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Rapid start-up of anaerobic ammonium oxidation  
(anammox) process for nitrogen removal from wastewater

**MUHAMMAD ALI**

**Supervisor: Professor Satoshi OKABE**

Division of Environmental Engineering  
Graduate School of Engineering, Hokkaido University  
Doctor Dissertation  
March 2015

# Rapid start-up of anaerobic ammonium oxidation (anammox) process for nitrogen removal from wastewater

Muhammad Ali

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Engineering

Examination Committee: Prof Satoshi OKABE (Supervisor)  
Prof Naoyuki FUNAMIZU (Member)  
Prof Masahiro TAKAHASHI (Member)

Division of Environmental Engineering  
Graduate School of Engineering, Hokkaido University  
March 2015

## ABSTRACT

The dissertation is associated with the field of biological wastewater treatment and more precisely focus on nitrogen removal from wastewater. The nutrients removal (mainly N and P) from wastewater is necessary in order to avoid the eutrophication of the surface waters. Nitrogen compounds ( $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) removal is commonly performed by means of biological processes due to the lower cost as compared to chemical treatment. The conventional nitrogen removal process consists of two steps, nitrification and denitrification (N&DN). During nitrification, ammonium is oxidized to nitrite by ammonia oxidizing bacteria (AOB), then nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). In denitrification process, heterotrophic denitrifying bacteria reduce nitrate to nitrogen gas by utilizing organic carbon as an electron donor under anoxic condition. However, this process is not the most appropriate to treat  $\text{NH}_4^+$ -rich wastewater streams. While treating  $\text{NH}_4^+$ -rich wastewaters by N&DN process, more oxygen is required and an external carbon source has to be added that result in high operational cost.

Autotrophic nitrogen removal by anaerobic ammonium oxidizing (anammox) bacteria is an alternative to conventional nitrogen removal process for treatment of  $\text{NH}_4^+$ -rich wastewater streams. This biological process was first discovered in nitrifying reactor about two decade ago in Netherlands. Anammox process oxidize ammonium, with nitrite as an electron acceptor, to dinitrogen gas under anoxic condition. Currently, this process is being used for the treatment of reject water from anaerobic digesters and industrial wastewaters with high  $\text{NH}_4^+$  concentrations and low organic matter content. Anammox process has several advantages over conventional N&DN process *e.g.* high nitrogen removal rates (NRRs), less oxygen demand, no external carbon source required, less sludge production rate, and less/no greenhouse gas ( $\text{N}_2\text{O}$ ) production etc.

However, growth rate of anammox bacteria is extremely slow and takes about one to two weeks to get double. Slower growth rate of anammox bacteria results in longer start-up period of anammox treatment plant. This doctoral endeavour was to lessen start-up period of anammox process. In order to achieve the rapid and stable start-up of anammox process sufficient amount of active anammox biomass is required. In order to secure enough seeding biomass, inoculation with highly active preserved anammox biomass could be one of possible solutions. Chapter three of the dissertation focuses on simple, rapid and effective preservation and reactivation of anammox bacterium "*Candidatus Brocadia sinica*". Subsequently, chapter four presents innovative immobilization technique for anammox bacteria to achieve rapid and successful start-up of anammox process. In order to shorten start-up period of anammox process it was essential to understand ecophysiology of anammox bacteria. Chapter five highlights the physiological characteristics of anammox bacterium related to genus "*Ca. Jettenia*" one of the six proposed genera in the taxonomic group of anammox. Further, this chapter describes proteomics and cellular metal analysis of the same genus. Finally, chapter six details conclusions and recommendations drawn during doctoral endeavour.

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# 1. INTRODUCTION

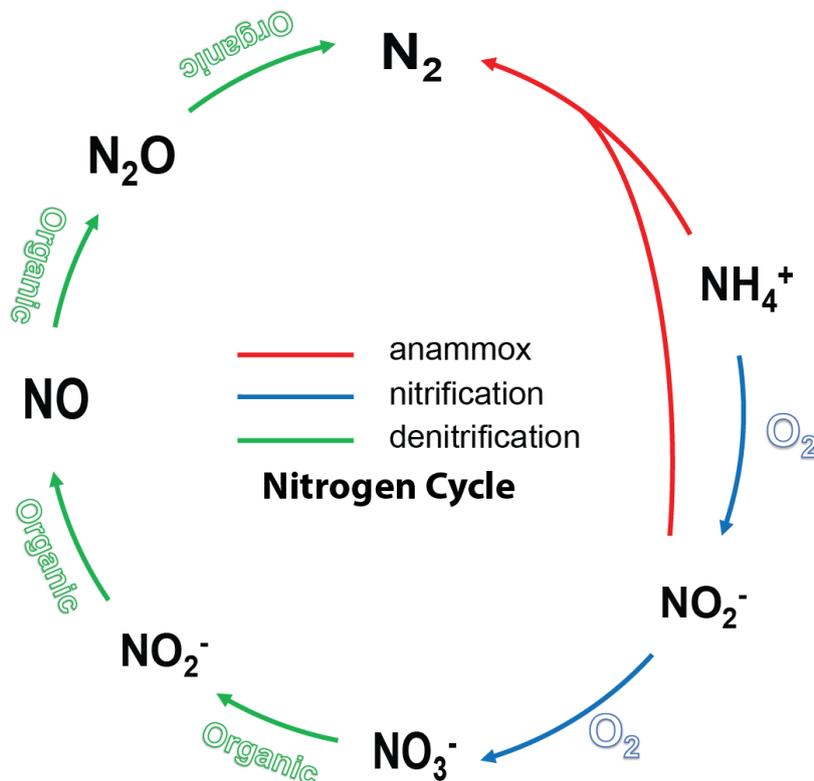
## 1.1. Background

About 70 per cent of the earth surface is cover with water. However, only less than one per cent of the water on the earth is available for human consumption. This small amount of water satisfy agricultural, industrial, recreational and domestic needs of 7.2 billion people of the world. According to the United Nations, over one billion people do not have access to safe water while about two and half million people still lack access to improved sanitation (UNESCO, 2009). This precious natural resource is being contaminated by the discharge of untreated wastewaters. In particularly, excessive release of nitrogen to the natural waters can lead to eutrophication, which can in turn encourage the overgrowth of weeds and algae. The algae numbers are unsustainable and eventually most of them die. The decomposition of the algae by bacteria uses up so much of the oxygen in the water that most or all of the animals die, which creates more organic matter for the bacteria to decompose. In addition to causing deoxygenation, some algal species produce toxins that contaminate drinking water supplies.

To ensure sustainable wastewater discharge to natural waters, it should be treated and nitrogen should be removed before release to environment. Anaerobic ammonium oxidation (anammox) process is more economical and environmental friendly as compared to conventional nitrification and denitrification (N&DN) process (**Fig. 1.1**). About two decade ago, biologists assumed that the microbial nitrogen cycle was essentially completed. However, discovery of anammox bacteria open new avenue and indicated that our understanding about N-cycle microorganisms was still limited (Strous et al., 1999). Further, genome sequencing of several co-culture through next generation sequencing (Strous et al., 2006) provided examples that the enormous biodiversity hidden in various natural and man-made ecosystems. It was revealed that anammox process is responsible for more than 50% nitrogen removal in ocean (Brandes et al., 2007; Long et al., 2013; Zhu et al., 2013). The contribution of the process to  $N_2$  production is however strongly site specific and range between a few per cent in some shallow water sediments (Trimmer et al., 2003) to almost 100% in the water column at the Benguella upwelling region (Kuypers et al., 2005). In addition to global nitrogen contribution, anammox process was considered to be effective and economical treatment method for  $NH_4^+$ -rich wastewaters and was first applied for the treatment of reject water produced from sludge treatment facility in Rotterdam, The Netherlands (van der Star et al., 2007).

However, integration of anammox process into wastewater treatment plant is still a challenge because of extremely slow growth rate of anammox bacteria and long startup period. First full-scale anammox treatment plant took three and half year to start-up and under normal circumstances, later took more than three months to start-up if inoculated with sufficient anammox biomass (Ni and Zhang, 2013). My main motivation was to develop an economical, durable and efficient immobilization technology for anammox bacteria which can enable rapid start-up of anammox process in wastewater treatment plant. Overall this endeavor was a unique

combination of microbiology and process engineering, focused on developing the next generation of wastewater treatment system.



**Fig. 1.1:** The turnover of nitrogen compounds in the biosphere (nitrogen cycle)

## 1.2. Research Objective

The main goal of this endeavor was to reduce the start-up time of anammox process. In order to achieve this goal various research objectives were established as mentioned followings:

### **I. Investigate simplest, rapid and effective methods for preservation of anammox bacteria;**

This experiment was aimed to develop a long-term preservation technique for mass storage of anammox biomass at practical scale.

### **II. Reactivation of preserved anammox bacteria;**

Preserved anammox biomass was reactivated in up-flow column reactors by immobilizing in polyvinyl alcohol (PVA) and sodium alginate (SA) gel.

### **III. Determination of optimum amount of inoculum required for rapid start-up of anammox process;**

Influence of biomass content on start-up period and performance of anammox process was investigated. Further, comparison of nitrogen removal performances between naturally aggregated and artificially immobilized anammox biomass was performed.

### **IV. Physiological characterization of anammox bacterium “Ca. Jettenia caeni”**

We cannot achieve rapid start-up of anammox process unless ecophysiology of anammox bacteria was explored. Anammox bacterial cells related to genus “Ca. Jettenia” was enrichment in membrane

bioreactor. Phylogenetic analysis revealed that enriched biomass was related to anammox genus “*Ca. Jettenia*” and named as “*Ca. Jettenia caeni*”. Physiological characteristics of enriched anammox culture of “*Ca. Jettenia caeni*” were investigated. Besides, proteomics and cellular metal analysis were also performed for the same enrichment culture to further investigate biochemistry of this anammox bacterium.

### **1.3. Structure of Thesis**

#### **1.3.1. Chapter 1**

This chapter states dissertation overview and broader research questions that were addressed during doctoral endeavour. Further, detail research objectives were outlined in this chapter.

#### **1.3.2. Chapter 2**

This chapter presents systematic review of previous studies focusing on practical application of anammox process. It was revealed during literature review that there are more than 114 reported anammox full-scale plants around the world and the number is increasing rapidly. However, still there are many problems in application of anammox process at full-scale *e.g.* longer start-up period, limited mainstream application and poor effluent water quality. The literature review aims to present a comprehensive summary of researches on anammox microbiology, ecophysiology and full-scale application potentials, problems and prospects. Beside, an integrated wastewater treatment system was proposed for sustainable and energy generating wastewater treatment.

#### **1.3.3. Chapter 3**

It was revealed in literature review that it is still the biggest challenge to secure enough seeding biomass for rapid start-up of full-scale anammox process due to slow growth. Preservation of active anammox biomass could be one of the solutions. This chapter focuses on long term preservation and reactivation of anammox bacterium, “*Ca. Brocadia sinica*”. Main aim of this study was to develop simple, rapid and effective preservation and reactivation technique that can be applied for mass scale storage. It was revealed that anammox biomass can be stored for up to 150 d at room temperature in simple nutrient medium. Furthermore, the 90-d-stored biomass was successfully reactivated in up-flow column reactors by immobilizing in PVA and SA gel.

#### **1.3.4. Chapter 4**

In previous experiment, we found that artificial immobilization of anammox biomass can help early start-up of anammox process. However, no guideline was available on optimum content of anammox biomass inside immobilized biomass. This chapter focuses on the determination of optimum biomass concentration for immobilization to ensure rapid start-up of the process. In this study, six column reactors (10-ml volume) were operated containing artificially immobilized biomass with different biomass concentrations in order to determine threshold biomass concentration for start-up of anammox process. Further, this study focused on

performance comparison between naturally and artificially aggregated biomass. Further, mechanism of higher nitrogen removal by immobilized biomass was studied with fluorescence *in-situ* hybridization, microelectrode profiles of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , and spatial distribution of  $^{13}\text{C}$ -bicarbonate incorporation by anammox bacterial cells. It was revealed that core of granular biomass was not metabolically active whereas anammox bacterial cells present in the core of immobilized biomass were metabolically active.

### 1.3.5. Chapter 5

In order to expedite start-up of anammox process, it was essential to understand ecophysiology of this bacteria. Since discovery of anammox bacteria (in 1990s), six candidate genera have been proposed for taxon of anammox, and numerous studies have been conducted to understand their ecophysiology. This study focused on the physiological characterisation of anammox bacterium related to genus “*Ca. Jettenia*”. In this study the ecophysiology of anammox bacterium “*Ca. Jettenia caeni*” (previously known as “*Ca. Planctomycete KSU-1*”) was characterised. In addition, proteomic and cellular trace metal analysis was performed to examine underlying core metabolisms and corresponding metal cofactors. This study was a unique combination of physiological characteristics, proteomics analysis and cellular trace metal analysis that helped to further understand the anammox bacterium “*Ca. Jettenia caeni*”.

### 1.3.6. Chapter 6

In this chapter findings of previously conducted studies were compiled and recommendations for future prospective were highlighted.

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## 2. LITERATURE REVIEW

Excess release of nitrogen into waters has become a severe environmental problem. Conventionally, biological nitrification and denitrification (N&DN) process is being used to remove nitrogen from wastewaters. Anammox is a biological process capable of anaerobic transformation of  $\text{NH}_4^+$  to dinitrogen gas with  $\text{NO}_2^-$  as an electron acceptor (Kartal et al., 2013). Anammox process proved better substitute of conventional N&DN, due to 60% decrease in oxygen demand, 100% decrease in carbon source demand and less/no  $\text{N}_2\text{O}$  (global warming potential GWP 310) production (Graaf et al., 1995; Kartal et al., 2008; Kuenen, 2008; Okabe et al., 2011). Above and beyond, 90% less sludge is generated in anammox process as compared to conventional N&DN process, which results in less recurring cost (Strous et al., 1999b).

Nitrogen removal from wastewaters by anammox process is reliant on partial oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$ . About half of the  $\text{NH}_4^+$  is oxidized to  $\text{NO}_2^-$  through partial nitrification and subsequently remaining  $\text{NH}_4^+$  is oxidized with  $\text{NO}_2^-$  to nitrogen gas by anammox process under anoxic condition, this combination often known as partial nitrification and anammox (PN&A). In beginning, PN&A process were applied separately in two stages, first stage partial nitrification process and followed by anammox process in second stage. Later partial nitrification and anammox processes were introduced in single stage reactor (Third et al., 2001).

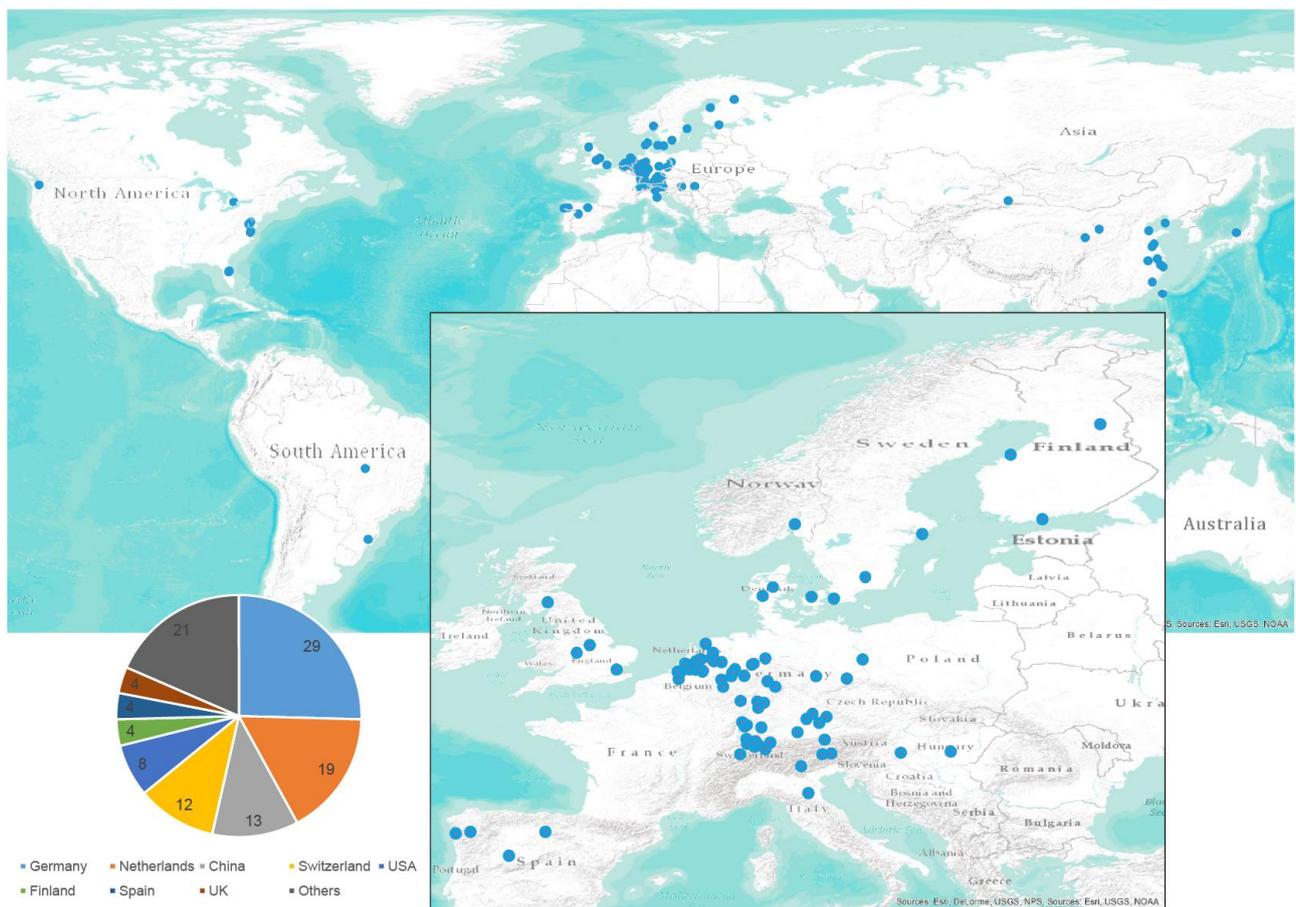
Anammox activities and/or bacterial 16S rRNA gene have been detected in various natural ecosystems, such as anoxic environments including marine sediments, freshwater and terrestrial ecosystems, and manmade ecosystems, such as constructed wetlands, wastewater treatment plants (WWTPs) and petroleum reservoirs (Ali et al., 2013). Anammox process is known to be a major contributor of nitrogen loss in these ecosystems (Kartal et al., 2010).

The biggest hurdle for the application of anammox process is the slow growth rate (maximum specific growth rate,  $\mu_{\text{max}}$ , ranged between  $0.0020 \sim 0.0041 \text{ d}^{-1}$ ; doubling time,  $T_{1/2}$ , ranged between  $7 \sim 14$  days), causing slow start-up of the process at full-scale (Ali et al., 2014a; Awata et al., 2013; Cho et al., 2011; Oshiki et al., 2011; Park et al., 2010b; Schmid et al., 2005; Schmidt et al., 2003; Strous et al., 1998; van der Star et al., 2007). In order to secure enough seeding biomass, inoculation with preserved and/or immobilized anammox biomass could be one of the possible solutions. Recently, a simple, economical and effective methodology was successful developed for long-term (up to five months) storage of anammox biomass in simple nutrient medium with addition of molybdate at room temperature, which would expedite the practical use of anammox process for wastewater treatment (Ali et al., 2014b).

In this chapter, ecophysiology and application of anammox process for wastewater treatment was comprehensively reviewed. In addition, an integrated wastewater treatment system has been proposed for domestic sewage for net energy-positive wastewaters treatment through coupling of high rate anammox process with microbial fuel cell (MFC).

## 2.1. Application of anammox process

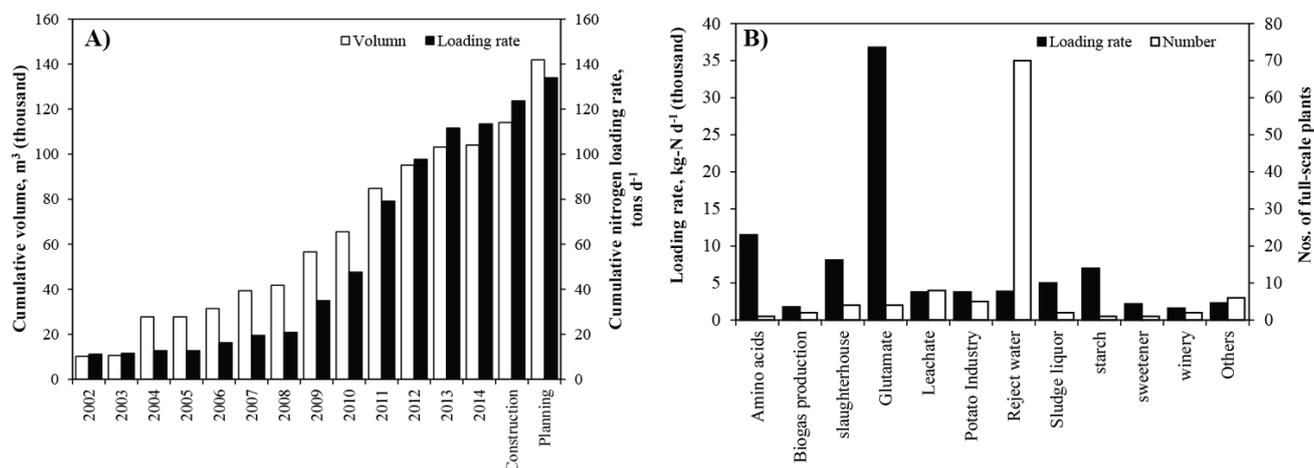
To our knowledge, there are 114 (including ten under construction and eight at design phase) reported full-scale anammox installation around the world (personal communication with Adriano Joss and Paques Inc.) and (Lackner et al., 2014). Overwhelming majority of anammox plants, about 88 out of 114, were constructed in Europe and the rest were established in China, North America, and rest of the world as shown in **Fig. 2.1**. In total, more than 142,000 m<sup>3</sup> volume capacity of anammox treatment plants are already in system to cater for 134 tons per day of nitrogen load and the number is increasing rapidly while looking at the installations under planning, design and construction (**Fig. 2.2A**). Overwhelming majority of these full-scale treatment plants have been applied to treat reject water streams generated from sludge treatment facilities. Though, in terms of nitrogen loading rates (NLRs), mainly anammox treatment process have been treating industrial wastewaters generated from slaughterhouse, amino acids and glutamate industries etc. (**Fig. 2.2B**). Nevertheless, application of anammox process for mainstream treatment of domestic wastewaters is still scarce.



**Fig. 2.1:** Geographical distribution of full-scale anammox plants around the world.

The first full-scale anammox reactor was established for the treatment of reject water at Waterboard Hollandse Delta, in Rotterdam, Netherland and took 3.5 years to achieve 90 ~ 95% removal of the nitrogen load corresponding to conversion rate of 0.25~0.50 kg-N m<sup>-3</sup> d<sup>-1</sup> (Abma et al., 2007). Recently, there are many

examples of start-up (in three month) of full-scale anammox plants (Ni and Zhang, 2013). The main challenges for applying anammox process to the main wastewater stream are high C/N ratio and low temperature wastewaters, longer start-up period, and poor effluent water quality. Recently, some researchers demonstrated the ability of anammox bacteria to grow at mainstream conditions at low temperature (10~20°C) (Gilbert et al., 2014; Lotti et al., 2014). New dimension of anammox process has been explored and would further accelerate its application for nitrogen removal from domestic wastewater streams.



**Fig. 2.2:** Treatment capacities and number of current full-scale anammox treatment plants. A) Cumulative volume and nitrogen loading rate (NLR) of full-scale anammox treatment plants over period of time. B) NLR and number of full-scale anammox treatment plants treating wastewater from various sources. These figures were prepared after compiling the information published in (Lackner et al., 2014) and the information obtained through personal communication with Andiano Joss (Eawag, Swiss Federal Institute for Aquatic Science and Technology, Switzerland) and Maxime Remy (Paques Inc. The Netherlands).

### 2.1.1. Reactor configurations

Initially, two-stage anammox reactor configurations was developed. Now focus has been shifted mainly to single-stage configuration due to its less capital cost and footprint (personal communication with Paques Inc.). About 90% full-scale anammox installations were realized as single stage reactor. There were mainly two formations of single-stage nitrogen removal reactor *e.g.* Completely Autotrophic Nitrogen-removal Over Nitrite (CANON) (Third et al., 2001) and Oxygen Limited Autotrophic Nitrification Denitrification (OLAND) (Windey et al., 2005). Current full-scale implementations include the moving bed biofilm reactor (MBBR), granular sludge reactor and sequential batch reactor (SBR). The SBR technology is the most commonly applied reactor type (more than 50% of all PN&A systems) followed by granular systems and MBBRs (Lackner et al., 2014).

### 2.1.2. Operating conditions

Various operating parameters including influent and effluent water quality of 23 full-scale anammox plants are listed in **Table 2.1**. Volumetric NLR varies between 0.1 ~ 7.0 kg-N m³ d⁻¹. While, typical range of NLR used for single-stage PN&A reactor is 2~2.4 kg-N m³ d⁻¹ (personal communication with Dr Maxime Remy, Process Specialist, Paques Inc. Netherland). Usually single-stage reactors are operated under micro-aerobic conditions. Typical range of DO in single-stage reactor is 0.2~1.5 mg-O₂ L⁻¹. Optimum operating temperature

of anammox process is 37°C, however anammox bacteria can also synchronize at low temperature environment (Gilbert et al., 2014; Z. Hu et al., 2013; Isaka et al., 2008a; Winkler et al., 2012) even anammox activity was observed at 4°C during batch experiment (Oshiki et al., 2011).

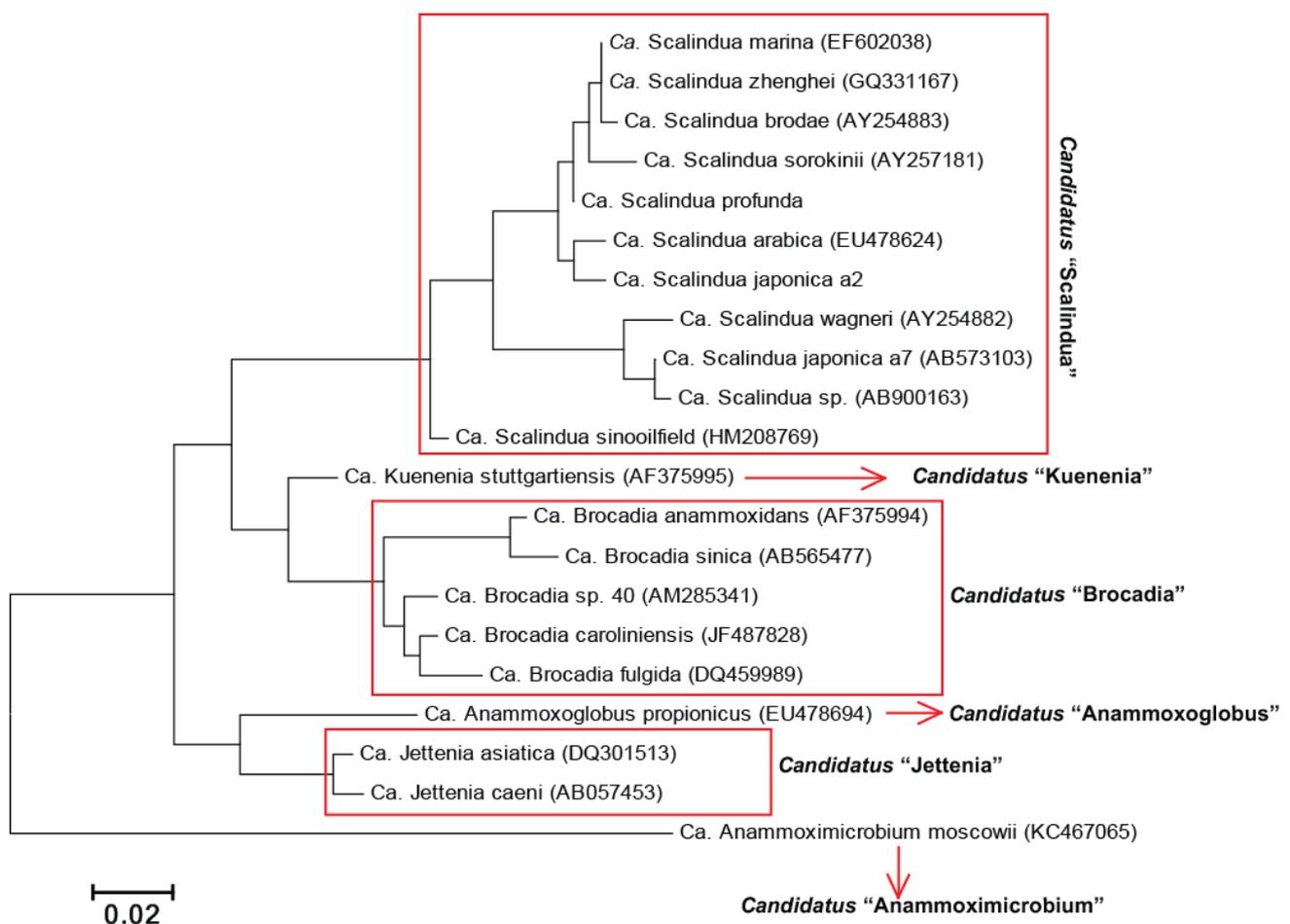
Anammox process is suitable for the treatment of NH<sub>4</sub><sup>+</sup>-rich wastewater streams and influent ammonium concentrations range between 500 ~ 3000 mg-N L<sup>-1</sup> (Table 1). Only concern while feeding high ammonium to PN&A reactor is free ammonia (NH<sub>3</sub>) concentration which is known to be inhibitor for aerobic ammonium-oxidizing bacteria (AOB) and anammox bacteria (Jaroszynski et al., 2012; Li et al., 2012). Reactor configuration, operating conditions and design can be tailored to accommodate ultrahigh NH<sub>4</sub><sup>+</sup> loading rates in the treatment system. Another, very important parameter is COD/N ratio which ranged between 0.2~3.8. Almost, every kind of wastewaters contain organic carbon and particularly typical wastewater has high COD/N ratio 10~20 (Metcalf&Eddy, 2003) and some of the organic compound completely/partially inhibit anammox process (Ali et al., 2014a; Guven et al., 2005; Oshiki et al., 2011). At this moment, one of the biggest concern in application of anammox process at mainstream wastewater treatment is high COD/N ratio and poor effluent quality, which is clearly depicted in **Table 2.1**. For mainstream application, post-treatment is essential to polish effluent water quality. Up to now full-scale anammox treatment plant has not been applied at mainstream for domestic sewage and even for industrial sewage application effluent from anammox treatment plant directed to conventional WWTP for further polishing. However, while using anammox process as side stream poor effluent quality is not concern because effluent would be recirculated back into WWTP.

## 2.2. Ecophysiology and niche partitioning of anammox bacteria

Anammox bacteria are affiliated with the bacterial phylum *Planctomycetes* (Strous et al., 1999a). Anammox activities and/or anammox bacterial 16S rRNA gene sequences have been ubiquitously detected from anoxic environments including marine, freshwater and terrestrial ecosystems, where the anammox process significantly (up to almost 100%) contributed to nitrogen loss (Brandes et al., 2007; Z. Y. Hu et al., 2013; Long et al., 2013). To date, about nineteen species and broadly six genera have been identified in the taxonomic group of anammox bacteria (**Fig. 2.3**). There is no pure culture of any anammox species, however, several anammox bacterial cultures were enriched as monospecies in laboratory by using different culturing techniques *e.g.* rotating biological contactor (Egli et al., 2001), SBR (Strous et al., 1998), up-flow column reactor (Oshiki et al., 2011) and membrane bioreactor (MBR) (Ali et al., 2014a; Awata et al., 2013; van der Star et al., 2008b). Anammox bacterial species “*Ca. Jettenia caeni*” (Ali et al., 2014a), “*Ca. Brocadia sinica*” (Oshiki et al., 2011), “*Ca. Scalindua japonica*” (Awata et al., 2013), “*Ca. Kuenenia stuttgartiensis*” (van der Star et al., 2008b) were reported to be enriched with more than 90% purity.

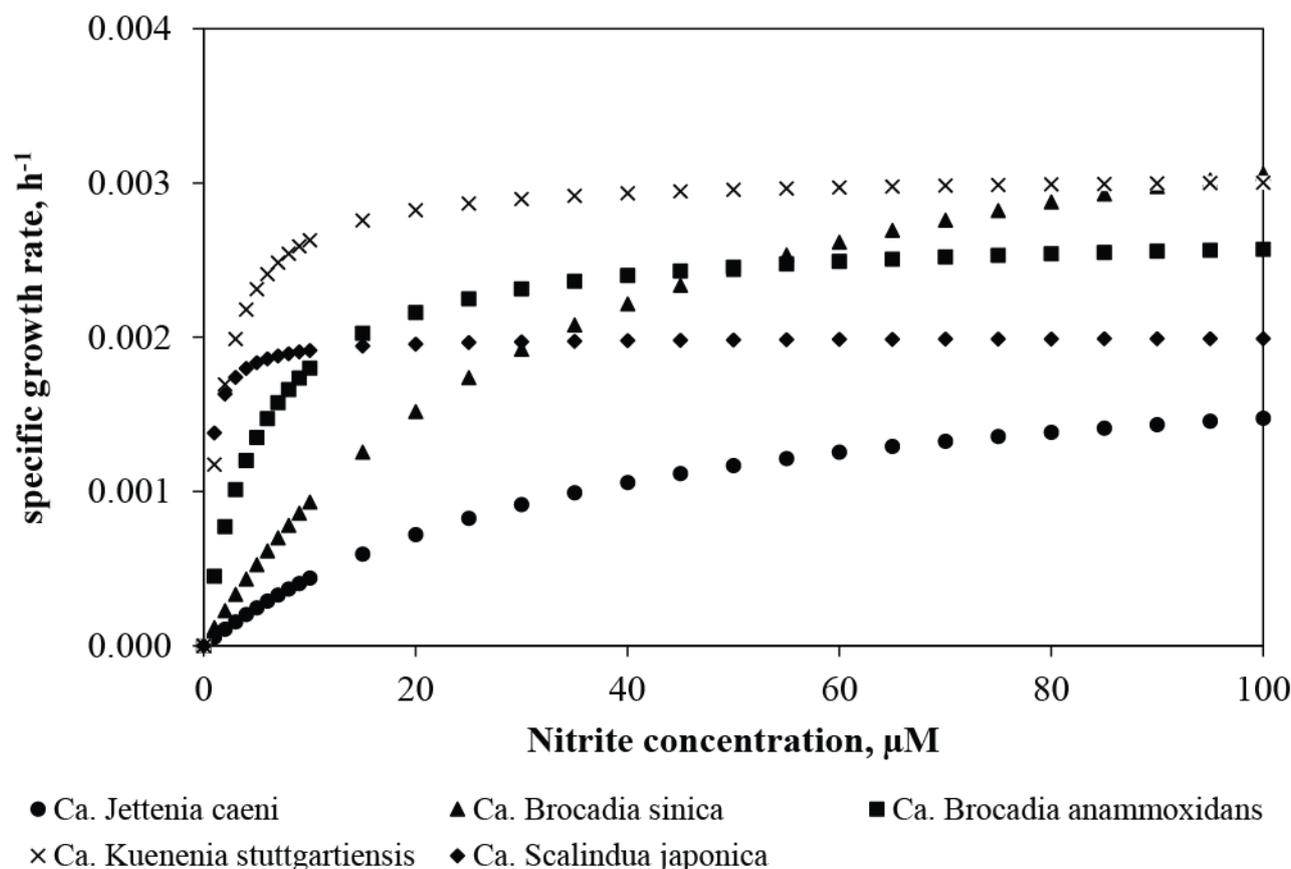
Now, comprehensive datasets of physiological characteristics, genome sequences, and proteome are available for the genera “*Ca. Kuenenia*”, “*Ca. Brocadia*”, “*Ca. Scalindua*”, and “*Ca. Jettenia*”. Growth kinetics and other physiological parameters are presented as **Table 5.1** (see chapter five) for the anammox bacteria affiliated with the genus “*Ca. Jettenia*”, “*Ca. Brocadia*”, “*Ca. Kuenenia*” and “*Ca. Scalindua*”. The datasets are

useful to explore niche differentiation among these anammox bacteria in natural and man-made ecosystems. While enriching anammox as monospecies culture, one anammox species outcompeted with coexisting bacterial species present in the parent inoculum (Huang et al., 2013; Park et al., 2010b; van der Star et al., 2008b, 2007). Each anammox bacterial strain has a dissimilar physiology and their abundance in the eco-systems depend on growth kinetics and tolerance to various environmental conditions. For instance, anammox bacteria affiliated with the genus “*Ca. Brocadia*” and “*Ca. Kuenenia*” are possibly growth-rate and affinity strategists, respectively, and thus the latter can overgrow under the conditions where  $\text{NH}_4^+$  and/or  $\text{NO}_2^-$  concentrations are low (Ding et al., 2013; van der Star et al., 2008b). Specific growth rate of various anammox species were simulated and presented as a function of substrate concentration (**Fig. 2.4**). This growth curve were simulated by using the Monod equation and nitrite concentration was used as a limiting substrate. Growth and competition between anammox species could be predicted by using these simulated growth curves. However, growth kinetic parameters are not the only key factor for determination of population dynamic in any ecosystem. There may be various other parameters involved in the population dynamic of mixed anammox culture in any ecosystem e.g. salinity, sulfide, temperature, organics concentrations etc.



**Fig. 2.3:** Biodiversity of anammox bacteria. 16S rRNA gene sequences of various anammox bacteria were retrieved from National Center for Biotechnology Information (NCBI) database and aligned by using the ClustalW program (version 1.83). Phylogenetic tree was constructed using MEGA 6.06 software by the neighbor joining (NJ) (Poisson

model with 1,000 iterations) method by using 16S rRNA gene. GenBank accession numbers are indicated in brackets. Scale bar represents 2% sequence divergence.



**Fig. 2.4:** Simulated specific growth rate of various anammox bacterial species on different nitrite concentration (while nitrite was considered as limiting substrate). These specific growth rates were simulated by using monad equation,  $\mu = \mu_{max} \frac{S}{K_s + S}$ , where  $\mu$  is the specific growth rate;  $\mu_{max}$  is the maximum specific growth rate;  $S$  is the concentration of the limiting substrate (nitrite was taken as limiting substrate);  $K_s$  is the apparent half-saturation constant for nitrite. The values of  $\mu_{max}$  and  $K_s$  for respective anammox bacterial strain were taken from **Table 5.1**.

Metadata of over 6000 anammox 16S rRNA gene sequences revealed that the global distribution of anammox bacteria is mainly governed by salinity (Sonthiphand et al., 2014). Anammox activity of “*Ca. Scalindua sp.*” was lost under absence of salinity (Awata et al., 2013). Conversely, anammox activity of “*Ca. Jettienia caeni*” was very sensitive to salinity; *e.g.*, The half maximal inhibitory concentration ( $IC_{50}$ ) was 68 mM (chloride) salinity, depicting that the “*Ca. Jettienia caeni*” is likely a freshwater strain (Ali et al., 2014a) and almost all the 16S rRNA gene sequences affiliated with the genus “*Ca. Jettienia*” were retrieved from freshwater ecosystems (Sonthiphand et al., 2014). Successive changes of anammox bacterial community structures with salinity gradient have been described from the Yodo River (Amano et al., 2007) and Cape Fear River estuaries (Dale et al., 2009).

Similarly, it was observed that sulfide also influenced geographical distribution anammox activity as anammox activity in anoxic water column of the Black Sea disappeared under the presence of micro-molar concentration ( $< 6 \mu\text{M}$ ) of sulfide (Jensen et al., 2008). The  $IC_{50}$  of sulfide has been examined using different enrichment cultures of anammox bacteria to be in ranges of 30 to 300  $\mu\text{M}$  (Carvajal-Arroyo et al., 2013; Dapena-

Mora et al., 2007; Jin et al., 2013) whereas  $IC_{50}$  of “*Ca. Jettenia caeni*” was higher than these values; i.e., 540  $\mu$ M (Ali et al., 2014a).

Temperature also influence anammox activity and abundance *e.g.* marine anammox bacteria affiliated with the genus “*Ca. Scalindua*” has optimum growth temperature around 25°C whereas maximum growth of freshwater anammox bacterial species were observed at about 37°C.

The “*Ca. Anammoxoglobus propionicus*” and “*Ca. Brocadia fulgida*” were known to oxidize propionate and acetate for reduction of  $NO_3^-$ , respectively; and dominate under constantly availability of propionate or acetate (Kartal et al., 2008, 2007). Generally, anammox bacteria were known to be inhibited by high organic concentration particularly methanol which was considered to be universal inhibitor for anammox bacteria. Methanol has been regarded as a strong inhibitor for anammox bacteria, and addition of 0.5 mM (Güven et al., 2005) or 3.3 mM methanol (Jensen et al., 2007) completely inhibited anammox activities of enrichment culture and marine sediments, respectively. However, anammox activity of the “*Ca. Jettenia caeni*” cells was not strongly inhibited by addition of 1 mM methanol (Ali et al., 2014a). On the other hand, high tolerance to methanol was found in enrichment cultures constituted of “*Ca. Kuenenia stuttgartiensis*” and “*Ca. Jettenia caeni*” cells, where addition of 5 mM methanol decreased only 29% of anammox activities (Isaka et al., 2008b). Nonetheless, practical evidences are required to further nail down ecological niche partitioning of anammox bacteria in various ecosystems and vital to study microbial competition among various anammox bacterial strains during long-term cultivation.

### 2.3. Preservations of anammox biomass

Inoculation of sufficient amount of active anammox biomass is essential to achieve the rapid and stable start-up of anammox process for wastewater treatment. However, it is difficult to cultivate and maintain a large amount of active anammox biomass in advance. In order to secure enough seeding biomass, inoculation with preserved anammox biomass could be one of possible solutions. Around 38 preservation techniques were investigated for the long-term storage of various anammox bacterial species under different conditions (**Table 2.2**). Previously, anammox activity was inhibited after two-month storage, at -20°C, of anammox biomass harvested from an OLAND reactor (Vlaeminck et al., 2007). Further, it was reported that pre-freezing with liquid nitrogen (-200°C) was required for long-term preservation of anammox bacteria via lyophilization (Rothrock et al., 2011). Anammox bacteria immobilized in PVA were preserved for short period of 17 h at -8 °C (Magrí et al., 2012). A cryopreservation protocol was also introduced for long-term storage of planktonic anammox bacterial cells by dimethyl sulfoxide (DMSO) as a cryoprotective agent (CPA) (Heylen et al., 2012). However, lyophilization and cryopreservation seem impracticable at mass scale due to complex procedures and costly chemicals. Recently, a simple, rapid and effective preservation technique (at room temperature) was introduced for anammox bacterial species “*Ca. Brocadia sinica*”, that is feasible at a practical scale (Ali et al., 2014b). Storage in nutrient medium containing 3 mM of molybdate at room temperature with periodical (every 45 days) supply of  $NH_4^+$  and  $NO_2^-$  was proved to be the most effective storage technique for “*Ca. Brocadia*

sinica” biomass. Furthermore, preserved biomass was successfully reactivated by immobilizing in PVA and SA gel and achieved NRR of  $7 \text{ kg-N m}^{-3} \text{ d}^{-1}$  within 35 days of operation. Combination of preservation and immobilization of anammox biomass would help in acceleration of anammox process application at full-scale WWTP. However, this technique should be test to other phylogenetically different anammox bacterial species before mass scale application.

## 2.4. Immobilization of anammox biomass

Integration of anammox process into conventional wastewater treatment system is still hindered due to its slow growth rate (Awata et al., 2013; Isaka et al., 2006; Jetten et al., 1999; Oshiki et al., 2011; Strous et al., 1998), resulting in longer start-up period of anammox process (Park et al., 2010a; Schmid et al., 2005; van der Star et al., 2007). In addition, washout of biomass due to the production of  $\text{N}_2$  gas is notorious in anammox processes (Chen et al., 2010; Dapena-Mora et al., 2004; Strous et al., 1998), which causes more long-term operation for initiation of anammox process. To account for these issues, immobilization of anammox biomass offers a potential for the improvement of anammox process efficiency. The gel immobilization of anammox biomass has following advantages; i.e., the continuous process can be carried out under high loading rate without washing out of anammox bacterial cells, it is easy to separate liquid and solid phase in the reactor (Isaka et al., 2006; Vogelsang et al., 1999). Gel entrapment techniques have been used to immobilize autotrophic nitrogen cycle bacteria *i.e.* nitrifying bacterial cells (Isaka et al., 2011, 2008a; Kimura et al., 2013) and anammox bacterial cells (Ge et al., 2009; Isaka et al., 2008b, 2007; Magrí et al., 2012; Quan et al., 2011). In these previous studies, anammox bacteria were immobilized in PVA, or SA or mixture of both, or polyethylene glycol (PEG) and inoculated into reactors for treatment of synthetic or real wastewater.

### 2.4.1. Immobilization techniques

Primarily, three different kinds of immobilization techniques were used to entrap anammox bacterial cells for wastewater treatment (**Table 2.3**). Initially, anammox biomass was immobilized by PEG (10%, w/v) and tetramethylenediamine (0.5%, w/v), later to start polymerization potassium per sulfate was added (Isaka et al., 2007). Then, PVA (7.5%, w/v) and SA (1.0%, w/v) solution was used to entrap anammox biomass and  $\text{NaNO}_3$  (50%, w/v) and  $\text{CaCl}_2$  (2%, w/v) solution was used for solidification of gel beads (Quan et al., 2011). Recently, a much simpler technique was introduced to immobilize anammox biomass by immobilization solution containing PVA (3%, w/v) and SA (1%, w/v) then just  $\text{CaCl}_2$  (2%, w/v) solution was used for solidification (Ali et al., 2014b). Another immobilization technique, in which anammox biomass was simply mixed in PVA (10%, w/v) solution, was used and for solication of immobilized biomass it was frozen at  $-8^\circ\text{C}$  for 17 hours (Magrí et al., 2012). However, anammox activity was reversibly inhibited by freezing at  $-8^\circ\text{C}$  for 17 hours. A study was conducted to evaluate immobilization with PVA, SA and combination of PVA and SA and found that immobilized anammox biomass entrapped in solution of PVA and SA was more rigid and effective for nitrogen removal than immobilization only with PVA or SA (Zhu et al., 2014).

#### 2.4.2. *Anammox biomass*

It is also pertinent to mention that different researchers used different anammox bacterial strains for immobilization *i.e.* “*Ca. Brocadia caroliniensis*” (Magrí et al., 2012), “*Ca. Jettenia caeni*” (Furukawa et al., 2009; Isaka et al., 2011, 2008a, 2007; Kimura et al., 2013; Quan et al., 2011; Zhu et al., 2014) and “*Ca. Brocadia sinica*” (Ali et al., 2014b). Phylogenetically different anammox strain may behave different under different immobilization conditions. A study should be conducted on the behaviour of phylogenetically different anammox bacterial strains immobilized with various immobilization technique. Such comparison may give us an opportunity for selection of appropriate anammox bacterial strain and immobilization techniques for practical use.

Previously, various anammox biomass concentrations ranging from 0.5 g-volatile suspended solids (VSS) L<sup>-1</sup> to 3.8 g-VSS L<sup>-1</sup> were employed for immobilization (**Table 2.4**). However, no clear recommendations were available on biomass concentration in immobilized biomass for quick start-up of anammox reactors. Optimum and efficient use of anammox biomass can reduce required inoculum amount for practical application. Therefore, optimum biomass concentration inside immobilized biomass needs to be investigated for general guideline and application of immobilization technique in full-scale treatment systems.

#### 2.4.3. *Performance evaluation*

Bench- or pilot-scale reactors containing immobilized anammox biomass were reported to have significant NRRs ranging from 0.5 to 10 kg-N m<sup>-3</sup> day<sup>-1</sup> under different operating conditions (**Table 2.3**). Previously, 8.2 kg-N m<sup>-3</sup> day<sup>-1</sup> of NRR was reported after 100 days of operation (Quan et al., 2011). Similarly, about 4 kg-N m<sup>-3</sup> day<sup>-1</sup> of NRR were observed in a very short period of 1 ~ 2 months while treating real wastewater (Furukawa et al., 2009; Isaka et al., 2011). However, nitrogen removal performance comparison between immobilized anammox biomass and naturally aggregated granular biomass is still required to convince practitioners and designer of full-scale anammox plant. This information would be very essential for use of immobilized biomass for practical application.

#### 2.4.4. *Practical application of immobilized biomass*

Recently, a Japanese company, Hitachi Plant Service Co. Ltd. established first two stage full-scale anammox plant (volume of anammox reaction: 100m<sup>3</sup>) for treatment of wastewater from a local chemical industry. Both AOB and anammox bacteria were immobilized separately into a high-molecular-weight polymer and 3-mm cube gel carriers were prepared. Later gel carriers were inoculated separated into nitrification and anammox reaction tanks. This process was expected to treat 500 m<sup>3</sup> d<sup>-1</sup> NH<sub>4</sub><sup>+</sup> rich (700 mg-N L<sup>-1</sup>) stream with 80% nitrogen removal efficiency. Successful application of this immobilized anammox biomass would open a new era for using of immobilization technology for autotrophic biomasses for wastewater treatment application. Above all information was retrieved from homepage of Hitachi Plant Service Co. Ltd.

## 2.5. Greenhouse gas emission

Nitrous oxide (N<sub>2</sub>O) is a strong greenhouse gas (GHG) with high GWP of about 310. Although, global N<sub>2</sub>O emissions accounts for only about 0.03% among all GHGs but its impact on global warming has been estimated up to 10% (Bates, 2008). According to IPCC guidelines value of 0.5% of the nitrogen load as an estimate of the N<sub>2</sub>O emission from wastewater, regardless of whether the nitrogen is converted in WWTPs or in effluent-receiving rivers and estuaries (Eggleston et al., 2006).

N<sub>2</sub>O emissions is growing concern during biological treatment of sewage, manure and industrial wastewaters in general and biological nitrogen removal processes in particular (Desloover et al., 2012). Various studies reported N<sub>2</sub>O production from biological nitrogen removal processes at bench- and full-scale WWTPs (**Table 2.4**). Large variations in the N<sub>2</sub>O emissions were reported in lab-scale WWTPs (0–95% of nitrogen load) and full-scale WWTPs (0–14.6% of nitrogen load) (Kampschreur et al., 2009). Both nitrification and denitrification processes can lead to emission of N<sub>2</sub>O. However, N<sub>2</sub>O emissions are extremely variable and depend on many operational parameters such as dissolved oxygen (DO) and nitrite (NO<sub>2</sub><sup>-</sup>) concentrations in both nitrification and denitrification stage (Itokawa et al., 2001; Kampschreur et al., 2008; Park et al., 2000) and carbon availability (low COD/N ratio) in the denitrification stage (Itokawa et al., 2001; Park et al., 2000).

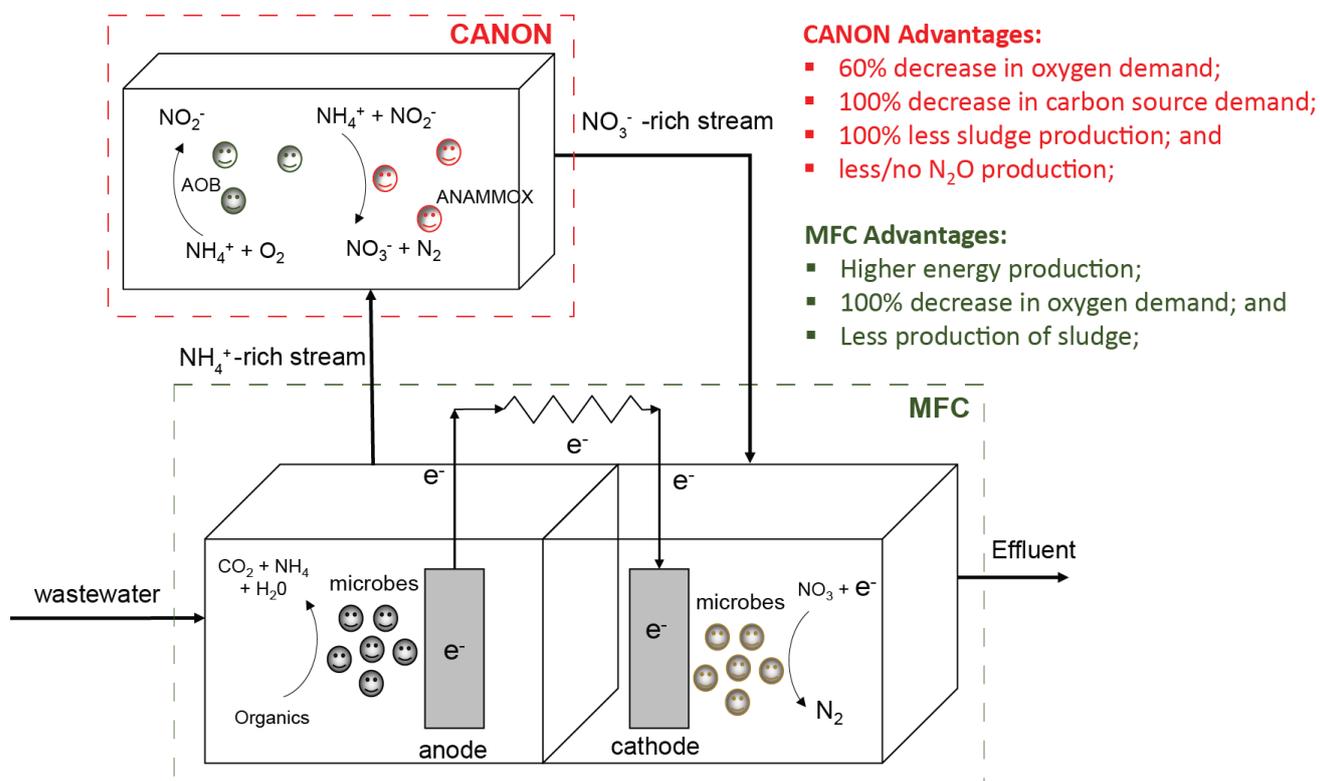
Aerobic partial nitrification (PN) granules, containing heterotrophic denitrifiers in the core, were reported to emit 5.6% N<sub>2</sub>O of NLR (Ishii et al., 2014). On the other hand, autotrophic partial nitrification reactors were reported to emit averagely 2.5% N<sub>2</sub>O (0.8~6.1%) of NLR (Graaff et al., 2010; Joss et al., 2009; Kampschreur et al., 2008; Kong et al., 2013; Okabe et al., 2011; Rodriguez-Caballero and Pijuan, 2013). Averagely 0.27% N<sub>2</sub>O production were reported from anammox reactors (Desloover et al., 2012; Kampschreur et al., 2008; Okabe et al., 2011). However, it was confirmed that anammox bacteria was not the source of N<sub>2</sub>O by using heterotrophic denitrifiers specific inhibitor (penicillin G) (Okabe et al., 2011).

## 2.6. Mainstream application of anammox process

Mainstream application of anammox process for domestic wastewater is still a challenge needs to be addressed thoroughly in further studies. Recently, a study demonstrated the feasibility of mainstream application of anammox process for nitrogen removal from domestic sewage containing synthetic nitrite to support anammox process (Lotti et al., 2014). This research has open many avenues for mainstream application of anammox process. Researchers should investigate some feasible integrated wastewater treatment system for mainstream application and better effluent quality. Earlier it was considered that sewage treatment is an energy intensive process. Only in US, wastewater treatment accounts for around 3% of total electricity demand (William F. Owen, 1982). However, nowadays researchers are considering domestic wastewater treatment as a potential energy resource (McCarty et al., 2011).

Previously, it was demonstrated that anammox process could reduce energy requirement from 44 Wh p<sup>-1</sup> d<sup>-1</sup> to 21 Wh p<sup>-1</sup> d<sup>-1</sup> by increasing hydraulic retention time (HRT) of primary clarifier to facilitate more biogas

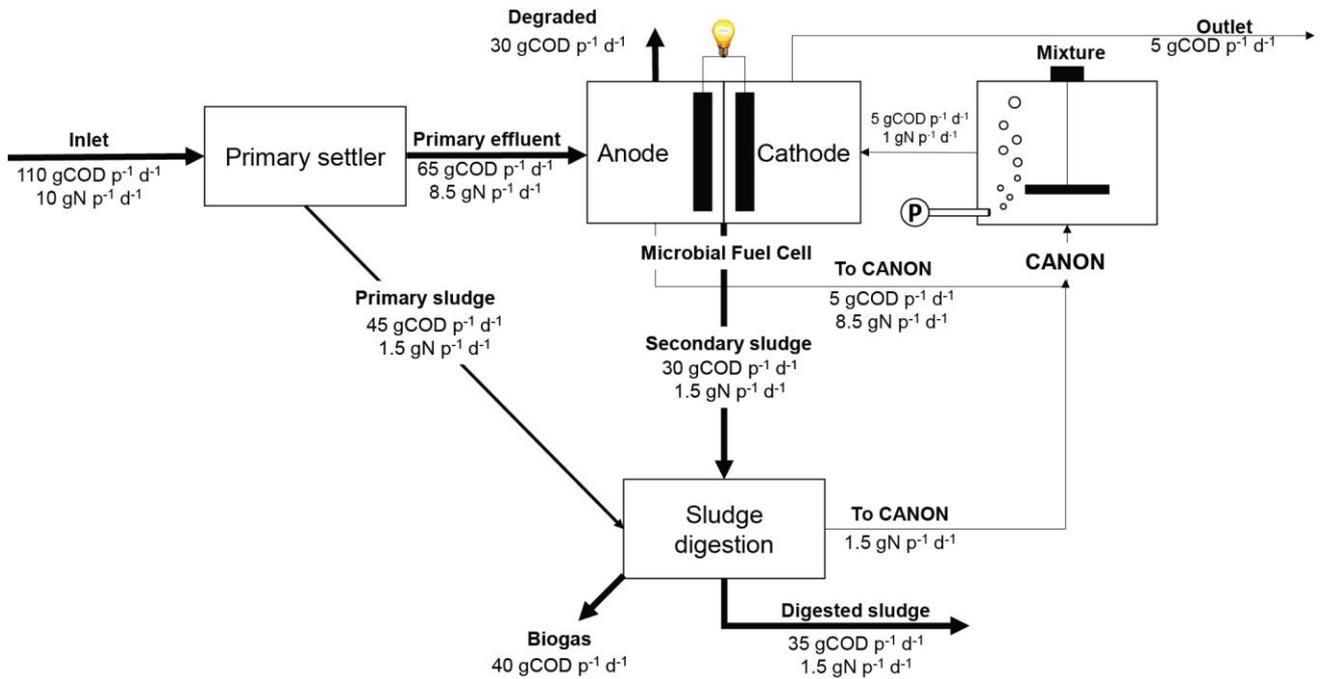
production (Siegrist et al., 2008). Lower HRT of primary clarifier was provided to deliberately bypass organic loading to aeration tank to aid heterotrophic denitrification. Nevertheless, introduction of autotrophic nitrogen removal by PN&A allowing us to increase HRT of primary clarifier to recover more biogas. Further, it was established that the wastewater treatment process, coupling anaerobic digestion with anammox process on the mainstream, could yield  $24 \text{ Wh p}^{-1} \text{ d}^{-1}$  (Kartal et al., 2010) and recently it was practically demonstrated by treating effluent of A-stage by anammox process (Lotti et al., 2014).



**Fig. 2.5:** Proposed integrated wastewater treatment system. Microbial fuel cell (MFC) and completely autotrophic nitrogen-removal over nitrite (CANON) reactor is proposed to couple in way that raw wastewater will be introduced to anode chamber of MFC, then effluent from anode compartment ( $\text{NH}_4^+$ -rich stream) will be fed to CANON reactor, further  $\text{NO}_3^-$ -rich stream (CANON reactor effluent) will be treated in cathode compartment of MFC reactor.

We presented an innovative integrated wastewater treatment system for efficient energy recovery and better effluent quality by combining MFC and anammox process (**Fig. 2.5**). Raw domestic wastewater will be introduced to anode compartment of MFC. Microorganisms produce an energy rich biogas in anaerobic digestion from an organic substrate, whereas electrons are produced in the anode compartment of MFC (He and Angenent, 2006). Microorganisms present in anode compartment are known to oxidize electron donors such as carbohydrates, volatile fatty acids, and other organic compounds (Rabaey et al., 2005). Mostly organics will be removed in anode compartment and  $\text{NH}_4^+$  rich (low C/N ratio) effluent will be fed to CANON reactor. CANON reactor capable of  $\text{NH}_4^+$  oxidation under oxic and anoxic condition simultaneously by aerobic ammonium oxidation (AOB) and anammox bacteria, respectively (Vázquez-Padín et al., 2010). Effluent of CANON containing  $\text{NO}_3^-$  will be supplied to the MFC cathode and  $\text{NO}_3^-$  will be reduced to nitrogen gas by bioelectrochemical process (Wang et al., 2009). Mass balance of COD and nitrogen of the proposed integrated

wastewater treatment system presented as **Fig. 2.6**. Energy calculations, shown as **Table 2.5**, depicted that the coupling of MFC with anammox process could yield more energy ( $53 \text{ Wh p}^{-1} \text{ d}^{-1}$ ) as compared to other previously proposed treatment system ( $24 \text{ Wh p}^{-1} \text{ d}^{-1}$ ).



**Fig. 2.6:** Chemical oxygen demand (COD) and nitrogen mass balance of proposed integrated wastewater treatment system for treatment of domestic wastewater. It was assumed that unit COD and nitrogen approaching to wastewater treatment system were 110 g-COD and 10 g-nitrogen per person per day, respectively. Similarly, untreated COD and nitrogen leaving from wastewater treatment system in the effluent and digested sludge were 5 g-COD and 1.5 g-nitrogen per person per day, respectively.

**Table 2.1:** Operational parameters of selected full-scale anammox reactors. Information at S. No. 1 ~ 10 was gathered through personal communication with Andiano Joss (Eawag, Swiss Federal Institute for Aquatic Science and Technology, Switzerland) and rest was compiled from the data mentioned in (Lackner et al., 2014).

S. No.	Location	Reactors [no.]	Volume [m <sup>3</sup> ]	Loading rate [kg-N m <sup>-3</sup> d <sup>-1</sup> ]	DO [mg-O <sub>2</sub> L <sup>-1</sup> ]	Temp. [°C]	Influent			Effluent			
							NH <sub>4</sub> <sup>+</sup> [mg-N L <sup>-1</sup> ]	COD/NH <sub>4</sub> <sup>+</sup>	pH	NH <sub>4</sub> <sup>+</sup> [mg-N L <sup>-1</sup> ]	NO <sub>3</sub> <sup>-</sup> [mg-N L <sup>-1</sup> ]	COD [mg L <sup>-1</sup> ]	pH
1	Bazenheid	2	400	1.00			3050	0.2	9.2				
2	Glarnerland	1	400	0.50		26-27	700 - 900		8.1				7.7
3	Limmattal	2	250		0.2 - 0.3		1100	0.5	8	70			7.6
4	Niederglatt	1	160	0.35		29+/-1	760	3.8	7.7	20	50	70	7.8
5	Schönau	2	330										
6	St. Gallen	2	300	0.72		18 - 30	890	1.2		73	80	325	8
7	Thunersee	1	700	0.29	<0.4	15 - 35	1100		7.9	155	80		6.5
8	Werdhölzli	2	1400	0.89		30+/-3	650	1.0	7.8	30	5	400	7.1
9	Pfannenstiel	1	320	1.25	0.5	28	750						
10	Amersfort	1	780	0.65	0.3		>500	-	-	150	<25	-	-
11	Apeldoorn	1	2400	0.54	0.3		>1000	1.8	6.8	100	50	<1000	6.8
12	Balingen	1	705	0.04-0.11	0.3		>500	1.6	7.1	<100	<50	-	7.1
13	Heidelberg	2	2 x 570	0.2	0.35		1000	-	7.1	<50	50	-	7.1
14	Ingolstadt	2	2 x 560	0.18-0.20	1		1000	0.7	-	150	<100	250	-
15	Nieuwegein	1	450	0.61	0.2		1000	0.6	-	200	<100	-	-
16	Plettenberg	1	134	0.45	0.25		>500	-	7.1	<100	<50	-	7.1
17	Zurich	2	2 x 1400	0.4	0.05		700	0.9	-	<50	<20	-	-
18	Malmo	4	4 x 50	1.0-1.2	1.5		1000	0.7	-	<100	<100	-	-
19	Olburgen	1	600	1.0-2.33	-		<500	1.1	8	<25	<25	<200	8

S. No.	Location	Reactors [no.]	Volume [m <sup>3</sup> ]	Loading rate [kg-N m <sup>-3</sup> d <sup>-1</sup> ]	DO [mg-O <sub>2</sub> L <sup>-1</sup> ]	Temp. [°C]	Influent			Effluent		
							NH <sub>4</sub> <sup>+</sup> [mg-N L <sup>-1</sup> ]	COD/NH <sub>4</sub> <sup>+</sup>	pH	NH <sub>4</sub> <sup>+</sup> [mg-N L <sup>-1</sup> ]	NO <sub>3</sub> <sup>-</sup> [mg-N L <sup>-1</sup> ]	COD [mg L <sup>-1</sup> ]
20	Lichtenvoorde	2	150 + 75	0.89-1, 0.78	1.5	500	2.0	-	25	25	<250	-
21	Landshut	2	288 + 495	1.11, 0.65	-	>1500	0.3	-	750/100	10/50	-	-
22	Rotterdam	2	1800 + 70	0.27, 7.03	-	1000	1.5	-	500/<50	<100	-	-
23	Bergen Op Zoom	4	7920 (2370, 1650, 1600, 2300)	0.1 (average)	0.9	<500	3.7	-	<5	<10	-	-

**Table 2.2:** Preservation techniques for various anammox species. The information was extracted from various studies *i.e.* serial number 1~17 (Ali et al., 2014b), 18~26 (Heylen et al., 2012), 27~32 (Rothrock et al., 2011) and 33~37 (Vlaeminck et al., 2007), and composed into following tabular form.

S. No.	Storage temp.	Biomass type	Anammox species	Preservation media	Storage period (days)	Reactivation (%)
1	-80°C	Granule	Ca. Brocadia sinica	-	45	59
2	-80°C	Immobilized	Ca. Brocadia sinica	-	45	3
3	4°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup>	45	51
4	4°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , Molybdate	90	7
5	4°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , NO <sub>3</sub> <sup>-</sup>	90	14
6	4°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup>	90	4
7	4°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , Molybdate	90	5
8	4°C	Immobilized	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> ,	90	6
9	4°C	Immobilized	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , Molybdate	90	9
10	4°C	Immobilized	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , NO <sub>3</sub> <sup>-</sup>	90	33

S. No.	Storage temp.	Biomass type	Anammox species	Preservation media	Storage period (days)	Reactivation (%)
11	15~25°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> ,	150	25
12	15~25°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , Molybdate	150	65
13	15~25°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , Penicillin G	45	44
14	15~25°C	Granule	Ca. Brocadia sinica	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , NO <sub>3</sub> <sup>-</sup>	150	22
15	-80°C	Granule	Ca. Kuenenia stuttgartiensis	-	150	40
16	-80°C	Planktonic	Ca. Kuenenia stuttgartiensis	-	150	4
17	-80°C	Planktonic	Ca. Scalindua sp.	-	150	1.5
18	-80°C	Granule	Ca. Kuenenia stuttgartiensis	DMSO	203	62
19	-80°C	Planktonic	Ca. Kuenenia stuttgartiensis	DMSO	203	21
20	-80°C	Planktonic	Ca. Scalindua sp.	DMSO	203	22
21	-80°C	Granule	Ca. Kuenenia stuttgartiensis	DMSO, trehalose, TSB	203	63
22	-80°C	Planktonic	Ca. Kuenenia stuttgartiensis	DMSO, trehalose, TSB	203	52
23	-80°C	Planktonic	Ca. Scalindua sp.	DMSO, trehalose, TSB	203	30
24	-80°C	Granule	Ca. Kuenenia stuttgartiensis	DMSO, trehalose	203	60
25	-80°C	Planktonic	Ca. Kuenenia stuttgartiensis	DMSO, trehalose	203	42
26	-80°C	Planktonic	Ca. Scalindua sp.	DMSO, trehalose	203	36
27	- 60°C	Biofilm	Ca. Brocadia caroliniensis	Skim milk + glycerol	120	18
28	- 60°C	Biofilm	Ca. Brocadia caroliniensis	Skim milk	120	17
29	- 60°C + Lyophilization	Biofilm	Ca. Brocadia caroliniensis	Skim milk + glycerol	120	7 ~ 11
30	- 60°C + Lyophilization	Biofilm	Ca. Brocadia caroliniensis	Skim milk	120	7 ~ 11

S. No.	Storage temp.	Biomass type	Anammox species	Preservation media	Storage period (days)	Reactivation (%)
31	-200°C + Lyophilization	Biofilm	Ca. Brocadia caroliniensis	Skim milk + glycerol	120	7 ~ 11
32	-200°C + Lyophilization	Biofilm	Ca. Brocadia caroliniensis	Skim milk	120	7 ~ 11
33	-20°C	OLAND	<i>n.d.</i>	-	150	<i>n.d.</i>
34	-20°C	OLAND	<i>n.d.</i>	Glycerol	150	<i>n.d.</i>
35	4°C	OLAND	<i>n.d.</i>	-	150	55
36	4°C	OLAND	<i>n.d.</i>	Nitrate	150	30
37	20°C	OLAND	<i>n.d.</i>	Nitrate	150	31

*n.d.*; not determined.

**Table 2.3:** Various immobilization techniques used for immobilization of anammox bacteria;

S. No.	Biomass concentration	Dominant anammox species	NRRs [kg-N m <sup>-3</sup> day <sup>-1</sup> ]	Start-up period [day]	Temp. [°C]	Reactor Type	Immobilization material	Stirring speed [rpm]	Packing volume [%]	Reference
1	1.79 g-VSS L <sup>-1</sup>	Ca. Brocadia caroliniensis	0.5	30	33	CSTR	10% PVA	100	20%	(Magrí et al., 2012)
2	3.8 g-VSS L <sup>-1</sup>	Ca. Jettenia caeni	8.2	100	33	CSTR	7.5% PVA + 1% SA	100	30%	(Quan et al., 2011)
3	0.9 g-VSS L <sup>-1</sup>	Ca. Jettenia caeni	4	35	30	CSTR	10% PEG	100	30%	(Furukawa et al., 2009)
4	1.34 g-VSS L <sup>-1</sup>	Ca. Jettenia caeni	3.8	65	30	CSTR	10% PEG	60-70	20%	(Isaka et al., 2011)
5	0.61 g-VSS L <sup>-1</sup>	Ca. Jettenia caeni	6	15	36	CSTR	10% PEG	80	20%	(Isaka et al., 2008a)
6	0.55 g-SS L <sup>-1</sup>	Ca. Jettenia caeni	3.7	67	36	CSTR	10% PEG	80	30%	(Isaka et al., 2007)

S. No.	Biomass concentration	Dominant anammox species	NRRs [kg-N m <sup>-3</sup> day <sup>-1</sup> ]	Start-up period [day]	Temp. [°C]	Reactor Type	Immobilization material	Stirring speed [rpm]	Packing volume [%]	Reference
7	1.34 g-VSS L <sup>-1</sup>	Ca. Jettenia caeni	3.8	65	30	CSTR	10% PEG	60-70	20%	(Kimura et al., 2013)
8	0.10 g-SS L <sup>-1</sup>	Unknown	4.4	100	30	CSTR	10% PVA	Unknown	20%	(Ge et al., 2009)
9	0.32 g-VSS L <sup>-1</sup>	AOB:Anammox=1:2	1.69	180	33~35	CSTR	7.5% PVA + 1% SA	Unknown	20%	(Qiao et al., 2013)
10	0.7 g-VSS L <sup>-1</sup>	Ca. Jettenia caeni	0.58	60	35	SBR	3% PVA + 1% SA	80	40%	(Zhu et al., 2014)
11	1.0 g-VSS L <sup>-1</sup>	Ca. Brocadia sinica	8.03	35	37	UPBR	3% PVA + 1% SA	N/A	70%	(Ali et al., 2014b)

**Table 2.4:** Nitrous oxide production rate observed in various studies during biological nitrogen removal process

S. No.	Reactor Scale	Reactor Type	Nitrogen loading rate (NLR)	The ratio of N <sub>2</sub> O production to NLR	Reference
			[kg-N m <sup>-3</sup> d <sup>-1</sup> ]	[%]	
1	Lab-scale	Aerobic PN Granules	1.22	5.6	(Ishii et al., 2014)
2	Full-scale	Anammox	7.14	0.6	(Kampschreur et al., 2008)
3	Lab-scale	Anammox	11.6±1.2	0.1 ± 0.07	(Okabe et al., 2011)
4	Full-scale	Anammox	0.26	0.1	(Desloover et al., 2012)
5	Full-scale	PN&A	2	1.2	(Kampschreur et al., 2008)
6	Lab-scale	PN&A	0.1315	0.1	(Sliekers et al., 2002)
7	Lab-scale	PN&A	0.30-0.74	0.4-2	(Yang et al., 2013)
8	Lab-scale	PN&A	0.025	2.45	(Z. Hu et al., 2013)
9	Lab-scale	PN&A	0.5	0.41-1.53	(Xiao et al., 2013)

S. No.	Reactor Scale	Reactor Type	Nitrogen loading rate (NLR)	The ratio of N <sub>2</sub> O production to NLR	Reference
			[kg-N m <sup>-3</sup> d <sup>-1</sup> ]	[%]	
10	Full-scale	PN&A	1.5	0.8	(Joss et al., 2009)
11	Pilot-scale	PN&A	0.58-2.7	3.0-6.4	(Clippeleir et al., 2013)
12	Full-scale	PN	2.8	1.7	(Kampschreur et al., 2008)
13	Lab-scale	PN	0.5194	1.9 ± 0.53	(Graaff et al., 2010)
14	Lab-scale	PN	1.2502	1.50 ± 0.22	[120]
15	Lab-scale	PN	2.996	0.80 ± 0.19	(Kong et al., 2013)
16	Lab-scale	PN	2.5	4.0 ± 1.5	(Okabe et al., 2011)
17	Full-scale	PN	0.18-0.22	5.1-6.6	(Desloover et al., 2012)
18	Pilot-scale	PN	0.85	6.1-2.2	(Rodriguez-Caballero and Pijuan, 2013)
19	Lab-scale	PN-SBR	0.994	0.8 ± 0.4	(Rathnayake et al., 2013)
20	Lab-scale	PN-SBR	8	1.0 ± 0.1	(Law et al., 2011)

PN; partial nitrification.

PN&A; partial nitrification and anammox

**Table 2.5:** Calculation of net energy consumption based on mass fluxes for various treatment options

Description	Mass flux (g p <sup>-1</sup> d <sup>-1</sup> )				Energy (Wh p <sup>-1</sup> d <sup>-1</sup> )			
	Case A	Case B	Case C	Case D	Case A	Case B	Case C	Case D
COD removal	40	30	15	30	-40	-30	-15	38
Aeration for nitrogen removal	22	22	16	16	-22	-22	-16	-16
Pumping/mixing energy					-20	-20	-15	-20
Methane/energy production	30	40	55	40	38	51	70	51
Net Energy					-44	-21	24	53
Reference	(Siegrist et al., 2008)		(Kartal et al., 2010)	This study	(Siegrist et al., 2008)		(Kartal et al., 2010)	This study

## 2.7. References

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### 3. PRESERVATION AND REACTIVATION OF ANAMMOX BIOMASS

#### 3.1. Introduction

Anammox has revolutionized treatment of wastewater streams highly concentrated in  $\text{NH}_4\text{-N}$ , due to lower oxygen, no external carbon source demand and less  $\text{N}_2\text{O}$  production (Kartal et al., 2010; Kuenen, 2008; Okabe et al., 2011a; Mulder et al., 1995). On the other hand, integration of anammox process into full-scale wastewater treatment system is still a challenge, due to its slow growth rate with doubling time typically 1-2 weeks (Awata et al., 2013; Jetten et al., 2005; Oshiki et al., 2011; van der Star et al., 2008), causing slow start-up of the process (Cho et al., 2011; Park et al., 2000; Schmid et al., 2005; Strous et al., 1998; Tsushima et al., 2007b; van der Star et al., 2007).

Inoculation of sufficient amount of active anammox biomass is essential to achieve the rapid and stable start-up of anammox process for wastewater treatment. However, it is difficult to cultivate and maintain a large amount of active biomass in laboratory or industry in advance. In order to secure enough seeding biomass, inoculation with preserved anammox biomass could be one of possible solutions. Several techniques are available for the preservation of microbial cultures e.g. refrigeration, freezing ( $-20^\circ\text{C}$  to  $-200^\circ\text{C}$ ), lyophilization, or any combination of these. Although these techniques have been successfully used to preserve the cultures of various micro-organisms, only a few researches have attempted preserving anammox bacteria. Vlaeminck et al. (2007) demonstrated that two-month storage of anammox biomass obtained from an oxygen limited autotrophic nitrification denitrification (OLAND) process at  $-20^\circ\text{C}$  resulted in irreversible inactivation of anammox bacteria. Rothrock et al. (2011) have reported that pre-freezing with liquid nitrogen ( $-200^\circ\text{C}$ ) was required for long-term preservation of anammox bacteria via lyophilization. Recently, Magri et al. (2012) preserved anammox biomass immobilized in PVA gel at  $-8^\circ\text{C}$  for 17 h but anammox activity was severely damaged.

In addition, anammox activity was not accurately evaluated in these researches because only  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  or  $\text{NO}_3^-$  concentration was measured to evaluate the anammox activity. Anammox activity can be evaluated accurately by culturing with  $^{15}\text{N}$ -ammonium and  $^{14}\text{N}$ -nitrite, which produces mixed labeled  $^{14+15}\text{N}_2$ . Production of  $^{14+15}\text{N}_2$  is the true evidence of anammox reaction and distinguished from the heterotrophic denitrification which produces only  $^{14+14}\text{N}_2$  (Kartal et al., 2007). More recently, Heylen et al. (2012) introduced a protocol for long-term cryopreservation of single cell anammox biomass using DMSO as a cryoprotective agent (CPA). Unfortunately, lyophilization and cryopreservation with CPA seems to be impracticable at mass scale due to complex procedures and costly chemicals. Therefore, a simple, effective and economical long-term preservation and storage method of anammox biomass is urgently needed.

The aim of this study was to develop a simple, rapid and effective preservation method for anammox biomass, which is feasible at a practical scale. Granular anammox biomass and anammox biomass immobilized in PVA and sodium alginate (SA) were preserved in various culture media for up to 150 days under  $-80^\circ\text{C}$ ,  $4^\circ\text{C}$  and ambient temperature ( $15\sim 25^\circ\text{C}$ ). In addition, the influence of the periodical nutrient ( $\text{NH}_4^+$  and  $\text{NO}_2^-$ ) supply and replacement of the entire culture media during the preservation periods on the maintenance of anammox

activity was examined. The anammox activity was measured after 45, 90 and 150 days of preservation by determining 1) specific  $^{14+15}\text{N}_2$  production rates and 2) transcription levels of *hzsA* gene encoding hydrazine synthase alpha subunit. Furthermore, anammox biomass preserved for 90 days was immobilized in PVA and SA gel and then inoculated in up-flow column reactors, and the nitrogen removal performances were monitored for 35 days to demonstrate whether the preserved biomass can be reactivated rapidly and used as a seeding biomass.

## 3.2. Materials and Methods

### 3.2.1. Anammox biomass

Granular anammox biomass was harvested from a continuous up-flow column bioreactor being operated in our laboratory for many years (Tsushima et al., 2007a). Operating conditions and other details about the parent reactor were presented previously (Tsushima et al., 2007b). In the granular anammox biomass, an anammox bacterium, “*Ca. Brocadia sinica*”, accounted for more than 90% of total biomass as determined by fluorescence *in-situ* hybridization analysis (Cho et al., 2010; Kindaichi et al., 2007) with AMX820 and EUB mix oligonucleotide probes (Oshiki et al., 2011).

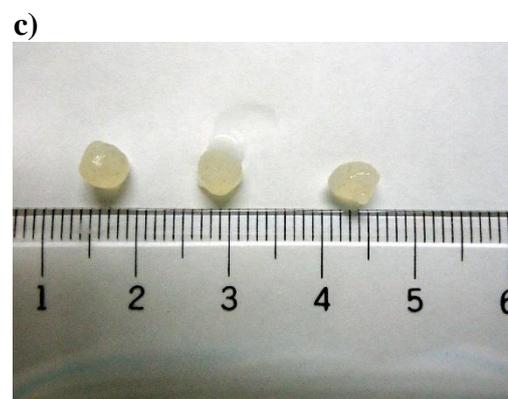
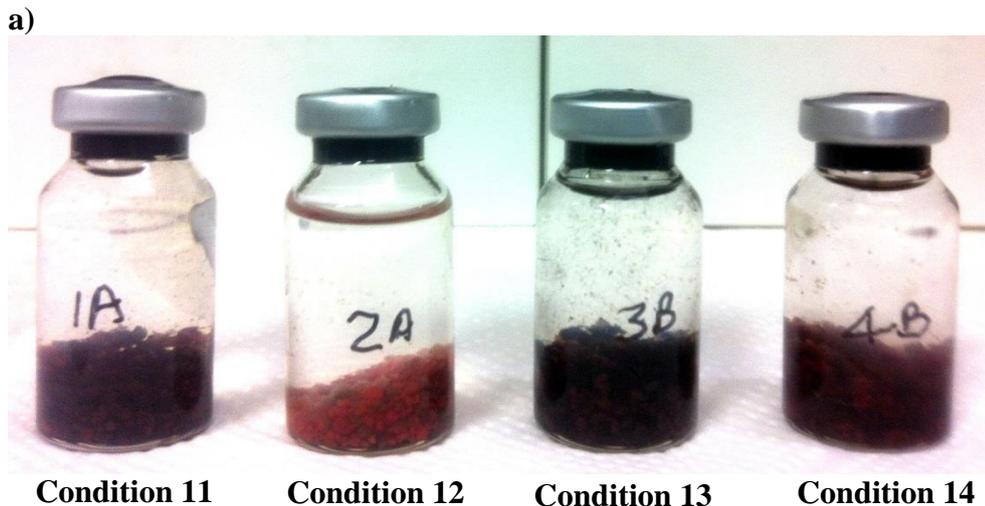
The immobilized anammox biomass was harvested from another up-flow column reactor (volume; 220 ml) (Bio column reactor, Fujisaki, Osaka, Japan) operated at 37°C with continuous supply of inorganic media containing  $\text{NH}_4^+$  and  $\text{NO}_2^-$  (5mM each). Immobilized anammox biomass was prepared from granular anammox biomass as described in section 3.2.3. and inoculated in the up-flow reactor with 70% (v/v) packing ratio. The up-flow column reactor was operated with a hydraulic retention time ranged between 3.0~0.4 h, resulting in the NRR of 6.1 kg-N  $\text{m}^{-3}\text{d}^{-1}$  at 27 days of operation. Immobilized anammox biomass was harvested at 27 days of operation by using a spatula and subjected to preservation experiment.

### 3.2.2. Inorganic media

Inorganic media used in the present study contained;  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{CaCl}_2$  (100 mg  $\text{L}^{-1}$ ),  $\text{MgSO}_4$  (300 mg  $\text{L}^{-1}$ ),  $\text{KH}_2\text{PO}_4$  (30 mg  $\text{L}^{-1}$ ),  $\text{KHCO}_3$  (500 mg  $\text{L}^{-1}$ ) and trace element solution (van de Graaf et al., 1996). Concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were set in the range 0~5 mM. Prepared media was purged by  $\text{N}_2$  for more than 30 min to remove dissolved oxygen.

### 3.2.3. Gel immobilization of anammox bacterial cells

Granular anammox biomass was manually dispersed into floccular biomass (<100  $\mu\text{m}$  diameter) by a mortar-pestle style tissue homogenizer (Thomas Scientific, Swedesboro, NJ, USA) and suspended in inorganic media at concentration of 4.0 g-VSS  $\text{L}^{-1}$ . The biomass suspension was mixed well with an equal volume of immobilization support solution containing PVA (6%, w/v) and sodium alginate (SA) (2%, w/v). The gel beads were prepared by dropping mixed solutions into a solution of  $\text{CaCl}_2$  (4% w/v) by using a peristaltic pump (Eyela Roller Pump RP, Tokyo, Japan) equipped with a Phar Med BPT tube (ID 3.1 mm). Those procedures enabled to form spherical gel beads with average diameter of 4~5 mm (**Fig. 3.1**). The gel beads were cured in the same  $\text{CaCl}_2$  solution for 12 h to enhance physical strength of the gel beads. The prepared immobilized anammox biomass were rinsed with sterilized water three times, and then used for inoculation.



**Fig. 3.1:** a) Granular anammox biomass stored under conditions 11 ~ 14 at room temperature after 90 days. Molybdate (3 mM) was added in the condition 12 to inhibit sulfate reduction. Therefore, the color of biomass stored at the condition 12 for 90 days was red, indicating no FeS formation as a result of sulfate reduction. b) Up-flow column anammox reactors packed with the immobilized anammox biomass that was stored at room temperature for 90 days (at 0 day of operation). The anammox biomass stored at condition 11 for 90 days was immobilized in the PVA-SA solution (PVA 6% w/v; SA 2% w/v) and then inoculated in Reactor 1. Similarly, the biomass stored at condition 12, 13 and 14 were immobilized and inoculated in Reactor 2, 3 and 4, respectively. c) The gel beads were prepared by dropping the immobilization solution containing preserved biomass into CaCl<sub>2</sub> solution (4% w/v) through a peristaltic pump (Eyela Roller Pump RP, Tokyo, Japan) equipped with a Phar Med BPT tube (ID 3.1 mm), forming perfect spherical gel beads of 4~5 mm.

#### 3.2.4. Preservation experiments

Granular anammox biomass and immobilized anammox biomass were washed with inorganic media without NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> to remove any residual nutrients prior to preservation. The washed biomass (2.5 g-VSS)

was dispensed into 10-ml glass serum vials (Nichiden-Rika Glass, Kobe, Japan), which were sealed with butyl rubber stoppers and aluminum caps and preserved under conditions indicated in **Table 3.1**.

**Table 3.1:** Preservation conditions tested in this study

Condition No.	Temp.	Biomass	NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> (mM)	NO <sub>3</sub> <sup>-</sup> (mM)	Molybdate (mM)	Penicillin G (mM)	Media exchange
1	-80°C	Granule	-	-	-	-	-
2		Immobilized	-	-	-	-	-
3		Granule	5	-	-	-	EM*
4	4°C	Granule	5	-	3 mM	-	EM*
5		Granule	5	5.7	-	-	EM*
6		Granule	5	-	-	-	-
7		Granule	5	-	3 mM	-	-
8		Immobilized	5	-	-	-	-
9		Immobilized	5	-	3 mM	-	-
10		Immobilized	5	5.7	-	-	-
11		Granule	5	-	-	-	+ NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup>
12	Room temp. (15 ~ 25°C)	Granule	5	-	3 mM	-	+ NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup>
13		Granule	5	-	-	1.5	+ NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup>
14		Granule	5	5.7	-	-	+ NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup>

- : not applied

\*: Entire media (EM) was exchanged after every 15 days

+ NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>: Only NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> were supplemented after 45 days

As for biomasses preserved at 4°C and ambient temperature, 7.0 ml of anoxic inorganic media containing 5 mM of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> was dispensed into vials. In addition, NO<sub>3</sub><sup>-</sup> (5.7 mM) and sodium molybdate (3 mM) were supplemented to some vials as redox buffer and an inhibitor of microbial sulfate reduction, respectively. Inorganic media was not dispensed into vials preserved at -80°C. During preservation, periodical exchange of entire inorganic media and supplementation of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> were further conducted for some vials. For granular anammox biomass preserved at 4°C, entire inorganic nutrient media was exchanged every 15 days. NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> (final concentration each 5 mM) was supplemented to granular anammox biomass preserved at ambient temperature after 45 days of incubation. Triplicate vials were prepared for each preservation condition.

### 3.2.5. Activity test

Biomass preserved for 45, 90 and 150 days was harvested from vials and subjected to SAA measurement as previously described by Oshiki et al. (2013). Briefly, the preserved vials were left on desk until temperature inside of vials was equivalent to room temperature (20~25°C). No heating of vials were undertaken to relieve heat shock stress of biomass. Biomass collected from the vials were dispersed by mortar-pestle style tissue homogenizer into small floccular biomass (< 100 µm diameter) and diluted with inorganic media without NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> to final concentrations of 0.5~1.0 mg-VSS ml<sup>-1</sup>. Two millilitre of biomass suspension was dispensed into 10-ml glass serum vials, which were sealed with butyl rubber stoppers and aluminum caps. All these

procedures were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake Charter Township, MI) where oxygen concentration was < 1 ppm. Headspace in the vials was exchanged with highly pure He gas (>99.9999%) by vacuuming and purging.  $^{15}\text{N-NH}_4\text{Cl}$  and  $^{14}\text{N-NaNO}_2$  (each 2.5 mM) were added from anoxic stock solutions to each vial, and then the vials were incubated under anoxic condition at 37°C for 5 h. Fifty microliter of headspace gas was collected after every hour by a gas tight syringe (VICI, Baton Rouge, LA, USA) and injected to a gas chromatograph. All the batch tests were performed in triplicate for each preservation condition.

### 3.2.6. *Transcription analysis of hzsA gene*

Total RNA was extracted by using Rneasy Mini Kit (Qiagen, Valencia, CA, USA) with minor modification. Biomass sample was suspended in 0.5 ml of PBS buffer and 0.75 ml of phenol:chloroform:isoamyl alcohol (125:24:1, pH 4.0) and transferred into a lysing matrix E tube (MP Biomedicals, Tokyo, Japan). The tube was agitated in a FastPrep bead-beating system (MP Biomedicals, Tokyo, Japan) for 90s at 5.5 m/s. After centrifugation at 15,000 g for 10 min at 4°C, the supernatant was mixed with 0.4 ml of buffer RLT and 0.8 mL of 70% EtOH. The suspension was passed through a Rneasy column, and the column was subsequently washed with buffer RW1 and buffer RPE. Finally, extracted total RNA was eluted from the column by using Dnase-free distilled water. All buffers were prepared and used as following an instruction manual provided by manufactures (Qiagen, Valencia, CA, USA). Contaminating DNA molecules were removed by using Rnase-free Dnase Set included in a Rneasy Mini Kit (Qiagen, Valencia, CA, USA). RNA concentration was determined by using a NanoVue Plus spectrophotometer (GE Healthcare, Tokyo, Japan).

Reverse transcription was performed using PrimeScript<sup>TM</sup> II 1<sup>st</sup> strand cDNA Synthesis Kit (TaKaRa Bio, Otsu, Japan) with random hexamers, in accordance with manufacturer's instructions. The synthesized cDNA was subjected to quantitative PCR analysis using Applied Biosystems 7500 Real-time PCR System (Applied Systems, California, USA). PCR mixture consisted of 12.5  $\mu\text{l}$  of 2 x SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> (Perfect Real Time; TaKaRa Bio), 0.2  $\mu\text{mol/l}$  of each primer, and template cDNA (2.5 ng) at a final volume of 25  $\mu\text{L}$ . Primers used for *hzsA* gene expression analysis were *hzsA\_F* (5'-CACGTGACCGGTAATTCTCT-3') and *hzsA\_R* (5'-GGTATTTTGAAGGAGACTGG-3') (Harhangi et al., 2012). The thermal profile used for PCR started with an initialization step of 10 min at 94°C, followed by 35 cycles (15s at 94°C and 1min at 60°C), followed by melting curve analysis. All samples were quantified in triplicate. Amplification of *hzsA* was ascertained by determining the nucleotide sequences of PCR amplicon by Sanger sequencing method.

In order to normalize the level transcription of *hzsA*, abundance of *hzsA* in total genomic DNA was also determined by quantitative PCR analysis. Total genomic DNA was extracted by using Fast DNA SPIN kit (MP Biomedicals, Tokyo, Japan) according to the standard protocol provided by the manufacturer. Concentration of DNA molecules was determined spectrophotometrically, and 2.5 ng of total genomic DNA was used in 25  $\mu\text{l}$  PCR mixture and subjected to quantitative PCR analysis.

### 3.2.7. Start-up of anammox reactor by using preserved biomass

Granular anammox biomass preserved at ambient temperature for 90 days was immobilized into gel bead as described in the section 3.2.3, and inoculated to up-flow column reactors (volume; 10 ml) with a packing ratio of 70 % (v/v). The column reactors were operated at 37°C with continuous supply of inorganic media containing  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . The NRR was monitored for 35 days of operation. The reactors were operated with a hydraulic retention time of 0.42 h. Concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  in the inorganic media were set at 2.5 mM until 24 days of operation, and then increased to 5 mM, which corresponded to the NLRs of 4.5 and 9.0  $\text{kg-N m}^{-3} \text{d}^{-1}$ , respectively.

### 3.2.8. Chemical Analysis

Concentrations of  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  gas were determined by gas chromatography mass spectrometry analysis (Waki et al., 2010; Yoshinaga et al., 2011). Headspace gases were analyzed by a gas chromatograph GCMS-QP2010SE (Shimadzu, Japan) equipped with a CP-Pora Bond Q fused silica capillary column (Agilent Technologies, Santa Clara, CA, USA), and  $m/z = 29$  and  $30$  were monitored. Amount of  $^{29}\text{N}_2$  gas was quantified by a standard curve prepared with  $^{30}\text{N}_2$  standard gas (>98% purity) (Cambridge Isotope Laboratories, Tewksbury, MA, USA) (Oshiki et al., 2013).

Biomass concentration was determined as protein concentration as previously described (Oshiki et al. 2011). Briefly, biomass was pelleted by centrifugation (18,200 g for 10 min), and the biomass pellet was suspended in 10% (w/v) sodium dodecyl sulfate solution. After boiling for 10 min, the suspension was centrifuged at 18,200 g for 10 min. Protein concentration in the supernatant was determined by Lowry method (Lowry *et al.*, 1951) using DC Protein Assay Kit (Bio-Rad, Tokyo, Japan) and bovine serum albumin as protein standard. Conversion of protein concentration to VSS concentration was performed with conversion coefficient 1.64 (Oshiki et al. 2011).

Concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  were determined using ion chromatographs (IC-2010, TOSOH, Tokyo, Japan) equipped with TSKgel IC-Anion HS or TSKgel IC-Cation columns (TOSOH, Tokyo, Japan) after filtration using 0.2  $\mu\text{m}$ -pore-size membranes (Okabe et al., 2011b).

### 3.2.9. Linear regression analysis

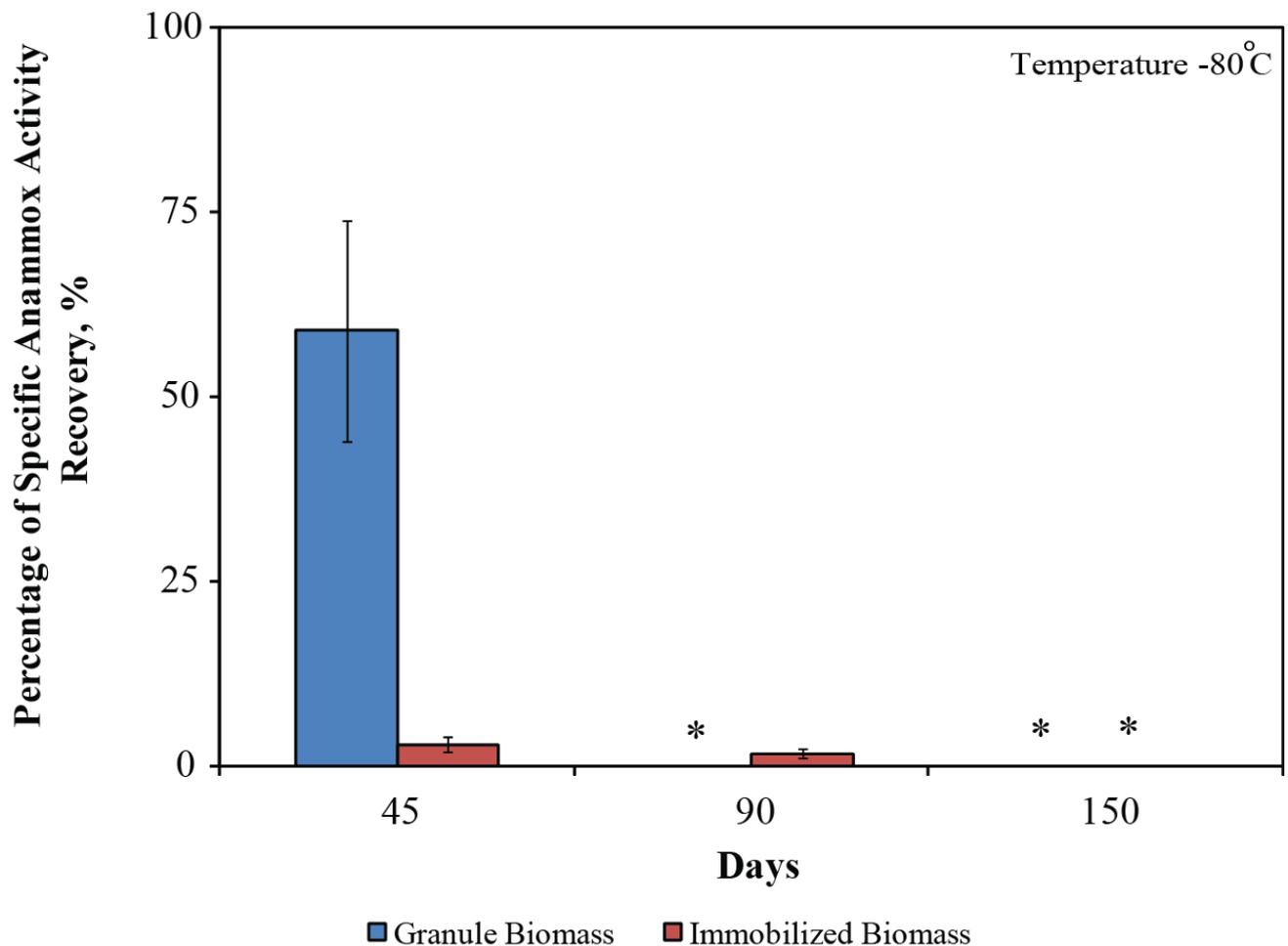
Linear regression analysis was performed to examine a correlation between transcription level of *hzsA* and specific anammox activities. The correlations were examined using Microsoft Excel 2013.

## 3.3. Results and Discussions

### 3.3.1. Storage at -80°C

The specific anammox activities (SAAs) of fresh granular and immobilized biomass before storage were ascertained as  $302.7 \pm 57.6$  and  $554.0 \pm 61.6$   $\mu\text{mole-N g-VSS}^{-1} \text{h}^{-1}$ , respectively. The percentage of SAA recovered from the granular and immobilized anammox biomass after storage at -80°C was determined (**Fig 3.2**). Granular biomass showed 59% recovery of SAA after 45 days but no activity was observed after 90 and 150 days. Heylen et al. (2012) observed about 40% recovery of SAA of granular biomass of “*Ca. Kuenenia*

stuttgartiensis” after storing at -80°C for about 200 days. However, it was also reported that preservation success was always strain dependent as entirely different SAA recoveries were observed for “*Ca. Kuenenia*” and “*Ca. Scalindua*” even under the same preservation condition (Heylen et al., 2012).



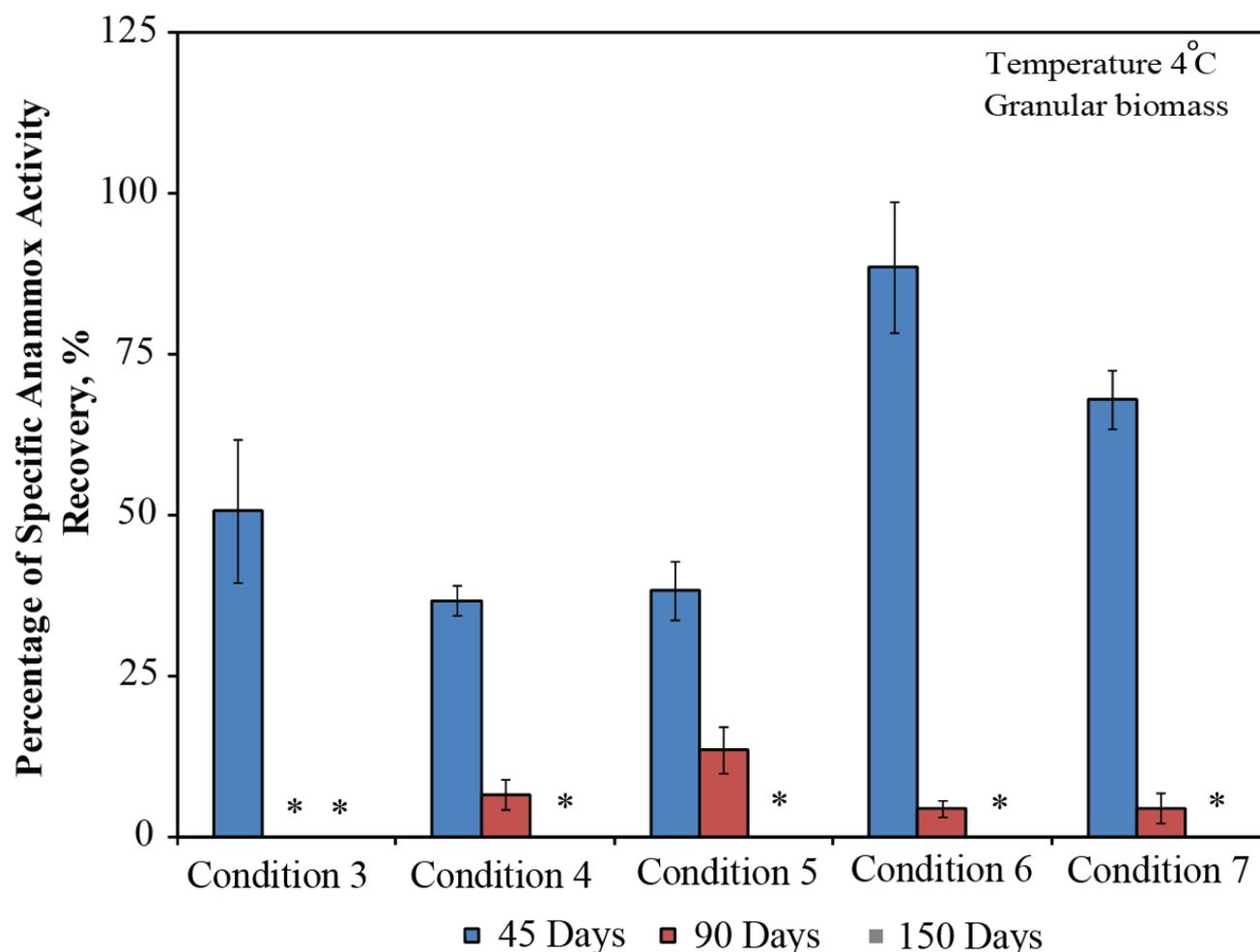
**Fig. 3.2:** Per cent recovery of the specific anammox activity (SAA) of biomass stored at -80°C after 45, 90 and 150 days. Anammox activity was measured based on <sup>29</sup>N-N<sub>2</sub> production rate from <sup>15</sup>N-ammonia and <sup>14</sup>N-nitrite. (\*) represents no activity was observed. Error bars indicate the standard deviations (SD) of triplicate samples.

Immobilized anammox biomass stored at -80°C could recover < 5% of SAA after preservation. Previously, it was observed that anammox biomass immobilized in PVA gel lost about 90% of SAA after storing at -8°C only for 17 h (Magri et al., 2012). Freezing at -80°C could result in concentration of dissolved solutes in the immobilizing gel around the cell, which would cause a leakage of water from the cell to maintain identical solutes concentration around the cell membrane (Pegg, 2007). This would be a possible reason why the freezing of immobilized anammox biomass at -80°C was unsuccessful.

### 3.3.2. Storage at 4°C

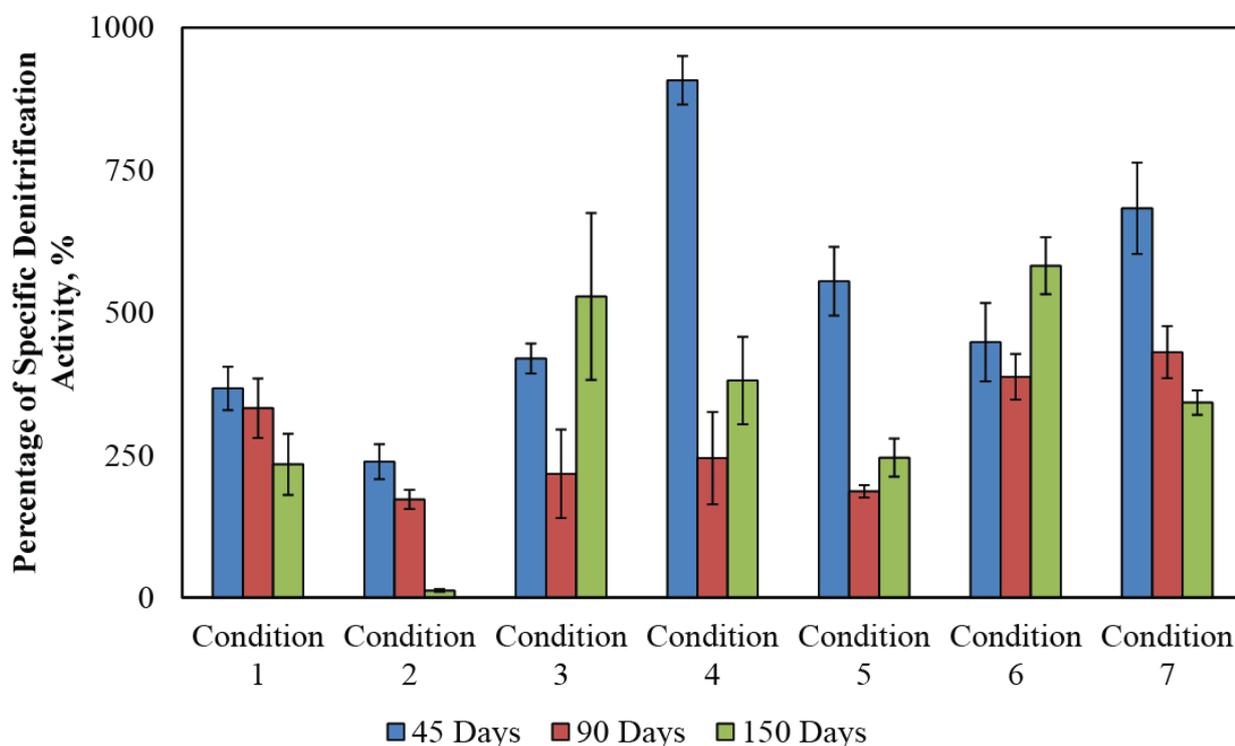
Granular biomass stored under condition 6 showed a maximum SAA recovery of 89% after 45 days, whereas less than 15% of SAA was observed after 90 days (**Fig 3.3**). Similarly, SAA could not be maintained under any condition at 4°C after 150 days of storage. On the contrary, Vlaeminck et al. (2007) showed anammox activity was increased by 25 to 30% during storage from 60 and 150 days under condition similar to condition

5. This is probably because anammox activity was over estimated due to an increase in denitrification activity since the anammox activity was not determined by  $^{15}\text{N}$  isotopic labelling experiment. In our study, significant increase in heterotrophic denitrification activity was observed during the storage (Fig. 3.4).



**Fig. 3.3:** Per cent recovery of the specific anammox activity (SAA) of granular biomass stored at 4°C after 45, 90 and 150 days. Anammox activity was measured based on  $^{29}\text{N-N}_2$  production rate from  $^{15}\text{N}$ -ammonia and  $^{14}\text{N}$ -nitrite. (\*) represents no activity was observed. Error bars indicate the standard deviations (SD) of triplicate samples.

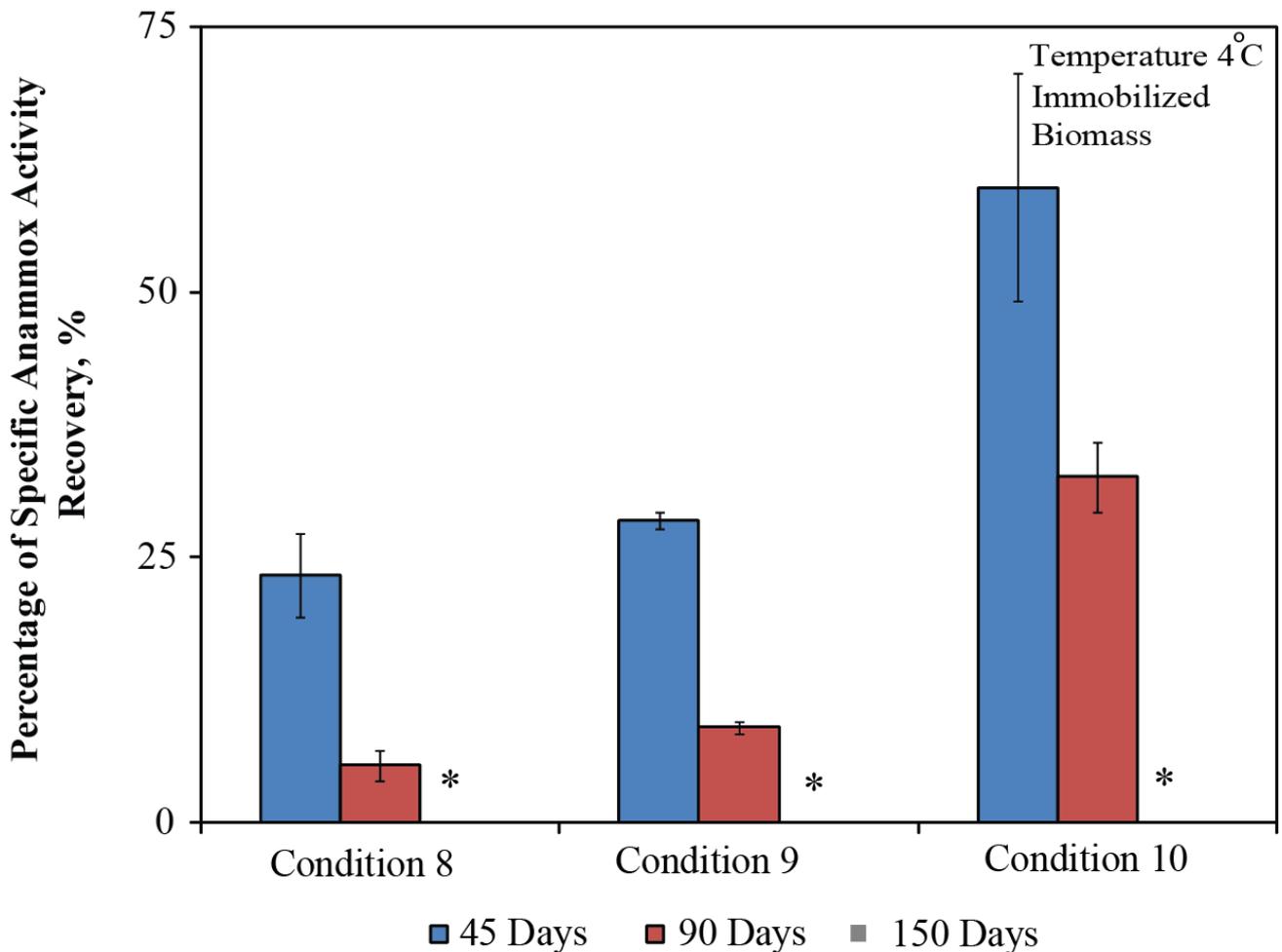
Although the entire nutrient media in vials was exchanged after every 15 days (condition 3, 4 and 5), relatively low recoveries of SAA (about 51, 37 and 38%, respectively) were observed. Exchange of entire media most likely caused contamination of oxygen into the vials which led to the decrease in SAA. Therefore, entire media exchange strategy was not applied to any of the storage conditions for immobilized anammox biomass. Immobilized anammox biomass stored under condition 10 showed the SAA recovery of 60 and 33% after 45 and 90 days of storage, respectively (Fig 3.5). Recovery of SAA from immobilized biomass is relatively low as compared to granular biomass. These experimental results suggest that the storage of granular biomass is better due to the good recovery of SAA and no immobilization steps.



**Fig. 3.4:** Per cent recovery of specific heterotrophic denitrification activity of preserved biomass after 45, 90 and 150 days. Heterotrophic denitrification activity was determined by measuring  $^{30}\text{N-N}_2$  production rate from  $^{15}\text{N}$ -nitrite. Error bars indicate the standard deviations (SD) of triplicate samples.

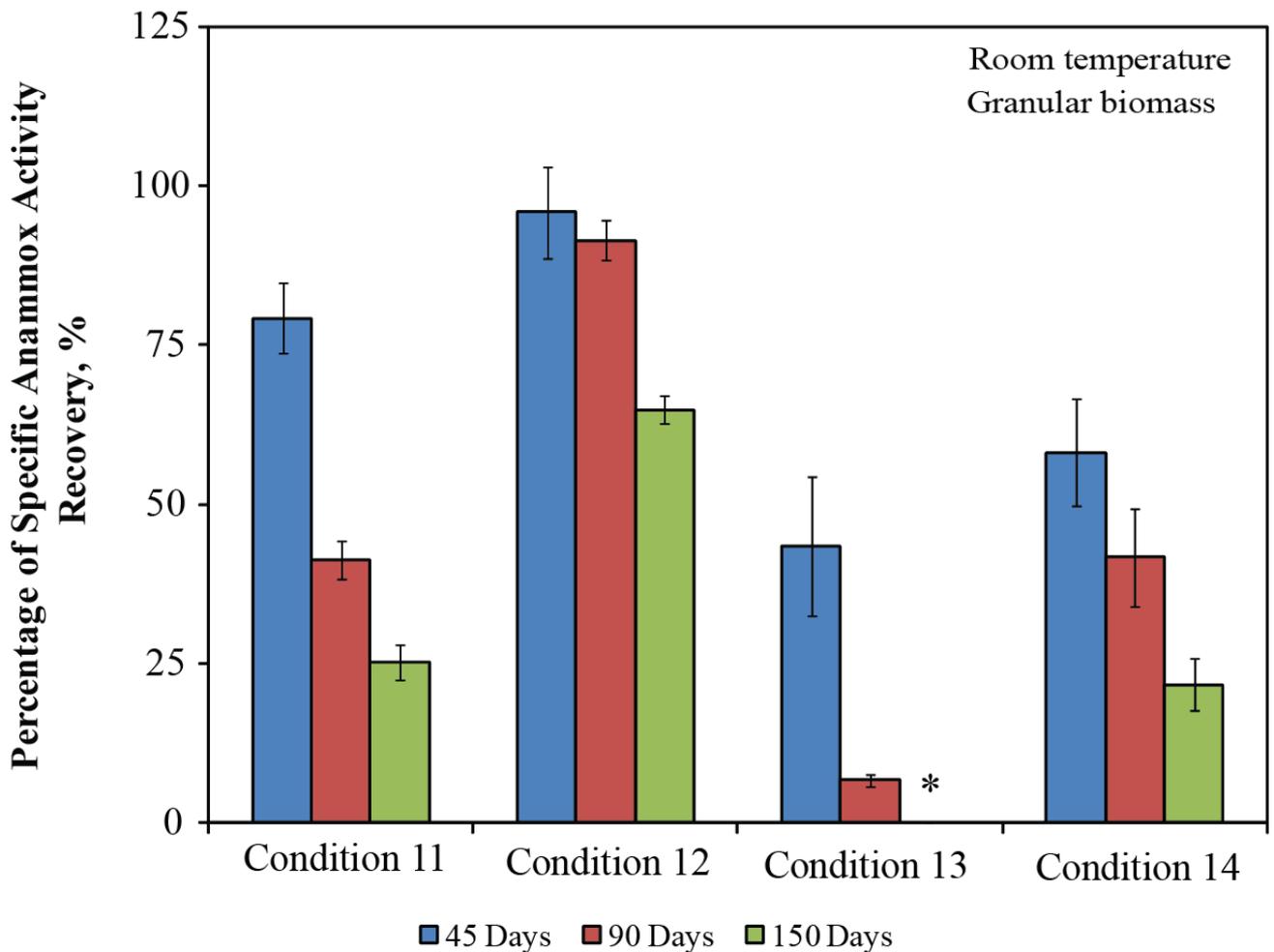
### 3.3.3. Storage at room temperature

In the previous storage experiments, it was observed that heterotrophic denitrification activity increased significantly (**Fig. 3.4**). To suppress the heterotrophic denitrification activity during storage, Penicillin G was added as an inhibitor (van de Graaf et al., 1996) under condition 13. Granular biomass stored under condition 12 showed the maximum SAA recovery of 96, 92 and 65% after 45, 90 and 150 days of storage, respectively (**Fig 3.6**). Under condition 12, sodium molybdate was added as inhibitor for sulfate-reducing bacteria (SRB) at a final concentration of 3 mM as recommended by Fukui et al. (1997) and  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were supplemented after every 45 days at a final concentration of 5 mM. The color of anammox biomass, except for biomass stored under condition 12, turned into black after 90 days, indicating sulfate reduction occurred without molybdate addition (Fig. S1). It was reported previously that 1~2 mM of sulfide reduced SAA by 60% and the sulfide concentration above 5 mM completely inhibited anammox activity (Dapena-Mora et al., 2007). Addition of 3 mM of molybdate effectively inhibited sulfate reduction, resulting in the salvation of anammox activity at room temperature for even 150 days. Nitrate addition, instead of molybdate addition, could be an alternative way to inhibit sulfate reduction. For example, about 30 and 31% of anaerobic ammonium removal activity were recovered after 60 and 150 days of storage, respectively, under the condition similar to condition 14 with nitrate addition (80 mg-N/L) every 15 days (Vlaeminck et al., 2007).



**Fig. 3.5:** Per cent recovery of the specific anammox activity (SAA) of immobilized biomass stored at 4°C after 45, 90 and 150 days. Anammox activity was measured based on  $^{29}\text{N-N}_2$  production rate from  $^{15}\text{N}$ -ammonia and  $^{14}\text{N}$ -nitrite. (\*) represents no activity was observed. Error bars indicate the standard deviations (SD) of triplicate samples.

Instead of the entire media exchange, only  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were supplemented to each vial after every 45 days at a final concentration of 5 mM, which lead to maintain high SAA even after 150 days of storage. Addition of nitrate after every 15 days was more laborious as compared to preservation condition of our study in which molybdate was added only once at the start of preservation. Storage at room temperature in nutrient medium (with periodical supply of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  every 45 days) containing molybdate (3 mM) was proved to be the most effective condition among those tested in this study.

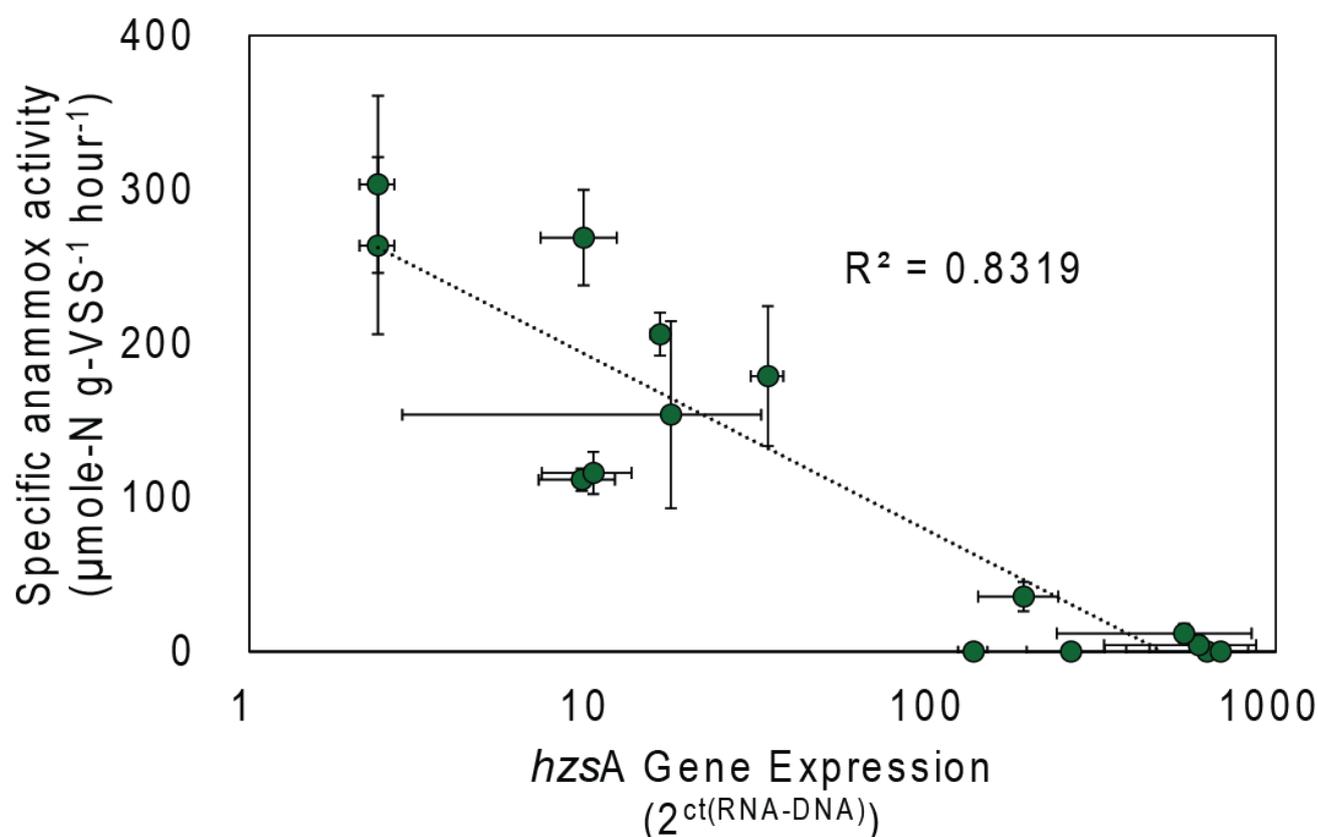


**Fig. 3.6:** Per cent recovery of the specific anammox activity (SAA) of granular biomass stored at room temperature (15 ~ 25°C) after 45, 90 and 150 days. Anammox activity was measured based on  $^{29}\text{N-N}_2$  production rate from  $^{15}\text{N}$ -ammonia and  $^{14}\text{N}$ -nitrite. (\*) represents no activity was observed. Error bars indicate the standard deviations (SD) of triplicate samples.

#### 3.3.4. Comparison of *hzsA* gene expression with $^{29}\text{N}_2$ production rate

Total RNA was extracted from preserved biomass under the conditions 1 and 3 ~ 7, and the transcription level of *hzsA* was determined by quantitative RT-PCR. **Fig. 3.7** represented a correlation between the transcription level of *hzsA* normalized by DNA and specific anammox activity (SAA). In the **Fig 3.7**, Transcription level of *hzsA* was expressed as Ct values, where the Ct values are inversely proportional to the log 10 of the copy number present; *i.e.*, lower values of  $2^{\text{ct(RNA-DNA)}}$  means that *hzsA* was more transcribed in biomass. Linear regression analysis revealed a significant correlation ( $R^2 = 0.83$ ) between the transcription level of *hzsA* and specific anammox activities, showing that the transcription level of *hzsA* was higher in active biomass (*i.e.*, the biomass showing higher specific anammox activities). Along with several other methods, the real-time PCR is a very useful technique to detect the presence and activity of anammox bacteria (Tsushima et al. 2007a). Though, 16S rRNA gene is not always related to the physiology or activity of the target microorganism, and the current anammox primers do not capture all diversity (Schmid et al., 2005). However, hydrazine synthase (*hzsA*) primers are more specific than the primers targeting the 16S rRNA, hydrazine

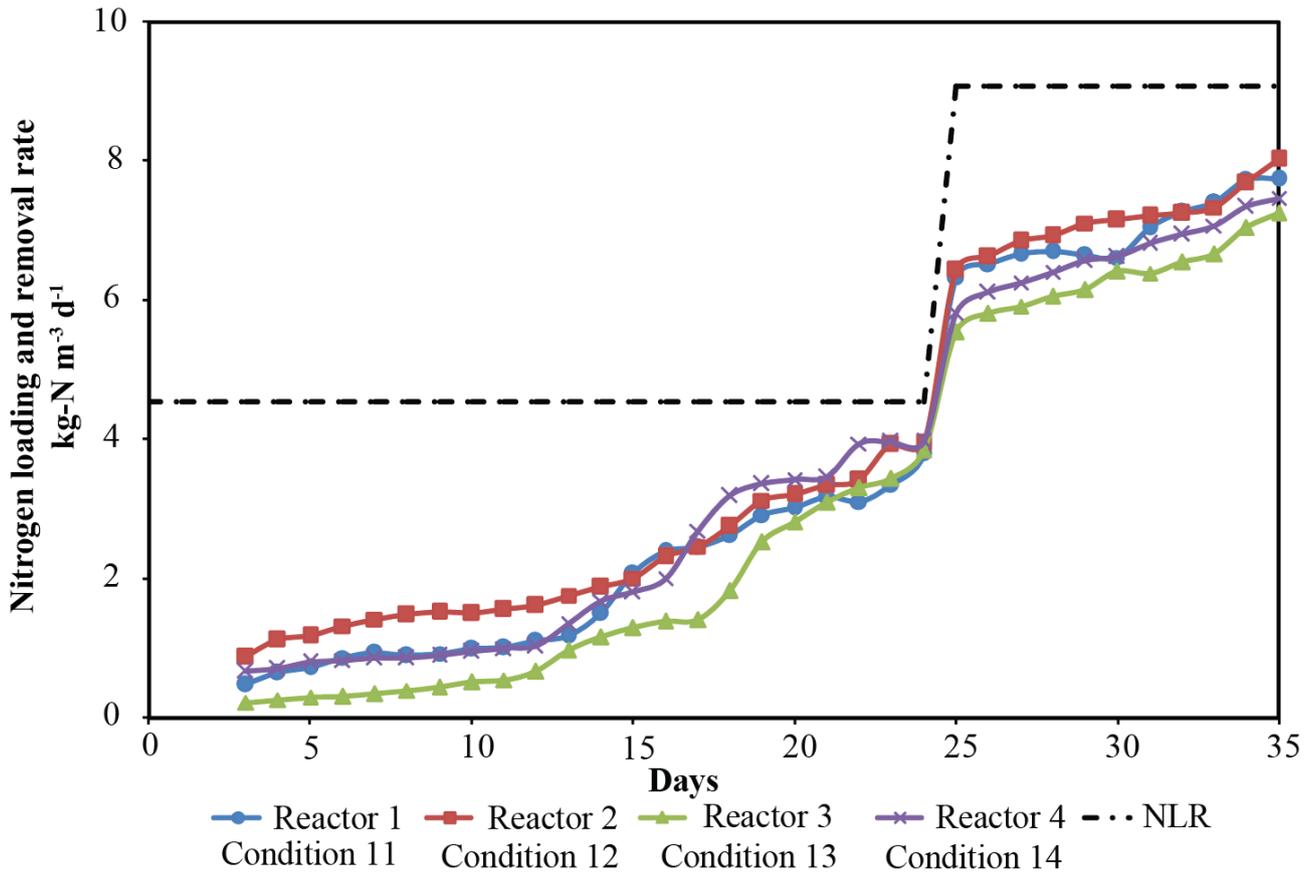
oxidoreductase (*hzo*) or nitrite reductase (*nirS*) genes, because these genes are also present in other bacteria (Harhangi et al., 2012; Li et al., 2010). Hence, the *hzsA* gene expression analysis validated the results of SAA obtained through  $^{29}\text{N}_2$  production rate.



**Fig. 3.7:** Relationship between the relative transcription levels of *hzsA* gene and specific anammox activity (SAA) derived from  $^{29}\text{N}_2$  production rate. This result was used to confirm the SAA determined by  $^{29}\text{N}_2$  production rate for storage conditions 1 and 3 ~ 7 (Fig. 3.2 & Fig. 3.3, respectively).

### 3.3.5. Reactivation of the stored biomass

Gel immobilization technique for anammox biomass provides various advantages, *i.e.*, the high NLR can be achieved at short hydraulic retention times without nitrite inhibition and washout of cells, it is easy to separate liquid and solid phase in the reactor, and a small amount of initial biomass is required (Isaka et al., 2011; Kimura et al., 2013; Magri et al., 2012; Tsushima et al., 2007b). Therefore, 90-day- stored biomass under conditions 11, 12, 13 and 14 (SAA of  $133.0 \pm 9.7$ ,  $208.6 \pm 6.8$ ,  $21.4 \pm 3.4$ , and  $69.8 \pm 13.2 \mu\text{mole-N g-VSS}^{-1} \text{h}^{-1}$ , respectively) were immobilized in PVA-SA gel and inoculated in the reactor 1, 2, 3 and 4, respectively (**Fig. 3.1**). Time courses of NRRs of these reactors were presented in **Fig 3.8**. The stoichiometric ratios of the consumed  $\text{NO}_2^-$  to the consumed  $\text{NH}_4^+$  ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) and produced  $\text{NO}_3^-$  to the consumed  $\text{NH}_4^+$  ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) were in the range of 1.1~1.4 and 0.21~0.27, respectively. These stoichiometric ratios are close to theoretical values of 1.32 and 0.26 for  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ , indicating anammox was responsible for nitrogen removal in these reactors.



**Fig. 3.8:** Reactivation of the 90-day-stored biomass under conditions 11~14. Each biomass was immobilized in PVA-SA gel and packed in up-flow column reactors 1 ~ 4, respectively. Nitrogen removal rate (NRR) of each reactor was monitored for 35 days. Hydraulic retention time was 0.4 h and nitrogen loading rate (NLR) was maintained at 4.5 and 9.0 kg-N m<sup>-3</sup> d<sup>-1</sup> in each reactor. Operating temperature was 37°C.

After 20 days of operation, the NRR of each anammox reactor became comparable, regardless of their different preservation conditions, namely different SAA. The NRRs increased significantly and ultimately reached 7 kg-N m<sup>-3</sup> d<sup>-1</sup> at the end of operation (after 35 days), showing the rapid reactivation of 90-day-stored anammox biomass. Likewise, Magri et al. (2012) revealed that about 70% of SAA was lost during storage, but the SAA was recovered after one month of reactivation. Based on this experimental result, anammox activity lost during storage at room temperature can be recovered within a short time period, suggesting that the stored anammox biomass could be directly used for a rapid start-up of anammox process without any additional reactivation steps.

### 3.4. Conclusion

In this study, it was shown that anammox biomass can be stored effectively for longer period of time at room temperature in nutrient medium containing molybdate (3 mM) with only supplementation of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> every 45 days. Using this preservation condition, 96, 92 and 65% of the initial SAA were recovered after 45, 90 and 150 days, respectively. Storage at room temperature does not require any special equipment and skill and thus is a simple, easy and cost-effective method for biomass preservation. Furthermore, rapid reactivation of the preserved biomass could be achieved by immobilizing in PVA-SA gel. The combination of room

temperature storage and PVA-SA immobilization techniques would be feasible at a practical scale and thus accelerate the practical use of anammox process for wastewater treatment. However, these preservation and reactivation conditions were tested by using anammox biomass with overwhelming major of “*Ca. Brocadia sinica*”, and further verification by using phylogenetically different anammox bacteria is still required to establish a general storage protocol for anammox bacteria.

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## 4. IMMOBILIZATION OF ANAMMOX BIOMASS

### 4.1. Introduction

Anaerobic ammonium oxidation (anammox) process has been regarded as an efficient, cost effective and environmental friendly alternative to conventional nitrogen removal process due to lower oxygen demand, no external carbon source requirement, lower sludge production and less N<sub>2</sub>O gas emission (Kartal et al., 2010; Kuenen, 2008; Okabe et al., 2011). Anammox process has been installed for various wastewaters treatment, and there are presently more than 100 full-scale anammox plants around the world (Lackner et al., 2014). One of the remaining important challenges in practical application of anammox process is slow and unstable start-up due to their slow growth rate (Ali et al., 2014a; Awata et al., 2013; Jetten et al., 1999; Oshiki et al., 2011; Park et al., 2010; van der Star et al., 2007). To further promote the full-scale anammox installations, it is required to establish a more rapid and reliable start-up method. For rapid and successful start-up of anammox process, a sufficient amount of seeding biomass is essential, and the inoculated biomass must be efficiently retained in the reactor. Biomass washout due to production of N<sub>2</sub> gas bubbles was another cause of the failure of process start-up (Chen et al., 2010; Dapena-Mora et al., 2004).

In order to secure enough seeding biomass, the preservation of anammox biomass could be one of possible solutions (Ali et al., 2014b; Heylen et al., 2012; Rothrock et al., 2011; Vlaeminck et al., 2007). Ali et al. (2014b) recently reported that anammox biomass could be stored for five months at room temperature in nutrient medium containing 3 mM molybdate with periodical supplementation of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup>. However there is a limitation of biomass storage capacity in practice. It is therefore desirable to determine the necessary minimal quantity of anammox biomass for successful start-up.

In order to efficiently retain the inoculated biomass in a reactor, biomass could be immobilized in gel beads, which allows us to operate the reactor at high nitrogen loading rates (*i.e.*, at short hydraulic retention time) without nitrite inhibition and biomass washout (Isaka et al., 2006; Magrı et al., 2012; Vogelsang et al., 1999). This reactor operation is suitable for cultivation of anammox bacteria (Tsushima et al., 2007a). Anammox bacteria were successfully immobilized either in polyvinyl alcohol (PVA) (Magrı et al., 2012), sodium alginate (SA) (Zhu et al. 2009), mixture of PVA and SA (Ali et al., 2014b; Quan et al., 2011), or polyethylene glycol (PEG) (Isaka et al., 2011), and the reactors consisting of these immobilized gel beads could achieve the nitrogen removal rates (NRRs) of 0.58 ~ 12 kg-N m<sup>-3</sup> d<sup>-1</sup> within time periods of 35 ~ 180 days. In these studies, the initial biomass concentrations were varied; 1.8 g-volatile suspended solids (VSS) (L of reactor volume)<sup>-1</sup> (Magrı et al., 2012), 3.5 g-VSS L<sup>-1</sup> (Zhu et al., 2009), 5 g-SS L<sup>-1</sup> (Quan et al., 2011), and 5.2 g-VSS L<sup>-1</sup> (Isaka et al., 2011). Although the minimal biomass concentration that is necessary for successful start-up of anammox process must be an important factor to reduce the required seeding biomass, it has not been studied and reported so far. In addition, a detailed comparison of biological and physicochemical properties between naturally aggregated granular and artificially immobilized anammox biomass has not been characterized in the previous studies.

The objective of this study was, therefore, to determine the minimal concentration of anammox biomass immobilized in gel beads that is necessary for rapid and successful start-up of anammox process. For practical application, the criteria of the rapid and successful start-up of anammox reactor was defined as follows; an acceptable start-up time period of anammox reactors is about a month and a target volumetric nitrogen removal rate is 10 kg-N m<sup>-3</sup> d<sup>-1</sup>. The minimal concentration of anammox biomass that is necessary for meeting these criteria was determined in this study. For this purpose, different amounts of anammox biomass were immobilized in a mixture gel of PVA and SA, and the immobilized gels were packed in up-flow column reactors. The influence of the initial biomass concentration on the start-up and performance of anammox process were investigated and compared with the reactor containing the naturally aggregated granular biomass. Furthermore, the detailed comparison of biological and physicochemical properties such as effective diffusion coefficient and anammox activity in the immobilized gel beads and granules was made on the basis of various *in situ* analyses: fluorescence *in situ* hybridization (FISH) analysis, microelectrode measurements, and <sup>13</sup>C-bicarbonate incorporation analysis by secondary ion mass spectrometry (SIMS).

## **4.2. Materials and methods**

### **4.2.1. Anammox biomass**

Naturally aggregated granular anammox biomass (average 5-mm of diameter) (hereafter referred as granular biomass), was harvested from an up-flow anammox reactor (hereafter referred as parent reactor) (Tsushima et al., 2007b) (**Fig. 4.1A**). The granular biomass contains mono species of anammox bacterium, “*Candidatus Brocadia sinica*”, and they accounted for more than 94% of total bacterial cells as determined by FISH (Oshiki et al., 2011).

### **4.2.2. Immobilization technique**

Granular biomass was dispersed by a mortar-pestle style tissue homogenizer (50cm<sup>3</sup>, Φ 6 × 290 × 195 mm) into small floccular biomass (< 100 μm diameter) and diluted with culture medium (described below in section 2.3) to six different biomass concentrations *i.e.* 4.8, 4.1, 3.3, 2.3, 0.9 and 0.5 g-VSS L<sup>-1</sup>. Autoclaved immobilization support solutions of 6% (w/v) PVA and 2% (w/v) SA were mixed with an equal volume of the prepared biomass suspension as described previously (Ali et al., 2014b). The mixture was dropped into a solution of 4% (w/v) CaCl<sub>2</sub> by using a peristaltic pump (Eyela Roller Pump RP, Tokyo, Japan) equipped with a PharMed BPT tube (ID 3.1 mm), forming spherical gel beads with 4~5-mm diameter (**Fig. 4.1B**). The immobilized gel beads were cured in the same CaCl<sub>2</sub> solution for 12 h to enhance their mechanical stability. The immobilized anammox biomass (hereafter referred as immobilized biomass) were collected and rinsed thrice with deionized water and inoculated in up-flow column reactors.

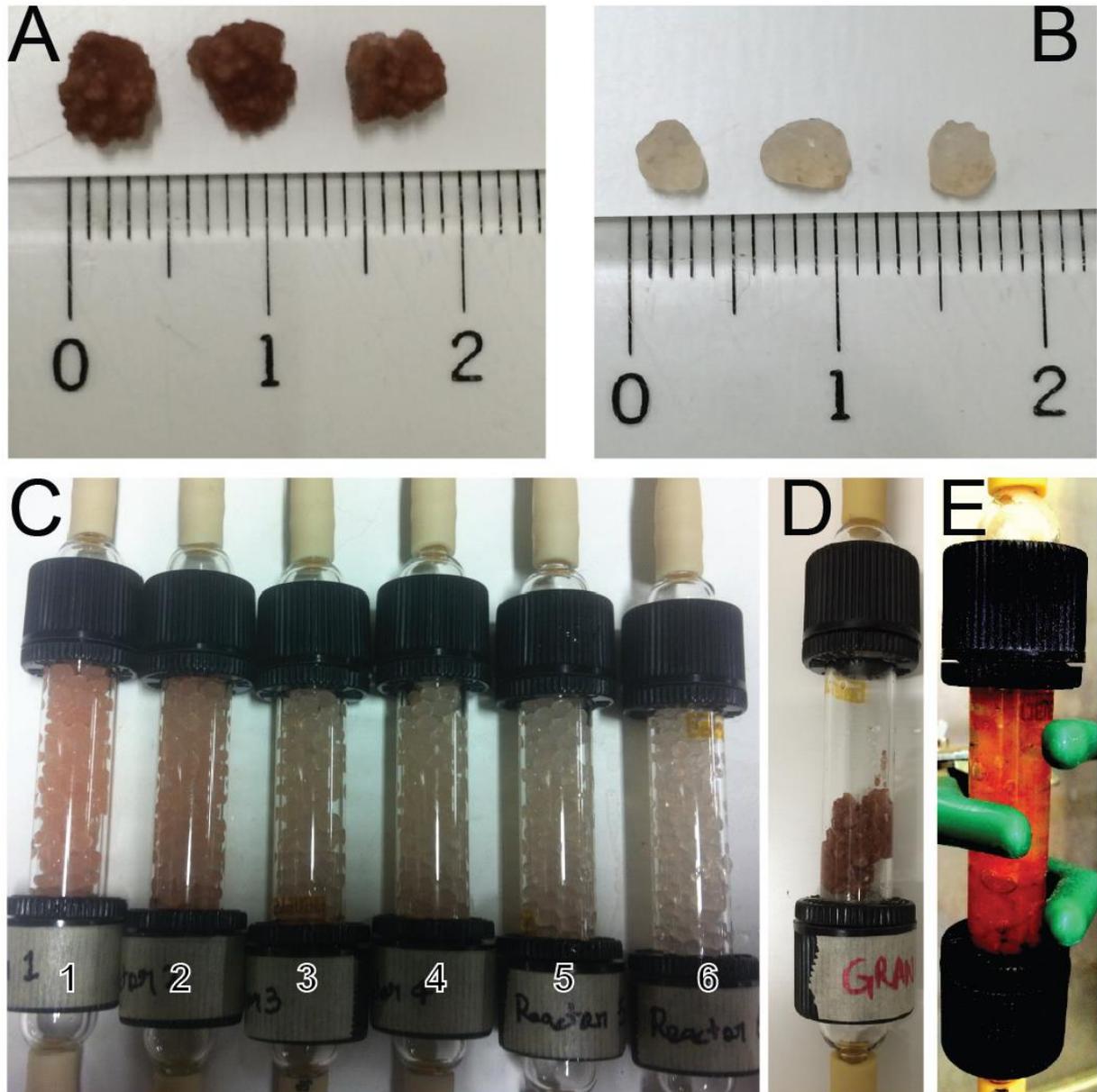
### **4.2.3. Reactors establishment and operation**

Immobilized biomass with different initial biomass concentrations were separately inoculated into six up-flow glass column reactors (10-mL, Φ18 × 80 mm, FujiRika, Osaka, Japan) with a packing ratio of 70 %

(v/v) (**Fig. 4.1C**). Anammox biomass concentrations in the reactors were 1.67, 1.45, 1.15, 0.82, 0.33 and 0.16 g-VSS L<sup>-1</sup> (“L<sup>-1</sup>” here and hereafter referred to the volume of the reactor) in reactor 1 to 6, respectively (Table S1). Likewise, granular biomass (Φ 4~5 mm) was harvested from the parent reactor and transferred to the same 10-ml glass column reactor at the biomass concentration of 2.5 g-VSS L<sup>-1</sup> (**Fig. 4.1D**). These column reactors were fed with synthetic medium and operated for 35 days at 37°C. The synthetic medium used in the present study contained; NH<sub>4</sub><sup>+</sup> (2.5~7.5) mM, NO<sub>2</sub><sup>-</sup> (2.5~7.5) mM, CaCl<sub>2</sub> 100 mg L<sup>-1</sup>, MgSO<sub>4</sub> 300 mg L<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 30 mg L<sup>-1</sup>, KHCO<sub>3</sub> 500 mg L<sup>-1</sup> and trace element solution (van de Graaf et al., 1995). Concentrations of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> were set at 2.5 mM for 0~14 days, and increased to 5.0 and 7.5 mM after 15- and 30-day operation, which resulted in nitrogen loading rates (NLRs) of 4.3, 8.1 and 12.1 kg-N m<sup>-3</sup> d<sup>-1</sup>, respectively (**Table 4.1**). Hydraulic retention time (HRT) was set at 0.42 h. Concentrations of NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> in the influent and effluent of the reactors during operation were determined using ion chromatographs (IC-2010, TOSOH, Tokyo, Japan) equipped with TSKgel IC-Anion HS and TSKgel IC-Cation columns (TOSOH) after filtration using 0.45-μm-pore-size membranes (Okabe et al., 2011).

**Table 4.1:** Operating conditions of reactors containing immobilized and granular biomass used for this study.

Parameters	Reactor 1	Reactor 2	Reactor 3	Reactor 4	Reactor 5	Reactor 6	Granular biomass reactor
<b>Biomass concentration (g-VSS L<sup>-1</sup>)</b>	1.67	1.45	1.15	0.82	0.33	0.16	2.5
<b>Packing volume (%)</b>				70			35
<b>HRT (h)</b>				0.42			
<b>NLR (kg-N m<sup>-3</sup> d<sup>-1</sup>)</b>			4.3 (0-14 d), 8.1 (15-29d), & 12.1 (30-34d)				
<b>Operating temperature (°C)</b>				37			



**Fig. 4.1:** **A)** Naturally grown granular biomass (diameter 4~5 mm) harvested from a parent anammox reactor (Tsushima et al., 2007b). **B)** The homogenized anammox biomass were immobilized in PVA-SA gel beads (PVA 6 % w/v, SA 2 % w/v, diameter 4~5 mm). **C)** Up-flow column reactors packed with the gel beads immobilized different initial biomass concentrations at the beginning of operation. More reddish gel beads indicate higher anammox biomass content. **D)** Up-flow column reactor packed with the naturally aggregated granular biomass at the start of operation. **E)** The up-flow column reactor after several days of operation. The gel beads exhibited vivid red color, indicating anammox bacteria have grown actively in the reactor.

#### 4.2.4. Specific anammox activity

Specific anammox activity (SAA) was determined by measuring  $^{29}\text{N}_2$  production rate as previously described (Ali et al., 2014b; Oshiki et al., 2013). Briefly, granular and immobilized biomass were suspended into the anammox culture medium (without  $\text{NH}_4^+$  and  $\text{NO}_2^-$ ), and then 5 ml of each biomass suspension was dispensed into serum vials (12.5 ml, Nichiden-Rika Glass, Kobe, Japan) separately and sealed with butyl rubber

stoppers and aluminium caps. To avoid oxygen contamination, all biomass was handled in an anaerobic chamber (Coy Laboratory Products, Grass Lake Charter Township, MI, USA), and the headspace gas was exchanged with ultra-pure He gas (>99.9999%) by using a gas exchange machine (model IP-8, SANSHIN, Yokohama, Japan). The vials were pre-incubated at 37°C for at least 6 hr, and then  $^{15}\text{N-NH}_4\text{Cl}$  and  $^{14}\text{N-NaNO}_2$  were supplemented to each vial at a final concentration of 2.5 mM each and incubated at 37°C. Fifty microliter of headspace gas was collected after every hour by a gas tight syringe (VICI, Baton Rouge, LA, USA) and injected to a gas chromatograph (GCMS-QP2010SE, Shimadzu, Japan) equipped with a CP-Pora Bond Q fused silica capillary column (Agilent Technologies, Santa Clara, CA, USA), and  $m/z = 28, 29$  and  $30$  were monitored. Concentrations of  $^{28}\text{N}_2$ ,  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  gas were determined by gas chromatography mass spectrometry analysis (Amano et al., 2007; Waki et al., 2010). Specific ammonium oxidation rate was determined by measuring  $^{29}\text{N}_2$  gas production rate. Amount of  $^{29}\text{N}_2$  gas was quantified by a standard curve prepared with  $^{30}\text{N}_2$  standard gas (>98% purity, Cambridge Isotope Laboratories, Tewksbury, MA, USA) (Oshiki et al., 2013). This experiment was performed in triplicate for each type of biomass. Biomass concentration was determined as protein concentration and converted to g-VSS  $\text{L}^{-1}$  as previously described (Oshiki et al., 2011). Briefly, biomass was pelleted by centrifugation (18,200  $g$  for 10 min), and the biomass pellet was suspended in 10% (w/v) sodium dodecyl sulfate solution. After boiling for 10 min, the suspension was centrifuged at 18,200  $g$  for 10 min. Protein concentration in the supernatant was determined by Lowry method (Lowry et al., 1951) using DC Protein Assay Kit (Bio-Rad) as described in the manufacture instruction.

#### **4.2.5. FISH analysis of granular and immobilized biomass**

Granular biomass and immobilized biomass (harvested from reactor 3) were fixed with 4% (w/v) paraformaldehyde in phosphate-buffer saline (PBS; 10mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) at 4°C for 6 hr. Biomass was then washed thrice with PBS and embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) at -30°C. Thin sections (20  $\mu\text{m}$  in thickness) of biomass were prepared using a cryostat (Reichert-Jung Cryout 1800, Leica, Bensheim, Germany) and pasted on teflon coated glass slides. FISH was performed using the probes EUB mix (FITC) and AMX 820 (TRITC) as described previously (Okabe et al., 1999b) to identify total and anammox bacterial population, respectively. Hybridized samples were observed using confocal laser-scanning microscope (TCS SP8, Leica, Japan) equipped with Ar ion and He-Ne laser. Later, obtained microscopic images were processed and analysed by Leica Application Suite Advanced Fluorescence, (LAS AF, version 4.4).

#### **4.2.6. Microelectrode measurements**

The steady state concentration micro-profiles of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and pH in granular and immobilized biomass were measured using liquid ion exchanger (LIX) type microelectrodes (de Beer et al., 1997a). The microelectrodes were prepared, calibrated, and operated as previously described (Okabe et al., 1999a; Rathnayake et al., 2013; Satoh et al., 2007). The synthetic medium used for microelectrode measurements was of same composition as described in section 2.3. The granular and immobilized biomass (harvested from reactor

3) were incubated in a flow chamber (2.5 L) containing anoxic medium for at least 3 h before microelectrode measurements to ensure that steady-state profiles were obtained. Microelectrodes were inserted almost perpendicular to the surface of the biomass. The liquid-biofilm interface was identified by a high precision stereo microscope (Stemi 2000, Carl Zeiss, Germany). Temperature of the anoxic medium was maintained at 37°C throughout the experiments. At least three profiles were measured for each chemical species using different aggregate as previously described (Song et al., 2013).

#### 4.2.7. *Effective diffusion coefficient*

Effective diffusion coefficients of the granular and immobilized biomass were directly measured by using LIX-type  $\text{NH}_4^+$  specific microelectrode as suggested previously (Stewart, 2003). Briefly, the granular and immobilized biomass were washed thrice with deionized water before measurements. Both the granular and immobilized biomass were incubated in a small flow chamber (300-mL volume) filled with 100 mL of medium containing 1 mM  $\text{NH}_4^+$  (without  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ). An ammonium specific microelectrode was inserted perpendicular to the respective biomass at 500  $\mu\text{m}$  depth from the surface. At steady microelectrode reading, 10 mL of 100 mM  $\text{NH}_4^+$  solution was added to increase  $\text{NH}_4^+$  concentration of bulk solution to 10 mM. The steady reading was defined as the point at which fluctuation in reading for 20 seconds became lower than 0.1 mV. The time was recorded to attain the reading corresponding to 90% of bulk solution concentration (9 mM  $\text{NH}_4^+$ ). Strict anoxic condition was maintained by purging  $\text{N}_2$  gas to avoid oxidation of  $\text{NH}_4^+$  by nitrifies. Three measurements were recorded for each type of biomass. Effective diffusion coefficients were calculated by using the following equation:

$$D_e = 0.37 \times d^2 / t_{90\%} \quad (\text{Eq. 1})$$

Where,  $D_e$  is effective diffusion coefficient in the biomass,  $d$  is the penetration depth and  $t_{90\%}$  is the time required for the solute to attain 90% of the bulk solution concentration at the penetration depth  $d$ . The values of ( $D_{aq}$ ), diffusion coefficients in pure water for  $\text{NH}_4^+$ ,  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , were taken from literature (Yi et al., 2011). The relative effective diffusivity, described as a ratio  $D_e/D_{aq}$ , was determined for  $\text{NH}_4^+$ . Same relative effective diffusivity was used to determine effective diffusion coefficient for  $\text{NO}_2^-$  and  $\text{NO}_3^-$ . Furthermore, volumetric  $\text{NH}_4^+$  and  $\text{NO}_2^-$  consumption and  $\text{NO}_3^-$  production rates were estimated from the respective solute concentration profiles by using the Fick's second law of diffusion as described previously (Lorenzen et al., 1998; Nakamura et al., 2004). Following equation (Eq. 2), derived from the Fick's second law of diffusion, was used to simulate concentration profiles on the basis of volumetric  $\text{NH}_4^+$  and  $\text{NO}_2^-$  consumption and  $\text{NO}_3^-$  production rates, ( $A_n \times D_e$ ). The derivation and terms used in this equation was previously explained elsewhere (Lorenzen et al., 1998).

$$C_{n+1} = C_n + h \times [\partial C / \partial z_{n-1} + h \times A_{n-1}] \quad (\text{Eq. 2})$$

#### 4.2.8. *Isotope microscopic analysis*

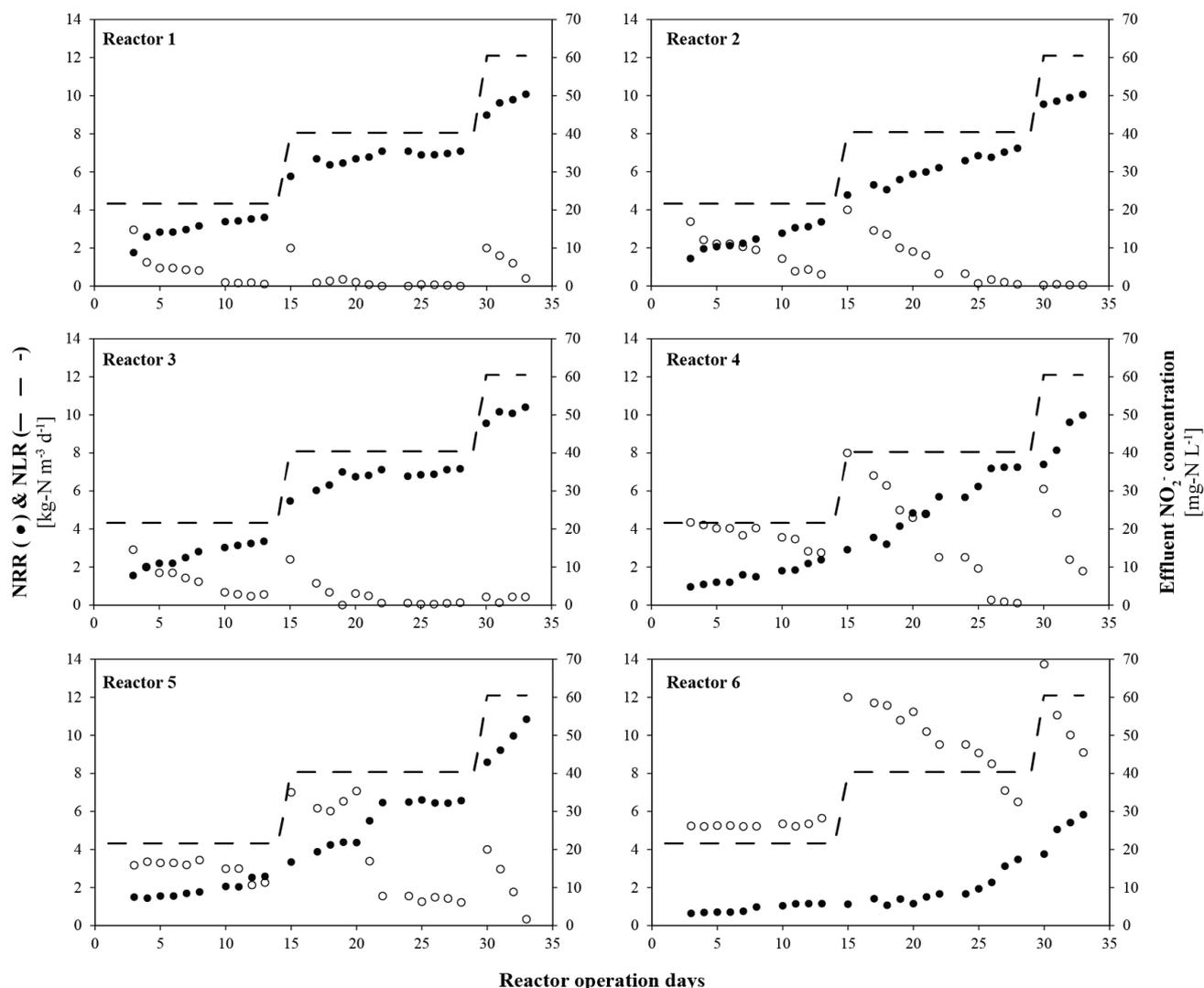
Granular and immobilized biomass were separately inoculated in up-flow column reactors and operated under the same operating conditions as previously mentioned in section 2.3. Biomass concentrations in the

granular and immobilized biomass reactors were 1.15 and 2.5 g-VSS L<sup>-1</sup>, respectively. These reactors were continuously fed with <sup>13</sup>C-NaHCO<sub>3</sub> (97% chemical purity, Cambridge Isotope Laboratories, MA, USA) as a sole carbon source. After 35-day operation, granular and immobilized biomass were harvested and washed thrice with phosphate buffered saline solution. Washed biomass were embedded in Tissue-Tek OCT compound and sectioned into slices (10 μm in thickness) by using a cryostat (Reichert-Jung Cryout 1800, Leica, Bensheim, Germany). These thin sections were pasted on silica wafers. An *in situ* survey of spatial distribution of <sup>13</sup>C-carbon incorporation by anammox bacterial cells in the granular and immobilized biomass was performed by line profiling analysis using a secondary ion mass spectrometry SIMS (ims-1270, CAMECA, France) equipped with an ion detector (stacked CMOS-type active pixel sensor; SCAPS)(Sakamoto et al., 2007).

### 4.3. Results and discussions

#### 4.3.1. Influence of initial biomass content on start-up of anammox processes

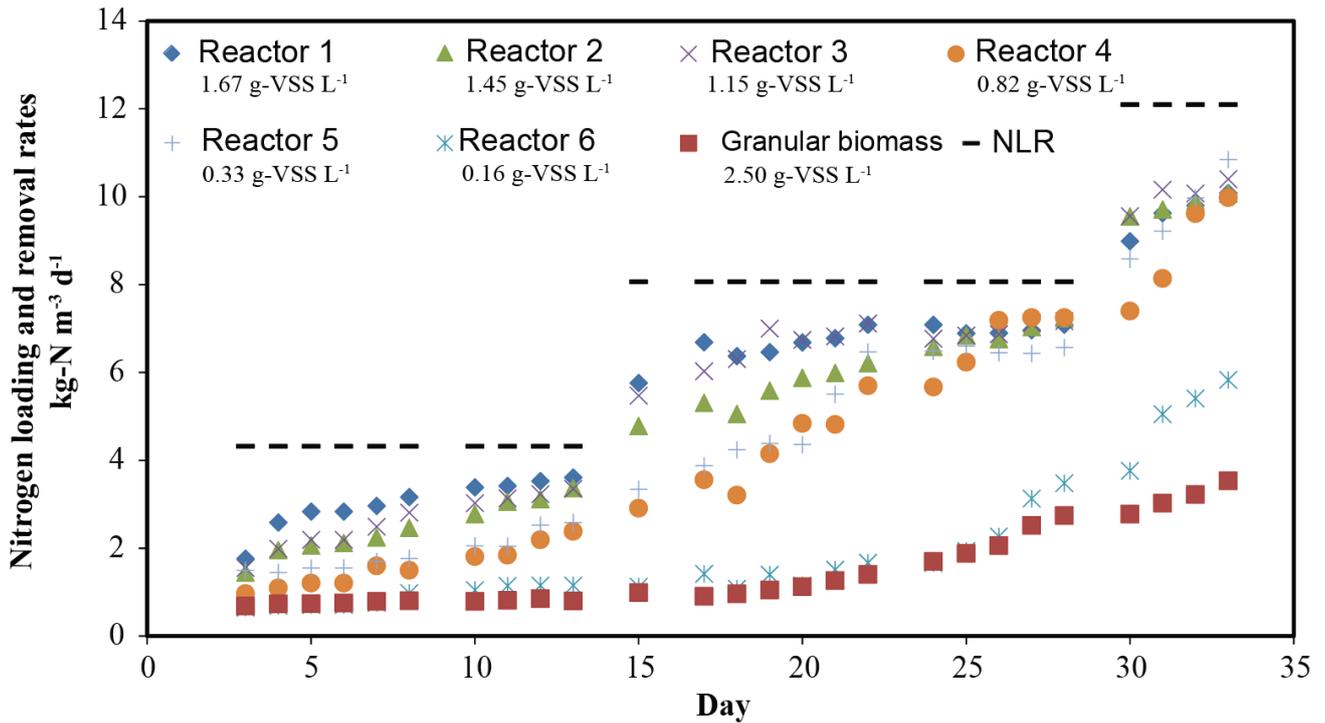
Six up-flow column reactors packed with the immobilized anammox biomass (containing biomass concentrations of 0.16~1.67 g-VSS L<sup>-1</sup>) were operated for 35 days at 37°C. As for comparison, a column reactor containing the granular biomass of 2.5 g-VSS L<sup>-1</sup> was also operated under the same conditions. Nitrogen loading rates were increased when the effluent NO<sub>2</sub><sup>-</sup> concentrations in all reactors except for reactor 6 became below 15 mg L<sup>-1</sup> to avoid nitrite inhibition (**Fig. 4.2**). NRRs increased with time in all the reactors (**Fig. 4.3**). Stoichiometric ratios of consumed NO<sub>2</sub><sup>-</sup> to consumed NH<sub>4</sub><sup>+</sup> ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) and produced NO<sub>3</sub><sup>-</sup> to consumed NH<sub>4</sub><sup>+</sup> ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) in the reactors were in the range of 1.1~1.4 and 0.16~0.22, respectively. Those stoichiometric ratios are close to the theoretical stoichiometric ratios of anammox reaction (*i.e.* 1.15 and 0.16 for  $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$  and  $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ , respectively) (Lotti et al., 2014), indicating anammox was responsible for nitrogen removal in these reactors. During the operation of the column reactors, the color of the immobilized biomass was changed from pale white to reddish color, indicating the growth of anammox bacteria (**Fig. 1E**). For initial few days, NRRs were dependent on the initial biomass content: the higher initial biomass concentrations resulted in higher NRRs (**Fig. 4.3**). The reactor 1 (containing biomass concentration of 1.67 g-VSS L<sup>-1</sup>) could achieve NRR of 3 kg-N m<sup>-3</sup> d<sup>-1</sup> just after 7-day operation, whereas the NRR of the reactor 5 (containing 0.33 g-VSS L<sup>-1</sup>) was about 50% of the reactor 1. However, the difference in NRR gradually became small as time passed. Almost the same NRR (approximately 7 kg-N m<sup>-3</sup> d<sup>-1</sup>) was achieved for all reactors after 25-day operation and then reached above 10 kg-N m<sup>-3</sup> d<sup>-1</sup> after 33 days, except for the reactor 6 (containing the lowest biomass concentration of 0.16 g-VSS L<sup>-1</sup>) and granular biomass reactor (containing 2.5 g-VSS L<sup>-1</sup>). The NRR of the reactor 6 was slowly but steadily increased to 5.8 kg-N m<sup>-3</sup> d<sup>-1</sup>, whereas the granular biomass reactor could attain only NRR of 3.5 kg-N m<sup>-3</sup> d<sup>-1</sup> at the end of operation. Based on these experimental results, the minimal concentration of anammox biomass that is necessary for meeting the criteria of rapid and successful start-up of anammox process was 0.33 g-VSS L<sup>-1</sup> in this study. It should be noted that only about 10% of the granular biomass is good enough for rapid and successful start-up when the biomass is immobilized in PVA-SA gel beads.



**Fig. 4.2:** Nitrogen removal rates (NRRs), nitrogen loading rates (NLRs) and effluent  $\text{NO}_2^-$  concentrations of six column reactors containing artificially immobilized anammox biomass. These reactors were operated at  $37^\circ\text{C}$  for 35 days.

#### 4.3.2. Specific activity derived from batch test

To explain the better performance of the immobilized biomass reactor, specific anammox activity (SAA) was determined after 35-day operation by measuring the production of mixed labelled  $^{14+15}\text{N}_2$  from the cultures incubated with  $^{15}\text{N}$ -ammonium and  $^{14}\text{N}$ -nitrite. Production of  $^{29}\text{N}_2$  is the true evidence of anammox process and can be distinguished from the heterotrophic denitrification and other biological reactions (Holtappels et al., 2011; Kartal et al., 2007). The SAA of the immobilized biomass was  $278.5 \pm 30.9 \mu\text{mol-}^{29}\text{N}_2 \text{ g-VSS}^{-1} \text{ h}^{-1}$ , which was significantly higher than that of the granular biomass ( $184.7 \pm 30.9 \mu\text{mol-}^{29}\text{N}_2 \text{ g-VSS}^{-1} \text{ h}^{-1}$ ) ( $P < 0.05$ ) (**Table 4.2**). These values were within the range of previously determined SAA values for anammox biomass (Dapena-Mora et al., 2007; Isaka et al., 2008; Magrı et al., 2012). The higher SAA of the immobilized biomass supported the higher NRRs of the immobilized biomass reactors with lower biomass concentration.



**Fig. 4.3:** Time courses of nitrogen loading rates (NLRs) and removal rates (NRRs) of six up-flow column reactors containing immobilized anammox biomass and granular biomass. These reactors were operated under the same conditions at 37°C for 35 days.

**Table 4.2:** Specific anammox activity (SAA) of granular and immobilized biomass harvested from up-flow column after 35 days of operation. Anammox activity was measured based on  $^{29}\text{N}_2$  production rate from  $^{15}\text{N}$ -ammonium and  $^{14}\text{N}$ -nitrite.

Description		Granular Biomass	Immobilized Biomass
$^{29}\text{N}_2$ Production rate ( $\mu\text{mole h}^{-1} \text{ vial}^{-1}$ )	AVG	0.23	0.27
	SD	0.03	0.03
Biomass concentration <sup>3</sup>	mg-protein vial <sup>-1</sup>	0.74	0.59
	mg-VSS vial <sup>-1</sup>	1.22	0.97
Specific anammox activity ( $\mu\text{mole-}^{29}\text{N}_2 \text{ g-VSS}^{-1} \text{ h}^{-1}$ )	AVG	184.73	278.48
	SD	30.92	30.94
	$n^1$	3	3
	$t$	-3.71	
	$df^2$	4	
	$p$ -value	0.020	

<sup>1</sup> “ $n$ ” indicates number of samples.

<sup>2</sup> “ $df$ ” represents degree of freedom.

#### 4.3.3. Effective diffusion coefficient in immobilized biomass

The effective diffusion coefficients,  $D_e$ , in the immobilized and granular biomass were determined to explain the higher SAA in the immobilized biomass. It is speculated that  $D_e$  in the immobilized biomass is different from that in the granular biomass because  $D_e$  is highly dependent on the biomass matrix such as gel polymer or bacterial biomass including microbial exopolymers (de Beer et al., 1997b; Stoodley et al., 1994;

Stewart, 1998). The  $D_e$  of  $\text{NH}_4^+$  at 37°C in the granular and immobilized biomass were determined to be  $8.6 \pm 2.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  and  $29.0 \pm 6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ , respectively (**Table 4.3**). Accordingly, the ratios of  $D_e/D_{aq}$  were calculated as 0.23 and 0.77, respectively. These values of  $D_e/D_{aq}$  were comparable to the values for biofilms (Stewart, 2003). The determined  $D_e$  values were used for the following calculation of volumetric  $\text{NH}_4^+$  and  $\text{NO}_2^+$  consumption rates and  $\text{NO}_3^-$  production rate on the basis of concentration profiles determined by microelectrode measurements (**Fig. 4.4**).

#### 4.3.4. Spatial distribution and activity of anammox bacteria

In order to explain the higher SAA in the immobilized biomass than the granular biomass, the spatial distributions of anammox activity and bacteria within the immobilized and granular biomass were analyzed using microelectrode measurement and FISH analysis. The steady state concentration profiles of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and pH in the granular and immobilized biomass were measured up to a depth of 1100  $\mu\text{m}$  with 100  $\mu\text{m}$  step size (**Fig. 4.4A and B**). The  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations concomitantly decreased while the concentration of  $\text{NO}_3^-$  and pH increased inside the granular and immobilized biomass, which indicates typical evidence of anammox activity.

**Table 4.3:** Effective diffusion coefficients,  $D_e$ , and relative effective diffusivity,  $D_e/D_{aq}$ , for granular and immobilized biomass.

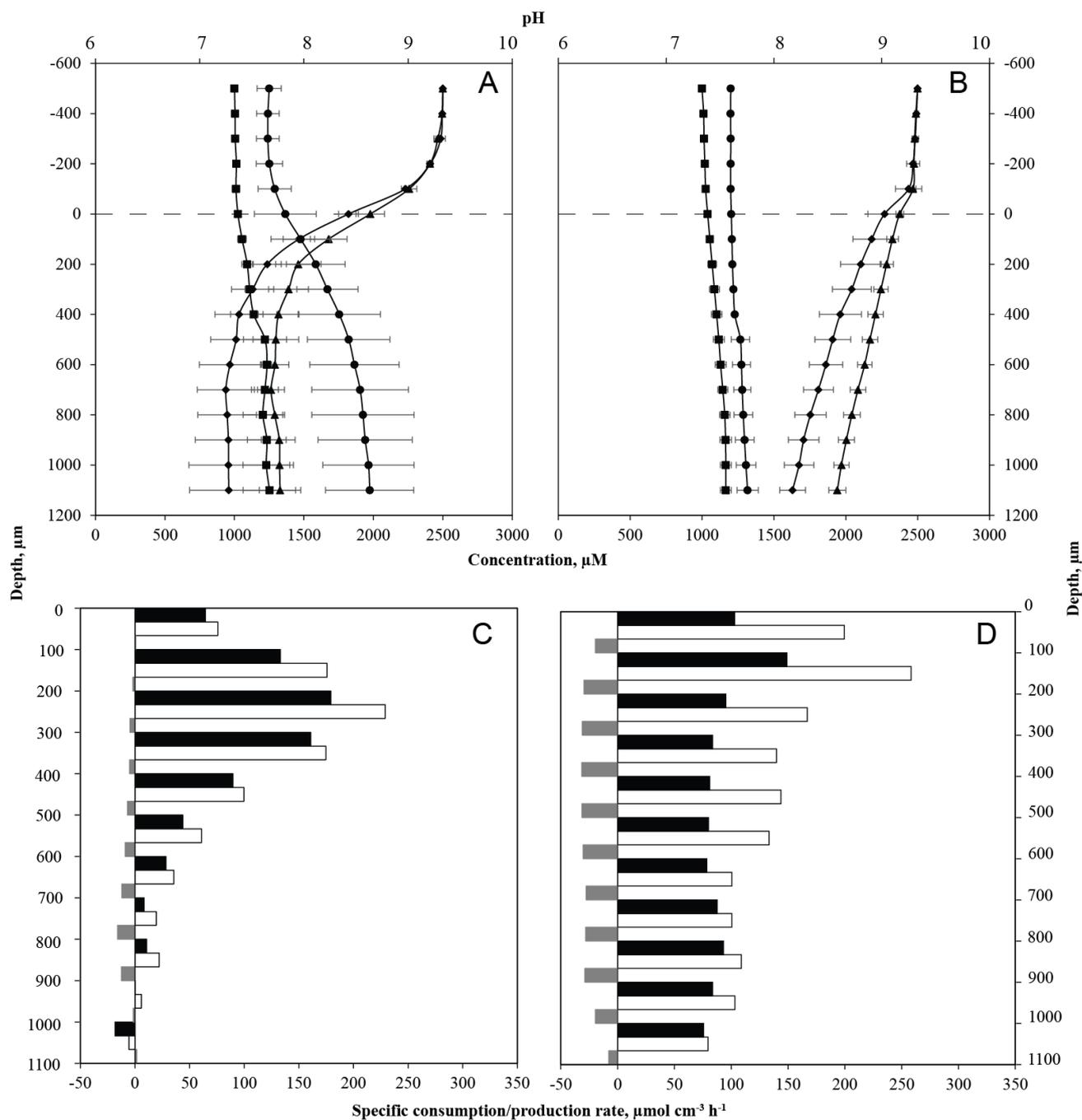
Description	Effective diffusion coefficient, $D_e$ ( $\times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ )	
	Granular biomass	Immobilized biomass
$\text{NH}_4^+$	8.61	28.99
$\text{NO}_2^-$	8.31	27.96
$\text{NO}_3^-$	8.26	27.82
	Relative effective diffusivity	
$D_e/D_{aq}^1$	0.23	0.77

<sup>1</sup> “ $D_e/D_{aq}$ ” indicates relative effective diffusivity and have no units.

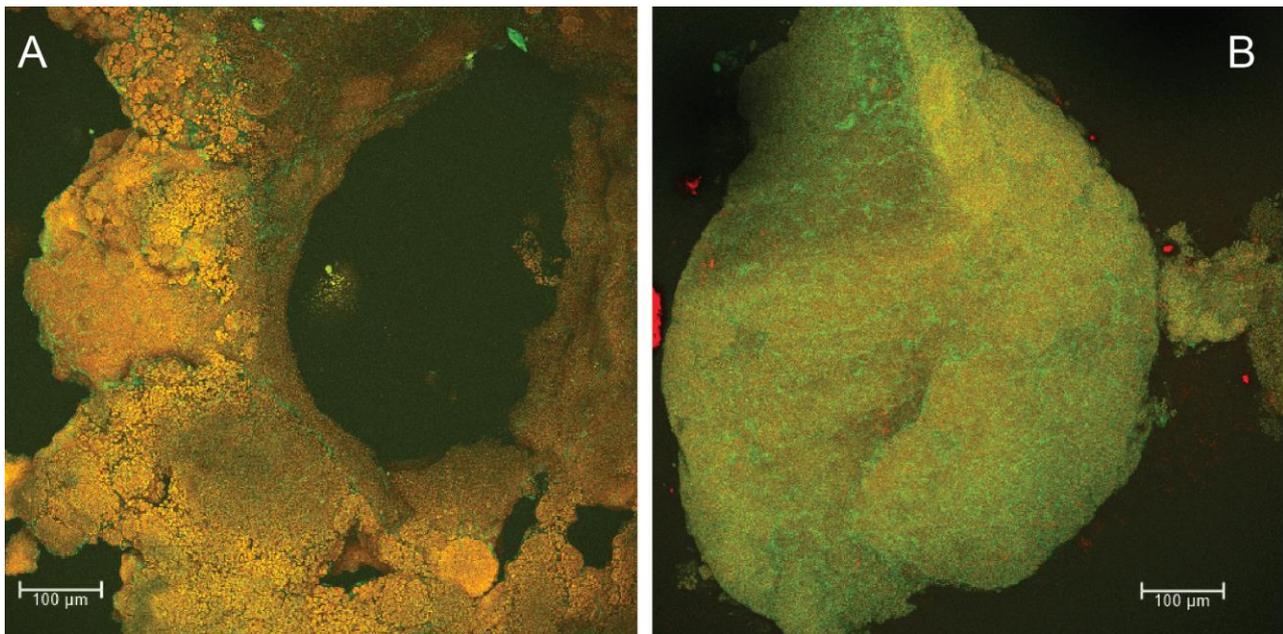
In the granular biomass, high  $\text{NH}_4^+$  and  $\text{NO}_2^+$  consumption rates were observed only in the outer 600  $\mu\text{m}$  (**Fig. 4.4C**). Even though  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations still remained at high levels, no significant anammox activity was observed below 600  $\mu\text{m}$  depth. This is probably because pH was increased up to 8.5 and higher in the inner layer (below 600  $\mu\text{m}$ ), which probably inhibited the anammox activity. It was reported that anammox activity was reduced to half (Awata et al., 2013; Oshiki et al., 2011) and even to quarter (Ali et al., 2014a) at pH 8.5. Similar localizations of anammox activity,  $\text{NH}_4^+$  and  $\text{NO}_2^-$  consumptions,  $\text{NO}_3^-$  production and increase in pH mainly in the outer 0 ~ 500  $\mu\text{m}$  layer, were observed in the anammox biofilms (Cho et al., 2011; Kindaichi et al., 2007; Tsushima et al., 2007a).

In contrast, moderate  $\text{NH}_4^+$  and  $\text{NO}_2^-$  consumption and  $\text{NO}_3^-$  production rates were evenly observed throughout the immobilized biomass (down to 1,100  $\mu\text{m}$ ) (**Fig. 4.4D**). pH around 7.6~7.8 was unchanged throughout the immobilized biomass. These experimental data suggested that substrates and bicarbonate were rapidly replenished from the bulk solution since the effective diffusion coefficient in the immobilized biomass

was three times higher than one in the granular biomass (Table 4.3). To our knowledge this is the first report presenting experimental evidence to explain the high anammox activity in the immobilized biomass.

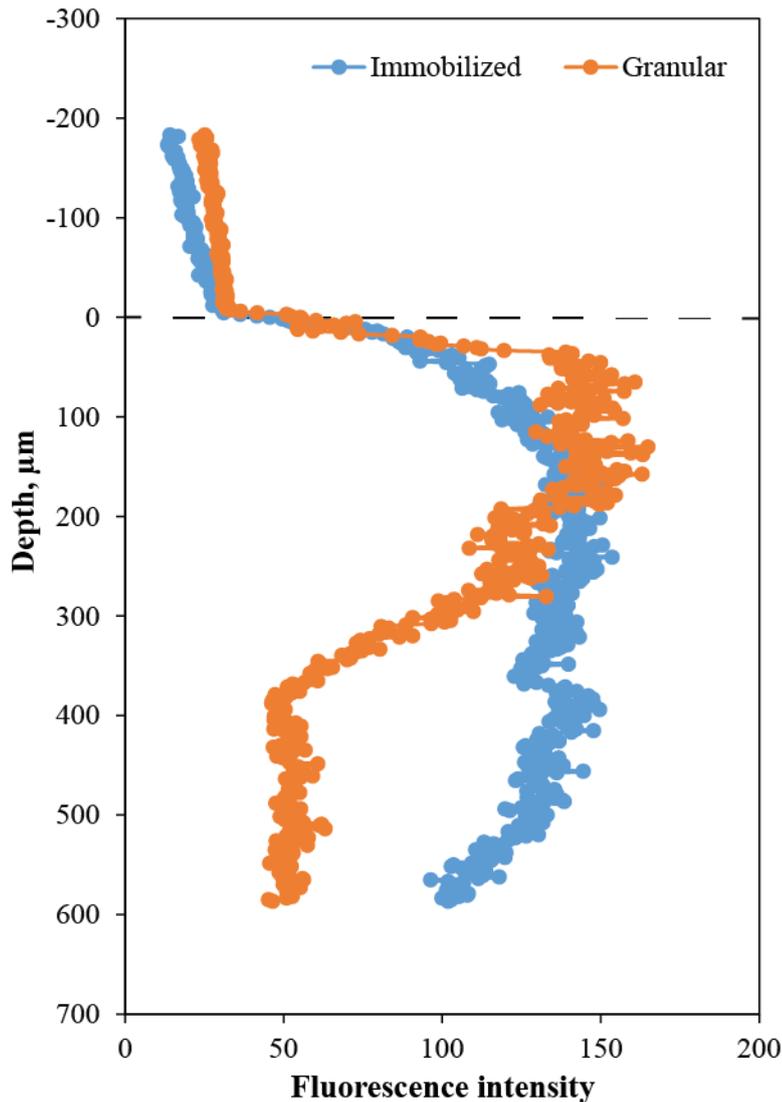


**Fig. 4.4:** Steady state concentration profiles of ammonium (triangle), nitrite (diamond), nitrate (square) and pH (circle) in granular biomass (A) and immobilized biomass (B). Dashed line represents a liquid-solid interface of granular or immobilized biomass. These measurements were performed under anoxic condition that was maintained by purging N<sub>2</sub> gas. Three profiles were measured for each chemical species using three granules. Error bars indicate the range of standard deviations (SD) derived from triplicate measurements. Spatial distributions of the volumetric consumption and production rates of ammonium (black filled bars), nitrite (empty bars), and nitrate (grey filled bars) of the granular and immobilized biomass are calculated from the average measured concentration profiles (A and B) and the newly determined effective diffusion coefficients ( $D_e$ ) (Table 4.3) using Microsoft EXCEL 2013 add-ins solver, respectively (C and D). Positive and negative values indicate the consumption and production rates, respectively.



**Fig. 4.5:** Confocal laser-scanning microscope image of thin cross-section of the granular anammox biomass (**A**) and immobilized anammox biomass (**B**), showing *in situ* spatial organization of anammox bacteria (yellow) and coexisting other bacteria (green). Fluorescence *in situ* hybridization was performed with FITC-labeled EUB mix probe composed of equimolar EUB338, EUB338II, and EUB338III (green) for most members of Eubacteria and TRITC-labeled amx820 probe (red) for anammox bacteria. Both probes hybridized with anammox bacteria, resulting in yellow signal. Higher abundance of anammox bacteria is detected in the outer layer of the granular biomass, whereas more homogeneous distribution was found in the immobilized biomass. Scale bars represent 100  $\mu\text{m}$ .

Substrate transport limitation due to the low diffusivity in the granular biomass resulted in the localization of anammox activity (**Fig. 4.4A and 3C**) and consequently bacterial cells (biomass) in the outer layer (**Fig. 4.5 and Fig. 4.6**). Similar localizations of bacterial cells or biomass density in the anammox granules and biofilms were reported previously (Kindaichi et al., 2007; Lin et al., 2013; Tsushima et al., 2007b). High biomass density and low effective diffusion of substrates created the sharp decrease in  $\text{NH}_4^+$  and  $\text{NO}_2^+$  concentrations and pH increase in the granular biomass (**Fig. 4.4A**). Anammox biomass in the inner part of the granule biomass did not contribute to the overall nitrogen removal. In contrast, due to the higher diffusivity the substrate transport was accelerated to sustain the activity of anammox bacteria throughout the immobilized biomass, which leads to the homogeneous distribution of anammox biomass (**Fig. 4.5B and Fig. 4.6**) and consequently the higher SAA than the granular biomass (**Table 4.2**). It should be noted that biomass concentration is important for the immobilized biomass reactors whereas specific surface area is more important than biomass concentration for the granular biomass reactor due to substrate transport limitation.

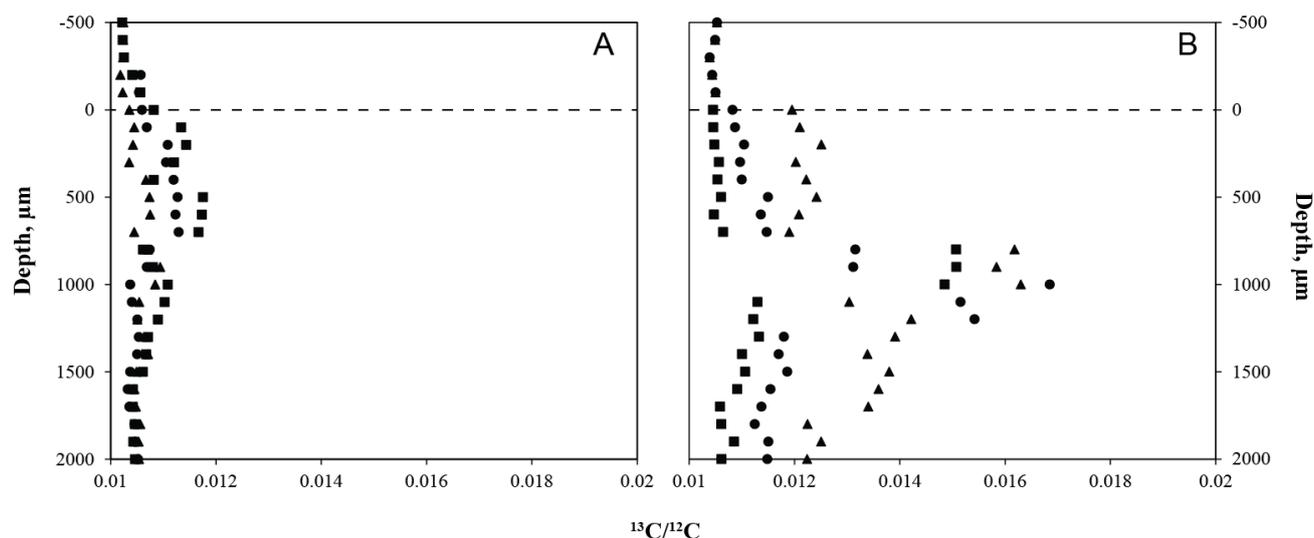


**Fig. 4.6:** Fluorescence intensity profile of granular and immobilized biomass. Dashed line represent surface of the biomass and positive depths are inside of the biomass. Ten different line profiles were randomly chosen for fluorescence intensity analysis from the FISH images of both types of biomass by using image processing software, (LAS AF, version 4.4). Shown fluorescence intensity profiles represent average values of ten randomly chosen fluorescence intensity profiles for each type of biomass.

#### 4.3.5. $^{13}\text{C}$ -bicarbonate incorporation

The spatial distributions of *in situ* anammox activity of the immobilized and granular biomass were further investigated by measuring  $^{13}\text{C}$ -bicarbonate incorporation of anammox bacterial cells by using a secondary ion mass spectrometry (SIMS) (**Fig. 4.7**). Much higher incorporation of  $^{13}\text{C}$ -bicarbonate was observed in the immobilized biomass than the granular biomass. Anammox bacteria present even in the inner part of the immobilized biomass significantly incorporated  $^{13}\text{C}$ -bicarbonate, but not in the granular biomass. Adsorption and entrapment of  $^{13}\text{C}$ -bicarbonate in gel matrix was not observed for the immobilized gel beads incubated as negative control. These results were in good agreement with the results of the microelectrode measurements and FISH analysis.

For this experiment, selection of isotope-labelled substrate and incubation time were important to avoid cross-feeding of isotope (Kindaichi et al., 2004; Okabe et al., 2005).  $^{13}\text{C}$ -bicarbonate was used as a labelling substrate (carbon source) in this study, because anammox bacteria dominated in both the biomass (more than 94% of total bacteria) and all reactors were operated under anoxic condition to exclude the contribution of ammonium-oxidizing bacteria (AOB). Considering the doubling time and growth yield of *Ca. Brocadia sinica* (Oshiki et al., 2011) and preliminary experimental results, 35-day incubation period was chosen, in which the  $^{13}\text{C}$  cross-feed to heterotrophic bacteria likely did not occur.



**Fig. 4.7:** Carbon isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ) of the granular (A) and immobilized biomass (B) analysed by secondary ion mass spectrometry (SIMS) through line profiling technique with a step size of 100  $\mu\text{m}$ . Dashed lines (0  $\mu\text{m}$  depth) represent the surface of the granular and immobilized biomass. Positive depths indicate inside of the biofilm. The values of  $^{13}\text{C}/^{12}\text{C}$  at negative depths are considered as natural abundance of carbon isotope ratio. In preliminary experiments, adsorption/entrapment of  $^{13}\text{C}$ -bicarbonate in gel matrix was not observed for the immobilized biomass. Granular and immobilized biomass were separately inoculated in up-flow column reactor and fed with nutrient medium containing  $^{13}\text{C}$ -bicarbonate as sole carbon source for 35 days. Triplicate measurements were taken for each type biomass represented by triangle, circle and square.

#### 4.4. CONCLUSION

- For rapid and successful start-up of anammox process, anammox biomass was immobilized in a mixture gel of 6% (w/v) PVA and 2% (w/v) SA, which were then packed in up-flow column reactors. The minimal initial concentration of anammox biomass that is necessary for meeting the criterion of rapid and successful start-up (achieving the nitrogen removal rate (NRR) of higher than  $10 \text{ kg-N m}^{-3} \text{ d}^{-1}$  within a month) was determined.
- The column reactor containing the immobilized biomass ( $0.33 \text{ g-VSS L}^{-1}$ ) was started up much faster than the reactor containing the granular biomass ( $2.5 \text{ g-VSS L}^{-1}$ ) and achieved higher NRR ( $10.8 \text{ kg-N m}^{-3} \text{ d}^{-1}$ ) after 35-day operation. This result indicates that PVA-SA gel immobilization is an efficient strategy to initiate anammox reactors with minimal quantity of anammox biomass.
- The effective diffusion coefficient in the immobilized biomass (gel beads) was directly determined by microelectrodes and found to be more than three times higher than one in the naturally aggregated granular

biomass. The substrate transport was accelerated to sustain the activity of anammox bacteria in the inner part of the immobilized biomass, which consequently resulted in the higher nitrogen removal rate than the granular biomass.

A considerably small amount of anammox biomass is enough for successful establishment of anammox reactors if PVA-SA gel immobilization technology is used. It often needs long periods of time and many efforts to grow anammox granules. Even though granules were developed, anammox biomass inside of the granules would not contribute to nitrogen removal due to diffusion limitation as presented in this study. Although the gel immobilization is an efficient method to establish anammox reactors, the durability of the immobilized biomass for long-term operation should be investigated in future study.

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## 5. ECOPHYSIOLOGY OF ANAMMOX BACTERIUM “*CANDIDATUS JETTENIA CAENI*”

### 5.1. Introduction

Anammox activities and/or anammox bacterial 16S rRNA gene sequences have been ubiquitously detected in anoxic environments, including marine, freshwater, and terrestrial ecosystems, areas in which anammox significantly contributes to nitrogen loss up to nearly 100% (Brandes et al., 2007; Long et al., 2013; Zhu et al., 2013). In addition to its geochemical significance, the anammox process has attracted a great deal of attention as an environmentally friendly wastewater treatment process (Kartal et al., 2010; Ali et al., 2013).

Anammox bacteria are monophyletically affiliated with the bacterial phylum Planctomycetes (Strous et al., 1999a); six candidate genera have been provisionally proposed to be in this bacterial lineage, including “*Ca. Brocadia*” (Kuenen and Jetten, 2001), “*Ca. Anammoxoglobus*” (Kartal et al., 2007a), “*Ca. Jettenia*” (Quan et al., 2008), “*Ca. Kuenenia*” (Egli et al., 2003), “*Ca. Scalindua*” (Kuypers et al., 2003), and “*Ca. Anammoximicrobium*” (Khramenkov et al., 2013). Although no pure culture is currently available, several enrichment cultures have been obtained. Interestingly, anammox bacteria have been enriched as monospecies, with one anammox species outcompeting the others in the original biomass (van der Star et al., 2007; Park et al., 2010; Huang et al., 2013). Therefore, each anammox bacterial species (or genus) may have distinct physiological features. For instance, anammox bacteria affiliated with the genus “*Ca. Brocadia*” and “*Ca. Kuenenia*” may be growth-rate and affinity strategists, respectively, and thus the latter can overgrow under low  $\text{NH}_4^+$  and/or  $\text{NO}_2^-$  conditions (van der Star et al., 2008; Ding et al., 2013). “*Ca. Anammoxoglobus propionicus*” and “*Ca. Brocadia fulgida*” show a great ability to oxidize propionate and acetate with reduction of  $\text{NO}_3^-$ , respectively; therefore, they can overgrow in the conditions in which propionate or acetate is constantly available (Kartal et al., 2007a; Kartal et al., 2008). “*Ca. Scalindua*” is regarded as a marine genus as its 16S rRNA genes have been retrieved mainly from marine ecosystems (Schmid et al., 2007; Woebken et al., 2008), and as “*Ca. Scalindua* sp.” lost the anammox activity in the absence of salinity (Awata et al., 2013).

In contrast, little is known about the physiological characteristics of the genus “*Ca. Jettenia*” presently. The 16S rRNA gene sequences affiliated with the genus “*Ca. Jettenia*” have primarily been retrieved from freshwater ecosystems (Humbert et al., 2010; Yoshinaga et al., 2011; Zhu et al., 2011; Humbert et al., 2012; Wang et al., 2012; Hu et al., 2013a; Long et al., 2013; Shen et al., 2013), suggesting that “*Ca. Jettenia*” is a freshwater genus.

Recently, the nearly complete genome sequences of the genus “*Ca. Jettenia*” (i.e., planctomycete KSU-1 and “*Ca. Jettenia asiatica*”) (Hira et al., 2012; Hu et al., 2012) were determined, which would provide an insight into the underlying biochemical mechanisms of anammox process. Reduction of  $\text{NO}_2^-$  to nitric oxide (NO) is the first reaction in the anammox process (Kartal et al., 2013), and genomic data suggested that “*Ca. Jettenia*” uses a copper-containing nitrite reductase (NirK) for this reaction (Hira et al., 2012). In contrast, anammox bacteria affiliated with the genus “*Ca. Kuenenia*” or “*Ca. Scalindua*” reduce  $\text{NO}_2^-$  to NO using a

cytochrome cd<sub>1</sub>-type nitrite reductase (NirS) (Strous et al., 2006; Kartal et al., 2011; van de Vossenberg et al., 2013). In addition to nirK, genes encoding hydrazine synthase (Hzs) and hydrazine dehydrogenase (Hdh) are also present in the “*Ca. Jettenia*” genomes, suggesting that “*Ca. Jettenia*” cells metabolize hydrazine as an intermediate of the anammox process. In addition to the genes responsible for the anammox process, nrfA, encoding pentaheme cytochrome c nitrite reductase, is located in the genome, suggesting that “*Ca. Jettenia*” cells can conduct respiratory ammonification; i.e., the second step of dissimilatory nitrate reduction to ammonium. Although the genomic data enable determination of the above physiological traits, these data can only suggest potential metabolic capabilities. Therefore, proteomic analysis examining global protein expression patterns in microbial cells is required to complement the genomic studies. In “*Ca. Kuenenia stuttgartiensis*” and “*Ca. Scalindua profunda*” cells, Hzs and Hdh were highly expressed, indicating that these anammox bacterial cells can synthesize and oxidize hydrazine as an intermediate (Kartal et al., 2011; van de Vossenberg et al., 2013). Furthermore, physiological experiments are essential for analyzing the deduced metabolic capabilities in the “*Ca. Jettenia*” genomes; i.e., hydrazine metabolism and respiratory ammonification. In addition, anammox bacteria have unique intracellular structures with three separated cellular compartments, including the periplasm, riboplasm, and anammoxosome (van Niftrik and Jetten, 2012), while the cellular structure of “*Ca. Jettenia*” cells remains unknown. Moreover, quinone is an electron carrier synthesized by prokaryotes; anammox bacteria utilize menaquinone (MK) for the transfer of electrons extracted from hydrazine by dehydrogenation to membrane-bound protein complexes that generate proton-motive force and/or relay electrons to reductive processes in the periplasm or anammoxosome (Kartal et al., 2013). The MK molecule possesses an isoprenoid side chain with species-specific lengths (Hiraishi et al., 1998); the nature of the quinone side chain in anammox bacteria including “*Ca. Jettenia*” remains hitherto unknown.

Trace metals are essential cofactors for a variety of enzymes. “*Ca. Kuenenia stuttgartiensis*” and “*Ca. Scalindua profunda*” cells highly expressed heme proteins, including Hzs, Hdh, and hydroxylamine dehydrogenase (Hao) (Kartal et al., 2011; van de Vossenberg et al., 2013), suggesting that large amounts of iron are necessary for coordination at the center of the heme molecules (Klotz et al., 2008; Bali et al., 2011). Iron is also required for some redox-active molecules other than the heme, including the iron-sulfur cluster (Ayala-Castro et al., 2008). In addition, CO dehydrogenase/acetyl-CoA synthase (Acs), which is responsible for CO<sub>2</sub> fixation in anammox bacterial cells, was also found to be highly expressed (Kartal et al., 2011; van de Vossenberg et al., 2013). The beta/alpha subunits of this protein complex require nickel and/or copper as metal cofactors. Furthermore, “*Ca. Jettenia*” genomes appear to encode copper-containing NirK instead of cytochrome cd<sub>1</sub>-containing NirS as canonical NO-forming nitrite reductases. Thus, copper appears to be an abundant trace metal in “*Ca. Jettenia*” cells. The metalloproteins are clearly involved in the core metabolism of anammox bacteria, although the abundance of these metals in anammox bacterial cells has not been determined.

Consequently, the objective of this study was to investigate the physiological characteristics of anammox bacteria affiliated with the genus “*Ca. Jettenia*”. An enrichment culture of planctomycete KSU-1 (>90% of the total biomass) was successfully established. Phylogenetic analysis of 16S-23S rRNA gene

sequences indicated that the planctomycete KSU-1 represents a new species in the genus “*Ca. Jettenia*”, and thus the name “*Ca. Jettenia caeni*” sp. nov. was proposed (See Experimental procedures.). In the present study, the physiological characteristics of “*Ca. Jettenia caeni*”, including 1) the temperature and pH ranges of anammox activities; 2) apparent half-saturation constants ( $K_S$ ) for  $\text{NH}_4^+$  and  $\text{NO}_2^-$ ; 3) inhibition of anammox activity by  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , salinity, sulfide, organic matter (i.e., methanol, ethanol, propanol, formate, acetate, and glucose), and a copper chelator, diethyldithiocarbamate (DDC); 4) maximum specific growth rate ( $\mu_{\max}$ ) calculated from biomass yield ( $Y$ ) and maximum anaerobic ammonium oxidation rate ( $q_{\max}$ ); 5) hydrazine biosynthesis with consumption of hydroxylamine; 6) respiratory ammonification; and 7) respiratory quinone species were investigated. Cellular structures and abundance of trace metal elements in the whole cell lysate were examined using transmission electron microscopy and inductively coupled plasma atomic emission spectroscopy (ICP-AES), respectively. Proteomic analysis was carried out using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nanoscale liquid chromatography tandem mass spectrometry (nanoLC/MS/MS).

## 5.2. Results

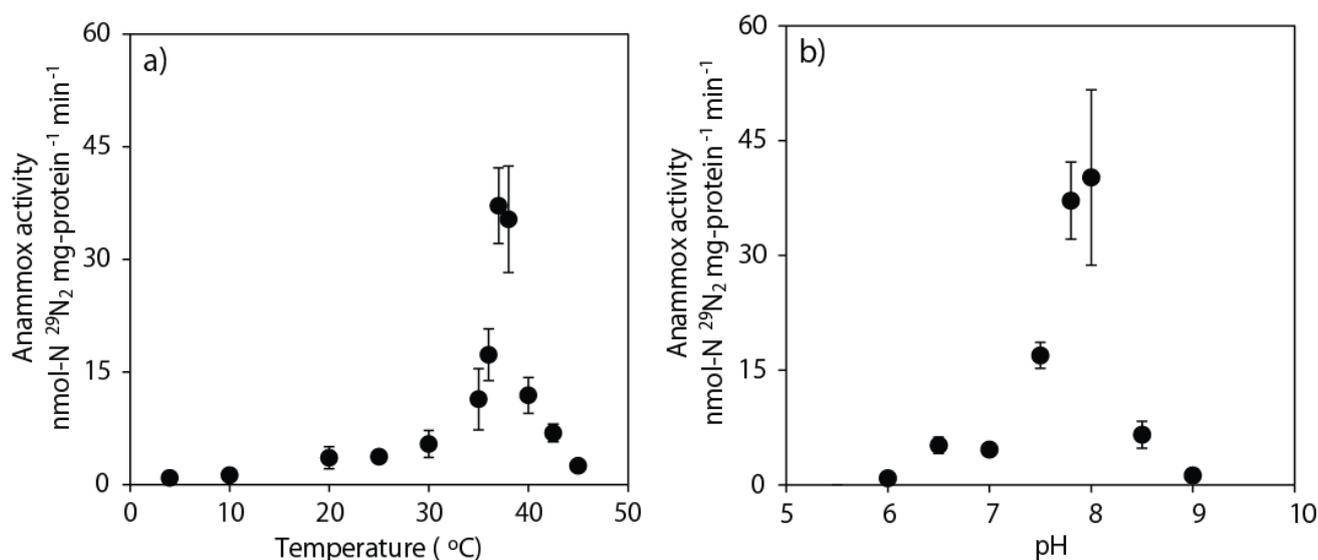
### 5.2.1. Physiological characteristics

The cells of “*Ca. Jettenia caeni*” were anoxically incubated in the presence of  $^{15}\text{NH}_4^+$  and  $^{14}\text{NO}_2^-$ , and anammox activities were specifically determined as  $^{29}\text{N}_2$  gas production rates. Physiological characteristics of “*Ca. Jettenia caeni*” are summarized in **Table 5.1**, and detailed explanations are described below. Anammox activities were detected at 20–42.5°C and pH 6.5–8.5, with maximum anammox activity detected at 37°C and pH 8 (**Fig. 5.1a and b**). Mean ( $\pm$  standard deviation, SD) activation energy was  $75.1 \pm 8.4$  kJ/M, as determined from an Arrhenius plot of rate constants for anammox activities. The mean ( $\pm$  SD)  $K_S$  values for  $\text{NO}_2^-$  and  $\text{NH}_4^+$  were  $35.6 \pm 0.92$  and  $17.1 \pm 4.3$   $\mu\text{M}$ , respectively, based on the Monod equation (**Fig. 5.2**).

Salinity (sodium chloride), sulfide, and  $\text{NO}_2^-$  inhibited anammox activity, and 50% inhibitory concentrations ( $\text{IC}_{50}$ ) were 68 mM, 540  $\mu\text{M}$ , and 11 mM, respectively (**Fig. 5.3**). Similarly, anammox activity was inhibited by the addition of formate, propanol, and ethanol but not by acetate, glucose, and methanol (**Table 5.2**). Specifically, anammox activity was reduced by >90% with the addition of 1 mM ethanol or propanol. Addition of 12.5 mM DDC reduced anammox activity by 59%.

The  $\mu_{\max}$  was calculated from  $Y$  and  $q_{\max}$ .  $Y$  was determined following incorporation of  $^{14}\text{CO}_2$  during anaerobic ammonium oxidation, which was  $0.056$  mol-C (mol- $\text{NH}_4^+$ ) $^{-1}$  (**Fig. 5.4**).  $q_{\max}$  was determined by incubating “*Ca. Jettenia caeni*” cells in an up-flow column reactor while continuously supplying  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . The  $q_{\max}$  during the 45 d of operation was  $2.35$  kg-N  $\text{NH}_4^+ \cdot \text{m}^{-3} \cdot \text{d}^{-1}$  (**Fig. 5.5**), where biomass concentration in the reactor was  $5.4$  g-VSS  $\text{L}^{-1}$ ; therefore,  $q_{\max}$  was  $0.031$  mol- $\text{NH}_4^+$  g-VSS $^{-1} \cdot \text{d}^{-1}$ . The  $q_{\max}$  was converted to  $0.871$  mol  $\text{NH}_4^+ \cdot \text{mol C}^{-1} \cdot \text{d}^{-1}$  using a conversion factor of  $0.0357$  mol C g-VSS $^{-1}$ , which was derived based on the elemental composition of anammox bacterial cells as  $\text{CH}_2\text{O}_{0.5}\text{N}_{0.15}$  (Strous et al., 1998). Finally,  $\mu_{\max}$  was calculated to be  $0.002$  h $^{-1}$ , corresponding to a doubling time of 14.2 d. The  $\mu_{\max}$  of “*Ca. Jettenia caeni*” cells was

comparable to values determined using anammox bacterial cells enriched in other bioreactors (Strous et al., 1999b; Egli et al., 2001; Awata et al., 2013).



**Fig. 5.1:** Temperature (a) and pH dependency (b) of anammox activities in “Ca. Jettenia caeni”: The cells were anoxically incubated at 4–45°C (a) or pH 6.0–9.0 (b), and anammox activity was determined as <sup>29</sup>N<sub>2</sub> gas production rate. Error bars represent the standard deviations from triplicate vials.

Hydrazine biosynthesis was examined by incubating the cells with NH<sub>4</sub><sup>+</sup> and hydroxylamine (3 mM each). Hydrazine accumulated in the culture concurrently with consumption of hydroxylamine (**Fig. 5.6**). Hydrazine accumulation continued until hydroxylamine had been depleted (i.e., 180 min of incubation); thereafter, hydrazine concentration gradually decreased.

Respiratory ammonification was examined by incubating the cells with <sup>15</sup>NO<sub>3</sub><sup>-</sup> and <sup>13</sup>CH<sub>3</sub>COOH (2 mM and 4 mM, respectively). The <sup>14</sup>NO<sub>2</sub><sup>-</sup> (2 mM) or <sup>14</sup>NH<sub>4</sub><sup>+</sup> (5 mM) was also supplemented as a pool substrate, which would be preferentially utilized and result in higher accumulation of <sup>15</sup>NO<sub>2</sub><sup>-</sup> and <sup>15</sup>NH<sub>4</sub><sup>+</sup>. The “Ca. Jettenia caeni” cells reduced <sup>15</sup>NO<sub>3</sub><sup>-</sup> with the oxidation of <sup>13</sup>CH<sub>3</sub>COOH, resulting in accumulation of <sup>13</sup>CO<sub>2</sub> in the headspace of the vials (data not shown). Reduction of <sup>15</sup>NO<sub>3</sub><sup>-</sup> resulted in accumulation of <sup>15</sup>NO<sub>2</sub><sup>-</sup>, <sup>15</sup>NH<sub>4</sub><sup>+</sup>, <sup>29</sup>N<sub>2</sub>, and <sup>30</sup>N<sub>2</sub> gas (**Fig. 5.7**). In the vials with added <sup>14</sup>NO<sub>2</sub><sup>-</sup> or <sup>14</sup>NH<sub>4</sub><sup>+</sup>, 35% or 3% of <sup>15</sup>NO<sub>3</sub><sup>-</sup> was recovered as <sup>15</sup>NO<sub>2</sub><sup>-</sup> or <sup>15</sup>NH<sub>4</sub><sup>+</sup>, respectively. The nitrate reduction rate by “Ca. Jettenia caeni” cells was 2.0 ± 0.4 nmol·mg protein<sup>-1</sup>·min<sup>-1</sup>, which was slightly higher than that of “Ca. Brocadia sinica”; i.e., 1.3 ± 0.1 nmol·mg protein<sup>-1</sup>·min<sup>-1</sup> (Oshiki et al., 2013a).

Quinones were extracted from “Ca. Jettenia caeni” cells using chloroform and methanol, which were analyzed by reversed-phase high performance liquid chromatography (HPLC). Major respiratory quinone was identified as MK-7 (**Fig. 5.8**).

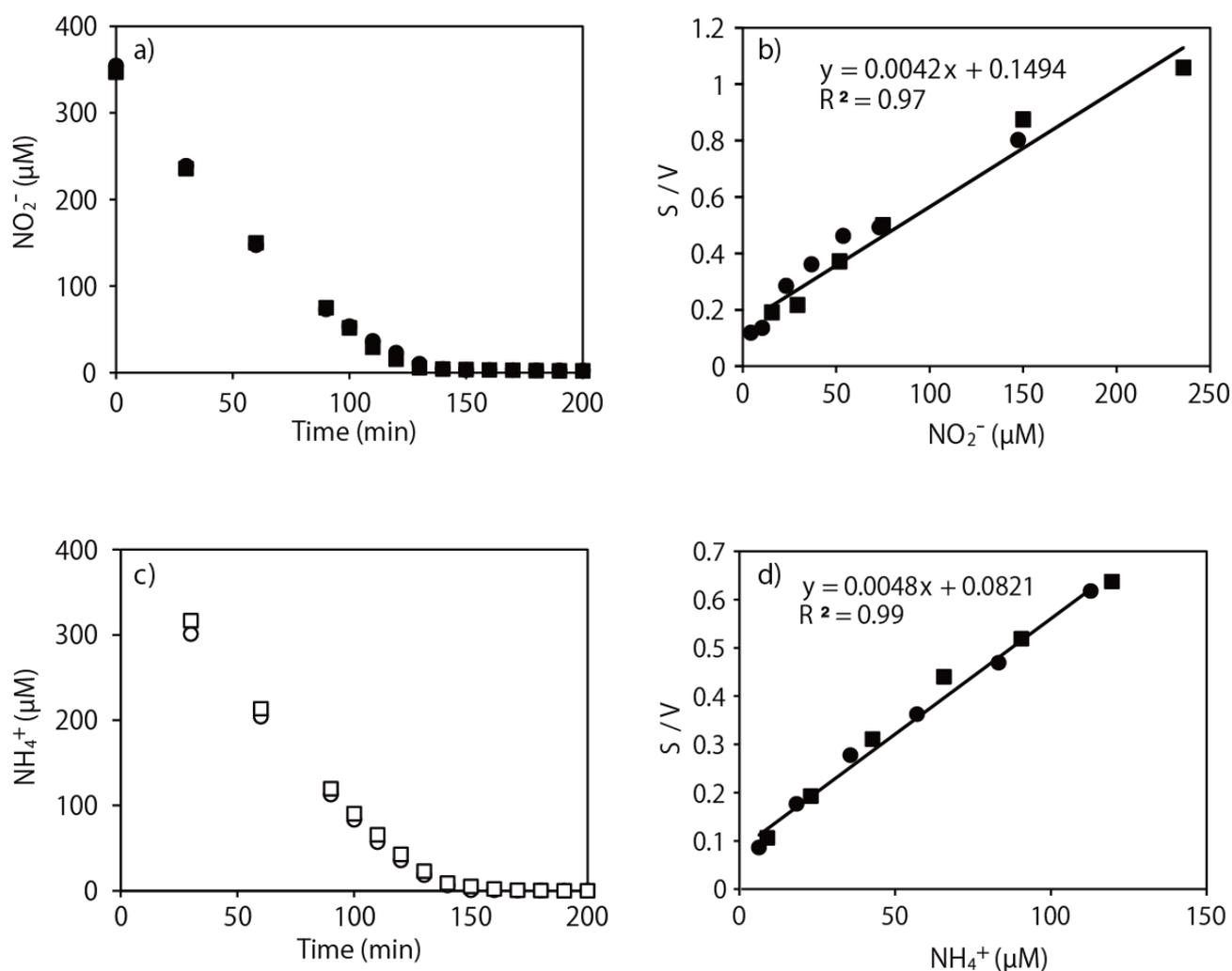
**Table 5.1. Physiological characteristics of “*Candidatus* Jettenia kumamotensis”: *n.d.*; not determined.**

Parameter	“ <i>Ca. Jettenia kumamotensis</i> ”	“ <i>Ca. Brocadia sinica</i> ”	“ <i>Ca. Brocadia anammoxidans</i> ”	“ <i>Ca. Kuenenia stuttgartiensis</i> ”	“ <i>Ca. Scalindua sp.</i> ”	“ <i>Ca. Scalindua profunda</i> ”
<b>Growth Temp.</b> (°C)	20 - 42.5	25 - 45	20 - 43	25 - 37	10 - 30	15 - 45
<b>Growth pH</b>	6.5 - 8.5	7.0 - 8.8	6.7 - 8.3	6.5 - 9.0	6.0 - 8.5	7.4
<b>Biomass yield</b> (mmol-C [mmol-NH <sub>4</sub> <sup>+</sup> ] <sup>-1</sup> )	0.056	0.063	0.07	<i>n.d.</i>	0.03	<i>n.d.</i>
<b>μ<sub>max</sub></b> (h <sup>-1</sup> )	0.002	0.0041	0.0027	0.0026 - 0.0035	0.002	<i>n.d.</i>
<b>Affinity constant (K<sub>s</sub>)</b>						
<b>NH<sub>4</sub><sup>+</sup></b> (μM)	17.1 ± 4.3	28 ± 4	5	<i>n.d.</i>	3	<i>n.d.</i>
<b>NO<sub>2</sub><sup>-</sup></b> (μM)	35.6 ± 0.92	34 ± 21	5	0.2 - 3	0.45	<i>n.d.</i>
<b>Activation energy</b> (kJ mol <sup>-1</sup> )	55.4 ± 6.8	56 ± 3	70	<i>n.d.</i>	81.4 ± 3	<i>n.d.</i>
<b>NO<sub>3</sub><sup>-</sup> reduction activity*</b> (nmol mg-protein <sup>-1</sup> min <sup>-1</sup> )	2.0 ± 0.4	1.3 ± 0.1	<i>n.d.</i>	0.3 ± 0.02 fmol cell <sup>-1</sup> d <sup>-1</sup>	1.45 ± 0.05	7 nmol-formate mg-protein <sup>-1</sup> min <sup>-1</sup>
<b>Tolerance</b>						
<b>NO<sub>2</sub><sup>-</sup></b> (mM)	11	<16	7	13, 25	7.5	<i>n.d.</i>
<b>NH<sub>4</sub><sup>+</sup></b> (mM)	>20	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	>16	<i>n.d.</i>
<b>Sulfide</b> (μM)	540	<i>n.d.</i>	110	10 - 300	<i>n.d.</i>	<i>n.d.</i>
<b>Salinity</b> (mM) <sup>†</sup>	68 mM <sup>†</sup>	<513 mM <sup>†</sup>	<i>n.d.</i>	50 - 200 mM <sup>†</sup>	1.5 - 4.0% (w/v) <sup>‡</sup>	3.3% (w/v) <sup>‡</sup>
<b>Reference</b>	This study	Oshiki <i>et al.</i> , 2011	Jetten <i>et al.</i> , 2005	Egli <i>et al.</i> , 2001; Dapena-Mora <i>et al.</i> , 2007; van de Star <i>et al.</i> , 2008; Russ <i>et al.</i> , 2014	Awata <i>et al.</i> , 2013	van de Vossenberg <i>et al.</i> , 2008

\*Activities were determined by using acetate for “*Ca. Jettenia kumamotensis*”, formate for “*Ca. Kuenenia stuttgartiensis*” and “*Ca. Scalindua profunda*”, and ferrous iron for “*Ca. Brocadia sinica*” and “*Ca. Scalindua sp.*”, respectively.

<sup>†</sup> Concentration of chloride ions.

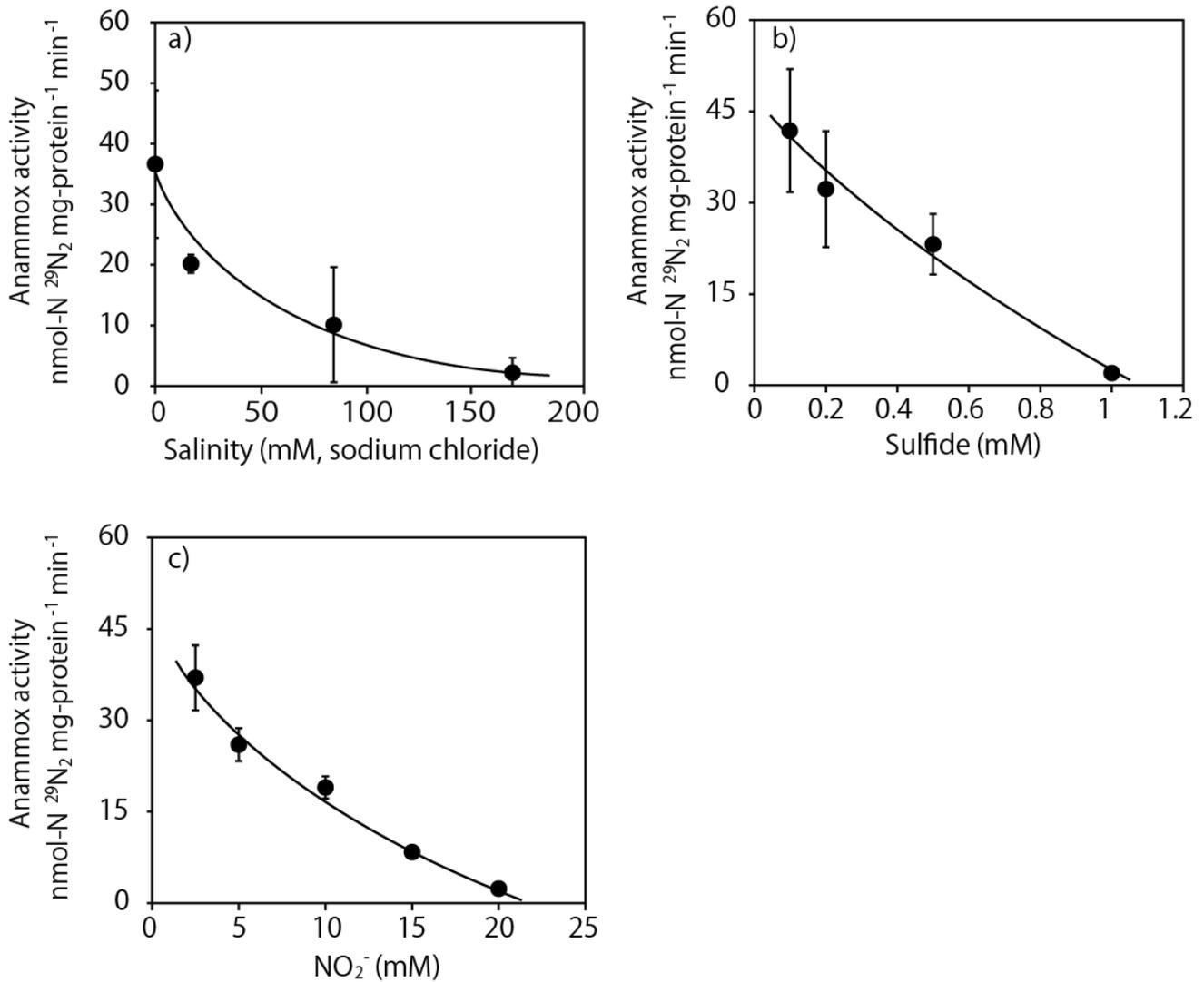
<sup>‡</sup> Growth salinities for “*Ca. Scalindua sp.*” and “*Ca. Scalindua profunda*”. The value for “*Ca. Kuenenia stuttgartiensis*” was determined without acclimatization to salinity condition.



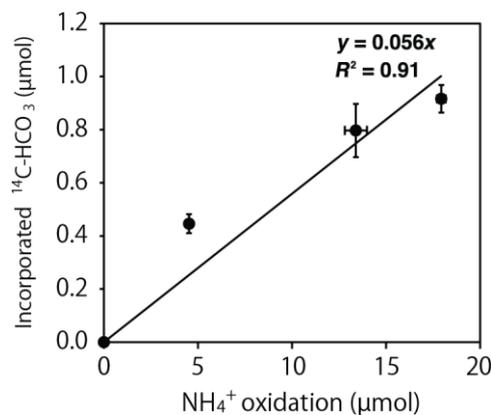
**Fig. 5.2:** Apparent affinity constant ( $K_s$ ) for nitrite and ammonium: The cells of “*Ca. Jettenia caeni*” were anoxically incubated under  $\text{NO}_2^-$ - (panel a) or  $\text{NH}_4^+$ - limiting conditions (panel c). In panels b and d, S and V represent the concentration and consumption rate of nitrogenous compounds, and  $K_s$  for  $\text{NO}_2^-$  and  $\text{NH}_4^+$  were calculated from the Hanes-Woolf plot to be 35.6  $\mu\text{M}$  and 17.1  $\mu\text{M}$ , respectively. Incubations were performed in duplicate as indicated by circle and square symbols, respectively.

**Table 5.2:** Effect of organic matters on specific anammox activity (SAA) of the “*Ca. Jettenia caeni*” cells: Organic matters were supplemented at a final concentration of 1 mM. Standard deviation (SD) represents the standard deviation derived from triplicate vials. *ra*: relative activity against the control vial without addition of organic matter.

Compound	anammox activity (nmol-N $^{29}\text{N}_2$ mg-protein $^{-1}$ per min)		
	average	SD	<i>ra</i> (%)
Acetate	31.1	10.5	83.7
Glucose	20	6	53.7
Formate	7.4	0.9	19.8
Propanol	2.7	0.3	7.3
Methanol	18.6	6.4	50.1
Ethanol	1.1	0.4	3
Control	37.2	5	100

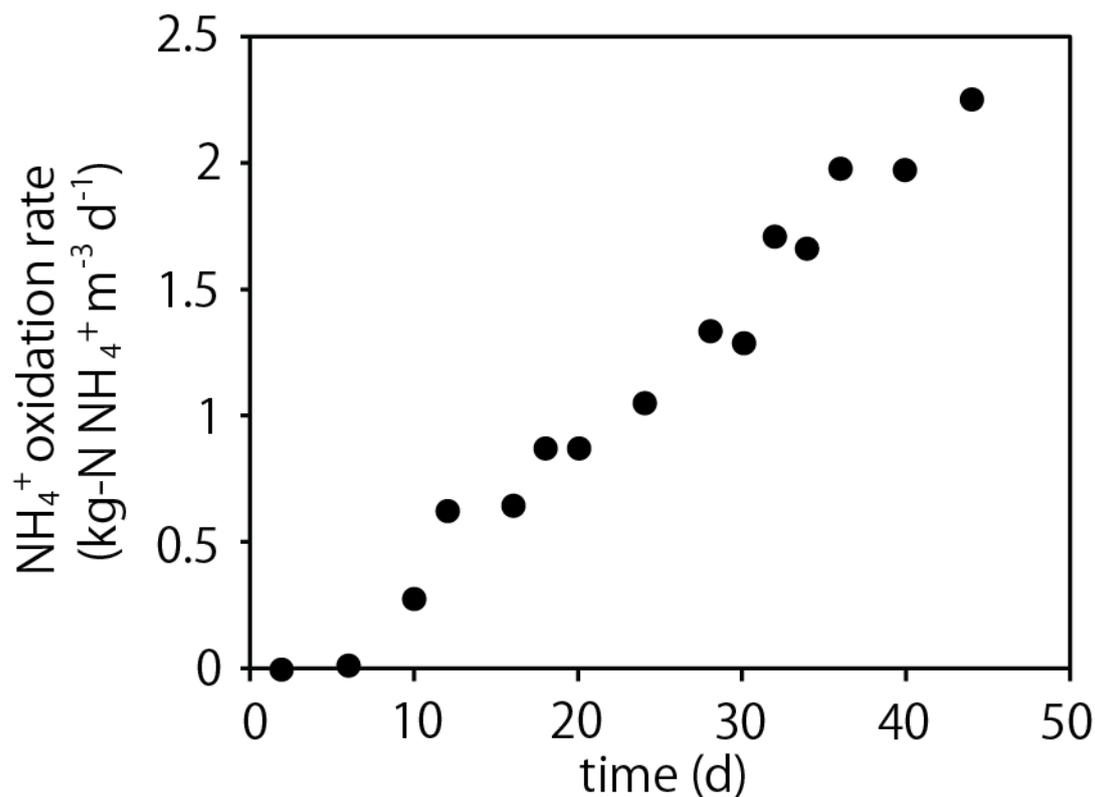


**Fig. 5.3:** Influence of a) salinity, b) sulfide, and c) nitrite on anammox activities of “*Ca. Jettenia caeni*”: Anammox activities were determined as the <sup>29</sup>N<sub>2</sub> production rate. IC<sub>50</sub> concentrations for salinity (sodium chloride), sulfide, and nitrite were determined to be 68 mM, 540 μM, and 11 mM, respectively. Markers represent the average values and error bars represent the standard deviation from triplicate vials.

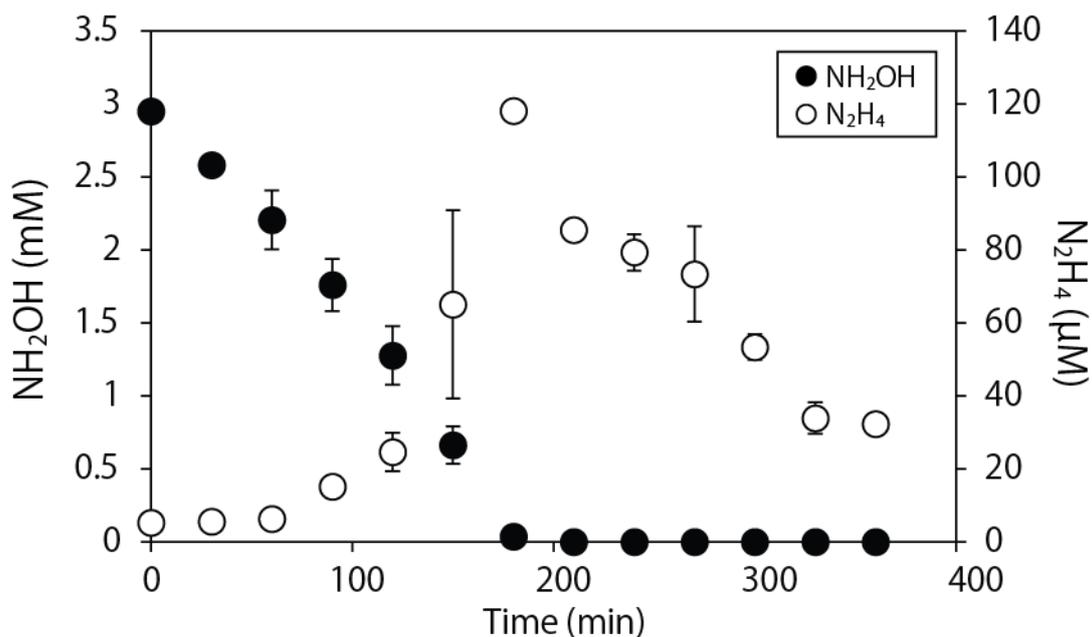


**Fig. 5.4:** Incorporation of [<sup>14</sup>C] bicarbonate by “*Ca. Jettenia caeni*” cells: The cells were anoxically incubated at 37°C for 3 d in the presence of NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup> (6 mM each), and [<sup>14</sup>C] bicarbonate (20 μCi vial<sup>-1</sup>). Incorporation of [<sup>14</sup>C] bicarbonate into the cells was examined in a liquid scintillator. The same incubation was repeated in the absence of [<sup>14</sup>C] bicarbonate, and oxidation of NH<sub>4</sub><sup>+</sup> was measured by ion chromatography. Biomass yields (Y) were obtained

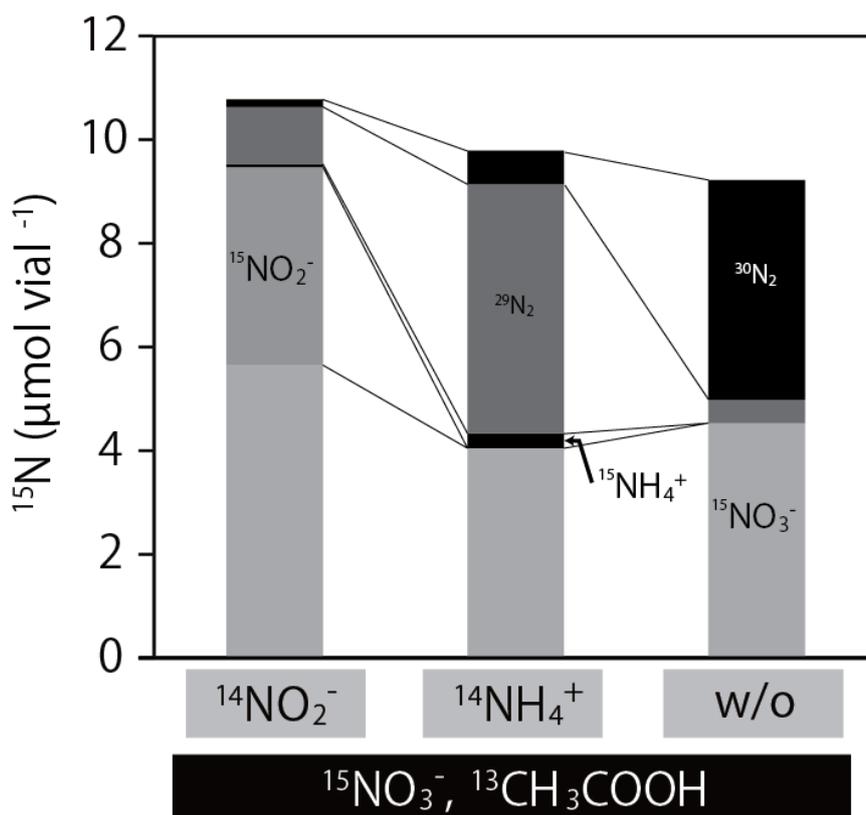
as a slope of the linear regression curve, which was  $0.056 \text{ mol C (mol NH}_4^+)^{-1}$ . Error bars represent the standard deviations from triplicate vials.



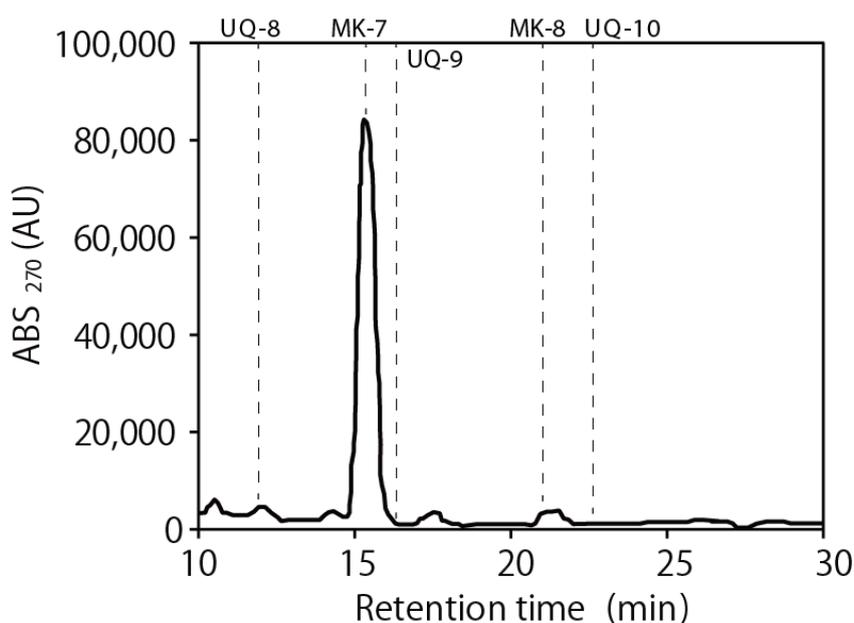
**Fig. 5.5:** Maximum ammonium oxidation rate ( $q_{\max}$ ) of “*Ca. Jettenia caeni*” cells: The cells were cultivated at  $37^\circ\text{C}$  in an up-flow column reactor with a continuous supply of  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . Loading rate of ammonium in the reactor was increased stepwise from  $2.6\text{--}6.4 \text{ kg N}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$ . Ammonium oxidation rate increased to  $2.35 \text{ kg N NH}_4^+\cdot\text{m}^{-3}\cdot\text{day}^{-1}$  at 44 d of operation, where the concentration of volatile suspended solid (VSS) was  $5.4 \text{ g VSS/L}$ ; therefore,  $q_{\max}$  was found to be  $0.031 \text{ mol NH}_4^+\cdot\text{g VSS}^{-1}\cdot\text{d}^{-1}$ .



**Fig. 5.6:** Time course accumulation and consumption of hydrazine after addition of hydroxylamine: The cells of “*Ca. Jettenia caeni*” were anoxically incubated in the presence of  $\text{NH}_4^+$  and hydroxylamine ( $3 \text{ mM}$  each). Liquid samples were collected at 30-min time intervals, and hydroxylamine (filled circles) and hydrazine concentrations (open circles) were determined colorimetrically. Error bars represent the standard deviation of duplicate incubations.



**Fig. 5.7:** Nitrate reduction to ammonium via nitrite by respiratory ammonification: The 5-mL cell suspension was dispensed into 12.5-mL glass vials and incubated for 8 h after adding 2 mM  $^{15}\text{NO}_3^-$  and 4 mM  $^{13}\text{CH}_3\text{COOH}$ . The  $^{14}\text{NO}_2^-$  (2 mM) or  $^{14}\text{NH}_4^+$  (5 mM) was also supplemented as a pooled substrate, which would be preferentially utilized and promote accumulation of  $^{15}\text{NO}_2^-$  and  $^{15}\text{NH}_4^+$ . As a control, the same incubation was repeated in the absence of pooled substrates, which is designated as “w/o”. The heights of the bars represent the mean values of duplicate batch tests.

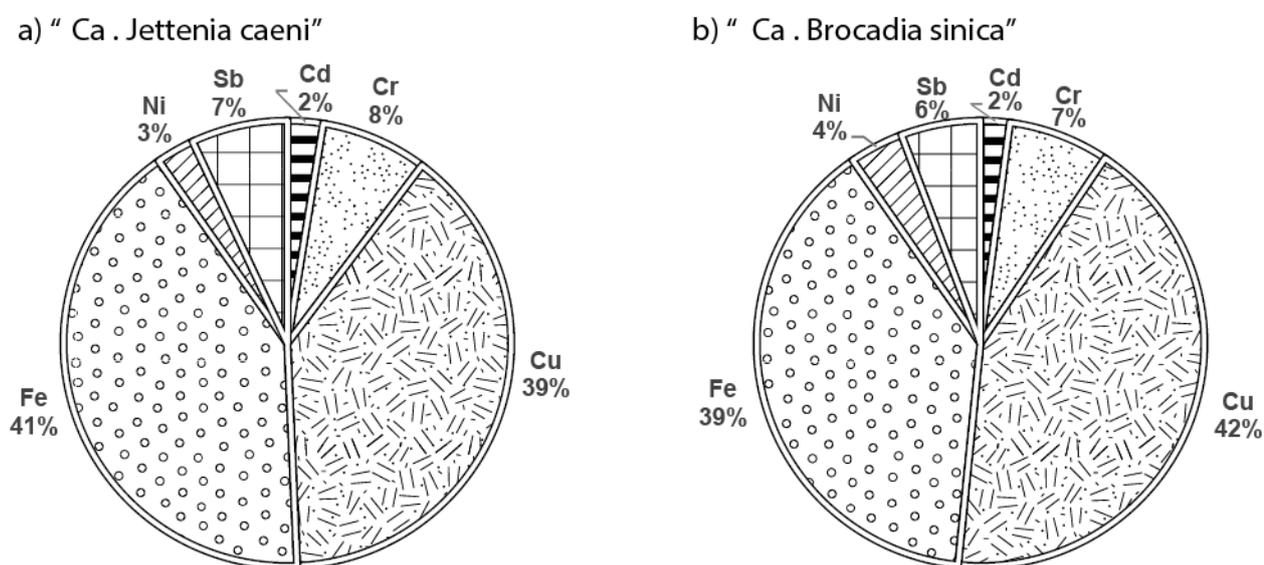


**Fig. 5.8:** Quinone analysis of the “*Ca. Jettenia caeni*” cells: Quinone was extracted using a chloroform-methanol mixture and analyzed by reversed-phase high performance liquid chromatography (HPLC). Dotted lines indicate the retention times of ubiquinone (UQ)-8, menaquinone (MK)-7, UQ-8, MK-8, and UQ-10, respectively, which were

determined by analyzing quinone standards prepared from known species of bacteria and pure chemicals. Extracts prepared from “*Ca. Jettenia caeni*” cells showed a prominent peak at a retention time of 15.5 min, corresponding to MK-7. The vertical axis indicates the absorbances at a wavelength of 270 nm in arbitrary units (AU).

### 5.2.2. Intracellular metal composition

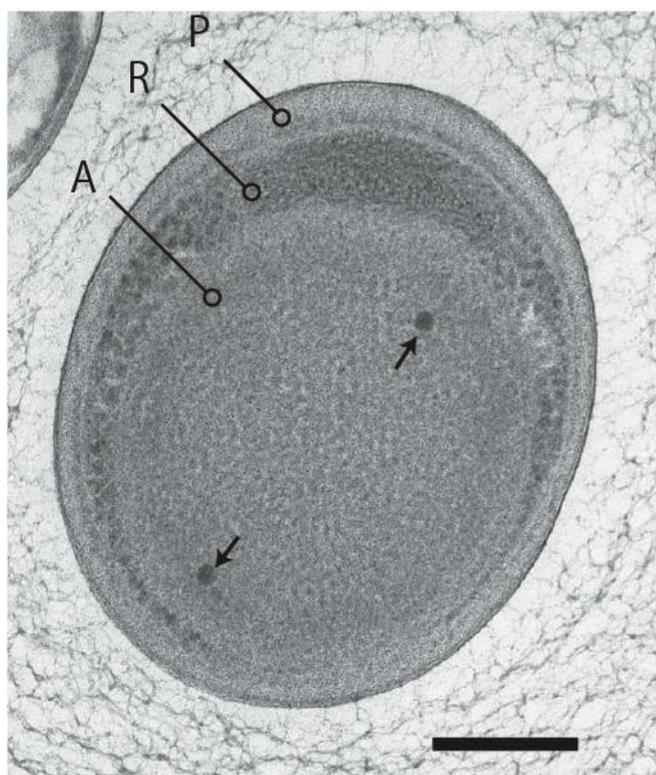
The abundance of cellular metal elements was investigated by lysing “*Ca. Jettenia caeni*” cells in the presence of HNO<sub>3</sub> and subsequently determining metal concentrations in the cell lysates using ICP-AES. The cells were thoroughly washed with deionized water prior to cell lysis to remove trace metals derived from the liquid media. Iron and copper were present in large amounts, accounting for 80% of the total metal weight (**Fig. 5.9a**). In addition, nickel, antimony, cadmium, and chromium were detected from “*Ca. Jettenia caeni*”. The same measurement was performed for the phylogenetically-distinct anammox bacterial cells “*Ca. Brocadia sinica*”. Similarly to the above finding from the “*Ca. Jettenia caeni*” cells, iron and copper were the dominant metal species in “*Ca. Brocadia sinica*” cells (**Fig. 5.9b**).



**Fig. 5.9:** Intracellular metal composition in “*Ca. Jettenia caeni*” (a) and “*Ca. Brocadia sinica*” cells (b): The cells were disrupted by ultrasonication and lysed sequentially with nitric acid. The abundance of metal elements (Fe, Cu, Cr, Cd, B, Ni, Sb, and Zn) in the cell lysates was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Pie charts indicate the abundance of metal elements in total metal weight. Zinc and boron were below the detection limit (<0.001 mg/L) in both lysates prepared from “*Ca. Jettenia caeni*” and “*Ca. Brocadia sinica*” cells.

### 5.2.3. Transmission electron microscopy (TEM)

The cellular structure of “*Ca. Jettenia caeni*” was examined by TEM after staining with uranyl acetate and lead citrate. The cells contained three separate cellular compartments, anammoxosome, riboplasm, and paryphoplasm, which have been observed in other anammox bacterial cells (**Fig. 5.10**) (van Niftrik et al., 2008). The anammoxosome contained a few electron-dense particles.



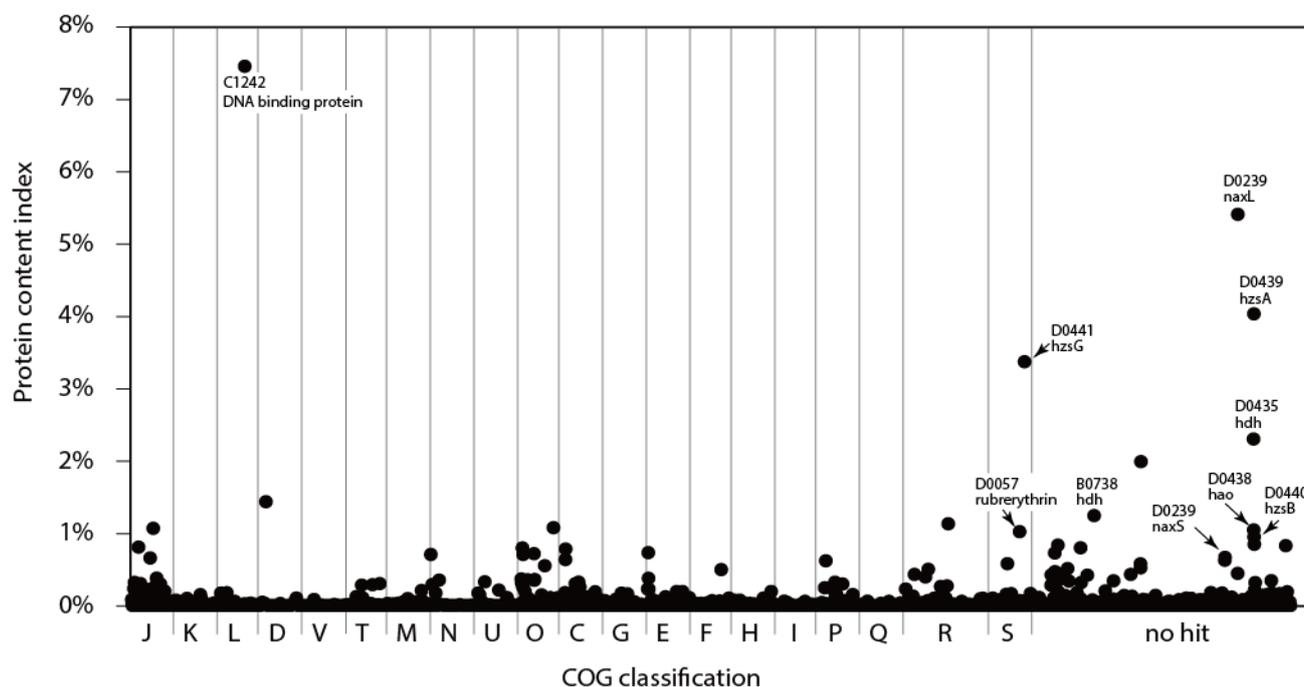
**Fig. 5.10:** Transmission electron micrograph of “*Ca. Jettenia caeni*” cell: The cells of “*Ca. Jettenia caeni*” were cryofixed and an ultrathin section was examined using a transmission electron microscope JEM-1200EX at 80 kV. P, paryphoplasm; R, riboplasm; A, anammoxosome. Arrows indicated electron-dense particles inside the anammoxosome. Scale represents 200 nm.

#### 5.2.4. Proteomic analysis

Total protein extracted from “*Ca. Jettenia caeni*” was analyzed by SDS-PAGE and nanoLC/MS/MS after in-gel tryptic digestion. Proteins were identified based on the peptide mass fingerprints using the MASCOT search program. Their abundances were semi-quantitatively evaluated as the protein content index (PCI) calculated from the exponentially modified protein abundance index (emPAI). The planctomycete KSU-1 genome (GenBank accession number, BAFH0100000 to BAFH01000004) contains 3601 protein-coding sequences, and “*Ca. Jettenia caeni*” cells expressed 919 proteins (this information can be accessed at this URL. <http://onlinelibrary.wiley.com/doi/10.1111/1462-2920.12674/supinfo>). The identified proteins were classified based on the clusters of orthologous groups (COGs) (**Fig. 5.11**), where proteins involved in the following core metabolism mechanisms were highly expressed: hydrazine biosynthesis catalyzed by Hzs, hydrazine oxidation by Hdh, oxidation of  $\text{NO}_2^-$  to  $\text{NO}_3^-$  by nitrite/nitrate oxidoreductase (Nxr), and  $\text{CO}_2$  fixation via the reductive acetyl-CoA pathway by Acs. The high expression of these proteins was also observed in previous proteomic analyses for “*Ca. Kuenenia stuttgartiensis*” (Kartal et al., 2011) and “*Ca. Scalindua profunda*” (van de Vossenberg et al., 2013). In addition, an  $\text{NH}_4^+$ -forming nitrite reductase, NrfA, was also expressed in “*Ca. Jettenia caeni*”. In contrast, NirK expression was not detected in proteomic analysis.

Because the above ICP-AES analysis revealed that copper was one of the most abundant metal species in “*Ca. Jettenia caeni*”, orthologs encoding known copper-containing enzymes were examined through a blastP search. The genes encoding protein subunits of Acs, multicopper oxidase, and NirK were identified as copper-

containing enzymes (this information can be accessed at this URL. <http://onlinelibrary.wiley.com/doi/10.1111/1462-2920.12674/supinfo>). Of these, the KSU1\_D0227, KSU1\_D0226, and KSU1\_B0271 genes were highly or moderately expressed.



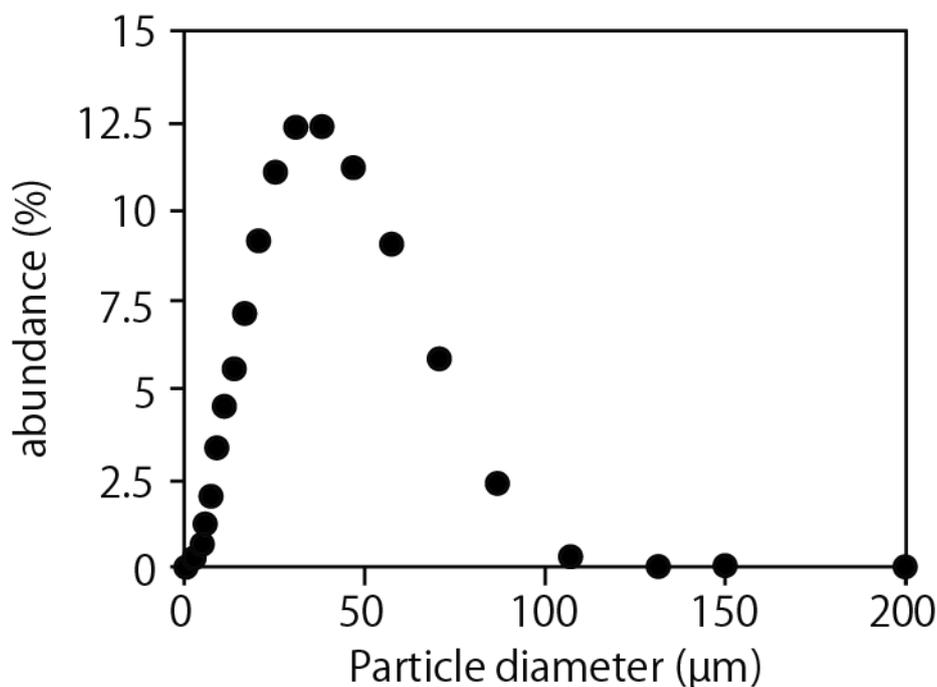
**Fig. 5.11:** Proteome of the “*Ca. Jettenia caeni*” cells: Total proteins were extracted, separated on a polyacrylamide gel containing sodium dodecyl sulfate (SDS), and peptide mass fingerprints were obtained using nanoscale liquid chromatography tandem mass spectrometry (nanoLC/MS/MS) after in-gel tryptic digestion. Proteins were identified from the peptide mass fingerprints using a MASCOT search program (version 2.3.01) and amino acid sequences of protein-coding genes located in the planctomycete KSU-1 genome. Protein abundance is represented as the protein content index (PCI), which was calculated from the exponentially modified protein abundance index (emPAI) provided by the MASCOT program. Proteins were classified into clusters of orthologous groups (COGs), and the descriptions of COG functional categories are as follows: [J] translation, ribosomal structure, and biogenesis; [K] transcription; [L] replication, recombination and repair; [D] cell cycle control, cell division, and chromosome partitioning; [V] defense mechanisms; [T] signal transduction mechanisms; [M] cell wall/membrane/envelope biogenesis; [N] cell motility; [U] intracellular trafficking, secretion, and vesicular transport; [O] posttranslational modification, protein turnover, chaperones; [C] energy production and conversion; [G] carbohydrate transport and metabolism; [E] amino acid transport and metabolism; [F] nucleotide transport and metabolism; [H] coenzyme transport and metabolism; [I] lipid transport and metabolism; [P] inorganic ion transport and metabolism; [Q] secondary metabolites biosynthesis, transport and catabolism; [R] general function prediction only; and [S] function unknown. Category “no hit” contained proteins for which no COG functional category was annotated. Some identified proteins are highlighted with locus tag numbers and gene/product names.

### 5.3. Discussion

#### 5.3.1. Physiology of “*Ca. Jettenia caeni*”

Comprehensive physiological characteristics were determined for the anammox bacteria affiliated with the genus “*Ca. Jettenia*” (**Table 5.1**). “*Ca. Jettenia caeni*” cells produced  $^{29}\text{N}_2$  gas through coupling of  $^{15}\text{NH}_4^+$  and  $^{14}\text{NO}_2^-$ . TEM analysis revealed the presence of the anammoxosome inside the cells (**Fig. 5.10**). In addition, “*Ca. Jettenia caeni*” cells synthesized hydrazine with the consumption of hydroxylamine (**Fig. 5.6**), which was previously shown to be catalyzed only by anammox bacterial cells (van de Graaf et al., 1997). Furthermore, the proteins Hzs, Hdh, Nxr, and Acs required for core metabolism in anammox bacterial cells were abundant in

“*Ca. Jettenia caeni*” cells (**Fig. 5.11**). Therefore, the genus “*Ca. Jettenia*” shares common physiological and structural features of anammox bacteria. It should be noted that the physiological characteristics of “*Ca. Jettenia caeni*” were investigated in small aggregating cells. The average diameter of biomass aggregates was 24  $\mu\text{m}$  (80% of the aggregates had mean diameters less than 50  $\mu\text{m}$ ) (**Fig. 5.12**), which is comparable to those used in previous studies (Strous et al., 1999b; Dapena-Mora et al., 2007; Oshiki et al., 2011). This unavoidable experimental condition may lead to the overestimation of  $K_s$  and  $IC_{50}$  values because of substrate diffusion limitations in the aggregates (Okabe et al., 1999; Stewart, 2003; Kindaichi et al., 2007; Song et al., 2013).



**Fig. 5.12:** Aggregation size distribution of biomass in the MBR: Circles represent the aggregation size distribution of biomass dispersed by vigorous stirring. After disruption, the average particle diameter was  $24 \pm 0.33 \mu\text{m}$ .

The  $K_s$  values for  $\text{NO}_2^-$  and  $\text{NH}_4^+$  were comparable to those of “*Ca. Brocadia sinica*” (Oshiki et al., 2011), but higher than those of other anammox bacteria. This result appears to be reasonable as the “*Ca. Jettenia caeni*” cells were originally obtained in an up-flow column reactor operated at relatively high  $\text{NH}_4^+$  and  $\text{NO}_2^-$  loads and concentrations; i.e.,  $<1.25 \text{ kg N}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$  and 17 mM each, respectively (Fujii et al., 2002).

The anammox activity of “*Ca. Jettenia caeni*” was very sensitive to salinity; the  $IC_{50}$  was 68 mM for sodium chloride, suggesting that salinity greatly impacts microbial community structures (Lozupone and Knight, 2007; Logares et al., 2009), even for anammox bacteria (Sonthiphand et al., 2014). Successive changes in anammox bacterial community structures with a salinity gradient have been described in the Yodo River (Amano et al., 2007) and Cape Fear River estuaries (Dale et al., 2009). In addition, the impact of salinity on the anammox bacterial community structure was demonstrated for mangrove sediment slurry in a laboratory (Wang and Gu, 2013). High sensitivity against salinity suggests that “*Ca. Jettenia caeni*” is a freshwater strain. In fact, nearly all 16S rRNA gene sequences affiliated with the genus “*Ca. Jettenia*” have been retrieved from freshwater ecosystems (Kartal et al., 2006; Humbert et al., 2010; Yoshinaga et al., 2011; Zhu et al., 2011; Humbert et al.,

2012; Wang et al., 2012; Hu et al., 2013a; Long et al., 2013; Shen et al., 2013). Therefore, salinity is likely a key environmental parameter that determines the geographical distribution of anammox bacteria affiliated with the genus “*Ca. Jettenia*”. However, anammox bacteria can acclimate to high salinity conditions during long-term cultivation (Kartal et al., 2006). For example, the anammox bacterium affiliated with “*Ca. Jettenia*” adapted to high salinity (Liu et al., 2009). In this previous study, salinity was increased stepwise over 5–10 d, and anammox activity was maintained even up to a salinity of 513 mM sodium chloride (Liu et al., 2009). This finding highlights questions regarding how well physiological characteristics determined during short-term cultivation (typically within 1 d) reflect the behaviors of the anammox bacterial population in natural ecosystems. Further studies investigating the long-term population dynamics of anammox bacteria and examination of the microbial competition among anammox bacteria should be conducted as have been conducted using nitrifying bacteria or heterotrophs (van Niel et al., 1993; Nogueira and Melo, 2006; Füschnlin et al., 2012).

Sulfide is a strong inhibitor in anammox bacteria and limits the geographical distribution of anammox activity in marine ecosystems (Jensen et al., 2008), and anammox activity in an anoxic water column of the Black Sea disappeared in the presence of <6  $\mu\text{M}$  concentrations of sulfide. The  $\text{IC}_{50}$  of sulfide has been examined using enrichment cultures of anammox bacteria over ranges of 10–300  $\mu\text{M}$  (Dapena-Mora et al., 2007; Carvajal-Arroyo et al., 2013; Jin et al., 2013; Russ et al., 2014). The  $\text{IC}_{50}$  of “*Ca. Jettenia caeni*” in the present study was 540  $\mu\text{M}$ , which was higher than these values. However, as described above, physiological experiments were conducted using aggregated biomass of “*Ca. Jettenia caeni*”, which may lead to the higher  $\text{IC}_{50}$  values because of the diffusion limitation in the biomass. Furthermore, sulfide may be oxidized by “*Ca. Jettenia caeni*” or by coexisting heterotrophs capable of sulfide-dependent denitrification, as was recently observed in anoxic marine sediments (Prokopenko et al., 2013).

Interestingly, the anammox activity of “*Ca. Jettenia caeni*” cells was not strongly inhibited by the addition of 1 mM methanol. Methanol is considered to be a strong inhibitor in anammox bacteria, and addition of 0.5 mM (Güven et al., 2005) or 3.3 mM methanol (Jensen et al., 2007) completely inhibited anammox activities of an enrichment culture and marine sediments, respectively. However, high tolerance to methanol was observed in enrichment cultures comprised of “*Ca. Kuenenia stuttgartiensis*” and “*Ca. Jettenia caeni*” cells, in which addition of 5 mM methanol decreased anammox activities by only 29% (Isaka et al., 2008). These observations suggest that methanol is not a universal inhibitor of anammox bacteria.

“*Ca. Jettenia caeni*” cells catalyzed respiratory ammonification with oxidation of  $^{13}\text{CH}_3\text{COOH}$ . The production of  $^{15}\text{NO}_2^-$  and  $^{15}\text{NH}_4^+$  (**Fig. 5.7**) clearly indicated that the “*Ca. Jettenia caeni*” cells reduced  $^{15}\text{NO}_3^-$  to  $^{15}\text{NH}_4^+$  via  $^{15}\text{NO}_2^-$ . Notably, this experiment was performed in the presence of penicillin G, which is an inhibitor of most heterotrophs, but does not affect anammox bacteria (Jetten et al., 1999; Okabe et al., 2011; Hu et al., 2013b); therefore, the reduction of  $^{15}\text{NO}_3^-$  to  $^{15}\text{NH}_4^+$  via  $^{15}\text{NO}_2^-$  was likely mediated by “*Ca. Jettenia caeni*” cells. The simultaneous occurrence of  $^{13}\text{CO}_2$  production and  $^{15}\text{NO}_3^-$  reduction indicated that  $^{13}\text{CH}_3\text{COOH}$  was utilized as an electron donor for  $\text{NO}_3^-$  reduction. Expression of NrfA and Nxr supported the respiratory

ammonification by “*Ca. Jettenia caeni*” cells. NrfA is a canonical  $\text{NH}_4^+$ -forming nitrite reductase involved in respiratory ammonification by heterotrophic denitrifiers (Simon, 2002; Corker and Poole, 2003). The involvement of NrfA may not be a common trait among anammox species because the ortholog is missing in the “*Ca. Kuenenia stuttgartiensis*” genome, which is composed of 4 contigs (Strous et al., 2006; Speth et al., 2012), although this anammox bacterium was also capable of respiratory ammonification (Kartal et al., 2007b). Nxr, a member of type-II DMSO reductase family enzymes (Lücker et al., 2013), is another candidate enzyme involved in respiratory ammonification; i.e.,  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$ . Nitrospira-type Nxr is constituted of three subunits, nxrABC (Lücker et al., 2010; 2013), and the planctomycete KSU-1 genome encodes the orthologs of nxrA (the KSU1\_B0257 and KSU1\_D0577 genes), nxrB (the KSU1\_B0260 gene) and nxrC (the KSU1\_B0261). The Nitrospira-type Nxr could mediate  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$ , while the reaction was not electrogenic (Ehrich et al., 1995); therefore, further study is needed to demonstrate the involvement of Nxr in the respiratory ammonification by anammox bacterial cells. Apart from the NrfA and Nxr, involvement of multiheme proteins (kustc0392–kustc0395 proteins) in respiratory ammonification by “*Ca. Kuenenia stuttgartiensis*” has been suggested (Kartal et al., 2007b), and the orthologs of these genes are located in the planctomycete KSU-1 genome; i.e., the KSU1\_D0377–KSU1\_D0380 genes.

### 5.3.2. *Involvement of NirK in NO<sub>2</sub><sup>-</sup> reduction by the KSU-1 enrichment*

*NirK* expression was not detected in our proteomic analysis, which was somewhat surprising as the reduction of  $\text{NO}_2^-$  to NO is the first reaction in the anammox process (Kartal et al., 2011) and it is thought that anammox bacteria affiliated with the genus “*Ca. Jettenia*” conduct this reaction using NirK (Hira et al., 2012; Hu et al., 2012). The KSU1\_D0929 gene encoding nirK was previously expressed in *Escherichia coli* cells, and the specific enzymatic activity of  $\text{NO}_2^-$  reduction was determined to be  $570 \pm 50 \mu\text{mol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$  (Hira et al., 2012). The specific activity of NirK and specific cellular anammox activity determined in the present study (i.e.,  $>37 \text{ nmol N}^{29}\text{N}_2 \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ ) (Fig. 5.1) suggested that NirK accounts for up to 0.00649% of the protein weight in “*Ca. Jettenia caeni*” cells. However,  $\text{NO}_2^-$  reduction by “*Ca. Jettenia caeni*” cells was significantly inhibited by the addition of DDC. Copper is a cofactor of NirK and is required for  $\text{NO}_2^-$  reduction, while it is loosely associated with the enzyme (Heylen et al., 2006; Lawton et al., 2013). Addition of DDC forms a copper chelate and suppresses the  $\text{NO}_2^-$ -reducing activity of NirK-dependent heterotrophic denitrifiers (Shapleigh and Payne, 1985; Coyne et al., 1989). Therefore, inhibition by DDC suggests that “*Ca. Jettenia caeni*” cells utilize NirK for  $\text{NO}_2^-$  reduction. Taken together, NirK is likely a nitrite reductase employed in “*Ca. Jettenia caeni*” cells, although the protein expression level was low. This low expression is likely the result of high specific enzymatic activity of NirK ( $570 \pm 50 \mu\text{mol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ , Hira et al., 2012), which is 3 orders of magnitude higher than that observed in the downstream reaction; i.e., hydrazine biosynthesis (the specific enzymatic activity of Hzs was  $0.33 \text{ nmol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ , Kartal et al., 2011). In contrast,  $\text{NO}_2^-$  reduction was observed even in the presence of DDC, suggesting the potential involvement of nitrite reductase other than the NirK.  $\text{NO}_2^-$  may have been reduced to  $\text{NH}_4^+$  by NrfA (Simon, 2002; Corker and Poole, 2003), a cryptic NO-forming nitrite reductase (i.e., octaheme cytochrome c nitrite reductase including Hao-like proteins) (Atkinson

et al., 2007; Tikhonova et al., 2012; Hanson et al., 2013; Kozłowski et al., 2014; Kraft et al., 2014), and/or a set of penta-, deca-, and another pentaheme proteins (Kartal et al., 2007b).

### 5.3.3. *Iron- and copper-containing enzymes*

Iron was found to be abundant in “*Ca. Jettenia caeni*” cells. This is reasonable because these cells contained abundant heme proteins, including Hzs, Hao, and Nxr. Additionally, TEM indicated that the anammoxosome in “*Ca. Jettenia caeni*” cells contained electron-dense particles (**Fig. 5.10**), which have been also found in the anammoxosome of phylogenetically-distinct anammox bacterial cells and identified as iron particles by energy dispersive X-ray spectroscopy (van Niftrik et al., 2008). Therefore, the presence of iron particles further supports the abundance of iron in “*Ca. Jettenia caeni*” cells. Copper was also a dominant metal component. Proteomic analysis revealed that some copper-containing enzymes, including KSU1\_D0226, KSU1\_D0227, KSU1, and\_B0271, were highly or moderately expressed in the proteome. Because the KSU1\_D0226 and KSU1\_D227 proteins are catalytic enzymes involved in the reductive acetyl-CoA pathway, high expression of these proteins was reasonable. However, the association of copper in Acs remains controversial (Can et al., 2014). It has been suggested that a copper-nickel site is the active center of Acs, while a nickel-nickel site was later identified (Bramlett et al., 2003; Seravalli et al., 2004; Svetlitchnyi et al., 2004). Therefore, the association between copper and nickel in the Acs of “*Ca. Jettenia caeni*” cells should be further explored in protein purification and crystallography studies. The physiological role of the KSU1\_B0271 protein remains unclear. The KSU1\_B0271 gene encodes a multicopper oxidase, which is responsible for some oxidation/reduction reactions. The KSU1\_B0271 gene is located upstream of the Nxr gene cluster (KSU1\_B0256–KSU1\_B0269 genes), suggesting that the KSU1\_B0271 protein plays a role in the oxidation of  $\text{NO}_2^-$  to  $\text{NO}_3^-$ , however, this should be verified in the future. In addition to iron and copper, antimony was also found in the cell lysates of “*Ca. Jettenia caeni*” cells. Antimony can accumulate in both microbial cells and extracellular polymeric substances (Zhang et al., 2011), but its role as metal cofactor of protein remains unclear.

The present study has illuminated the physiological characteristics of anammox bacteria affiliated with the genus “*Ca. Jettenia*”, suggesting that these cells are freshwater anammox bacteria and prefer  $\text{NH}_4^+$ - and/or  $\text{NO}_2^-$ -rich habitats. Now, comprehensive data sets of detailed physiological characteristics, genome sequences, and proteome are available for the genera “*Ca. Kuenenia*”, “*Ca. Brocadia*”, “*Ca. Scalindua*”, and “*Ca. Jettenia*”. The data sets are useful to explore niche differentiation among these anammox bacteria in natural and man-made ecosystems.

## 5.4. **Experimental procedures**

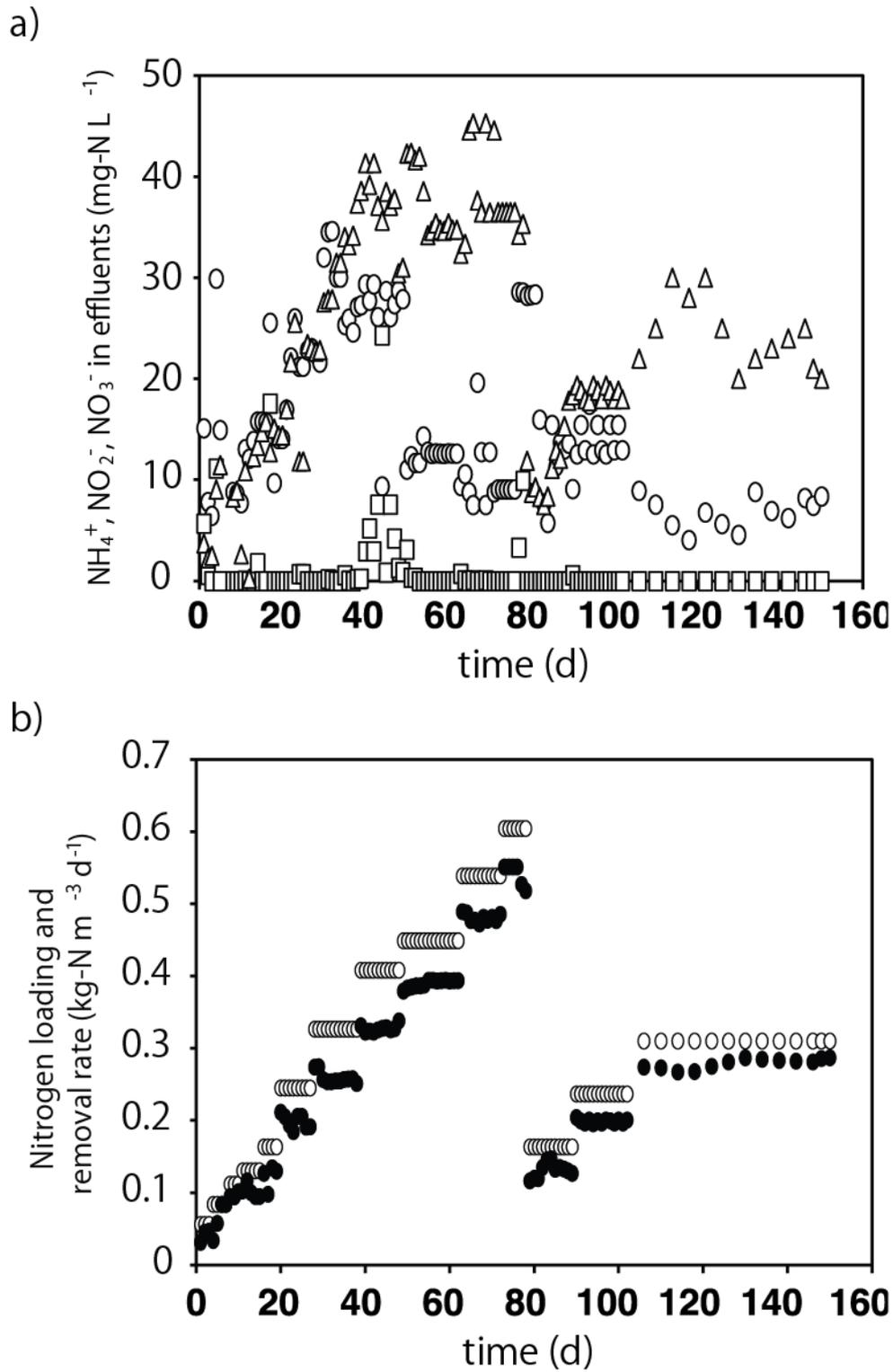
### 5.4.1. *Anammox bacterial cells*

An enrichment culture of anammox bacteria examined in the present study was obtained by cultivating the biomass containing planctomycete KSU-1 cells using a MBR. The planctomycete KSU-1 cells were originally enriched at Kumamoto University (Fujii et al., 2002) from activated sludge collected from a wastewater treatment plant operated in the Kumamoto city, Japan. The cells were cultivated in an up-flow column reactor with non-woven biomass carrier. Inorganic nutrient media containing  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were fed

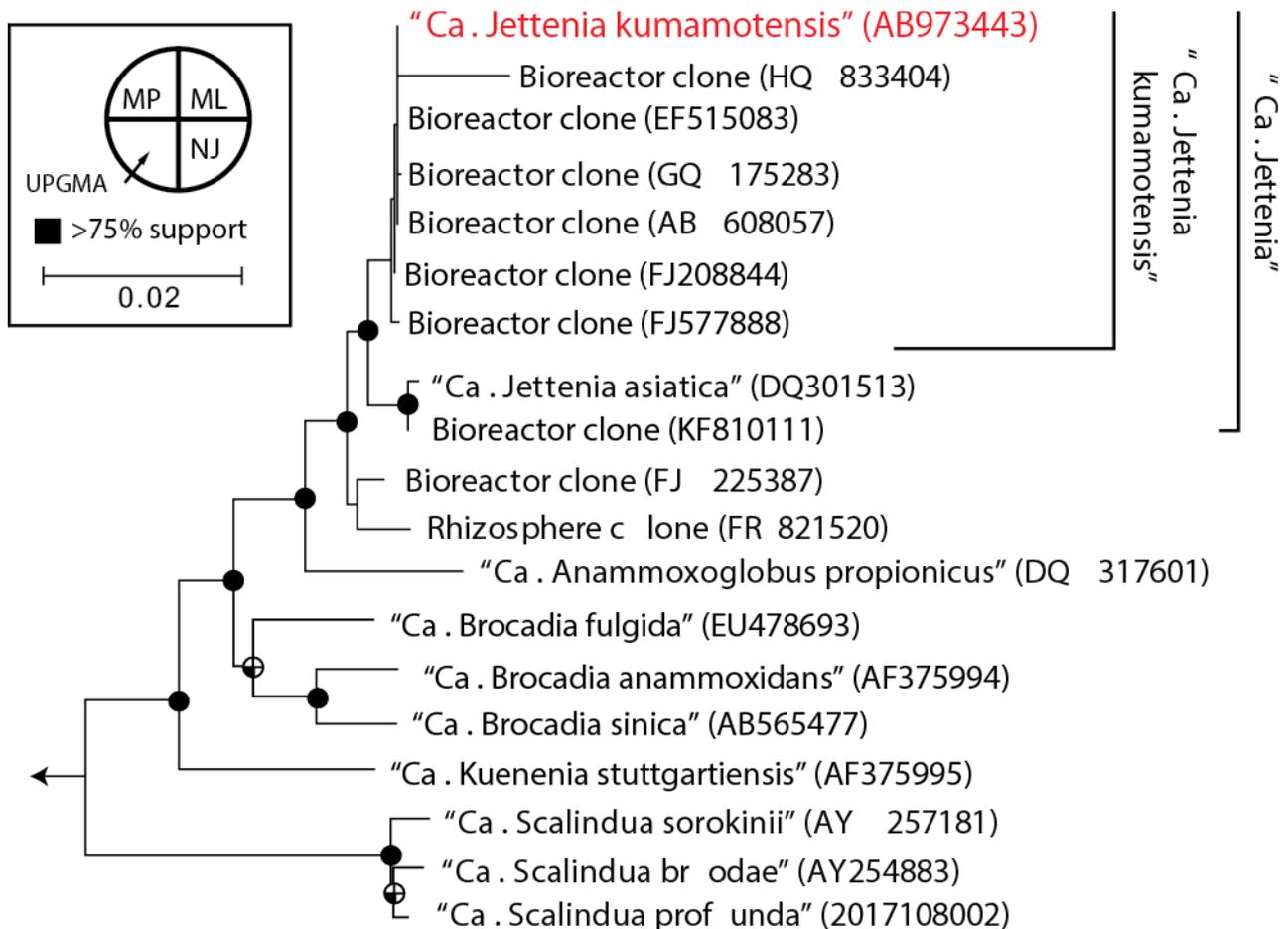
to the reactor, and continuously operated for more than 10 years. Planctomycete KSU-1 genome was determined by using the genomic DNA extracted from biofilms attached to the biomass carrier (Hira et al., 2012).

The biofilms were gently homogenized by shaking and inoculated in a 3-L jar fermenter MBF-500ME (EYELA; Tokyo, Japan) equipped with a hollow-fiber membrane module (polyethylene membrane with pore size of 0.1  $\mu\text{m}$ ). Cultivation conditions were similar to those previously described for the cultivation of “*Ca. Brocadia sinica*” (Oshiki et al., 2013b). Briefly, the MBR was operated at  $37 \pm 1^\circ\text{C}$  and pH 7.5–8.0 in the dark with mixing by a metal propeller at a stirring speed of 150 rpm. Anoxic conditions were maintained by continuous purging with a mixture of argon and  $\text{CO}_2$  gas (95:5) at a rate of 10 mL/min. Inorganic medium was fed continuously at a rate of 3.33 L/d. The medium contained the following inorganic substances and was prepared using groundwater;  $\text{NH}_4(\text{SO}_4)_2$ , 35–238 mg N/L;  $\text{NaNO}_2$ , 35–280 mg N/L;  $\text{KHCO}_3$ , 125 mg/L;  $\text{KH}_2\text{PO}_4$ , 57 mg/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 9 mg/L; EDTA·2Na, 5 mg/L (Rouse et al., 1999). Yeast extract (BD Biosciences; Franklin Lakes, NJ, USA) was also supplemented at a final concentration of 1 mg/L after 30 d of operation. The MBR was operated for more than 150 d with continuous addition of inorganic media at a nitrogen-loading rate of 0.06–0.60  $\text{kg N} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ . The NRR increased gradually from 0.03 to 0.55  $\text{kg N} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$  (**Fig. 5.13**). The concentration of  $\text{NO}_2^-$  in the effluent was generally maintained below 1 mg N/L. Stoichiometric ratios of consumed  $\text{NO}_2^-$  to consumed  $\text{NH}_4^+$  ( $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ) and of produced  $\text{NO}_3^-$  to consumed  $\text{NH}_4^+$  ( $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ) were 1.00–2.12 (average, 1.26) and 0.10–0.37 (average, 0.22), respectively. Those stoichiometric ratios agreed with the theoretical values of the anammox reaction; i.e., 1.32 and 0.26, respectively (Strous et al., 1998). Although the enrichment culture was established for 150 d of operation, anammox bacterial cells proliferated as flocculated biomass (average diameter, 150  $\mu\text{m}$ ).

The phylogenetic affiliation of the anammox bacterium in the MBR was examined by 16S-23S rRNA gene sequencing analysis. The nucleic acid sequence of 16S-23S rRNA genes was retrieved and both strands were sequenced using the direct Sanger sequencing method. The nucleic acid sequence was identical to that of the 16S rRNA, intergenic spacer region (ISR), and 23S rRNA gene sequences of the planctomycete KSU-1 genome (GenBank accession number: BAFH01000003); therefore, the anammox bacterium enriched in the present study was the planctomycete KSU-1. Levels of the 16S rRNA gene, ISR, and 23S rRNA gene sequence similarity between the planctomycete KSU-1 and other known anammox bacteria are shown in **Table 5.3**. The nucleic acid sequence similarities of the 16S rRNA gene between the planctomycete KSU-1 and “*Ca. Jettenia asiatica*” were 98%, which is below the 98.7–99% threshold suggested for species delineation (Stackebrandt and Ebers, 2006). Phylogenetic analysis based on the nearly full-length 16S rRNA gene sequences also suggested that a bacterial clade of planctomycete KSU-1 was separated from that of “*Ca. Jettenia asiatica*” (**Fig. 5.14**), which was supported by another phylogenetic analysis based on the *hzsA* gene sequences (Harhangi et al., 2012). As the planctomycete KSU-1 was invalid regarding the terminology of bacterial name and in order to avoid confusion between the planctomycete KSU-1 and the anammox bacterium enriched in the present study, the authors revised the taxonomic name and tentatively proposed the taxonomic classification of “*Ca. Jettenia caeni*” sp. nov. (See below species description.).



**Fig. 5.13:** Nitrogen removal performance of the MBR: a) Concentrations of  $\text{NH}_4^+$  (circles),  $\text{NO}_2^-$  (squares), and  $\text{NO}_3^-$  (triangles) in the effluent. b) Rates of influent nitrogen-loading (empty circles) and nitrogen removal (filled circles).



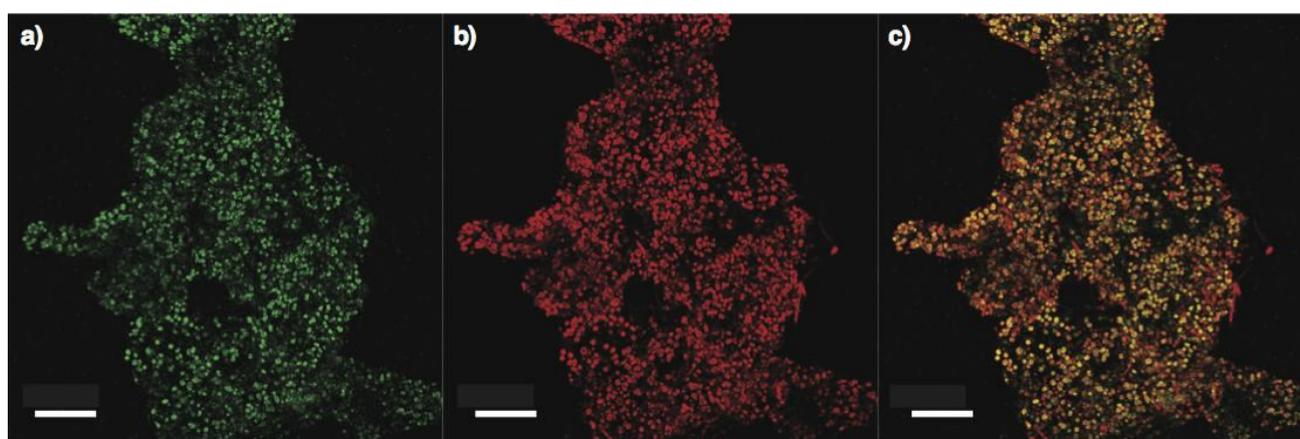
**Fig. 5.14:** Phylogenetic affiliation of "Ca. Jettenia caeni" estimated based on nearly full-length 16S rRNA gene sequence: Total DNA was extracted using a Fast DNA spin kit for soil (MP Biomedicals; Tokyo, Japan) according to the manufacturer's instructions. Concentration of the DNA was determined using a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences; Little Chalfont, UK). Planctomycetal 16S-23S rRNA gene was amplified by PCR with the oligonucleotide primer set Pla46f and 1037r as previously described (Oshiki *et al.*, 2011). The nucleic acid sequence of the PCR amplicon was determined by the direct Sanger sequencing method using oligonucleotide primers Pla46f, 357f, 968f, 1392f, 1892f, 2399f, 2891f, 3316f, 3747f, 530r, 907r, 1500r, 2091r, 1035r, 2810r, 1020r, 3801r, and 1037r as previously described (Oshiki *et al.*, 2011). 16S rRNA gene sequences (*Escherichia coli* positions 67–1389) were aligned using the ClustalW program (version 1.83) and the phylogenetic tree was constructed using MEGA 6.06 software. The tree was constructed with the maximum likelihood (ML) (Jones-Taylor-Thornton model with 500 bootstrap iterations), neighbor joining (NJ) (Poisson model with 1000 iterations), maximum parsimony (MP) (close neighbor interchange on random trees search algorithm with 500 iterations), and unweighted pair group methods with arithmetic mean (UPGMA) (maximum composite likelihood model with 1000 iterations) using the 16S rRNA gene of *Rhodopirellula baltica* (GenBank accession number EF012750) as an outgroup (sequence is not shown in the tree). The NJ tree is shown here. Pie charts at nodes represent the confidence of branch topology, and bootstrap values greater than 75% are filled in black (MP method for upper-left sector, ML method for upper-right sector, UPGMA method for bottom-left sector, and NJ method for bottom-right sector). Phylogenetic position of the genus "Ca. Jettenia" is indicated in the right bracket, and the nucleic acid sequence of the 16S rRNA gene determined in the present study is shown in red. GenBank accession numbers are indicated in brackets. Taxon Object ID at the Integrated Microbial Genomes with Microbiome Samples is shown for "Ca. Scalindua profunda". Scale bar represents 2% sequence divergence.

ksu203 probe  
 "Ca . Jettenia kumamotoensis" (AB973443)  
 "Ca . Jettenia asiatica" (DQ30151)  
 "Ca . Anammoxoglobus propionicus" (EU47869)  
 "Ca . Broccdia fulgida" (EU478693)  
 "Ca . Broccdia anammoxidans" (AB375994)  
 "Ca . Broccadia sinica" (AB759554)  
 "Ca . Kuenenia stuttgartiensis" (AF37599)  
 "Ca . Scalindua sp." (AB822932)  
 "Ca . Scalindua wagneri" (AY254882)

```

ATGGAACCTTT CAGCCCC
ATGGAACCTTT CAGCCCC
ATGGAACCTTT CAGCCCC
ATGGAGCITTC-- ACITCC
ATGAAATCTTTCGACCCC
ATGAAATCTTACGATTCC
ATGGACCITTT CAGATCC
AAGGAAC-- -AGAATTCC
AAGATA-- -TTGCTACCCCT
AAGGCA-- -TTGCTGCCCT
  
```

**Fig. 5.15:** Alignment of oligonucleotide probe ksu203 and anammox bacterial 16S rRNA gene sequences.



**Fig. 5.16:** Microscopic images of fluorescence *in situ* hybridization (FISH) analysis: a) Biomass collected from the MBR after 45 d of operation. The cells were fixed in 4% (w/v) paraformaldehyde, and hybridized with both fluorescein isothiocyanate (FITC)-labelled ksu203 probe (green) for “*Ca. Jettenia caeni*” and b) tetramethylrhodamine (TRITC)-labelled EUB mix probe composed of equimolar EUB338, EUB338II, and EUB338III (red) for most members of eubacteria. c) Yellowing signals result from overlaying both probes into one image. An Olympus FV-300 confocal laser-scanning microscope equipped with an Ar ion laser (488 nm) and a HeNe laser (543 nm) (Olympus; Tokyo, Japan) was used for microscopy. The abundance of “*Ca. Jettenia caeni*” in the total biomass was determined using ImageJ software (Schneider *et al.*, 2012). Scale bar = 20  $\mu$ m.

The abundance of “*Ca. Jettenia caeni*” cells in the enrichment culture was determined by fluorescence *in situ* hybridization (FISH) analysis using the oligonucleotide probe ksu203 recently designed by our research group (e.g., L. Zhang, unpublished data). The nucleic acid sequence of the ksu203 probe was 5'-ATGGAACCTTTTCAGCCCC-3', corresponding to *Escherichia coli* positions 203–217. The ksu203 probe contained some mismatch bases against anammox bacterial 16S rRNA other than those affiliated with the genus “*Ca. Jettenia*” (**Fig. 5.15**). The optimal formamide concentration for specific hybridization of the ksu203 probe was determined to be 20% by hybridization with the “*Ca. Jettenia caeni*”, “*Ca. Broccadia sinica*”, and “*Ca. Scalindua sp.*” cells as previously described (Oshiki *et al.*, 2012). The “*Ca. Jettenia caeni*” cells accounted for more than 90% of the total biomass (**Fig. 5.16**), indicating that “*Ca. Jettenia caeni*” cells were the dominant bacterial population in the enrichment culture.

**Table 5.3:** Nucleic acid sequence similarities of *16S rRNA*, intergenic spacer region (ISR), and *23S rRNA* genes of “*Ca. Jettenia caeni*” and 6 anammox species. The following nucleotide sequences were used as representatives of the species: “*Ca. Jettenia asiatica*” (DQ301513), “*Ca. Kuenenia stuttgartiensis*” (AF375995), “*Ca. Anammoxoglobus propionicus*” (EU478694), “*Ca. Brocadia anammoxidans*” (AF375994), “*Ca. Brocadia fulgida*” (EU478693), and “*Ca. Scalindua brodae*” (AY254883). ND: not determined because the comparable nucleotide sequence was unavailable. Table S4. Nucleic acid sequence similarities of *16S rRNA*, intergenic spacer region (ISR), and *23S rRNA* genes of “*Ca. Jettenia kumamotoensis*” and 6 anammox species. The following nucleotide sequences were used as representatives of the species: “*Ca. Jettenia asiatica*” (DQ301513), “*Ca. Kuenenia stuttgartiensis*” (AF375995), “*Ca. Anammoxoglobus propionicus*” (EU478694), “*Ca. Brocadia anammoxidans*” (AF375994), “*Ca. Brocadia fulgida*” (EU478693), and “*Ca. Scalindua brodae*” (AY254883). ND: not determined because the comparable nucleotide sequence was unavailable.

	“ <i>Ca. J. asiatica</i> ”			“ <i>Ca. K. stuttgartiensis</i> ”			“ <i>Ca. A. propionicus</i> ”			“ <i>Ca. B. anammoxidans</i> ”			“ <i>Ca. B. fulgida</i> ”			“ <i>Ca. S. brodae</i> ”			
	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	
“ <i>Ca. J. caeni</i> ”	98%	88%	98%	90%	88%	88%	93%	80%	86%	92%	81%	90%	93%	80%	89%	86%	ND	ND	
“ <i>Ca. J. asiatica</i> ”				93%	87%	87%	93%	88%	85%	92%	89%	89%	92%	86%	86%	89%	ND	ND	
“ <i>Ca. K. stuttgartiensis</i> ”							92%	89%	90%	93%	88%	88%	93%	89%	94%	90%	ND	ND	
“ <i>Ca. A. propionicus</i> ”										91%	90%	90%	91%	87%	87%	89%	ND	ND	
“ <i>Ca. B. anammoxidans</i> ”													94%	84%	86%	89%	ND	ND	
“ <i>Ca. B. fulgida</i> ”																	91%	ND	ND
“ <i>Ca. S. brodae</i> ”																			

“*Ca. Brocadia sinica*” cells were also harvested from another MBR for analysis of intracellular metal composition and were cultivated as previously described (Oshiki et al., 2013b). In the culture, “*Ca. Brocadia sinica*” accounted for more than 90% of the total biomass as determined by the FISH analysis (Tsushima et al., 2007a).

#### 5.4.2. Activity tests

Standard anaerobic techniques were conducted in an anaerobic chamber (Coy Laboratory Products; Grass Lake Charter Township, MI, USA) (Ito et al., 2002; Awata et al., 2013). Anoxic stock solutions and buffers were prepared by repeatedly vacuuming and purging with He (>99.99995%) gas. The cells of “*Ca. Jettenia caeni*” were dispersed by stirring at 250 rpm for 5 h; the cells were washed twice and suspended in the inorganic medium containing 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.8). Composition of the inorganic medium was the same as that supplied into the MBR. Next, a 5-mL cell suspension was dispensed into 12.5-mL glass serum vials (Nichiden-Rika Glass; Tokyo, Japan), which were sealed with butyl rubber stoppers and aluminum caps. Biomass concentration in the vials ranged from 0.1–0.6 mg protein/mL. The headspace was replaced by repeatedly vacuuming and purging with He gas. These vials were incubated overnight at 37°C to remove any trace amounts of substrates and oxygen. The activity test was initiated by adding  $^{15}\text{NH}_4\text{Cl}$  (Isotec Inc.; Miamisburg, OH, USA) and  $\text{Na}^{14}\text{NO}_2$  at a final concentration of 2.5 mM using a gas-tight syringe (VICI; Baton Rouge, LA, USA). The vials were incubated in triplicate at 37°C in the dark for up to 8 h.

The temperature dependency of anammox activity was examined by incubating the vials at 4–45°C. Activation energy was calculated based on the temperature dependency of SAA as previously described (Oshiki et al., 2011). pH dependency was examined at pH 6.0–9.0. Initial pH was adjusted with 0.5 M  $\text{H}_2\text{SO}_4$  or 1 M NaOH, and pH was not controlled during incubation.

Inhibition of anammox activity by  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , salinity, sulfide, and organic matter was examined by spiking these chemicals into the vials. The initial concentrations of  $\text{NO}_2^-$  and  $\text{NH}_4^+$  were 2.5–15 mM and 2.5–20 mM, respectively. Salinity and sulfide concentrations were 17–684 mM (sodium chloride) and 0.1–1 mM, respectively. The  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  solution was prepared in nitrogen-bubbled deionized water in an air-tight bottle and subsequently dispensed into vials to reach the final sulfide concentration.  $\text{IC}_{50}$  values for  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ , salinity, and sulfide were calculated based on the production rate of  $^{29}\text{N}_2$ . Inhibition by acetate, glucose, formate, propanol, methanol, and ethanol was examined by adding these chemicals separately at a final concentration of 1 mM.

Values of  $K_S$  for  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were determined under  $\text{NO}_2^-$ - or  $\text{NH}_4^+$ -limiting conditions, respectively, where the initial  $\text{NO}_2^-$  and  $\text{NH}_4^+$  concentrations were 350  $\mu\text{M}$  and 1000  $\mu\text{M}$  or 1500  $\mu\text{M}$  and 450  $\mu\text{M}$ .  $K_S$  values were calculated according to the Michaelis-Menten kinetics.

Hydrazine biosynthesis was examined by incubating the cells at 37°C for 6 h and adding NH<sub>4</sub><sup>+</sup> and hydroxylamine (3 mM each). The liquid samples for the determination of hydrazine and hydroxylamine concentrations were collected over time using a 1-mL disposable syringe.

Respiratory ammonification activity was examined by incubating the vials with Na<sup>15</sup>NO<sub>3</sub><sup>-</sup> (2 mM) (Shoko Co., Ltd.; Tokyo, Japan) and <sup>13</sup>CH<sub>3</sub>COOH (4 mM) (Cambridge Isotope Laboratories Inc.; Cambridge, MA, USA). For pooled substrates, <sup>14</sup>NH<sub>4</sub>Cl and Na<sup>14</sup>NO<sub>2</sub><sup>-</sup> were also supplemented separately at final concentrations of 5 mM and 2 mM, respectively. To inhibit the activities of most of the heterotrophs, penicillin G (Sigma-Aldrich; St. Louis, MO, USA) was added at a final concentration of 500 μM.

#### 5.4.3. *Maximum specific growth*

The value of μ<sub>max</sub> was calculated using the following equation:

$$\mu_{\max} = q_{\max} \times Y,$$

where

μ<sub>max</sub> = maximum specific growth rate, h<sup>-1</sup>,

q<sub>max</sub> = maximum specific anaerobic ammonium oxidation rate, mol NH<sub>4</sub><sup>+</sup> (mol C)<sup>-1</sup> d<sup>-1</sup>, and

Y = biomass yield, mol C (mol NH<sub>4</sub><sup>+</sup>)<sup>-1</sup>.

The value of q<sub>max</sub> was determined by cultivating “*Ca. Jettenia caeni*” cells in an up-flow glass column reactor and determining the maximum specific anaerobic ammonium oxidation rate in the reactor. The biomass suspension harvested from the MBR was inoculated into a 46-mL column reactor equipped with 1-μm-pore-size glass filters to avoid biomass washout. This column reactor was operated at 37°C and pH 7.5–7.8 with a continuous supply of inorganic medium containing NH<sub>4</sub><sup>+</sup> (2–5 mM) and NO<sub>2</sub><sup>-</sup> (2–5 mM). The hydraulic retention time (HRT) in the reactor was set to 0.52 h, which resulted in NLRs of 2.6–6.4 kg N·m<sup>-3</sup>·d<sup>-1</sup>. The ammonium oxidation rate was calculated based on the NH<sub>4</sub><sup>+</sup> concentrations of the influent and effluent and the HRT. The column reactor was operated for 45 d; thereafter, all biomass was harvested and subjected to determination of VSS concentration (Tsushima et al., 2007b).

The value of Y was determined from the rates of <sup>14</sup>CO<sub>2</sub> incorporation and NH<sub>4</sub><sup>+</sup> oxidation as previously described (Oshiki et al., 2011; Awata et al., 2013). Briefly, the cells were incubated at 37°C for 3 d with the addition of NH<sub>4</sub><sup>+</sup> and NO<sub>2</sub><sup>-</sup> (6 mM each). The <sup>14</sup>CO<sub>2</sub> was supplemented at a final concentration of 20 μCi/vial, corresponding to 740 kBq/vial. The cell suspension was collected every day, washed three times with phosphate-buffered saline, and mixed with scintillation cocktail (Clear-sol I; Nacalai Tesque; Kyoto, Japan). Radioactivity was determined in an LSC-5100 liquid scintillation counter (Hitachi-Aloka Medical; Tokyo, Japan) (Okabe et al., 2005). Additional incubation in the absence of <sup>14</sup>CO<sub>2</sub> was performed in parallel to determine the rate of NH<sub>4</sub><sup>+</sup> oxidation. The value of Y was obtained by dividing the incorporation rate of <sup>14</sup>CO<sub>2</sub> by the oxidation rate of NH<sub>4</sub><sup>+</sup>.

#### **5.4.4. Identification of respiratory quinone**

Quinone was extracted with a chloroform-methanol mixture and analyzed by reversed-phase HPLC (L-7000, Hitachi) as previously described (Hiraishi et al., 1998). Briefly, the cells were pelleted by centrifugation (10,000 rpm, 10 min), which was suspended in phosphate buffer (50 mM, pH 6.8) containing 1 mM ferricyanide. Quinone was extracted three times in an equal volume of chloroform-methanol solution (2:1, v/v), evaporated using a rotary evaporator, and re-extracted with n-hexane-water (1:1, v/v). The extracted quinone was injected into the HPLC system and the retention times of peaks were compared to those of quinone standards. The ubiquinone (UQ) and MK standards, including UQ-8, -9, MK-7, and -8, were prepared from known bacterial species (Hiraishi et al., 1998), and UQ-10 was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan).

#### **5.4.5. Electron microscopy**

Dispersed cells were cryofixed, and freeze-substitution was performed with osmium tetroxide and acetone as previously described (van Niftrik et al., 2008; Oshiki et al., 2011). After dehydration with acetone, the cells were embedded in Quetol 651 resin. Ultrathin sections (80–90 nm) were obtained using an ultramicrotome. These sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope JEM-1200EX (JEOL; Tokyo, Japan) at 80 kV.

#### **5.4.6. Analysis of intracellular metal composition**

The cell lysate was prepared using HNO<sub>3</sub> as previously described (Cameron et al., 2012). Briefly, 50 mL of the cell suspension was centrifuged (8000 rpm, 15 min at 10°C), and the cell pellet was washed twice with deionized water to remove residual metals from the inorganic media. The cell pellet was dried at 50°C to a constant weight; the dried pellet was resuspended in deionized water at a concentration of 0.33 g VSS L<sup>-1</sup>. The bacterial cells in the suspension were disrupted by sonication with an ultrasonicator at 100 W for 1 min. The sonication step was repeated twice and the cell suspension was incubated on ice for 3 min after each treatment to avoid heating. The cell suspension was centrifuged at 5000 rpm for 30 min and the supernatant was filtered through 0.2-µm syringe filters (Advantec; Tokyo, Japan). The supernatant was mixed with ultrapure HNO<sub>3</sub> (Wako Pure Chemical Industries; Tokyo, Japan) at a 1:9 volume ratio and incubated at 4°C overnight. Metal content in the cell lysates was examined by ICP-AES (ICPE-9000; Shimadzu; Kyoto, Japan). For quantitative analysis, standard curves for Fe, Cu, Cd, B, Cr, Ni, Sb, and Zn were prepared by diluting standard trace metal solutions purchased from Wako Pure Chemical Industries with 0.1 N HNO<sub>3</sub> and by measuring in the same manner. Standard curves were prepared in range of 0–1 mg/L.

#### **5.4.7. Proteomic analysis**

Total protein was extracted by suspending the cells (80 mg wet mass) in sample buffer A containing 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (4%, w/v), Triton X-100 (2%, w/v), urea (7 M), thiourea (2 M), and dithiothreitol (60 mM). After 1 h of incubation on ice and centrifugation (25,000 ×g for 1 h at 4°C), the supernatant was recovered as soluble protein fraction A. The remaining biomass pellet was resuspended in sample buffer B containing SDS (4%, w/v), glycerol (20%, v/v), Tris-HCl (0.125 M, pH 8.4), and dithiothreitol (20 mM) to extract proteins from the remaining biomass. After boiling for 15 min and

centrifugation (25,000 ×g for 1 h at 20°C), the supernatant was recovered as soluble protein fraction B. Proteins in the soluble protein fractions A and B were separated on SDS-containing polyacrylamide gels (NuPAGE 4-12% Bis-Tris gel) (Life Technologies; Carlsbad, CA, USA), and stained with Simplyblue staining solution (Life Technologies) according to the manufacturer's instructions. Gel lanes (7 cm in height) were cut into 10 gel slices, which were destained with acetonitrile and subjected to nano-LC/MS/MS analysis after in-gel tryptic digestion (Kasahara et al., 2012). Peptide mass fingerprints were analyzed using the MASCOT search program version 2.3.01 (Perkins et al., 1999). The amino acid sequences of protein-coding genes located in the planctomycete KSU-1 genome (GenBank accession number; BAFH0100000 to BAFH01000004) were used as the reference database. Calculations of the exponentially modified protein abundance index (emPAI) and protein content index (PCI) were conducted as previously described (Ishihama et al., 2005; Shinoda et al., 2009).

#### 5.4.8. Analytical techniques

Concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  were determined using ion chromatographs (IC-2010, TOSOH; Tokyo, Japan) equipped with TSKgel IC-Anion HS or TSKgel IC-Cation columns (TOSOH; Tokyo, Japan) after filtration with 0.2  $\mu\text{m}$ -pore-size cellulose acetate membranes (Okabe et al., 2011). The  $\text{NH}_3$  and  $\text{NO}_2^-$  concentrations were determined colorimetrically to find  $K_S$  values.  $\text{NH}_3$  concentration was determined using the indophenol method (American Public Health Association et al., 2012), and absorbance at a wavelength of 600 nm was determined using multilabel plate readers (ARVO MX 1420-01J; PerkinElmer; Waltham, MA, USA). The  $\text{NO}_2^-$  concentration was determined using the naphthylethylenediamine method (American Public Health Association et al., 2012) at an absorbance wavelength of 540 nm.

The concentrations of hydrazine and hydroxylamine were determined colorimetrically as previously described. Briefly, hydrazine was derivatized using p-dimethylaminobenzaldehyde, and absorbance at 460 nm was measured (Watt and Chrisp, 1952). For hydroxylamine, liquid samples were mixed with 8-quinolinol solutions, heated at 100°C for 5 min, followed by measuring absorbance at 705 nm (Frear and Burrell, 1955).

The concentrations of  $^{29}\text{N}_2$ ,  $^{30}\text{N}_2$ , and  $^{13}\text{CO}_2$  gas were determined by gas chromatography mass spectrometry (GC/MS) analysis (Waki et al., 2010; Yoshinaga et al., 2011). Fifty microliter of headspace gas was collected using a gas-tight syringe (VICI; Baton Rouge, LA, USA), immediately injected into a gas chromatograph GCMS-QP2010SE (Shimadzu) equipped with a CP-Pora Bond Q-fused silica capillary column (Agilent Technologies; Santa Clara, CA, USA), and  $m/z = 29$ , 30, and 45 were monitored. The amounts of  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  gas were determined using a standard curve prepared with  $^{30}\text{N}_2$  standard gas (>98% purity) (Cambridge Isotope Laboratories; Tewksbury, MA, USA).

Stable isotopic compositions of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  were determined as previously described (Isobe et al., 2011). Briefly, the cell suspension was filtered through a 0.2- $\mu\text{m}$  cellulose acetate filter (Advantec; Tokyo, Japan) and the filtrates were dispensed into sealable glass vials.  $\text{NO}_2^-$  was chemically reduced to  $\text{N}_2\text{O}$  gas using sodium azide, and the  $^{15}\text{N}/^{14}\text{N}$  ratio of the  $\text{N}_2\text{O}$  gas ( $m/z = 44$ , 45, and 46, respectively) in the headspace was examined by GC/MS.  $\text{NH}_4^+$  was recovered as  $(\text{NH}_4)_2\text{SO}_4$  using the ammonia diffusion method with an acidified

glass fiber membrane (Holmes et al., 1998), which was subsequently oxidized to  $\text{NO}_3^-$  by persulfate. The  $\text{NO}_3^-$  was biologically reduced to  $\text{N}_2\text{O}$  gas by the *Pseudomonas chlororaphis* subsp. *aureofaciens* ATCC13985<sup>T</sup> strain, and the  $^{15}\text{N}/^{14}\text{N}$  ratio of  $\text{N}_2\text{O}$  gas was examined by GC/MS. The  $^{15}\text{N}/^{14}\text{N}$  ratios were quantified using standard curves prepared from  $\text{NH}_4\text{Cl}$  and  $\text{NaNO}_2^-$  solution containing the  $^{15}\text{N}$  atom at known ratios; i.e., 0.37, 1.35, 5.31, 10.3, 20.1, 49.8, and 99.2 atom%  $^{15}\text{N}$ .

Biomass concentration was determined as protein concentrations, which were converted to VSS concentrations. Protein concentration was determined as previously described (Oshiki et al., 2011). Briefly, cells were pelleted by centrifugation ( $18,200 \times g$  for 10 min), and the pellet was suspended in 10% (w/v) SDS solution. After boiling for 10 min, the suspension was centrifuged at  $18,200 \times g$  for 10 min. Protein concentration in the supernatant was determined using the Lowry method with a DC protein assay kit (Bio-Rad; Hercules, CA, USA) and bovine serum albumin as a protein standard. Unit conversion from protein concentrations to VSS concentrations was conducted by multiplying a factor of  $1.67 \text{ g-VSS g-protein}^{-1}$ , which was determined previously (Oshiki et al., 2011).

Size distribution of cell aggregates was determined using an ultraviolet laser diffraction particle size analyzer SALD-7100 (Shimadzu).

#### **5.4.9. Nucleic acid sequence accession number**

The 16S-23S rRNA gene sequence of “*Ca. Jettenia caeni*” was deposited in the DDBJ nucleic acid sequence database under the accession number AB973443.

#### **5.4.10. Description of “*Ca. Jettenia caeni*” sp. nov.**

“*Ca. Jettenia caeni*” (cae’ni. L. gen. neut. n. caeni of sludge, pertaining to the biomass of origin).

Cells are cocci (0.8-1.2  $\mu\text{m}$ ) and contain three separated cellular compartments, including the paryphoplasm, riboplasm, and anammoxosome. Grows anoxically with  $\text{NH}_4^+$  and  $\text{NO}_2^-$  as an electron donor and acceptor, respectively.  $\text{CO}_2$  is used as a carbon source. Nitrogen gas and nitrate are the products of anammox process. Anammox activity occurs at mesophilic (20–42.5°C) and neutrophilic (pH 6.5–8.5) conditions (optimally at 37°C and pH 8.0). Reduce  $\text{NO}_3^-$  to  $\text{NH}_4^+$  with the oxidation of acetate. Accumulate hydrazine in the culture with the consumption of hydroxylamine. The predominant menaquinone is MK-7.

The type strain, KSU-1 was enriched from activated sludge collected from a wastewater treatment plant installed in the Kumamoto city, Japan. Here, the taxonomic classification of “*Ca. Jettenia caeni*” is proposed for the bacterial clade of the KSU-1. The bacterial clade of “*Ca. Jettenia caeni*” contained the nearly full-length 16S rRNA genes retrieved from five bioreactors (Fig. S7). The GenBank accession number of the “*Ca. Jettenia caeni*” KSU-1 genome is BAFH0100000 to BAFH01000004. The G+C content of the genomic DNA is 40.1 mol%, and the genome size is 4.09 Mbp.

Other synonym: planctomycete KSU-1

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## 6. CONCLUSIONS AND RECOMMENDATIONS

### 6.1. Preservation and reactivation of anammox biomass

It is still the biggest challenge to secure enough seeding biomass for rapid start-up of anammox process due to slow growth. Preservation of active anammox biomass could be one of the solutions. In this study, biomass of anammox bacterium, “*Ca. Brocadia sinica*”, immersed in various nutrient media were preserved at -80°C, 4°C and room temperature. Storage in nutrient medium containing 3 mM of molybdate at room temperature with periodical (every 45 days) supply of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  was proved to be the most effective storage technique for “*Ca. Brocadia sinica*” biomass. Using this preservation condition, 96, 92 and 65% of the initial SAA was sustained after 45, 90 and 150 days of storage, respectively. Furthermore, preserved biomass was successfully reactivated by immobilizing in PVA-SA gel and then inoculated to up-flow column reactors. Total NRRs rapidly increased to  $7 \text{ kg-N m}^{-3} \text{ d}^{-1}$  within 35 days of operation. Based on these results, the room temperature preservation with molybdate addition is simple, cost-effective and feasible at a practical scale, which will accelerate the practical use of anammox process for wastewater treatment.

### 6.2. Immobilization of anammox biomass

Rapid start-up of anaerobic ammonium oxidation (anammox) process in up-flow column reactors was successfully achieved by immobilizing minimal quantity of biomass in polyvinyl alcohol (PVA)-sodium alginate (SA) gel beads. The changes in the reactor performance (*i.e.*, nitrogen removal rate; NRR) were monitored with time. The results demonstrate that the reactor containing the immobilized biomass concentration of  $0.33 \text{ g-VSS L}^{-1}$  achieved NRR of  $10.8 \text{ kg-N m}^{-3} \text{ d}^{-1}$  after 35-day operation, whereas the reactor containing the granular biomass of  $2.5 \text{ g-VSS L}^{-1}$  could achieve only NRR of  $3.5 \text{ kg-N m}^{-3} \text{ d}^{-1}$ . This indicates that the gel immobilization method requires much lower seeding biomass for start-up of anammox reactor. To explain the better performance of the immobilized biomass, the biological and physicochemical properties of the immobilized biomass were characterized and compared with the naturally aggregated granular biomass. Effective diffusion coefficient ( $D_e$ ) in the immobilized biomass was directly determined by microelectrodes and found to be three times higher than one in the granular biomass. High anammox activity (*i.e.*,  $\text{NH}_4^+$  and  $\text{NO}_2^-$  consumption rates) was evenly detected throughout the gel beads by microelectrodes due to faster and deeper substrate transport. In contrast, anammox activity was localized in the outer layers of the granular biomass, indicating that the inner biomass could not contribute to the nitrogen removal. This difference was in good agreement with the spatial distribution of microbes analysed by fluorescence *in situ* hybridization (FISH). Based on these results, PVA-SA gel immobilization is an efficient strategy to initiate anammox reactors with minimal quantity of anammox biomass.

### 6.3. Ecophysiology of anammox bacterium “*Ca. Jettenia caeni*”

Six candidate genera of anammox bacteria have been identified in the taxonomic group of anammox, and numerous studies have been conducted to understand their ecophysiology. In this study, physiological

characteristics of an anammox bacterium belong to the genus “*Ca. Jettenia*” were investigated. “*Ca. Jettenia caeni*” was found to be a mesophilic (20–42.5°C) and neutrophilic (pH 6.5–8.5) bacterium with a maximum growth rate of 0.0020 h<sup>-1</sup>. Bacterial cells of “*Ca. Jettenia caeni*” showed typical physiological and structural features of anammox bacteria; *i.e.*, <sup>29</sup>N<sub>2</sub> gas production by coupling of <sup>15</sup>NH<sub>4</sub><sup>+</sup> and <sup>14</sup>NO<sub>2</sub><sup>-</sup>, accumulation of hydrazine with the consumption of hydroxylamine, and the presence of anammoxosome. In addition, the cells were capable of respiratory ammonification with oxidation of acetate. Notably, the cells contained menaquinone-7 as a dominant respiratory quinone. Proteomic analysis was performed to examine underlying core metabolisms, and high expressions of hydrazine synthase, hydrazine dehydrogenase, hydroxylamine dehydrogenase, nitrite/nitrate oxidoreductase, and CO dehydrogenase/acetyl-CoA synthase were detected. These proteins require iron or copper as a metal cofactor, and both were dominant in planctomycete KSU-1 cells.

#### 6.4. Future outlook

In this endeavour, simple, rapid and effective long-term preservation (at room temperature) and reactivation technique for anammox biomass was successfully developed. Later, rapid start-up of anammox process was demonstrated by immobilizing preserved biomass in PVA-SA gel. Storage at room temperature does not require any special equipment and skill and thus is a simple, easy and cost-effective method for biomass preservation. The combination of room temperature storage and PVA-SA immobilization technique would be feasible at a practical scale and thus accelerate the practical use of anammox process for wastewater treatment. However, these preservation and reactivation conditions were tested by using anammox biomass with overwhelming major of “*Ca. Brocadia sinica*”. In future this preservation technique should be tested by using phylogenetically different anammox bacteria to establish a general storage protocol for anammox bacteria.

In addition, rapid start-up of anammox process was achieved with very less biomass content (0.33 g-VSS L<sup>-1</sup>) by immobilizing in PVA-SA gel. Nitrogen removal performance of immobilized biomass was better than granular biomass. Further, it was revealed that the core of granular biomass was not metabolically active because of high pH, >8.5, in the inner layer below 500 μm depth from the surface. Whereas, anammox bacterial cells in the core of immobilized biomass proved their metabolic activity by incorporating <sup>13</sup>C-bicarbonate during incubation for 35 days. This study would be useful for researchers and professionals while choosing optimum amount of inoculum for establishment of anammox reactor with immobilized biomass. Nevertheless, long-term operation of immobilized biomass should be investigated in the future study.

Rapid start-up of anammox process cannot be achieved until we have through understanding of anammox ecophysiology. During doctoral endeavour, physiological of anammox bacterium belong to genus “*Ca. Jettenia*” was characterised comprehensively. The physiological characteristics of this anammox bacteria illuminated that these cells are freshwater anammox bacteria and prefer NH<sub>4</sub><sup>+</sup>- and/or NO<sub>2</sub><sup>-</sup>-rich habitats. Now, comprehensive data sets of detailed physiological characteristics, genome sequences, and proteome are available for the genera “*Ca. Kuenenia*”, “*Ca. Brocadia*”, “*Ca. Scalindua*”, and “*Ca. Jettenia*”. The datasets are

useful to explore niche partitioning among these anammox bacteria in natural and man-made ecosystems. However, these physiological traits were mainly investigated during batch test incubation. Long-term continuous incubation study is still required to further pin down the dominance of phylogenetically different anammox species under different environmental conditions.

Though application of anammox process at full-scale treatment plants is growing rapidly, still anammox process is not very popular chose as compared to N&DN process. There are three major challenges highlighted in this dissertation; 1) longer start-up period; 2) limited mainstream application; and 3) poor effluent water quality of anammox process. Anammox inoculum can be available from already established full-scale anammox treatment plant. In addition, start-up period can also be reduced up to acceptable period of about one month by using appropriate storage and immobilization technique for anammox biomass. However, mainstream application of anammox process and poor effluent quality are still issues warrant thorough investigation and attention in the further studies. Recent studies demonstrated the feasible application of anammox process on main line of domestic sewage treatment (Gilbert et al., 2014; Hu et al., 2013; Lotti et al., 2014a; Lotti et al., 2014b). Nevertheless, there is a lot of room in this research area that requires further attention from the researchers. An integrated wastewater treatment system has been introduced (see chapter two) by coupling anammox process with MFC for effective and energy-positive wastewater treatment. Although application of proposed treatment system presents great challenges in terms process optimization. However, it is feasible scientifically and expect other researchers to take up the challenge for applying proposed treatment system for effective and energy-positive wastewater treatment.

## **6.5. References**

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