kind of carbon fixation which could avoid global warming. Third, nitrite is the electron donor and also the electron acceptor. Nitrite reduces to ammonium as electron acceptor and oxidizes to nitrate to provide energy for assimilation biomass.

Since the Anammox bacteria growth rate is very low, researchers investigated the Anammox bacteria doubling time to evaluate the performance of Anammox bacteria growth [11-15]. The doubling time could be assessed by measuring the end product, i.e. nitrogen gas. When production of nitrogen gas is twice than the initial value, the period could be considered as doubling time. The first present the doubling time was 30 days [12]. Following the cultivation improved, the doubling time was shorted from 30 days to 11 days [11].

The bacteria, which found in the fluidized bed reactor in The Netherlands, were confirmed as a species of planctomycete. Planctomycete has been supposed only by a few organtrophs. Formerly the planctomycetes were considered to be of limited environmental importance. But this view changed as a molecular microbial ecology repeatedly providing new evidence that these bacteria are ubiquitous. Planctomycetes have the single- or double-membrane-bounded compartments separating their chromosome in the cytoplasm. They lack of peptidoglycan in their cell wall and are insensitive to ampicillin [16].

To seek out the origin of Anammox bacteria in the biggest anoxic basin, the researchers sampled sea water at different depth from the "Black Sea" [17]. They found that Anammox bacteria grow abundantly at the depth of 90 meters in sea. This evidence proved scientists the conversion of nitrite to nitrogen gas by heterotrophic bacteria and not by the major dinitrogen mechanism. From the sea water samples (depth of 100 meters), the 16S rRNA gene sequences were performed and the phylogenetic analysis was carried out. The results showed the Anammox bacteria are related to member of order Planctomycetales. The Anammox contributes at least 30% of the total nitrogen turnover in this inland sea. Anammox bacteria are abundant and important in the nitrogen cycle of the Black Sea. The species found in the Black Sea were identified as *Candidatus* Scalindua sorokinii [17]. The mystery of missing nitrogen in the ocean was solved by investigation in the "Black Sea". Anammox activity is also responsible for a major part of global nitrogen turnover [18].

The first discovered and identified Anammox bacteria is *Candidatus* Brocadia anammoxoidans, which found in a wastewater treatment plant [11]. Consequently, more Anammox bacteria were found in the wastewater treatments plants including *Candidatus* Kuenenia stuttgartiensis [19], *Candidatus* Scalindua brodae [20], *Candidatus* Scalindua wagneri [20], *Candidatus* Anammoxoglobus propionius [21], *Candidatus* Brocadia [22], and *Candidatus* Jettenia asiatica [23].

A comparison of nitrification and Anammox is given in Table 1 [13, 24]. The major differences between nitrification and Anammox are the maximum specific ammonium consumption rate and growth rate. The ammonium consumption rate of Anammox is 22.5 times more than nitrification, but 1/100 less growth rate than nitrification. Furthermore, the Anammox cannot tolerate nitrite concentration over 20 mM for 12 hours.

The optimum Anammox growth temperature is between 20-43^{o}C. For example, a rotating biological contactor (RBC) handling Anammox was successfully operated at temperature 20^{o}C [25]. This result was similar to Isaka et al. where they operated an anaerobic biological filtrated (ABF) reactor for Anammox [26]. Moreover, many researchers analyzed the marine Anammox samples, and reported reasonably measurable activities at low temperature. Dosta et al. indicated that the maximum activity of Anammox was found at 35-40^{o}C; but if the temperature higher than 45^{o}C, an irreversible loss of the activity was observed due to the biomass lysis [24].

Table 1 Comparison of nitrification and Anammox

Parameters	Nitrification	Anammox
Biomass yield	0.08 mol C (mol ammonium)$^{-1}$	0.066 ± 0.01 mol C (mol ammonium)$^{-1}$
Maximum specific ammonium consumption rate	2 nmol (min mg protein)$^{-1}$	45 ± 5 nmol (min mg protein)$^{-1}$
Maximum specific growth rate	0.04 h^{-1} (doubling time 0.73 days)	0.0027 h^{-1} (doubling time 11 days)
Temperature range	28-36°C	20-43°C (with an optimum at 40°C)
pH range	pH 7.5-8.0	pH 6.7-8.3 (with an optimum at pH 8)
Inhibition of Nitrite concentration	-	> 20 mM (non-Anammox activity in 12 h, suboptimum > 10 mM)

Hydrazine and hydroxylamine were the two most important intermediates of Anammox [13, 27]. Hydrazine is an extremely toxic compound and contained high potential energy, considered as a rocket fuel. The hydrazine has not been found in microorganism synthesis due to its high toxicity. This unique compound firstly was found in the Anammox bacteria. The Anammox bacteria have a special membrane "anammoxosome" to resist the toxicity of hydrazine. The synthesis pathway of ammonium and nitrite in Anammox was shown in Fig. 6. The electron acceptor nitrite is reduced to hydroxylamine, which reacted with ammonium as electron donor for forming hydrazine. The end product of Anammox was oxidation of hydrazine to produce nitrogen gas and provides four electrons to reduce nitrite for forming hydroxylamine. The oxidation and reduction enzymes involved in the reaction include hydrazine dydrolase (HH), hydrazine-oxidizing enzyme

(HZO), and nitrite-reducing enzyme (NR)[27].

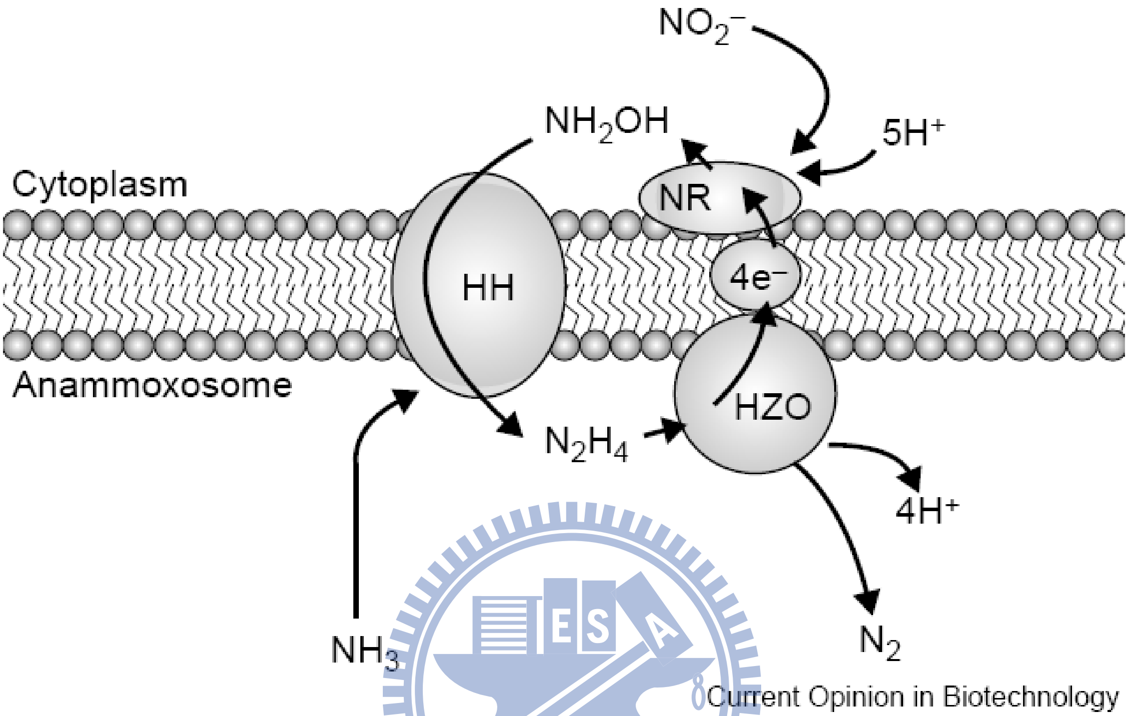


Fig. 6 Mechanism of Anammox [27].

2.3 Single reactor system for high ammonium removal over nitrite (SHARON)

Anammox process requires ammonium and nitrite as shown in the eq. (9). SHARON process was developed to treat high ammonium concentration wastewater [28-31]. The concept of SHARON was only 50% of ammonium need to be converted to nitrite (eq. 11). If the wastewater contains high ammonium and low COD, the combination of SHARON and Anammox is an appropriate system for ammonium removal.

$$NH_4^+ + HCO_3^- + 0.75O_2 \rightarrow 0.5NH_4^+ + 0.5NO_2^- + CO_2 + 1.5H_2O \quad (11)$$

If only 50% ammonium was converted, the wastewater treatment does not need to supply extra bicarbonate to compensate alkalinity consumption in the nitrification process. The converted nitrite combined residual ammonium could carry out Anammox. The SHARON process provides nitrite not nitrate by the operation strategy of controlling sludge retention time (SRT), ammonium and free ammonia concentration, and dissolved oxygen level. The ammonium oxidizer growth rate is lower than nitrite oxidizer. A proper SRT could lead AOB to be retained in the reactor and NOB be washed out from the reactor. The SHARON process was operated in a single reactor without any sludge retention [29], meaning the SHARON process SRT equals to HRT. This operation strategy keeps a full-scale SHARON process operation for more than two years. Furthermore, the reactor temperature was controlled at $35^{o}C$, AOB growth rate is higher than NOB [29]. The SHARON process takes advantages of the high temperature, enabling high specific growth rate, and result in SRT consisting with HRT.

For AOB ammonia is the actual substrate rather than ammonium, and hydroxylamine. But, the AOB is inhibited by ammonia concentration at 10-100 mg-N/L, higher ammonia toxicity tolerance than NOB by ammonia concentration at 1-10 mg-N/L. Keeping the higher influent ammonium concentration could inhibit NOB growth but not AOB. Lower DO concentration, around 0.2-0.5 mg/L, was another possible condition to stop nitrification at nitrite [7, 32]. For the oxidation of ammonium to nitrite, 25% oxygen was saved than complete oxidation of ammonium to nitrate.

The SHARON plus Anammox is an economically feasible process compared to nitrification followed by denitrification. Only 1.7 kg O_2 is needed for 1 kg ammonium removal and no additional organic carbon source is needed in the SHARON plus Anammox process. In the nitrification and denitrification process 4.6 kg O_2 needed for complete conversion of ammonium to nitrate and 4.5 kg COD for denitrification [28].

The disadvantages of the SHARON process are building and energy costs. Beside, two separate reactors are necessary, i.e. one for partial nitrification and the other for Anammox. Moreover, the temperature of partial nitrification reactor has to be controlled above 37^{o}C, which could enhance the operation cost.

2.4 Oxygen-limited autotrophic nitrification-denitrification process (OLAND)

In the SHARON process, two reactors are needed to perform partial nitrification and Anammox. In some countries, land was limited especially in high-density population city. To perform partial nitrification and Anammox in a single reactor, oxygen-limited autotrophic nitrification-denitrification (OLAND) process was developed [33]. The advantage of OLAND was saving half of nitrification/denitrification cost and building only in a set of reactor. Two group bacteria which are aerobic ammonium oxidizing bacteria (AerAOB) and anaerobic ammonium oxidizing bacteria (AnAOB) coexist in OLAND process. The AerAOB oxidized ammonium into nitrite in aerobic condition, which is similar to AOB as illustrated previously, whereas AnAOB oxidized ammonium into dinitrogen gas in anaerobic condition, which is similar to Anammox bacteria. The reactions of AerAOB and AnAOB are given in eq. (12) and eq. (13).

$$1.32NH_4^{+}+1.98O_2 \rightarrow 1.32NO_2^{-}+1.32H_2O+2.64H^{+} \quad (12)$$

$$NH_4^{+}+1.32NO_2^{-}+0.13H^{+} \rightarrow 0.26NO_3^{-}+1.02N_2+2.03H_2O \quad (13)$$

However, Anammox requires in an absolutely anaerobic reaction. Only 0.5% air saturation oxygen level is able to inhibit Anammox activity [34]. The oxygen-limited condition was operated in OLAND keeping the Anammox bacteria survived without oxygen toxicity. Recently, Anammox was found to survive in the anoxic condition and the low dissolved oxygen could enrich Anammox bacteria. Therefore, Anammox process can be rewritten as “Anoxic” ammonium oxidation.

$$NH_4^+ + 0.85O_2 \rightarrow 0.44N_2 + 0.11NO_3^- + 1.08H^+ + 1.44H_2O \quad (14)$$

2.5 Completely autotrophic nitrogen removal over nitrite in one single reactor (CANON)

The CANON process was first published by Third et al.[35]. A two liters SBR was employed to treat a synthetic wastewater containing high ammonium concentration and without COD. The CANON process included nitrifying bacteria and Anammox bacteria for completing the partial nitrification and Anammox reactions. Nitrifying bacteria was used to convert ammonium to nitrite, and nitrite was utilized immediately in a single reactor. Well-controlled DO level in the reactor successfully developed the CANON process. The limitation of CANON was influent ammonium concentration and DO level in the reactor. If ammonium concentration is higher than nitrifying bacteria ability, the effluent showed poor ammonium removal. Moreover, higher DO concentration will complete nitrification resulting in ammonium to nitrate and also inhibit Anammox activity. The Anammox bacteria is difficult to cultivate due to its slow grow rate. However, the CANON process could be accelerated (cultivation in 3.5 month) by inoculating the activated sludge [36]. The well-operated CANON provides a good methodology to get Anammox bacteria and also provides an efficient choice for ammonium removal with the less land requirement and less final efforts. However, COD could not be removed in this system. Another consideration was AOB produce N_2O and NO at low oxygen concentration. To clarify this point, CANON demonstrated negligible N_2O production in low oxygen concentration (less than 0.1%)[37].

The CANON process was still evaluated in laboratory scale. Table 2 compares the CANON process operated in different laboratories. The maximum working volume applied in the CANON system was two liters only, but the maximum nitrogen loading rate reached 0.6 kg N/m^3/d. The most common reactor used in the CANON process was the SBR, which could save land requirement and the capital cost.

Table 2 Comparison of CANON processes

Reactor	Working volume (L)	N removal rate (kg N/m^3/day)	Reference
SBR	2	0.27	[35]
SBR	2	0.32	[37]
SBR	1	0.6	[36]
SBR	0.87	0.45	[15]

2.6 Simultaneous partial nitrification, Anammox and denitrification process (SNAD)

Nitrifying bacteria and Anammox bacteria are extremely slow growers. If the SRT is not longer enough, the nitrifying and Anammox bacteria will be washed out from the reactor. Under organic carbon rich wastewater, heterotrophic bacteria can significant grow. Selection of influent is an important issue when applying the Anammox process. Alternatively, the development of the Anammox and denitrification in a single reactor can facilitate the simultaneous nitrogen and carbon removal. Recently, the simultaneous partial nitrification, Anammox and denitrification (SNAD) has been developed following the concepts of Anammox and shortcut nitritation-denitritation (SND)[38-39]. Chen et al. demonstrated the feasibility of the SNAD process, and this process was successful in operation for an organic loading of up to 0.34 kg COD/m^3/d [38]. This finding widens the application of Anammox for wastewater treatment, i.e. high ammonium and low organic carbon content. Anammox removes 90% ammonium and leaves 10% nitrogen in the form of nitrate. Fortunately, organic carbon in the Anammox effluent could be supplied for denitrification. Nitrate produced from Anammox could be reduced to nitrite and nitrite to nitrogen gas through oxidization of organic carbon in the wastewater. The advantage of SNAD is (i) organic carbon could be removed by heterotrophic bacteria, (ii) and avoid the toxicity of organic carbon to Anammox. The application of the SNAD species observed in the full-scale plant to the detailed investigation in laboratory/full scale is still under research.

2.7 Energy capture in nitrogen removal

The microorganism captures energy released from oxidation-reduction reactions [40]. Electrons are provided from the electron donor and transferred to the intracellular electron carriers. The electron acceptor receives the electron transported from carriers. The transfer steps have a free-energy release that the cells capture in the form of energy carriers.

The free energy of nitritation, nitratation, nitrification, denitrification, Anammox including different electron donor and acceptor are listed in Table 3. The free energy of denitrification is always higher than nitrification and Anammox even using different electron acceptor. When influent contains organic compound, the denitrification will start working first. If nitrite and nitrate coexists in the same environment, denitrifying bacteria consumes the nitrate (free energy of consuming nitrate is higher than nitrite). After denitrification consumed up the biodegraded organic compounds, nitrite is better than nitrate for Anammox because more energy could be gained by Anammox considering nitrite as the electron acceptor. Moreover, the nitritation harvested more energy than nitratation when oxygen is the electron acceptor. Heterotrophic bacteria always have higher energy capture than autotrophic bacteria (referring the Table 3).

Table 3 Stoichiometric equation of N-removal processes

Reaction (donor/acceptor)	Stoichiometric equation	Free energy (kJ/mol)
Nitritation (ammonium/oxygen)	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$	-275
Nitratation (nitrite/oxygen)	$NO_2^- + 0.5O_2 \rightarrow NO_3^-$	-74
Nitrification (ammonium/oxygen)	$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$	-348
Anammox (ammonium/nitrite)	$NH_4^+ + NO_2^- \rightarrow N_2 + H_2O$	-358
Anammox (ammonium/nitrate)	$NH_4^+ + 0.6NO_3^- \rightarrow 0.8N_2 + 0.4H^+ + 1.8H_2O$	-297
Denitrification (nitrate/methanol)	$NO_3^- + 0.83CH_3OH + H^+ \rightarrow 0.5N_2 + 0.83CO_2 + 2.17H_2O$	-545
Denitrification (nitrite/methanol)	$NO_2^- + 0.5CH_3OH + H^+ \rightarrow 0.5N_2 + 0.5CO_2 + 1.5H_2O$	-388
Denitrification (nitrate/acetate)	$NO_3^- + 0.625CH_3COO^- + H^+ \rightarrow 0.5N_2 + 0.625CO_2 + 0.625HCO_3^- + 1.125H_2O$	-498
Denitrification (nitrite/acetate)	$NO_2^- + 0.375CH_3COO^- + H^+ \rightarrow 0.5N_2 + 0.375CO_2 + 0.375HCO_3^- + 0.875H_2O$	-360
Denitrification (nitrate/glucose)	$NO_3^- + 0.208C_6H_{12}O_6 + H^+ \rightarrow 0.5N_2 + 1.25CO_2 + 1.75H_2O$	-568
Denitrification (nitrite/glucose)	$NO_2^- + 0.125C_6H_{12}O_6 + H^+ \rightarrow 0.5N_2 + 0.75CO_2 + 1.25H_2O$	-402

2.8 Anammox application and comparison

Table 4 summarizes the application of the Anammox process so far reported in the literature. Most of the Anammox processes are applied to laboratory scale reactor, reactor size less than 10 L. Otherwise, one pilot scale [41] and three full scale reactors [39, 42-43] were established around the world. The synthetic wastewater was popular at the acclimation stage of Anammox bacteria. Anammox enrichment is easily achieved through inoculating Anammox biomass. Also, it can reduce the acclimation time. Strous et al. [11] indicating that SBR is a powerful tool for slow growing Anammox bacteria. Most researchers followed this role for Anammox acclimation. In Table 4, 15 articles used SBR for experiment set up, and especially one full scale SBR was applied for landfill-leachate treatment [43].

The suspended growth is most common found in the Anammox enrichment, but the attached growth was favorable in the recent years. Attached growth could overcome the wash out of sludge and could retain the Anammox biomass in the reactor. Also, Anammox bacteria prefer to attached growth on a carrier. Several researches used materials like the fixed bed or biofilm reactors for Anammox growth. To prevent wash-out the extremely growth Anammox bacteria, membrane bioreactor was also used for acclimation.

Table 4 Summary of Anammox application in literatures

Process	Inoculation	Reactor (Volume)	Medium	Reference
Anammox	Denitrifying fluidized-bed sludge	Fluidized-bed (23 L)	Backer's yeast wastewater	[2]
Anammox	Denitrifying fluidized-bed sludge	Serum bottles (0.5 L)	Effluent from the denitrifying bed reactor supplemented with ammonium and nitrate	[10]
Anammox	Denitrifying fluidized bed sludge	Fluidized bed (2.5 L) Fixed bed (2 L)	Synthetic wastewater and sludge digestion effluent	[44]
Anammox	Anammox fluidized bed reactor	SBR (15 L)	Synthetic	[11]
SHARON	_____	CSTR (1.5 L)	Effluent of the sludge digestion	[29]
Anammox	Anammox biomass	Serum bottles (0.05 L) Biofilm reactors (0.33 L)	Synthetic	[45]
CANON	Anammox biomass-SBR	SBR (2 L) Chemostat (2 L)	Synthetic	[35]
Anammox	Anammox biomass	SBR (2 L)	Synthetic	[46]
Anammox	Denitrification process sludge	CSTR (2.5 L)	Synthetic	[47]
CANON	Anammox biomass-SBR	SBR (2 L)	Synthetic	[37]
Partial nitrification followed Anammox	Activated sludge	CSTR (2100 L)	Real wastewater	[41]
Anammox and denitrification	Activated sludge	Serum bottles (0.5 L)	Poultry manure	[48]
Anammox	Denitrification process sludge	CSTR (2.5 L and 14 L)	Synthetic	[49]
Anammox and CANON	Anammox biomass-SBR	Gas-lift (1.8 L)	Synthetic	[50]
Anammox	Anammox biomass	Gas-lift (3 L) SBR (1 L)	Synthetic	[51]
Anammox	Municipal WWTP	SBR (1 L)	Synthetic	[52]

OLAND	Nitrifying bacteria and Anammox biomass	MBR (1.5 L)	Digested sludge dewatering wastewater	[53]
CANON	Activated sludge + RBC sludge	SBR (3 L)	Synthetic	[54]
CANON	Anammox biomass	RBC (50 L) Fixed-film bioreactor (8.1 L)	Synthetic	[55]
CANON	Activated sludge	Modified SBR (1 L)	Synthetic	[36]
SHARON	Biological nitrogen removal reactors	SBR (1 L) Chemostat (4 L)	Anaerobic sludge reject water	[31]
Anammox	Anammox biomass	SBR (2.5 L)	Synthetic	[56]
Anammox	Activated sludge	Continuous up-flow reactor (0.5 L)	Synthetic	[57]
Anammox	Anammox biomass-SBR	MSBR (5 L)	Synthetic	[14]
DEAMOX	Expanded granular sludge bed	UASB (2.53 L)	Backer's yeast wastewater	[58]
Anammox	Anammox biomass	SBR (1 L)	Synthetic	[59]
Anammox	Anammox biomass	Glass bottle (0.7 L)	Deorderization wastewater	[60]
Anammox	Mixed culture (sludge of UASB, activated sludge, anaerobic sludge digestion)	SBR (7 L)	Synthetic	[61]
Anammox	Nitrifying sludge and Anammox biomass	Gas-lift (70000 L)		[42]
Anammox	Sludge from 11 different wastewater treatment plants	Up-flow fixed-bed glass biofilm column (0.8 L)	Synthetic	[62]
Anammox	Anammox biomass	SBR (500000 L)	Reject water from digested-sludge dewatering	[43]
SNAD	Anaerobic granule sludge	UASB (0.2 L)	Synthetic	[63]
CANON	Anammox biomass	SBR (1.87 L)	Synthetic	[15]
SNAD	Anammox biomass	Nonwoven RBC (1.2 L)	Synthetic	[38]
SNAD	Activated sludge	Continuous flow (400000 L)	Landfill-leachate	[39]
SNAD	SNAD sludge	SBR (18 L)	Synthetic	[64]
Anammox	SNAD sludge	UASB (50 L)	Synthetic	[65]

2.9 Summary

The various nitrogen removal methods are introduced in the previous sections. The optimum choice depends on the purpose and need. The wastewater characteristics, operational cost and maintaining cost should be well-evaluated before developing a method. Schmidt et al. summarizes some nitrogen treatment methods as showed in Table 5 [3]. Besides, the SNAD process is also listed in this table to compare with other nitrogen treatment methods.

Table 5 Summary of biological nitrogen removal processes [66]

	Conventional nitrification denitrification	Anammox	SHARON	CANON/OLAND	SNAD
AOB	exist	absent	exist	exist	exist
NOB	exist	absent	absent	exist	exist
Anammox	absent	exist	exist	exist	exist
Denitrifying bacteria	exist	absent	absent	absent	exist
Ammonium loading (kg $N/m^3/d$)	2-8	10-20	0.5-1.5	2-3	0.5-0.7
N-removal efficiency	95%	90%	90%	90%	99%
Application status	established	Full scale plant	Full scale plant	Laboratory	Full scale plant
Investment costs	medium	low	medium	medium	medium
Operational costs	high	Very low	low	low	low

Chapter 3 Materials and Methods

3.1 Description of the full-scale landfill-leachate reactor

Anammox bacteria could be enriched from different sludges, sediments and soils. So far, Anammox bacteria have been found in high ammonium containing wastewater. A sludge sample was collected from an anaerobic digestion unit treating a pig waste for enriching Anammox bacteria. For the cultivation of Anammox bacteria, the landfill-leachate containing high ammonium and low COD is suitable. To collect and enrich Anammox bacteria from the landfill-leachate, a full-scale landfill-leachate treatment plant which is located in Taiwan was identified. This treatment plant is has been operating since 2006 with an average flow of 304 m^3/d. The landfill site was used to dispose domestic waste from 1992 to 2005. The schematic diagram of the treatment plant is shown in Fig. 7 and the influent and effluent qualities from the aeration tank (the SNAD process) are summarized in Table 6. The phenomena of simultaneous partial nitrification, denitrification and Anammox occurs in two parallel aeration tanks (15.6 m *L* by 4.1 m *W* by 3 m *D*) corresponding to a working volume of 384 m^3 and operating at a hydraulic retention time (HRT) of 1.26 d with . The sludge retention time (SRT) in the aeration tank was maintained between 12 and 18 d. The concentrations of the mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) in the aeration tanks were 2110 and 1506 mg/L, respectively. The aeration tanks were equipped with a couple of fine bubble tubular diffusers. The DO concentration in the reactor was maintained at approximately 0.3 mg/L, which facilitated the co-existence of ammonium oxidizing bacteria (AOB), Anammox bacteria and denitrifiers. The pH in the aeration tanks was around 7.4 and the temperature was found to be fluctuated under the influence of ambient temperature within 30-33°C during the course of the sample collection process (October – December, 2008). Influent and effluent samples were collected from the aeration tanks on a regular basis and analyzed in both the on-site lab in the landfill-leachate treatment plant and the lab in Institute of Environmental Engineering, NCTU, Taiwan. The average COD, ammonium and nitrate concentrations at the upstream end of the bioreactor, i.e., influent, were 554, 634 and 3 mg/L, respectively; whereas, nitrite concentration was below the detectable limit at all times.

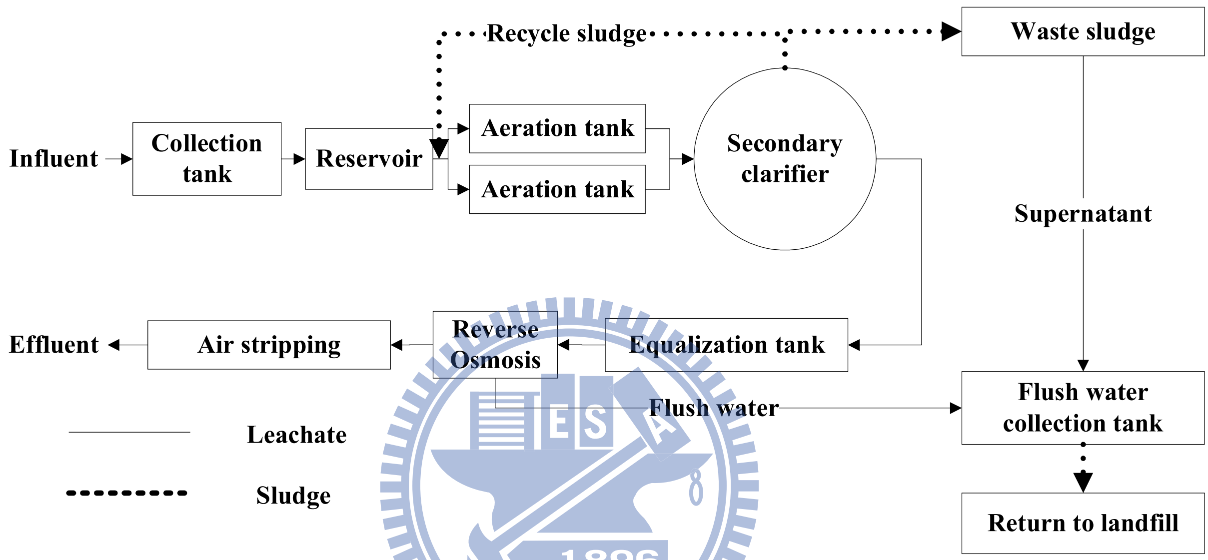


Fig. 7 Schematic diagram of the landfill-leachate treatment plant

Table 6 Main characteristics of leachate

Parameters	Influent to aeration tank	Effluent off aeration tank
pH	7.9	7.3
TS, mg/L	2610±21.8	1970±21.8
VS, mg/L	554±26.8	448±26.8
COD, mg/L	554, mg/	399, mg/
BOD, mg/L	57.3±0.72	8.70±0.1
NO_2^--N, mg/L	ND<0.0	0.40.0/
NO_3^--N, mg/L	3.0 mg/	22.7mg/L3
NH_4^+-N, mg/L	634 mg/	126 mg/L
TKN, mg/L	676±55.9	114±9.4
PO_4^{3-}, mg-P/L	3.80±0.50	1.75±0.66
TOC, mg-C/L	159±0.0	70.2±0.0

Note: ND - not detectable, Sample size (n) = 3 except pH.

3.2 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 [67]. The thermal cycling consisted of 10 min at 94 ^{o}C followed by 35 cycles each of 90 sec at 94^{o}C, 45 sec at 52^{o}C, 120 sec at 72^{o}C and ended by an additional 10 min at 72^{o}C. 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 the 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') [47](referred as primer III) with a 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 at68^{o}C and ended by an additional 10 min at 72^{o}C.

3.3 Fluorescence in situ hybridization (FISH)

The 16S rRNA-targeted oligonucleotide probe used in this study was Amx820 [68] 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. [69]. A 100X objective Olympus BX51 microscope (Olympus Optical Co., Japan) fitted with a mercury bulb and blue, green and red filter sets was used for observing the slides. The photomicrograph was made using an Olympus U-CMAD 3 camera (Olympus Optical Co., Japan) with the exposure times of 0.05 sec for DAPI and 0.5 sec for Amx820.

3.4 Lab scale SNAD system and operation strategy

The SNAD process was developed in a SBR with a working volume of 2.5 L and using the inoculated biomass from the full-scale landfill-leachate treatment plant in Taiwan [39]. The schematic diagram and photographs of SBR are shown in Fig. 8 and Fig. 9, respectively. The reactor was set by using a 3-L beaker keeping inside an incubator and equipped with influent and effluent pumps, pH, ORP and DO probes. An agitator was installed in the reactor with two blades and controlled the rotating speed at 100 rpm for mixing the mixed liquor completely. The SBR was operated in a cycle of 24 h with 12 h influent/reaction, 11.5 h reaction, 0.25 h settling and 0.25 h decanting. The HRT of the reactor was maintained at 2.5 d by feeding the reactor at a flow rate of 1 L influent per day. The SBR was operated in such a way to maintain a MLSS concentration of around 5,000 mg/L. The SRT of the SBR was maintained at infinitive to retain the slow growing Anammox bacteria. A fine bubble form diffuser was installed in the reactor and was cleaned by acid solution for a period to remove the attached biomass. In addition, a DO control system was installed in the SBR to supply/adjust the desired DO level precisely. The DO control system composes of a DO meter, air flow valve and PID controller. The application of the DO control system is useful to maintain a DO level in the SBR as accurate as ~0.1 mg/L, which is also helpful for preventing the rapid nitrite accumulation and nitrite oxidation to nitrate. Throughout the course of the study period, the SBR was operated at 35^{o}C and the reactor contents are mixed uniformly using an agitator.

The real-time landfill-leachate was used as the feed for the SBR. The real-time landfill-leachate was collected four times (Stages I to IV) in a calendar year to monitor/quantify the real wastewater influent, stored in a refrigerator and used for the SBR study. The SBR reactor was operated continuously using the landfill-leachate samples collected at various stages, i.e. stage-I for 0-38 d, stage-II for 39-54 d, stage-III for 55-147 d, and stage-IV for 148-191 d. The composition of the landfill-leachate wastewater at various stages (I to IV) is shown in Table 7. In the first stage, the influent ammonium concentration of the leachate was 295 mg/L corresponding to a C/N ratio of 0.85. However, the influent ammonium concentration increased gradually in the subsequent samplings with a maximum of 700 mg/L in the stage IV. On the other hand, the COD/TN ratio was maintained as constant (0.85) in the stages I and II, decreased gradually in the stage III (0.55) and again increased in the stage IV (0.71). This data showed that NH_4^+-N and COD concentrations fluctuated drastically in the study period. However, the nitrite and nitrate concentrations in the leachate were close to zero (0-4 mg/L) irrespective of the sampling time.

Table 7 the composition of the real-time landfill-leachate wastewater at various stages

Stages	Time of operation (d)	NH_4^+-N (mg/L)	NO_2^--N (mg/L)	NO_3^--N (mg/L)	BOD (mg/L)	COD (mg/L)	COD/TN (BOD/TN) ratio	OLR (mg-COD/L/d)	NLR (mg-N/L/d)
I	38	295	2	0	88	250	0.85 (0.35)	100	118
II	16	590	4	0	164	500	0.85 (0.33)	200	236
III	93	660	0	0	155	365	0.55 (0.42)	146	264
IV	44	700	0.5	1.8	178	500	0.71 (0.36)	200	280

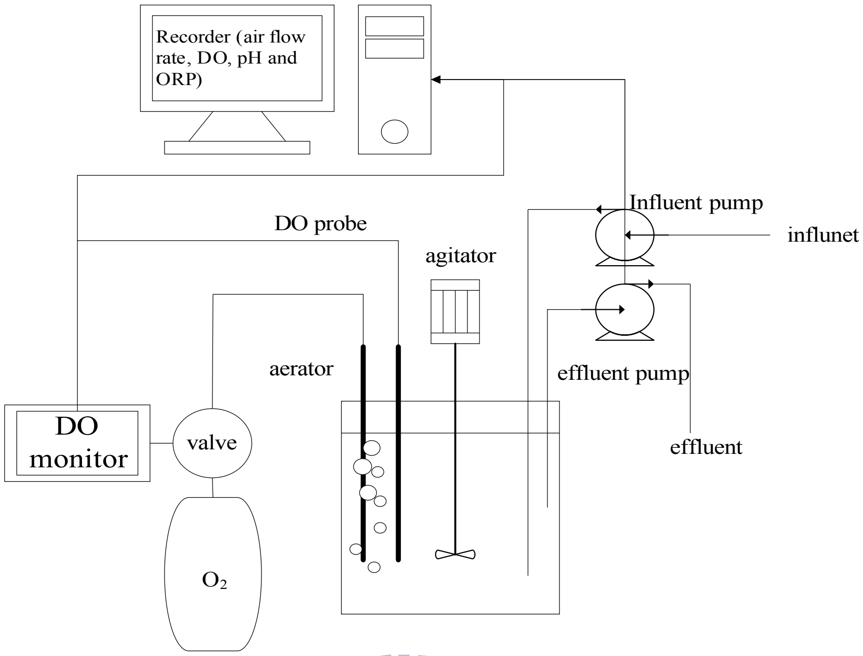


Fig. 8 Schematic diagram of SBR

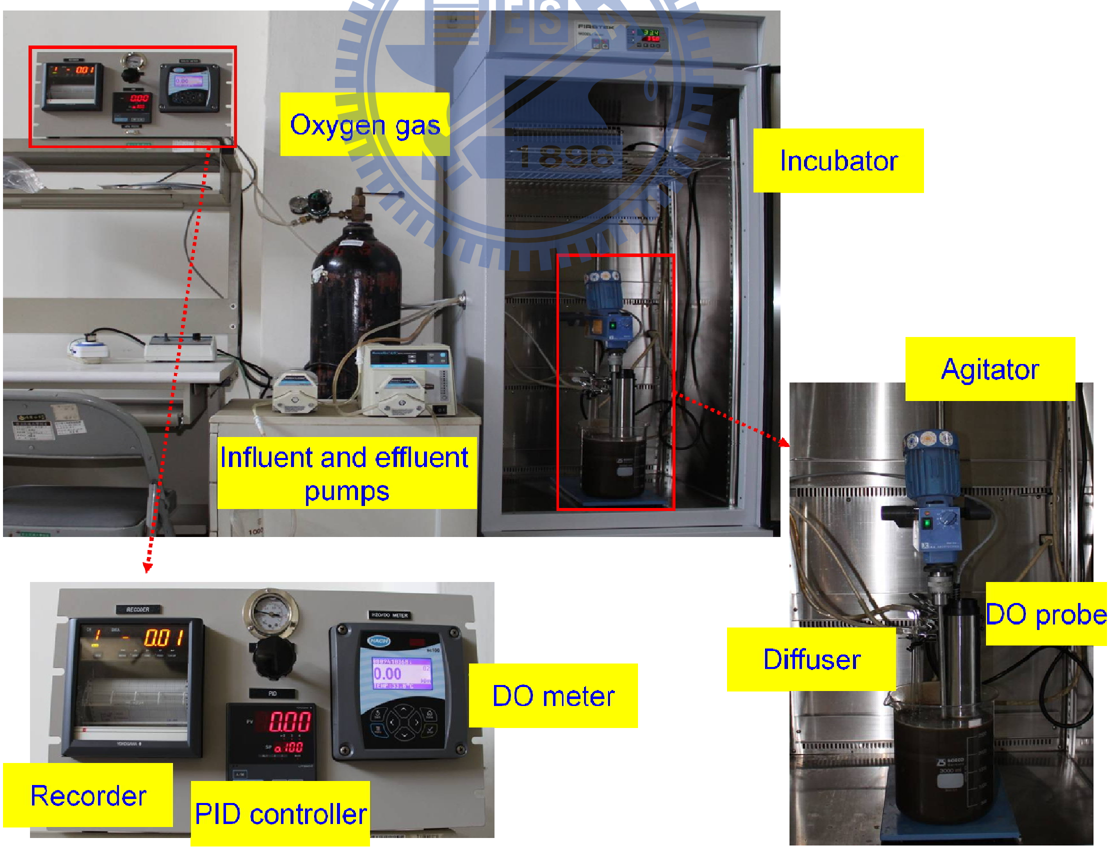


Fig. 9 Photographs of SBR and PID control system

3.5 Anammox bacteria preservation and viability test

Anammox bacteria collected from the landfill-leachate treatment plant was flushed twice using degassed synthetic medium. The Anammox granules were ground by tissue grinder (Knotes, USA). The ground sample was filled up using a cryogenic vial and added with glycerin. The cryogenic vial was frozen at -80^{o}C, which preserved of Anammox bacteria.

After removal of the cryogenic vial from freezing compartment, the frozen vial was put in a water bath at 37^{o}C. When the vial temperature reached 37^{o}C, it was centrifuged for 10 min at 125x g. The outside part of the vial was disinfected by 70% ethanol before moving to an anaerobic glove box. Then, the cap of the vial was opened and the supernatant of the vial in the anaerobic glove box was removed. The Anammox biomass in the vial was moved to a 100-mL anaerobic tube filling with the culture medium [12] shown in Table 8. The final volume of anaerobic tube was 50 mL.

Table 8 Culture medium compositions

Medium Composition	mg/L	Trace element	mg/L
$(NH_4)_2SO_4$	42	EDTA	1500
$NaNO_2$	15	$ZnSO_4$ $7H_2O$	430
$KHCO_3$	1250	$CoCl_2$ $6H_2O$	240
NaH_2PO_4	50	$MnCl_2$ $4H_2O$	990
$CaCl_2 \cdot 2H_2O$	200	$CuSO_4$ $5H_2O$	250
$MgSO_4 \cdot 7H_2O$	200	$NaMoO_4$ $2H_2O$	220
$FeSO_4$	14	$NiCl_2$ $2H_2O$	190
EDTA	18.6	$NaSeO_4$ $10H_2O$	210
		H_3BO_4	14
Trace element	1 (mL/L)	$NaWO_4$ $2H_2O$	50

The anaerobic tube was incubated in an incubator controlling temperature at 35^{o}C for 24 h. After incubation, the sample was put under UV exposure with a wavelength between 350 nm and 600 nm. A significant peak at the wavelengths between 419 and 553 nm was observed. Dosta et al.

indicated that Anammox bacteria show a maximum peak between 400-410 nm [24]. Meanwhile, 520 and 550 nm adsorption signals were also indicate the activity of the Anammox bacteria [70-71].

3.6 Analytical techniques

The pH and ORP in the SBR were recorded using the digital pH and ORP meters (Suntex PC320, Taiwan), respectively. All chemical analyses were performed according to the Standard Methods [72]. Ammonium and nitrite concentrations were determined by the colorimetric method, whereas nitrate was measured spectrophotometrically. The organic matter content of the wastewater was analyzed according to the Standard Methods and expressed as COD. Moreover, the solids in the SBR including suspended solid (SS), volatile suspended solid (VSS), MLSS and MLVSS were determined by drying in an oven and muffle furnace described from the Standard Methods [72].

Chapter 4 Results and Discussion

4.1 Performance of landfill-leachate treatment plant

Fig. 10 (a-c) shows the influent and effluent concentrations of nitrogenous compounds in the aeration tank from the full-scale landfill-leachate treatment plant. The nitrite concentration in the aeration tank influent is below the detectable limit. However, a decrease in ammonium concentration with simultaneous increase nitrate concentration is observed in the aeration tank. This implies a conventional chemolithoautotrophic oxidation of ammonium to nitrite by AOB and subsequently from nitrite to nitrate by nitrite oxidizing bacteria (NOB). The low DO concentration in the aeration tank may inhibit the microbial activity so that a complete nitrification is not observed. Alternatively, ammonium concentration can be consumed by Anammox bacteria. Thermodynamically, Anammox is favorable at two molar ratios of ammonium to nitrite, i.e., 1:1 and 1:1.67 [2, 9, 73]. When the Anammox becomes predominant, two interlinked processes can be hypothesized to occur: (i) partial nitrification, i.e., ammonium to nitrite by the AOB, followed by (ii) Anammox. Oxygen limited conditions can provide an adequate environment for a stable interaction between Nitrosomonas-like aerobic microorganism and Planctomycete-like anaerobic bacteria [38, 41, 74]. Moreover, the concept of CANON process is also the combination of partial nitrification and Anammox in a single reactor. Kuai and Verstreate [33] and Hippen et al. [75] used the concepts of CANON process in OLAND and aerobic deammonification processes, respectively [73]. Therefore, AOB is believed to oxidize ammonium to nitrite initially in the aeration tank by consuming DO to create an anaerobic/anoxic microenvironment. The produced nitrite is utilized along with the remaining ammonium by the Anammox bacteria to be converted into nitrogen gas in the anaerobic environment [19]. However, quantitative analyses on the ammonium converting to nitrite by AOB and the ammonium utilized in Anammox are highly complicated.

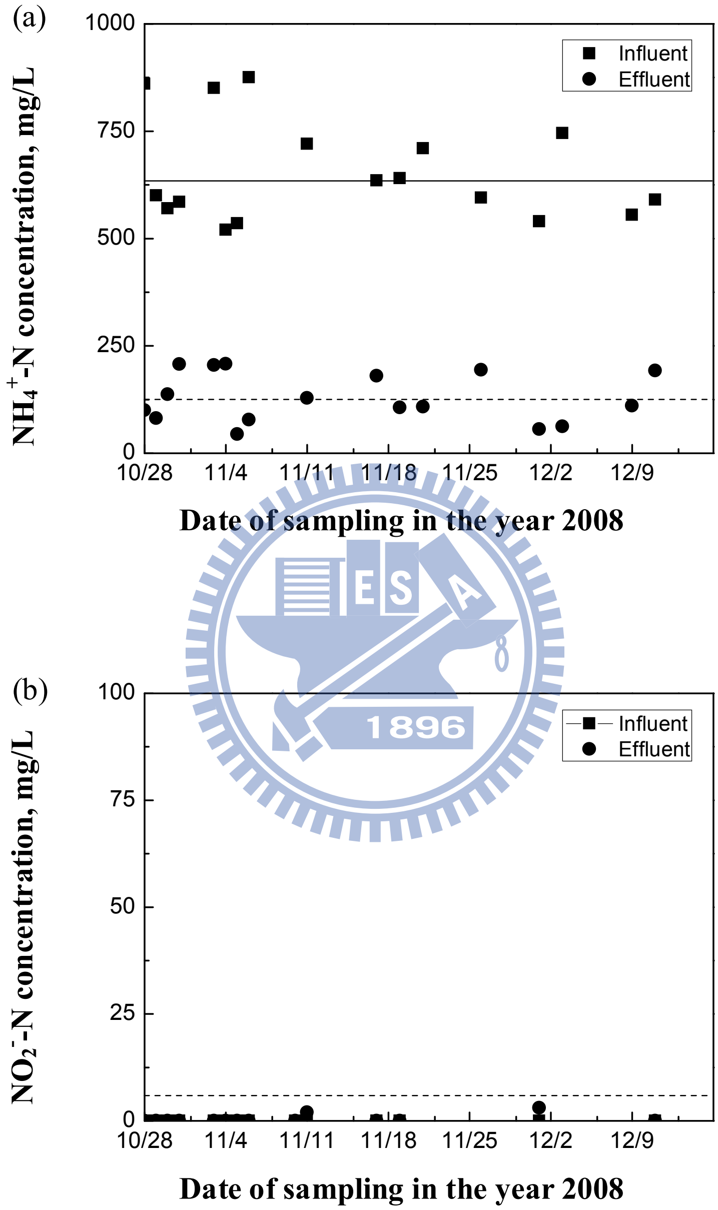
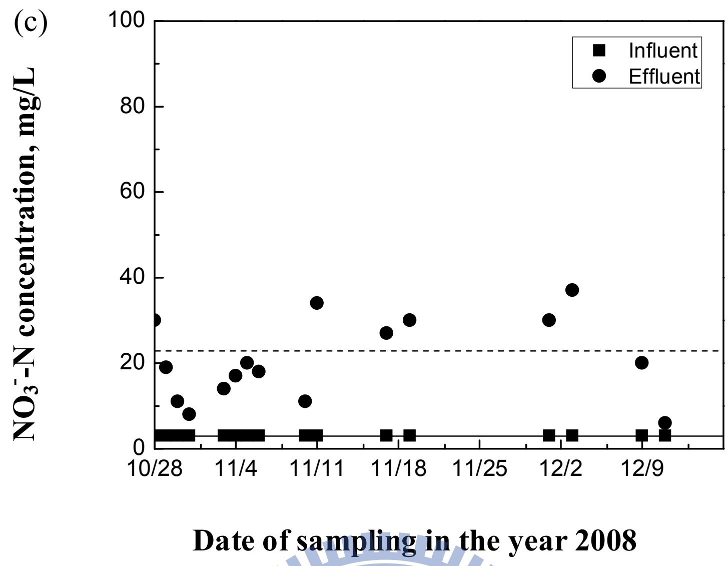


Fig. 10 The influent and effluent profile of (a) NH_4^+-N and (b) NO_2^--N in the aeration tank. (Note: Solid and dash lines indicate the average of influent and effluent concentrations, respectively.)



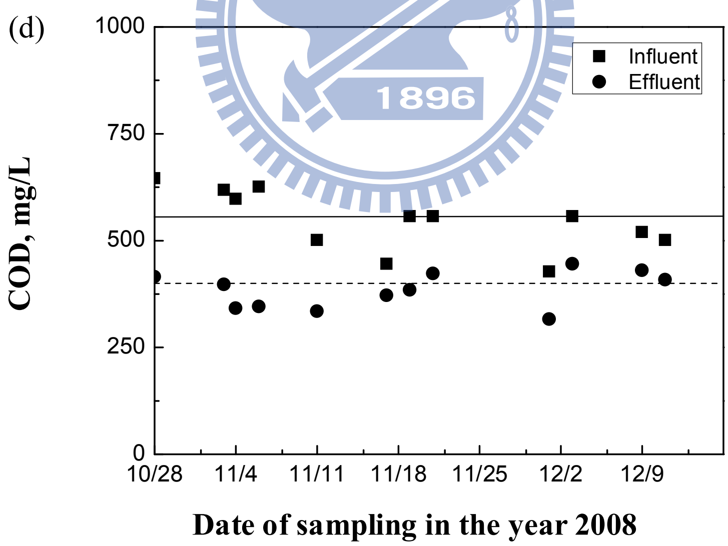


Fig. 10 The influent and effluent profile of (c) NO_3^--N and (d) COD in the aeration tank. (Note: Solid and dash lines indicate the average of influent and effluent concentrations, respectively.)

The maximum removal efficiencies of total nitrogen (TN) and ammonium in the aeration tank were approximately 76% and 80%, respectively. Interestingly, a decrease in COD was also observed (Fig. 10 (c)) in the aeration tank with simultaneous reduction of the ammonium concentration. The COD is used by heterotrophic bacteria as carbon and energy source during denitrification, whereas nitrite and/or nitrate are used as electron acceptors. The affinity between nitrite and Anammox bacteria is much higher than that between nitrite and denitrification bacteria [76]. Therefore, nitrite produced from partial nitrification could be consumed by the Anammox bacteria immediately. Thus, the majority of nitrite is consumed by the Anammox bacteria, and the nitrate produced from Anammox is consumed by heterotrophic denitrification with simultaneous COD removal. Subsequently, the nitrite produced from partial nitrification is also consumed by Anammox bacteria. Denitrification and Anammox occur under anoxic condition in the presence of electron donors. Several researchers reported the possibility of denitrification/partial denitrification and Anammox in one reactor [48, 73, 77]. Moreover, Anammox was first identified in a methanogenic reactor [2].

Recently, Chen et al. identified the SNAD process for the simultaneous nitrogen and COD removal using a small scale non-woven rotating biological contactor (RBC). The mechanism of SNAD is believed to account for the simultaneous removals of nitrogen and COD in the aeration tank of the landfill-leachate treatment plant. The stoichiometric relationship of partial nitrification, Anammox and denitrification are used to estimate the quantities of nitrogen and COD consumed in the treatment plant. The performance of the SNAD system and other partial nitrification followed by ananmox processes in the full-scale level were compared and listed in Table 9.

Table 9 Comparisons of Anammox applied full-scale wastewater treatment plants

Process	Location	Volume (L)	Target	NLR (g/L/d)	References
SHARON+ Anammox	The Netherlands	70,000	Rejection water of digested sludge dewatering	10	Abma et al., 2007
DEMON	Australia	500,000	Real-time wastewater	0.68	Wett, 2007
SNAD	Taiwan	384,000	Landfill-leachate	0.5	Wang et al., 2010

4.2 Model based evaluation of partial nitrification, Anammox and denitrification

If the aeration tank is considered as a black box (Fig. 11 (a)), the quantity of nitrogen consumed by partial nitrification, Anammox and denitrification can be modeled based on the stoichiometric equations. The monthly average data are used for modeling and the outcomes are shown in Fig. 11 (b).

The model assumes the stoichiometric relationships for several biological activities in the aeration tanks: (i) partial nitrification occurs in the aeration tank followed by Anammox and denitrification, (ii) in partial nitrification, the molar ratio of ammonium to nitrite is 1:1, (iii) the molar ratio of ammonium to nitrite consumes in Anammox is 1:1.32 and produces 0.26 mol of nitrate, subsequently that is utilized in denitrification, (iv) the organic matter composition of the influent is $C_{1.6}H_{3.3}O_{1.1}N_{0.02}$ (based on the elemental analysis of the landfill-leachate) and therefore, (v) theoretically, denitrification can utilize 1 mol of nitrate per mol of COD consumption as shown in eq. 15.

$$C_{1.6}H_{3.3}O_{1.1}N_{0.02} + 1.6NO_3^- \rightarrow 0.8N_2 + 1.6CO_2 + 1.1H_2O + 0.02NH_3 + 1.6OH^- \quad (15)$$

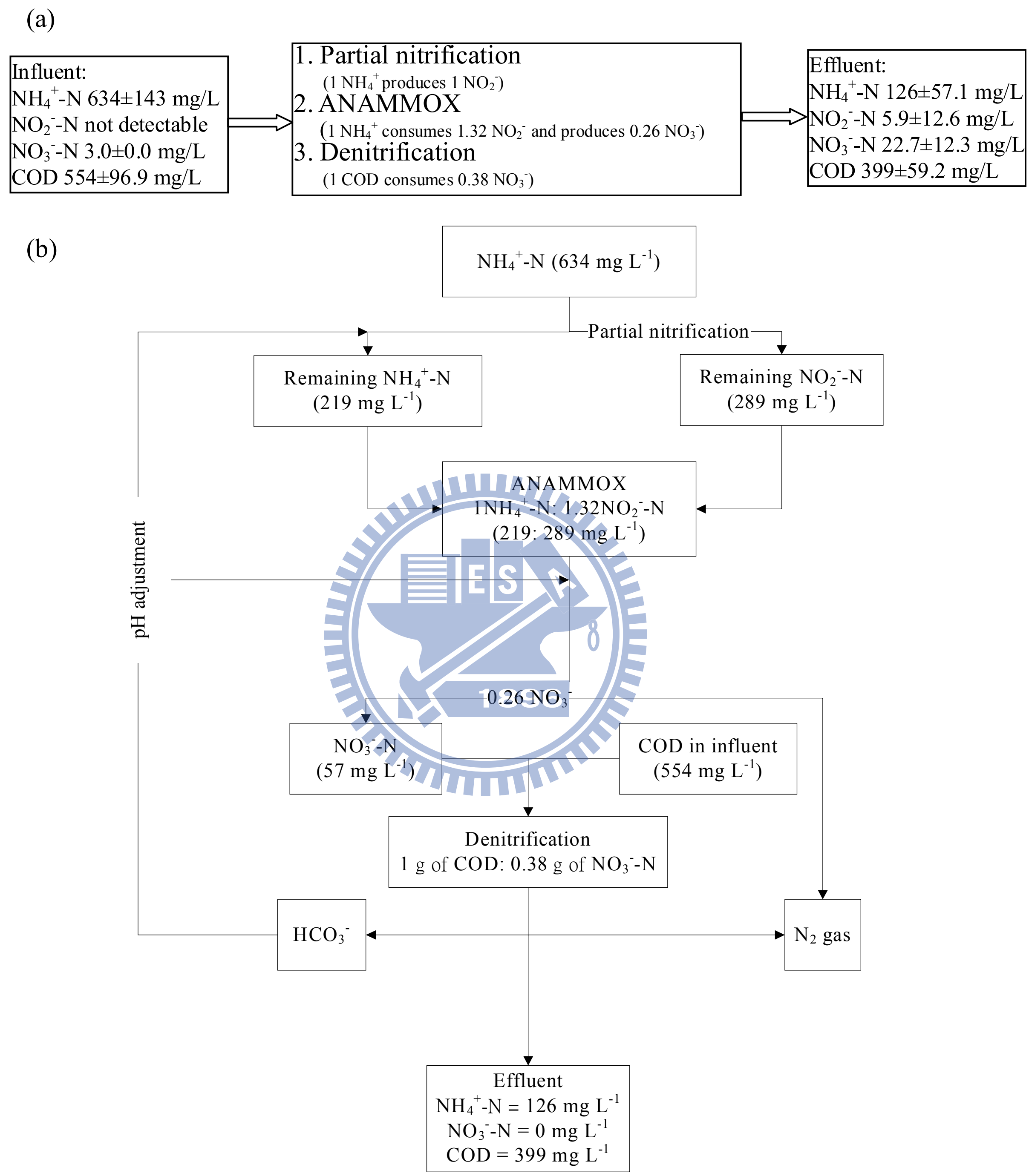


Fig. 11 Schematic diagrams showing (a) The leachate characteristics feeding into the aeration tank, and (b) the model based evaluation of SNAD process in the aeration tank

It can be seen from Fig. 11 (b) that based on the stoichiometric model the combined partial nitrification and Anammox could remove about 68% of TN from the aeration tank. On the other hand, heterotrophic denitrification is responsible for the removal of 8% TN and 23% COD in the aeration tank. Whereas, the total COD removal in the aeration tank is 28%, i.e., COD reduced from 554 to 399 mg/L (Fig. 11 (b)). The gap between the COD removals (23%) could be due to the COD consumption by the other heterotrophic organisms in the aeration tank. Denitrification and Anammox produce alkalinity to maintain at a stable pH range while partial nitrification occurs. The stoichiometric calculations of ammonium, nitrate and nitrite concentrations shown in Fig. 11 (b) are in a good match with the corresponding the experimental results.

4.3 Observation of microbial community

At the time of sampling collection for biotechnological analysis, several images of the bacterial granules existed in the aeration tank were captured (Fig. 12). The images show the red granules, which were found to be in Anammox reactors typically [43, 62, 78]. The average diameter of Anammox granules found in the field was 5 mm as shown in Fig 12 (d). Compared to the diameter of granules in the first full-scale Anammox reactor, 1.4 mm granules were found [79]. Another granule formation (diameter 2.5 mm) was found in a UASB, which was inoculated with activated sludge [65]. Subsequently, the FISH analysis confirmed the occurrence of Anammox bacteria in the aeration tank (Fig. 13). The total bacteria containing DNA was stained with the DAPI that represents roughly all bacteria as shown in Fig. 13 (a). The Fig. 13 (b) represented the Anammox bacteria hybridized with probe Amx820. Through the comparison of Fig. 13 (a) and (b), the major population in the granule was Anammox bacteria. The morphology of Anammox bacteria was spherical-shaped/coccus-shaped.

(a)

(b)

flask.

(c)

(d)

Fig. 12 (a) Granules in the aeration tank, (b) Anammox granules attached on a carrier, (c) attached growth of Anammox bacteria on the aeration tank wall and (d) Anammox granules in a flask.

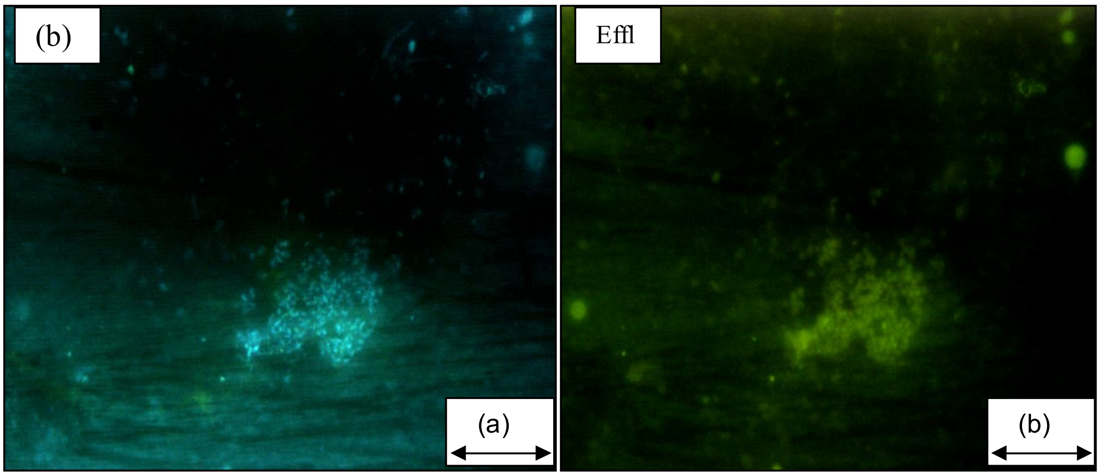


Fig. 13 fluorescence micrographs of bacteria granules collected from the aeration tank (a) DAPI, (b) Amx820

Moreover, the results of PCR showed clear bands around 1500 bp marker in lane I (primer pair I), between the 200 and 300 bp marker in lane II (primer pair II) and 1500 bp marker in lane III (primer pair III)(Fig. 14). The results of sequence analysis are shown in Table 10. FISH and PCR results confirm the occurrence of many well-known Anammox species. The detail sequences are provided in appendix A.

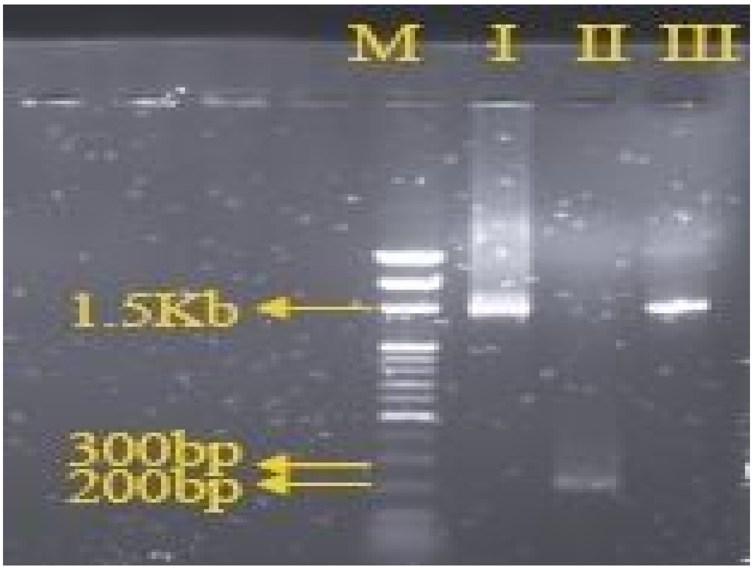


Fig. 14 Electrophoresis profiles of the PCR-amplified DNA fragments (I, II and III represent primer pair I, II and III, respectively)

Table 10 Outcomes of sequence analysis

Species Identified	Similarity (%)	NCBI No.	Species reported by
Candidatus Kuenenia stuttgartiensis	99	CT573071.1	[80]
Candidatus Kuenenia stuttgartiensis	99	AF375995.1	[81]
Anaerobic ammonium oxidizing planctomycete KOLL2a	99	AJ250882.1	[78]
Candidatus Brocadia fulgida	93	EU478693.1	[82]
Planctomycete KSU-1	93	AB057453.1	[47]
Candidatus Brocadia fulgida	93	DQ459989.1	[83]
Candidatus Jettenia asiatica	92	DQ301513.1	[23]

4.4 Preservation and viability of Anammox bacteria

The reddish granules found in the landfill-leachate treatment plant contain abundant Anammox bacteria. To preserve these bacteria, the granules were ground into suspended solid form. These samples were stored in a refrigerator at -80^{o}C. After a week, the temperatures of the frozen samples were increased slowly for further analysis. Then, the Anammox bacteria were removed to an anaerobic vial to demonstrate the Anammox bacteri. A UV-spectroscopy was used to analyze the cytochrome *c*. The cytochrome *c* usually present in cell extracts of Anammox bacteria. The maximum adsorption of cytochrome *c* at 410 nm was found for the oxidized form of the protein. Huston et al. found another two small peaks around 520 and 550 nm. The result of viability of Anammox bacteria is shown in Fig. 15. The major peak in Fig. 15 was at 419 nm, which is in a good agreement with the result of Dosta et al. [24]. The other two small peaks were found at 525 and 553 nm that were similar to Huston et al. [70]. These results demonstrate that even the stored Anammox bacteria cells at -80^{o}C can be recovered for its original activity. Moreover, the UV-spectroscopy could provide an easy and convenient method to monitor activity of Anammox bacteria. The Anammox bacteria samples have been sent for preservation at Bioresource Collection and Research Center (Hsinchu, Taiwan), and a preservation number was obtained "980011".

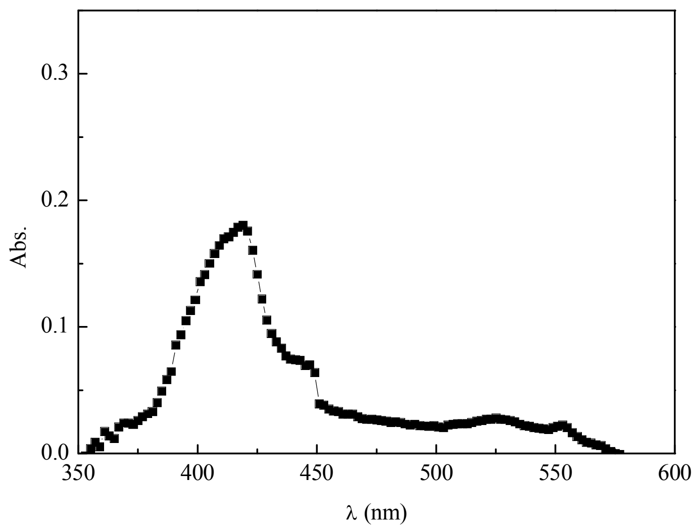


Fig. 15 The result of viability of Anammox bacteria after deep freezing

4.5 Performance of SNAD system in the SBR

Table 11 shows the influent characteristics of the landfill-leachate at various stages of the SBR operation. Using the influent data, the nitrogen loading rate (NLR) and organic loading rate (OLR) are calculated using the eq. (16) and eq. (17), respectively.

$$NLR = \frac{Inf.\{(NH_4^+ - N) + (NO_2^- - N) + (NO_3^- - N)\}}{HRT} \quad (16)$$

$$OLR = \frac{Inf.COD}{HRT} \quad (17)$$

It can be seen in Table 11 that the NLR was gradually increasing from stages I to IV. On the other hand, the OLR was fluctuating greatly due to the random fluctuation of COD in the landfill-leachate samples. The BOD levels in the landfill-leachate also varied significantly from the stages I to IV. However, the BOD/TN value of the landfill-leachate remains almost the similar ratios (0.33 to 0.42) in all stages due to the elevated ammonium concentration. This information reveals that (i) maintaining consistent influent characteristics to any landfill-leachate treatment system is highly impossible, and (ii) the treatment system could be designed in such a way to withstand various influent concentration and/or shock loading.

The effluent concentrations of TN, COD and their removal efficiencies are shown in Table 11. In the SNAD system, a part of ammonium is converted to nitrite by the conventional chemolithoautotrophic AOB. The successive oxidation of nitrite to nitrate is not possible owing to the low DO concentration in the aeration tank (~0.1 mg/L). Subsequently, the produced nitrite is utilized along with the remaining ammonium by the Anammox bacteria to nitrogen gas. Finally, the nitrate produced in the Anammox activity is utilized by the heterotrophic denitrifiers. The profiles of nitrogen species under all the stages are shown in Fig 16. The ammonium removal in the reactor was unstable in the beginning period of stage I; however, complete NH_4^+-N removal was observed in the stage II. This indicates that doubling the NLR (118 to 236 mg-N/L/d) and OLR (100 to 200 mg/L/d) has no significant effect on the SBR system. However, the subsequent increased on the NLR causing the decreased SBR performance. The higher effluent of NH_4^+-N concentration was an evidence to prove the decreasing the SBR performance. This could be due to the limitation of partial nitrification process under low DO concentration in the SBR. A decrease in DO concentration of as low as 0.04 mg/L was observed in an CANON process operating in SBR mode [37]. The DO in this study was controlled precisely around 0.1 mg/L through PID controller. Therefore, it is envisaged that the major rate-limiting step in the SBR is probably the transfer of oxygen from the gas-phase to the liquid-phase [50].

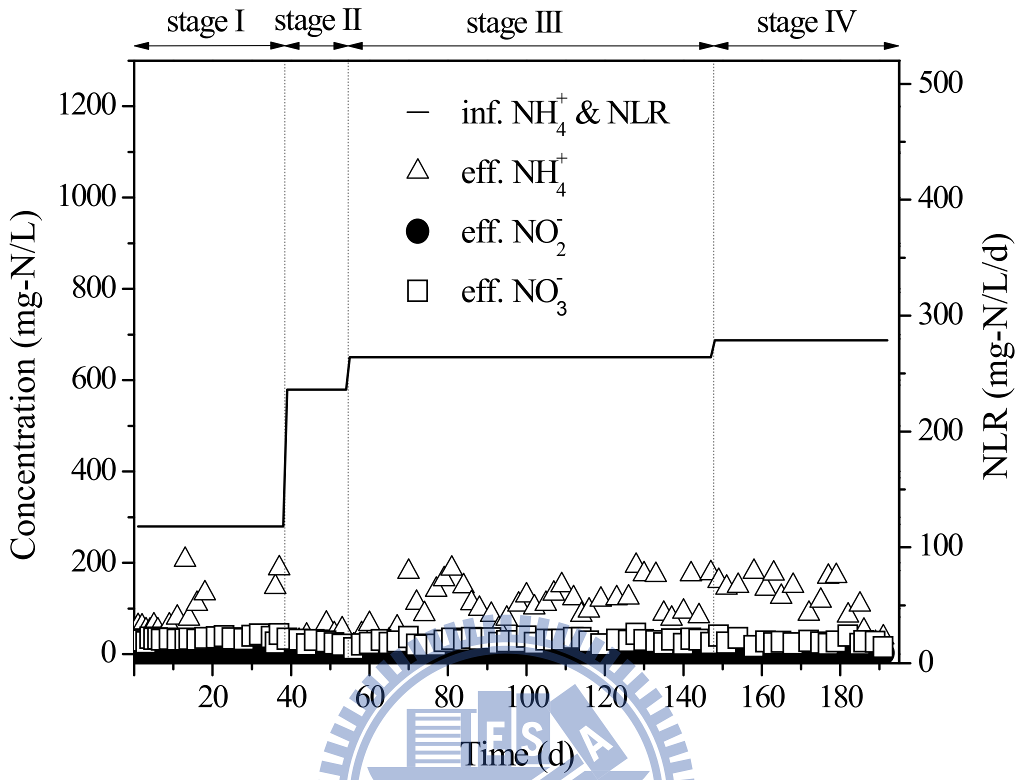


Fig. 16 Profiles of influent and effluent nitrogen compounds in the landfill-leachate

Table 11 Characteristics of real-time landfill-leachate before and after treatment

Stages	Time of operation (d)	NH_4^+-N (mg/L)		NO_2^--N (mg/L)		NO_3^--N (mg/L)		Inf. BOD (mg/L)	COD (mg/L)		*Inf. COD/TN (BOD/TN) ratio	OLR (mg/L/d)	NLR (mg-N/L/d)	*Eff. TN (mg/L)	NH_4^+-N removal (%)	COD removal (%)
		Inf.	Eff.	Inf.	Eff.	Inf.	Eff.		Inf.	Eff.						
I	38	295	53	2	1.54	0	36.5	88	250	198	0.85 (0.35)	100	118	91	82	21
II	16	590	37	4	2.23	0	26.5	164	500	329	0.85 (0.33)	200	236	66	94	34
III	93	660	111	0	0.68	0	28.9	155	365	252	0.55 (0.42)	146	264	140	83	31
IV	44	700	126	0.5	0.61	1.8	26.5	178	500	279	0.71 (0.36)	200	280	153	82	45

*TN is the sum of NH_4^+-N, NO_2^--N and NO_3^--N

The removal efficiencies of TN and ammonium are shown in Fig. 17. On the other hand, the nitrite concentration was close to zero and the nitrate concentration was less than 36 mg/L (Fig. 16) in all of the stages indicating that the Anammox bacteria were highly efficient in the SNAD process. The COD presented in the SBR was used by heterotrophic bacteria as carbon and energy sources during denitrification; the consistent removal of COD (Fig. 18) proved the activity of the denitrifiers in the SBR. The COD removal profiles under the stages I to IV indicate that the SBR was not affected by the increase or decrease of the OLR. Theoretically, the molar ratio of NH_4^+-N: NO_2^--N consumes from Anammox is 1:1.32 and produces 0.26 mole of NO_3^--N, and subsequently that is utilized in denitrification. In our previous study, it was observed that 1 g of COD is consumed for 0.38 g of NO_3^--N removal [39]. The increase in the NLR could increase the stoichiometric production of NO_3^--N, which was directly related to the COD consumption in the SBR. Therefore, the COD consumption in the SBR was based on the performance of partial nitrification and Anammox. However, the excess COD or the presence of non-biodegradable organic matter in the landfill-leachate had no significant effect on the performance of the SBR. On the other hand, the successful autotrophic nitrogen removal processes, for example CANON or Anammox alone, is not stable in the presence of COD; however, the application of SNAD system could completely remove nitrogen and a part of COD or majority of BOD. As a whole, the SNAD system widens the anammox process including the treatment of low C/N wastewater/landfill-leachate.

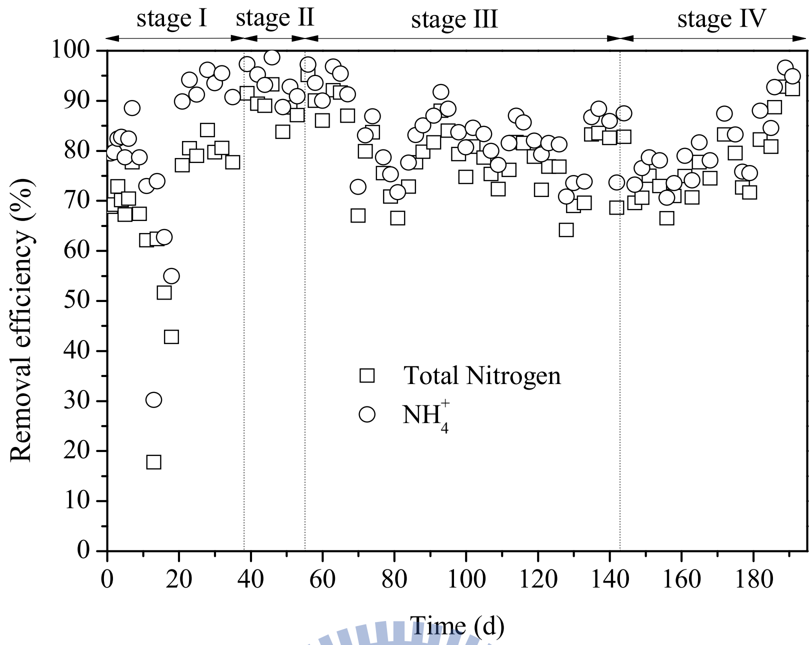


Fig. 17 Profiles of total nitrogen and ammonium removal

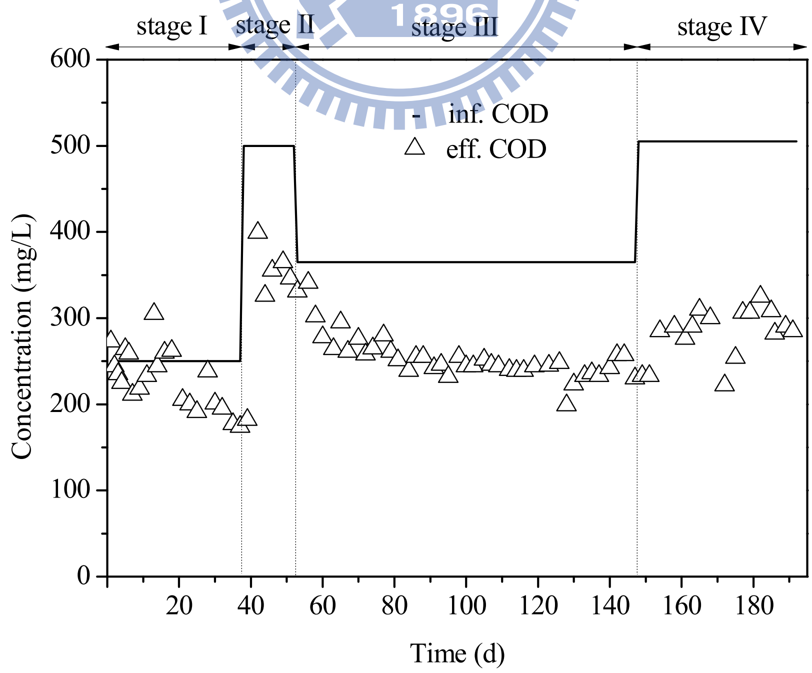


Fig. 18 Profiles of influent and effluent COD

4.6 Evaluation of the performances of different processes in the SNAD

A simple stoichiometric model was applied to evaluate the performance of each process, i.e. partial nitrification, Anammox and denitrification, in nitrogen removal [39]. Similarly, the model was applied in this study to evaluate the performance of each process at various stages of the SBR operation, and the outcomes are shown in Table 12. The results reveal that partial nitrification and Anammox are the main nitrogen removal processes in the SNAD. On the other hand, heterotrophic denitrification is responsible for the removal of nitrate and COD, and resulted in the TN removal of around 6 to 9% (Table 12). The performance of the SBR in partial nitrification and Anammox at various stages of treatment are shown in Fig. 19. In the stages of I to III, the trends of all three profiles are similar indicating that nitrogen removal is in a good correlation with organic matter removal irrespective of the variations in OLR and NLR. However, a decrease in the efficiency of TN removal by partial nitrification and Anammox was observed at the highest NLR (stage IV); whereas, the COD removal continues to increase due to the production of higher nitrate in the stage IV. These facts indicate that the SNAD system is more sensitive to NLR than the OLR under the conditions investigated in this study. Subsequently, the response of the SNAD system under various OLR and NLR was evaluated based on the sensitivity index as shown in Eq. (18)[84].

$$SI = \frac{O_{max} - O_s}{O_s} \qquad (18)$$

Where, O_{max} is the maximum concentration of substrate in the effluent from the stages II to IV (mg/L), and O_s is the normal concentration of substrate in the effluent at the stage I (mg/L). The values of SI for all nitrogen species and COD are shown in Table 13. The SI values indicate that the SNAD process was affected more by the influent ammonium compared to COD. Moreover, the negative values for nitrite and nitrate reflects that the conversion from ammonium to nitrite and nitrate (since no nitrite and nitrate in the influent) had no significant effect on the performance of the SBR.

Table 12 Nitrogen consumption in the various processes of SNAD system

Item	Stage I	Stage II	Stage III	Stage IV
TN removal by partial nitrification + anammox (%)	62.7	80	72.6	67.7
TN removal by denitrification (%)	6.2	8.9	6	7.6
Overall TN removal (%)	68.9	88.9	78.6	75.3

Table 13 Sensitivity of the SNAD system to various influent C/N ratios

Stages	Sensitivity index (SI) for various parameters*			
	COD	NH_4^+-N	NO_2^--N	NO_3^--N
Stage II	0.66	-0.30	0.45	-0.27
Stage III	0.27	1.09	-0.56	-0.21
Stage IV	0.41	1.38	-0.6	-0.27

*Effluent quality of stage-I is kept as the reference for SI calculation

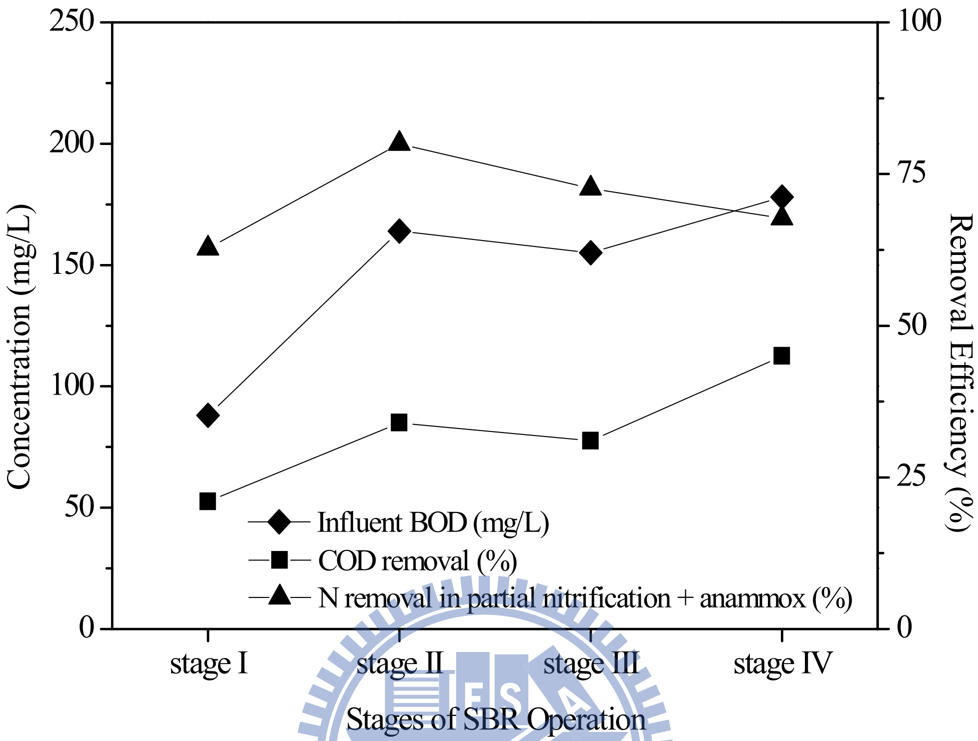


Fig. 19 Performance of SNAD process at various stages of SBR operation

4.7 Comparison of various nitrogen removal processes

In this section, several nitrogen removal processes are compared with SNAD system. The alkalinity plays an important role in balancing the pH of the biological treatment system. In the biological treatment systems, pH should be maintained at a constant value. Alkalinity could buffer the variation of pH, especially in the alkalinity consuming process. The autotrophic bacteria utilize the bicarbonate as carbon source and cause a pH drop.

The SNAD system could is simply divided into three nitrogen transfer mechanisms including nitritation, Anammox and denitrification. Nitritation and Anammox will consume alkalinity to grow the biomass as carbon source; dentrification will produce alkalinity as OH^-. The alkalinity of SNAD could be calculated from the stoichiometric equation. Alkalinity consumption or production in these three nitrogen transfer mechanisms was summarized in Table 14. The Oxygen and additional organic matter consumption are compared in different processes in the Table 14. The nitritation-denitrification process needs complete oxidation of nitrogen from ammonium to nitrate and then to nitrogen gas. Oxygen supply requirement from it is the highest (4.18 g O_2/g N) among all ofthe processes. SHARON-Anammox, CANON and SNAD processes convert nearly 50% influent ammonium to nitrite causing the less oxygen consumption than complete or partial nitrification process.

Table 14 Comparison of nitrogen removal process

Process Requirement	Oxygen (g O_2/g N)	Alkalinity (g $CaCO_3$/g N)	Organic* (g COD/g N)	References
Nitrification-Denitrification	4.18	3.53	3.7	[40]
Nitritation-Denitrification	3.16	3.53	2.3	[40]
SHARON-Anammox	1.58	3.42	X	[85]
CANON	1.95	3.93	X	[50]
SNAD	1.95	3.54	X	This study

*Additional organic carbon

Due to the complete oxidation of nitrogen, 7.07 g $CaCO_3$/g N is consumed in nitrification. When nitrate reduced to nitrogen gas, 3.53 g $CaCO_3$/g N is recovered in denitrification. The total alkalinity consumed in the nitrification-denitrification process is 3.53 g $CaCO_3$/g N. This result is similar to the nitritation-denitrification process. In the SHARON-Anammox, CANON and SNAD processes, 50%, 57% and 57% ammonium is well controlled to form nitrite, respectively. The Anammox reaction only compensate for 0.23 g $CaCO_3$ per gram nitrogen removal. Organic matter plays an electron acceptor in heterotrophic denitrification. Additional organic is needed for operating the nitrification-denitrification process. But, autotrophic Anammox bacteria are not consuming any organic matter. Due to this, no organic matter is neither required nor consumed in the SHARON-Anammox process.

Chapter 5 Conclusion and suggestion

The nitrogenous and carbonaceous compounds present in the aeration tank were removed by the SNAD process. The overall removal efficiencies of nitrogenous and carbonaceous compounds in the SNAD process was calculated using stoichiometric equations are 75.7% and 28%, respectively. The microorganism from the landfill-leachate treatment aeration tank was confirmed to be Anammox bacteria using the FISH and PCR analyses.

The SNAD process also was successfully developed in a SBR. The influence of OLR and NLR on the performance of the SBR was investigated. The experimental outcomes indicate that the performance of the SBR is affected more by the NLR compared to the OLR. The influent BOD has a good correlation with the TN removal in the system. Partial nitrification and anammox were responsible for majority of total nitrogen removal in the SBR. The landfill-leachate contains less bioavailable COD; therefore, the effluent from the SNAD process still needs some treatment with regard to organics. The maximum ammonium removal in the SBR was close to 94%, however, it can be improved by increasing the oxygen-transfer efficiency from gas to liquid phase. As a whole, the experimental findings indicate that the SNAD process is more suitable for the landfill-leachate treatment with high nitrogen and low COD content.

Recommendations for future work

Based on the findings of this study, some suggestions are summarized as follows:

1. The reactor used for SNAD process could be improved by sealing the reactor exposing to the atmosphere. A completely covered SBR is a better reactor design for estimating the gas production (nitrogen gas, nitrous oxide) and oxygen aerating rate.
2. The model based on simple assumptions could be improved through consideration of more detailed aspects.

3. Continuous flow of a reactor design could be implemented to ehance the performance in the future study.

4. According to the slowly growth of Anammox bacteria, attached growth could provide Anammox bacteria retaining in the reactor and reduced the DO exposure chance.

5. The ratio and quantity of AOB, NOB, Anammox bacteria and heterotrophic denitrifier could be identified using biological molecular technologies. The results of this examination could explain the performance of the SNAD process.

6. An appropriate HRT and SRT might be estimated in the future study.

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Appendix A: 16S rRNA sequence

tggcg aaagggtgag
taatgcattg ataacctgcc tttgagatgg gaataactgc gtttcgagca atcggaacta
ccgaaagggc tgctaatacc caataatact ataggtgcaa aagcacttgt ggtcaaatgc
taggaattct gttccttgtg cttaaagagg ggttaatgtc ctatcagcta gttggtgggg
taatggccta ccaaggcaaa gacgggtagc cggcttgaga gggtggtcgg ccacactggg
actgagacac tgcccagact cctacgggag gctgcagtcg agaatctttc gcaatgcccg
aaagggtgac gaagcgacgc cgcgtgtggg aagaaggcct tcgggttgta aaccactgtc
gggagttagg aaatgcaggt gcgttaatag cgcacttgct tgactaaggc tccagaggaa
gccacggcta actctgtgcc agcagccgcg gtaatacaga ggcggcaagc gttgttcgga
attattgggc gtaaagagca cgtaggcggc cttgcaagtc agttgtgaaa gccttccgct
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ctggtggagc ggtgaaatgc gtagatatca gaaggaacgc cggcggcgaa agcgactctc
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ccttaccggg gcttgacatg gtagaagtag aatcctgaaa gggtgacgat cggtatccag
tccgaagcta tcacaggtgt tgcatggctg tcgtcagctc gtgtcgtgag acgttgggtt
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agagagactg ccgtcgttaa gacggaggaa ggtggggacg acgtcaagtc atcatggccc
ttatgtcccg ggctgcacac gtgctacaat ggtcggtaca aagggatgct aagtcgcgag
atggtgcgaa acctataaag ccgatctcag ttcagattgg aggctgaaac tcgcctccat
gaagttggaa tcgctagtaa tcgcggatca gctacgccgc ggtgaatatg ttcccgagcc
ttgtacacac cgcccgtcaa gccacccaag caagatgcac ccaaaatcgc ctgcctaacc
cgtaagggag ggaagtgcct aagg

Appendix B: The original data of SBR

Time (d)	Supply O_2 (L)	pH	ORP (mV)	Inf. NH_4^+ (mg-N/L)	Eff. NH_4^+ (mg-N/L)	Eff. NO_2^- (mg-N/L)	Eff. NO_3^- (mg-N/L)	Eff. COD (mg/L)	Supply $KHCO_3$ (g)	Eff. alk. (mg CaCO3/L)	Eff. SS (mg/L)	Eff. VSS (mg/L)	Eff. MLSS (mg/L)	Eff. MLVSS (mg/L)
1	22.915	7.95	105	295	60.8	ND	31.5	273	2.5				3577	2573
2	17.129	7.99	91	295	60	1.1	29.8	244	2.5					
3	18.923	7.93	107	295	52	2.8	25.4	235	2.5					
4	17.617	7.95	100	295	51	1.5	35.8	225	2.5					
5	15.696	8.21	86	295	63	0.7	33	264	2.5					
6	17.397	8.18	97	295	52	1.3	34.1	259	2.5					
7	21.227	7.91	108	295	34	1	31	211	2.5	973	68	49	3930	2850
8	16.513	7.91	99	295					2.5					
9	14.715	7.89	108	295	63	0.1	33.3	218	2.5					
10	12.332	7.72	113	295					2.5					
11	7.673	8.22	85	295	80	1.2	30.8	233	2.5					
12	0.979	8.18	92	295					2.5					
13	23.509	7.99	95	295	206	4.7	32	305	2.5					
14	16.444	8	71	295	77	1	33	244	2.5				4373	3220
15	11.458	7.88	103	295					2.5					
16	7.735	7.95	83	295	110	1.58	31.2	260	2.5	733	51			
17	9.083	7.85	110	295					2					
18	8.782	8.02	96	295	133	1.3	34.6	262	1					
19	11.621	8.03	112	295					0.5					
20	12.576	7.89	120	295					0	315				
21	16.5	7.63	137	295	30	1.77	36	205	0		25			

22	13.249	7.48	152	295					0				3320	2670
23	13.597	7.31	162	295	17.3	1.77	38.6	200	0	245	45	28		
24	11.381	7.61	142	295					0					
25	18.376	7.43	116	295	26	2.2	34	191	0.5	218	160	90		
26	2.733	7.87	82	295					1					
27	4.071	7.7	90	295					2.5					
28	3.715	8.05	73	295	11.38	2.92	32.7	238	1	923	18			
29	6.78	7.97	91	295					1				3323	2483
30	13.949	7.89	103	295	19.3	1.18	39.4	201	1	495	31	18		
31	21.329	7.82	117	295					1					
32	15.042	7.92	117	295	13.4	1.64	42.4	195	1	490	26	13		
33	8.197	8.11	107	295					1					
34	12.298	8	121	295					1					
35	10.939	7.96	116	295	27.5	1.56	37	177	1	545	23	11		
36	9.987	7.92	118	295	147	0	25		1				3387	2507
37	5.323	7.9	114	295	188	0.8	43.6	174	1	553	21	16		
38	25.071	7.61	143	295					1					
39	31.139	7.33	194	590	16.4	0.7	33.2	182	2	180	16	15		
40	34.473	8	113	590					2					
41	34.448	7.85	126	590					2					
42	36.303	7.6	144	590	28.3	3.1	31.3	399	2	547	23	22		
43	9.115	7.89	80	590					2					
44	33.246	7.93	102	590	40.6	3.6	21	326	2	885	40	27		
45	20.874	8.03	85	590					2				3080	2200
46	36.664	7.93	115	590	8.12	1.8	30	355	2	662	70	31		
47	36.634	8.24	85	590					2					

48	35.471	8.01	88	590					2					
49	32.691	7.94	99	590	66.6	2.6	27	365	2	967	35	22		
50	29.929	8.02	86	590					2					
51	28.836	8.26	81	590	43	1.4	23	346	2	860	37	29		
52	37.574	8.04	76	590					1				2753	2237
53	33.035	7.83	115	590	54	2.4	20	331	1	770	30	21		
54	27.153	7.89	105	590					1					
55	27.541	7.93	114	660					1					
56	31.932	8	83	660	18.4	1.9	12.2	341	1					
57	13.563	8.2	73	660					1					
58	33.829	8.04	109	660	43	1.8	21.15	302	1	645	23.33	23		
59	35.013	8.27	91	660					1				1973	1520
60	34.136	8.15	99	660	66	2	24.3	278	1	650	24	24		
61	15.9	8.17	78	660					1					
62	32.916	7.69	89	660					1					
63	34.379	7.79	97	660	21.2	1.13	30.34	264	1	540	38	28		
64	33.644	7.89	94	660					1					
65	38.206	7.94	116	660	30.4	1.3	23.8	295	1	705	29	19		
66	34.716	7.96	115	660					1				4713	3537
67	28.434	7.98	115	660	58.14	1.02	27	261	1	750	27	14		
68	29.317	8.31	120	660					1					
69	32.678	8.01	138	660					1					
70	40.361	5	142	660	180	0.8	37	276	1	781	18	17		
71	42.587	7.99	150	660					1					
72	33.101	7.94	153	660	112	1	20	258	1	665	29	9		
73	40.609	7.89	152	660					1				4427	3263

74	36.01	7.82	163	660	87	1.8	19	265	1	422	64	60		
75	37.088	7.87	159	660					1					
76	39.115	8.32	129	660					1					
77	36.445	8.09	134	660	141	1.1	20	280	0.5	975	6	6		
78	39.097	8.04	146	660					0.5					
79	36.608	8.27	142	660	163.5	1	28.5	261	0.5	860	18	0		
80	31.183	7.88	152	660					0.5				5703	3930
81	28.161	8.27	144	660	187	0.67	33.5	251	0	795	39	8		
82	32.844	8.47	141	660					0					
83	35.876	8.48	130	660					0					
84	38.203	8.34	133	660	148	1.5	30.3	239	0	985	11	11		
85	43.04	8.11	140	660					0					
86	40.14	8.18	132	660	111.6	1.2	35	255	0	685	10	3		
87	39.611	8.18	134	660					0				5573	3763
88	39.818	8.05	129	660	99	1.6	33	255	0	608	21	18		
89	35.435	8.06	135	660					0					
90	41.023	7.96	144	660					0					
91	38.962	7.85	148	660	86	1.2	34	242	0	395	17	14		
92	37.929	7.97	140	660					0					
93	44.236	7.95	142	660	55	0.5	24	246	0	437	0	0		
94	43.05	7.23	134	660					0				5353	3980
95	40.842	7.4	122	660	77	0.8	28	232	0	530	31	20		
96	41.159	7.43	123	660					0					
97	43.685	7.5	113	660					0					
98	39.974	7.44	107	660	108	0.8	28	255	0	610	28	23		
99	43.011	7.53	102	660					0					

100	46.316	7.51	112	660	128	0.8	38	244	0					
101	44.785	7.47	104	660					0				5653	4083
102	46.826	7.39	129	660	102	0.6	24	244	0	527	28	8		
103	39.348	7.51	108	660					0					
104	33.345	5.98	246	660					0					
105	43.699	6.86	179	660	110	0.3	31	252	0	162	35	16		
106	45.361	6.98	168	660					0					
107	45.602	7.19	148	660	132.4	0.75	30	246	0	292	14	0		
108	43.329	7.47	120	660					0					
109	45.606	7.5	131	660	151	0.95	31	244	0	530	51	23	5320	3823
110	43.95	7.43	137	660					0					
111	45.297	7.26	154	660					0					
112	46.116	7.12	154	660	122	0.48	35	240	0	285	12	12		
113	37.523	5.47	266	660					0					
114	45.19	5.74	256	660	86	0	35	239	0	20	9	9		
115	47.06	6.2	224	660					0				4843	3713
116	46.772	6.45	226	660	95	0	27	239	0	55	19	5		
117	44.049	6.77	199	660					0					
118	45.886	6.75	196	660					0					
119	35.125	5.39	272	660	119	0	21	244	0	10	25	15		
120	41.404	5.5	273	660					0					
121	38.508	5.47	277	660	137	0	47	231	0	15	25	14		
122	38.806	5.5	280	660					1				4600	3723
123	38.547	5.58	276	660	122	0	31	245	1	22	27	22		
124	43.784	6.66	202	660					1					
125	43.519	7.18	152	660					1					

126	46.858	7.15	151	660	124	0.3	29	248	1	210	38	12		
127	45.54	6.6	210	660					1					
128	44.918	5.62	271	660	193	0	44	199	1	12	38	13		
129	37.599	5.48	292	660					1					
130	35.733	5.5	281	660	175	0	30	223	1.5	12	30	11		
131	37.685	5.52	283	660					1.5					
132	40.173	5.58	280	660					2					
133	47.832	5.73	245	660	173	0	28	233	2	25	12	12		
134	46.958	6.66	208	660					2					
135	52.118	5.84	268	660	88	0	23	236	2	25	19	13		
136	48.985	5.75	265	660					2					
137	43.182	5.67	275	660	77	0	32	233	2	17	28	19		
138	43.662	5.68	274	660					2					
139	41.185	6.15	245	660					2					
140	46.685	5.82	254	660	93	0	22	242	2	22	14	-		
141	45.328	5.78	260	660					2					
142	50.326	6.38	234	660	174.4	0	33	257	2	52	18	-		
143	50.691	7.41	145	660					2				3776	2840
144	50.684	7.71	121	660	83	0	31	257	0	822	57	43		
145	51.591	7.61	131	660					0					
146	49.307	7.23	164	660					0					
147	48.481	5.81	257	660	177	0	24	230	2	22	40	13		
148	51.993	6.92	187	697					2					
149	53.193	7.26	150	697	160	0	40	233	2	307	16	16		
150	53.52	7.34	142	697					2				4590	3453
151	53.297	7.37	142	697	145	0	25	233	2	372	17	17		

152	55.175	7.36	142	697					2					
153	53.304	7.26	157	697					2					
154	51.824	7.58	122	697	149	0	35	285	2	497	25	25		
155	52.543	7.83	95	697					1					
156	53.96	7.68	110	697	200	0.68	27	276	1	575	14	6		
157	53.588	7.26	139	697					1				5057	3980
158	55.144	6.99	154	697	180	0.63	17	290	1	140	22	18		
159	54.638	6.57	192	697					1.5					
160	52.76	7.06	144	697					1.5					
161	51.61	7.09	136	697	143	0.35	27	276	1.5	165	22	22		
162	53.079	7.2	137	697					1.5					
163	54.189	7.37	135	697	176	0.62	23	290	1.5	252	12	12	4700	3650
164	51.911	7.37	132	697					1.5					
165	51.902	7.34	124	697	125	0.61	26	310	1.5	280	27	13		
166	52.118	7.42	122	697					1.5					
167	51.23	7.51	107	697					1.5					
168	51.401	7.42	75	697	149	1.39	23	300	1.5	292	35	23		
169	54.0.39	7.21	106	697					1.5					
170	53.678	5.93	209	697	116	0	26	222	2	22	19	19		
171		5.81	219	697					2				5073	3957
172	50.939	6.21	181	697	88	0	29	222	2	35	16	14		
173	54.056	6.92	112	697					2					
174	53.114	7.52	36	697					2					
175	52.519	7.71	6	697	117	0.67	25	254	2	452	66	62		
176	53.183	7.87	85	697					2					
177	54.139	7.71	73	697	169	0	22	306.2	2	590	15	8		

178	54.514	7.59	104	697					2				5163	3847
179	56.172	7.84	75	697	171	0	27	306.2	2	707	20	19		
180	57.053	7.96	64	697					2					
181	55.993	8	64	697					2					
182	55.807	8.05	62	697	84	0	40	324.8	2	1267	30	30		
183	56.613	8.07	57	697					2					
184	56.216	8.1	63	697					2					
185	47.614	7.75	93	697	108	3.8	22	308	2	502	31	22	5060	3887
186	50.311	5.92	214	697	51	0	28	282	3	30	18	17		
187	55.171	6.17	206	697					3					
188	54.735	6.15	216	697					4					
189	52.201	7	154	697	24	0.5	26	290	4	140	21	11		
190	48.943	7.61	79	697					4					
191	55.079	7.74	90	697	36	2.3	15	285	3.5	575	27	16		
192	47.336	8.01	64	697					3.5				5160	3820
193	25.158	8.27	47	697	108	3.4	19	290	3.5	1230	26	21		
194	46.052	7.78	76	697					3					
195	9.784	8.14	68	697					2					
196	21.539	8.15	53	697	181	3	23	298	2	1105	24	20		
197	39.244	7.77	80	697					2					
198	52.125	7.27	116	697	79	56	31	304	2.5	275	26	23		
199	20.972	7.85	76	697					2					
200	20.215	8.06	90	697	143	89	21	352	1	1160	32	21		
201	6.677	7.35	106	697					1					
202	50.956	6.9	95	697					2					
203	24.008	7.55	72	697	188	101	26	352	1	830	48	46		

204	44.016	7.49	55	697					1					
205	5.409	7.97	-28	697	202	132	19	422	0	1612	92	59		
206	1.491	8.09	-16	349					0				6300	4693
207	6.694	7.86	17	349	188	139	26	307	0	1042	88	65		
208	6.266	7.84	12	349					0					
209	5.936	7.68	83	349					0					
210	38.982	7.06	94	349	65	1.2	28	164	2.5	440	43	43		
211	41.084	7.39	168	523					4.5					
212	40.018	7.11	142	523	54	0.2	38	168	1	347	26	26		
213	16.405	7.45	107	523					0				5907	4497
214	38.506	6.95	148	523	145	0.1	40	198	0	207	23	21		
215	25.888	7.17	107	523					0					
216	14.38	7.45	93	523					0					
217	2.34	7.88	-13	523	162	0.5	42	203	0	857	24	24		
218	1.3	7.96	20	349					0					
219	4.986	7.86	73	349	313	0.6	77	230	0	930	61	58		
220	6.294	7.76	91	349					0					
221	5.05	7.82	73	349	195	0.6	77	208	0	800	42	39		
222	4.148	5.11	171	349					2					
223	26.78	7.14	127	349					2					
224	37.348	7.03	121	349	90	0.1	84	161	2	332	31	31		
225	19.554	7.16	94	523					2					
226	22.814	7.25	109	523	50	2	73	187	0	317	33	33		
227	22.991	7.32	107	523					0				5903	4517
228	11.147	7.53	91	523	138	2.2	84	200	0	417	33	28		
229	5.321	7.6	21	523					0					

230	5.321	7.28	49	523					0					
231	2.629	7.7	0	523	180	2.1	81	204	0	560	29	10		
232	1.087	7.75	4	523					0					
233	5.008	7.73	71	523	213	1.9	84	224	0	630	45	36		
234	1.245	7.77	28	523					0					
235	0.295	7.84	20	523	251	1.9	89	224	0	815	39	25		
236	1.769	7.85	66	523					0					
238	10.952	7.55	28	479	210	2	82	220	0	550	47	41		
239	0.281	7.82	2	479					0					
240	0.101	7.86	32	479	280	2	72	192	0	895	62	45		
241	0.451	7.94	21	319					0				5417	3983
242	1.109	7.91	68	319	267	1.5	76	165	0	847	79	50		
244	1.746	7.77	43	319					0					
245	0.795	7.81	71	319	246	1.9	98	132	0	682	66	36		
246	0.742	7.84	73	319					0					
247	0.966	7.86	73	319	196	0.8	68	150	0	790	56	42		
248	1.01	7.86	75	319					0					
249	0.851	7.95	73	319	216	1.2	64	163	0	835	54	48		
250	0.69	7.9	71	319					0					
251	0.762	7.87	68	319					0					
252	0.665	7.86	75	319	198	0.4	69	153	0	750	50	47		
253	0.641	7.86	79	319					0					
254	0.68	7.87	79	319	221	0.8	66	152	0	835	50	40		
255	0.624	7.54	100	319					0				4653	3413
256	0.596	7.78	82	319	239	0.7	70	159	0	662	39	22		
257	0.547	7.54	103	319					0					

258	12.615	7.3	154	319					1.5					
259	29.537	7.12	162	319	140	4.4	90	152	1.6	410	23	16		
260	58.775	7.07	177	319					0					
261	11.738	7.37	-34	319	138	2.8	180	187	0	500	66	55		
262	1.183	7.35	-18	319					0					
263	1.979	7.6	-10	319	181	2.4	96	173	0	490	68	43		
264	3.756	7.24	-4	319					0					
265	5.431	7.68	-17	319					0					
266	1.285	7.42	-1	319	232	0.4	80	168	0	470	57	38		