Application of novel acrylic resin biomass carrier for partial nitritation-anammox processes

(新規アクリル繊維性担体の部分亜硝酸化－Anammox プロセスへの適用)

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Abstract

Nitrogen compounds are responsible for a number of environmental problems, such as eutrophication, depletion of dissolved oxygen in water bodies, decaying smell, aquatic animal poisoning and methemoglobinemia. In order to eliminate or minimize these environmental problems, removal of nitrogen compounds in the wastewaters is necessary. Biological methods of nitrogen removal are widely accepted as methods to remove nitrogen compounds from waters and wastewaters. Removal of ammonium nitrogen under aerobic conditions is well established, however its removal under anaerobic conditions is relatively new and is in developing stage.

Anaerobic ammonium oxidation (Anammox) process, as a novel and simple nitrogen removal technique, was discovered about 12 years ago in a fluidized bed reactor. Anammox process could directly convert ammonium (electron donor) and nitrite (electron acceptor) to dinitrogen gas. Compared to the traditional nitrogen removal process, anammox process could save 50% of oxygen supply for nitrification and not need supplemental carbon source for denitrification.

Although many reactor types have been used for anammox research, the problem of anammox biomass washing out from the reactor has not been solved satisfactorily. Due to the long doubling time of anammox bacteria (11 days), only the systems, which could retain anammox biomass efficiently as possible, are compatible for this novel process. This study was undertaken with the objectives to investigate an efficient system with novel support materials, which could retain anammox biomass as much as possible. Furthermore, the possibility of practical wastewater treatment by partial nitritation-anammox process was also examined.

In the first part of this study, a novel acryl fiber biomass carrier (Biofill) was used for anammox research in an up-flow column reactor. This material could retain anammox

microorganisms efficiently and the average weight of attached sludge at the bottom part of the reactor was calculated to be 0.14 g-TSS/cm^3-Biofill. High removal efficiencies of ammonium, nitrite and total nitrogen (TN) were obtained at a high volumetric loading rate of 2.0 kg-TN/m^3/d. Furthermore, anammox activity did not decrease significantly with decreasing temperature, e.g., when the temperature decreased from 33.0℃ to 26.2℃, nitrite removal efficiencies decreased only by about 5% (from 94% to 89%). Scanning electronic microphotographs revealed that the microorganisms were in a compact state when attached to the support material. Based on the analysis of 16S rDNA, two close matches of anammox bacteria, KSU-1 and KU2, were detected.

In the second part of this study, two column-type reactors using different support materials namely, net type acrylic fiber (Biofix) and polyester nonwoven were used for anammox treatment. The nonwoven reactor was operated at 35℃ and the Biofix reactor at 25℃ (peak summer temperature, 31.5℃). During more than 330 days of operation, the nitrogen loading rates of the Biofix and nonwoven reactors were increased to 3.6 kg-N/m^3/d and 4.0 kg-N/m^3/d and T-N removal efficiencies reached to 81.3% and 86.3%, respectively. Protein matter was shown to be the most abundant extracellular polymeric substances (EPS) in the anammox sludge with almost three times more content in the granular sludge of the Biofix reactor than in the granular sludge of the nonwoven reactor. Considering the EPS levels and observation by scanning electron microscopy, the anammox granules in the Biofix reactor were denser than that in the nonwoven reactor. Results of DNA analyses indicated that the KSU-1 strain might prefer relatively low nutrient levels, while the KU2 strain might be better suited for the high substrate concentration. Other types of bacteria were also identified with the potential for consuming influent dissolved oxygen and facilitating anammox bacteria surviving under aerobic conditions.

In the third part of this study, partial nitritation (PN) and Anammox processes were applied for treatment of anaerobic sludge digester liquor from a municipal sewage treatment plant. Nitrite concentrations in PN effluent were about 1.9 times higher than effluent ammonium levels under a nitrogen loading rate of 3.2 kg-N/m^3/d at a temperature of 35℃ and pH of

7.55. The effects of free ammonia and free nitrous acid on the PN reaction were also investigated at conditions of no temperature and pH control. Under the same nitrogen loading rate of 3.2 kg-N/m^3/d, effluent nitrite levels were about 0.8 times of effluent ammonium. Average T-N removal efficiency of 71% was obtained after the PN reactor effluent was introduced into an anammox reactor. The BOD_5 removal efficiencies were stable and an average removal efficiency of 73% was observed despite changes in loading rates and operational conditions. Morphologic observation of the dense flocs formed in the partial nitritation reactor and low SVI values demonstrated satisfactory sludge settling capability, which was sufficient for retaining biomass using a settling tank.

In general, Biofill and Biofix carriers showed strong potential as materials for anammox biomass retention that could provide satisfactory nitrogen removal performances. The PN-Anammox process, as an alternative novel nitrogen removal technique, was proved to efficienctly remove nitrogen content of the actual anaerobic digester liquor. The overall results of this study demonstrated that Anammox process had great potential for application in order to control nitrogen pollution in the wastewaters.

List of acronyms and abbreviations

AOB	Ammonium oxidizing bacteria
BF	Biofringe
BL	Biofill
BX	Biofix
CANON	**C**ompletely **a**utotrophic **n**itrogen removal **o**ver **n**itrite
CLSM	Confocal laser scanning microscopy
DGGE	**D**enaturing **g**radient **g**el **e**lectrophoresis
FA	Free ammonia
FNA	Free nitrous acid
FISH	Fluorescence in-situ hybridization
KSU-1	Registration names of anammox strains in NCBI database
KU2	Registration names of anammox strains in NCBI database
MAP	Magnesium ammonium phosphate
NOB	Nitrite oxidizing bacteria
NLR	Nitrogen loading rate
OLAND	**O**xygen **l**imited **a**utotrophic **n**itrification-**d**enitrification
RBC	Rotating biological contactor
SHARON	**S**ingle reactor for **h**igh-rate **a**mmonium **r**emoval **o**ver **n**itrite
SNAP	**S**ingle-stage **n**itrogen removal using **a**nammox and **p**artial nitritation
UASB	**U**pflow **a**naerobic **s**ludge **b**lanket

Excluding chemical formula and common abbreviations such as DO, COD, etc.

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Chapter 1 Introduction

1.1 Nitrogen cycle

Nitrogen is one of the essential elements in nature. It is involved in biological survival, growth, and development processes. Usually, nitrogen exists in nature as the forms of NH_3 (-III), N_2 (0), N_2O (+I), NO (+II), NO_2^- (+III), NO_2 (+IV), and NO_3^- (+V). Biological processes cause nitrogen transition among different forms, which forms the nitrogen cycle, shown in Fig. 1-1.

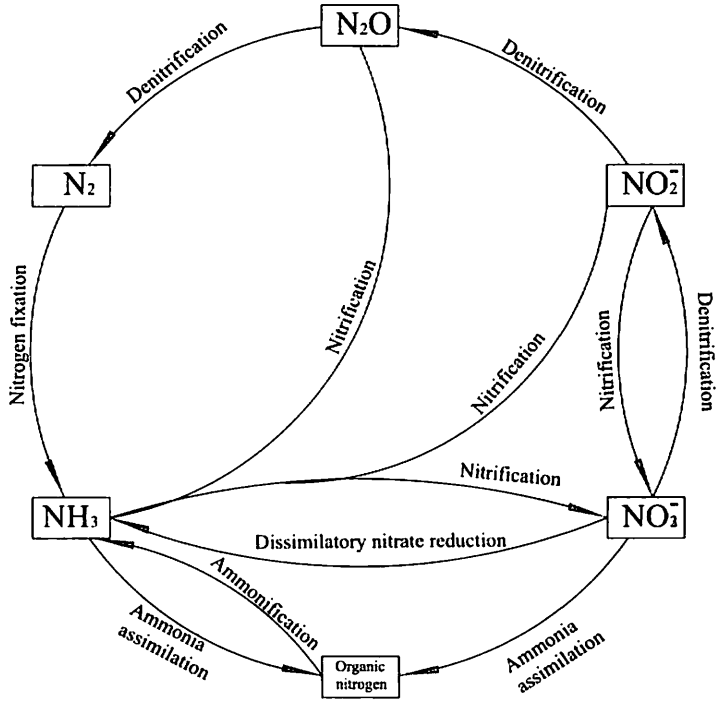


Fig. 1- 1 Nitrogen cycle (Kuenen *et al.*, 1988)

Atmosphere contains the most abundant dinitrogen gas, which accounts for about 79% of the whole atmosphere volume. However, dinitrogen gas can be assimilated by plants and also by animals after it is combined with hydrogen or oxygen. In nature, it is called nitrogen fixation, which is the biological reaction of reducing nitrogen gas to ammonia and this kind of reaction only exists in some pronucleus microorganisms. Ammonia assimilation is the biological reaction of transforming nitrogen to cellular material, which is the main biological method for fixing nitrogen. Ammonification is the reaction of

converting organic nitrogen to ammonia. The reaction of oxidizing ammonia to nitrite or nitrate is called nitrification, which can be divided into autotrophic nitrification and heterotrophic nitrification. After nitrification, nitrogen is more easily assimilated by plants and transported with water currents. Reduction of nitrite and nitrate to dinitrogen gas is called denitrificaiton, which is the primary pathway for the return of nitrogen to the atmosphere (Abeliovich, 1992). If nitrite or nitrate is not reduced to dinitrogen gas but further to ammonia, the reaction is defined as being dissimilatory reduction, which usually takes place in an anoxic environment deficient of electron acceptors (Tiedje, 1988).

1.2 Nitrogen contamination and its impacts

Nitrogen contamination is usually due to the nitrogen cycle being disturbed by human activities. For example, the exploitation, machining and utilizing of nitrogen bearing minerals or fuel will convert the nitrogen to the biosphere, which will increase the nitrogen content in local areas; nitrogen fixation in industry and agriculture accelerates the overall fixation speed, which can have a large impact on the nitrogen balance; the nitrogen input becomes higher than the output in the sphere of human activities, causing nitrogen accumulation and contamination.

Usually, municipal, industrial and agriculture wastewaters are the main sources of nitrogen pollution in the water environment, accounting for 49.0%, 30.0% and 11.1%, respectively, of the inputs. In fresh municipal wastewater, the total nitrogen consists of 60% organic and 40% ammonia forms, while in stale wastewater, the ammonia content will increase due to biodegradation reactions. There are many kinds of industrial wastewaters that contain ammonia, e.g., food processing and coking, and the concentrations can fluctuate over an extensive range. In addition, wastewater from livestock activities and landfill leachate contain high levels of ammonia nitrogen.

Plant and algae growth is limited by nitrogen and phosphorus contents in natural water bodies. However, continuous inflow of ammonia will not only cause eutrophication, but

also further bring a series of aftermaths: (1) hydrophyte and algae will reproduce abundantly and cover the water surface, which could deteriorate the landscape; (2) abundant algae growth will block the breathing of aquatic animals; (3) some algae can produce toxins, resulting in fish and shellfish poisoning; and (4) algae can also cause odor problems.

In addition, nitrification consumes dissolved oxygen (DO) in water bodies (4.6 mg-O_2/mg-NH_4^+). The saturated DO concentration in a water body is about 9.0 mg/L at a temperature of 20℃ and many fish cannot survive if the DO level becomes lower than 5.0 mg/L. Thus, DO consumption by ammonium oxidation can destroy an aquatic ecosystem. Furthermore, free ammonia of 0.1-10 mg-NH_3/L can result in fish poisoning. Since nitrates can lead to methemoglobinemia (blue baby syndrome) and gastric carcinoma, their concentrations are also strictly restricted.

1.3 Nitrogen control

Due to detrimental impacts of nitrogen on the environment, particularly on receiving water bodies, removal of nitrogen in the source wastewater has become increasingly important. A number of techniques have been developed for nitrogen removal primarily of physical-chemical and biological nature.

1.3.1 Physical-chemical processes

Physical-chemical processes for nitrogen removal involve principles of physical science and chemistry.

1.3.1.1 Ammonia stripping

In wastewater, the balance between free ammonia and ammonium is influenced by temperature and pH. For instance, free ammonia just accounts for 0.6% at a temperature of 25℃ and a pH of 7.0; while the percentage of free ammonia will increase to 98.2% when the pH increases to 11.0 at the same temperature. Similarly, improving temperature will

result in the same increment trend of free ammonia percentage.

Fig. 1-2 shows a schematic diagram of the ammonia stripping process. Firstly, the pH of the wastewater is adjusted to 10.5-11.5 by NaOH or CaO; then ammonia is stripped by aerating, which could be absorbed by H_2SO_4. Usually the stripping efficiency for municipal wastewater can be over 90% at a pH of 9.0 and a gas-water ratio of 3,600 m^3/m^3. However, there exist some problems for this process, e.g., low stripping rates in winter time, scale deposits due to pH adjustment using CaO, and air pollution due to the vented ammonia.

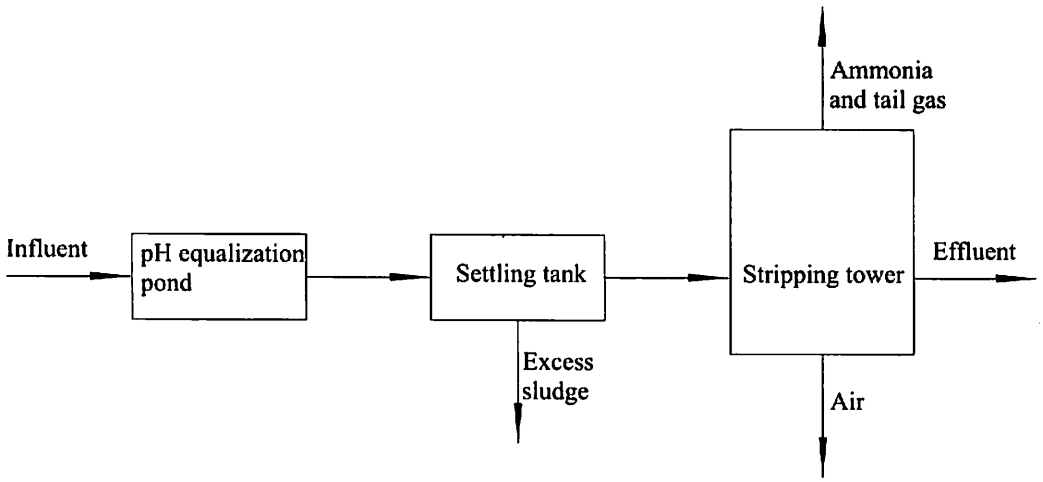


Fig. 1- 2 Ammonia stripping process

1.3.1.2 Selective ion exchange

Chinoptilolite is one kind of natural ammonium exchange materials, which has a higher selective exchange affinity to ammonium than other ions. Wastewater enters the ion exchange column with chinoptilolite and the ammonia will be absorbed by the exchange function. The process is shown in Fig. 1-3 and is usually used with an ion exchange column and stripping tower. When the column reaches the saturation state, its absorption capacity can be recovered by use of a regeneration reagent, such as NaCl, NaOH or $Ca(OH)_2$. Before entering the exchange column, the influent should be filtrated in order to avoid the clogging of the exchange column. This method can achieve about 90-97% ammonia removal efficiency but it has no removal mechanism for nitrite, nitrate and organic nitrogen.

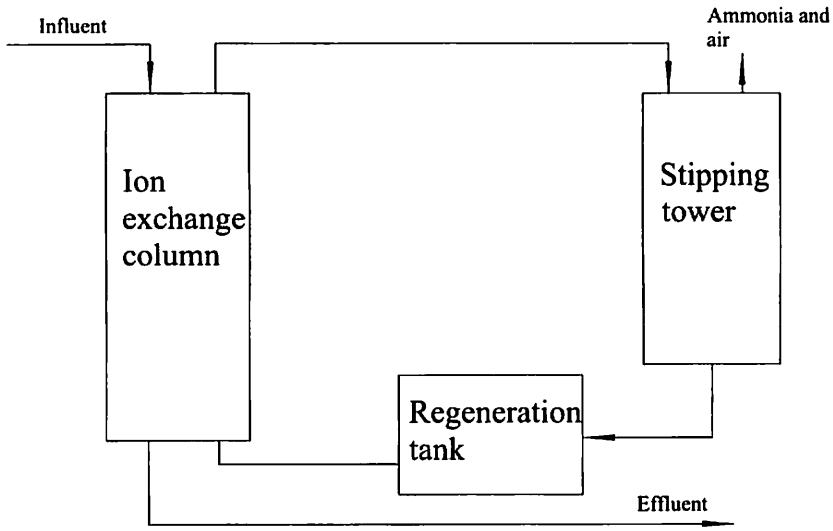


Fig. 1- 3 Selective ion exchange process for ammonia remvoal

1.3.1.3 Breakpoint chlorination

In this process, chlorine is added to the wastewater to oxidize ammonia to dinitrogen gas. The equations are shown as following:

$$NH_4^+ + 1.5HOCl \rightarrow 0.5N_2 + 1.5H_2O + 2.5H^+ + 1.5Cl^- \quad (1\text{-}1)$$

In this method, the residual chlorine and ammonia concentrations are associated with the input chlorine versus ammonia mass ratio. When the chlorine/ammonia ratio is between 0:1 and 5:1, the main product is NH_2Cl and the residual chorine concentration reaches a peak value at a ratio of 5:1. When ratio is between 5:1 and 7.6:1, the main product is $NHCl_2$and the residual chlorine and ammonia concentrations will decrease to the lowest level when the mass ratio of chlorine/ammonia reaches 7.6: 1 (the breakpoint). If the ratio goes up over 7.6: 1, the residual chlorine and ammonia concentrations will once again increase. The reaction speed is very fast and could finish the whole reaction within 15 seconds under a pH of 6.0-7.0 (USA EPA, 1991). In addition, SO_2 is applied for removal of residual chlorine as the mole ratio of 1: 1 according to the following equation:

$$SO_2 + HOCl + H_2O \rightarrow Cl^- + SO_4^{2-} + 3H^+ \quad (1\text{-}2)$$

1.3.1.4 Magnesium ammonium phosphate precipitation (MAP) process

MAP is produced by the addition of phosphate and magnesia into the wastewater, which

results in formation of MAP granules based on the following equation:

$$Mg^{2+} + PO_4^{3-} + NH_4^+ \rightarrow MgNH_4PO_4 \cdot 6H_2O \downarrow \quad (1\text{-}3)$$

Since MAP is a basic salt and is easy to dissolve under acidic condition, it is better to perform the reaction under basic conditions. The ideal dose ratio has been determined to be Mg: P: N=1.3: 1: 1 (Siegrist, 1996).

1.3.2 Biological processes

The use of biological processes is the most popular method for nitrogen removal, which consists of nitrification and denitrification processes. Nitrification is a series of biological reactions, which firstly oxidizes ammonia to nitrite and further to nitrate. Usually, all the responsible bacteria are called nitrifiers, but are more specifically divided into ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) according to the reaction mechanism. In fact, nitrogen process is involved with many metabolic approaches under the enzyme catalysis functions, with complicated substances and energy transformations. Ammonia, the real reaction substance (Hofman and Lees, 1953), converts to hydroxylamine by the ammonia monooxygenase enzyme, which was confirmed by isotope experiments using $^{18}O_2$ (Hollocher *et al.*, 1981). Hydroxylamine then converts to nitrite by two successive reactions, and the intermediates may be HNO or NO (Anderson and Hopper, 1983). There are no intermediates detected in the reaction of nitrite converting to nitrate and this reaction is thus considered to be a one-step reaction.

Denitrification is usually classified into assimilatory nitrate reduction and dissimilatory nitrate reduction based on whether or not the nitrogen is assimilated into bacterial cell mass. Nitrate reduction to nitrite is the first step in the denitrification process, followed by further successive reductive steps to NO, N_2O and N_2. The overall rate and extension of the denitrification process depends mainly on the biodegradability characteristics of the electron donor used and on the COD/N ratio in the reactor. Due to theses reasons, this process can be enhanced by adding readily biodegradable carbon sources, such as acetic acid and methanol, or carbon sources with high COD/N ratios relative to the wastewater.

Denitrification can remove oxidized nitrogen, but cannot remove ammonia directly. So the combined process of nitrification-denitrification is most commonly applied for nitrogen removal. However, the addition of a carbon source is expensive and increases the overall treatment cost of wastewater. In general, 1.0 kg ammonium converting to dinitrogen gas consumes 4.57 kg O_2 and 2-4 kg COD. Since both nitrification and denitrification processes should be developed in separate reactors, it leads to high costs of construction, operation and maintenance (Van Loodsdrecht *et al.*, 2005). Additionally, aeration is required in nitrification process, which further adds to the operational cost.

1.4 Anaerobic ammonium oxidation (anammox) process

1.4.1 Anammox principle

Three decades ago, Austrian chemist Broda predicted the existence of anammox bacteria (Broda, 1977), which he called "two kinds of lithotrophs missing in nature" that could oxidize ammonium using nitrite or nitrate as the electron acceptor according to the reaction standard free energy change(ΔG). From the three equations below, the energies released by reactions (1-5) and (1-6) are not less than reaction (1-4).

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ \qquad \Delta G\text{=-241kJ/mol} \qquad (1\text{-}4)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \Delta G\text{=-335kJ/mol} \qquad (1\text{-}5)$$

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \qquad \Delta G\text{=-278kJ/mol} \qquad (1\text{-}6)$$

However, it was not until twelve years ago that anammox process was discovered in a denitrifying fluidized-bed reactor (Mulder *et al.*, 1995). Subsequently, in another laboratory scale fluidized-bed reactor, it was reported that the removal efficiencies of ammonium and nitrite were 82% and 99%, respectively and the maximum total nitrogen removal rate was 1.5 kgN/m^3/d (Strous *et al.*, 1997). Subsequently, Strous *et al.* estimated the stoichiometric parameters for the anammox process (Strous *et al.*, 1997). The formula was shown to be as follows:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \qquad (1\text{-}7)$$

On the basis of this equation, when one mole ammonium is biodegraded, only 0.066 mole of biomass is generated, which is a very low rate for bacterial growth. In their research, Strous *et al.* also determined some other important parameters, such as a maximum specific rate of ammonium consumption of 45 nmol/mg-protein/min, a maximum specific growth rate of 0.0027 h^{-1} and a doubling time of 11 days (Strous *et al.*, 1998).

Compared with traditional nitrogen removal technologies, advantages of anammox are obvious. In the anammox reaction, nitrite is the electron acceptor and since anammox bacteria are autotrophs, they do not need additional organic carbon supplementation for denitrification and survival. Furthermore, oxygen completely inhibits anammox bacteria activity over 2 μM, so that the cost for oxygen supply can be saved. Besides, the extremely slow growth rate of anammox bacteria produces little excess sludge. Another advantage of anammox is that dinitrogen gas is the main product for anammox reaction, unlike greenhouse gases, which are harmful to the global environment (Jetten *et al.*, 2001).

Graaf *et al.*, through ^{15}N isotope tracer techniques, determined that hydroxylamine, which was decomposed from nitrite, was the most probable intermediate in anammox process (Van de Graaf *et al.*, 1997). Jetten *et al.*, however indicated that hydroxylamine and hydrazine were important intermediates of anammox reaction (Jetten *et al.*, 1999). Work carried out by Schalk *et al.* considered anammox process similar to Graaf's hypothesis after investigating anaerobic oxidation of hydrazine (Schalk *et al.*, 1998).

Brocadia anamoxidans and *Kuenen stuttgartiensis*, which were discovered in Netherlands, Germany and Switzerland, respectively, were the first two kinds of bacteria determined as anammox bacteria. Both of these branch from Planctomycetes. B. *anamoxidans* bacterium shows a spherical shape and, under electron microscope, exhibits some crateriform configuration on the cell wall (Kuenen and Jetten, 2001; Lindsay *et al.*, 2001). Furthermore, within the cytoplasm, there is a kind of cell organelle named anammoxosome, which plays an important role since it contains one of the key enzymes for anammox reaction, the hydrazine-oxidizing enzyme. At first, nitrite is reduced presumably to hydroxylamine by a

nitrite-reducing enzyme (NR) (Kuenen and Jetten, 2001). Then, ammonium and hydroxylamine are assembled by hydrazine hydrolase (HH) to form hydrazine. Subsequently, hydrazine is oxidized by a hydrazine-oxidizing enzyme (HZO), which has some identical characteristic as hydroxylamine oxidoreductase (HAO) of *Nitrosomonas europaea* (Jetten *et al.*, 1999). This redox happens inside the anammoxosome, producing dinitrogen gas, four protons and four electrons, as shown in Fig. 1-4.

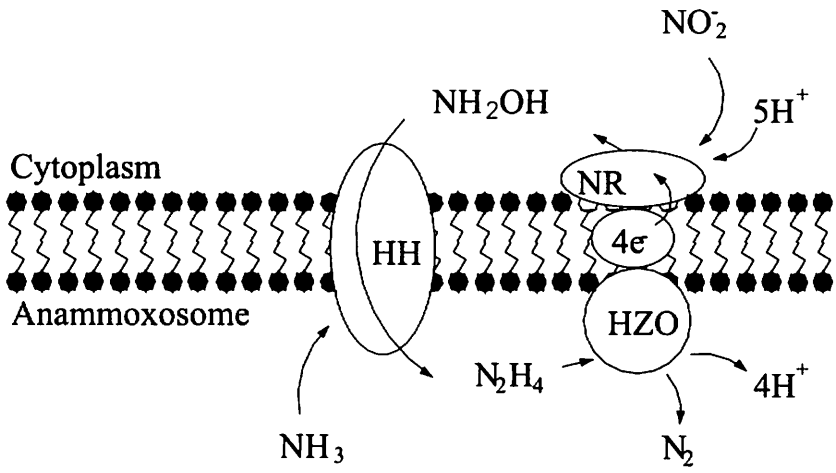


Fig. 1- 4 Biochemical mechanism of anaerobic ammonium oxidation (Jetten *et al.*, 2001) (HH: hydrazine hydrolase; HZO: Hydrazine-oxidizing enzyme; NR: Nitrite reducing enzyme)

Since recently, new types of annmmox bacteria have been discovered. Kuypers *et al.* reported that in the Black Sea a new species was detected, named *Scalindua sorokinii* (Kuypers *et al.*, 2003). Markus Schmid *et al.* also found two new genus anammox bacteria in a wastewater treatment plant treating landfill leachate in Pitse, UK (Schmid *et al.*, 2003). These anammox bacteria were shown to have high anammox activity of 5.0 nmol/mg-protein/min and could produce hydrazine from hydroxylamine. They were named provisionally as *Scalindua brodae* and *Scalindua wagneri.* Fujii *et al.* reported that two types of anammox bacteria were observed in a lab scale fixed biofilm reactor. With 16S rDNA approach and FISH technology, it showed that one sequence had a notable similarity (92.2%) to *Brocadia anamoxidans*; another one was a new genus, designated KSU-1, which was dominant among detectable members of the biofilm community (Fujii *et al.*, 2002).

1.4.2 Affecting factors

As far as biochemical reaction is concerned, pH is a key factor. Both NH_4^+ and NO_2^- react within a certain pH range; thereby making pH as an important factor in anammox process. Jetten *et al.* found that anammox process worked well between pH 6.7-8.3 and that the optimum was at pH 8.0 (Jetten *et al.*, 1999). In a study on *K. stuttgartiensis*, it was discovered that the pH range for this kind of bacterium was 6.5-9.0, with the optimum pH of 8.0 (Egli, 2001).

Temperature is another key affecting factor for biochemical reaction. Jetten *et al.* held that the temperature range for anammox bacteria was 20-43℃, with the optimum at 40℃ (Jetten *et al.*, 1999). For *K. stuttgartiensis*, the optimal was reported to be 37℃. No anammox activity was observed at a temperature greater than 45℃. Furthermore, even if the temperature re-decreased to 37℃, the anammox activity did not recover. Besides, the activity of *K. stuttgartiensis* was just as 24% at 11℃ compared to that at 37℃ and in the whole process only 15% nitrite was transformed to nitrate (Egli, 2001).

Ammonium and nitrite as substrates can also inhibit anammox activity. Jetten *et al.* found that anammox activity was completely inhibited under nitrite concentration of more than 20 mM. Moreover, if the anammox bacteria were exposed to high nitrite concentrations for more than 12 h, the anammox activity lost entirely (Jetten *et al.*, 1999). However, *K. Stuttgartiensis* was more tolerable to nitrite than *B.anammoxidans*, and showed higher activity in low biomass concentration (Egli, 2001). But in our laboratory, it was found that the inhibition would occur to KSU-1 strain if nitrite concentration was over 20 mg-N/L.

Strous *et al.* considered that the inhibition of DO was reversible. Through a series of experiments, they found that at 0.5-2.0% of atmosphere saturation level, anammox activity was inhibited completely. But after the air was blown off by argon, the anammox activity recovered completely (Strous *et al.*, 1997). It appears that anammox bacteria can survive under oxygen-limiting conditions by coexistence with DO consuming nitrifiers such that

nitrifiers oxidize ammonium to nitrite while consuming oxygen leading to reduced DO concentration.

1.4.3 Anammox performances in different systems

Many types of anammox reactors were tested for this novel nitrogen removal process. However, only the systems, which can retain biomass efficiently in the reactor, are suitable for anammox process. Most of these biomass-retaining reactors appear to have a characteristic of biofilm type reactor.

Strous *et al.* used one fixed-bed reactor and one fluidized-bed reactor for studying anammox reaction. For a fluidized-bed reactor treating synthetic waste, the ammonium removal rate was 0.8 kg NH_4^+-N/m^3/d and a nitrite removal efficiency of 99% whereas the combined ammonium and nitrite removal rate was 0.18 kg-N/kgVS/d.. This reactor was also used to treat sludge digester effluent with ammonium concentration of 1.1-2.1 kg NH_4^+-N/m^3 and nitrite of 0.07-0.84 kg NO_2^--N/m^3. The removal efficiencies of ammonium and nitrite were 82% and 99%, respectively, and the T-N removal rate was 1.5 kg-TN/m^3/d. In a study conducted in a fixed-bed reactor, the average removal efficiencies of ammonium and nitrite were 88% and 99%, respectively, whereas gas bubbles and clusters of grown-together flocs lead to washout (Strous *et al.*, 1997).

However, by using a gas-lift reactor treating ammonium and nitrite concentrations of 0.9 g/L of NH_4^+ and 1.1 g/L of NO_2^-, respectively the average total nitrogen removal efficiency was 88% and nitrite was completely removed (>99%). The maximum specific nitrogen removal rate was 1.15 g/g/d. Due to the nitrogen bubbles entrapped inside the granules or stuck to the surfaces, floatation of the anammox biomass occurred, which caused decrease in treatment efficiencies and accumulation of nitrite to approximately 200 mg/L. In order to avoid this phenomenon, a sequencing batch reactor (SBR) was as used for anammox process. During the operation, ammonium and nitrite concentrations both rose to 375 mg-N/L. The average total nitrogen removal efficiency was 78% and nitrite was completely consumed. The maximum specific nitrogen removal rate in SBR was 0.5 g/g/d,

lower than that of gas-lift reactor (Dapene-Mora *et al.*, 2004).

Sliekers *et al.* also examined the capacity of nitrogen conversion in a gas-lift reactor. Gas flow, consisting of 95% argon and 5% CO_2, was induced from the bottom and increased gradually to the maximum of 0.015 m/h (200 mL/min). In this research, T-N removal rate of 8.9 kg-TN/m^3/d was reported, along with ammonium and nitrite consumption rates of 3.8 kg NH_4^+-N/m^3/d and 5.1 kg NO_2^--N/m^3/d. This volumetric T-N conversion rate was higher than that of 4.8 kg-TN/m^3/d for fluidized bed reactor (van de Graaf *et al.*, 1996) and 7.0 kg-TN/m^3/d for sequencing batch reactor. It was reported that much higher T-N removal rate could be achieved for fluidized bed reactor with better biomass retention (Sliekers *et al.*, 2003).

Furukawa *et al.* used a 2.7-L lab scale reactor with a novel nonwoven matrix for biomass attachment to treat synthetic wastewater (Furukawa *et al.*, 2002; 2003). The reactor was seeded with sludge obtained from an industrial denitrification unit process. After 7 weeks, under autotrophic anoxic conditions, disappearance of ammonium and nitrite with production of low levels of nitrate was observed. Besides, an obviously red biomass growth developed which was dominant in the reactor. Ammonium and total nitrogen removal rates were 8.7 and 18.8 mg-N/L/h, respectively, at a HRT of 9 h. At influent ammonium and nitrite concentrations of approximately 250 mg-N/L, the T-N removal rate of 60% could be obtained with a hydraulic retention time of 7.5 hours. T-N and NH_4^+ volumetric removal rates were 40 and 20 mg-N/L/h, respectively. Subsequently, another larger reactor of 14-L volume was started-up with seed sludge from the smaller reactor and of the same packing material. After a fast and stable start-up, relatively high T-N removal rate of 300 mg/L/h could be achieved.

Fux *et al.* treated ammonium-rich wastewater by partial nitrification and anammox technology. The influent was digester supernatant from two different wastewater treatment plants (WWTPs) and anammox was carried out in a sequencing batch reactor (SBR) with suspended biomass inoculated with excess sludge from the Koelliken WWTP. Owing to

partial nitrification, the influent had a nitrite/ammonium ratio of 1.3. Over 90% of the nitrogen load was eliminated in the anammox reactor and the total nitrogen removal rates up to 2.4 kg T-N/m^3/d were achieved. Furthermore, the overall sludge production was negligible and a substantial amount of nitrate produced was denitrified by heterotrophs in the anammox reactor (Fux *et al.*, 2002).

High nitrogen (>87%) removal was reported in a wastewater treatment plant, located in Pistsea (UK), which is a nitrifying rotating disk reactor operated under oxygen limitating conditions. The ammonium concentration in the leachate was reduced from 349 mg/L to 3.5 mg/L, with a conversion rate of 5.8 g-N/m^2/d. In addition, specific anammox activity of sampled biomass in batch incubation test was calculated to be 5.0 nmol/mg protein/min (Schmid *et al.*, 2003).

1.4.4 Partial nitritation and anammox process

Compared to the traditional nitrogen removal process (nitrification and denitrification), the advantages of partitial nitritation include a lower oxygen requirement in nitrification step (25% less), and a lower or no organic carbon needed if denitrification or anammox is the following step (van Dongen *et al.*, 2001a; Shen *et al.*, 2003; Bernet *et al.*, 2005). According to this new concept, Single reactor High activity Ammonia Removal Over Nitrite (SHARON) process was developed by Delft University (Mulder *et al.*, 1997; Hellinga *et al.*, 1998).

There are many environmental factors affecting nitrification reaction and some of them are the basis of the development of partial nitrification process. The key point for partial nitrification is whether a stable nitrite accumulation can be obtained or not. Consequently, different strategies and appraoaches have been tested, including the control of temperature, hydraulic retention time, pH, dissolved oxygen and the presence of free ammonia (see in Table 1-1).

Temperature has different effects on the growth rate of AOB and NOB as shown in Table

1-1. Only at temperature above 25℃, AOB can effectively out-compete NOB. At suitable temperature control conditions, NOB can be selectively washed out with a low HRT and SRT. Selective competition of NOB is the main concept of SHARON process. The SHARON process operates at temperature above 26℃ without biomass retention, which means HRT is equal to the SRT. The effluent quality depends only on the growth rate of the bacteria involved and is independent of the influent concentrations (van Dongen *et al.*, 2001b). With high temperature and no sludge retention, the loading rate is set in such a way that the AOB can grow fast enough to stay in the reactor, while NOB is washed out.

Table 1- 1 Effect of the pH, temperature, free ammonia and nitrous acid on the nitrification process (Paredes *et al.*, 2007)

Factor	Effect
Temperature	
T>15℃	Ammonium oxidizers grow faster than nitrite oxidizers.
T=25℃	Ammonium oxidizers can out-compete nitrite oxidizers.
pH	
7.0-8.0	Optimum range for nitrification.
7.9-8.2	Optimum range for ammonium oxidizers (*Nitrosomomas*).
7.2-7.6	Optimum range for nitrite oxidizers (*Nitrobacter*).
Free NH_3 (mg/L)	
150	Inhibition of ammonium and nitrite oxidizers.
1.0-7.0	Inhibition of ammonium oxidizers and nitrite accumulation.
Long-term	Nitrite oxidizers (pure cultures of Nitrobacter and mixed cultures in biofilms) can be adapted to high free ammonia concentration (40 mg/L) and nitrite accumulation is reduced.
HNO_2 (mg/L)	
>2.8	Inhibition of ammonium and nitrite oxidizers

Considering the optimum pH ranges for nitrification, three types of effects of pH on the responsible bacteria have been understood: activation-deactivation of nitrifying bacteria;

nutritional effects, associated with the alkalinity; and inhibition through FA and FNA involving inorganic carbon compounds (Villaverde *et al.*, 1997). Activation-deactivation of nitrifying bacteria is linked to the binding of H or OH ions to the weak basic groups of the enzymes, blocking the active sites in a reversible way (Quinla *et al.*, 1984). Nutritional effects are mainly involved with the availability of the mineral carbon, which is required as a carbon source for the nitrifying autotrophic microorganisms. At a low pH, the predominant CO_2 species can be easily stripped from water. On the other hand, mineral carbon will mainly be present in the carbonate species at high pH levels, which can barely be assimilated. FA level increase at high pH levels while FNA concentration increases at a low pH.

Both FA and FNA can inhibit either AOB activity or NOB, but NOB is more sensitive than AOB to FA. However, the threshold level of FA at which the nitrite oxidation is inhibited increases with time. It was reported that pure cultures of *Nitrobacter* acclimatized to FA could tolerate high concentrations up to 40 mg/L of NH_3-N, while non-adapted cultures showed inhibition at concentrations as low as 3.5 mg/L NH_3-N (Wong-Chong *et al.*, 1978). Other studies showed that nitrite oxidizers could adapt to higher FA concentrations and, after a long time (6 to 12 months), the nitrite accumulation decreased while the nitrate concentration increased (Fux *et al.*, 2004).

Alkalinity is also an important factor in nitrification process. Partial nitrification can convert a fraction or entire ammonium into nitrite depending on the alkalinity of the wastewater. A molar ratio of 1:1 of ammonium/nitrite is considered suitable if the subsequent step is anammox process. Since the oxidation of 1 mole ammonium to nitrite consumes 2 moles of bicarbonate, and the reaction stops when the pH is lower than 6.5, a molar ratio of 1:1 of ammonium versus bicarbonate produces a transformation of approximately 50% of ammonium to nitrite and the residual remains as ammonium. So this molar ratio can be easily obtained by controlling the alkalinity in the wastewater. However, if the following step is denitrification, either full ammonium oxidation to nitrite is necessary, or an intermittent anaerobic step, by ceasing aeration and adding a carbon

source into the reactor is needed. For the first case, adequate alkalinity is crucial and another anammox reactor is required. On the other hand, the advantage is that there are no high requirements of alkalinity since the denitrificaiton step can produce bicarbonate (Brouwer *et al.*, 1996; Hellinga *et al.*, 1998; van Dongen *et al.*, 2001).

Control of DO concentration in the reactor is another possible way for improving nitrite accumulation. It is mainly based on the differences between the oxygen saturation coefficients of the Monod kinetics for ammonium oxidation and nitrite oxidation that are know to be 0.3 and 1.1 mg/L, respectively (Weismann, 1994). Another explanation for inthibition of nitrite oxidation by a low DO concentration is due to the accumulation of hydroxylamine, which is an intermediate production of the ammonium oxidation. In general, obligatory AOB gain their energy by oxidizing ammonium to nitrite in a two-step reaction with hydroxylamine as the intermediate. In the first step, oxidation of ammonium is catalyzed by ammonia monooxygenase, whereas the second step is the oxidation of hydroxylamine catalyzed by hydroxylamine oxidoreductase. Under low DO concentration and high ammonium level, hydroxylamine can be accumulated. Hydroxylamine can result in an inhibition, although nitrite oxidizers, at values of around 250 μM to over 2000 μM, inhibit the AOB activity. However, hydroxylamine is typically ignored in nitrification processes due to an implicit assumption that it will be available in significant levels in wastewater treatment (Yang *et al.*, 1992).

A number of investigations for partial nitrification control through DO have been carried out, including suspended and biofilm reactor systems, shown in Table 1-2. In suspended growth biomass system case, limited oxygen was crucial to obtain complete and stable ammonium conversion to nitrite, independent of the sludge age. However, the sludge age became the key parameter for partial nitrification without oxygen limitation. The results of biofilm system were similar to those of suspended biomass. The DO concentration gradient also selectively limited the nitrite oxidizers activity.

Table 1- 2 Effects of dissolved oxygen concentration on the nitrification processes (Paredes *et al.*, 2007)

System	DO (mg/L)	Effect
Suspended growth	0.5	Inhibition of nitrite oxidation and its accumulation.
	6.0	Full nitrification.
Activated sludge	<0.5	Nitrite and ammonium accumulation.
	0.7	Nitrite accumulation up to 67% of the applied NH_4^+.
	1.0	80% oxidation of NH_4^+, 80% as NO_2^-.
	1.4	99% oxidation of NH_4^+, 70% as NO_2^-.
	>1.7	Full nitrification.
	2.4	99% oxidation of the applied NH_4^+, 10% as NO_2^-.
Biofilm airlift reactor	1.0	Stable and 100% nitrite accumulation.
	<1.0	Low ammonium conversion and low NO_2^- and NO_3^- accumulation.
	1.5	50% of ammonium conversion to nitrite.
	>2.5	Full nitrification, NH_4^+ oxidation depended on applied ammonium load.
Biological aerated filter	2.0-5.0	Nitrite accumulation up 60% of total ammnonia conversion.
Completely stirred biofilm reactor	0.5	Stable nitrite accumulation (90%) and 100% ammonium removal
	>0.5	Increasing dissolved oxygen increases nitrate concentration in the effluent. A further reduction produces nitrite accumulation again
Moving bed biofilm reactor	3.0	50% of ammonium conversion to nitrite. After 11 months full nitrification took place.

High salinity concentrations have negative effect on organic carbon, nitrogen and phosphorus removal in wastewater treatment. Moussa *et al.* concluded that ammonia oxidizers were more sensitive to short- and long-term salt stress and therefore leading to inhibition of nitrification process (Moussa *et al.*, 2006). In saline environments, the

adaptation of a microbial cenosis to high salinity values has a significant effect on their species diversity. Using oligonucleotide probes in the case of AOB, *Nitrosomonas europaea* was detected in higher numbers in saline environments compared to the levels in freshwater system (Tal *et al.*, 2003; Moussa *et al.*, 2006). High salt concentrations strongly inhibited the activity of higher organisms such as protozoa. Furthermore, high salt concentrations lead to a satisfactory settling capability (Moussa *et al.*, 2006).

1.5 Advanced nitrogen removal processes

1.5.1 Aerobic deammonification

Aerobic deammonification was discovered at Hannover Uviversity when treating the landfill leachate liquor in a rotating biological contactor (Hipper *et al.*, 1997). Later, similar experiments for landfill leachate treatment were conducted that proved the occurrence of aerobic deammonification phenomenon (Siegrist *et al.*, 1998b). The nitrification rate and T-N removal rate increased by 80% and 150%, respectively, when the O_2 pressure increased from 19% to 28%; the nitrification rate and T-N removal rate were reduced by about 30-40% if the discs rotating speed decreased from 1 rpm to 0.66 rpm (similar to decrease the DO concentration). It was deduced that T-N removal might be closely associated with the nitrification process (Siegrist *et al.*, 1998a). The essence of aerobic deammonification is the biological nitrogen removal with inorganic materials as the electron donor.

Fig. 1-5 shows the postulated reaction mechanism of synergetic nitrification-denitrification model. Based on this model, aerobic deammonification process is realized by the synergetic effect of AOB and Anammox bacteria. *Nitrosomonas* is mainly distributed on the surface of biofilm, and could get enough oxygen to oxidize ammonium to nitrite. Then, nitrite and ammonium diffuse into the interior of biofilm, in which ammonium combines with nitrite to dinitrogen gas by anammox reaction under anoxic conditions (Koch *et al.*, 2000).

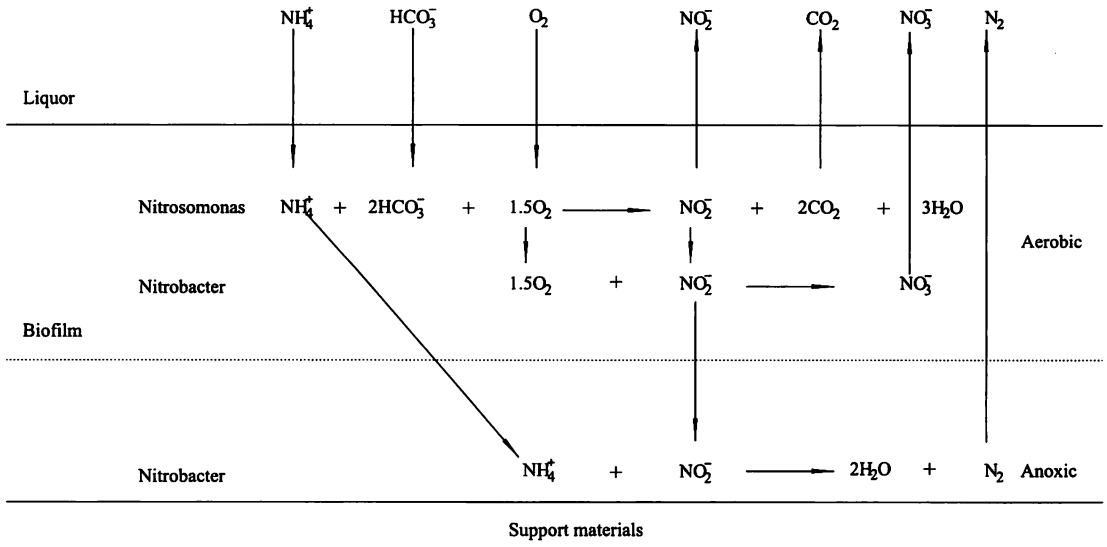


Fig. 1- 5 Synergetic model of nitrification and denitrification

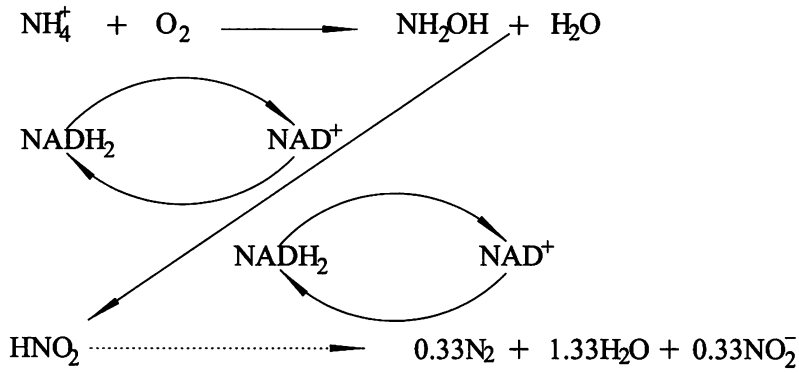


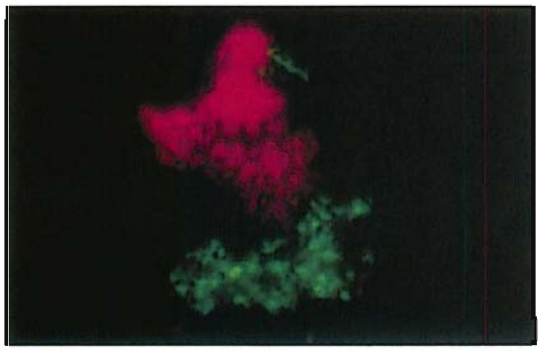
Fig. 1- 6 Independent model of nitrification and denitrification process

Another independent model for nitrification and denitrification process was postulated mainly by ammonium oxidizing bacteria. According to this model, *Nitrosomonas* on the biofilm surface oxidizes ammonium to nitrite with oxygen as the electron acceptor. Then the diffused nitrite is reduced to dinitrogen by $NADH_2$ in the bacteria as the electron donor under anoxic conditions inside the biofilm as shown in Fig. 1-6. In this reaction, hydroxylamine is the critical intermediate, supplying electron donor to $NADH_2$. Other studies showed that *Nitrosomonas* had anammox activity, which could convert ammonium and nitrite to dinitrogen under anoxic conditions. However, the anammox activity of *Nitrosomonas* is very low, only about 1/10 of *Brocadia anammoxidans*. Detailed information is available in the publication by Siegrist *et al.*, (1998b).

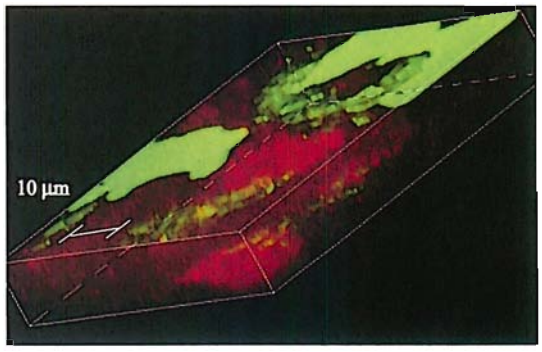
1.5.2 CANON and SNAP processes

CANON (Completely Autotrophic Nitrogen removal Over Nitrite) process was first developed at Delft University as a new type of nitrogen removal process (Sliekers *et al.*, 2002). This process utilizes AOB for oxidizing ammonium to nitrite and then anammox bacteria convert the rest of ammonium and nitrite to dinitrogen gas. Since both AOB and anammox bacteria belong to autotrophic group, CANON process does not need external organic carbon. However, oxygen is required for ammonium oxidation to nitrite and can inhibit the activities of anammox bacteria . Therefore the CANON process should be operated under low dissolved oxygen concentration conditions. The stoichiometric relation of CANON process is showed in Eq. 1-8. SBR and gas-lift reactors were used for examining CANON process and satisfactory results were obtained (Sliekers *et al.*, 2002; Sliekers *et al.*, 2003). SNAP (Single stage Nitrogen removal using Anammox and Partial nitritation) process was developed at Kumamoto University as a novel technique for nitrogen removal (Furukawa *et al.*, 2006). SNAP process, having the same reaction principle as that of Canon process, was tested using a novel acrylic resin material (Biofix, NET Co. Ltd., Japan) as the support material to retain biomass. Compared with CANON process, SNAP process could be established in a stable form at a relatively higher loading rate of 0.96 kg-N/m^3/d versus 0.13 kg-N/m^3/d of CANON process in SBR; while the T-N removal efficiency of SNAP process could reach 78.5% versus 48.9% of CANON process in SBR (Lieu *et al.*, 2005; Slierkers *et al.*, 2002). The superior support material (Biofix, NET Co. Ltd., Japan) of SNAP process could be attributed to the satisfactory removal performance since the support material had the superior capacity of biomass retaining. In the two processes, it was found that the anammox bacteria were enclosed by the NOB, showed in Fig. 1-7, and they could successfully co-operate with each other to removal ammonium-rich wastewater. DO, pH, and temperature controlling is necessary to achieve satisfactory performances in two processes.

$$NH_3 + 0.85O_2 \rightarrow 0.44N_2 + 0.11NO_3^- + 1.43H_2O + 0.14H^+ \quad (1\text{-}8)$$



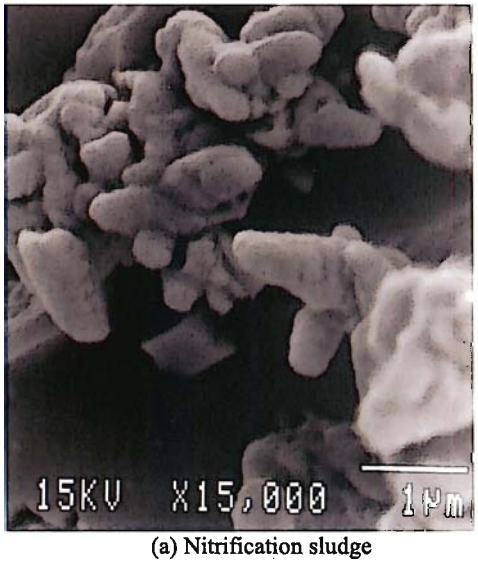
(a) Fish observation of CANON sludge



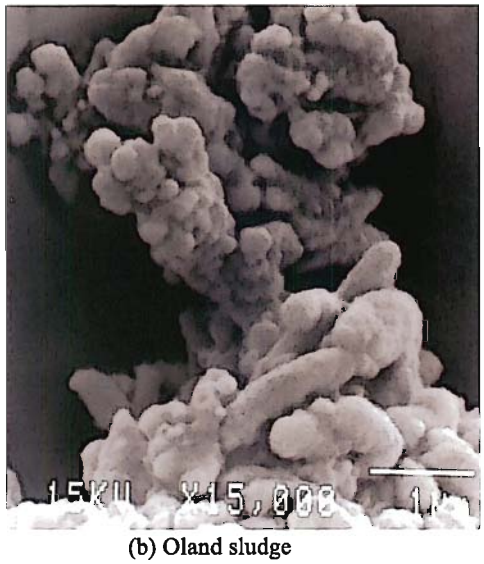
(b) CLSM observation of SNAP sludge

Fig. 1- 7 Observation comparison of CANON and SNAP sludge

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



(a) Nitrification sludge



(b) Oland sludge

Fig. 1- 8 Comparing SEM observation of nitrification and OLAND sludge (Kuai *et al.*, 1998)

OLAND process was first developed at Ghent University in 1998 and it was found that *Nitrosomonas eutropha* could utilize hydrogen or ammonia as electron donor for nitrite reduction (Bock *et al.*, 1995). Kuai *et al.* considered that autotrophic AOB could partially oxidize ammonium to nitrite with oxygen as electron acceptor and reduce nitrite to dinitrogen gas with ammonium as electron donor (Kuai *et al.*, 1998). It was reported that OLAND process could save 63% oxygen and all the organic carbon comparing with traditional nitrification-denitrification process. Low growth rate of OLAND biomass (2 mg/g/d) appears to be the most serious challenge in OLAND process, which is currently in

the laboratory research stage. OLAND sludge showed white color and the VSS/TSS ratio was similar to that of nitrification sludge. However, the activity of OLAND sludge was 1/12 to 1/3 times lower than the nitrification sludge. The OLAND sludge did not show much difference compared to nitrification sludge according to the SEM observation shown in Fig. 1-8.

1.6 Research objectives of this study

The main objectives of this study were to develop attached-biomass in Anammox process for nitrogen removal by using novel but effective types of support materials. The main objectives are listed below:

1. To apply novel acrylic fiber support material (Biofill, NET Co., Ltd., Japan) for synthetic nitrogen rich wastewater treatment by anammox process and to investigate the operational performance of anammox system and the capacity of the support material for retaining anammox biomass.

2. To compare anammox performance of two types of support materials, nonwoven and Biofix, under different operational conditions by:
   - comparing the biomass characteristics of two reactors in light of extracellular polymeric substances (EPS) analysis and scanning electron microscopic (SEM) observation; and
   - determining the main bacterial population in two reactors by 16S rDNA approach.

3. To combine partial nitritation and anammox processes for practical anaerobic sludge digestion liquor treatment by:
   - examining the optimum operational conditions for organic carbon and nitrogen removal simultaneously; and
   - studying the effect of free ammonia (FA) and free nitrous acid (FNA) on the

partial nitritation process performance.

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Chapter 2 Anammox treatment potential in an up-flow column reactor using a novel acrylic fiber biomass carrier

2.1 Introduction

Recently, a newly discovered biologically mediated segment of the nitrogen cycle consisting of anaerobic ammonium oxidation (Mulder et al, 1995; Van der Graaf *et al.*, 1995; Kuenen and Jetten, 2001) has been of increasing interest as a potentially useful means of nitrogen abatement. In this reaction, ammonium serves as the electron donor using equal molar amounts of nitrite as the electron acceptor directly resulting in production of dinitrogen gas. The potential existence of microorganisms capable of this energetically favorable reaction had been predicted three decades ago by Broda (1977). Only several years ago, an organism capable of the process was identified for the first time as a deeply branching *planctomycete* (Strous *et al.*, 1999) and the stoichiometry of the combined catabolic and anabolic reactions was determined by Strous *et al.* (1998) to be:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (2\text{-}1)$$

As shown in Eq. (2-1), a relatively small amount of nitrate is produced, evidently from the oxidation of nitrite, by which electron equivalents are generated for the reduction of inorganic carbon into biomass (Furukawa *et al.*, 2003).

However, the growth rate of anammox bacteria is extremely slow with a doubling time of about two weeks, thus the anammox process needs a reactor system with highly efficient biomass retention. It is therefore of importance for anammox process development to find a suitable biomass support material to retain the bacteria as efficiently as possible.

The purpose of this chapter is to investigate the suitability of a novel acrylic fiber biomass carrier (Biofill) for anammox study as a packing material in an up-flow column reactor.

2.2 Material and Methods

2.2.1 Experimental setup

The reactor used in this research was constructed of polymethyl methacrylate having an inner diameter of 140 cm and height to the effluent port of 985 cm, shown in Fig. 2-1. The liquid volume was 17.3 L and the reaction zone, including influent distribution and biomass retention sections, was 15.2 L. Experiments were conducted at 35℃, controlled thermostatically using a hot water jacket and darkness was maintained with a black-vinyl sheet enclosure. The feeding solution was introduced from the bottom in an up-flow mode by a peristaltic pump (Eyela Co., Ltd., Tokyo). Dinitrogen gas purging was used to remove dissolved oxygen (DO) from the influent. The DO concentrations in the influent were consistently maintained below 1.0 mg/L. Acrylic fiber biomass carriers (Biofill: NET Co., Ltd., Kawanishi, Japan) shown in Fig. 2-2 were used as biomass carrier, and 700 pieces of Biofill were placed in the reaction zone. One Biofill was approximately 1.07 g and the average volume of one Biofill was about 6.0 cm^3 with a specific surface area was 146.5 m^2/m^3.

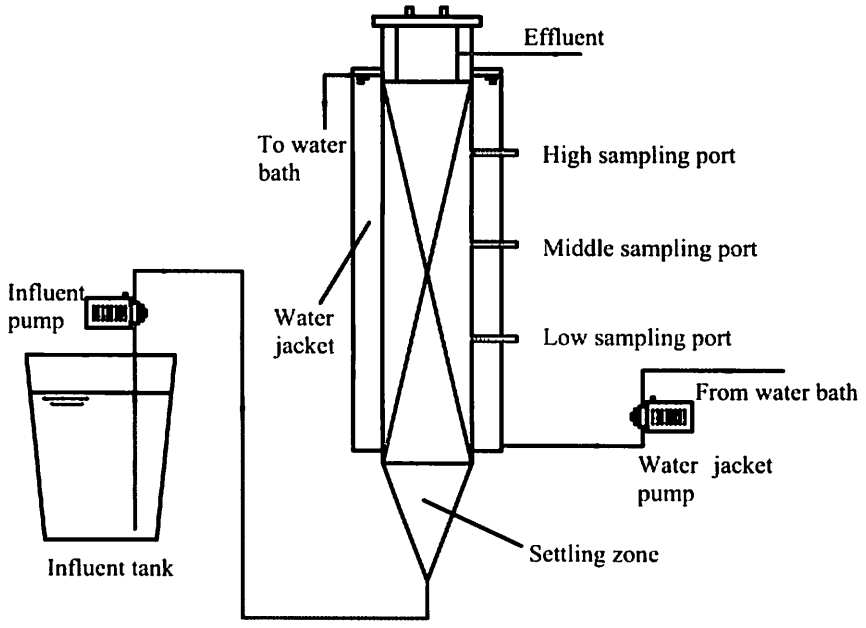


Fig. 2- 1 Schematic diagram of the up-flow anammox column reactor

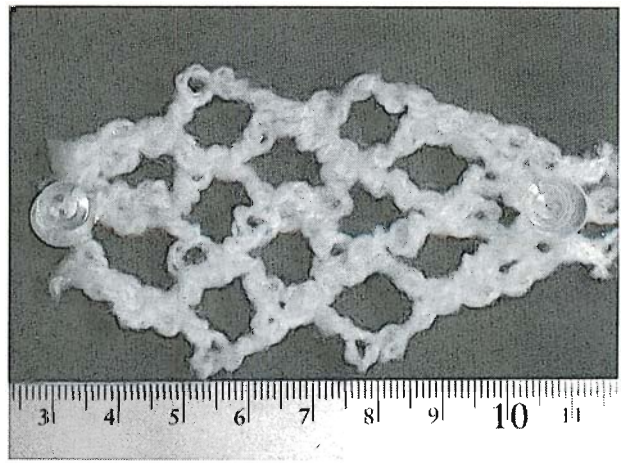


Fig. 2- 2 Photo of the acrylic fiber biomass carrier (Biofill)

2.2.2 Synthetic wastewater

Table 2- 1 Composition of synthetic wastewater

Compound	Concentration (mg/L)
$(NH_4)_2SO_4$	20-200 (as N)
$NaNO_2$	20-200 (as N)
$KHCO_3$	125
KH_2PO_4	54
$FeSO_4 \cdot 7H_2O$	9
EDTA · 2Na	5
Trace element solution#	1.0 mL/L
Mine salt solution#	2.0 mL/L

Table 2- 2 Trace element and mine salt solution compositions

Trace element solution compositions		Mine salt solution compositions	
Compound	Concentration (mg/L)	Compound	Concentration (mg/L)
$CoCl_2 \cdot 6H_2O$	0.24	$CaCl_2$	700
$CuSO_4 \cdot 5H_2O$	0.25	KCl	700
H_3BO_3	0.014	MgSO4	500
$MnCl_2 \cdot 4H_2O$	0.99	NaCl	500
$Na_2MoO_4 \cdot 2H_2O$	0.22		
Na_2SeO_4	0.11		
$NiCl_2 \cdot 6H_2O$	0.19		
$ZnSO_4 \cdot 7H_2O$	0.43		

The reactor was fed with synthetic wastewater, whose composition was shown in Table 2-1. In this research, $(NH_4)_2SO_4$ and $NaNO_2$ were used as the ammonium and nitrite sources, in

equal molar amounts. The other influent components are shown in Table 2-2. All chemicals were analytical grade.

2.2.3 Operational conditions

For starting up of the reactor, seed sludge was taken from another upflow column reactor using nonwoven as biomass carriers (Furukawa *et al.*, 2003). 6.1 g anammox sludge, which was equivalent to about 400 mg/L of MLSS in the reactor, was seeded.

Table 2- 3 Operational conditions

	A-I	A-II	A-III	A-IV	A-V	B-I	B-II	B-III	B-IV	B-V
Days	1-7	8-16	17-23	24-53	54-109	110-128	129-141	142-150	151-198	199-261
Flow rates (L/h)	0.64	0.64	0.64	0.64	0.64	1.00	1.00	1.25	1.25	1.92
HRT (h)	23.4	23.4	23.4	23.4	23.4	15.0	15.0	12.0	12.0	7.81
T-N (mg/L)	40.0	100	140	180	240	240	240	300	400	400
NLR (kg-T-N/m^3/d)	0.04	0.10	0.16	0.22	0.27	0.41	0.48	0.60	0.80	1.20

Table 2-3 (continued)

	C-I	C-II	C-III	C-IV	C-V	C-VI
Days	262-269	270-277	278-308	309-347	348-364	365-392
Flow rates (L/h)	1.92	1.92	1.92	2.55	3.40	4.25
HRT (h)	7.81	7.81	7.81	5.88	4.41	3.53
T-N (mg/L)	300	200	300	300	300	300
NLR (kg-TN/m^3/d)	0.90	0.60	0.90	1.20	1.60	2.00

In the first phase (A), nitrogen loading rate (NLR) was increased stepwisely from 0.04 kg-TN/m^3/d to 0.27 kg-TN/m^3/d by increasing the influent concentrations as shown in Table 2-3. During the second phase (B), experimental strategies of decreasing the hydraulic retention time (HRT) and increasing influent concentrations were employed to increases in NLR for investigation of the treatment potential of Biofill in an anammox application. In phase B, the NLR was increased to 1.2 kg-TN/m^3/d, while influent ammonium and nitrite concentrations finally reached to 200 mg/L. In the phase C, the NLR was decreased to 0.6 kg-TN/m^3/d via 0.9 kg-TN/m^3/d due to inferior performance at the end of phase B. After

adaptation, NLR was once again increased in order to evaluate the tolerance and recovery capacity to NLR shock loading.

2.2.4 Analytical methods

Ammonium was quantified based on the indophenol reaction with o-pheniphenole (Kanda, 1995). Nitrite was quantified by the colorimetric method (APHA, 1991) and nitrate by the ultraviolet spectrophotometric screening method (APHA, 1991). DO was measured using a DO meter (HORIBA, pH/DO meter D-55).

For scanning electron microscopy (SEM), samples were first washed in a 0.1 M phosphate buffer solution (pH 7.4) for 5 min each time. Then samples were hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the buffer solution. Next, samples were washed in the buffer solution three times for 10 min and then fixed for 90 min in a 1.0% OsO_4 solution prepared with the buffer solution. After washing samples three times for 10 min each in the buffer solution, they were dewatered for 10 min each in serially graded solutions of ethanol at concentrations of 10, 30, 50, 70, 90, 95, 99, and 99.5%. SEM observations were conducted using a scanning electron microscope (JEOL, JSM-5310LV).

DGGE using 16S rDNA was performed for bacterial community analysis. An amount of sample taken from one support material was centrifuged at 12,000×g for 5 min and these harvested cells were used for the extraction of DNA. DNA extraction and purification were performed by UltraClean Soil DNA Isolation Kit (Mo Bio, West Carlsbad, CA, USA). PCR reaction mixture was prepared with 2 μl of the extracted DNA, 10 μl of Taq-& Go ready mix (Qbiogene, Irvine, CA, USA), 1 μl of each 25μM forward and reverse primer solution. The forward primers were GM-5F with GC-clamp (5'-CGCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGGGGGCCTACGGGAGGCTGCAG-3') and Akuf1 (5'-CCTA CGGGAGGCTGCAG-3') (made by our laboratory); the reverse primers were DS907R (Weisburg *et al.*, 1991) (5'-CCCCGTCAATTCCTTTGAGTTT-3') and Akur1 (5'-CCCCG TCAATTCTTTTGAGTTT-3') (made by our laboratory). PCR was performed according to hot-start (Chou *et al.*, 1992) and touch-down (Thompson *et al.*, 1991) methods. The PCR

tubes were set in PC708 thermal cycler (ASTEC, Fukuoka) and kept at 94℃ for 2 min, then the temperature was maintained at 80℃ and 2 μl of the above extracted DNA was added as a template. The reaction was continued as follows: annealing (65℃ for 30 sec.), elongation (72℃ for 1.5 min) and denaturation (94℃ for 20 sec.); the PCR cycle was repeated with annealing temperature being lowered to 55℃ in 1.0℃ increments (total of 19 cycles) and 10 cycles were continued at 55℃ for annealing. Amplified DNA was verified by an agarose gel electrophoresis using Mupid S electrophoresis system (Advance, Tokyo). DGGE gel was made by SJ-1060GF gradient gel maker (ATTO, Tokyo) with 40–50% formamide concentration gradient. Electrophoresis was performed using AE-6290E system (ATTO) at 200 V for 6 h according to the manufacturer's protocol. After electrophoresis, the gel was stained with SYBR GREEN I for 30 min and DNA bands were verified using EM-20E UV-transilluminator (UVP, Upland, CA, USA). Distinct DNA bands were cut out from the gel using sterilized plastic pipette tips. Each gel section was placed into a PCR tube and the DNA fragment was reamplified. The cycle sequence was performed with the same forward primers and DTCS Quick Start Kit (Beckman coulter, USA), and temperature conditions were as follows: 94℃(20 sec.) -57℃(30 sec.) -72℃ (1.5 min)×35 cycles. The products were purified by Ultra Clean PCR Clean-up Kit (Mo Bio). DNA sequencing was performed using DTCS Quick Start Kit and CEQ8000 (Beckman Coulter, Fullerton, CA, USA) in accordance with manufacturer's protocol. The sequences of DNA bands were compared with BLAST DNA database. The sequence alignment and phylogenetic analysis were performed using the ClustalW software (Thompson *et al.*, 1991).

2.3 Results and discussions

2.3.1 Phase A: Start up

In the first period of this phase, operation was focused on successful startup. The influent flow rate was set at a low level of 0.64 L/h (HRT of 23.4 h) and the initial influent concentrations of ammonium and nitrite were both 20 mg/L. During the first 23 days, the influent nitrite levels were increased stepwisely to 70 mg/L and the T-N removal

efficiencies of more than 80% were constantly observed. The results of phase A are summarized in Fig. 2-3. Before the influent nitrite concentration increased to 120 mg/L, the nitrite removal efficiencies were stable and almost over 90%. In this stage, the average reaction ratio of NH_4-N: NO_2-N was 1:1.11, which was a little lower than the stoichiometric ratio of 1.32 determined by others (Strous *et al.*, 1998). The influent nitrite was then increased to 120 mg/L for a NLR of 0.27 kg-TN/m^3/d near the end of phase A, and the ammonium, nitrite and T-N removal efficiencies were 73%, 87% and 68%, respectively.

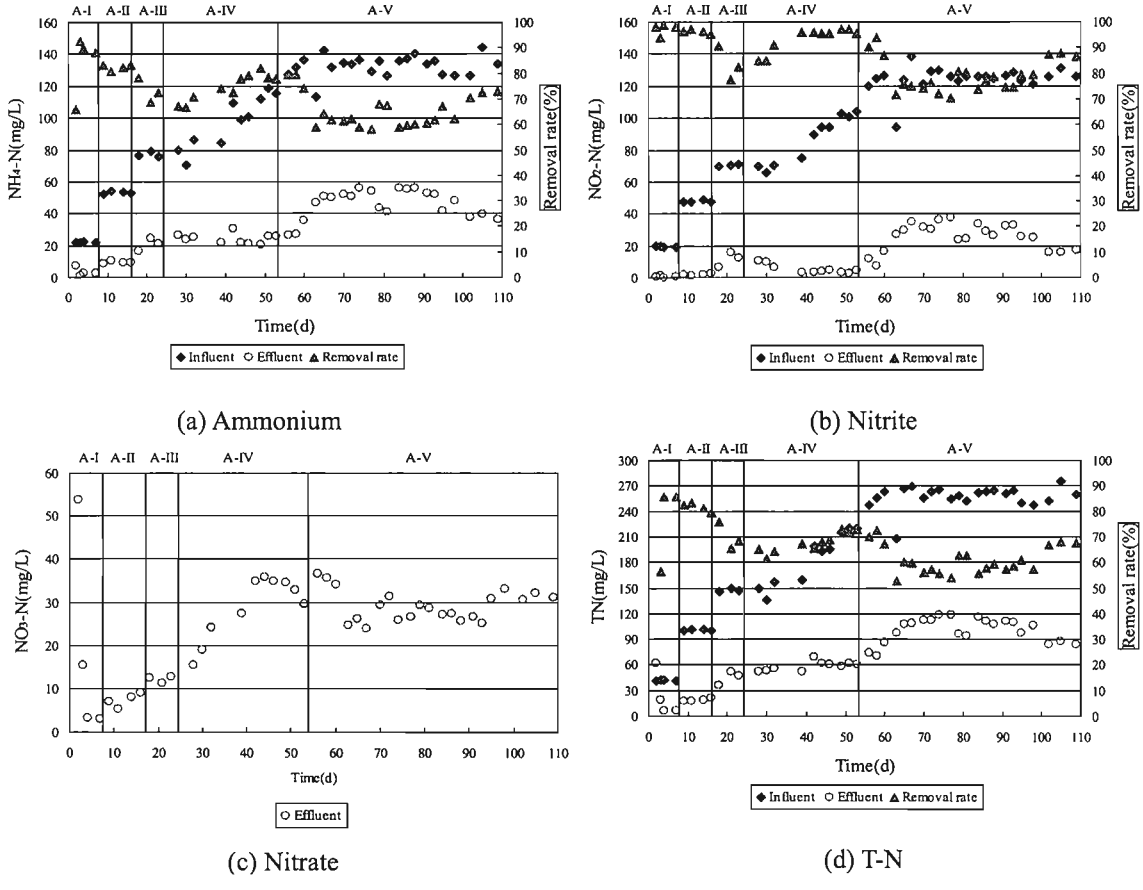
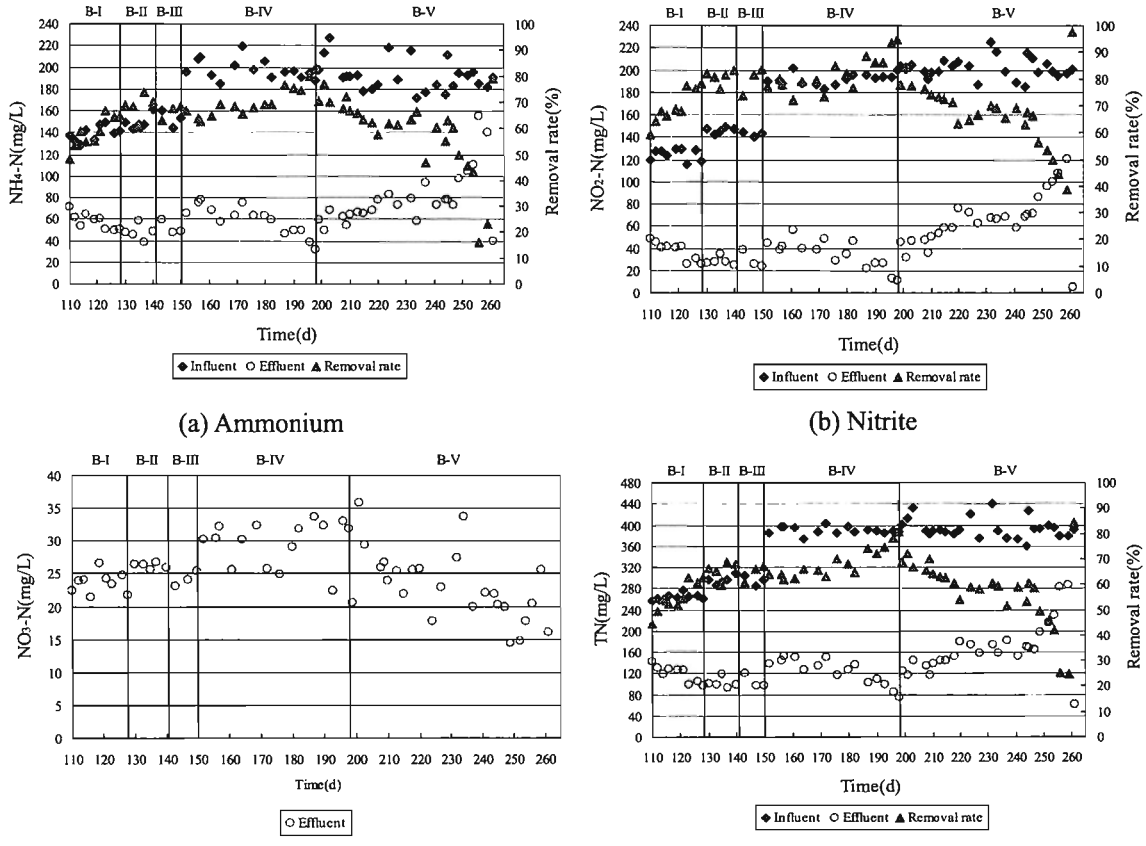


Fig. 2- 3 Time courses of nitrogen concentrations during phase A

2.3.2 Phase B: Effects of NLR

After successfully establishing of the anammox process, experiments were conducted to evaluate tolerance capacity to shock loading. In this phase, the NLR was increased by adjusting influent concentrations and HRT, alternatively. During the first 40 days of this

period, the NLR had increased to 0.6 kg-TN/m^3/d with a HRT of 12 h and influent T-N of 300 mg/L. When the influent T-N concentration was increased to 400 mg-N/L for a NLR of 0.8 kg-TN/m^3/d, it took another 40 days for the adaptation. Once the process adapted to high NLR, the ammonium, nitrite and T-N removal efficiencies reached 83%, 95% and 81%, respectively. However, the effluent nitrogen concentration fluctuated during the following two months after decrease in HRT to 8.0 h (NLR of 1.2 kg-TN/m^3/d). The anammox activity gradually became inhibited due to high nitrite concentrations in the reactor with the removal efficiencies of ammonium, nitrite and T-N decreasing to 23%, 39% and 24%, respectively. But the removal efficiencies recovered to 79%, 97% and 84%, respectively, after one day stopping of influent. These results suggest that this kind of inhibition is reversible and anammox activity can recover given enough time for nutrients degradation.



(a) Ammonium (b) Nitrite

(c) Nitrate (d) T-N

Fig. 2- 4 Time courses of nitrogen concentrations during phase B

Concentration profiles of nitrogenous compounds in the reactor at a NLR of 0.8

kg-TN/m^3/d are shown in Fig. 2-5. From these results, it was found that most nitrogen was removed at the inlet part of the reactor. The ammonium, nitrite and T-N removal efficiencies at the inlet part of the reactor were 70%, 83% and 70%, respectively, while the removal rates in the effluent were 75%, 86% and 72%, respectively. Consequently, it is apparent that most of the anammox activity was concentrated in the inlet part, which could be attributed to the high filling density and good absorption capacity of the support material.

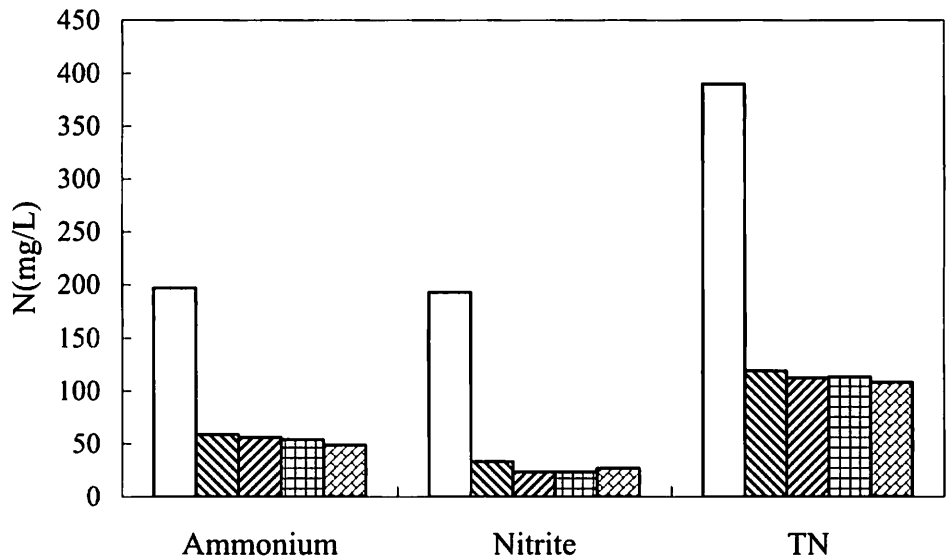


Fig. 2- 5 Changes in nitrogen concentrations at different height level (0.8 kg-TN/m^3/d)
□ Influent, ▨ Low port, ▨ Middle port, ▦ High port, ▨ Effluent

2.3.3 Phase C: Removal capacity

Due to inferior performance in the previous phase, the NLR was reduced to 0.9 kg-TN/m^3/d at the beginning of phase C by decreasing influent ammonium and nitrite concentrations from 200 mg/L to 150 mg/L. And the NLR decreased to 0.9 kg-TN/m^3/d from 1.2 kg-TN/m^3/d, T-N removal efficiency increased to 34%, only 10% higher than that of 1.2 kg-TN/m^3/d, not as expected. So the NLR was decreased to 0.6 kg-TN/m^3/d by further reducing the influent ammonium and nitrite to 100 mg/L each. After another seven days of operation, the anammox activity began to improve greatly. The NLR was restored to 0.9 kg-TN/m^3/d, and the removal efficiencies of ammonium, nitrite and T-N increased to

78%, 90% and 71%, respectively. Subsequently, the influent concentrations were kept consistent and NLRs were increased by decreasing HRT. On the basis of accumulation of sufficient anammox biomass, the NLRs increased again to 1.20 kg-TN/m^3/d and then to 1.6 kg-TN/m^3/d in a short time, with maintaining nitrite removal efficiencies as high as 95% and 83%, respectively. In addition, the nitrogen removal efficiencies were not fluctuated after the NLR was increased to 2.0 kg-TN/m^3/d. During the following 28 days of operation for this NLR, the ammonium, nitrite and T-N removal efficiencies as high as 74%, 93% and 73%, were obtained, respectively.

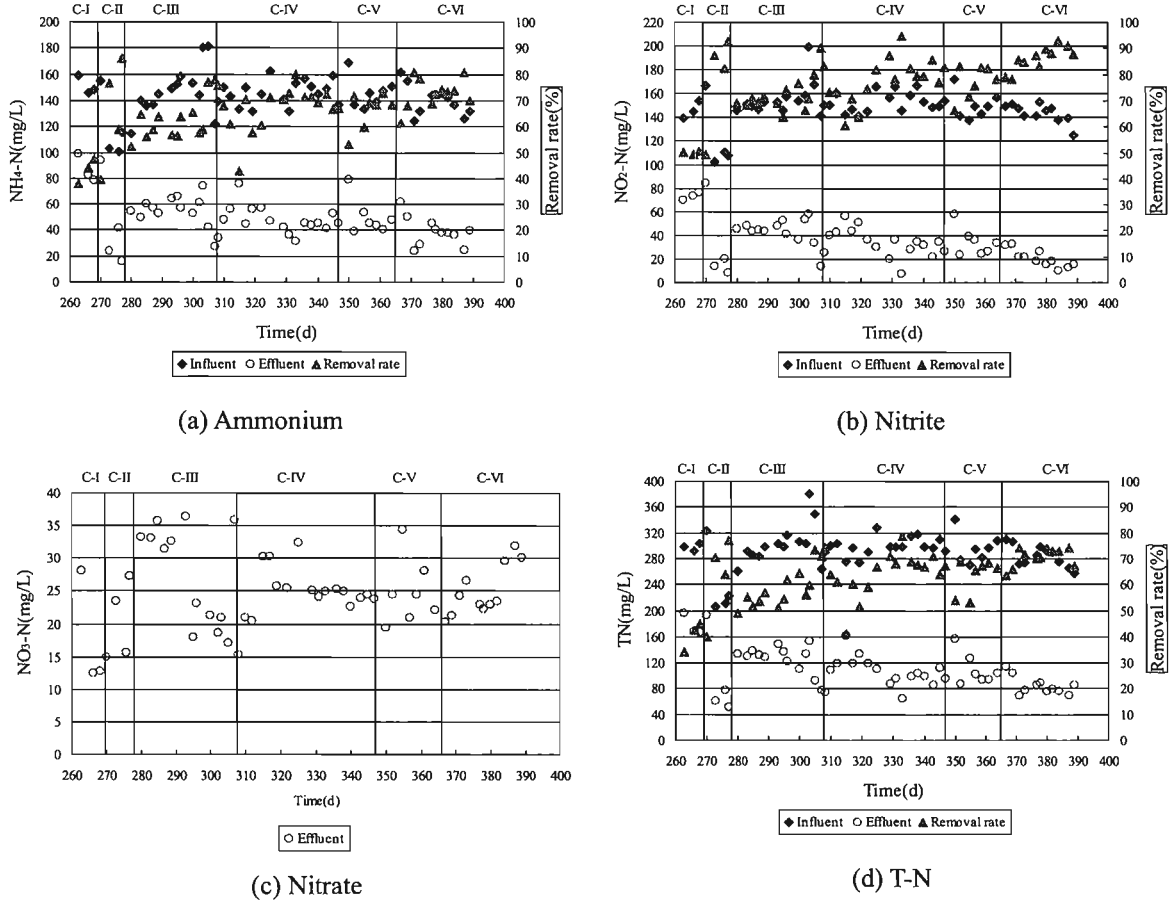


Fig. 2-6 Time courses of nitrogen concentrations during phase C

2.3.4 Temperature effect on anammox performances

After achieving stable T-N removal efficiencies at a high NLR of 2.0 kg-TN/m^3/d, the effects of temperature were evaluated by varying reactor temperatures from 15.3℃ to 33.0℃. Figure 2-7 shows the changes in nitrite removal rates at different height levels under various operational temperatures. After wastewater passed the low and middle parts

of the reactor, almost 50% and 70% of the nitrite were removed, respectively, and the nitrite removal efficiencies were kept fairly stable, especially in the low part of the reactor. This could be attributed to the thick layer of anammox biomass accumulated in the bottom and middle parts of the reactor. It was suggested that high enough anammox biomass concentration would reduce the effect of temperature and benefit the stable nitrogen removals. With decreasing temperature, the anammox activity was not greatly affected with a high nitrite removal efficiency of 89% at 26.2℃. This nitrite removal efficiency was only 5% lower than that obtained at 33.0℃. When reactor was operated without temperature control, the nitrite removal efficiency decreased to 60% at ambient temperature of 15.3℃. However, the nitrite removal efficiency recovered to 80% within one day after increasing reactor temperature to 23℃. This result indicated that anammox activity can quickly recover from low operational temperature conditions. Furthermore, similar results were observed for the removal profiles of ammonium and T-N.

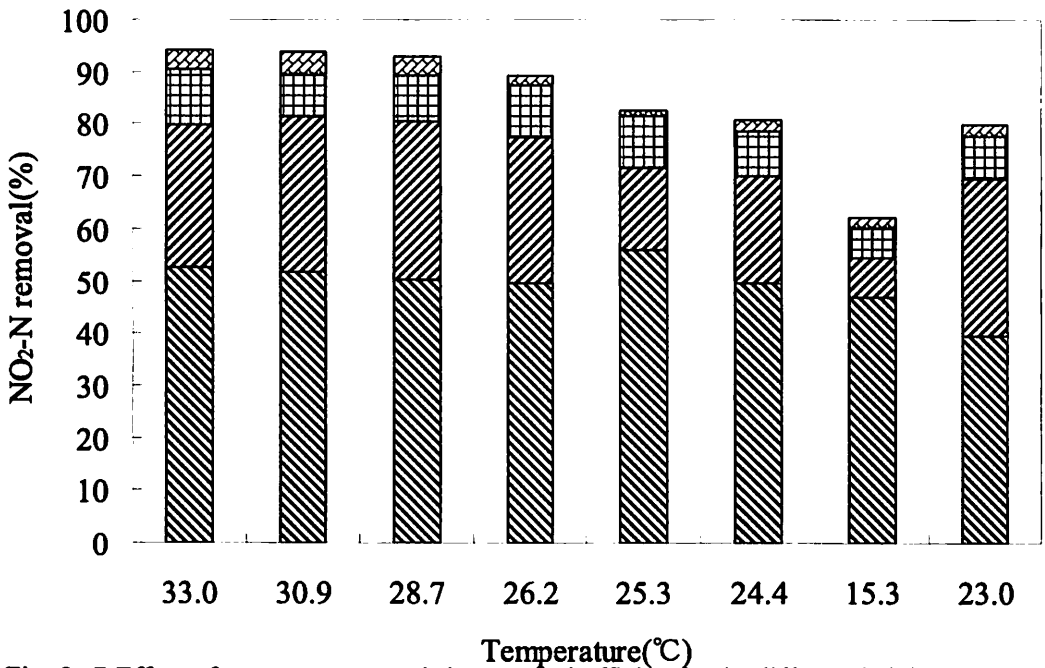


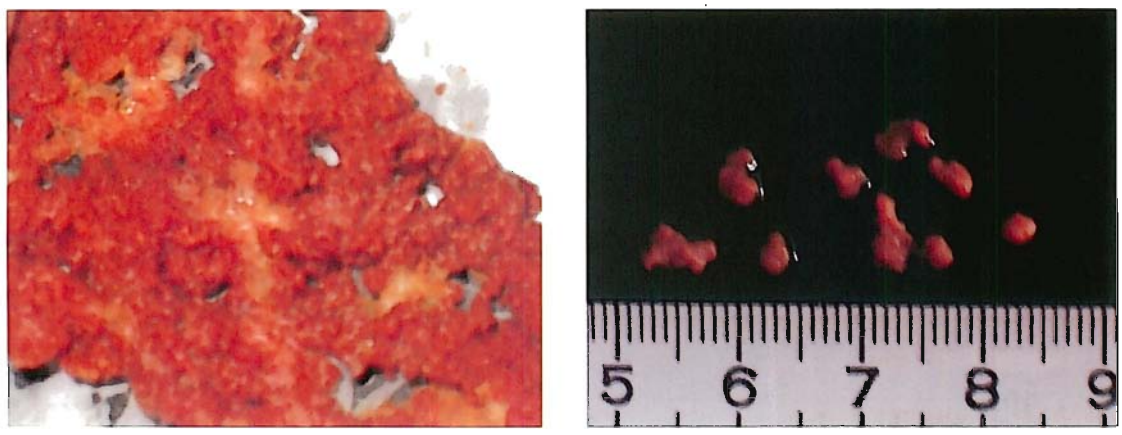
Fig. 2- 7 Effect of temperature on nitrite removal efficiencies in different height levels under NLR of 2.0 kg-TN/m^3/d

Low port, Middle port, High port, Effluent

For Fig. 2-5 (on day 190), the slow growth rate of anammox biomass would make most anammox microorganism concentrating at the bottom part since seeding and long HRT of

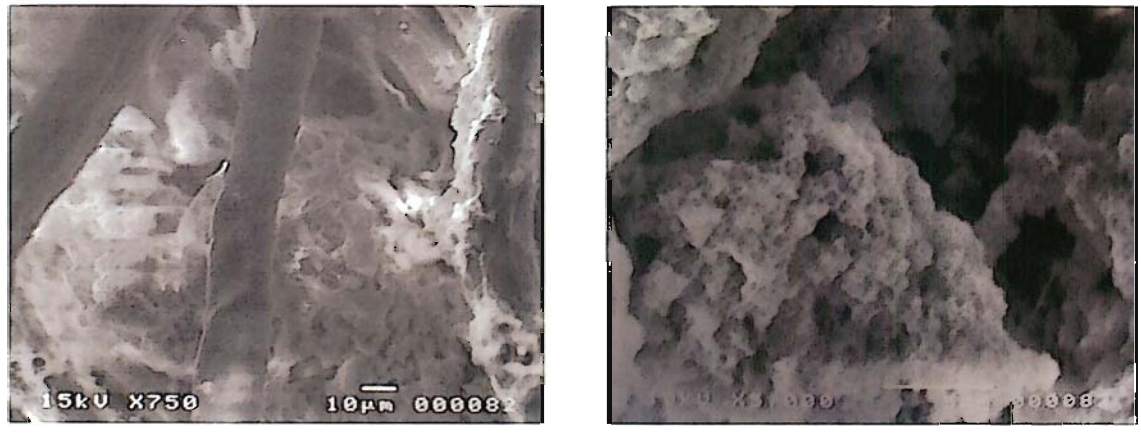
12 h will also benefit anammox bacteria of bottom part for degrading nutrients. But results in Fig. 2-7 were determined on day 390. It was possible for anammox biomass to expand inside the reactor, but the short HRT of 3.53 h made it difficult to degrade most nitrogen only by bottom part. Therefore, the extended anammox biomass in middle part worked and caused the difference between Fig. 2-5 and Fig. 2-7.

2.3.5 Anammox sludge



(a) (b)

Fig. 2- 8 Anammox sludge immobilized on Biofill



(a) (b)

Fig. 2- 9 SEM of anammox sludge attached on Biofill

Fig. 2-8 shows the attached anammox sludge on Biofill. Compared with the original appearance of Biofill, a large amount of anammox bacteria was attached on the support material, on both the inside and the outside of Biofill. Granular sludge formed in the inside of Biofill was shown in Fig. 2-8 (b). The extraordinary merit of this kind material for stable retaining of anammox sludge was clearly shown. Figure 2-9 shows the scanning electron

micrographs (SEM) of anammox sludge on Biofill. From these SEM photographs, it was found the microorganisms attached on the support materials in a compacted state. On day 284, all the carriers was taken out of the reactor and five pieces of Biofill were chose randomly for determining the weight of biomass. The average weight of attached biomass was determined to be 0.035 g-TSS/cm^3-Biofill. On day 390, with the same means, other five pieces of Biofill from the bottom part samples were used for measuring the biomass weight on the carriers. The average weight was calculated as 0.14 g-TSS/cm^3-Biofill.

2.3.6 DNA analysis

In Fig. 2-10, Lane 1 and Lane 3 were samples from the sludge in the settling zone and support material in the bottom area of reactor, respectively, using the same primer of Akuf1-Akur1; Lane 2 and Lane 4 were samples from the sludge in the settling zone and support material in the bottom area of reactor, respectively, using the same primer of GM5F-DS907R. At first GM5F-DS907R (Weisburg *et al.*, 1991) were tried for anammox bacteria identification, but failed. Later we designed the primer set of Akuf1-Akur1 for specific detection of anmmox bacteria consulting other references (Egli *et al.*, 2001; Strous *et al.*, 1999). So both primers were applied for anammox bacteria identifying in that time. On the support material, two close matches to anammox bacteria were detected by the phylogenetical analysis of 16S rDNA sequences. One was KU2 (AJ250882) and the other was KSU-1(AB057453). KU2 was first isolated from a rotating disk contactor in Switzerland and is quite similar to *Candidatus B. anammoxidans*, though it exhibits higher tolerances to phosphate (up to 20 mM) and to nitrite (up to 13 mM) and was active at lower cell densities. Its optimum pH was reported to be 8.0 and optimum temperature was 37℃ (Egli *et al.*, 2001). KSU-1 was discovered from a laboratory-scaled reactor using a polyester nonwoven biomass carrier in Japan; by fluorensence imaging,

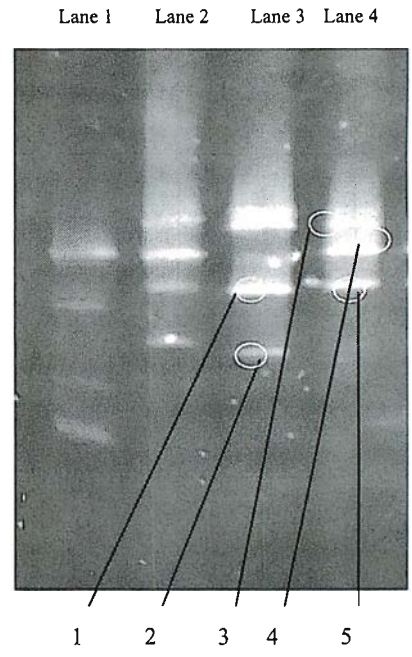


Fig. 2- 10 DGGE profile of partial 16S rDNA amplified by PCR

KSU-1 has been shown to form spherical clusters wrapped in a thin layer of *Zoogloea* sp. (Fujii *et al.*, 2002). Furthermore, the KSU-1 strain was found dominated in the sludge from Single-stage Nitrogen removal using Anammox and Partial nitritation (SNAP) process (Furukawa et al, 2006). Table 2-4 showed all the strains in this reactor.

Table 2- 4 Results of PCR analysis of bacterial community

Band	Homology (Accession No.)	Identidy (%)
1	Anaerobic ammonium-oxidizing palnctomycete KOLL2a partial 16S rRNA gene (AJ250882)	325/331(98%)
	Candidatus Kuenenia Stuttgartiensis 16S ribosomal RNA gene (AF375995)	322/351(97%)
2	Planctomycete KSU-1 gene for 16S rRNA (AB057453)	367/370(99%)
	Candidatus Kuenenia Stuttgartiensis 16S ribosomal RNA gene (AF375995)	364/370(98%)
3	Beta proteobacterium Rufe9b 16S rRNA gene (AY235688.1)	485/518(93%)
	Beta proteobacterium Rufe9 16S ribosomal RNA gene (AY235687.1)	485/518(93%)
4	Anaerobic filamentous bacterium GOMl-1 gene for 16S rRNA (AB243672)	448/503(89%)
	Anaerobic filamentous bacterium KOME-1 gene for 16S rRNA (AB243673)	433/489(88%)
5	Dehalococcoides sp. BHI80-15 16S rRNA gene (AJ431246)	363/425(85%)
	Caldilinea aerophila gene for 16S rRNA (AB067647)	359/425(84%)

2.4 Conclusions

The novel acrylic fiber biomass carrier (Biofill) demonstrated a high anammox sludge retention capacity in an up-flow column-type reactor. At a high NLR of 2.0 kg-TN/m^3/d, T-N removal efficiency as high as 73% was obtained. With a decrease in reactor temperature, nitrogen removal efficiencies did not decrease sharply. And even without temperature control, ammonium, nitrite and T-N removal efficiencies of 48%, 60% and 48%, respectively, were obtained at ambient temperature of 15.3℃. In addition, the removal efficiencies of ammonium, nitrite and T-N increased to 64%, 80% and 64%, respectively, within one day later after increasing operational temperature to 23℃.

Through the SEM observation, it was found that the anammox sludge had a quite compact state when attached on the support material and the average weight of attached biomass in the bottom part of the reactor was revealed to be 0.14 g-TSS/cm^3-Biofill. DNA analysis revealed the existence of two close matches of anammox bacteria identified as KSU-1 and KU2. This new acrylic resin fiber biomass carrier also has a high potential for nitrogen removal of wastewaters with relatively low levels of nitrogen under a high loading rate.

2.5 References

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Chapter 3 Comparison of anammox treatment performances using different kinds of biomass carriers

3.1 Introduction

Anammox, as a potentially useful autotrophic biological processes, was discovered about 10 years ago (Mulder *et al.*, 1995; van der Graaf *et al.*, 1995). In this process, nitrite serves as an electron acceptor, combining with ammonium to produce dinitrogen gas, the only environmentally friendly form of nitrogen. The species responsible for this nitrogen conversion has been identified as a deeply branching planctomycete with a doubling time of 11 days (Strous *et al.*, 1999a). The stoichiometry of the anammox reaction was determined by Strous *et al.* (1998) to be:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \qquad (3\text{-}1)$$

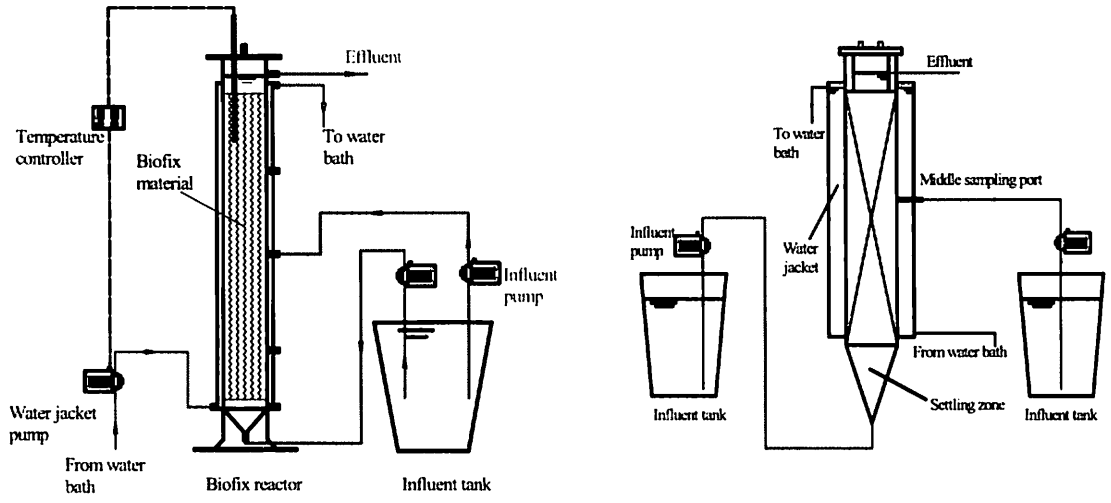
Biofilm reactors have been applied for anammox research, such as fixed bed, fluidized bed and gas lift reactors (Depena-Mora *et al.*, 2004; Sliekers *et al.*, 2003; Strous *et al.*, 1997; Van der Graaf *et al.*, 1996). In order to improve the retention of anammox biomass, the SBR reactor has also been applied for anammox sludge cultivation; however, mechanical stirring could not adequately provide solid-gas separation and the sludge floating became a serious problem, which resulted in deterioration of effluent quality (Depena-Mora *et al.*, 2004; Strous *et al.*, 1997; Strous *et al.*, 1999b). In our laboratory, a nonwoven biomass carrier was successfully used for cultivation of the slowly growing anammox microorganisms (Furukawa *et al.*, 2003). Trigo *et al.* used a membrane sequencing batch reactor for anammox research and demonstrated that this kind of membrane reactor could be suitable for nitrogen removal (Trigo *et al.*, 2006).

Although these kinds of biofilm systems could obtain good performances during anammox laboratory studies, it was inevitable that valuable biomass would washout from the reactors. In this study, two up-flow column-type reactors, one with the nonwoven carrier and the other

with the Biofix carrier, were applied for anammox research at the operational temperature of 35℃ and 25℃ (in summer season the temperature of Biofix reactor was over 25℃), respectively. The nonwoven material used here has demonstrated effectiveness in retention of biomass in unit processes designed for nitrification, nitrophenol degradation, and anammox (Bhatti et al., 2002; Furukawa et al., 2002; 2003). Although the Biofix material is applied here for the first time in an anammox study, it has shown to have potential for single-stage nitrogen removal by combining anammox and partial nitritation (SNAP) process (Furukawa et al., 2006). The anammox treatment performances of two systems were compared under various operational and temperature conditions. Extracellular polymeric substances (EPS) and scanning electron microscopy (SEM) of anammox biomass were also utilized for characterization the microorganisms. In addition, 16S rDNA analyses were applied for determining the species of the microorganism in the reactors.

3.2 Materials and methods

3.2.1 Biofix experiment set-up



(a) Biofix reactor (b) nonwoven reactor

Fig. 3- 1 Schematic diagram of Biofix and nonwoven reactors

An up-flow column type reactor was used for the anammox study including Biofix as the support material as shown in Fig. 3-1. The reactor had a square (15-cm by 15-cm) cross section and height (to effluent port) of 102 cm. The reaction zone, including influent

distribution and biomass retention sections, was 18.8 L. The Biofix biomass carrier was made of acrylic resin with a specific surface area of 113.8 m^2/m^3. Five bundles of the Biofix material were inserted in the reactor for a volume of 10.6 L (56.2% pack). The system was usually operated at 25℃, controlled thermostatically in a water bath, and the thermostat heater was not used in summer season due to the room temperature being continuously in excess of 25℃ (peak 31.5℃). In addition, dark conditions were maintained with a black-vinyl sheet enclosure.

3.2.2 Nonwoven experiment set-up

The anammox reactor utilizing the nonwoven material was made by acrylic resin. The reactor had an inner diameter of 24.0 cm and height (to effluent port) of 112 cm. The liquid volume was 50.7 L and the reaction zone was 54.3 L. The reactor was capped and fitted with an effluent trap. The nonwoven carrier was a porous polyester material (100×2 cm strips, 1.0 cm thickness) coated with a pyridinium type polymer (US patent, 5185415; Japan Vilene Co., Ltd.) designed to enhance retention of microorganisms. In the reaction zone, there were 36 vertically suspended nonwoven strips for a total-one sided sheet area of 7,200 cm^2 (matrix volume, 7,200 cm^3). The reactor was operated at 35℃, thermostatically controlled by water jacket. In addition, dark conditions were maintained with black-vinyl sheet enclosures.

3.2.3 Inocula and operational conditions of reactors

The Biofix reactor was inoculated from another laboratory scale fix-bed anammox reactor operated at the Kumamoto University (Furukawa et al., 2002). The initial MLVSS concentration for the Biofix reactor was 1,706 mg/L. The nonwoven reactor was also inoculated from the same anammox reactor with an initial MLVSS concentration of 1,034 mg/L.

For the two reactors, both increment of influent ammonium and nitrite concentrations and decrease in hydraulic retention time (HRT) were alternatively applied for increasing the nitrogen loading rate (NLR). Increase in NLR was performed after the limiting substrate nitrite removal efficiency of 80% or greater was obtained.

3.2.4 Feeding media

Both reactors were fed with synthetic media, with $(NH_4)_2SO_4$ and $NaNO_2$ serving as influent ammonium and nitrite, respectively. $KHCO_3$, KH_2PO_4, $FeSO_4 \cdot 7H_2O$ and EDTA·2Na were also added as shown in Table 3-1.

Table 3- 1 Composition of synthetic medium

Compound	Concentration (mg/L)	
	Biofix reactor	Nonwoven reactor
$(NH_4)_2SO_4$	50-300	30-330
$NaNO_2$	50-300	30-330
$KHCO_3$	125	125
KH_2PO_4	54	54
$FeSO_4 \cdot 7H_2O$	9	9
EDTA · 2Na	5	5

3.2.5 Analytical methods

The chemical analyses were same with describing Chapter 2 (Page 20).

For EPS measuring, proteins were measured using the method of Lowry et al. (1951) and carbohydrates by the method of Dubois et al. (1956). Nucleic acids (combined RNA and DNA) were estimated by the UV absorption method (Experimental Guidelines for Biotechnology, 1992) using the following equation:

$$Nucleic\ acids\ (g/L) = 30.98\ A\ /\ (10{,}000 \cdot (0.09)\ b) \qquad (3\text{-}2)$$

where, 30.98 is the gram molecular weight of phosphorous, A is the absorbance of the sample solution at 260 nm, 10,000 is the constant of proportionality (*absorbtivity*) of phosphorous in nucleic-acid form (average of the RNA and DNA components), 0.09 is the weight fraction of phosphorous in nucleic acids and b is the absorbance light path (1.0 cm in this study).

The abundance of bacteria in the community of the sludge used was estimated from the image of denaturing gradient gel electrophoresis (DGGE). Metagenomic DNA was extracted from the sludge using ISOIL kit (Nippon gene Co., Ltd., Tokyo, Japan). Partial 16S rRNA genes in the metagenome were amplified by PCR with a primer set, 357F with a GC-clamp and 534R

(Muyzer *et al.*, 1998). The amplified fragments were resolved by DGGE for 16 hr at 100V at 60℃ using DCode system (Bio-Rad Laboratories, Hercules, CA, USA). An 8% acrylamide gel with a 30-to-65% denaturing gradient was used, where 100% denaturant was defined as 7 M urea and 40% formamide. The gel was stained with SYBR-Gold solution (Invitrogen Corp., Carlsbad, CA, USA), and visualized as a 16-bit gray-scale image using FLA-2000 system (Fuji Photo Film Co., Ltd., Tokyo, Japan). The intensities of all bands were quantified using a software, Image Gauge v3.4, included in the system. KSU-1 population content was calculated from the corresponding band intensity and the sum of all band intensities (Muyzer *et al.*, 1998).

3.3 Results and discussion

3.3.1 Biofix reactor

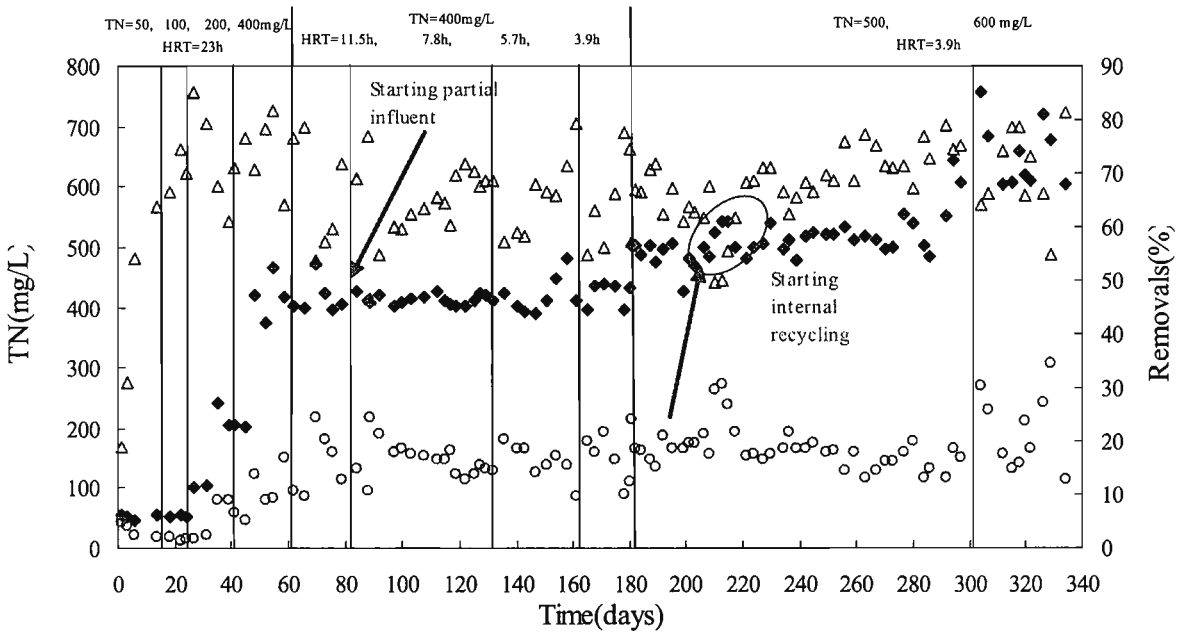


Fig. 3- 2 Time courses of T-N concentration and removal rates

◆ Influent, ○ Effluent, △ Removal rates

Operation was continued about 340 days without interruption. The NLRs were increased from 0.05 to 3.6 kg-N/m^3/d by means of increasing the concentrations of nitrogen compounds (NH_4^+ and NO_2^-) and shortening the HRT as shown in Fig. 3-2. In the first phase, NLRs were increased by increasing influent T-N concentrations stepwise at a constant HRT of 23 h.

Because the reactor was initially at an ambient temperature of about 25℃, the anammox biomass could not adapt well and had very low activities. It took 24 days for the T-N removal efficiency to progress from 19% to 78%. Subsequently, stable anammox activities were maintained and average T-N removal rates of 75% was observed even the influent T-N concentration was increased to 400 mg/L (NLR of 0.4 kg-TN/m^3/d). Moreover, the removal efficiencies did not drop sharply even when the T-N level was doubled. It was concluded that increasing the NLRs by increasing the influent T-N concentration was not harmful to anammox treatment performance. In phase II, T-N levels were kept stable and HRT was shortened to increase the NLRs over about 120 days operation. In the end, the NLR was increased to 2.5 kg-TN/m^3/d and the highest T-N removal of 77% was achieved. Compared with phase I, changing the NLR by shortening the HRT resulted in more harmful influences on anammox treatment performance. For example, when the HRT was shortened from 11.5 h to 7.8 h and from 5.7 to 3.9 h, T-N removal efficiencies decreased by 31% and 25%, respectively.

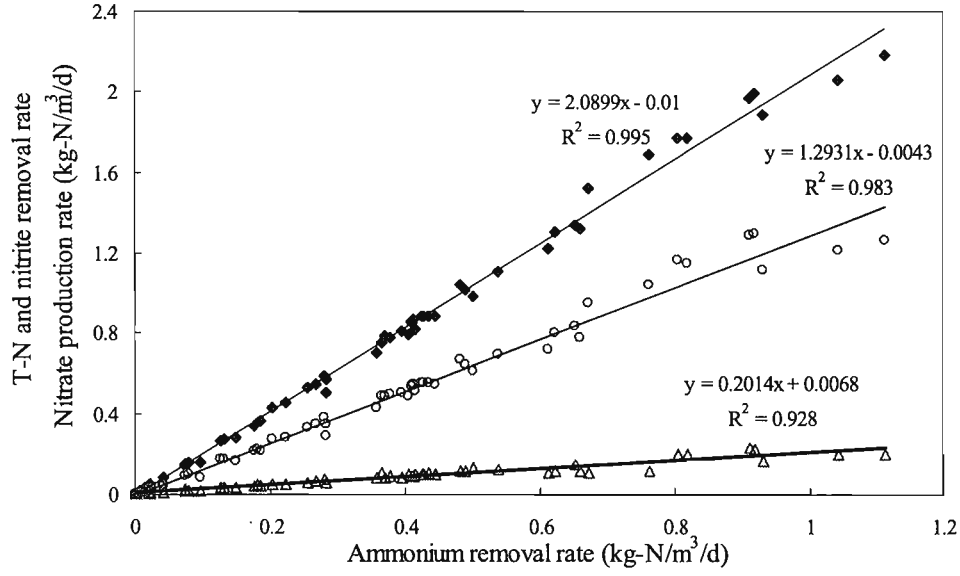


Fig. 3- 3 T-N and nitrite removals and nitrate production as functions of ammonium removals
◆ T-N ○ Nitrite △ Nitrate

A relatively high influent T-N of 500 mg/L was associated with unstable nitrogen removal performances. N removal efficiencies were diminished owing to the washout of granular sludge, formed by the detached anammox sludge, from day 210 to day 220. Internal recycling

was applied to mitigate the problem on day 238. During this period, the highest T-N removal efficiency of 81.3% was achieved at a NLR of 3.6 kg-N/m^3/d (T-N of 600 mg/L, HRT of 3.9 h). In Fig. 3-3, T-N removal, nitrite removal and nitrate production rates are shown, which are in good agreement with the stoichiometric equation of the anammox reaction (Eq. 3-1). Though the results are described on a mass concentration basis, conversion to a mole basis would yield the same results.

3.3.2 Nonwoven reactor

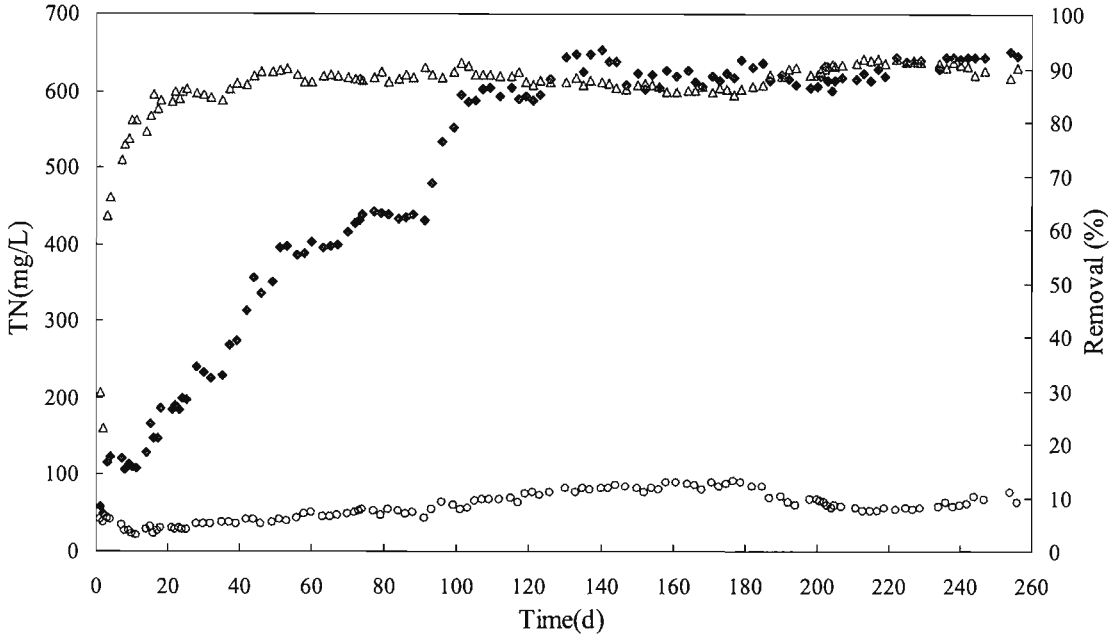


Fig. 3- 4 Nitrogen removal performances for nonwoven reactor

◆ Influent ○ Effluent △ Removal rate

Fig. 3-4 shows the T-N removal performance in nonwoven reactor. In the initial period, very low removal efficiencies were obtained, but increasing trend was clarified; e.g., the T-N removal efficiency reached above 80% after 10 days. In the following about 90 days (until day 103), high and stable removal performances were always observed and the peak removal efficiency of 91% was obtained on day 103 (NLR of 2.2 kg-N/m^3/d). Later, removal performance had become stagnating until day 177 and removal performance showed a slow declining trend, e.g., the lowest T-N removal efficiency of 85% was observed on day 177. Then, removal performance recovered and exhibited higher removal efficiencies of about 90% during the following period even NLR rose to 4.0 kg-N/m^3/d. Considering the

relationship between nitrite removal, nitrate production and ammonium removal, it is obviously that the ratio among nitrogen of 1:1.22:0.18 was little lower than the results determined by Strous (Strous *et al.*, 1998). Furthermore, observed T-N removal efficiencies above 90% were higher than the theoretical value of 88% according to the anammox reaction equation (Strous *et al.*, 1998). It might be in relation to other kinds of biomass, which could biodegrade nitrite or nitrate under anoxic conditions.

Comparing with the Biofix reactor, the nonwoven reactor demonstrated a shorter startup time and greater treatment stability under a relatively high loading and relatively high operational temperature. For the nonwoven reactor, startup was completed successfully within 2-3 weeks and NLRs could be increase to 3.0 kg-N/m^3/d within 4 months of operation with T-N removal rates of about 87%. These T-N removal performances revealed a strong N removal capability for the nonwoven reactor despite the continuous increase in NLRs. When the NLR reached 4.0 kg-N/m^3/d (T-N of 650 mg/L, HRT of 2 h), a high T-N removal rate of 90% was observed.

3.3.3 EPS content

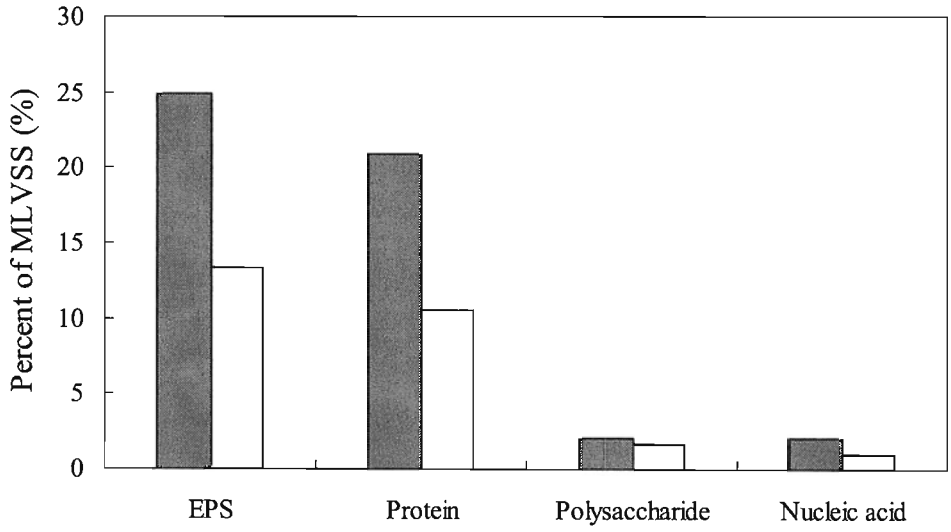


Fig. 3- 5 EPS comparison of granular and attached biomass in Biofix reactor

EPS assists in the formation of microbial aggregates whether the biomass is in suspended or biofilm states. EPS is thought to support the metabolic cooperation among cells in aggregate

form. Moreover, it benefits surface adhesion, cell aggregation in flocs and biofilms, stabilization of biofilm structure, etc (Wingender *et al.*, 1999). Fig. 3-5 shows a comparison of EPS levels in anammox granular sludge and attached biomass of the Biofix reactor. From this figure, it is clear that protein was the predominated component in the EPS of anammox sludge. Furthermore, EPS levels for granular sludge were almost two times higher than those of attached biomass, which suggests that high EPS levels would be beneficial for the formation of granular sludge.

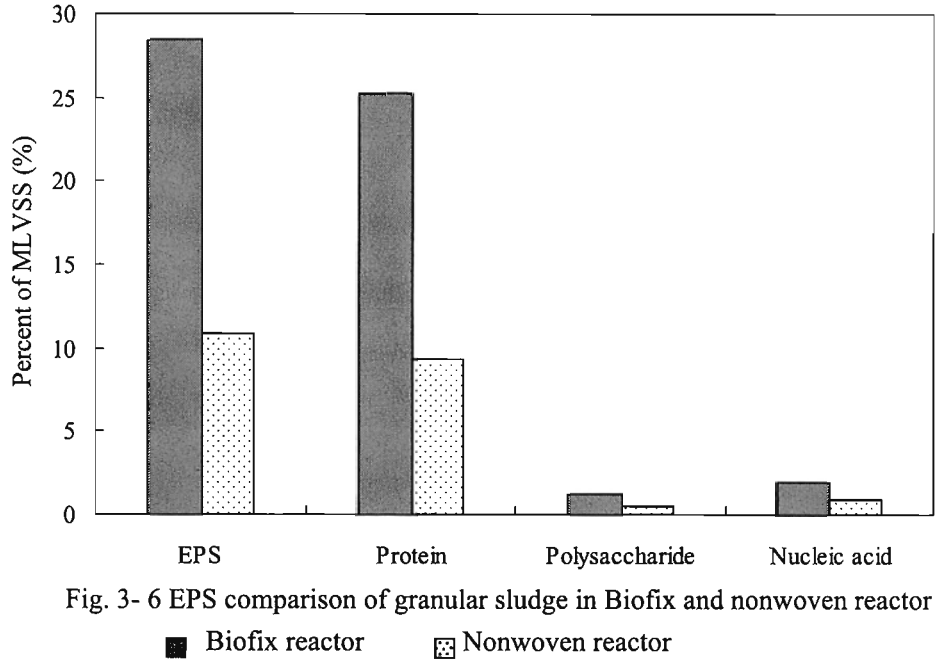
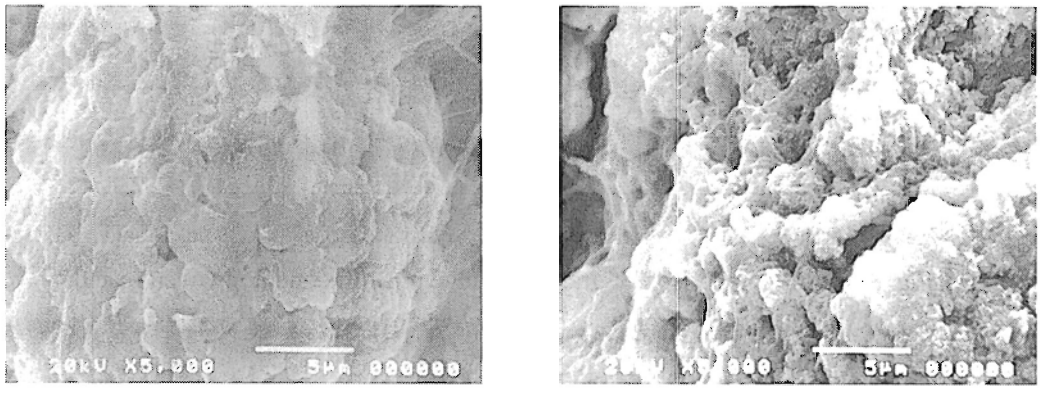


Fig. 3- 6 EPS comparison of granular sludge in Biofix and nonwoven reactor

Previously, most researchers reported that polysaccharide was the most abundant component EPS fraction (Costerto *et al.*, 1981). However, protein and nucleic acid are also significant components in EPS and possibly the largest fraction. Nielsen *et al.* and Dignac *et al.* quantified the EPS composition of activated sludge in different wastewater treatment systems, and found that protein was dominating (Dignac *et al.*, 1998; Nielsen *et al.*, 1997). Laspidou and Rittmann considered that due to the high content of negatively charged amino acids, protein was more involved than sugars in electrostatic bonds with multivalent cations, a key factor in stabilizing aggregate structure (Laspidou *et al.*, 2002). The other significant function of extracellular protein is as enzymes performing the digestion of macromolecules and particulate material in the microenvironment of embedded cells, which could trap, bind, and

concentrate organic materials in close proximity to the cells (Laspidou *et al.*, 2002). Extracellular enzymes are also localized near the cells and could hydrolyze the adsorbed organic materials. Wingender and Hoffman considered that the proximity of extracellular hydrolysis to the cells could favor efficient inception of low-formula hydrolysis products by reducing diffusion loss of products to the surrounding water (Hoffman *et al.*, 1999; Wingender *et al.*, 1999). The EPS levels of granular sludge in the two reactors were also compared (Fig. 3-6). It was demonstrated that extracellular protein was the main fraction enabling the formation of granular sludge according to the above discussion. From this figure, it is evident that the EPS level of the granular sludge in the Biofix reactor was more than double that of the nonwoven reactor, which suggests that the granular sludge in the Biofix reactor would probably have a more compact structure.

3.3.4 SEM observation

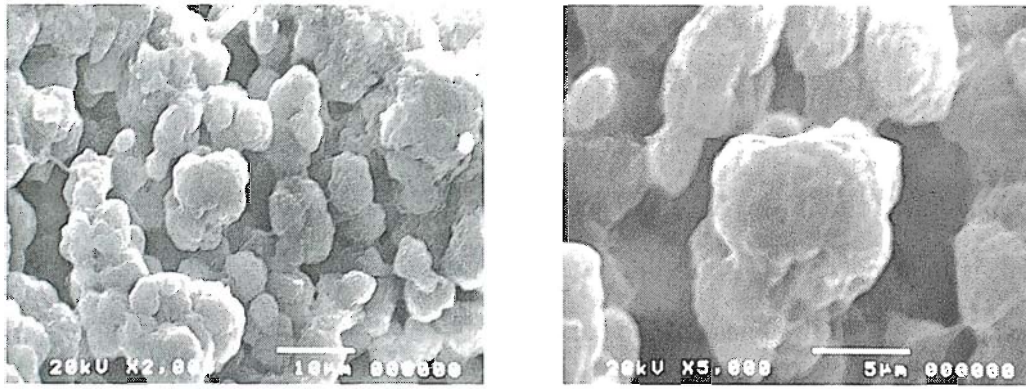


(a) Biofix (b) Nonwoven

Fig. 3- 7 Comparison of surface condition of granular sludge

The SEM photos of the granular sludge in the Biofix reactor show a high degree of compactness (Fig. 3-7 (a)). Each micro-element was tightly integrated with other parts and there was little interspace between them. However, there existed a lot of branch-like micro-structures on the surface of the granular sludge in the nonwoven reactor and the spaces between the micro units were larger than those of the Biofix reactor. Based on the SEM observation of the granular anammox sludge of two reactors, it was concluded that the micro-organization structure in the Biofix reactor presented higher compactness than those in nonwoven reactor. Fig. 3-8 shows the SEM photos of the attached biomass on Biofix

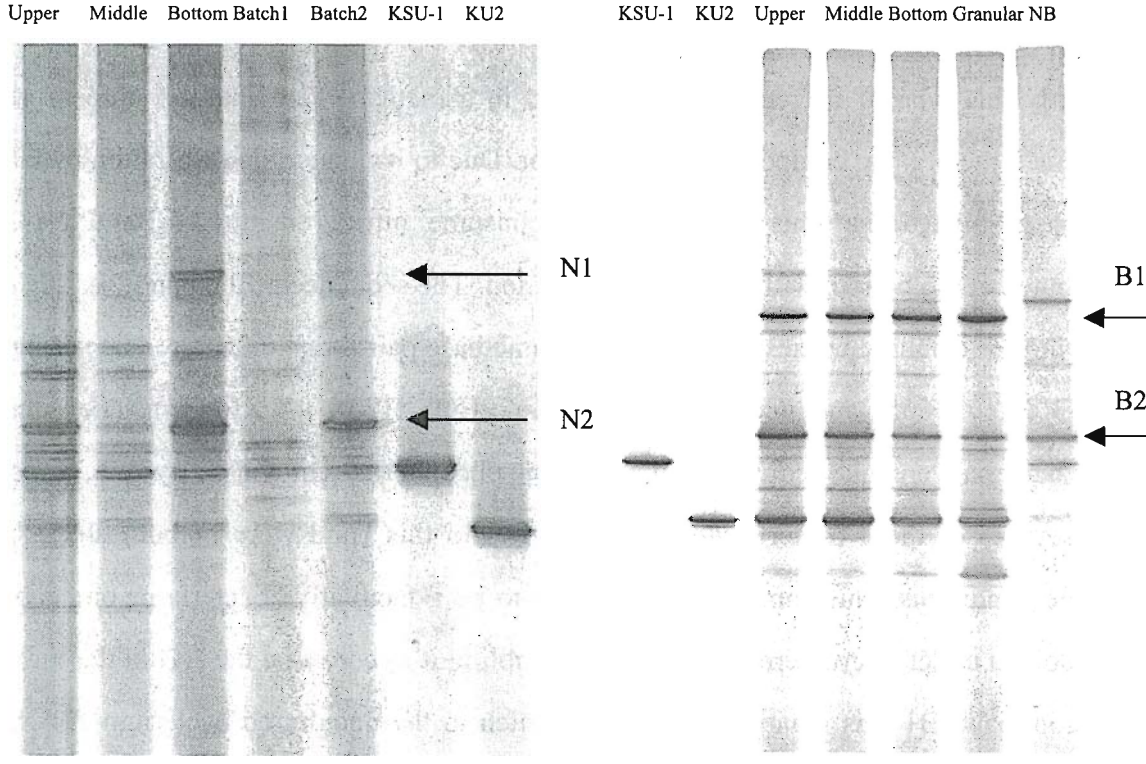
materials. The micro-organization structure exhibited sphericity in the microcosmic point of view. This structure may be formed with the shear forces caused by the upward flow of gas bubbles and water currents in the spaces between support materials. The Biofix materials with its net-type structure provided a favorable environment for the attached biomass allowing for effective contact with nutrients, gas and water current.



(a) (b)

Fig. 3- 8 SEM photos of attached biomass on Biofix

3.3.5 DNA analysis



(a) nonwoven (b) Biofix

Fig. 3- 9 DGGE results of different parts in Nonwoven and Biofix reactors

Note: Line Batch 1 and Batch 2 mean the samples were taken from the upper and bottom parts of Nonwoven reactor for batch experiment; Line NB means the samples from bottom part of nonwoven reactor just for comparing.

Fig. 3-9 shows DGGE photos of the nonwoven and Biofix reactors operated at an NLR of 3.0 kg-N/m^3/d to 3.2 kg-N/m^3/d. Samples were taken out of the reactors from the bottom, middle and. upper parts and the granular sludge of the Biofix reactor was also investigated. For the nonwoven reactor, relative amounts of the anammox bacterium KSU-1 strain (Fujii *et al.*, 2002) exhibited a decreasing tendency from top to bottom parts, e.g, there was about 20% in the upper and middle parts and only 15% in the bottom part; while the anammox bacterium KU2 strain showed the reverse trend. For the Biofix reactor, the KU2 strain was dominant and the four KU2 bands of the different parts demonstrated almost identical concentrations; while the KSU-1 concentrations in the Biofix reactor showed much lower levels relative to KU2; however, an increasing trend from the bottom upward of the KSU-1 strain could be distinguished based on the thickness of the bands.

Since bands N2 and B2 are at almost the same level, they were identified as *Chloroflexi* belonging to the green non-sulfur bacteria (AB113620, identity of 100%; AB113606, identity of 100%). The *Chloroflexi* bacteria are facultative anaerobes frequently found in UASB reactors that are filamentous and often associated with sludge granulation and bulking. Recently this type of bacteria was also found in a methane fermentation process where it consumed the organic compounds in the reactor. Due to its anaerobic respiration metabolism, it was postulated that this bacteria could consume nitrite or nitrate. Band N1 mainly distributed at the bottom with a high concentration. The sequence of the band was matched to two kinds of database entries, which had the same base pair array, uncultured bacterium clone K4_33 (AY793665) and uncultured bacterium PHOS-HE36 (AF314435). The sources of these bacteria were lake sediment and an aerobic phosphorus-removal ecosystem, respectively. The sources of these bacteria with high matches to this band were of both anaerobic and aerobic conditions, thus band N1 was thought to be a facultative bacteria or one capable of metabolism under micro-aerobic conditions, enabling it to consume the DO in the influent. In addition, band B1 was identified as a close match to the uncultured bacterium PHOS-HE36 (identity of 97%, accession: AF314435). This type of bacteria could also consume the DO in the influent and survive under the anaerobic and anoxic conditions.

3.4 Conclusions

Two column-type upflow anammox reactors with different carrier materials showed high nitrogen removal performances. In a Biofix reactor, a T-N removal efficiency of 81.3% was observed with a NLR of 3.6 kg-N/m^3/d at a temperature of 25℃, while for a nonwoven reactor, a high T-N removal efficiency of 86.3% was obtained even with a NLR of 4.0 kg-N/m^3/d. Comparing the levels of EPS in the anammox granular sludge in two reactors, it was found that protein was the dominant fraction and the EPS content of anammox granular sludge in the Biofix reactor was almost three times higher than that in the nonwoven reactor (28.4% versus 10.9%). Based on the analysis of EPS and SEM observation, the anammox granular sludge in the Biofix reactor was revealed to have a more compact state than that in the nonwoven reactor. The microstructure of anammox sludge attached on the Biofix material exhibited a spherical form in SEM photos, which may be due to shear forces caused by the upward flow of gas bubbles and water through the support materials. DNA analyses revealed four unknown sequences. Two DNA bands were identified as *Chloroflexi* belonging to the green non-sulfur bacteria, which have the function of consuming nitrite or nitrate in the reactor. One of these bands was matched to two database entries of the uncultured bacterium clone K4_33 (AY793665) and the uncultured bacterium PHOS-HE36 (AF314435). The other band was identified as a close match to the uncultured bacterium PHOS-HE36 (identity of 97%, accession: AF314435). All these bacteria can serve the function of consuming DO in the influent, thereby potentially assisting the anammox microorganism to function under macro-aerobic conditions.

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Chapter 4 Application of partial nitritation-anammox process for nitrogen removal of anaerobic digester liquor treatment

4.1 Introduction

In anaerobic digester liquor, the low level of organic carbon is hardly treated by biodegradation, while the high concentration of ammonium almost accounts for 15-20% of the nitrogen load in a wastewater treatment plant due to digester liquor recycling (van Kempen *et al.*, 2001; Wett and Alex, 2003). It is necessary to find a separate and effective process to treat this kind of wastewater because the regulations are becoming stricter. The traditional nitrogen removal process (nitrification-denitrificaiton) firstly oxidizes ammonium to nitrite and nitrate and then reduces to dinitrogen gas via nitrite as shown in Eq. 4-1 and Eq. 4-2, respectively.

$$NH_4^+ + 2O_2 \xrightarrow{nitrification} NO_3^- + H_2O + 2H^+ \quad (4\text{-}1)$$

$$6NO_3^- + 5CH_3OH + CO_2 \xrightarrow{denitrification} 3N_2 + 6HCO_3^- + 7H_2O \quad (4\text{-}2)$$

Nitrogen removal by partial nitritation (PN) combined with the anammox process can save 62.5% O_2, 50% of alkalinity, and 100% organic carbon as compared with the traditional method as revealed by comparing Eq. 4-3, 4-4 with Eq. 4-1, 4-2. In addition, the biomass production rate of this combined process is much lower than that of the traditional method (3 g $COD_{biomass}$/mol NH_4^+ versus 20 g $COD_{biomass}$/mol NH_4^+), which further reduces the treatment cost by reducing the excess sludge (Fux *et al.*, 2002).

$$NH_4^+ + 0.75O_2 \xrightarrow{partial-nitritation} 0.5NO_2^- + 0.5H_2O + H^+ + 0.5NH_4^+ \quad (4\text{-}3)$$

$$NH_4^+ + NO_2^- \xrightarrow{anammox} N_2 + 2H_2O \quad (4\text{-}4)$$

The purpose of this research was to treat the anaerobic digester liquor by the partial nitritation /anammox process using a novel biomass carrier. The optimized operational conditions were also investigated, and the effects of free ammonia (FA) and free nitrous acid (FNA) were

studied as well.

4.2 Materials and methods

4.2.1 Experimental set-up

The PN reactor containing biofringe (BF) material used in this study was constructed of acryl resin, having downdraft and updraft sections in a parallel upright arrangement as shown in Fig. 4-1. It also had clear zones of approximately 70 mm at the bottom and 30 mm at the top (below and above the biofringe reaction zone in the downdraft section). The working volume was 10.8 L and influent was introduced deeply within the updraft section using a peristaltic pump. Air was also introduced near the base of the updraft section, serving to mix and oxygenate the wastewater while circulating it through the reactor. The air flow rate was fixed at 6 L/min. Anammox reactor used in this part was same with that in Chapter 3.

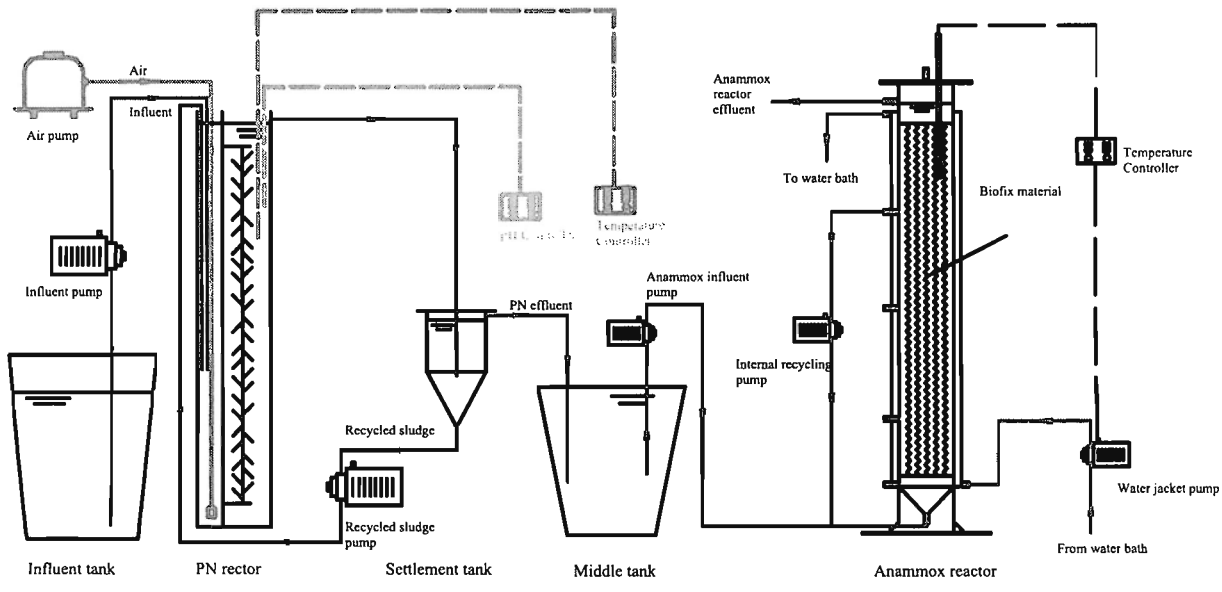


Fig. 4- 1 Schematic diagram for PN-Anammox apparatus

The settling tank was also made of acryl resin with a 2.5 L working volume and 0.017 m^2 of water surface area. The effluent from the outlet of the BF vessel was fed into the center of the settling tank by the gravity. The underflow was drawn from the central bottom of the settler

and returned to the BF reactor at a 100% recycle rate.

The BF material consists of fringe yarns (NET Co. Ltd, BF, diameter, *ca.* 3 mm) attached to a support filament as shown in Fig. 4-2. The fringe yams are symmetrically attached, extending equal distances beyond each side the support filament, and twisted to give an even 3-dimsonal distribution.

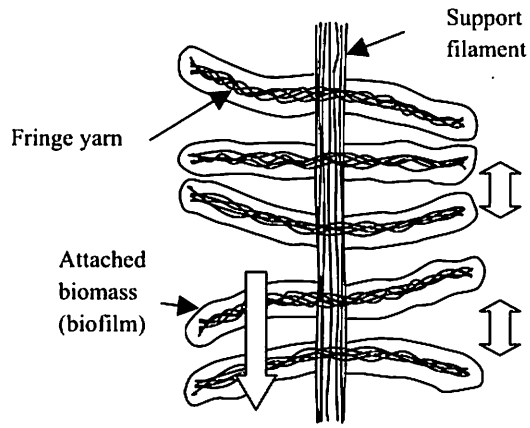


Fig. 4- 2 Configuration for biomass carrier

The staple fiber of the BF fringe yarns is a hydrophilic acrylic composite. The material has a rough texture with a porosity surface, which allows for a great amount of sludge to attach on it. BF with a flexible fringe yam matrix in a fix position is induced by water flow to flex, which causes a 'swimming' motion that enhances mass transfer of nutrients to the bioflm. So that, BF materials combines the advantages of fix-bed and fluidized-bed processes. It also eliminates the losses with absence of clogging and channeling, which cannot be easily avoided in most fix-bed processes. The BF process can be continuously operated without dependence on hydrodynamic conditions to avoid settling or floating of the attachment medium or the requirement of screens or traps to prevent washout, which can be difficult to achieve in fluidized-bed processes.

4.2.2 Inocula and feeding media

The reactors were initially seeded using activated sludge from a lab-scale fill-and-draw batch

reactor with a mixed-liquor suspended solids (MLSS) concentration of 3000 mg/L. The digester liquor used in this study was taken from the Kumamoto East Wastewater Treatment Plant (Kumamoto, Japan). The detailed information of the digestion liquor is shown in Table 4-1.

Table 4- 1 Water quality of the anaerobic digestion liquor

Compounds	Concentration (mg/L)
BOD_5	150.1-200.8
COD	178.1-274.3
NH_4-N	428.0-1012.5
NO_2-N	0
NO_3-N	0
SS	20-40
pH	8.16-9.37

4.2.3 Analytical methods

Nitrogen and SEM measuring were same with the describing in Chanter 2 (Page 20). EPS measuring was same with the describing in Chapter 3 (Page 32).

The suspended solids (SS) content was determined according to *Standard Methods* (2540 D; APHA *et al.*, 1995). The total sludge content was estimated as mixed-liquor suspended solids (MLSS) and biomass as mixed-liquor volatile suspended solids (MLVSS). For the determination of MLSS, a sludge sample of know volume was washed twice by centrifuging at 1,000×*g* for 15 min, decanting and resuspending in deionized water and then dried to a constant weight at 105℃ (with cooling under desiccation). MLVSS and mineral (ash) contents of MLSS samples were determined following ignition at 550℃ for 1 h. The pH level was measured by the electrometric method using a pH meter (IM-22P; TOA Electronics, Ltd., Tokyo, Japan). DO was measured using a DO meter (HORIBA, pH/DO meter D-55).

4.3 Results and discussion

4.3.1 Sludge characteristics and organic carbon removal

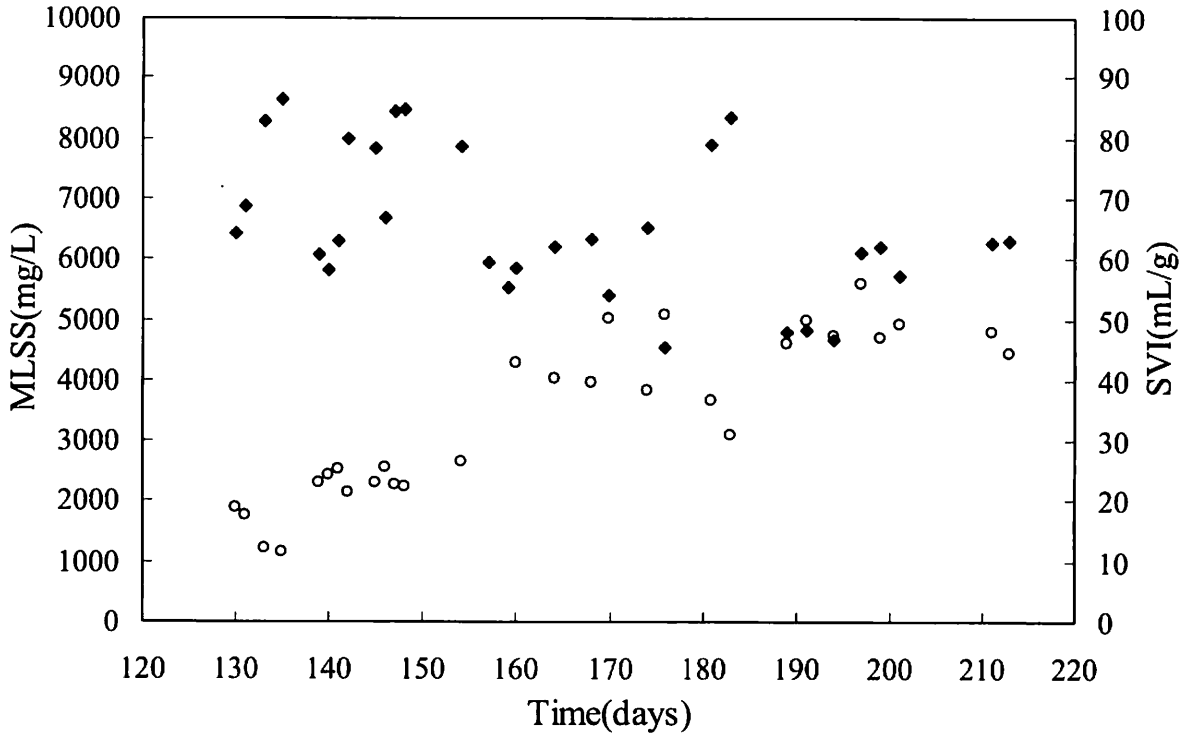


Fig. 4- 3 Time courses of MLSS and SVI levels in PN reactor

The MLSS concentration was initially set at 3,000 mg/L, which decreased gradually to about 100 mg/L within 3 weeks as shown in Fig. 4-3. Considering the low levels of effluent SS (almost zero), the biomass was considered mostly attached on the packing materials. The effluent SS concentrations were kept at low levels (peak of 90.2 mg/L) during the following 100 days. On day 130, some sludge detached from bottom part of BF materials due to shaking caused by sludge sampling for SEM analyses and then the suspended solids concentration immediately jumped to about 6,400 mg/L. Subsequently, MLSS levels were always over 5,800 mg/L and SVI values were very low (average of 29 mL/g), which implies that this system could efficiently retain microorganisms using a setting tank with sludge recycling due to the effective sludge settling. For BOD_5 removal, stable performance was obtained with an average removal efficiency of 73% achieved over 200 days operation. On day 100, the removal efficiency decreased suddenly when pH and temperature control was stopped, and

then gradually recovered under room temperature conditions. In addition, NLRs did not have much effect on organic carbon removal; e.g., removal efficiencies decreased only 5% (from 82% to 77%) when BOD_5 loading rates increased from 0.26 to 0.59 kg-$BOD_5/m^3/d$.

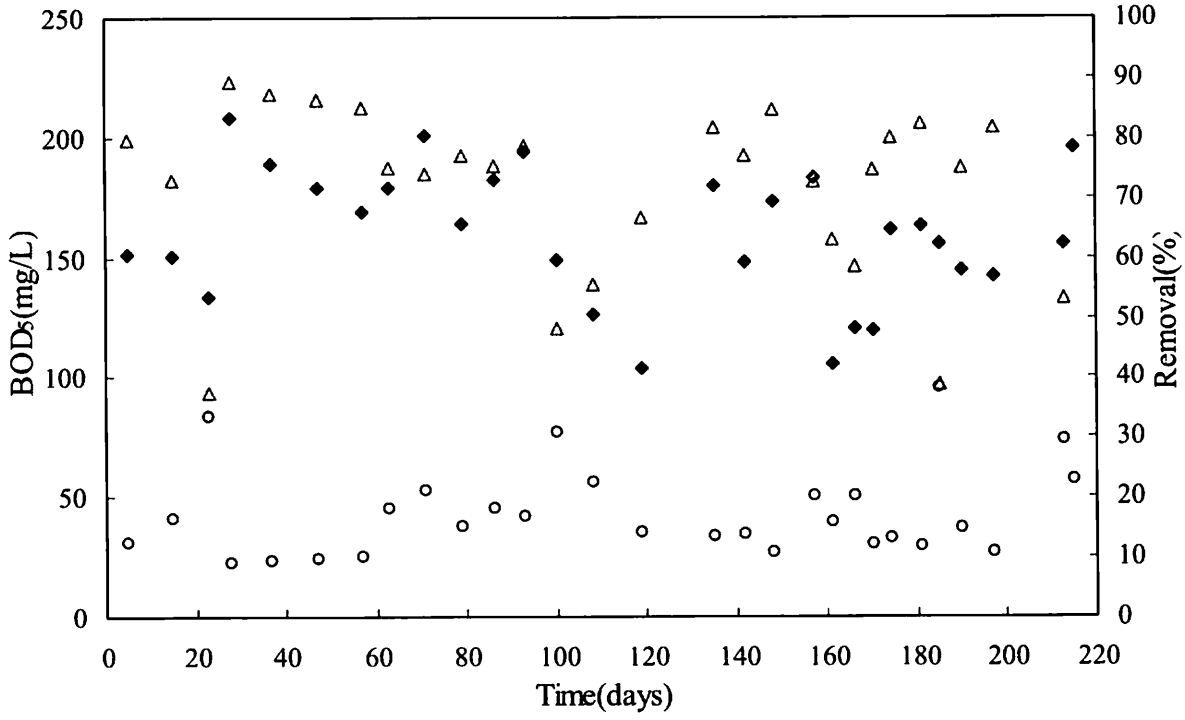


Fig. 4- 4 Time courses of BOD_5 removal performances in PN reactor

◆ Influent ○ Effluent △ Removal

4.3.2 Nitrogen conversion

In the initial period of 20 days, the partial nitritation performance was unsatisfactory due to the high pH level in the reactor (average of 8.88). Effluent nitrite levels increased gradually to 479 mg/L after adjusting reactor pH to a suitable range (pH 7.5) (effluent NO_2-N/NH_4-N ratio of 1.15). Subsequently, the effects of pH on the partial nitritation were investigated by adjusting reactor pH from 7.4 to 8.0. It was observed that the effluent nitrite concentrations increased with increasing pH and the peak effluent NO_2-N/NH_4-N ratio of 5.71 was observed during this period (days 36-100). In the following 20 days (period 11), the reactor was run at a constant HRt without pH, DO, and temperature control (the room temperature was about 25℃ due to continuous air-conditioning). Satisfactory performances were obtained and the average effluent NO_2-N/NH_4-N ratio was 1.16 in this period, which demonstrated that the partial nitritation reaction could successfully be achieved under a relatively low NLR of 0.53

kg-N/m^3/d even without control of running conditions. Considering the treatment potential of this system, the NLRs were increased to 1.20 kg-N/m^3/d after operational conditions were resumed. The effluent nitrite levels only slightly decreased and the treatment capacity soon began to recover. On day 130, biomass detachment from BF materials resulted from SEM sampling, which caused the suspended solids level to rise more than before (from 37 mg/L to 6400 mg/L). The effluent NO_2-N/NH_4-N ratio on day 130 was 3.31 and the peak value of 61.8 was observed later under the same NLR of 1.20 kg-N/m^3/d, shown in Fig. 4-5. It was postulated that there existed a large number of ammonium oxidizing bacteria (AOB) in the reactor and the attached state restricted the treatment potential of AOB. It was also considered that a suspended state could improve the contacting chances between microorganism and nutrients, and therefore the performances of partial nitritation were strengthened greatly. Soon, NLRs were increased via 2.40 kg-N/m^3/d to 3.20 kg-N/m^3/d, and the average effluent nitrite/ammonium ratio reached about 1.9 with temperature and pH control.

Table 4- 2 Summary of different operational conditions and performances

Period (day)	Flow rate (L/h)	HRT(h)	NLRs (kg-N/m^3/d)	pH (reactor)	Temperature (℃)	DO (mg/L)	Effluent NO_2-N/NH_4-N
1(1-19)	0.30	36.0	0.53	8.88±0.09	34.8±0.30	6.40±0.40	0.02±0.001
2(20-35)	0.30	36.0	0.53	7.52±0.69	34.7±0.29	6.46±0.68	0.72±0.027
3(36-43)	0.30	36.0	0.53	7.50±0.00	34.7±0.19	7.10±0.75	1.08±0.53
4(44-49)	0.30	36.0	0.53	7.60±0.00	34.6±0.33	6.76±0.04	1.54±0.01
5(50-55)	0.30	36.0	0.53	7.40±0.00	34.7±0.25	6.90±0.23	1.15±0.11
6(56-65)	0.30	36.0	0.53	7.70±0.00	34.7±0.34	6.77±0.44	2.14±0.01
7(66-73)	0.30	36.0	0.53	7.80±0.00	34.7±0.32	6.28±0.57	6.32±0.20
8(74-81)	0.30	36.0	0.53	7.90±0.00	34.8±0.33	6.80±0.04	3.56±0.23
9(82-92)	0.30	36.0	0.53	8.00±0.00	34.8±0.26	---	4.66±0.49
10(93-100)	0.30	36.0	0.53	7.55±0.00	34.9±0.24	6.26±0.01	3.73±0.52
11(101-120)	0.30	36.0	0.53	7.08±0.21	---	7.32±0.52	1.16±0.31
12(121-137)	0.66	16.4	1.20	7.47±0.18	34.7±0.25	4.16±0.69	12.52±2.03
13(138-141)	1.35	8.0	2.40	7.46±0.00	34.8±0.41	0.70±0.10	1.85±0.28
14(142-154)	1.80	6.0	3.20	7.54±0.00	34.9±0.22	1.43±0.82	1.90±0.39
15(155-164)	1.80	6.0	3.20	7.16±0.07	23.0±0.42	3.59±0.31	0.80±0.20
16(165-172)	1.56	6.9	2.13	6.85±0.00	23.9±0.29	3.63±0.24	0.72±0.05
17(173-183)	1.20	9.0	2.78	6.88±0.41	23.8±0.35	4.29±1.47	4.64±6.87
18(184-213)	1.80	6.0	3.20	7.12±0.55	31.8±4.0	1.27±0.17	1.37±0.13

Note: Influent average ammonium concentration was looked as 800 mg-N/L to calculate the NLRs.

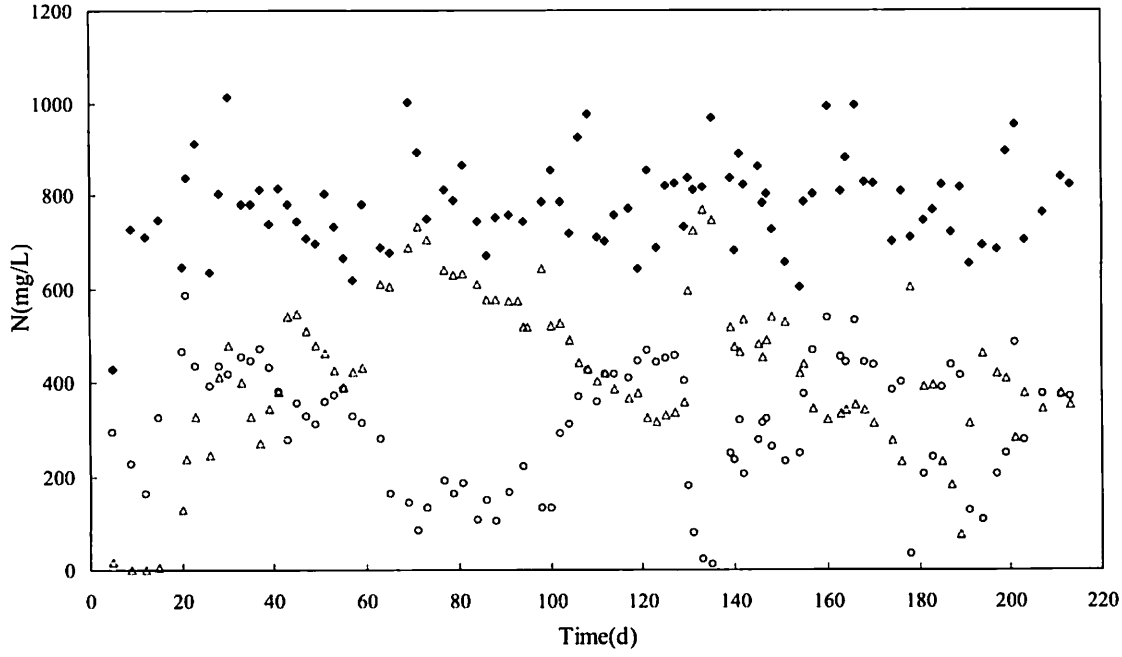


Fig. 4- 5 Time courses of nitrogen concentrations in PN reactor

◆ Influent ammonium ○ Effluent ammonium △ Effluent nitrite

From period 15 (day 155-164), the PN reactor was operated once again without pH, DO, and temperature control in order to investigate the partial nitritation capacity under variable conditons with a high NLR of 3.2 kg-N/m^3/d. Due to the beneficial effects of FA and FNA, satisfactory partial nitritation performance was achieved and the average effluent nitrite/ammonium ratios were 0.80 and 0.72 in period 15 and 16, respectively. From day 161, the effluent of the PN reactor was introduced into the anammox reactor and the resulting removal performance is showed in Fig. 4-6. Since the volumes of the two reactors were different, the NLRs of the anammox reactor was only 1.85 kg-N/m^3/d (HRT of 10.4 h) even at the same flow rate. However, high nitrite removal efficiencies (99.9%) and low nitrate production (effluent nitrate of 49 mg/L) were observed and an average T-N removal efficiency of 78% was obtained in this period. But high nitrite concentrations and a relatively long HRT stimulated the growth of NOB, resulting in greatly increased of nitrate production in the system, with the highest effluent nitrate concentrations of the two reactors being 261.5 and 312.1 mg/L, respectively. Simultaneously, the lower pH value due to nitritation caused the FA and FNA concentrations in the PN reactor changing, resulting in the reverse effects for AOB and NOB. Although there were high effluent nitrate concentrations, most ammonium and

nitrite were removed by anammox reaction and an average T-N removal efficiency of 72% was achieved.

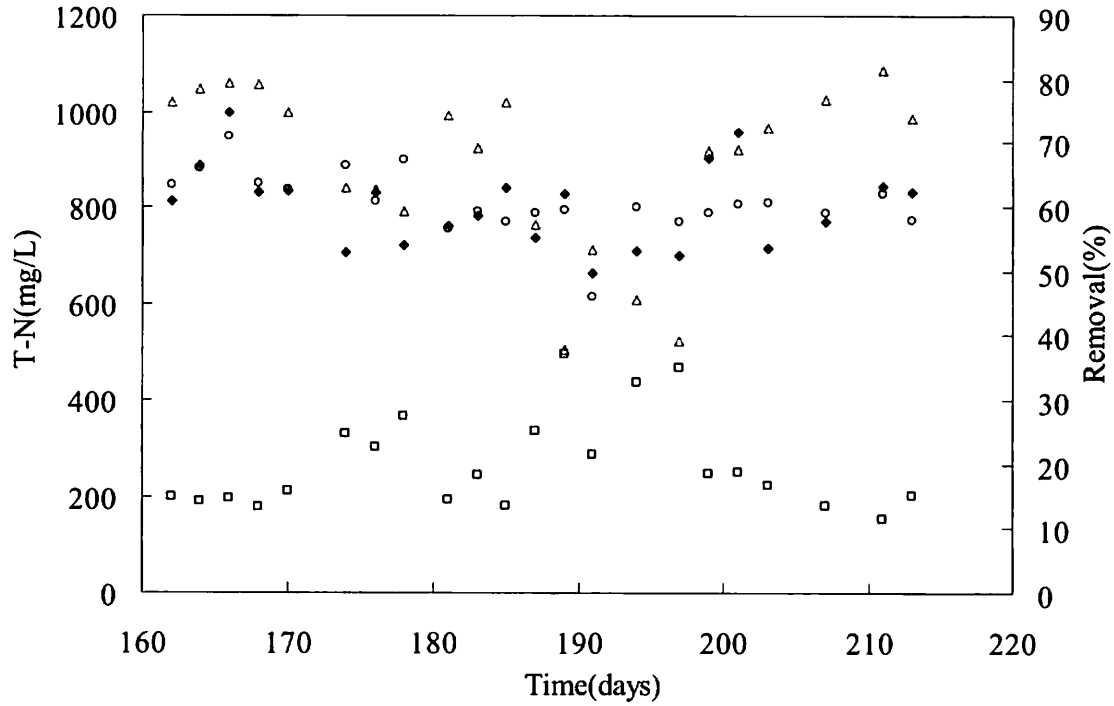


Fig. 4- 6 Time courses of T-N concentrations and removals in PN and Anammox reactor

◆ Influent ○ PN Effluent □ Anammox effluent △ Removal efficiency

4.3.3 Effects of FA and FNA

$$FA(NH_3, mg/L) = \frac{17}{14} \times \frac{[NH_4^+] \times 10^{pH}}{e^{[6344/(273+t)]} + 10^{pH}} \quad (4\text{-}5)$$

$$FNA(HNO_2, mg/L) = \frac{46}{14} \times \frac{[NO_2^-]}{e^{[-2300/(273+t)]} \times 10^{pH}} \quad (4\text{-}6)$$

Free ammonia can not only accelerate the ammonium oxidation reaction rate, but can also inhibit the activity of AOB and NOB. It was reported that AOB activity was inhibited at FA levels between 10 to 150 mg/L, whereas NOB activity was already greatly reduced at FA levels between 0.1 and 10 mg/L (Anthonisen *et al.*, 1976). The inhibition of FNA to *N. eutropha* was much weaker than to *Nitrobacter*; e.g., the activity of *Nitrobacter* was reduced by 40% while there was no distinct influence on the activity of *N. eutropha* when the nitrite concentration was over 1400 mg/L. In addition, the activity of *Nitrobacter* was totally inhibited at FNA level ranges between 0.22 and 2.60 mg/L (Sharma and Ahler, 1977).

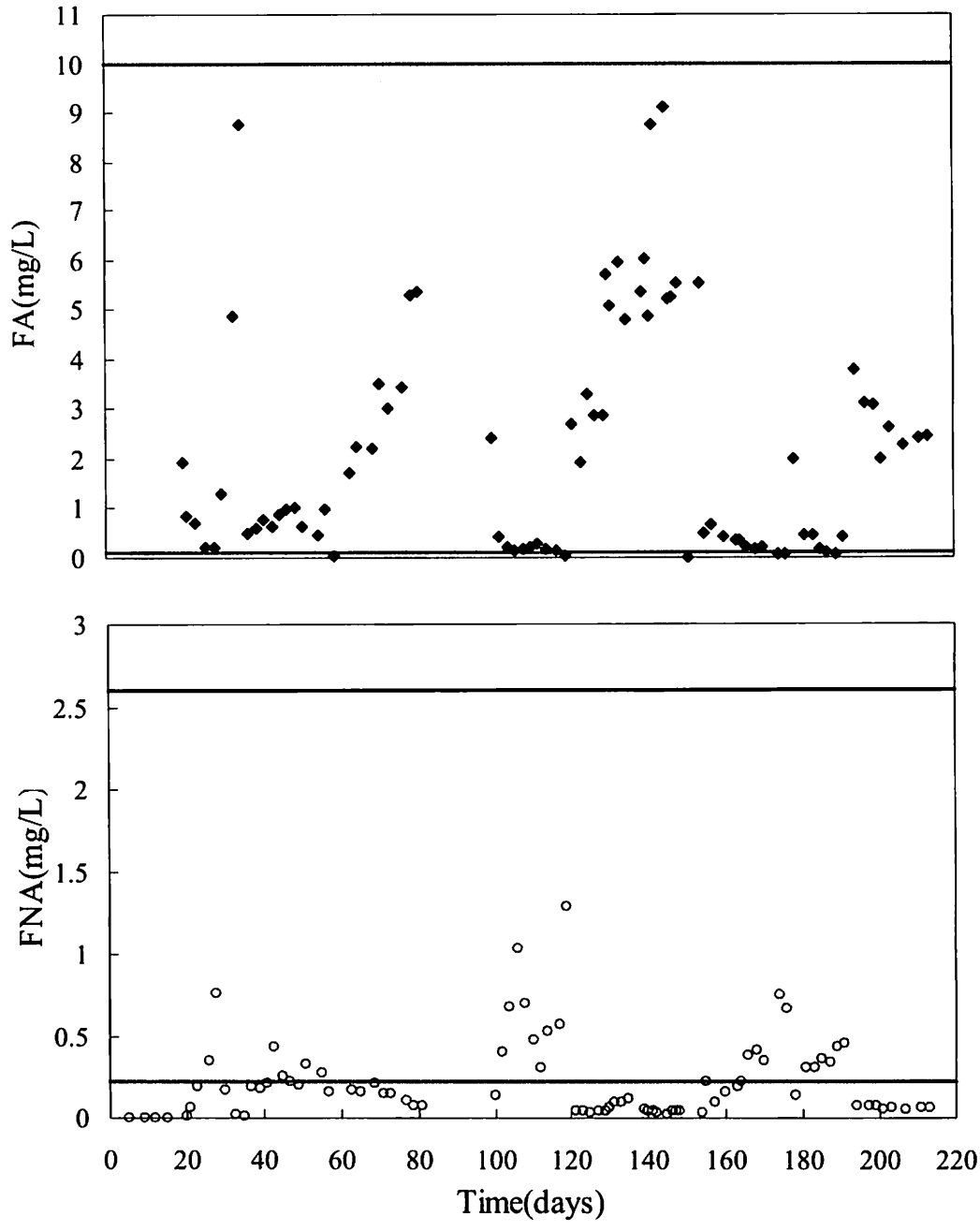


Fig. 4- 7 Time courses of FA and FNA concentrations in PN reactor

◆FA ○FNA

Fig. 4-7 shows FA and FNA fluctuations over time as calculated based on Eqs. 6-7. In the initial 20 days, high FA concentrations suppressed the activity of both AOB and NOB, therefore the effluent nitrite levels were very low. In the subsequent 140 days, FA concentrations fluctuated between 0.1 and 10 mg/L, which appeared to inhibit the NOB activity successfully and facilitate the accumulation of nitrite even without pH, DO and temperature control. The detailed mechanisms for FA inhibition to NOB activity are still not clear. Vadivelu *et al.* deduced that it may be due to a direct inhibitory effect of FA on the

nitrite oxidoreductase, or an enzyme involved in the electron transport or proton translocation. However, inhibition of FA on ATP production (with the use of proton motive force) may result in a similar effect (Vadivelu *et al.*, 2007). It was also assumed that the hydroxylamine formed by ammonia oxidizers might suppress the growth of nitrite oxidizers, though no nitrate formation was observed (Stüven and Bock, 2001).

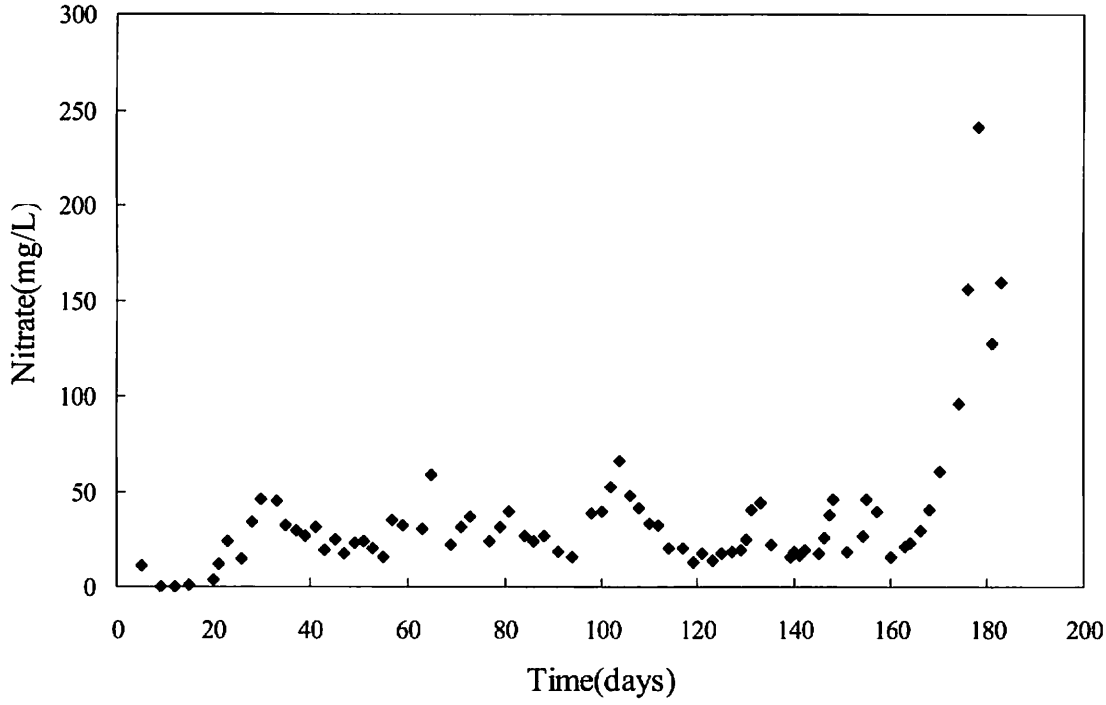
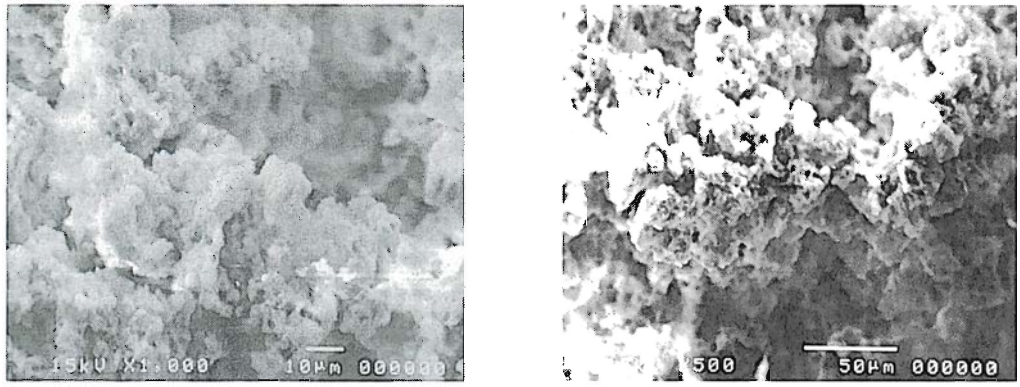


Fig. 4- 8 Time course of effluent nitrate concentrations in PN reactor

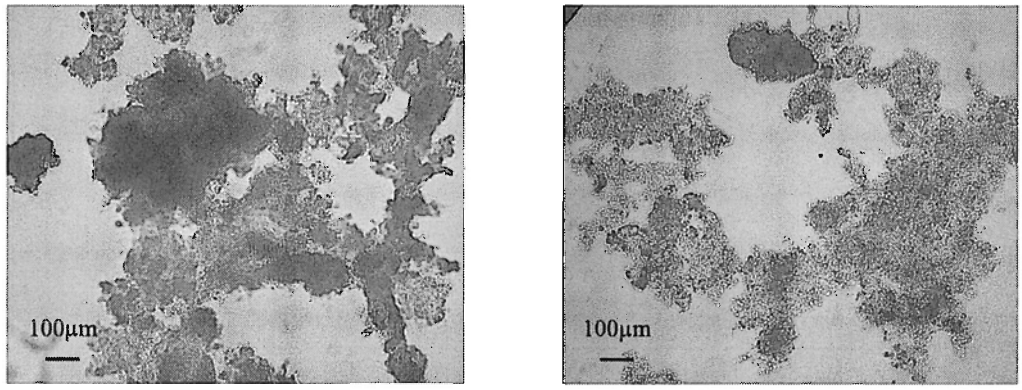
Fig. 4-8 shows the course of the effluent nitrate concentrations in the PN reactor. From the beginning to day 160, nitrate was at a low level with a high value of only 66 mg/L due to the inhibition effects of the NOB by FA and FNA. Later, a longer HRT (HRT of 9 h) supplied enough reaction time to the NOB and improved their growth. Simultaneously, the nitrification reaction without pH control resulted in lower pH values (the lowest of 6.43) in the PN reactor, which further caused FA levels to decrease below 0.1 mg/L and FNA to increase above 0.2 mg/L. AOB activity was gradually inhibited by the changes of FA and FNA levels in the PN reactor. From day 170, the effluent nitrate levels increased gradually and reached a peak value of 262 mg/L on day 178. Later, pH control was applied (pH of 7.55) again in order to increase the FA concentration and further inhibit the NOB activity. Although the effluent nitrite levels increased, the effluent nitrate maintained a relative high level of over 100 mg/L.

4.3.4 Biomass morphology



(a) (b)

Fig. 4- 9 SEM observation of attached biomass



(a) (b)

Fig. 4- 10 Microscopic observation of biomass in PN reactor

The seed sludge was conventional activated sludge, cultured in our laboratory using synthetic wastewater. Initially, the sludge showed light brown color, and then the color changed to black due to humic substances. Fig. 4-9 shows the SEM photos of attached biomass on day 130 indicating a large amount was retained on the support materials. The detached sludge still agglomerated together and formed dense flocs, improving the sludge settling capacity with low SVI values (in Fig. 4-3). Microscopy observations of the dense flocs in the PN reactor are shown in Fig. 4-10. The EPS content accounted for 8.7% of the MLVSS, which consisted of proteins, polysaccharides and nucleic acids at 6.1%, 2.7% and 0.002%, respectively. Extraceluller protein was the dominant component of the EPS contents compared with the other two substances. The high extraceluller protein level was considered facilitate activated sludge congregating and dense flocs formation (Rouse *et al.*, 2004; Cheng *et al.*, 2007).

4.4 Conclusions

After about 200 days of operation, partial nitritation was successfully achieved under suitable operational conditions. The PN reactor effluent nitrite/ammonium ratio reached 1.9 at a NLR of 3.2 kg-N/m^3/d with a temperature of 35℃ and pH of 7.55. Even without pH, temperature and DO control, an effluent nitrite/ammonium ratio of 0.8 was observed at the same NLR. When effluent from the PN reactor was introduced into the anammox reactor, an average T-N removal efficiency of 78% was obtained in the annmmox reactor at a NLR of about 1.8 kg-N/m^3/d despite fluctuations in the PN effluent nitrite/ammonium ratio. FA and FNA have critical effects on partial nitritation performances, which were utilized to effectively inhibit NOB activity in a suitable concentration range. SEM observations showed a lot of sludge attached on the support materials and that detached biomass agglomerated together and formed dense flocs, improving sludge settling capability.

4.5 References

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Chapter 5 Conclusions and recommendations

5.1 Conclusions

Anammox process, as a new alternative to traditional nitrification-denitrification technique, with novel acrylic fiberbiomass carriers (Biofill and Biofix) showed strong potential for nitrogen contained wastewater treatment. According to the experimental results obtained in this study, the following conclusions were summarized:

1. In the first part of this study, the novel acrylic biomass carriers of Biofill were applied for anammox research. T-N removal efficiency of 73% was observed under a NLR of 2.0 kg-TN/m^3/d. Temperature decrease did not show much impact on anammox activity. Even at a temperature of 15.3℃, T-N removal rate of 48% was achieved. Anammox activity recovered by increasing the operational temperature, e.g., T-N removal efficiency increased to 64% after one day with increasing operational temperature to 23℃. The attached biomass on Biofill was calculated to be 0.14 g-TSS/cm^3 of Biofill, and SEM observation showed that majority of anammox biomass was attached to the biomass carriers. DNA analyses supplied two close matches of anammox bacteria identified as KSU-1 and KU2.

2. In the second part of this study, two types of biomass carriers, Biofix and nonwoven, were utilized for investigating potential of anammox process under different operational conditions. Although the nonwoven reactor showed high nitrogen removal potential with short acclimation, satisfactory TN removal performances were obtained for Biofix reactor at a temperature of 25℃. The T-N removal efficiency of 81.3% was achieved at a NLR of 3.6 kg-N/m^3/d for Biofix reactor at a temperature of 25℃. On the other hand, T-N removal efficiency of 86.3% was obtained for nonwoven reactor at a NLF of 4.0 kg-N/m^3/d and temperature of 35℃. EPS analyses and SEM observation revealed that the the anammox granular sludge in the Biofix reactor was in a more compact state than that in the nonwoven reactor. DNA analyses not only found

anammox bacteria, but also revealed four unknown sequences. The four kinds of sequences had the same functions of consuming the influent DO or the residual nitrite and nitrate in the solution. The symbiosis with other bacteria in anammox sludge could supply a suitable cultural condition for anammox bacteria, even in the macro aerobic environment.

3. Since partial nitritation (PN)-Anammox combined process has the competitiveness to conventional techniques for nitrogen removal treatment, the combined process was applied for ammonia removal of anaerobic digester sludge liquor of municipal sewage treatment plant. At a temperature of 35℃ and pH of 7.55, effluent nitrite concentrations were about 1.9 times higher than PN effluent ammonium concentrations at a NLR of 3.2 kg-N/m^3/d. At the same NLR, the effluent nitrite/ammonium ratio of 0.8 was obtained without pH, temperature and DO control. Average T-N removal efficiency of 70.3% was achieved after PN reactor effluent was introduced into the anammox reactor with the same flow rate. FA and FNA were proved to have the function of inhibiting AOB and NOB activities at suitable ranges. EPS data showed that extracelullar protein was the main component in the activated sludge, which could facilitate detached biomass agglomeration and formation of dense flocs, further improving the sludge settling capability.

5.2 Recommendations

In order to further understand and examine the applicability of anammox process for nitrogen control in wastewaters, the following recommendations are made:

1. All the reactor configurations in this research were identical, i.e., of the up-flow column type fixed bed biofilm configuration. Other configurations could be used to demonstrate if reactor has any effect on the performance of anammox process. For example membrane reactor, SBR, and fluidized bed with granular anammox sludge may be tested.

2. Application of actual wastewater to anammox process, instead of synthetic wastewater, is recommended so as to examine various aspects of reactor start-up, reactor performance, inhibition kinetics, and operational and maintenance problems.

3. Concerning the PN-Anammox combined process, further work is recommended to determine the effects of FA and FNA on AOB and NOB activities. Moreover, optimum operational conditions of PN process need to be investigated. Also, the possibility of PN reaction without temperature and pH control could be further investigated.

Appendix: Publication related to this dissertation

1. **Qiao S.**, Cheng Y.J., Liu Z.J., Kawagoshi Y., Fujimoto A., Koyama T., Furukawa K., 2007, Anammox treatment potential in an up-flow column reactor using a novel acrylic fiber biomass carrier, Japanese Journal of Water Treatment Biology, **43(1)**, 31-41.

2. **Qiao S.**, Hata K., Cheng Y.J., Inatomi Y., Nishiyama T., Fujii T., Koyama T., Furukawa K., 2007, Comparison of anammox treatment performances using different kinds of biomass carriers, Japanese Journal of Water Treatment Biology (accepted).

3. Cheng Y.J., Watanabe Y., **Qiao S.**, Koyama T., Furukawa K., 2006, Comparison of treatment capacity of swim-bed technology and conventional activated sludge process for domestic wastewater treatment, Japanese Journal of Water Treatment Biology, **42(3)**, 129-137.

4. Cheng Y.J., Yazaki D., **Qiao S.**, Watanabe Y., Koyama T., Kawakami N., Furukawa K., 2007, Excess sludge reduction and biomass characteristics in swim bed wastewater treatment process, Japanese Journal of WaterTreatment Biology, **43(2)**, 73-82.

5. **Qiao S.**, Cheng Y.J., Koyama T., Furukawa K., 2007, Application of partial nitrification and Anammox process for anaerobic digester liquor treatment, Japanese Journal of Water Treatment Biology (submitted).

6. **Qiao S.**, Furukawa K., Koyama T., 2006, Studies on the start-up of anammox process using net type acrylic fiber biomass carrier Presentation at the Annual Meeting 2006 of Japanese Society of Water Treatment Biotechnology, Sendai (Japan), November, **26**, 26 (日本水処理生物学会大会講演要旨集)

7. 部分亜硝酸化を用いた消化脱離液の処理に関する研究
   土木学会西部支部研究発表会講演概要集 2007, 3, 877-878.
   喬 森、 小山 登一郎、 古川 憲治

8. 部分亜硝酸化/Anammox による都市下水場嫌気性消化脱離液の窒素除去
   日本水環境学会シンポジウム、2007, 9, 169-170.
   古川 憲治、 喬 森