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ENRICHMENT, GROWTH KINETICS AND ECOPHYSIOLOGY OF ANAMMOX BACTERIA

HOKKAIDO
UNIVERSITY

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Engineering

Declaration

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

Signed:_____

Date:_____

ABSTRACT

Anaerobic ammonium oxidizing (anammox) bacteria have the unique metabolic ability to convert ammonium and nitrite to dinitrogen gas. This discovery led to the knowledge that activity of these bacteria were responsible for a substantial part (50 – 100%) of the enormous nitrogen losses observed in the freshwater and marine environments. However, ecophysiology and niche differentiation of anammox bacteria are still the enigma that remains due to the time-consuming enrichment methods, insufficient/inaccurate knowledge of growth kinetics and various environmental factors involved that yet to be identified. In this thesis, I focus on the eco-physiological characteristics of anammox bacteria to address their niche differentiation and ecological significance. In chapter 3, since it is still not possible to achieve pure culture of anammox bacteria, lack of appropriate culture has become the biggest hurdle for physiological study of anammox bacteria requiring high purity planktonic culture. Such culture has once been obtained using membrane bioreactor before, but took considerably amount of time and efforts. This chapter focuses on the development of a novel method for rapid cultivation of free-living anammox cells using immobilization technique. It was demonstrated that active free-living planktonic anammox cells with purity > 95% was successfully developed in the MBR with overall only 35 days operation time. In chapter 4, it was acknowledged that the specific niche of certain microorganism is mostly determined from their kinetics including maximum specific growth rate. Inaccurate information of their kinetics would inevitably disturb the interpretation of their behaviour in complex eco-systems. Specifically, in the case of anammox bacteria, whose maximum specific growth rate is always in debate. In this chapter, a reliable re-evaluation procedure for maximum specific growth rates of three anammox species was developed and showing that anammox bacteria may not appropriate to be considered as slow-growing bacteria any more. In chapter 5, previously the issue of niche differentiation of anammox bacteria has been addressed by analysing the database of so far published 16S rRNA gene sequences, illustrating their niche partitioning and global distribution. However, information regarding their true dynamic behaviour could not be obtained. Enriched culture-dependent competition under given conditions is a direct way to illustrate their niche differentiation. In this chapter, the microbial competitions for a common substrate (nitrite) among three anammox species (*i.e.* "*Ca.* Brocadia sinica", "*Ca.* Jettenia caeni" and "*Ca.* Kuenenia

stuttgartiensis") were systematically investigated in nitrite-limited gel-immobilized column reactors (GICR) and membrane bioreactors (MBRs) under different nitrogen loading rates (NLRs). 16S rRNA gene-based population dynamics revealed that "*Ca*. J. caeni" could proliferate only at low NLRs, whereas "*Ca*. B. sinica" outcompeted other two species at higher NLRs in both types of reactors, demonstrating NLR was one of factors determining ecological niche differentiation of "*Ca*. B. sinica" and "*Ca*. J. caeni". On the other hand, a statistical study looking at over 6000 anammox 16S rRNA gene sequences from the public database, indicated that salinity was the most important factor governing anammox bacterial distributions, with "*Ca*. Scalindua" dominated in saline environments while "*Ca*. Brocadia" were mostly found in freshwater environments. Though it is strongly suggested, physiological verification of salinity as a niche factor for anammox bacteria has never been conducted. Detailed understanding of the extent of the effect of salinity on adaptation, genetic basis and ecological significance are all completely lacking at this moment. In this chapter, we conducted both batch and continuous experiment combined with mass analysis and genetic identification aiming to address the question whether and how salinity becomes one of the key factor in the niche differentiation between "*Ca*. Brocadia" and "*Ca*. Scalindua". Results obtained suggest that trehalose associated different response to osmostress in "Ca. B. sinica" and "Ca. S. japonica" might be one of the reason shaping the distinct niche in between. In the final chapter findings of previously conducted studies were compiled and recommendations for future prospective were highlighted.

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1 INTRODUCTION

The anaerobic ammonium oxidation (anammox) process has been recognized as one of the main biological nitrogen processes, where ammonium and nitrite react and convert into dinitrogen gas directly under anaerobic condition (without oxygen) (Strous et al., 1999a). The discovery of anammox process revolutionized our classic understanding of global nitrogen cycle, in addition, the traditional model system of wastewater treatment process regarding biological nitrogen removal was also challenged (Ali and Okabe, 2015; Francis et al., 2007). During the first decades after anammox process was discovered, lot of studies were carried out on application of this novel process into nitrogen removal, more specifically, side-stream wastewater including excess sludge digestion supernatant that contains high concentration of ammonium (Kartal et al., 2010; van der Star et al., 2007). However, studies working on the biochemistry and physiology of anammox process and bacteria were still very limited, even after 30 years of discovery and in spite of the advancing of molecular technology. Therefore, our understanding regarding ecophysiology on this particular microorganism is extremely lacking at this moment. The reasons should be the so far unavailability of pure culture for anammox bacteria (Strous et al., 1999b) and the difficulty in the enrichment of anammox culture. Here anammox culture refers to highly enriched planktonic cell culture (van der Star et al., 2008). When anammox bacteria was found in the very beginning, its doubling time (specific growth rate) has been characterized as long as more than two weeks. Although later on more and more studies point out the intrinsic growth rate of anammox bacteria should be much faster and may

close to a doubling time of two days, still, at that time, to enrich an anammox culture is far from an easy job. It is already quite difficult to enrich the culture, not to mention to study the characterises, which is why physiology-related study are such limited (Zhang et al., 2017b). While for bio-chemical study of anammox bacteria, some methods like cell separating (Percoll separation) has enabled the separation of anammox cells from other bacterial cells, even reach a purity more than 99% (Kartal et al., 2011). However, the yield of this method is relatively low and therefore, require large amount of anammox biomass. In addition, the separated cells can be used for further extraction of DNA, RNA and protein, all biochemistry-related materials, but could not be used for long term cultivation because along with the operation it inevitably introduces other microbial members into the system. Therefore, some biochemical studies have been carried out, with the situation of physiological study largely in blank.

There is no need to question the importance and necessity in the pursuit of anammox bacteria physiology and one more step, their ecology in order to understand their contribution to global nitrogen cycle (Oshiki et al., 2015; Zhang et al., 2017a). As definition, bacterial physiology is a scientific discipline that concerns the life-supporting functions and processes of bacteria, which allow bacteria cells to grow and reproduce. Simply, the information of bacterial physiology is used to interpret, predict the life style of specific bacteria, the optimum condition that this microorganism demonstrates its maximum specific growth rate. While this is closely related to the ecological status of the microorganism in nature, which is determined from its physiological requirements.

In this thesis, the main objective is to study the ecophysiology of anammox bacteria. This objective was approached through the following chapters, briefly explained as below.

Outline of Thesis

- Chapter 1:

  This chapter states the overview of current dissertation and broader research questions that were addressed during doctoral endeavour. In addition, the details of each research objectives were outlined in this chapter.

- Chapter 2:

  This chapter presents a critical review of previous studies focusing on physiology and niche differentiation of anammox bacteria. It was revealed during literature review that number of publications regarding the physiology and ecology of anammox bacteria so far was considerably fewer compared with the application part of anammox process. There are many issues remained to be addressed before more studies can be carried out on the study of physiology and ecology of anammox bacteria. The literature review aims to present a comprehensive summary of researches on how to enrich appropriate culture of anammox bacteria, determination of key kinetics for anammox bacteria and their niche differentiation. Besides, a detailed summary of the specific niche where so far identified anammox species were enriched was established.

- Chapter 3:

  It was revealed in literature review that it is still not possible to achieve pure culture of anammox bacteria. This became the biggest hurdle for physiological study of anammox bacteria requiring high purity planktonic culture. Such culture has once been obtained using membrane bioreactor before, but took considerably amount of time and efforts. This chapter focuses on the development of a novel method for rapid cultivation of free-living anammox cells using immobilization technique. It was demonstrated that active free-living planktonic anammox cells with purity > 95% was successfully developed in the MBR with overall only 35 days operation time.

- Chapter 4:

  It was acknowledged that the specific niche of certain microorganism is mostly determined from their kinetics including maximum specific growth rate. Inaccurate information of their kinetics may misinterpret their behaviour in complex eco-systems. In the case of anammox bacteria, whose maximum specific growth rate is always in debate. In this study, a reliable re-evaluation procedure for maximum specific growth rates of three anammox species was developed and showing that anammox bacteria may not appropriate to be considered as slow-growing bacteria any more.

- Chapter 5:

  Previously the issue of niche differentiation of anammox bacteria has been addressed by analysing the database of so far published 16S rRNA gene sequences, illustrating their niche partitioning and global distribution. However, information regarding their actual dynamic behaviour could not be obtained. Enriched culture-dependent competition is the most direct way to illustrate their niche differentiation regarding certain factors. In this study, the microbial competitions for a common substrate (nitrite) among three anammox species (*i.e.* "*Candidatus* Brocadia sinica", "*Candidatus* Jettenia caeni" and "*Candidatus* Kuenenia stuttgartiensis") were systematically investigated in nitrite-limited gel-immobilized column reactors (GICR) and membrane bioreactors (MBRs) under different nitrogen loading rates (NLRs). 16S rRNA gene-based population dynamics revealed that "*Ca*. J. caeni" could proliferate only at low NLRs, whereas "*Ca*. B. sinica" outcompeted other two species at higher NLRs in both types of reactors, demonstrating NLR was one of factors determining ecological niche differentiation of "*Ca*. B. sinica" and "*Ca*. J. caeni".

- Chapter 6:

  A statistical study looking at over 6000 anammox 16S rRNA gene sequences from the public database, indicated that salinity was the most important factor governing anammox bacterial distributions, with "*Ca*. Scalindua" dominated in saline environments while "*Ca*. Brocadia" were mostly found in freshwater environments. Though it is strongly suggested, physiological verification of salinity as a niche factor for anammox bacteria has never been conducted. Detailed understanding of the extent of the effect of salinity on adaptation, genetic basis and ecological significance are all completely lacking at this moment. In this chapter, we conducted both batch and continuous experiment combined with mass analysis and genetic identification aiming to address the question whether and how salinity becomes one of the key factor in the niche differentiation between "*Ca*. Brocadia" and "*Ca*. Scalindua". Results obtained suggest that the osmoadaptive strategy employed by "*Ca*. B. sinica" limited its competition with "*Ca*. S. japonica", shaped the distinct niche in between.

- Chapter 7:

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

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2 LITERATURE REVIEW

This chapter has been summarized for submission as:

Zhang, L, Narita, Y and Okabe, S. Enrichment, growth kinetics and niche differentiation of anammox bacteria.

2.1 Abstract

Anaerobic ammonium oxidizing (anammox) bacteria have the unique metabolic ability to convert ammonium and nitrite to dinitrogen gas. This discovery led to the knowledge that activity of these bacteria were responsible for a substantial part (50 – 100%) of the enormous nitrogen losses observed in the freshwater and marine environments. However, ecophysiology and niche differentiation of anammox bacteria are still the enigma that remains due to the time-consuming enrichment methods, insufficient/inaccurate knowledge of growth kinetics and various environmental factors involved that yet to be identified. In this review, we summarized state of the art information on culture enrichment and growth kinetics that are essential for deciphering the ecophysiology of anammox bacteria. In addition, details of the enriching conditions for several anammox species were summarized and potential factors for niche partitioning were discussed.

2.2 INTRODUCTION

The discovery of missing autotroph, anaerobic ammonium oxidizing (anammox) bacteria, has radically revolutionized our stereotypical view of biological nitrification and denitrification and stimulated the hunt of "impossible" microbes (Mike S M et al., 1999; Strous et al., 1999a). Anammox bacteria, belongs to the order of Planctomycetales (Strous et al. 1999a), has been known to catalyze the oxidation of ammonium by nitrite as the electron acceptor under anaerobic condition (van de Graaf et al., 1996). So far five candidatus genera (*Brocadia, Kuenenia, Jettenia, Scalindua and Anammoxoglobus*) and 19 species have been reported (Ali and Okabe, 2015; Kartal et al., 2007a; Oshiki et al., 2016; Z. X. Quan et al., 2008; Schmid et al., 2003; Strous et al., 1999a, 1998) with anammox activities being detected from various natural and man-

made ecosystems (Hu et al., 2011; Humbert et al., 2012; Jetten et al., 2003; Marcel M M Kuypers et al., 2003; Oshiki et al., 2016; Sonthiphand et al., 2014).

Anammox bacteria received considerable attention in industrial application owing to its great economical potential from their distinct metabolism and physiology (Ali and Okabe, 2015; Kartal et al., 2010), but were assumed to be minor players in the N cycle within natural ecosystems before. In 2002, anammox process was found to be responsible for 24 - 67% of N loss in marine sediments (Thamdrup and Dalsgaard, 2002), and 20 - 40% of N loss has been proposed to be attributed to anammox in the suboxic water columns of the Black Sea and Gulfo Dulce (Dalsgaard et al., 2003; Marcel M. M. Kuypers et al., 2003). Recent studies indicate an even greater percentage (up to 100%) of marine N loss may come from anammox process (Hamersley et al., 2007; Kuypers et al., 2005; Schmid et al., 2007; Trimmer et al., 2013). Combined with universal detection of anammox activity in various ecosystems, ubiquitous of this process was revealed (Francis et al., 2007; Hu et al., 2011). Thus, knowledge of ecological biogeography, more specifically, ecological niche differentiation (Vandermeer, 1972) of anammox bacteria, is essential for understanding the contribution of anammox process to global nitrogen cycle. However, although number of publications related to anammox is increasing exponentially (**Fig. 1A**), research focus in recent years has been placed on the application and process optimization of anammox for industrial and municipal wastewaters (**Fig. 1B**). While the investigations for its ecology and physiology, the main disciplines illustrating the natural importance of anammox bacteria, were extremely limited (**Fig. 1C and 1D**).

Physiological characteristics are essential for understanding and interpreting the ecological niche of anammox bacteria (Oshiki et al., 2015, 2011). The main reason that physiological study of anammox bacteria was quite limited is the lack of appropriate culture with free living cells and a high purity (> 90%) (van der Star et al., 2008). So far anammox bacteria have been enriched as aggregates like granules and biofilm due to its low specific growth rate (Strous et al., 1998; Tsushima et al., 2007b). However, the good retention capacity of aggregates would to some extent slower the selection rate between anammox species or even, in some case, made it possible for two species to co-exist in one eco-system due to the substrate diffusion limitation (Ali et al., 2015c; Zhang et al., 2017a). This disturbs interpretation on niche partitioning in between. Unavailability of planktonic cell cultures, information of kinetic parameters could neither be determined accurately (Lotti et al., 2014).

Here, in view of recent knowledge, status regarding the enrichment of anammox culture, the growth kinetics of anammox bacteria and niche differentiation between certain anammox species were summarized. Firstly, the methods so far used for enriching anammox bacteria were described with some new progresses in achieving completely suspended planktonic anammox cells. Then, the growth kinetics including the μ_{max} and *K*s obtained so far for various anammox species were summarized and discussed. Lastly, a discussion on niche differentiation between anammox species was conducted based on the competition among anammox species and physical environmental parameters used for enrichment.

2.3 ENRICHMENT OF ANAMMOX CELLS

Despite that anammox bacteria are key players in the global nitrogen cycle, no pure cultures is still available (Strous et al., 1999b). However, anammox bacteria can be selectively enriched by supplying specific substrate under anoxic condition (*i.e.* ammonium and nitrite) (van de Graaf et al., 1996). Due to the relatively slow growth rate as a doubling time more than 2 days (Zhang et al., 2017b), biomass retention is the key factor for a successful enrichment of anammox bacteria (Strous et al., 1998). Different reactor configurations have been tested so far to achieve a robust but yet reproducible enrichment strategy including sequencing batch reactor (Strous et al., 1998), up flow column reactor (Tsushima et al., 2007a), immobilized upflow column reactor (Isaka et al., 2007) and up flow reactor packed with nonwoven porous polyester materials (Fujii et al., 2002), etc. (**Table 1**). Those configurations enable the formation of biomass aggregates by supplying a selection pressure as settling down and wash out, attachment to supporting materials and immobilization using artificial materials. Although large amount of anammox biomass could be enriched in those systems, they are not in ideal form for fundamental investigation on anammox bacteria (*i.e.* eco-physiological and biochemical characteristics). Firstly, satisfactory purity (>90%) could not be achieved in aggregated biomass due to the complex microbial community induced by substrate diffusion limitation and aggregation (Lawson et al., 2017; Tsushima et al., 2007b), making it difficult to draw definite conclusion regarding the physiological phenomenon. Secondly, heterogeneous distribution of specific anammox activity in the aggregates. Lastly, the extracellular polymeric substance (EPS) as the supporting material in aggregates, was found to trigger analyzing difficulties in protein purification process (Cirpus et al., 2006; Ni et al., 2010).

To overcome above issues, growth of anammox bacteria as completely planktonic cells is essential. As the pioneer and breakthrough, in 2007, MBR was introduced to cultivate an anammox bacterium, "*Ca.* K. stuttgartiensis", in the form of planktonic cells and unprecedented high purity (97.6%) from which precise measurement of physiological characteristics became possible (**Table 1**) (van der Star et al., 2008). Later, phylogenetically-different anammox species have been also cultivated in the form of the planktonic cells in MBRs; *i.e.*, "*Ca*. Jettenia" (Ali et al., 2015a), "*Ca*. Brocadia" (Lotti et al., 2014; Narita et al., 2017; Mamoru Oshiki et al., 2013) and "*Ca*. Scalindua"

(Awata et al., 2013a; Oshiki et al., 2011) and more and more physiological and biochemical information was revealed (Oshiki et al., 2015).

2.3.1 Rapid cultivation as planktonic cells

However, this MBR cultivation still takes an enormous amount of time to achieve free-living cells; usually more than 100 – 200 days (Ali et al., 2015a; Lotti et al., 2014; Mamoru Oshiki et al., 2013; van der Star et al., 2008). This time-consuming cultivation process is apparently a bottle neck of physiological, biochemical and kinetic studies of anammox bacteria. A recent study was able to provide a new procedure where completely suspended planktonic cell culture could be achieved within 35 days (Zhang and Okabe, 2017). It was found that anammox bacteria cells grown in the gel beads did not form rigid aggregates and were easily dispersible. This suggests that the biomass grown in the gel beads could be used as an inoculum of MBR to obtain free-living planktonic cells. The combination of gel immobilization technique as pre-culture and MBR cultivation was proved as effective for rapid cultivation of planktonic cell cultures (**Fig. 2**). It is believed that with much easier access to anammox planktonic cell culture, more and more physiological studies will be carried out.

2.4 MICROBIAL GROWTH KINETICS OF ANAMMOX BACTERIA

Microbial growth kinetics, *i.e.*, the relationship between the specific growth rate (μ) of a microbial population and the limiting substrate concentration (s) (also known as the Monod kinetics), is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology, or biotechnology (Kovarova-Kovar and Egli, 1998). It has been extensively used to predict, systematically evaluate and interpret the competitions among the microorganisms of interest for their niche partitioning (Bollmann et al., 2002; French et al., 2012; Füchslin et al., 2012; Kindaichi et al., 2006; Martens-Habbena et al., 2009; Ngugi et al., 2016; Nogueira and Melo, 2006; Nowka et al., 2015; Zhang et al., 2017a). Despite their importance, μ_{max} and *K*s of anammox bacteria are still debatable issues due to experimental difficulties with a lack of planktonic enrichment cell cultures with high purity (Strous et al., 1999b; van der Star et al., 2008).

2.4.1 Maximum species growth rate (μ_{max})

The μ_{max} of anammox bacteria was so far mainly determined by either controlling the sludge retention time (SRT) or measuring the biomass yield and maximum specific substrate consumption rate (Lotti et al., 2015; Oshiki et al., 2011). A recent study reevaluated the maximum specific growth rates (μ_{max}) of three phylogenetically distant anammox bacterial species (*i.e.* "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. S. japonica") by directly measuring the time course increase of 16S rRNA gene copy numbers as planktonic cells or immobilized biomass using newly developed quantitative polymerase chain reaction (qPCR) assays (Zhang et al., 2017b). The μ_{max} of "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. S. japonica" were determined to be 0.33 ± 0.02 d^{-1}, 0.18 d^{-1} and 0.17 d^{-1}. These values were the fastest reported for these species so far. Before anammox bacteria has been recognized as slow growing bacteria and anaerobic ammonium oxidation itself was considered as simply a kinetically difficult metabolic strategy (Strous et al., 1998). Now it is considered that much lower μ_{max} obtained before

may result from the beyond-optimum condition for enrichment. By introducing more appropriate culture and measuring methods, it could be expected that more μ_{max} approaching the intrinsic ones of anammox bacteria will be revealed in the future.

Part of the μ_{max} values obtained from anammox bacteria, NOB and AOB were summarized in **Fig. 3** and **Table S1**. Most NOB possesses μ_{max} of 0.19 – 2.08 d^{-1} (Bock et al., 1990, 1983; Both et al., 1992; Ehrich et al., 1995; Laanbroek et al., 1994; Nowka et al., 2015; Spieck and Bock, 2005), AOB with a μ_{max} of 0.41 – 1.21 d^{-1} (Ahn et al., 2008; Chandran et al., 2008; Galí et al., 2007; Hanaki et al., 1990; Jubany et al., 2009; Kaelin et al., 2009; Katehis et al., 2002; Park and Noguera, 2007; Vadivelu et al., 2006), which are higher than anammox bacteria (μ_{max} of 0.05 – 0.33 d^{-1}) (Kartal et al., 2008, 2007b; Lotti et al., 2015; Narita et al., 2017; Puyol et al., 2013; Strous et al., 1998; van de Vossenberg et al., 2008a; van der Star et al., 2008; Zhang et al., 2017b). However, as discussed before, current information of μ_{max} from certain anammox species were considered as insufficient. While the re-evaluated μ_{max} from three anammox species ("*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. S. japonica") demonstrated a μ_{max} ranging from 0.17 to 0.33 d^{-1}, close to the lowest value of NOB and AOB. Since most values of AOB and NOB were obtained from pure culture, it is suggested that μ_{max} of anammox bacteria may closer to the ones of other autotrophic bacteria involved in natural nitrogen cycle than previously assumed.

2.4.2 Affinity constant to limiting substrate (nitrite)

Nitrite is the limiting substrate for anammox bacteria (Lotti et al., 2014; Strous et al., 1998). The affinity constant (*K*s, also known as half-saturation constant) is a key parameter in microbial ecology as well as the biological process in engineering field. So far a wide range (0.2 – 370 μM) of values have been reported for the *K*s of different anammox species **(Fig. 4)** (Ali et al., 2015a; Awata et al., 2013b; Carvajal-Arroyo et al., 2013; Kartal et al., 2008, 2007a; Lotti et al., 2014; Narita et al., 2017; Mamoru Oshiki et al., 2013; Oshiki et al., 2017; Strous et al., 1999b; van der Star et al., 2008). Either continuous cultures (Mamoru Oshiki et al., 2013; van der Star et al., 2008) or batch experiments (Ali et al., 2015a; Strous et al., 1999b) has been employed for *K*s identification. It is not surprising to see this high variability by considering the species-

specific difference, various cultivation methods, purity of the biomass and its aggregation status (Lotti et al., 2014). Among those values, the five that obtained from planktonic cell cultures ("*Ca*. K. stuttgartiensis", "*Ca*. B. sinica", "*Ca*. B. sp. 40", "*Ca*. S. japonica" and "*Ca*. B. sapporoensis") (Awata et al., 2013a; Lotti et al., 2014; Narita et al., 2017; Mamoru Oshiki et al., 2013; van der Star et al., 2008) are of highly importance since they represent the most accurate measurement. Based on available *K*s, one could easily predict the result of interspecific competition if substrate is the only factor involved. For instance, "*Ca*. B. sinica" might be the *r*-strategist while "*Ca*. K. stuttgartiensis" be the *K*-strategist due to its higher affinity but lower μ_{max} (Oshiki et al., 2015).

We also summarized some reported *K*s values for nitrite from nitrite oxidizing bacteria (NOB) and *K*s for ammonium/ammonia from ammonium oxidizing bacteria (AOB) (**Fig. 4**) (Button, 1985; Jiang and Bakken, 1999; Keen and Prosser, 1987; Nowka et al., 2015; Suwa et al., 1994; Suzuki et al., 1974; Ward, 1987). *K*s values from NOB and AOB were mostly obtained from pure culture. The estimated *K*s for NOB vary considerably, ranging from 9 to 544 μM, but higher than the ones from certain anammox species ("*Ca*. K. stuttgartiensis", "*Ca*. B. anammoxidians", "*Ca*. B. sp. 40", "*Ca*. S. japonica" and "*Ca*. B. sapporoensis"). Similarly, *K*s of AOB ranged between 6 to 4000 μM, higher than the ones of "*Ca*. B. anammoxidians" and "*Ca*. S. japonica". It should be noted that some *K*s from anammox bacteria were measured using aggregates, much lower intrinsic *K*s value is expected and thus it might turn out that more anammox species possess higher affinity with substrate than AOB and NOB.

*K*s is not only important for ecological niche differentiation as interspecific competition, it is also a valuable reference for the competition of anammox bacteria with other microorganisms sharing same nutrients in natural and man-made ecosystems. In one stage partial nitrification/denitrification systems, NOB compete with anammox bacteria for nitrite that converted by AOB from ammonium (Lackner et al., 2014; Winkler et al., 2012). Overgrow of NOB may outcompete the anammox bacteria and produce residual nitrate, which is not desirable for nitrogen removal. Considering the *K*s value of anammox bacteria, strict population controlling strategy may not be necessary if appropriate anammox species was selected (Higher affinity with substrate, *e.g.* "*Ca*. K. stuttgartiensis" and "*Ca*. S. japonica"). Competition between AOB and anammox bacteria is less since in granules or biofilm, AOB was always proliferate at the outside of the layer while anammox bacteria resident at inner part depending on dissolved

oxygen concentration. Based on the lower *K*s value for ammonium, anammox bacteria could efficiently scavenge the remaining ammonium from AOB to create an ideal ecosystem for complete nitrogen removal (Sliekers et al., 2002). Oxygen is another important parameter between the competition of anammox bacteria and AOB or NOB. Interestingly, one recent study investigated the oxygen sensitivity of anammox and coupled N-cycle process in oxygen minimum zones (OMZ) found that activity of anammox reaction was only moderately affected by changing oxygen concentration, further, aerobic ammonia oxidation was active at an oxygen concentration of non-detectable level (Kalvelage et al., 2011). These intriguing findings may suggest a fiercer and more frequent competition between anammox bacteria and AOB, NOB. In addition, if we only consider the *K*s and μ_{max}, the whole group of anammox bacteria could be simply interpreted as *k*-strategist while the AOB and NOB groups are more likely to be the *r*-strategist since they possess higher μ_{max} but lower affinity (higher *K*s value). This property, to some extent, secured the advantageous position of anammox bacteria in frequently occurred famine conditions in natural environment (Roszak and Colwell, 1987). This is also supported by the universal detection of anammox activity in open ocean and estuary areas where the ammonium concentrations are less than 0.03 – 1 μM (Beman et al., 2008; Herfort et al., 2007; Könneke et al., 2005; Wuchter et al., 2006) and 22 – 45 μM to 115 μM when receiving agricultural run-off (Beman and Francis, 2006; Santoro et al., 2008), respectively, with an even lower concentrations of nitrite (~ 0.5 μM) (Beman et al., 2013).

*K*s of anammox bacteria was also lower than the ones reported for denitrifiers (4 – 25 μM) (Almeida et al., 1995; Betlach and Tiedje, 1981). A higher affinity for nitrite would be advantageous for anammox bacteria to compete with denitrifiers. In fact, coexistence of nitrifying, anammox and denitrifying bacteria in a sequencing batch reactor has been successfully demonstrated with a high diversity (Langone et al., 2014), while the success of anammox bacteria in maintaining its population might be attributed to its high affinity to substrate. However, anammox bacteria was found to be susceptible for several kinds of organic compounds and may suffer an inhibition (e.g. 91% activity inhibition by 1 mM ethanol, 86% activity inhibition by 1 mM methanol and 36% activity inihibiyion by 1 mM formate from "*Ca*. B. sinica") (Jensen et al., 2007; Jin et al., 2012; Oshiki et al., 2011). Overgrow of denitrifiers over anammox bacteria under co-supplementary of organic compounds occurred under C/N ratio of 2.92 (Tang et al., 2010). Therefore, a simultaneous partial nitrification, Anammox and denitrification

system (*e.g.* SNAD) (Chen et al., 2009) that combines autotrophic nitrogen removal by anammox bacteria and heterotrophic nitrogen removal by denitrifiers needs careful process control and optimization (Takekawa et al., 2014).

2.5 INTERSPECFIC COMPETITION AMONG ANAMMOX BACTERIA

Competition is an excellent tool to decipher the niche partitioning between organisms of interest (Bollmann et al., 2002; Chakraborty et al., 2016; Füchslin et al., 2012; Koeppel and Wu, 2014; Nowka et al., 2015; Vanniel et al., 1993; Winkler et al., 2017). In fact, population shifts between different anammox genera or species have been frequently reported in bioreactors: *e.g.,* from "*Ca.* B. fulgida" to "*Ca.* B. sp. 40" (Park et al., 2010a); from "*Ca.* B. fulgida" to "*Ca.* K. stuttgartiensis" (Park et al., 2015); from "*Ca.* B. sp." to "*Ca.* K. stuttgartiensis" (van der Star et al., 2008); and from "*Ca.* B. anammoxidians" to "*Ca.* A. propionicus" (Kartal et al., 2007a). Population shift is, however, not a systematic competition. The phenomenon observed could not be well interpreted due to various factors involved that may complexify the results. First, insufficiency of culture preparation. Inoculums of mixed cultures were directly subjected to certain conditions where a population shift was observed later. It remains unclear whether the result of population shift was due to the external factor or natural selection. Second, inappropriate form of biomass employed (aggregates). Third, inaccuracy/insufficient information regarding the kinetics of anammox bacteria so far.

Physical environmental factors have been found to have profound influence on niche partitioning of microorganisms. A recent review on niche differentiation among ammonia oxidizing archaea (AOA) and AOB has emphasized the importance of substrate concentration, cell structure, source of ammonia, pH, difference in its own activity, temperatures, zonation with water depth, oxygen deprivation and other factors that yet to be concluded (sulfide, phosphate, salinity, soil moisture, etc.) (Hatzenpichler, 2012). Another review covered the anammox bacteria and discussed some environmental parameters that have been suggested to be essential for its geographical distribution including nitrite concertation, salt concentration, temperature, sulfide and co-occurrence of organic compounds (e.g. formate, acetate, propionate, etc.) (Oshiki et al., 2015).

A recent study introduced a systematic competition using free-living planktonic cells of three anammox species demonstrated that nitrogen loading rate (NLR) could be one of the factors involved in niche differentiation between two

freshwater anammox species (*i.e.* "*Ca*. B. sinica" and "*Ca*. J. caeni") (Zhang et al., 2017a). Such information could be used to explain the difference in the abundance of each anammox species in different ecosystems (Sonthiphand et al., 2014). In fact, species "*Ca*. B. sinica" has been frequently detected in bioreactors operated at extremely high NLRs (26.0 – 76.7 kg-N m^{-3} day^{-3}) (Tang et al., 2011; Tsushima et al., 2007b) while "*Ca*. J. caeni" was enriched at much lower NLRs (0.3 – 0.6 kg-N m^{-3} day^{-3}) (Ali et al., 2015a).

Although several anammox species have been enriched so far (*i.e.* "*Ca*. B. anammoxidans", "*Ca*. B. fulgida", "*Ca*. B. sinica", "*Ca*. B. sapporoensis", "*Ca*. K. stuttgartiensis", "*Ca*. A. propionicus", "*Ca*. S. brodae", "*Ca*. S. profundal", "*Ca*. S. japonica", "*Ca*. Scalindua sp.", "*Ca*. J. caeni" and "*Ca*. J. asiatica") (Ali et al., 2015a; Egli et al., 2001; Fujii et al., 2002; Kartal et al., 2008, 2007b; Kindaichi et al., 2011; Lotti et al., 2014; Nakajima et al., 2008; Narita et al., 2017; Z.-X. Quan et al., 2008a; Tsushima et al., 2007b; van de Graaf et al., 1996; van de Vossenberg et al., 2008b), it is still far from possible to predict what anammox species could be enriched by manipulating physical environmental conditions (*i.e.* temperature, pH, salinity, etc.). Here we summarized the detailed enriching conditions for each species (**Table 3**). Enriched levels in aggregated granules or biofilms were mostly limited to less than 90%, except for marine anammox species ("*Ca*. S. brodae" and "*Ca*. S. profundal") since the saline condition has a higher selectivity. A recent study elucidated a metabolic network between anammox bacteria and Chlorobi-affiliated bacteria on protein degradation, extracellular peptides catabolization and nitrate recycling into nitrite in naturally aggregated granules (Lawson et al., 2017), while it remains unclear if such syntrophic relationship is essential for certain anammox species as growing criteria. For instance, *Syntrophothermus lipocalidus* and *Aminobacterium colombiense* has been successfully pure cultured by replacing an hydrogen-consuming syntrophic partner using an hydrogen-purging culture vessel (Adams et al., 2006). It is highly probable that some microorganisms could not be absent in order to secure the growth of certain anammox species. Secondly, most freshwater anammox species were enriched at temperature range from 27 °C to 37 °C while for marine anammox species, the temperature was 15 °C to 25 °C. This is a clear difference between freshwater and marine species, which should be correlated with the sea environment with mostly lower temperature compared with laboratory bioreactors. However, among the freshwater anammx species, temperature dependent specific anammox acitivity was not quite different with

maximum activity around 37 °C (Ali et al., 2015a; Oshiki et al., 2011). pH has been found to regulate the AOB and AOA abundance, diversity and activity in paddy soils (Li et al., 2015). However, it can be seen that all anammox species were enriched at pH of 6.8 – 8.5, therefore, may not be a key factor in niche differentiation. So far, anammox bacteria has been enriched from various source of biomass, including denitrifying sludge, activated sludge, nitrifying biomass, marine sediments or river sediments (Egli et al., 2001; Fujii et al., 2002; Kartal et al., 2008, 2007b; Kindaichi et al., 2011; Nakajima et al., 2008; Z.-X. Quan et al., 2008a; Tsushima et al., 2007b; van de Graaf et al., 1996; van de Vossenberg et al., 2008b). Recent studies have investigated the impact of inoculum and growth mode on microbial ecology of anammox bioreactor community, where they observed compared with inoculum and reactor configuration, composition of the feed and extant substrate concentration might play a more important role (Park et al., 2010a, 2010b). Similar phenomenon was also observed when enriching "*Ca*. B. fulgida" and "*Ca*. A. propionicus", where the authors used same inoculum but supplied different medium (containing 1 – 30 mM acetate and 0.8 – 15 mM propionate, respectively) (Kartal et al., 2008, 2007b). Therefore, source of inoculum may not be the determining factor. Thus, another parameter that should be considered is the medium composition including the water used for preparing the medium. Although most anammox studies used the medium composition same with the first enriching culture (**Table 3**) (van de Graaf et al., 1996), the amount (concentration) used was not always same, especially metal concentrations including iron (5.0, 6.3, 6.8 or 9.0 mg L^{-1}, respectively) (Ali et al., 2015b; Hsu et al., 2014; Strous et al., 1998; van de Graaf et al., 1996) and copper (0.25 mg L^{-1} or no addition) (Ali et al., 2015b; van de Graaf et al., 1996). Anammox bacteria is able to carry out nitrate dependent ferrous iron oxidation (M Oshiki et al., 2013) and additionally, ferrous iron has been found to have positive effect on anammox activity and specific growth rates (Liu and Ni, 2015; Qiao et al., 2013). Iron is deeply involved in anammox metabolism and vast majority of cellular iron in the form of cofactors within Fe-S proteins and multi-heme cytochromes participated in the oxidation of ammonium to dinitrogen gas (Ferousi et al., 2017; Kartal and Keltjens, 2016). A recent work analyzed the intracellular metal composition in "*Ca*. J. caeni", where they observed a slightly higher percentage of Fe compared with "*Ca*. B. sinica" (41% and 39%, respectively) (Ali et al., 2015a). On the other hand, copper was found to be another dominant metal component in anammox bacteria (39% and 42% from "*Ca*. J. caeni" and "*Ca*. B. sinica", respectively) and was involved in enzymes in reductive acetyl-CoA pathway for carbon fixation (Ali et al., 2015a; Kartal et al., 2011). It

remains unclear if there is a species-specific response to metal addition and whether this may become the selection factor. It is interesting that some anammox bacteria were enriched using ground water instead of demineralized water (Ali et al., 2015a; Kindaichi et al., 2011; Tsushima et al., 2007b). Indeed 16S rRNA genes of certain anammox species have been detected at groundwater environment (Sonthiphand et al., 2014). Composition of groundwater varies from regions and weather conditions, but mostly contains metal elements and nitrogen compounds due to biological contamination and rock dissolution, including ammonium, nitrite, nitrate, sulfate, chloride, potassium, sodium, calcium, magnesium, phosphate and bicarbonate (Dragon and Marciniak, 2010). These compounds are missing in the demineralized water. It requires and deserve further investigation on the difference between demineralized water and groundwater in the enrichment of anammox bacteria.

2.6 CONCLUSION

The wide distribution of anammox bacteria in the environment is currently well established. Their contribution to fixed nitrogen loss over denitrification is striking in many ecosystems. Eco-physiological niche has been indicated from universal detection at various environmental conditions. However, most studies were conducted using methods that target the abundance and/or expression of the molecular biomarkers. It is difficult to draw direct conclusion on the niche partitioning of anammox bacteria only by abundance and expression of genes due to their prolonged persistence. Culture-dependent competition study is still essential as a complementary tool to decipher their niche enigma. In this overview, we summarized the updated information of enrichment methods, growth kinetics of anammox bacteria that are essential for competition study and discussed some potential factors in niche differentiation. It is clear that we are in the very beginning stage in revealing the niche partitioning of anammox bacteria. The questions of why anammox bacteria has a species specific natural distribution and how they successfully compete with AOB, NOB or denitrifiers remain unclear. Nevertheless, it was suggested that anammox bacteria may possess much higher affinity with substrate and advantageously survive with AOB, NOB or denitrifiers. Interspecific differences in μ_{max} and *K*s may trigger their niche partitioning in terms of nutrients

availability. In addition, types of water and medium composition, especially metal concentrations are potential factors for niche partitioning. Definitely more highly enriched free living anammox cell cultures from various species are in great demand to explore the ecophysiology of this unique microorganism.

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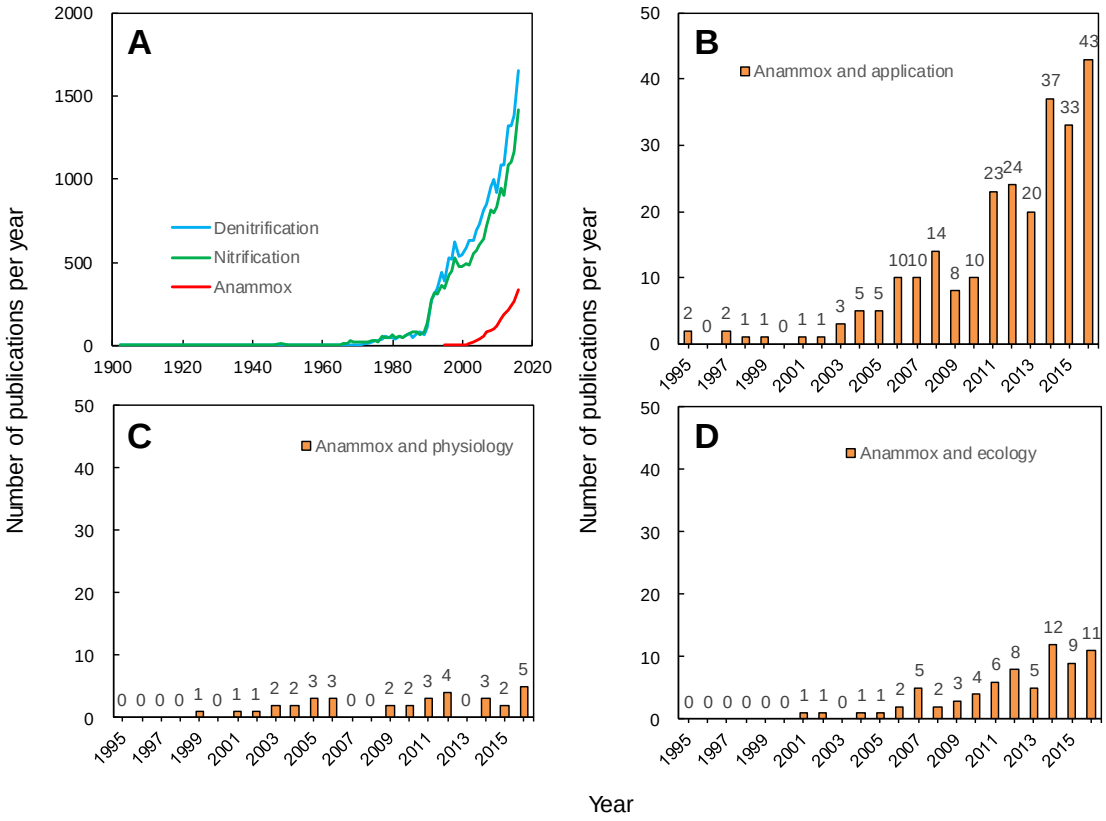

Figure 2.1 Research in the biological conversions of nitrogen including anammox from 1900 to 2016. (A) Articles that related to main biological process including nitrification, denitrification and anammox as the key words. (B), (C) and (D) Articles related to anammox and its subdiscipline. Articles with keywords "denitrification", "nitrification", "anammox", "anammox and application", "anammox and physiology", and "anammox and ecology" indexed by Web of Science were taken into account.

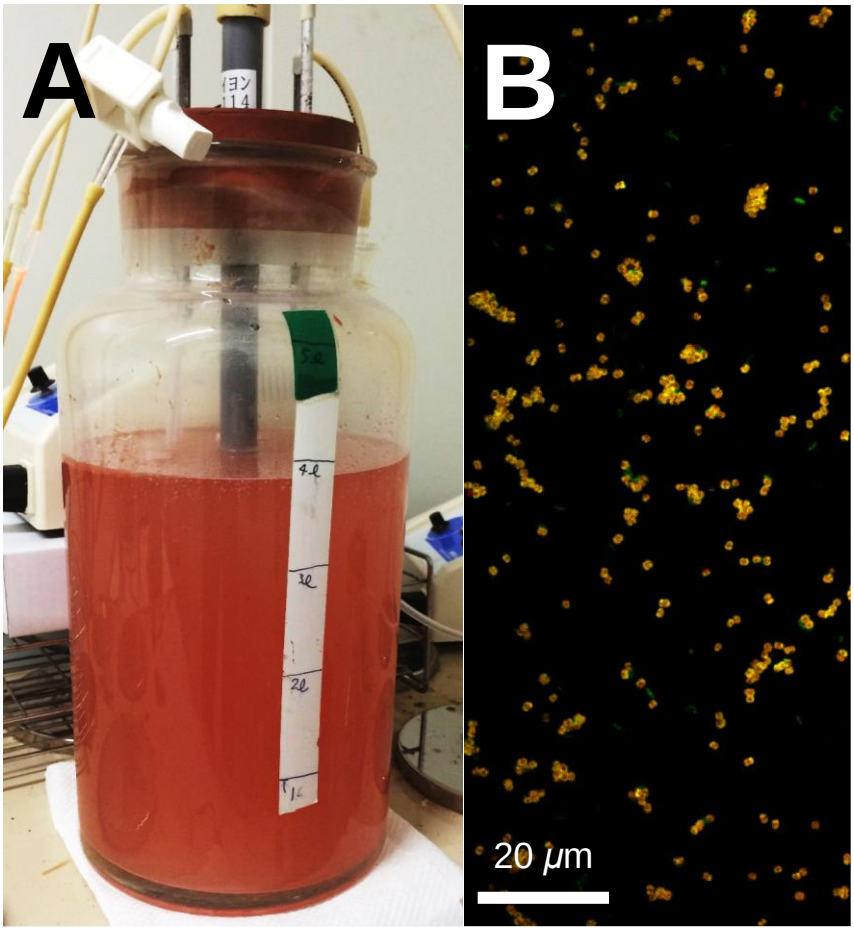

Figure 2.2 Enrichment of anammox bacteria as free-living cells in membrane bioreactor. (A) The anammox membrane bioreactor cultivating "*Ca.* Brocadia sinica". (B) Fluorescence in situ hybridization micrograph depicting highly enriched "*Ca.* Brocadia sinica" in yellow (combination of AMX820 probes counterstained with EUB 338 mix probes for most bacteria); scale bar = 20 *μ*m.

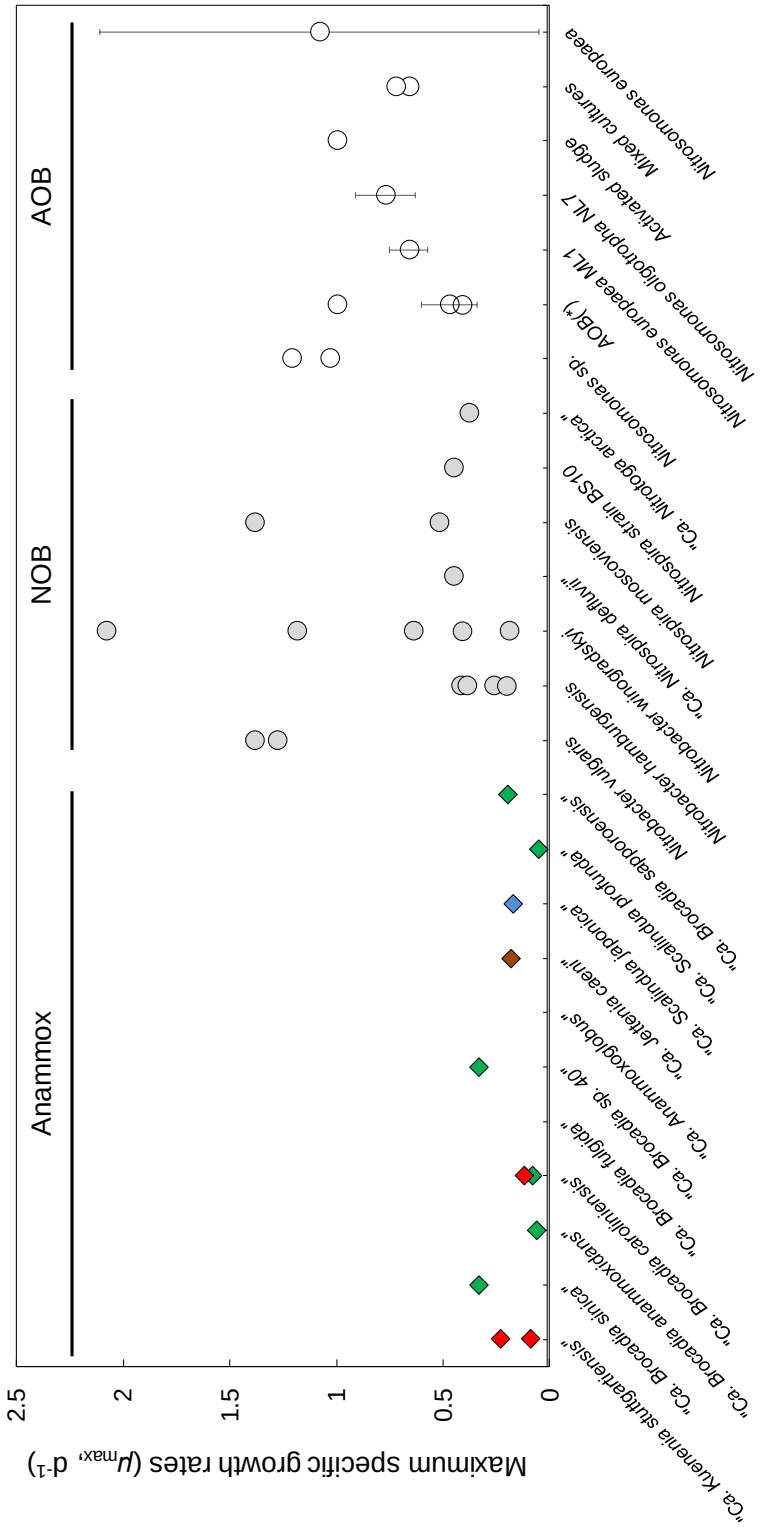

Figure 2.3 Maximum specific growth rates of anammox bacteria, NOB and AOB. For anammox bacteria, different genus was indicated in red ("*Ca.* Kuenenia"), green ("*Ca.* Brocadia"), blue ("*Ca.* Scalindua") and brown ("*Ca.* Jettenia"). *: AOB that was not identified as species.

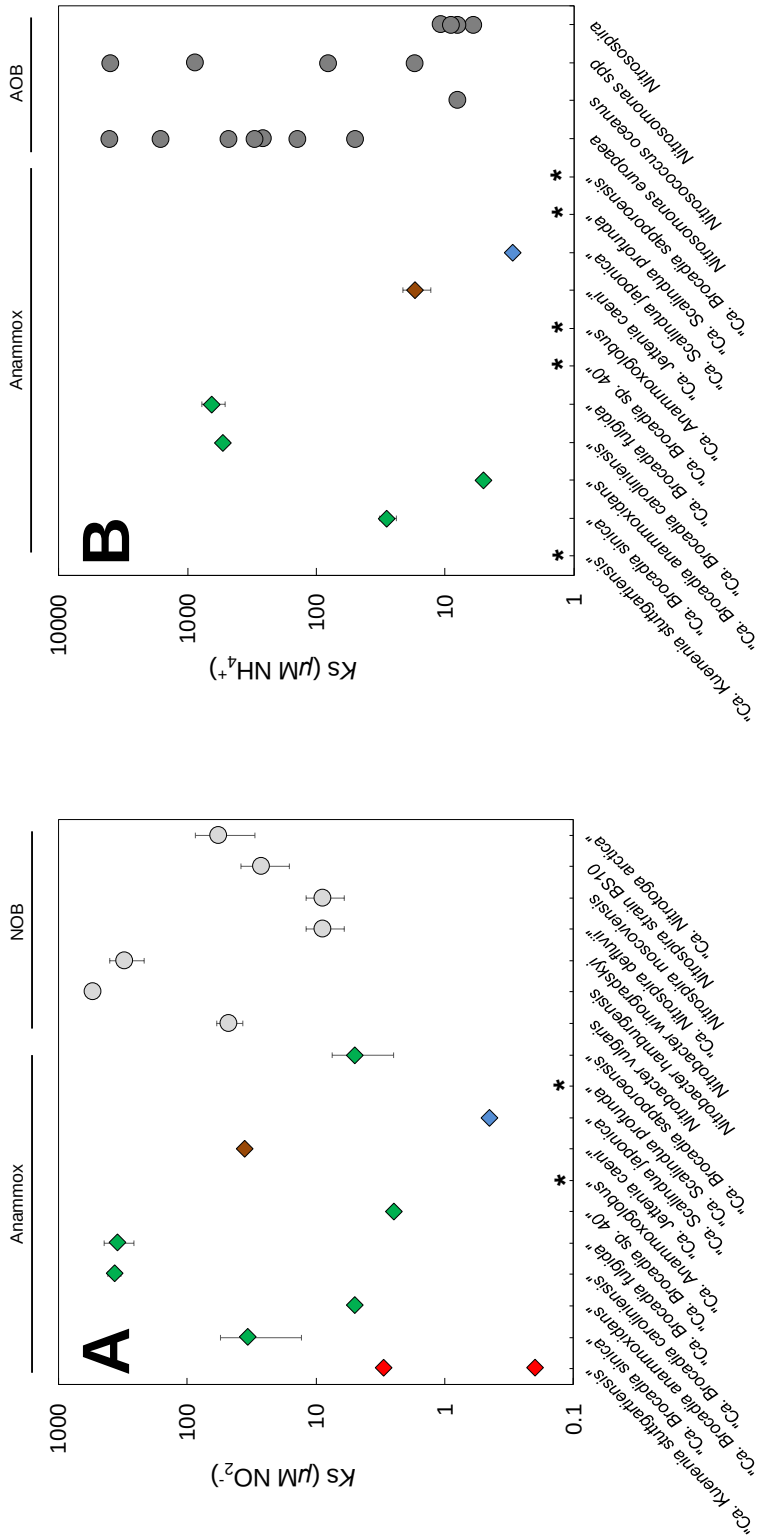

Figure 2.4 High affinity ammonium and nitrite oxidation by anammox bacteria compared with AOB or NOB. (A) *Ks* value for nitrite from anammox bacteria (diamond) and nitrite oxidizing bacteria (NOB) (circle). (B) *Ks* value for ammonium/ammonia from anammox bacteria (diamond) and ammonium/ammonia oxidizing bacteria (AOB) (circle). For anammox bacteria, different genus was indicated in red ("*Ca.* Kuenenia"), green ("*Ca.* Brocadia"),

blue ("*Ca.* Scalindua") and brown ("*Ca.* Jettenia"). *: information not available at the time of writing.

Table 2.1 Reactor types used for enriching anammox biomass.

Reactor type	Sequencing batch reactor (SBR)	Non-woven fabric assisted upflow column reactor	Immobilized gel reactor	Membrane bioreactor (MBR)
Form of biomass	Aggregated granules	Attached Biofilm	Dispersed biomass immobilized in artificial materials	Suspended planktonic cells
Advantages	1) Efficient biomass retention; 2) A homogeneous distribution of substrate, products and biomass aggregates over the reactor; 3) Reliable operation for more than one year (long term stability); 4) Stable conditions under substrate-limiting conditions.	1) Attached growth to reduce washout; 2) Fast start-up with supporting material; 3) Less energy input (no need for agitation or aeration to mix up).	1) Complete biomass retention; 2) Higher diffusion efficiency compared with granules and biofilm; 3) Different groups of microbes can be immobilized into one system.	1) Complete biomass retention; 2) No diffusion limitation; 3) Very high purity (>90%); 4) Possible to manipulate specific growth rate sensitively.
Disadvantages	1) Energy input is needed for floating granules; 2) Inert biomass at the inner part of granules; 3) Heterogeneity in substrate diffusion and specific activity; 4) Large amount of extracellular polymeric substance (EPS) disturbing the protein purification process;	1) Substrate diffusion limitation along with the biofilm; 2) Inert biomass at the inner part of granules; 3) Heterogeneity in substrate diffusion and specific activity; 4) Space needed for supporting materials in reactors; 5) Detachment from the	1) The strength of the immobilizing materials (easy broken); considering long-term operation; 2) Cost from the immobilizing materials; 3) Difficulty in measuring cellular composites including EPS due to	1) Less resistant to unfavorable conditions (e.g. oxygen contamination, organic loads); 2) Unsuitable for practical application; 3) Additional costs in membrane maintenance and aeration for anoxic condition, agitation; 4) Long time

	5) Mineral accumulation.	supporting materials during the operation.	the disturbance from immobilizing materials.	(6-12 months) needed for culture development.
Reference	(Ali et al., 2015c; Cirpus et al., 2006; Lin et al., 2013; Strous et al., 1998; Vlaeminck et al., 2010)	(Furukawa; et al., 2004; Kindaichi et al., 2007; Tsushima et al., 2007c)	(Ali et al., 2015c; Isaka et al., 2013; Magrí et al., 2012; Zhang et al., 2017b)	(Ali et al., 2015a; Lotti et al., 2015, 2014; Mamoru Oshiki et al., 2013; van der Star et al., 2008)

Table 2.2 Species specific enriching conditions.

Species	"Ca. B. anammoxidans"	"Ca. B. fulgida"	"Ca. B. sinica"	"Ca. B. sapporoensis"	"Ca. K. stuttgartiensis"	"Ca. A. propionicus"	"Ca. S. brodae"	"Ca. S. profundal"	"Ca. S. japonica"	"Ca. Scalindua sp."	"Ca. J. caeni"	"Ca. Jettenia asiatica"
Enrichment level (%)	74	80	70, 90	>90	90, >90	80, 65±5	~90	~90	85±4.5, >90	<67	72.8, >90	50
Reactor type	SBR*	SBR, MBR	UAB, MBR	MBR	Flask, SBR, MBR	SBR, batch mode reactor	SBR	SBR	UAB, MBR	UAB	UAB, MBR	UAB
Form of biomass	Granule	biofilm aggregates	Biofilm, suspended planktonic cells	suspended planktonic cells	Granule, suspended planktonic cells	Granule	Floc	Granule	Biofilm, suspended planktonic cells	Biofilm	Biofilm, suspended planktonic cells	Granule
Temperature	32-33	33	37, 30	30, 25, 37	30, 35, 38	33, 27-30	23	15-18	20	25	30, 37	30-35
pH	7.0-8.0	7.0-7.3	7.0-7.5, 7.6-7.8, 7.6-8.6	6.8-7.5,	7, 8.0 ± 0.1, 7.8-8.0, 7.1-7.5	7.0-7.3, 7-8	7-8	7-8		8.0	7.5-8.0	8.0-8.5
Inoculum	Denitrifying FBR sludge	Activated Sludge	Denitrification sludge, Anammox biofilm	Full-scale anammox reactor sludge, anammox UAB sludge	Nitrifying RBC, anammox SBR sludge, full-scale anammox reactor sludge	Activated sludge, landfill leachate	Marine sediment (Gullmar Fjord)	Marine sediment	Marine sediment, Anammox biofilm	Marine biofilm	Laboratory scale denitrification reactor sludge	River sediment
Location of enrichment	Delft (NLD)	Nijmegen (NLD)	Sapporo (JPN)	Delft (NLD), Sapporo (JPN)	Dübendorf, (CHE), Santiago de Compostela (ESP), Delft (NLD)	Nijmegen (NLD), Taichung (TWN)	Nijmegen (NLD)	Nijmegen (NLD)	Hiroshima (JPN)	Tsu (JPN)	Kumamoto (JPN), Sapporo (JPN)	Shanghai (CHN)
Medium composition **	A1, A2	A1	A1, A6	A5, B1	A3, A4	A1, B4	C1	C1	C2, C3	C4	B1, B2	B3
Type of water	Demineralized water	Not described	Ground water	Demineralized water, Ground water	Demineralized water	Demineralized water	Demineralized water	Demineralized water	Ground water	Deep sea water	Ground water	Not described
Influent substrate												
Ammonium (mM)	5	3-45	6.2-24.6, 16-30	60, 1-15	6, 26.8, 120	2.5-45, 13.1	1-45	1-45	0.58-6.3, 5-10	3.6-10.9	17.9, 2.5-16.8	15

Nitrite (mM)	5	3-45	3.7-22.8, 16-30	60, 1-15	6, 26.8, 120	2.5-45, 11.9	0.5-45	0.5-45	1.8-6.3, 5-10	3.6-14.1	17.9, 2.5-20	15
Nitrate (mM)	-	6	-	-	-	6, 0	0-1.5	0-1.5	-	-	- (3 mg-N/L in ground water)	-
Other	-	1-30 mM Acetate	-	-	-	0.8-15 mM propionate	-		-	Sulfide to adjust pH	-	-
SRT (d)	-	-	125, 30-60	12-15, 60	-	-	-	-	-	-	-	-
Reference	(Strous et al., 1998; van de Graaf et al., 1996)	(Kartal et al., 2008)	(Mamoru Oshiki et al., 2013; Oshiki et al., 2011; Tsushima et al., 2007b)	(Lotti et al., 2014; Narita et al., 2017)	(Dapena-Mora et al., 2004; Egli et al., 2001; van der Star et al., 2008)	(Hsu et al., 2014; Kartal et al., 2007a)	(van de Vossenberg et al., 2008a)	(van de Vossenberg et al., 2008a)	(Kindaichi et al., 2011; Mamoru Oshiki et al., 2013)	(Nakajima et al., 2008)	(Ali et al., 2015b; Fujii et al., 2002)	(Z.-X. Quan et al., 2008b)

*Abbreviations of reactor

SBR: sequencing batch reactor

MBR: membrane bioreactor

UAB: up-flow anaerobic biofilm

EGSB: expanded granular sludge bed

FBR: fluidized bed reactor

RBC: rotating biological contactor

**Medium composition

A group: variations of synthetic mineral medium proposed at first

A1. KHCO3, 500 mg L^{-1}; KH2PO4, 27.2 mg L^{-1}; MgSO4.7H2O, 300 mg L^{-1}; CaCl2.2H2O, 180 mg L^{-1}; trace element solution I (EDTA, 5 g L^{-1}; FeSO4, 5 g L^{-1}) 1 mL L^{-1}; trace elements solution II (EDTA, 15 g L^{-1}; ZnSO4.7H2O, 0.43 g L^{-1};CoCl2.6H2O, 0.24 g L^{-1}; MnCl2.4H2O, 0.99 g L^{-1}; CuSO4.5H2O, 0.25 g L^{-1}; NaMoO4.2H2O, 0.22 g L^{-1}; NiCl2.6H2O, 0.19 g L^{-1}; NaSeO4.10H2O, 0.21 g L^{-1}; H3BO4, 0.014 g L^{-1}), 1 mL L^{-1} (van de Graaf et al., 1996)

A2. $KHCO_3$, 1.25 g L^{-1}; NaH2PO4, 50 mg L^{-1}; CaCl2.2H2O, 300 mg L^{-1}; MgSO4.7H2O, 200 mg L^{-1}; FeSO4, 6.25 mg L^{-1}; ethylenediamine tetraacetic acid, 6.25 mg L^{-1}; trace elements solution I and II (same as A1), 1.25 mL L^{-1} (Strous et al., 1998)

A3. KHCO3, 250 mg L^{-1}; K2HPO4, 174.2 mg L^{-1}; MgCl2, 42.6 mg L^{-1}; CaCl2, 55.5 mg L^{-1}; trace element solution I (EDTA, 10 g L^{-1}; FeSO4, 5 g L^{-1}) 2 mL L^{-1}; trace elements solution II (EDTA, 15 g L^{-1}; ZnSO4.7H2O, 0.43 g L^{-1};CoCl2.6H2O, 0.24 g L^{-1}; MnCl2.4H2O, 0.99 g L^{-1}; CuSO4.5H2O, 0.25 g L^{-1}; NaMoO4.2H2O, 0.22 g L^{-1}; NiCl2.6H2O, 0.19 g L^{-1}; NaSeO4.10H2O, 0.21 g L^{-1}; H3BO4, 0.014 g L^{-1}), 1 mL L^{-1} (Egli et al., 2001)

A4. KHCO3, 1.5 g L^{-1}; KH2PO4, 24.5 mg L^{-1}; MgSO4.7H2O, 49.4 mg L^{-1}; CaCl2, 147 mg L^{-1}; EDTA, 14.6 mg L^{-1}; yeast extract, 1.0 mg L^{-1}; trace elements solution II (same as A1), 1 mL L^{-1} (van der Star et al., 2008)

A5. KHCO3, 0-1.5 g L^{-1}; KH2PO4, 24.5-2041 mg L^{-1}; MgSO4.7H2O, 49.4 mg L^{-1}; CaCl2, 73.5-147 mg L^{-1}; EDTA, 14.6 mg L^{-1}; vitamin solution, 0-10 mL L-1; trace elements solution I and II (same as A1), 1 mL L^{-1} (Lotti et al., 2014)

A6. KHCO3, 24.4 mg L^{-1}; KH2PO4, 24.4 mg L^{-1}; MgSO4.7H2O, 60 mg L^{-1}; CaCl2, 51 mg L^{-1}; yeast extract, 1.0 mg L^{-1}; trace elements solution I and II (same as A1), 0.5 mL (Mamoru Oshiki et al., 2013)

B group: Other medium for freshwater anammox bacteria

B1. NaHCO3, 84 mg L^{-1}; KH2PO4, 54 mg L^{-1}; MgSO4.7H2O, 1 mg L^{-1}; CaCl2.2H2O, 1.4 mg L^{-1}; FeSO4.7H2O, 9 mg L^{-1}; EDTA, 5 mg L^{-1}; NaCl, 1 mg L^{-1}; KCl, 1.4 mg L^{-1}

B2. KHCO3, 125 mg L^{-1}; KH2PO4, 54 mg L^{-1}; MgSO4.7H2O, 9 mg L^{-1}; CaCl2.2H2O, 1.4 mg L^{-1}; FeSO4.7H2O, 9 mg L^{-1}; EDTA, 5 mg L^{-1}; NaCl, 1 mg L^{-1}; KCl, 1.4 mg L^{-1}

B3. KH2PO4, 10 mg L^{-1}; trace elements (not described in detail) (Z.-X. Quan et al., 2008b)

B4. KHCO3, 1.36 mg L^{-1}; NaH2PO4, 65.2 mg L^{-1}; MgSO4.7H2O, 217 mg L^{-1}; CaCl2.2H2O, 326 mg L^{-1}; FeSO4.7H2O, 6.79 mg L^{-1}; EDTA, 6.79 mg L^{-1}; HCl, 1 mM; trace elements solution II (same as A1), 1 mL (Hsu et al., 2014)

C group: media for marine anammox bacteria

C1. Red sea salt, 33 gL^{-1}; FeSO4, 0-9 μM; KH2PO4, 0-0.2 mM; NaHCO3 (van de Vossenberg et al., 2008a)

C2. SEALIFE (artificial sea salt), 35 gL^{-1}; KHCO3, 500 mg L^{-1}; KH2PO4, 27 mg L^{-1}; MgSO4.7H2O, 300 mg L^{-1}; CaCl2.2H2O, 180 mg L^{-1}; trace elements solution I and II (same as A1), 1 mL (Kindaichi et al., 2011)

C3 SEALIFE (artificial sea salt), 35 gL^{-1}; KHCO3, 24.4 mg L^{-1}; KH2PO4, 24.4 mg L^{-1}; MgSO4.7H2O, 60 mg L^{-1}; CaCl2, 51 mg L^{-1}; yeast extract, 1.0 mg L^{-1}; trace elements solution I and II (same as A1), 0.5 mL (Mamoru Oshiki et al., 2013)

C4. KHCO3, 125 mg L^{-1}; KH2PO4, 54 mg L^{-1}; FeSO4.7H2O, 9 mg L^{-1}; EDTA, 5 mg L^{-1} (Nakajima et al., 2008)

3 RAPID CULTIVATION OF FREE-LIVING ANAMMOX CELLS

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3.1 ABSTRACT

It is still not successful to cultivate anaerobic ammonium oxidizing (anammox) bacteria, a key player in global nitrogen cycle, as pure culture. Planktonic cell culture with high purity is, therefore, essential for physiological and biochemical studies of anammox bacteria. However, development of such planktonic cell cultures requires an enormous amount of time and effort. Here we developed a novel method for cultivating free-living planktonic anammox cells rapidly. First, anammox granules were dispersed and immobilized in 6% polyvinyl alcohol – 4% sodium alginate (PVA-SA) gel beads for pre-incubation. Anammox bacteria grew rapidly as loosely connected micro-colonies in the gel beads. After 18 days of pre-cultivation, mature gel beads were harvested, physically dispersed by vortex and inoculated into a membrane bioreactor (MBR). The MBR was then continuously operated at a low nitrogen loading rate (< 0.9 kg-TN m^{-3} d^{-1}). After 17 days of operation, active free-living planktonic anammox cells with purity > 95% was successfully developed in the MBR. Total culture time (gel beads and MBR) to accomplish free-living planktonic anammox cells was only 35 days, which was significantly shorter than the previous reports. This new cultivation technique could greatly facilitate various microbial, physiological and biochemical studies of anammox.

3.2 INTRODUCTION

Highly enriched and free-living planktonic cells are essential for biochemical and physiological studies of anammox bacterium (Strous et al., 1999). Our understanding of biochemistry and physiology of anammox bacteria is still limited. (van der Star et al., 2008) was the first to successfully enrich an anammox bacterial species "*Ca.* Kuenenia stuttgartiensis" as planktonic cells using a membrane-assisted bioreactor (MBR). The MBR can cultivate anammox bacteria with complete biomass retention but without selection pressure such as settling ability and thereby free-living planktonic cells with high purity can be obtained in the MBR. The MBR has been considered as a useful and necessary tool for physiological and kinetic studies and used for various anammox bacterial species; *i.e.*, "*Ca.* Jettenia" (Ali et al., 2015a), "*Ca.* Brocadia" (Lotti et al., 2014; Oshiki et al., 2013) and "*Ca.* Scalindua" (Awata et al., 2013; Oshiki et al., 2011). In those studies, anammox granular biomass was directly inoculated into the

MBR and cultured in the standard anammox medium (van de Graaf et al., 1996) with low levels of divalent ions (i.e., calcium and/or magnesium) and addition of small amounts of yeast extract. However, this MBR cultivation still takes an enormous amount of time to achieve free-living cells; usually more than 100 – 200 days (Ali et al., 2015a; Lotti et al., 2014; Oshiki et al., 2013; van der Star et al., 2008). This time-consuming cultivation process is apparently a bottle neck of physiological, biochemical and kinetic studies of anammox bacteria.

Cell immobilization technology has been successfully applied to anammox bacteria (Ali et al., 2015b, 2014, Isaka et al., 2013, 2008, 2007; Zhang et al., 2017). Anammox bacteria could grow at almost the maximum rate in polyvinyl alcohol (PVA, 6% w/v)-sodium alginate (SA, 2% w/v) gel beads due to high substrate supply, no hydraulic shear stress and thereby less EPS production, and full retention of newly born cells (Zhang et al., 2017). Therefore, only a considerably small amount of anammox biomass was required for rapid successful start-up of anammox process if the biomass was immobilized in the PVA-SA gel (Ali et al., 2015b). However, the durability of the PVA-SA gel beads for long-term operation was a problem, because the gel beads tended to be broken due to insufficient hardness after long-term (*i.e.*, 1- 2 months) operation at 37 °C. We also found that anammox bacteria cells grown in the gel beads did not form rigid aggregates and were easily dispersible. This suggests that the biomass grown in the gel beads could be used as an inoculum of MBR to obtain free-living planktonic cells. The combination of gel immobilization technique as pre-culture and MBR cultivation has never been tested for rapid cultivation of planktonic cell cultures.

Therefore, the objective of current work is to develop a rapid and reliable method for achievement of free-living planktonic anammox bacterial cell culture with high purity (> 95%). Total culture time for this purpose in our method was only 35 days, which was significantly shorter than the previously reported values. In this technique, a pre-cultivation of physically-dispersed granular biomass using PVA-SA gel immobilization was performed before inoculating into MBR. Since we think the pre-culture in PVA-SA gel beads was a key to this new method, growth of anammox cells in the PVA-SA gel beads were investigated in details.

3.3 MATERIALS AND METHODS

3.3.1 Gel immobilization

"*Ca.* B. sinica" dominated anammox biomass was immobilized in PVA-SA gel beads as described previously (Ali et al., 2014). In brief, granular biomass harvested from an up-flow column reactor (Tsushima et al., 2007) was physically dispersed and mixed with the same volume of immobilization solution containing PVA (6%, w/v) and SA (2%, w/v). The initial biomass content in the gel bead was 1-3×10^8 copy number per gel bead. The mixed biomass solution was dropped into $CaCl_2$ (4%, w/v) solution by using a peristaltic pump (Eyela Roller Pump RP, Tokyo, Japan) equipped with a Phar Med BPT tube (ID 3.1 mm) to make spherical beads with diameter of 1 - 2 mm. The gel beads were cured in the $CaCl_2$ solution overnight to enhance the physical strength. After rinse with sterilized water at least three times, the gel beads were inoculated into an up-flow column reactor.

3.3.2 Operation of up-flow column reactor packed with PVA-SA gel beads

An up-flow column reactor (255 mL, Fujirika, Japan) was packed with immobilized gel beads and operated for 18 days at 37 °C and pH = 7.5 ± 0.5. The packing ratio of the gel beads was 70% (v/v). The column reactor was continuously fed with the anoxic inorganic synthetic medium (van de Graaf et al., 1996) at a hydraulic retention time (HRT) of 2.13 h. The concentrations of ammonium and nitrite in the feed were increased from 2 to 12 mM, corresponding to nitrogen loading rates of 1.3 to 7.6 kg-N m^{-3} d^{-1}, to avoid substrate limitation. The feeding medium was purged with N_2 gas to remove dissolved oxygen. A N_2 gas bag was connected to the medium tank to prevent oxygen contamination (Tsushima et al., 2007). Throughout the operation, gel beads samples were collected at the bottom of the reactor every week, and subjected to microbial analysis. In addition, influent and effluent water samples were also collected for chemical analyses on daily basis.

3.3.3 Operation of MBR

After 18 days of operation, highly active gel beads were harvested from the bottom of the column reactor and transferred to two 225 ml graduated conical tubes (Becton Dickinson, NJ, USA) containing 100 mL gel beads, respectively. Two tubes

were briefly vortexed for 1 min at maximum speed to disperse the gel beads, and then the dispersed gel beads were filtrated with a test sieve (710 μm, SANPO, Tokyo) to remove the debris of gel bead. The filtrated biomass suspension was inoculated into a MBR. The MBR consists of a wide mouth reagent bottle (2-liter, Sansyo, Japan), which was equipped with a hollow-fiber membrane unit composed of 300 polyethylene tubes (pore size, 0.1 μm; tube diameter, 1 mm; length, 70 mm, Mitsubishi Rayon, Japan). A peristaltic pump (MP-1000, EYELA, Tokyo, Japan), actuated by a water level sensor (HL–S1A, ASONE, Japan), was directly connected to the membrane unit to keep the volume of culture medium constant. The culture medium in the MBR was vigorously agitated with a magnetic stirrer working at 200 rpm. A mixed Ar and CO_2 (95:5) gas was continuously supplied to the culture medium at a flowrate of 10 mL min^{-1} to avoid oxygen contamination. pH was not controlled but ranged between 7.0 - 8.0. The same anoxic inorganic medium (van de Graaf et al., 1996) was continuously supplied at 2 mL min^{-1}. Sludge retention time (SRT) was not controlled. Nitrite concentration in the MBR was regularly monitored by colorimetric method according to standard methods (APHA, 1998) and was kept below detection limit to avoid nitrite inhibition. The membrane was washed routinely for every week.

3.3.4 Microbial analysis

Growth of "*Ca*. B. sinica" in the up-flow column reactor and MBR was monitored by quantitative PCR-based 16S rRNA gene copy number analysis. Gel beads and culture suspension in the MBR were collected, and genomic DNAs were extracted using Fast DNA spin kit for soil (MP Biomedical, Tokyo, Japan). The extracted DNAs were subjected to the PCR-based 16S rRNA gene copy number analysis, which was conducted with "*Ca*. B. sinica" specific PCR primers and TaqMan probe (**Table S1**) and Premix Ex Taq (Probe qPCR) (Takara Bio Inc., Japan) as described previously. More detailed qPCR procedure can be found elsewhere (Zhang et al., 2017). PCR amplification and fluorescence detection were conducted by ABI prism 7500 sequence detection system (Applied Biosystems, California, USA). "*Ca*. B. sinica" genome contains a single *rrn* operon (Oshiki et al., 2015).

FISH analysis was performed to determine the local cell density of "*Ca*. B. sinica" micro-clusters in the immobilized gel bead and naturally aggregated granules. The purity of the enriched MBR culture was also examined based on FISH. Briefly, the biomass was fixed in 4% paraformaldehyde (PFA) for up to 23 hr at 4 °C. The PFA-

fixed biomass was embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and then vertical thin sections with 20 μm of thickness were obtained using a cryostat (Reichert-Jung Cryocut 1800, Leica, Bensheim, Germany) (Okabe et al., 1999), which were mounted on Teflon-coated glass slides (Slide-glass, Matsunami Glass Ind., Ltd, Japan) and were subjected to *in situ* hybridization. For FISH analysis, fluoresceine isothiocyanate (FITC)-labeled AMX820 probe and tetramethylrhodamine-5-isothiocyanate (TRITC)-labeled EUB338 mixed probe (**Table S2**) were used for detection of "*Ca.* B. sinica" and most bacteria. Specimens were examined using a Fluoview FV 300 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with multi Ar laser (458 nm, 488nm, 514nm), HeNe laser (543nm) and HeNe laser (633nm). Fluorescence images were analyzed by the image-processing software Image-Pro Premier 9.1 (Media Cybernetics, USA).

To determine the local cell density of micro-clusters in the immobilized gel beads and naturally aggregated granules, number of fluoresced cells within certain regions (500 × 500 pixels) was counted as indicated as local cell density. In detail, at least three regions were selected for each micro-cluster and or each time point sample, minimum ten positions were selected and at each microscopic field, were selected for cell counting. Error bars shows standard deviation of local cell density.

3.3.5 Particle size distribution

Particle size distributions of biomass at different time points were determined using a laser diffraction particle size analyzer SALD-2000J (Shimadzu, Kyoto, Japan). Suspension from dispersed granules, biomass suspension in MBR were collected into 15 mL centrifuge tubes (Thermo Fisher Scientific, USA) and subjected to size determination. At three times were measured for each sample.

3.3.6 Scanning electron microscope of immobilized gel beads and naturally aggregated granules

Aggregated granules were prepared following the previous report (Van Neerven et al., 1990). In brief, freshly prepared PVA-SA gel beads and granules were washed three times for 10 min in the 0.025 M Na-cacodylate buffer (pH 6.8). A first fixation was performed in the same buffer containing 2% (w/v) glutaraldehyde for 3 h.

After washing three times for 10 min in the Na-cacodylate buffer, samples were placed for a second fixation for 1.5 h in this buffer containing 1.0% OsO_4. The beads were washed again in the buffer three times for 10 min and stored in the same buffer overnight prior to dehydration in a series of gradually increasing ethanol concentrations: 50, 60, 70, 80, 90 and two times 99.5%. Each step was allowed to equilibrate for 10 min; for cutting the beads, they were transferred to a cold metal block that was kept cool in a liquid N2 bath in such a way that the top of the block was in the vapor phase. The beads were split in halves with a precooled razor blade and a hammer. Subsequently, the split beads were quickly transferred again to 99.5% ethanol and subjected to critical point-drying and coated with Au. The samples were observed with SEM (JEOL JSM-6301F, Japan).

Day 15 samples were prepared following this second method because they became fragile and very weak for the chemical fixation used in the first method (Van Neerven et al., 1990). In brief, the beads were collected and briefly washed with demineralized water. Excess water was carefully removed by blotting lightly with filter paper. Subsequently, the gel beads were quickly plunged into liquid N_2, split and freeze-dried in a blazer apparatus equipped with the CRYO - SEM at -90 °C at 10^{-7} atm for 0.5 h. These lyophilized samples were directly coated with Au. Observation was directly executed using a CRYO - SEM (JEOL JSM-6701F, Japan).

3.3.7 Chemical analyses

The concentration of ammonium, nitrite and nitrate were measured with an ion-exchange chromatography (IC-2010, TOSOH, Tokyo, Japan) equipped with an TSKgel IC-Anion HS and TSKgel IC-Cation columns (TOSOH) after filtration with 0.2-μm-pore size membranes (Advantec Co., Ltd., Tokyo, Japan) (Tsushima et al., 2007).

3.4 RESULTS

3.4.1 Overall experiment procedure

Fig. 1 illustrated the change in the form of biomass in this newly proposed method. In detail, original biomass was collected from a granular up-flow column

reactor **(A)** and dispersed by vortexing in a 50mL centrifugation tube using a vortex mixer (Vortex-Genie 2 Mixer, Scientific Industries, Inc., USA). **(B)**. After standing still for *c.a.* 1 min, the biomass suspension (indicated using an arrow) was removed and immobilized using PVA-SA solution **(C)**. The initial biomass concentration was $1 – 3 \times 10^8$ copy numbers per gel bead. After 18 days of operation, the fully matured gel beads were collected and dispersed by vortexing for 30s (Vortex-Genie 2 Mixer, Scientific Industries, Inc., USA). **(D)**. The debris of gel beads was removed by passing through a sieve of 710 μm opening (SANPO, Tokyo) and sieved cell suspension was directly inoculated into a MBR **(E)**.

The MBR was operated at nitrogen loading rates (NLR) of 0.4 to 0.8 kg-N m^{-3} day^{-1}, and achieved >85% of nitrogen removal efficiencies right after start-up (**Fig. 3**). During the operation of the MBR, remaining debris of the dispersed gel beads was further eliminated by removing the biomass attached onto the membrane modules every day. Finally, the free-living planktonic biomass was remained in the MBR after 18 days of operation. FISH analysis revealed that >95% of total bacteria were "*Ca*. Brocadia sinica" cells in the MBR biomass (**Fig. 3**). Growth of "*Ca*. B. sinica" was also confirmed by the quantitative PCR analysis, which showed that an exponential growth (3.9×10^9 to 1.2×10^{10} copy numbers per mL) was observed as the nitrogen loading rate was increased **(Fig. 3)**. Phylogenetic analysis confirmed the dominance of "*Ca*. B. sinica" along with the experiment (data not shown).

Particle-size distributions of the dispersed granules (the inoculum for gel immobilization), biomass in the MBR on 0 day, and after 17 day of operation were determined and compared, respectively (**Fig. 2**). The average particle size of the dispersed granules was 184.7 μm, decreased to 69.0 μm after cultivation in the gel beads for 18 days and further decreased to 1.1 μm after cultivation in the MBR for 17 days, indicating that free-living planktonic anammox bacterial culture was achieved.

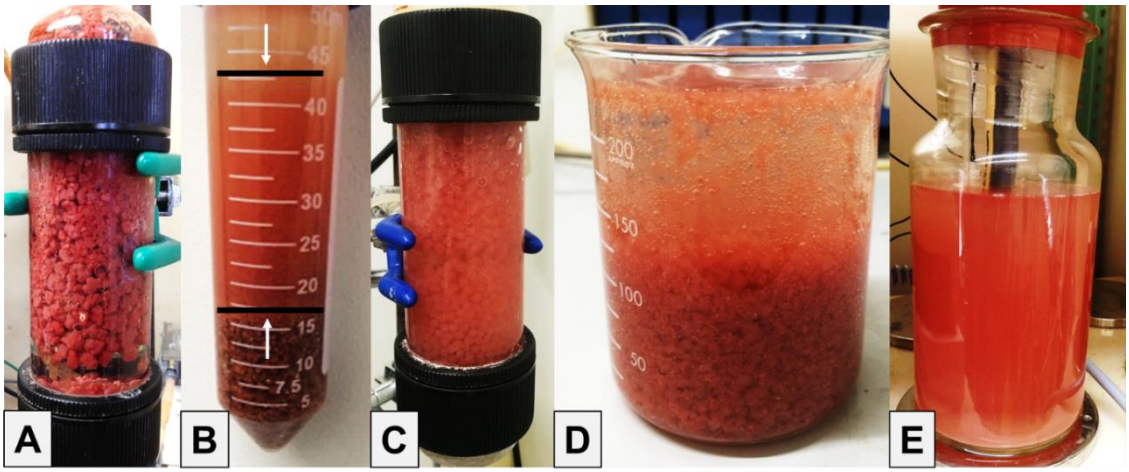

Figure 3.1: Change in the form of biomass. (A) An original up-flow granular column reactor: granules were collected from this reactor and dispersed. (B) Dispersed granules: only the upper part (suspended biomass, indicated using arrow) was used for PVA-SA gel immobilization. (C) A gel-immobilized column reactor: anammox biomass was immobilized in PVA-SA gel beads and cultivated in a column reactor for 18 days. (D) Gel beads after dispersion: the cultivated mature gel beads were collected from the column reactor. (E) Start-up of MBR: after physical dispersion of matured gel beads, the debris of gel matrix was removed by passing through a sieve of 710 µm opening (SANPO, Tokyo). The sieved biomass suspension was inoculated to MBR.

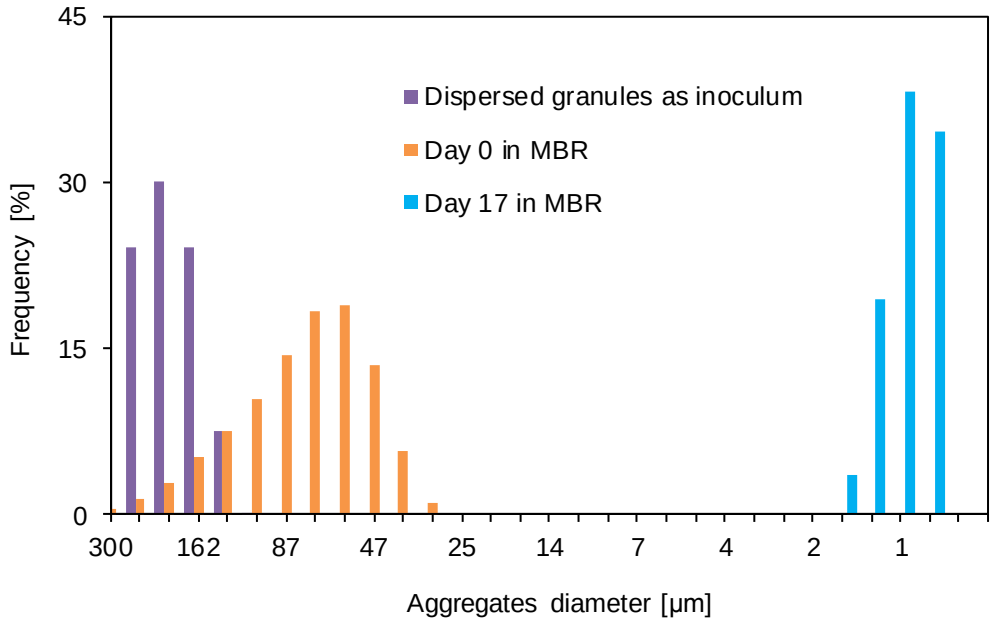

Figure 3.2: Size distribution of biomass particles indicated as frequency ([%], number of particles of total number of particles measured). The bars showed the particle distribution of dispersed granules (purple), day 0 (orange) and day 17 in MBR (blue). (For colored figure, the reader is referred to the web version of this article)

3.4.2 Growth pattern of "*Ca*. B. sinca" cells in gel beads

A key step of the rapid cultivation of free-living planktonic cells was cultivation in gel beads. The growth pattern of immobilized "*Ca*. B. sinica" cells in gel beads was, therefore, investigated in detail to understand the mechanism of easily dispersible biomass formation. "*Ca*. B. sinica" cells growing in the gel beads were visualized by FISH and compared with ones in naturally aggregated granules. (**Fig. 4A** and **4B**). "*Ca*. B. sinica" cells proliferated as micro-colonies in the gel beads. The micro-colonies became denser as they grew from day 10 to day 18, but were clearly looser than the naturally aggregated granules (**Fig. 4A** and **4B**). The local cell densities of micro-colonies in the gel beads increased from 334.2 ± 65.7 cells per 500 ×500 pixels at day 10 to 527.6 ± 135.9 at day18, accordingly the16S rRNA gene copy numbers increase from 2.1×10^9 at day 10 to 2.9×10^9 copy numbers per gel bead at 18 day. However, these local cell densities in the gel beads were significantly lower than that of the granules ($p < 0.01$, student *t*-test) (**Fig. 4**). The local cell density could not be measured for the gel beads on day 0 because it was too low.

In addition, the morphology of the micro-colony of "*Ca*. B. sinica" cells grown in the gel bead was analyzed using SEM and compared with that of naturally aggregated granules. In the 15-day-old gel bead, "*Ca*. B. sinica" cells were loosely aggregated with very less extracellular polymeric substance (EPS) - like matrix, whereas cells were densely packed and embedded in the EPS matrix (**Fig. 5D, 5E** and **5F**).

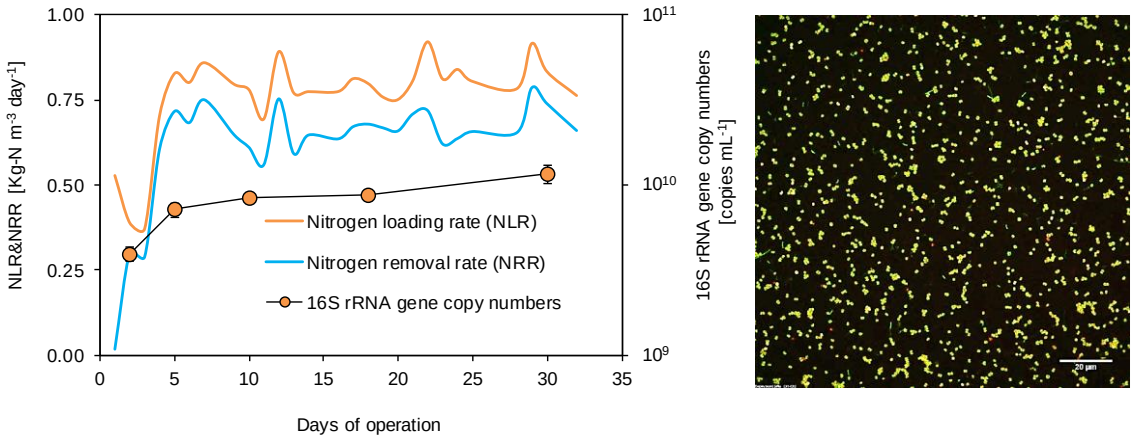

Figure 3.3: **Performance of the MBR after inoculation and planktonic free-living biomass after 17 days of cultivation.** *In situ* hybridization was performed with tetramethylrhodamine-5-isothiocyanate (TRITC) labeled AMX820 probe (red) for "*Ca*. B. sinica" and fluoresceine isothiocyanate (FITC) labeled EUB probes (green) for most of the bacteria. Bar indicates 20 μm.

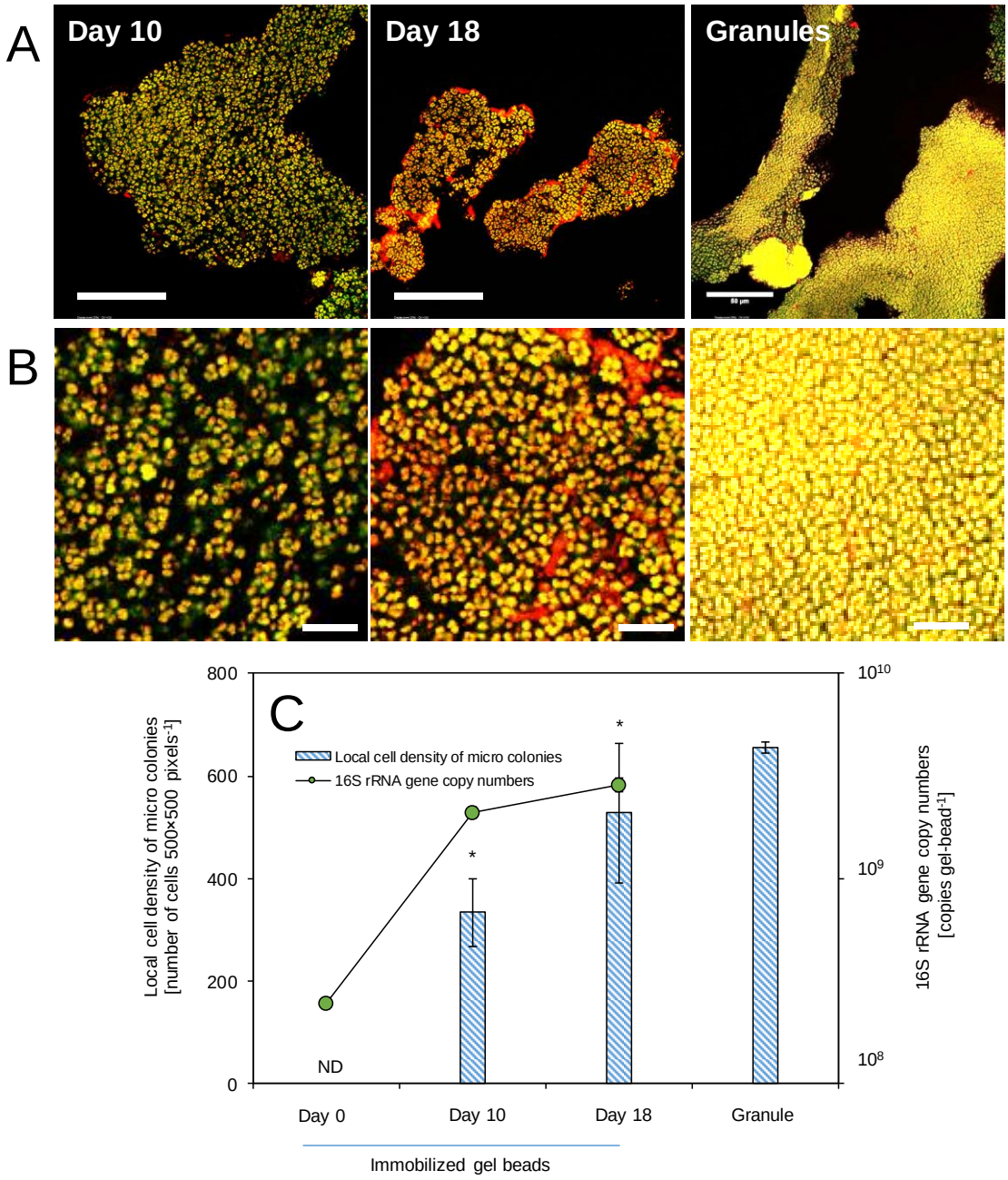

Figure 3.4: **Growth pattern of anammox bacteria in PVA-SA gel beads and granules.** "*Ca*. B. sinica" (yellow) grew as micro colonies inside the gel beads and compared with naturally aggregated granules (panel A). Local cell density of micro colonies increased with time in gel beads and compared with granules (panel B and C). Panel B is a close-up view of panel A. Panel C is the quantitative result of local cell density. White bars indicate 50 μm (panel A) and 10 μm (panel B), respectively. ND: not determined due to a low fluorescence intensity. *: $p<0.01$ (student *t* test). 16S rRNA gene copy numbers of "*Ca*. B. sinica" immobilized in gel beads at each time point were also measured. (For colored figure, the reader is referred to the web version of this article)

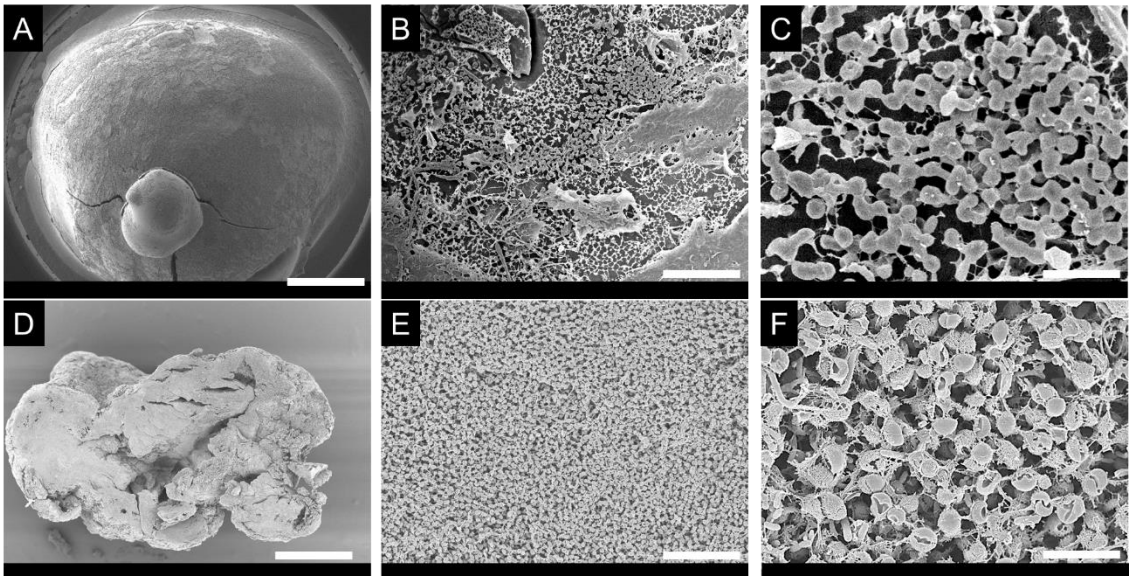

Figure 3.5: **SEM images of anammox cells grown in PVA-SA gel beads at day 0&15 and a naturally aggregated granule.** (**A**) Overview of the surface part of gel bead at day 15 (bar 1 mm). (**B**) A close-up view of the cell cluster on day 15 gel bead (bar 20 μm). (**C**) Enlarged anammox cell at day 15 (bar 4 μm). (**D**) Section view of the naturally aggregated granule (bar 0.6 mm). (**E**) A close-up view of the cell cluster in granule (bar 16 μm). (**F**) Enlarged anammox cell cluster in granule (bar 3 μm).

3.5 DISCUSSION

In general, anammox bacteria have strong self-aggregation ability, which is one of factors hampering advancement of physiological and biochemical studies of anammox bacteria. In this study, highly active and enriched anammox planktonic cell culture (> 95% and an average particle size of 1.1 μm) was successfully obtained within only one month period, which was much shorter than the previous studies where mostly at least 6 months were required (Ali et al., 2015a; Lotti et al., 2014; Oshiki et al., 2013; van der Star et al., 2008). In addition to the rapid cultivation, this proposed method required a relatively small amount of initial biomass that was immobilized in the gel beads (1 - 3×10^8 16S rRNA gene copy numbers per gel bead). The key step of this successful cultivation and its novelty compared with our previous work (Oshiki et al., 2013) was obviously a pre-culture in PVA-SA gel beads prior to MBR cultivation, which generates easily dispersible micro-colonies. Super high growth rates and activity of anammox cells have been observed when immobilized in PVA-SA gel beads (Ali et al., 2015b; Zhang et al., 2017). However, the growth pattern of anammox bacterial cells

in the gel beads was rarely investigated. A key question is why anammox bacteria immobilized in the gel beads grow as loosely aggregated (flocculated) micro-colonies. FISH and SEM results revealed that most immobilized anammox cells were in the form of micro-colonies inside the gel beads (Fig. 4A and 4B) and therefore, free from direct contact of water shear force, which is a strong stimulator for aggregation (Morgenroth et al., 1997). SEM analyses of denitrifying and nitrifying bacteria immobilized in gel beads have also showed micro-colonies with void spaces at the inner part of gel beads (Benyahia and Polomarkaki, 2005; Van Neerven et al., 1990). FISH results of the 10-day-old gel bead revealed that "Ca. B. sinica" cells were still present without squeezing each other in the gel beads, resulting in the significantly lower local cell density than naturally aggregated granules (Fig. 4C). In addition, less EPS-like matrix was observed in micro-colonies in the gel bead compared than those in the naturally aggregated granule (Fig. 5). These evidences might reflect the easily dispersible feature of micro-colonies in the immobilized gel beads. The super high growth rate of anammox bacteria was observed in the same immobilized gel beads (Zhang et al., 2017), which was probably attributable to less EPS production in loosely flocculated bacterial cells due to the absence of hydraulic shear stress. The hardness of PVA (6% w/v)-SA (2% w/v) gel beads seems to be an important factor controlling the growth pattern of anammox bacteria cells. The initial PVA-SA gel beads were strong sufficiently, but gradually became softer like grapes or fish eggs during 18 days of incubation. This would allow anammox bacteria cells to grow freely inside the gel beads. Rapid growth of loosely aggregated cells could be restricted due to substrate limitation inside the gel beads if the gel beads were made too harder. On the other hand, gel beads would be easily broken during pre-incubation if the gel beads were made too soft. Therefore, the optimum composition of PVA-SA or other materials should be determined in the future. In addition, quantification of EPS in immobilized gel beads is still necessary for verification.

Besides In addition to the property of easily dispersible micro-colonies, high activity of the immobilized cells is also essential for rapid achievement of planktonic cell cultures in MBR. It took quite a lot of time to resume the sufficient anammox activity when aggregated biomass was directly inoculated into MBR (Trigo et al., 2006). This is mainly because active cells occupied only at the outermost surface of granules (Ali et al., 2015b; Ni et al., 2009; Ni et al., 2010; Okabe et al., 2011). In the case of gel beads, most of the cells in the gel beads were equally active due to low diffusion

limitation (Fig. S23) (Ali et al., 2015b), which further promots the rapid growth of active free-living planctonic anammox bacteria cells in MBR.

3.6 CONCLUSION

In this study, authors have presented a technique for rapid cultivation of anammox planktonic cell culture. Immobilized technique was proved as a promising tool for obtaining freely grown anammox bacterial cells. Within one month period, planktonic free cell culture of anammox bacteria (more than 95% purity and an average particle distribution of 1.1 μm) could be obtained with adequate cell concentration for physiological, biochemical and microbial studies. This technique is also expected to be applied for other slow growing bacteria in the future.

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4 MAXIMUM SPECIFIC GROWTH RATE OF ANAMMOX BACTERIA REVISITED

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4.1 ABSTRACT

Anammox bacteria have long been considered to be slow-growing bacteria. However, it has recently been reported that they could grow much faster than previously thought when they were cultivated in a membrane bioreactor (MBR) with a step-wise decrease in the solid retention time (SRT). Here, we reevaluated the maximum specific growth rates (μ_{max}) of three phylogenetically distant anammox bacterial species (*i.e.* "*Ca*. Brocadia sinica", "*Ca*. Jettenia caeni" and "*Ca*. Scalindua sp.") by directly measuring 16S rRNA gene copy numbers using newly developed quantitative polymerase chain reaction (qPCR) assays. When free-living planktonic "*Ca*. B. sinica" and "*Ca.* J. caeni" cells were immobilized in polyvinyl alcohol (PVA) and sodium alginate (SA) gel beads and cultivated in an up-flow column reactor with high substrate loading rates at 37 ºC, the μ_{max} were determined to be 0.33 ± 0.02 d^{-1} and 0.18 d^{-1} (corresponding doubling time of 2.1 day and 3.9 day) from the exponential increases in 16S rRNA genes copy numbers, respectively. These values were faster than the fastest growth rates reported for these species so far. The cultivation of anammox bacteria in gel beads was achieved less than one month without special cultivation method and selection pressure, and the exponential increase in 16S rRNA gene numbers was directly measured by qPCR with high reproducibility; therefore, the resulting μ_{max} values were considered accurate. Taken together, the fast growth is, therefore, considered to be an intrinsic kinetic property of anammox bacteria.

4.2 INTRODUCTION

Anaerobic ammonium oxidation (anammox) has received a great attention as a promising environmentally friendly and energy efficient wastewater treatment process (Ali and Okabe, 2015). There are more than 100 full-scale anammox installations worldwide, and this number is growing rapidly (Lackner et al., 2014). Kinetics and stoichiometry are the two essential factors for the design and optimization of anammox-based wastewater treatment process (Lotti et al., 2014; van der Star et al., 2007). The relatively consistent stoichiometry of anammox metabolism has been reported from highly enriched anammox cultures regardless of biomass forms such as aggregated cells (Strous et al., 1998) or suspended (planktonic) cells (Lotti et al., 2014). However, kinetic parameters such as specific growth rate (μ) of anammox bacteria are still debatable issues due to experimental difficulties and a lack of planktonic enrichment

cell cultures with high purity (Strous et al., 1999; van der Star et al., 2008). Although anammox bacteria have long been regarded as slow-growing anaerobic chemolithoautotrophs (Strous et al., 1999), surprisingly a high maximum specific growth rate (μ_{max} = 0.33 d^{-1} at 30 ºC) was recently reported (Lotti et al., 2015). Lotti et al. (2015) suggested that the μ_{max} was not an intrinsic property and could be increased by optimizing the growth conditions (through increasing the biomass specific electron transfer capacity).

The μ_{max} of anammox bacteria was mainly determined in following two ways (Lotti et al., 2015; Oshiki et al., 2011);

(1) $\mu_{max} = q_{max} \times Y$ (Eq. 1)

(2) $\mu_{max} = 1 / SRT$ (Eq. 2)

where, q_{max} is maximum specific substrate uptake rate (g-NH_4^+-N g-VSS^{-1} d^{-1}), Y is the biomass growth yield (g-VSS g-NH_4^+-N^{-1}), and SRT is the solid retention time (d). The q_{max} can be calculated by dividing the maximum volumetric substrate removal rate (g-NH_4^+-N m^{-3} d^{-1}) by total biomass concentration in a reactor (g-VSS m^{-3}). For accurate determination of q_{max}, the reactor must be operated without any substrate limitation, which leads to underestimation of q_{max}, consequently μ_{max}. In some studies, however, aggregated biomass (granules, biofilms, or flocs) were used, which are more prone to confront substrate diffusion limitations (Okabe et al., 2011; Oshiki et al., 2011; Strous et al., 1998; Tsushima et al., 2007a; van de Graaf et al., 1996). Thus, kinetic parameters such as μ_{max} and *Ks* determined using aggregated biomass must be carefully reevaluated. The reported Y values were rather constant regardless of anammox species except for "*Ca.* Scalindua sp." (Oshiki et al., 2016).

Membrane bioreactors (MBRs) were recently used to determine the μ_{max}, because the MBR enables cultivation of free-living planktonic anammox bacterial cells (Ali et al., 2015a; Awata et al., 2013a; Lotti et al., 2014; Lotti et al., 2015; van der Star et al., 2008). In these studies, the SRT was step-wisely decreased close to the critical dilution rate (*i.e.*, maximum specific growth rate) by increasing the mixed liquor discharge rate, and thus the μ_{max} was estimated as an inverse of the SRT according to the model (Eq. 2). Although this approach enables to minimizing the effects of substrate limitation, maintenance, and decay, wall growth and biomass accumulation in hollow fiber membrane modules most likely occur in a long-term operation, which significantly overestimate the q_{max} and μ_{max}. Furthermore, the above mentioned μ_{max} determination

methods (Eq. 1 and 2) in general require relatively long experimental periods (a few months to a year) (Lotti et al., 2014; Lotti et al., 2015; van der Star et al., 2008). Hence, for better understanding of kinetic properties of anammox bacteria, it is necessary to revisit and reevaluate the μ_{max} values with a direct and rapid determination method with better accuracy.

In the present study, the μ_{max} of three phylogenetically distant anammox species (*i.e.* "*Ca*. Brocadia sinica", "*Ca*. Jettenia caeni" and "*Ca*. Scalindua sp.") were determined by directly measuring temporal increases in 16S rRNA gene copy numbers. The qPCR-based quantification assay of 16S rRNA gene copy number has been previously used to estimate μ_{max} of anammox bacteria (Tsushima et al., 2007; van der Star et al., 2007). First, the genera specific quantitative polymerase chain reaction (qPCR) assays were newly developed for quantification of each 16S rRNA gene copy numbers. According to the Monod equation, it is essential to cultivate the anammox biomass without any substrate limitation and inhibition to obtain the exponential growth, namely μ_{max}. Second, free-living planktonic cells were cultured in MBRs as a semi-batch mode with complete biomass retention and continuous substrate supply. Finally, planktonic cells were immobilized in polyvinyl alcohol and sodium alginate (PVA-SA) gel beads and cultured in up-flow column reactors with high substrate loading rates to avoid substrate limitation. The μ_{max} was then calculated from exponential increases in 16S rRNA gene copy numbers, and the highest μ_{max} was observed for immobilized anammox cells.

4.3 MATERIALS AND METHODS

4.3.1 Development of qPCR assays

Three TaqMan qPCR assays were developed to quantify the 16S rRNA genes copy numbers of "*Ca.* Brocadia sinica", "*Ca.* Jettenia caeni" and "*Ca*. Scalindua sp.", respectively. The primers and probes were designed using the Primer Express software ver. 2.0 (Applied Biosystems) (**Table 1**). The specificity of the primers was verified through Ribosome Database Project Seqmatch program (https://rdp.cme.msu.edu/seqmatch/ seqmatch_intro.jsp) (Cole et al., 2014). In addition, the specificity of the assays was experimentally verified using plasmids (10^6 copies μL^{-1}) containing the partial 16S rRNA genes of three species.

The qPCR mixture (20 µL) contained 10 µL of 2× Premix ExTaq (Takara Bio), 0.4 µL of each forward and reverse primers (10 µM), 0.8 µL of TaqMan probe (10 µM), 0.4 µL of the 50× ROX Reference Dye II (Takara Bio), and 2 µL of template DNA. The qPCR was performed in MicroAmp Optical 96-well reaction plates (Applied Biosystems) with an ABI prism 7500 sequence detection system (Applied Biosystems) in the following reaction conditions: initial annealing at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, and primer extension at 60°C for 34 sec. Serial dilutions (10^3 to 10^7 copies μL^{-1}) of the plasmid DNA containing the target 16S rRNA gene were prepared for the quantification standard. The amplification efficiencies were calculated as described previously (Ishii et al., 2013).

4.3.2 Determination of maximum specific growth rate in MBR

Three membrane bioreactors (MBRs) were operated to determine the maximum specific growth rates of "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. Scalindua sp.", respectively (Oshiki et al., 2013). Each anammox biomass with a purity of > 90%, which was previously enriched in our laboratory (Ali et al., 2015a; Awata et al., 2013; Oshiki et al., 2013), was used as an inoculum. Anammox cultures consisted of completely free-living planktonic cells as indicated by fluorescence *in situ* hybridization (FISH) (**Fig. 1**). A membrane module composed of 300 polyethylene hollow fiber membranes (pore size, 0.1 μm; tube diameter, 1 mm; length, 70 mm, Mitsubishi Rayon, Japan) was installed on a wide mouth reagent bottle (2-liter, Sansyo, Japan). A peristaltic pump (MP–1000, EYELA, Tokyo, Japan), actuated by a water level sensor (HL–S1A, ASONE, Japan), was directly connected to the membrane module to keep a constant water level in the MBR. The MBR culture was continuously agitated at 200 rpm using a magnetic stirrer. A mixed Ar and CO_2 (95:5) gas was continuously supplied to the cell culture at 10 mL min^{-1} to avoid oxygen contamination. pH was not controlled but ranged between 7.0 - 8.0. In the case of "*Ca*. B. sinica" and "*Ca*. J. caeni", the constitution of trace elements in the influent medium was (mg L^{-1}): $FeSO_4 \cdot 7H_2O$ 9.0, EDTA·2Na 5.0, NaCl 1.0, KCl 1.4, $CaCl_2 \cdot 2H_2O$ 1.4, $MgSO_4 \cdot 7H_2O$ 1.0, $NaHCO_3$ 84, KH_2PO_4 54 and 0.5 mL L^{-1} trace elements solution II (van de Graaf et al., 1996). In the case of "*Ca*. Scalindua sp.", the inorganic nutrient medium was supplied with 25 g L^{-1} of SEALIFE (Marine Tech, Tokyo, Japan), an artificial sea salt, $CaCl_2 \cdot 2H_2O$ (114 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (99 mg L^{-1}), $KHCO_3$ (500 mg L^{-1}), KH_2PO_4 (24.4 mg L^{-1}), 0.5 mL L^{-1} trace element solution I & II (van de Graaf et al., 1996). The detailed information of the artificial mineral medium

of SEALIFE was described in the previous work (Kindaichi et al., 2011; Oshiki et al., 2013). In all experiments, nitrite concentration was set as a limiting substrate.

The concentrations of ammonium, nitrite and nitrate were determined using an ion-exchange chromatography (IC-2010, TOSOH, Tokyo, Japan) equipped with an TSKgel IC-Anion HS and TSKgel IC-Cation columns (TOSOH) after filtration with 0.2-μm-pore size membranes (Advantec Co., Ltd., Tokyo, Japan) (Tsushima et al., 2007b). Nitrite concentration in MBR was also regularly monitored by colorimetric method according to standard methods (APHA, 1998). Water quality of "*Ca*. Scalindua sp." culture was measured only using colorimetric method according to standard methods (APHA, 1998) due to the high ionic strength of the medium.

To obtain an exponential growth of anammox bacteria, planktonic biomass was inoculated at time zero and the culture medium was continuously supplied into the MBR. The initial biomass concentration of "*Ca*. B. sinica", "*Ca*. Scalindua sp." and "*Ca*. J. caeni" was 3.7×10^9, 1.2×10^{10}, and 8.2×10^8 16S rRNA gene copies mL^{-1}, respectively. The exponential growth can be observed when substrate and nutrients are present in excess. As growth proceeds, more substrates are consumed and required. Thus, the substrate (ammonium and nitrite) loading rate must be gradually increased with time to avoid substrate limitation. In this study, the substrate loading rate was adjusted by substrate concentrations and feeding rate. It should be noted that since anammox activity could be inhibited by high nitrite concentration (Carvajal-Arroyo et al., 2013; Lotti et al., 2012; Oshiki et al., 2016), the nitrite concentration must be carefully maintained in an appropriate range during the entire experiment. Solid retention time (SRT) of MBR was not controlled (at infinity) in all experiments.

4.3.3 Determination of maximum specific growth rate in immobilized gel beads

Free-living planktonic cells of "*Ca*. B. sinica" and "*Ca*. J. caeni" MBR cultures were immobilized in PVA (6%, w/v) and SA (2%, w/v) gel as described previously (Ali et al., 2014). After the gel beads were cured in the $CaCl_2$ solution overnight and washed with sterilized distilled water at least three times, the gel beads were inoculated into an up-flow column reactor (255 mL, Fujirika, Japan) with a packing ratio of 70% (v/v). The up-flow column reactor was operated at 37 °C, pH 7.5±0.5 and HRT 2.1 h as described previously (Ali et al., 2014). As anammox bacteria grew in the gel beads, the

substrate loading rate was gradually increased to avoid substrate limitation. The gel bead samples were always collected from the bottom of the reactor where the higher substrate concentrations were expected, and 16S rRNA gene copy number per gel bead was measured by qPCR as described below. In addition, the concentrations of NH_4^+-N, NO_2^- -N and NO_3^- -N in influent and effluent were measured on daily basis. Immobilization of "*Ca*. Scalindua sp." was not successful due to high ionic strength of the medium containing SEALIFE.

4.3.4 qPCR analysis

Maximum specific growth rates of "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. Scalindua sp." were determined from the exponential increase phases of 16S rRNA gene copy numbers. Culture suspensions in MBRs and gel beads in the column reactors were collected, and genomic DNAs were extracted using Fast DNA spin kit for soil (MP Biomedical, Tokyo, Japan) by following manufacturer's manual. The extracted DNAs were subjected to quantitative PCR analysis. Premix Ex Taq (Probe qPCR) (Takara Bio Inc., Japan) was used for the PCR, and the PCR mixture was prepared as described in the manufacturer's manual. "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. Scalindua sp." possesses one (Oshiki et al., 2015), two (Hira et al., 2012), and one (Oshiki et al., unpublished) *rrn* operon in their genomes, respectively.

4.3.5 EPS production of "*Ca*. B. sinica"

Production of extracellular polymeric substances (EPS) is a diversion of substrate (or energy) away from cellular growth, thereby might affect the specific growth rate. The EPS production of "*Ca*. B. sinica" was quantified during growth experiment in the MBR. As described above, a MBR was established and operated, and 16S rRNA gene copy numbers and extracellular protein and polysaccharide contents per cell were determined according to previous description with little modification (Tan et al., 2014). In briefly, 15 mL biomass were collected and centrifuged at 8,000 g for 10 min at 4°C. The pellets were subjected to freeze-drying for 24 h. After the dried biomass pellet was weighted and recorded, the biomass was resuspended in 1 mL of 0.9% NaCl (w/v) solution with 0.6% formaldehyde (v/v) for 1 h at 4°C, and then 400 μL of 1 M NaOH was added to the biomass suspension and incubated for 3 h at 4°C. Thereafter, EPS was collected at 17,000 g for 15 min at 4°C as the supernatant. The protein concentration was quantified by the Lowry method with bovine serum albumin as the

standard (Lowry et al., 1951). The polysaccharide concentration was measured using the phenol-sulfuric acid assay with glucose as the standard (DuBois et al., 1956).

4.3.6 EPS characterization using Fourier transform spectroscopy (FTIR)

Fourier transform spectroscopy (FTIR) was employed to analyze chemical and structural characteristics of EPS produced during different growth phases. Samples were prepared according to the manufacturer's protocol. FTIR spectra were collected using a FT/IR 660 plus spectrometer (JASCO, Japan). The operating range was from 4000 to 600 cm^{-1} with a resolution of 10 cm^{-1}. Based on the FTIR spectra, a principal component analysis (PCA) was performed with R 3.0.2 (R Development Core Team; Vienna, Austria) to illustrate the difference of EPS characteristics during the growth phases (Lammers et al., 2009).

4.4 RESULTS

4.4.1 Development of qPCR assay

Three TaqMan qPCR assays were newly developed for the quantification of "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. Scalindua sp.". The assays could specifically quantify target 16S rRNA genes as low as 10^3 copies μL^{-1} (**Fig. 2**). The standard curves for the three assays had the goodness-of-fit (r^2) values of >0.99 (**Fig. 2**) with the amplification efficiencies ranging between 97-109%. These results indicated high specificity and sensitivity of the qPCR assays.

4.4.2 Anammox bacterial growth in MBR

Free-living planktonic cells of "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. Scalindua sp." were cultivated in the MBRs to determine the maximum specific growth rate (μ_{max}). In order to obtain exponential growth (namely, μ_{max}), the nitrogen loading rate was carefully increased step-wisely to avoid substrate limitation as well as nitrite inhibition (**Fig. 3**).

In the MBR culture of "*Ca*. B. sinca", the bulk nitrite concentration was in the range 0 – 3.3 (average 0.38) mg-N L^{-1} (**Fig. 3, A1**). The 16S rRNA gene copy numbers increased from 3.7×10^9 to 2.9×10^{10}, in which the exponential increase was observed from day 8 to

day 19 (**Fig. 3, A2**). Accordingly, the μ_{max} and corresponding doubling time were calculated to be 0.17 d^{-1} and 4.1 d, respectively.

In the MBR culture of "*Ca*. J. caeni", the bulk nitrite concentrations fluctuated more widely from 0 – 5.1 (average 0.73) mg-N L^{-1} (**Fig. 3, B1**). Exponential increase of the 16S rRNA gene copy numbers was found rather late during day 33 to day 48 probably due to low initial biomass concentration and low nitrogen loading rate (**Fig. 3, B2**). The μ_{max} and corresponding doubling time were calculated to be 0.11 d^{-1} and 6.3 d, respectively.

In the MBR culture of "*Ca*. Scalindua sp.", the bulk nitrite concentration was in the range 0 – 1.8 (average 0.68) mg-N L^{-1} (**Fig. 3, C1**). Exponential increase of 16S rRNA gene copy numbers was observed from day 4 to day 10 (**Fig. 3, C2**). Accordingly, the μ_{max} and corresponding doubling time were calculated to be 0.17 d^{-1} and 4.1 d, respectively.

4.4.3 Anammox bacterial growth in immobilized gel beads

Free-living planktonic cells of "*Ca*. B. sinica" and "*Ca*. J. caeni" were immobilized in PVA-SA gels and cultivated in up-flow column reactors. In order to avoid both substrate limitation and inhibition, the nitrogen loading rate was carefully increased as the cells grew. The effluent nitrite concentrations were in the range 0.2 - 34 (average 11.3) mg-N L^{-1} for the "*Ca*. B. sinica" reactor and the range 2.4 - 27.9 (average 12.9) mg-N L^{-1} for the "*Ca*. J. caeni" reactor during the experiments (**Fig. 4, A1 and B1**). Exponential increase in 16S rRNA gene copy numbers of "*Ca*. B. sinica" was observed after 1 week, and the μ_{max} was calculated to be 0.34 d^{-1} (*i.e.*, doubling time of 2.1 d) accordingly (**Fig. 4, A2**). Similarly, exponential increase in 16S rRNA gene copy numbers of "*Ca*. J. caeni" was observed after 20 days, the μ_{max} was calculated to be 0.18 d^{-1} (*i.e.*, doubling time of 3.9 d) accordingly (**Fig. 4, B2**). These observed μ_{max} of both species were higher than the ones obtained from MBR cultures. The μ_{max} of "*Ca*. Scalindua sp." could not be determined due to unsuccessful immobilization.

4.4.4 Production of EPS

Extracellular protein and polysaccharide contents per biomass [μg mg-VSS^{-1}] were determined when planktonic "*Ca*. B. sinica" cells were cultured in a MBR (**Fig. 5, Fig. S1**). The MBR was operated with a step-wise increase in the nitrogen loading rate

to supply sufficient substrates without nitrite inhibition. The 16S rRNA gene copy numbers increased and reached a stable value after 30 days. Extracellular polysaccharide content was relatively constant (ranging from average 19 μg mg-VSS^{-1} during the exponential growth phase to 29 μg mg-VSS^{-1} during the stationary phase) throughout the cultivation, whereas extracellular protein content increased by 1.7 times from 61 to 108 μg mg-VSS^{-1} (**Fig. 5C**). PCA analysis of FTIR spectra revealed that there was a clear difference between the EPS produced during the exponential growth and stationary phase (**Fig. 5D**).

4.5 DISCUSSION

The maximum specific growth rates (μ_{max}) of anammox bacteria immobilized in PVA-SA gel beads were directly determined from exponential increases in 16S rRNA gene copy numbers. The μ_{max} measurement was conducted for "*Ca*. B. sinica" in triplicate and the consistent results were obtained (0.31 - 0.34 d^{-1}, **Fig. S2**, 95% confidence interval: 0.33 ± 0.02 d^{-1}), supporting the high reproducibility of this method. The qPCR-based quantification assay of 16S rRNA gene copy number is an easy, rapid, and specific alternative to quantification of target cells in environmental samples and mixed populations (Trung et al., 2011; Tsushima et al., 2007a). This approach is based on the assumption that a constant relationship exists between the cell number and the 16S rRNA gene copy number. It has been previously demonstrated that the cell numbers and 16S rRNA gene copy numbers was highly correlated during exponential growth phase under no stress condition (Chase and Harwood, 2011; Reichert-Schwillinsky et al., 2009). The qPCR-based quantification assay of 16S rRNA gene copy number has been previously used to estimate the μ_{max} of anammox bacteria (Tsushima et al., 2007; van der Star et al., 2007).

A limitation of this qPCR assay was, however, its inability to discriminate between active and inactive cells. Accumulation of inactive cells in gel beads and MBR cultures might lead to overestimation of μ_{max}. In the present study, μ_{max} was determined from exponential increase phases of 16S rRNA gene copy numbers, in which optimal growth conditions (no substrate limitation and inhibition) were maintained, and thus the growth rate is maximum and decay is negligible (Cupples et al., 2003). In fact, the decay rate of "*Ca*. B. sinica" has been reported to be 0.0029 - 0.0081 d^{-1} (Oshiki et al., 2013), which was < 3% of the μ_{max} values obtained in this study, suggesting the effect of decay rate

was negligible in this measurement. Therefore, the resulting μ values were considered to be μ_{max} and accurate.

According to the Monod equation, the limiting substrate (*i.e*., nitrite in this study) concentration in the up-flow column reactor must be carefully maintained in order to ensure the exponential growth (i.e., maximum specific growth rate). It should be also noted that high nitrite concentration inhibits anammox activity (Oshiki et al., 2016). The half-saturation constant (*Ks*) value for nitrite was 0.48 ± 0.29 mg-N L^{-1} for "*Ca*. B. sinica", 0.50 ± 0.013 mg-N L^{-1} for "*Ca*. J. caeni", and 0.0063 mg-N L^{-1} for "*Ca*. Scalindua sp.", respectively (Oshiki et al., 2016). The inhibition constant (*Ki*) value for nitrite was < 224 mg-N L^{-1} for "*Ca*. B. sinica", 154 mg-N L^{-1} for "*Ca*. J. caeni", and 105 mg-N L^{-1} for "*Ca*. Scalindua sp.", respectively (Oshiki et al., 2016). Therefore, the nitrite concentration must be maintained at least in the range between two times of its *Ks* value and a half of *Ki* value; 0.96 – 112 mg-N L^{-1} for "*Ca*. B. sinica", 1.0 – 78 mg-N L^{-1} for "*Ca*. J. caeni" and 0.0032 – 53 mg-N L^{-1} for "*Ca*. Scalindua sp.", respectively, to obtain the exponential increase in 16S rRNA gene copy numbers.

In the case of "*Ca*. B. sinica" gel bead column reactor, the effluent nitrite concentration was in the range 0.2 – 34.0 (average 11.3) mg-N L^{-1} during the experiment (**Fig. 4, A1**). In the case of "*Ca*. J. caeni" gel bead column reactor, the effluent nitrite concentration was in the range 2.4 - 27.9 (average 12.9) mg-N L^{-1} during the experiment (**Fig. 4, B1**). This indicates that the effluent nitrite concentration was mostly maintained above their *Ks* values and below *Ki* value throughout the experiment. In addition, the gel bead samples were always taken from the bottom part (medium inlet side) of the column reactor, thus, the higher nitrite concentrations than the effluent concentration could be expected due to the concentration gradient developed along with the column height (Kindaichi et al., 2007). Furthermore, the substrate diffusivity in PVA-SA gel beads was relatively high (77% of diffusivity in water) (Ali et al., 2015b). Taken together, nitrite was present in excess in the gel beads; therefore, the resulting μ_{max} values were considered reliable.

As a result, the μ_{max} values of 0.33 ± 0.02 d^{-1} for "*Ca*. B. sinica" and 0.18 d^{-1} for "*Ca*. J. caeni" were obtained from the immobilized cells at 37 ºC, which were higher than the previously reported μ_{max} values for corresponding anammox species (Ali et al., 2015a; Oshiki et al., 2011) (**Table 2**). The highest previously reported μ_{max} value was 0.33 d^{-1} for anammox bacteria closely related to "*Ca*. Brocadia sp. 40" (Lotti et al., 2015), which was estimated as an inverse of SRT of MBR at 30 ºC (lower than the optimum

temperature). In their study, even though the SRT was gradually decreased to 3.0 days by increasing the flow rate of mixed liquor discharge for about 300 days, anammox activity (NO_2^- and NH_4^+ removal rates) remained relatively unchanged whereas the mixed liquor biomass concentration significantly decreased. Therefore, it was concluded that anammox bacteria have a remarkable capacity to increase the growth rate by maximizing the electron transfer capacity (biomass specific NH_4^+ oxidation rates [mg-NH_4^+-N g-VS^{-1} d^{-1}]). Under such short SRT (*i.e.*, high dilution rate) conditions and relatively long operation time, bacterial cells tend to attach and grow on the walls of MBR and/or membrane modules (Okabe et al., 1992; Okabe and Characklis, 1992), which results in significant overestimation of the biomass specific NH_4^+ oxidation rates, namely μ_{max}. In this study, the membrane and walls were vigorously shaken and cleaned to avoid biomass attachment everyday during the operation (usually a month), which resulted in no visible biomass attachment.

The μ_{max} values determined from the immobilized "*Ca*. B. sinica" (0.33 ± 0.02 d^{-1}) and "*Ca*. J. caeni" (0.18 d^{-1}) were higher than those obtained from planktonic free-living cultures in MBRs; 0.17 d^{-1} for "*Ca*. B. sinica", 0.11 d^{-1} for "*Ca*. J. caeni", and 0.17 d^{-1} for "*Ca*. Scalindua sp." (**Table 2**). A relatively small amount of anammox biomass was inoculated into the MBR culture, and the MBRs were operated as a semi-batch mode (no SRT control and continuous substrate supply) for a relatively short term (about a month). Temporal changes in 16S rRNA gene copy numbers were directly measured. Compared with the gel bead column reactors, it was difficult to maintain appropriate nitrite concentration in the MBR culture (**Fig. 3**). The average nitrite concentrations, especially for "*Ca*. B. sinica" and "*Ca*. J. caeni", were close to their *Ks* values during the experiment, suggesting occurrence of nitrite limitation. This is probably the reason why the lower μ_{max} values were observed in the MBR cultures.

In addition to the substrate limitation, all newly born daughter cells could be completely retained in PVA-SA gel beads and free from hydraulic shear stress. It is considered that free-living planktonic anammox cells need to produce more EPS than the immobilized cells, since hydraulic shear stress is a key stimulator of EPS production and consequently aggregation (Beun et al., 2002; Liu et al., 2010). Production of EPS requires investment of energy derived from the substrate utilization, approximately 7% of fixed carbon was secreted as soluble microbial products by "*Ca*. B. sinica" (Oshiki et al., 2011). The planktonic "*Ca*. B. sinica" cells produced 10^9 μg-extracellular protein

mg-VSS^{-1} and 29 μg-extracellular carbohydrate mg-VSS^{-1} at stationary growth phase, which were higher than the production at exponential growth phase (**Fig. 5, Fig. S1**).

Quantification of EPS production was not successful for immobilized cells due to high background of alginate matrix used for immobilization. Such allocation of energy to EPS production perhaps hinders the planktonic anammox bacteria from growing at a maximum rate, whereas immobilized cells could use most of the energy for cellular growth. However, this hypothesis must be verified in future.

4.6 CONCLUSIONS

The maximum specific growth rate (μ_{max}) of anammox bacteria was reevaluated by directly measuring exponential increases in 16S rRNA gene copy numbers. First, qPCR assays were developed for quantification of 16S rRNA gene copy numbers of three phylogenetically distant anammox bacteria; "*Ca*. Brocadia sinica", "*Ca*. Jettenia caeni" and "*Ca*. Scalindua sp.", respectively. The free-living planktonic anammox bacteria were immobilized in PVA-SA gel beads and cultivated in up-flow column reactors without substrate limitation, and temporal changes in 16S rRNA genes were monitored. The μ_{max} were determined to be 0.33 ± 0.02 d^{-1} for "*Ca*. B. sinica" and 0.18 d^{-1} for "*Ca*. J. caeni" at 37 ºC, respectively, which are higher than the highest previously reported values. These μ_{max} values were directly determined based on the exponential increase in 16S rRNA genes; therefore considered accurate. Furthermore, the direct 16S rRNA gene quantification of immobilized cells is relatively easy and rapid (takes only a month). In the present study, since special cultivation method and selection pressures were not applied, this fast growth rate is, therefore, considered to be an intrinsic kinetic property of anammox bacteria.

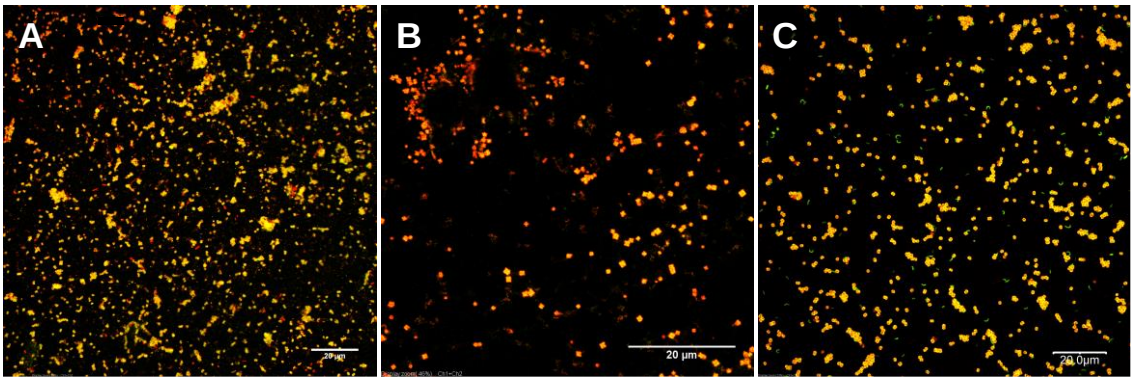

Figure 4.1 Confocal microscopic images of FISH analysis for enriched free-living planktonic cells of (A) "*Ca*. Brocadia sinica", (B) "*Ca*. Jettenia caeni" and (C) "*Ca*.

Scalindua sp." in MBRs. Cells were hybridized with combination of FITC-labeled AMX820 probe (green) and TRITC-labeled EUB mix probe (red) for "*Ca.* Brocadia sinica" (A), TRITC-labeled EUB338 mix probe (red) and FITC-labelled probe JEC152 (green) for "*Ca.* Jettenia caeni" (B), and FITC-labeled EUB338 mix probe (green) and TRITC-labelled probe Sca1129b for "*Ca.* Scalindua sp." (C). Anammox bacteria were shown in yellow for all panels. White bars indicate 20 µm.

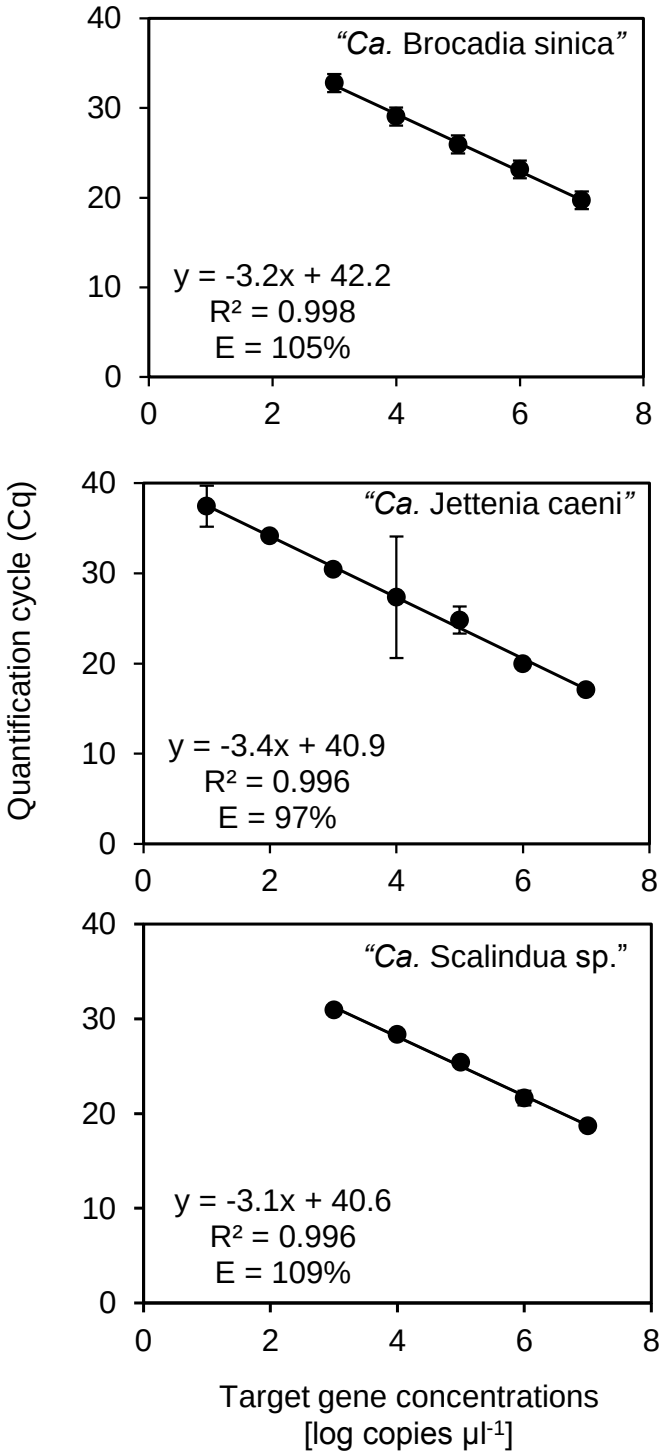

Figure 4.2 Standard curves of the qPCR assays developed in this study for "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca*. Scalindua sp.", respectively. The standard curves were prepared using serial 10-fold dilutions (from 10^7 to 10^3 or 10^1 copy numbers μL^{-1}) of the plasmid DNA carrying the partial 16S rRNA gene of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca*. Scalindua sp.", respectively. Goodness-of-fit (r^2) value, the slope of the standard curve, and amplification efficiency (*E*) are also shown. Error bars indicate the standard deviation of the triplicates.

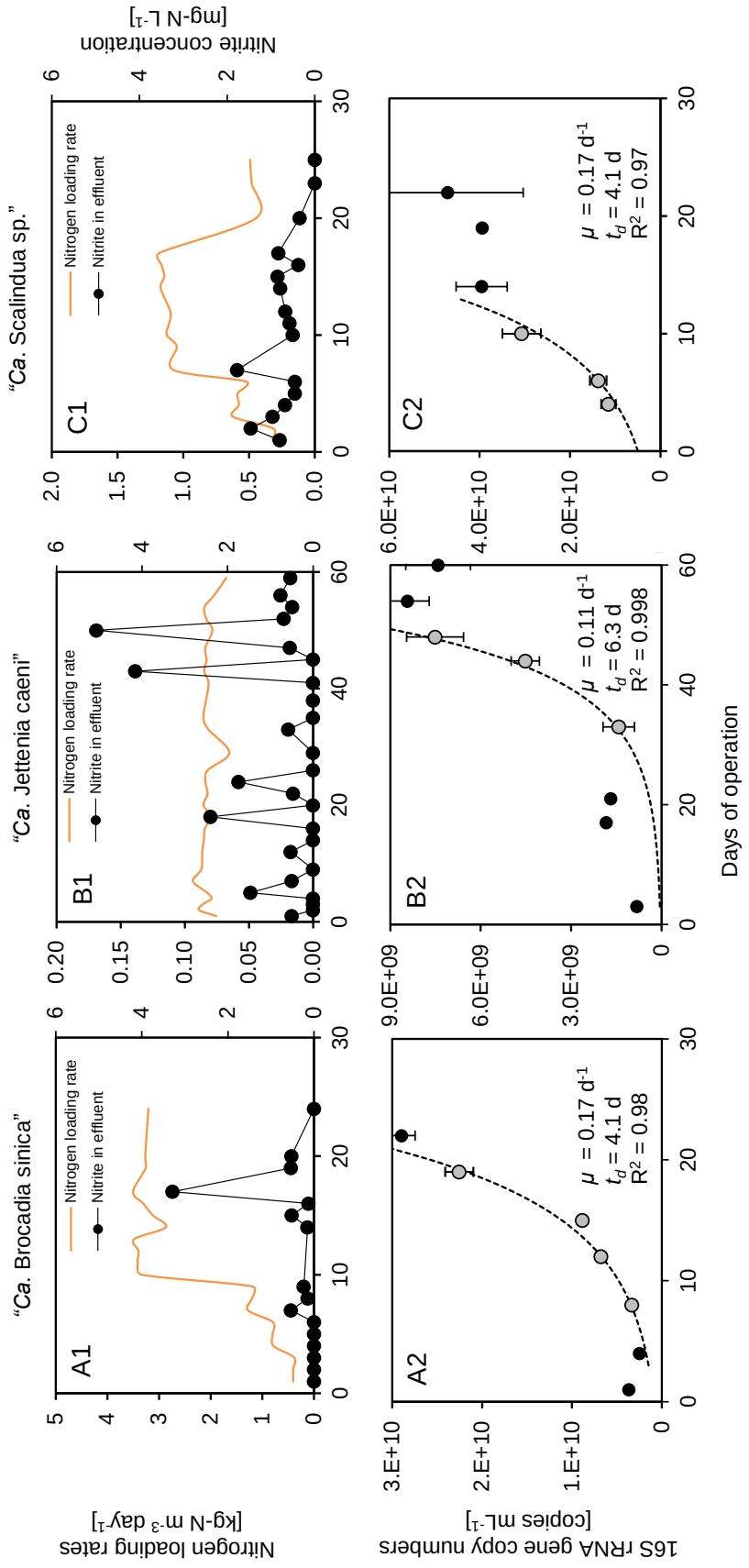

Figure 4.3 The reactor performance, nitrite concentration in bulk phase and growth dynamics of "*Ca*. B. sinica" (A1 and A2), "*Ca*. J. caeni" (B1 and B2) and "*Ca*. Scalindua sp." (C1 and C2) in a MBR, respectively. Maximum specific growth rate

(μ_{max}) was determined from the exponential increase in 16S rRNA gene copy numbers (gray plots). t_d : doubling time, t_d = ln 2 / μ.

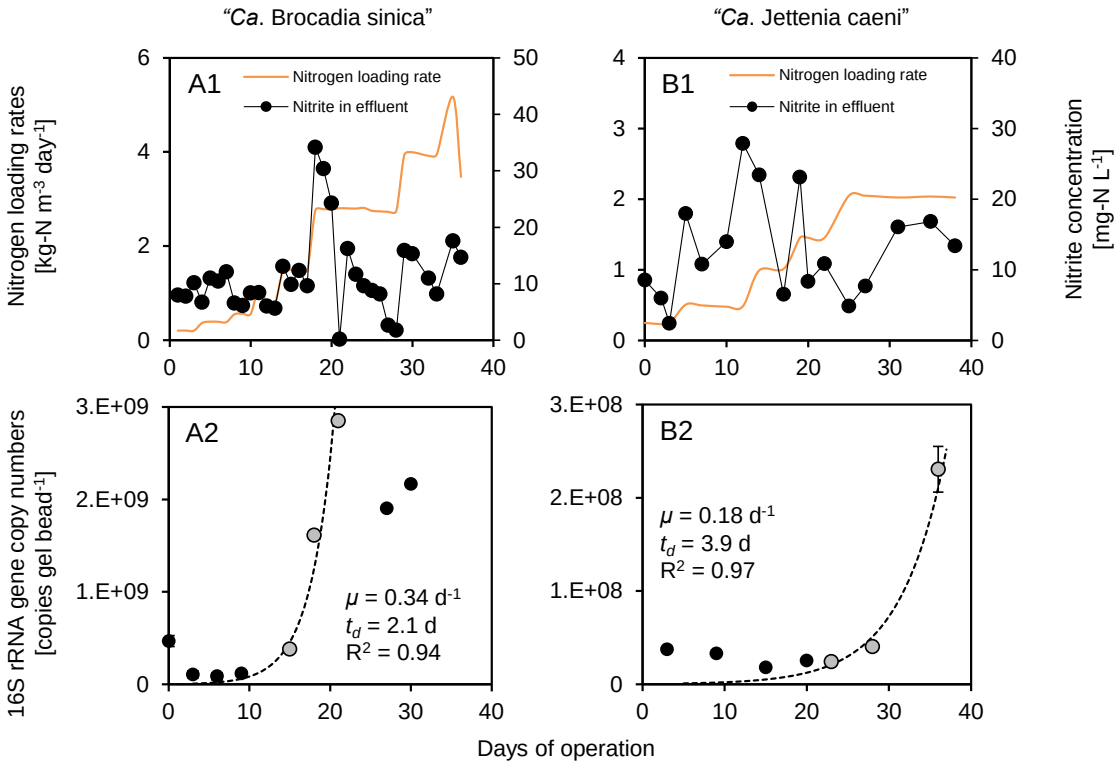

Figure 4.4 The reactor performance, effluent nitrite concentration and growth dynamics of "*Ca*. B. sinica" (A1 and A2) and "*Ca*. J. caeni" (B1 and B2) immobilized in gel beads, respectively. Maximum specific growth rate (μ_{max}) was determined from the exponential increase in 16S rRNA gene copy numbers (gray plots). t_d : doubling time, t_d = ln 2/μ.

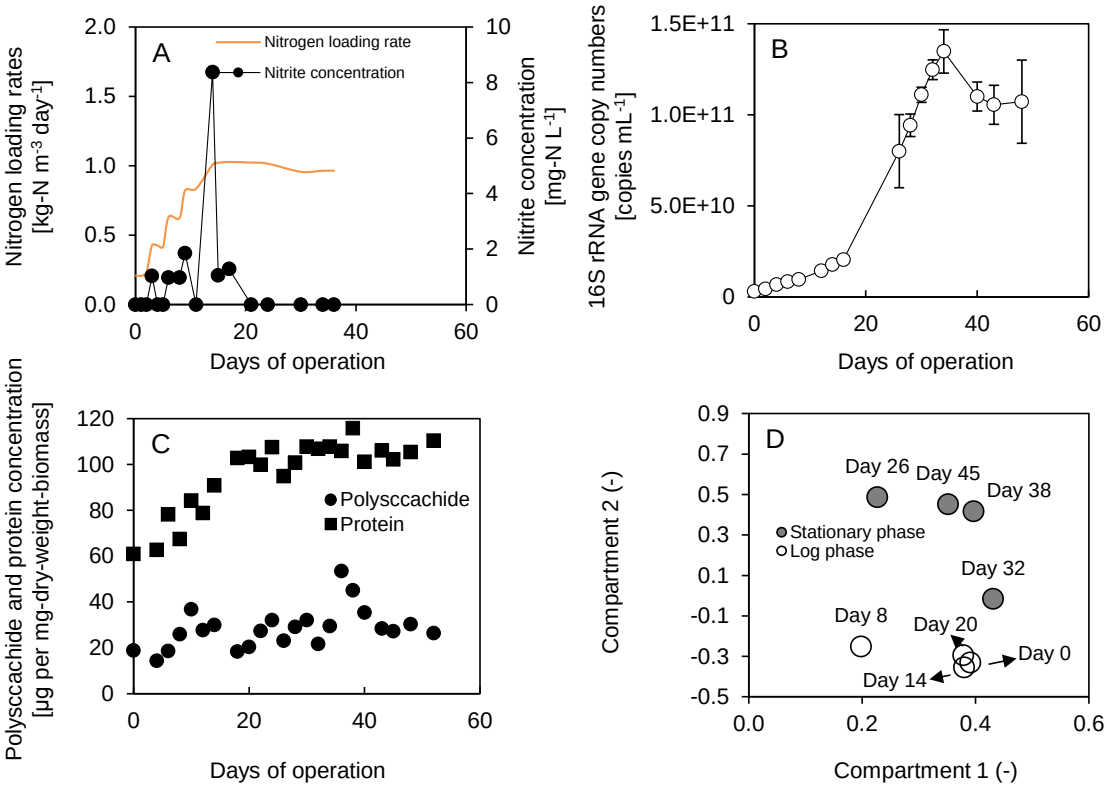

Figure 4.5 EPS production at different growth phases. Reactor performance and nitrite concentration in bulk phase in MBR (**A**). Temporal change in16S rRNA gene copy numbers of "*Ca.* B. sinica" (**B**). Time course changes in extracellular protein and polysaccharide content per biomass dry weight during cell growth (**C**). Principal component analysis (PCA) on the spectrum of Fourier transform infrared spectroscopy (FTIR) for EPS samples taken from different growth phases (**D**).

Table 4.1 Specific primers and probes designed in this study.

Target species	Name	Sequence (5'-->3')	Target site[a]
"*Ca.* Brocadia. sinica"	BRS95F	GATGGGAACAACAACGTTCCA	95-115
	BRS170R	TTCTTTGACTGCCGACACCA	170-189
	BRS130P	FAM-CCGAAAGGGTTGCTAATTCTCA-MGB-NFQ	130-151
"*Ca.* Scalindua sp."	SCJ447F	GTAAACAGGTTAATACCCTGT	447-467
	SCJ629R	TCAAGATCTACAGTTTCAGAT	629-649
	SCJ512P	FAM-CAGCAGCCGCGGTAATACAGA-MGB-NFQ	512-532
"*Ca.* Jettenia caeni"	JEC447F	GTAAGGGGGTGAATAGCCCTC	447-467
	JEC629R	TCCAGCCCTATAGTATCAACT	629-649
	JEC512P	FAM-CAGCAGCCGCGGTAATACAGA-MGB-NFQ	512-532

[a]: 16S rRNA position according to 16S rRNA gene sequence of Escherichia coli numbering.

Table 4.2 Summary of the maximum specific growth rates obtained in this study and reported values.

Anammox species	μ_{max} (d^{-1})	Biomass	Measuring methods	Temp. (°C)	Reference
"*Ca*. Kuenenia stuttgartiensis"	0.09-0.23	Planktonic cells	SRT control [1]	38	(van der Star et al., 2008)
"*Ca*. Brocadia sp.40"	0.33	Planktonic cells		30	(Lotti et al., 2015)
"*Ca*. Brocadia anammoxidans"	0.06	Aggregates	Maximum ammonium conversion rate and biomass yield [2]	32-33	(Strous et al., 1998)
"*Ca*. Brocadia sinica"	0.1	Granules		37	(Oshiki et al., 2011)
"*Ca*. Scalindua sp."	0.05	Planktonic cells		28	(Awata et al., 2013a)
"*Ca*. Jettenia caeni"	0.05	Planktonic cells		37	(Ali et al., 2015a)
"*Ca*. Brocadia sinica"	0.17	Planktonic cells	Temporal increase in 16S rRNA gene copy numbers as determined by qPCR	37	This study
	0.33	Immobilized cells			
"*Ca*. Scalindua sp."	0.17	Planktonic cells		22	
	(-)[3]	Immobilized cells			
"*Ca*. Jettenia caeni"	0.11	Planktonic cells		37	
	0.18	Immobilized cells			

[1] μ_{max} = 1 / SRT (Eq. 2)

[2] $\mu_{max} = q_{max} \times Y$ (Eq. 1)

[3] Immobilization of "*Ca*. Scalindua sp." using PVA and SA solution was not successful.

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5 MICROBIAL COMPETITION AMONG ANAMMOX BACTERIA IN NITRITE-LIMITED BIOREACTORS

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5.1 ABSTRACT

Phylogenetically diverse anammox bacteria have been detected in most of anoxic natural and engineered ecosystems and thus regarded as key players in the global nitrogen cycle. However, ecological niche differentiation of anammox bacteria remains unresolved despite its ecological and practical importance. In this study, the microbial competitions for a common substrate (nitrite) among three anammox species (*i.e.* "*Candidatus* Brocadia sinica", "*Candidatus* Jettenia caeni" and "*Candidatus* Kuenenia stuttgartiensis") were systematically investigated in nitrite-limited gel-immobilized column reactors (GICR) and membrane bioreactors (MBRs) under different nitrogen loading rates (NLRs). 16S rRNA gene-based population dynamics revealed that "*Ca*. J. caeni" could proliferate only at low NLRs, whereas "*Ca*. B. sinica" outcompeted other two species at higher NLRs in both types of reactors. Furthermore, FISH analysis revealed that "*Ca*. J. caeni" was mainly present as spherical microclusters at the inner part (low NO_2^- environment), whereas "*Ca*. B. sinica" was present throughout the gel beads and granules. This spatial distribution supports the outcomes of the competition experiments. However, the successful competition of "*Ca*. J. caeni" at low NLR could not be explained with the Monod model probably due to inaccuracy of kinetic parameters such as half saturation constant (K_s) for nitrite and a difference in the maintenance rate (m). In addition, the growth of "*Ca*. K. stuttgartiensis" could not be observed in any experimental conditions, suggesting possible unknown factor(s) is missing. Taken together, NLR was one of factors determining ecological niche differentiation of "*Ca*. B. sinica" and "*Ca*. J. caeni".

5.2 INTRODUCTION

Anaerobic ammonium-oxidizing (anammox) bacteria are chemolithoautotrophic bacteria that can oxidize ammonium to nitrogen gas with nitrite as the electron acceptor (Jetten et al., 2005; Neumann et al., 2014). Since its discovery, anammox activities and bacteria were detected from various natural and engineered ecosystems (Hu et al., 2011; Sonthiphand et al., 2014) and thus regarded as an essential contributor in the global nitrogen cycle (Kuypers et al., 2003).

Five candidatus genera (*Brocadia, Kuenenia, Jettenia, Scalindua and Anammoxoglobus*) and 19 species have been reported to date (Ali and Okabe, 2015; Kartal et al., 2007; Oshiki et al., 2016; Quan et al., 2008; Schmid et al., 2003; Strous et al., 1999, 1998).

Observations of anammox bacterial diversity have demonstrated that "*Ca.* Scalindua" dominated saline environments including marine sediments (i.e., Awata et al., 2013; Schmid et al., 2007; Sonthiphand et al., 2014; Villanueva et al., 2014), whereas the other four genera were mainly detected from engineered and fresh water ecosystems (i.e., Egli et al., 2001; Hu et al., 2011; Schmid et al., 2000; Sonthiphand et al., 2014; Strous et al., 1999). Although pure culture is still not available (Strous et al., 1999), some physiological studies have been conducted using anammox bacteria cultures enriched (>90%) in membrane bioreactors (MBRs) (Ali et al., 2015a; Awata et al., 2013; Lotti et al., 2014; Oshiki et al., 2013; van der Star et al., 2008). Ecological and physiological characteristics of anammox bacteria were recently summarized (Oshiki et al., 2016), and Monod growth kinetic parameters of three anammox species (*i.e.* "*Candidatus* Brocadia sinica", "*Candidatus* Jettenia caeni" and "*Candidatus* Kuenenia stuttgartiensis") were listed in **Table 1**.

Extensive statistical analysis of the anammox 16S rRNA gene sequences deposited in the public databases revealed distinct ecological niche differentiation of anammox bacteria in natural and engineered ecosystems (Sonthiphand et al., 2014). For example, "*Ca.* Brocadia" and "*Ca.* Kuenenia" was the second and third most abundant genus, respectively, and commonly retrieved from freshwater natural and engineered ecosystems. On the other hand, "*Ca.* Jettenia" was the lowest abundant genus among all anammox bacterial genera and the majority of them was retrieved from engineered ecosystems (e.g., wastewater treatment plants and bioreactors) (Sonthiphand et al., 2014). Based on this analysis, it is most probably that salinity was an important factor governing anammox bacterial distributions such as "*Ca.* Scalindua". However, very limited information is available to explain what and/or how extrinsic factors (i.e., DO, NO_2^- and NH_4^+ concentrations) affect ecological niche partitioning of anammox bacteria in freshwater-related ecosystems including engineered ecosystems.

In lab-scale bioreactors, population shifts of anammox bacteria have been frequently reported under various conditions: *e.g.*, from "*Ca.* B. fulgida"-dominated population to "*Ca.* Brocadia. sp.40"-dominated population (Park et al., 2010); similarly from "*Ca.* B. fulgida" to "*Ca.* K. stuttgartiensis" (Park et al., 2015); from "*Ca.* Brocadia sp." to "*Ca.* K. stuttgartiensis" (van der Star et al., 2008); and from "*Ca.* B. anammoxidians" to "*Ca.* Anammoxoglobus propionicus" (Kartal et al., 2007). This clearly suggested that genus- or species-specific niche differentiation and competition exist. However, there is no clear understanding about niche differentiation of anammox bacteria at present.

It has been hypothesized that these population shifts were caused by different kinetic and physiological properties of anammox bacteria (e.g., maximum specific growth rate, affinity to a limiting substrate (e.g., NH_4^+ or NO_2^-), susceptibility to various compounds or utilization of fatty acids) (Ali et al., 2015a; Kartal et al., 2007, 2008; Oshiki et al., 2016, 2011; Park et al., 2015; van der Star et al., 2008). Interestingly, "*Ca.* B. fulgida" and "*Ca.* A. propionicus" out-competed other anammox bacteria in the presence of acetate and propionate, respectively (Kartal et al., 2007; Kartal et al., 2008), suggesting that these fatty acids are the primary factor in determining the dominance of anammox bacteria. However, driving force (s) for above population shifts has never been systematically investigated and experimentally verified yet so far.

In this work, we therefore investigated the competition for a common substrate (nitrite) among three anammox genera; "*Ca*. Brocadia", "*Ca*. Jettenia", and "*Ca*. Kuenenia", which were commonly found in lab-scale bioreactors. Two different reactor systems, gel-immobilized column reactor (GICR) and membrane bioreactor (MBR), were inoculated with anammox enrichment cultures containing these three anammox species and continuously operated for a long time with different nitrogen loading rates. Population dynamics of each anammox species were monitored by measuring 16S rRNA gene copy numbers with quantitative PCR. In addition, specific growth rates of three species at different limiting substrate (nitrite) concentrations were simulated using the Monod model to explain the competition results. The niche differentiation among "*Ca*. Brocadia", "*Ca*. Jettenia", and "*Ca*. Kuenenia" was further discussed.

5.3 MATERIALS AND METHODS

5.3.1 Monod growth curve

Specific growth rates (μ) of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis" were simulated as a function of limiting substrate concentration using the classical Monod equation, $\mu = \mu_{max} \times S / (K_s + S)$, where μ is the specific growth rate (h^{-1}); μ_{max} is the maximum specific growth rate (h^{-1}), which was recently re-evaluated for "*Ca.* B. sinica" and "*Ca.* J. caeni" (Zhang et al., 2017); S is the concentration of the limiting substrate (nitrite in this study) (μM); K_s is the half-saturation constant (μM) (**Table 1**).

5.3.2 Anammox enrichment cultures

Planktonic free-living cells of "*Ca.* B. sinica" (>90%) and "*Ca*. J. caeni" (>90%) were obtained from 2-liter MBRs that have been operated in our laboratory (Oshiki et al., 2013a; Ali et al., 2015). "*Ca*. K. stuttgartiensis" coexisted in the MBR culture of "*Ca*. J. caeni", but their abundance was 1 - 3 orders of magnitude lower than that of "*Ca*. J. caeni" on the basis of 16S rRNA gene copy number measurement. In brief, the MBR was equipped with a hollow-fiber membrane unit composed of 300 polyethylene tubes (0.1 μm-pore, 1-mm diam., 70-mm long tubes). A peristaltic pump MP-1000 (EYELA, Tokyo, Japan) was directly connected to the membrane unit. Water level in the MBR was controlled using a water level sensor (HL-S1A, ASONE, Japan). The MBR culture medium was continuously mixed using a magnetic stirrer working at 200 rpm. To maintain anoxic condition, a mixed gas (Ar: CO_2 = 95:5) was purged into the culture medium at a flowrate of 10 ml min^{-1}. Throughout the cultivation, pH was not controlled but it was always maintained within the range of 7.0 - 8.0. The inorganic nutrient medium was supplied containing (mg L^{-1}): $FeSO_4 \cdot 7H_2O$ (9.0), EDTA·2Na (5.0), NaCl (1.0), KCl (1.4), $CaCl_2 \cdot 2H_2O$ (1.4), $MgSO_4 \cdot 7H_2O$ (1.0), $NaHCO_3$ (84), KH_2PO_4 (54) and 0.5 mL L^{-1} trace element solution II (van de Graaf et al., 1996). Biomass growth was induced by continuously supplying the nutrient medium, but the biomass was completely retained in the MBR by membrane filtration.

5.3.3 Long-term competition experiments

Competitions among "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis" for a common substrate (i.e., nitrite) were studied using three different forms of anammox biomass: gel-immobilized cells in up-flow column reactors, planktonic free-living cells in MBR and self-aggregated granules in an up-flow column reactor.

For the gel-immobilized column reactors (GICR), planktonic free-living anammox bacteria ("*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis") were immobilized in polyvinyl alcohol (PVA) (6%, w/v) - sodium alginate (SA) (2%, w/v) gel beads as described previously (Ali et al., 2015b). The gel beads were then inoculated into the column reactors (53.6 mL, Fujirika, Japan) with a packing ratio of 70% and cultivated at 37°C. The column reactors were continuously fed with the inorganic nutrient medium containing $(NH_4)_2SO_4$ and $NaNO_2$ at three different nitrogen loading rates (NLRs) of 0.5, 0.7 and 2.7 kg-N m^{-3} day^{-1} (defined as G1, G2 and G3), respectively. The NLR was

varied by changing the influent NH_4^+ and NO_2^- concentrations (0.7 mM, 1 mM and 4 mM). The hydraulic retention time (HRT) was fixed at 1 h. The feed medium was purged with nitrogen gas to maintain an anoxic condition.

For MBR experiments, enrichment cultures of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" were mixed and inoculated into four MBRs (the working volume of 950 mL each). The MBRs were operated at 37°C and four different NLRs of 0.07, 0.10, 0.14 and 0.17 – 0.42 kg-N m^{-3} day^{-1} (defined as M1, M2, M3, and M4), respectively. In MBR M1, M2 and M3, culture media containing different concentrations of NH_4^+ and NO_2^- were supplied at a fixed HRT of 15.8 h. In MBR M4, influent NH_4^+ and NO_2^- concentrations were increased from 4 mM to 12 mM in a stepwise manner when the residual nitrite concentration in the bulk liquid was close to a detection limit [around 1 μM]). Sludge retention time (SRT) was not controlled (except for sampling for water quality and molecular analysis) for all runs.

For the self-aggregated granular up-flow column reactor, the NLR was ranged from 4.3 to 8.6 kg-N m^{-3} day^{-1} by changing the feeding rate with fixed NH_4^+ and NO_2^- concentration at 10 mM.

5.3.4 Chemical analysis

Influent and effluent samples of each reactor were collected, filtered through 0.2-μm-pore membranes (Advantec, Tokyo, Japan), and analyzed for concentrations of NH_4^+, NO_2^- and NO_3^- using ion-exchange chromatography (DX-100, DIONEX, Sunnyvale, CA) with an Ionpac CS3 cation column and IonPac AS9 anion column (Okabe et al., 2011). In addition, NO_2^- concentration of MBR permeate was also measured colorimetrically according to the Standard Methods (Eaton et al., 2005). The detection limit of NO_2^- measurement was around 1 μM.

5.3.5 DNA extraction and quantitative PCR

Gel beads samples and self-aggregated granules at the bottom of column reactors (close to the influent port) were collected periodically. Suspended biomass (2 ml) in MBRs was collected on a daily basis. The biomass samples were stored at -80°C until used for DNA extraction. The DNA extraction was performed using Fast DNA spin kit (BIO101, Qbiogene, Carlsbad, CA) according to the manufacturer's instructions.

Two TaqMan qPCR assays were applied to quantify the 16S rRNA gene copy numbers of "*Ca.* B. sinica" and "*Ca.* J. caeni", respectively (Zhang et al., 2017). TaqMan qPCR assay for "*Ca*. K. stuttgartiensis" was newly developed in this study (**Table S1**). The qPCR mixture (20 μL) contained 10 μL of 2× Premix ExTaq (Takara Bio), 0.4 μL each forward and reverse primers (10 μM), 0.8 μL TaqMan probe (10 μM), 0.4 μL of 50× ROX Reference Dye II (Takara Bio), and 2 μL template DNA. The qPCR was run in MicroAmp Optical 96-well reaction plates (Applied Biosystems) and ABI prism 7500 sequence detection system (Applied Biosystems) with the following reaction conditions; 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 34 sec at 60°C. The standard curves for quantification were prepared by the 10-fold dilutions of plasmid DNA containing the target gene molecules ranging from 10^7 to 10^3 copies μL^{-1}. The amplification efficiencies were calculated as described previously (Ishii et al., 2013). "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. K. stuttgartiensis" possess one (Oshiki et al., 2015), two (Hira et al., 2012) and one (Strous et al., 2006) *rrn* operon in their genomes, respectively.

5.3.6 Fluorescence in situ hydridization

Fluorescence *in situ* hybridization (FISH) analysis was conducted to evaluate the spatial distribution of each anammox species in gel beads and self-aggregated granules. However, the percentage of "*Ca*. K. stuttgartiensis" was too low to be effectively detected by FISH for all samples. Oligonucleotide probe AMX820 (Schmid et al., 2001) or BRS211 (newly designed in current study, optimal formamide (FA) concentration was experimentally determined as 40%) was used to detect "*Ca.* B. sinica". A new probe (JEC152) for "*Ca.* J. caeni" was designed using the ARB software (Ludwig et al., 2004) (**Table S2**). Optimal FA concentration for JEC152 was determined to be 20% as described previously (Manz et al., 1992). EUB mix probe composed of an equimolar EUB338 I, II and III was used to target most member of *Eubacteria*. No cross hybridization occurred between each species was confirmed experimentally. Biomass samples were fixed in 4% paraformaldehyde solution as described previously (Pernthaler et al., 2001). Hybridization was performed according to the protocol described previously (Okabe et al., 1999; Tsushima et al., 2007a). Cells were observed under confocal laser scanning microscope (Fluoview FV 300, Olympus, Tokyo, Japan), equipped with multi Ar laser (458nm, 488nm, and 514nm) and HeNe

laser (543 nm and 633 nm). The images were analyzed by the software package (ver. 2.0) provided with the microscope.

5.4 RESULTS

5.4.1 Monod growth curves

The classical Monod model with the previously reported μ_{max} and *Ks* for nitrite was used to simulate the specific growth rates of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" as a function of nitrite concentration at 37°C (**Table 1** and **Fig. 1**). "*Ca.* B. sinica", which has the highest μ_{max}, would overgrow at a nitrite concentration of > 17 - 30 μM, below which "*Ca.* K. stuttgartiensis", which has the highest affinity to NO_2^- (lowest *Ks*), would win the competition. That is why it was hypothesized that "*Ca.* B. sinica" is a growth rate (*r*) strategist and "*Ca.* K. stuttgartiensis" is an affinity (*K*) strategist (Strous et al., 1999). In contrast, "*Ca.* J. caeni" seems to be outcompeted by other two species under any NO_2^- concentration, indicating the weakest competitor among the three anammox species.

5.4.2 Competition in gel-immobilized column reactors

Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" immobilized in PVA-SA gel beads cultured in up-flow column reactors were measured using qPCR assays. In reactor G1 (NLR = 0.5 kg-N d^{-1} m^{-3}) (**Fig. 2, A1 - C1**), a stable coexistence of "*Ca.* B. sinica" and "*Ca.* J. caeni" was established during the competition experiment. The two species similarly increased with time; hence their relative abundances were virtually constant during the entire experiment. Only "*Ca.* K. stuttgartiensis" population (one order of magnitude lower than "*Ca.* B. sinica" and "*Ca.* J. caeni") decreased gradually (**Fig. 2, B1**).

In reactor G2 (NLR = 0.7 kg-N d^{-1} m^{-3}) (**Fig. 2, A2 – C2**), only "*Ca.* B. sinica" population increased after a short lag phase of 8 d and dominated total anammox population, while both populations of "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" continuously decreased with time (**Fig. 2, B2**). The similar dominance of "*Ca.* B. sinica" over "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis was observed in reactor G3 (NLR = 2.7 kg-N d^{-1} m^{-3}) (**Fig. 2, A3 - C3**). It should be noted that the dominance of "*Ca.* B. sinica"

over "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis could not be reverted by lowering the NLR (data not shown).

5.4.3 Competition in MBRs

In MBR M1 (NLR = 0.07 kg-N d^{-1} m^{-3} and NO_2^- = 0.0 - 0.36 mM, **Fig. 3, A1**), interestingly only "*Ca.* J. caeni" population increased with time, outcompeted "*Ca.* B. sinica" and "*Ca*. K. stuttgartiensis", and dominated the anammox population (**Fig. 3, B1** and **C1**). "*Ca.* J. caeni" and "*Ca.* B. sinica" were unable to grow. The relative abundances of "*Ca.* J. caeni", "*Ca.* B. sinica", and "*Ca*. K. stuttgartiensis" after 60 days of cultivation were about 75%, 23%, and 2%, respectively.

In MBR M2 (NLR = 0.10 kg-N d^{-1} m^{-3} and NO_2^- = 0.0 - 3.88 mM, **Fig. 3, A2**), all three species decreased gradually from the beginning of incubation, because the NLR was not sufficiently high enough to support relatively high initial biomass concentrations (**Fig. 3, A2** and **B2**). However, a stable coexistence of "*Ca.* B. sinica" and "*Ca.* J. caeni" was observed. The relative abundance has remained unchanged (ca. 50% each) for about 25 days, but thereafter the "*Ca.* B. sinica" gradually displaced "*Ca.* J. caeni" (**Fig. 3, C2**). Unexpected high nitrite concentrations appeared at the beginning of operation was resulted from the lag phase of the anammox bacteria.

In MBR M3 (NLR = 0.14 kg-N d^{-1} m^{-3} and NO_2^- = 0.0 - 0.43 mM, **Fig. 3, A3**), only "*Ca.* B. sinica" could maintain its population, while the populations of "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis" gradually decreased due to low NLR (**Fig. 3, A3** and **B3**). After 60 days of cultivation, "*Ca.* B. sinica" dominated (83%) the anammox populations, and "*Ca.* J. caeni" accounted for 17% (**Fig. 3, C3**).

In MBR M4 (NLR = 0.17 - 0.42 kg-N d^{-1} m^{-3} and NO_2^- = 0.0 to 0.7 mM, **Fig. 3, A4**), after a short lag phase, only "*Ca.* B. sinica" gradually increased to 87% of total anammox population, whereas "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis" decreased (**Fig. 3, A4** and **B4**). The relative abundance of "*Ca.* J. caeni" decreased from ca. 50% to 13% after 40 days of cultivation (**Fig. 3, C4**).

5.4.4 Competition in granular reactor

Self-aggregated anammox granules containing three anammox species were established and cultivated in an up-flow column reactor to further investigate the population dynamics under high nitrogen loading rates (NLR= 4.3 – 8.6 kg-N d^{-1} m^{-3}

and NO_2^- = 9 - 11 mM at the bottom of reactor) (**Fig. 4A**). "*Ca.* B. sinica" and "*Ca.* J. caeni" were almost equally abundant in the original granules; however, the abundance of "*Ca*. K. stuttgartiensis" was one order magnitude lower than others (**Fig. 4B**). After 100 days of cultivation, "*Ca.* B. sinica" outcompeted "*Ca.* J. caeni" and "*Ca*. K. stuttgartiensis" and became the dominated species (94%) (**Fig. 4C**). This observation was in good agreement with the competition results of GICR and MBR operated at relatively high NLRs.

5.4.5 FISH analysis

FISH analysis revealed clear habitat segregation within gel beads and self-aggregated granules. Gel beads taken from the GICR G1 at day 69 demonstrated a spherical microcluster-like growth pattern of "*Ca*. J. caeni", which were surrounded by "*Ca*. B. sinica" cells (**Fig. 5A**). The microclusters of "*Ca*. J. caeni" were mainly present in the inner part of the gel beads. The cells of "*Ca.* B. sinica" and "*Ca.* J. caeni" distributed more or less evenly in the original granules at day 0 of competition experiment (**Fig. 5B**). However, after 90 days of cultivation, "*Ca*. B. sinica" was found to proliferate and dominate throughout granules, while "*Ca*. J. caeni" was mainly present at the inner part of granules as spherical microclusters (**Fig. 5C**), similar with the phenomenon observed in gel beads.

5.5 DISCUSSION

Different anammox genera or species have been seldom found in the same habitat or engineered reactors. In addition, population shifts were frequently observed during long-term cultivations in engineered reactors (Kartal et al., 2008, 2007, Park et al., 2015, 2010a, 2010b; van der Star et al., 2008). This clearly indicates a well-defined (but as yet unknown) ecological niche for each anammox bacteria. However, only little is known about the niche differentiation of anammox bacteria so far despite their ecological and practical importance. To our best knowledge, this is the first study to systematically investigate the interspecies competition of anammox bacteria. It was hypothesized that difference in kinetic and physiological characteristics is one of the important key factors determining the niche differentiation. To verify this hypothesis, population dynamics of three phylogenetically distant anammox bacterial species, "*Ca*. B. sinica", "*Ca*. J. caeni" and "*Ca*. K. stuttgartiensis", were experimentally measured in two types of nitrite-limited reactors, a gel-immobilized column reactor (GICR) and membrane bioreactor

(MBR), at different nitrogen loading rates (NLRs). In this study, anammox bacteria simply competed for a common single substrate (i.e., nitrite). Effluent ammonium concentrations in all competition experiments were higher than nitrite (0 mM - 3.88 mM) because the medium with a NH_4^+: NO_2^- ratio = 1 was used as the feed; therefore ammonium was not a limiting substrate . Hence, competition and kinetics are of crucial importance for the population dynamics.

The observed competition results demonstrated that the individual anammox species was able to develop specific niches due to their different responses to different NLRs. "*Ca*. B. sinica" was able to outcompete "*Ca*. J. caeni" and "*Ca*. K. stuttgartiensis" at high NLRs in both GICR and MBR, whereas "*Ca*. J. caeni" was able to proliferate only at low NLRs. The NLR is directly linked to the growth rate of anammox bacteria.

The specific growth rates of the three anammox species in nitrite-limited continuous reactors were also simulated using the classical Monod model with the currently available kinetic parameters K_s and μ_{max} (**Table 1**). Although Monod growth curve is not sufficient enough to describe the population dynamics of each species, it still could simulate the specific growth rates upon substrates, which is the vital parameter in the competition. As the simulation demonstrated, "*Ca*. B. sinica" possess higher specific growth rate than "*Ca*. J. caeni" under any nitrite concentrations (**Fig. 1**). However, this is in contradiction to the observed experimental results. The successful competition of "*Ca*. J. caeni" at low NLR could not be sufficiently explained with the difference in the currently available kinetic parameters, especially *Ks*, because the *Ks* values of both species were virtually the same (**Table 1**). The *Ks* values of "*Ca*. J. caeni" might not be accurate enough because it seems that it was determined from the flocculated biomass (the average diameter was 150 μm) (Ali et al., 2015) and thus regarded as apparent one. In fact, the *K*s value is significantly affected from the existence of substrate diffusion limitation (Lotti et al., 2014). In addition, the reported *K*s values for "*Ca*. B. sinca" and "*Ca*. K. stuttgartiensis" varied widely (**Fig. 1**).

A sensitivity analysis of substrate affinity to nitrite (*Ks*) for "*Ca.* J. caeni" with fixed maximum specific growth rate (μ_{max}) in **Table 1** was performed and compared with "*Ca.* B. sinica". The tested *Ks* were 0.1, 5 and 35.6 ± 0.92 (original) μM (**Fig. S1**). To grow over "*Ca*. B. sinica", whose *Ks* ranges from 13 – 55 μM, *Ks* for "*Ca*. J. caeni" had to be reduced to 5 μM and when nitrite concertation is below 4 μM, respectively. The nitrite concentration in M1 reactor during the initial period (~ day 4) was mostly below the detection limit (1 μM), makes it possible for "*Ca*. J. caeni" to grow opportunely by re-

considering the *Ks*. Taken together, sensitive analysis implied that expected competition result is greatly dependent on the accuracy of *Ks*. Therefore, a re-evaluation of *Ks* using planktonic free-living enriched cells for "*Ca*. J. caeni" without any substrate limitation is strongly recommended.

Kinetic parameters such as μ_{max} and *Ks* for "*Ca*. B. sinica", "*Ca*. J. caeni", and "*Ca*. K. stuttgartiensis" were determined at 37°C (**Table 1**), which was an optimum temperature of all three species (Ali et al., 2015; Oshiki et al., 2011; van der Star et al., 2008). The effect of temperature on the obtained competition results could be excluded. Furthermore, since both "*Ca*. B. sinica" and "*Ca*. J. caeni" have been successfully enriched and cultured using the same medium composition (Zhang et al., 2017), the medium composition should not be a differentiating factor for these two species.

Besides the possible inaccuracy of affinity constant determined previously, the discrepancy between the classical Monod model prediction and experimental data may also suggest an extension of the Monod model by incorporating the concept of maintenance metabolism (Fuchslin et al., 2012). Since anammox bacteria were growing at low NLRs (i.e., low growth rates) in this study, under such conditions the microbial maintenance (endogenous decay) rate significantly impacts on the microbial competition (Füchslin et al., 2012; Müller and Babel, 1996; Tros et al., 1996; van Bodegom, 2007). In M1 reactor, "*Ca*. B. sinica" was outcompeted but in M2 reactor, it could maintain the same percentage with "*Ca*. J. caeni", suggesting a higher minimum substrate concentration (S_{min}) for "*Ca*. B. sinica" to maintain and grow.

According to the extended Monod model, the growth rate of anammox bacteria is directly linked to the nitrogen removal rate and maintenance rate which was assumed to be proportional to biomass concentration (*X*) (Tros et al., 1996).

$$r_g = Y\, r_s - m\, X = \mu_{max}\, (S / K_s + S)\, X - m\, X \qquad \text{eq. (1)}$$

where r_g is biomass growth rate (g-biomass m^{-3} d^{-1}), *Y* is true growth yield (g-biomass formed g-substrate $consumed^{-1}$), r_s is substrate utilization rate (g-substrate m^{-3} d^{-1}), μ_{max} is true maximum specific growth rate (d^{-1}), *S* is a limiting substrate concentration (g m^{-3}), *Ks* is half saturation constant (g m^{-3}), *X* is biomass concentration (g-biomass m^{-3}), and *m* is specific maintenance rate (or endogenous decay rate) (d^{-1}). The net growth rate (μ_{net}) can be obtained by dividing eq. (1) by X as $\mu_{net} = \mu - m$. Thus, the biomass growth in the reactors was determined from the specific growth rate (μ) and specific maintenance rate (m).

The substrate concentration at which the net bacterial growth is zero (S_{min}) can be related to *m* and expressed as follows;

$$S_{min} = m\,Ks / (Y\,q_{max} - m) \qquad \text{eq. (2)}$$

where, q_{max} is maximum specific substrate utilization rate (g-substrate g-biomass^{-1}d^{-1}). However, the specific maintenance rate, *m*, of anammox bacteria species is currently lacking because of a limited numbers of enrichment cultures and experimental difficulty for slow-growing anammox bacteria (Fuchslin et al., 2012). Only a wide range of *m* values was reported for "*Ca*. B. sinica" (**Table 1**). In addition, the maintenance rate was considered to be strain, substrate and growth conditions dependent (Fuchslin et al., 2003; Tros et al., 1996). Thus, the *m* of anammox bacteria should be accurately determined or simulated under specific conditions in order to estimate the S_{min} and sufficiently predict the competition between "*Ca*. B. sinica" and "*Ca*. J. caeni" at very low nitrite concentrations.

Functionally similar anammox bacteria cannot coexist in the same environment due to competitive exclusion or niche differentiation unless spatial heterogeneity exists and/or environmental conditions temporally fluctuate. The growth or coexistence of two anammox species, "*Ca*. B. sinica" and "*Ca*. J. caeni", was, however, observed under low NLR conditions in both GICR (G1) and MBR (M1 and M2). In the GICR (G1), spatial gradient (variation) of NO_2^- concentration within the gel bead could be expected due to substrate transport limitation (i.e., low NO_2^- concentration inside gel beads), which may support the coexistence of both species. FISH analysis of gel beads revealed that "*Ca*. J. caeni" was mainly present in the inner part of gel (low NO_2^- environment) as spherical microclusters, while "*Ca*. B. sinica" was present throughout the gel beads (**Fig. 5, A1 – A3**). This spatial distribution supports the observed competition results that "*Ca*. J. caeni" was able to proliferate only under low NLR conditions, suggesting the inner part of gel beads might be suitable for the habitat of "*Ca*. J. caeni". Furthermore, a similar spatial distribution of two species was also observed in self-aggregated granules (**Fig. 5, C1 – C3**). The clustered (aggregated) growth of "*Ca*. J. caeni" could be simply related to nitrite limitation which stimulates bacteria to aggregate (Wimpenny and Colasanti, 1997) and/or explained as a self-protection process to create nitrite concentration scarcity when it was high (Nogueira and Melo, 2006). Other than this unique spatial distribution, the general outcomes of competitions were consistent, suggesting the results be independent of reactor type and biomass form.

The coexistence of both species "*Ca*. B. sinica" and "*Ca*. J. caeni" was also observed in the MBR M1 and M2, even though no significant temporal fluctuation of NO_2^- concentration was observed. In this study, bacterial cells were completely retained in the MBR (no biomass withdraw, meaning no SRT control), and the MBR was operated at very low NLRs. Thus, both species were growing at very low rates or barely maintaining their populations (the biomass growth rate could be nearly equal to or lower than the maintenance (endogenous decay) rate, see eq. (1)), suggesting low competitive exclusion force. Under such conditions, both species could coexist or survive even in MBR. However, "*Ca*. B. sinica" could slowly take over and dominate if the MBR was operated much longer period as seen in **Fig. 3 C2**, suggesting a difference in the species-specific maintenance rate.

Based on extensive analysis of the anammox 16S rRNA gene sequences in the public databases, "*Ca.* Jettenia" was found to be the lowest abundant genus among all anammox bacterial genera (Sonthiphand et al., 2014). Only two species ("*Ca*. j. caeni" and "*Ca*. J. asiatica") under the genus "*Ca*. Jettenia" have been identified and enriched so far (Ali et al., 2015a; Quan et al., 2008). The least detection frequency of "*Ca*. Jettenia" in natural and man-made ecosystems may related to the present competition results demonstrating that "*Ca*. Jettenia" was the weakest competitor among all anammox genera.

Even though the classical Monod model prediction revealed that "*Ca*. K. stuttgartiensis" could outcompete other two anammox species at low nitrite concentrations (< 17 - 30 μM), it failed to grow under all tested conditions in this study. One possible explanation for this result would be that the initial abundance of "*Ca*. K. stuttgartiensis" was the lowest (1 to 3 orders of magnitude lower than others) in all experiments. In our laboratory, "*Ca*. B. sinica", "*Ca*. J. caeni", "*Ca*. Brocadia sp.", and "*Ca*. Scalindua japonica" (a marine species) could be successfully enriched so far (Ali et al., 2015; Awata et al., 2013; Oshiki et al., 2013). However, the cultivation of "*Ca*. K. stuttgartiensis" was never successful in our laboratory regardless of more than 10 year's continuous efforts. This suggests that other unknown factor(s) are missing, for example, trace elements in water used for making culture medium might be missing. Apparently, resolving this missing factor(s) is essential to further understand the niche differentiation of anammox bacteria.

Application of the anammox process to main-stream municipal wastewater treatment is one of the most interesting and challenging topics currently (Ali and Okabe,

2015). In general, the main-stream municipal wastewater is characterized as low temperature and low nitrogen loading rates. It seems reasonable to suggest that "*Ca*. J. caeni", instead of "*Ca*. B. sinica" be selected as candidate species for main-stream application based on our observation, nevertheless, by considering a fluctuation in the influent nitrogen loading rates, "*Ca*. B. sinica" might be more versatile though it's actual performance, especially under low temperature, should be carefully assessed in the future.

5.6 CONCLUSIONS

Microbial competitions for a common substrate (nitrite) among three anammox bacteria species were investigated in nitrite-limited GICR and MBR at different nitrogen loading rates (NLRs). The following conclusions can be withdrawn;

- "*Ca*. J. caeni" could proliferate only at low NLRs, whereas "*Ca*. B. sinica" dominated anammox population at high NLRs in both reactors.
- FISH analysis revealed that "*Ca*. J. caeni" was mainly present as spherical microclusters in the inner part (low NO_2^- environment), whereas "*Ca*. B. sinica" was present throughout gel beads and granules. This spatial distribution could explain the coexistence of "*Ca*. B. sinica" and "*Ca*. J. caeni".
- The growth of "*Ca*. K. stuttgartiensis" could not be observed in all experimental conditions in this study, indicating possible unknown factor(s) was missing.

The classical and extended Monod models could not appropriately explain the outcomes of the competition experiments due to inaccuracy of kinetic parameters such as *Ks* for nitrite and missing information of maintenance rate (*m*). Further (re)evaluation of these kinetic parameters are required.

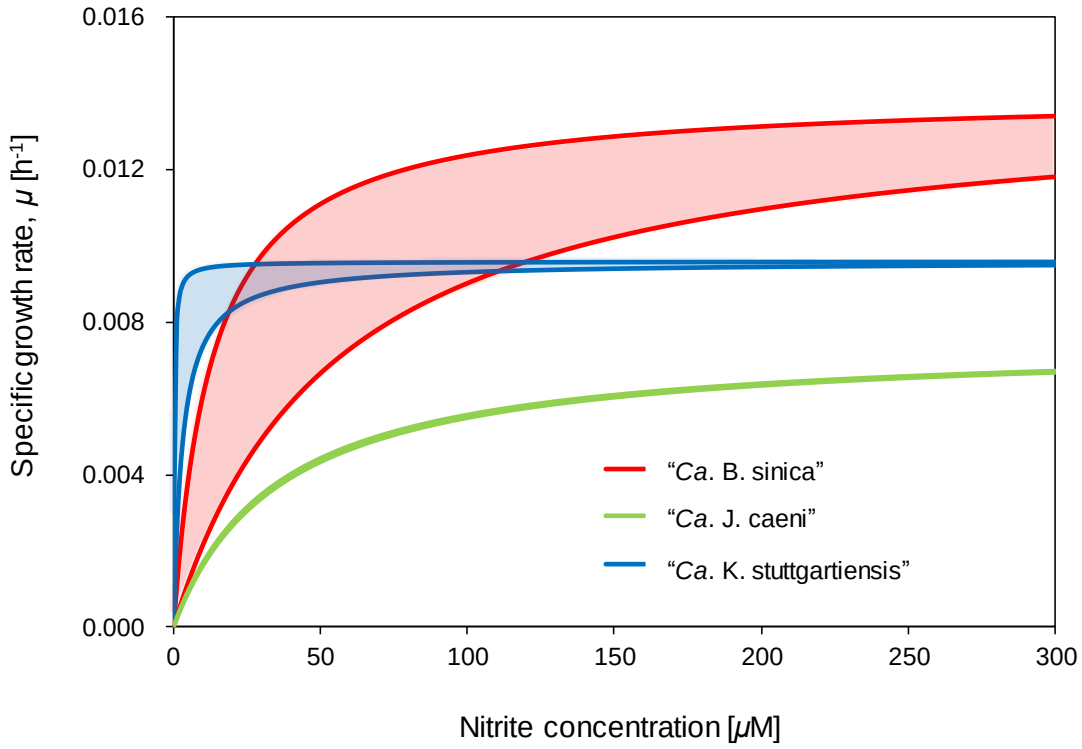


Figure 5.1 The Monod growth curves of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis". The specific growth rate (μ) was simulated as a function of a limiting substrate (nitrite) concentration by using the Monod equation. It should be noted that since the *Ks* values were associated with relatively high standard deviations, the Monod growth curves were calculated using the upper and lower limit of *K*s values for "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis".

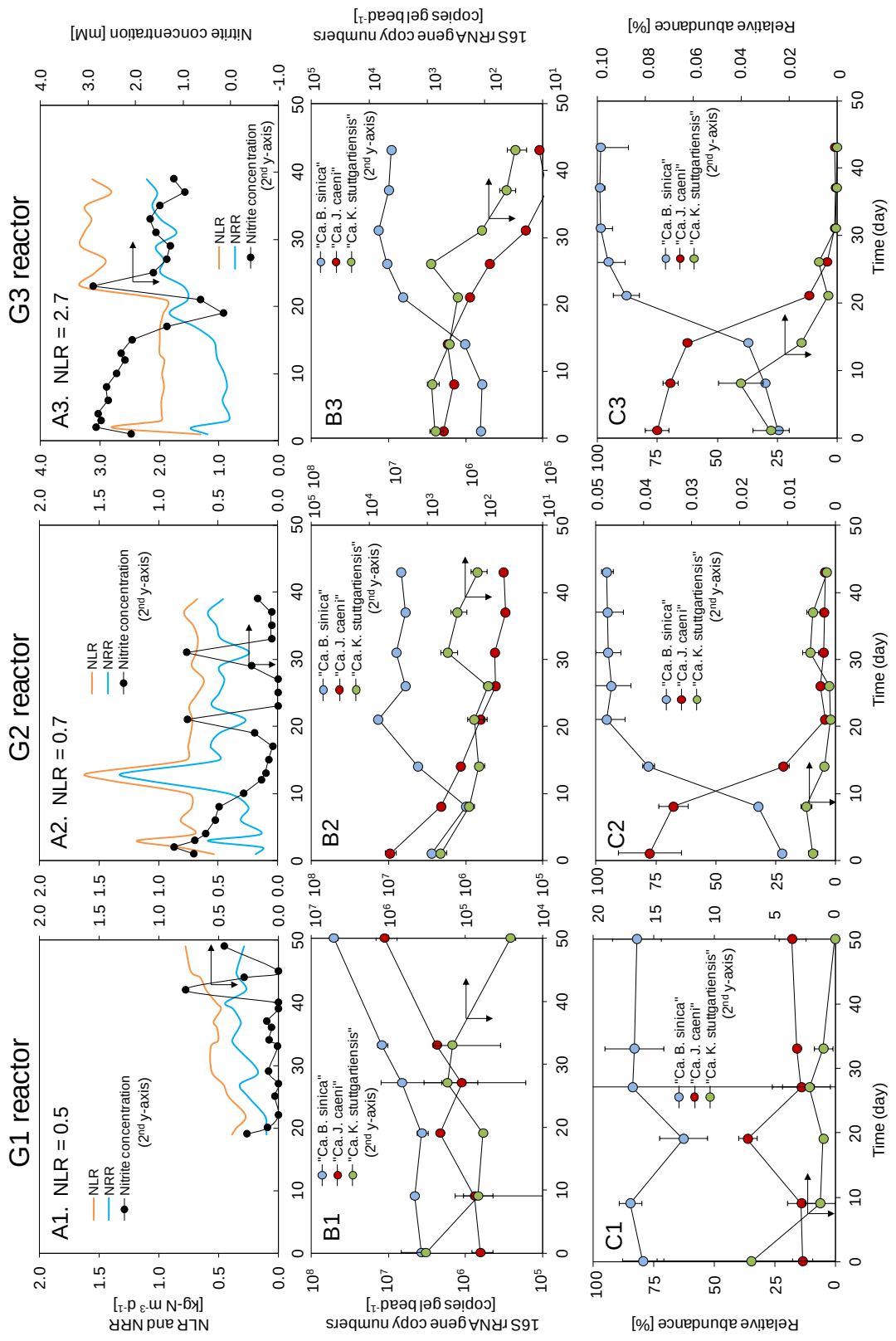
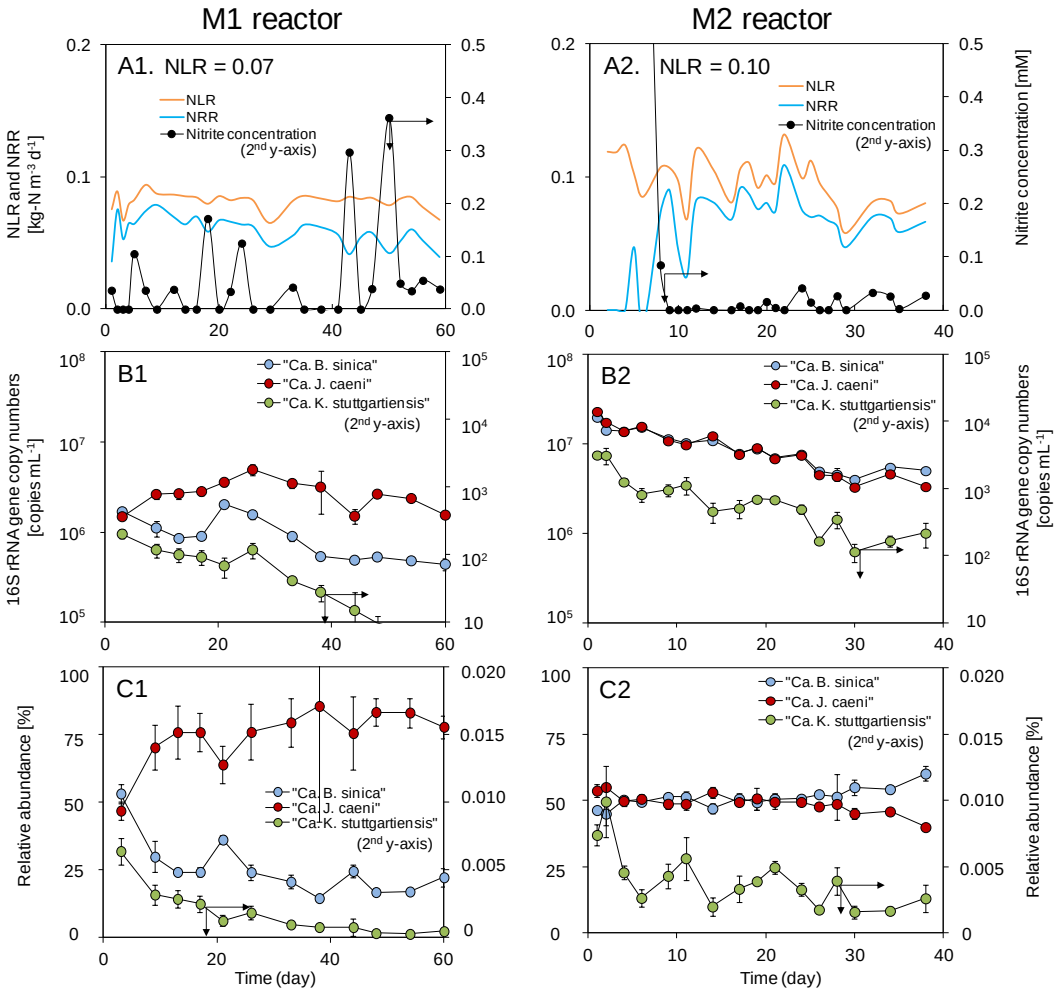


Figure 5.2 Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" in gel-immobilized column reactor (GICR) at 37°C. **A1- A3:** Nitrogen (NH_4^+ and NO_2^-) loading rate (NLR) and removal rate (NRR) and nitrite concentration

at the bottom of the column reactors. **B1- B3:** Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" in GICRs. **C1- C3:** Relative abundance of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis", which were calculated based on the 16S rRNA gene copy numbers.



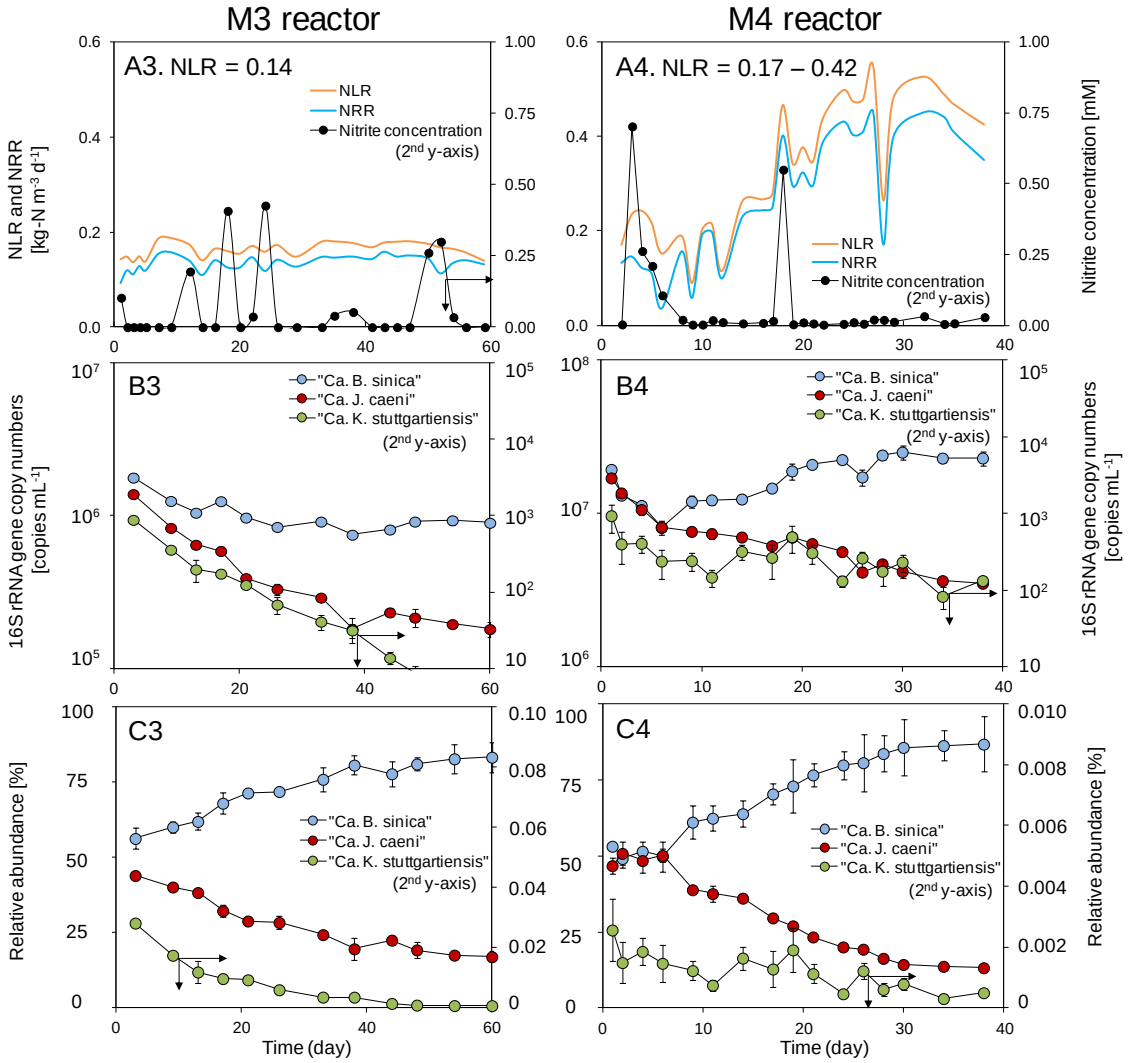


Figure 5.3 Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" in MBRs at 37°C. **A1- A4:** Nitrogen ($NH_4^+$and NO_2^-) loading rate (NLR) and removal rate (NRR) and nitrite concentration in the bulk phase. **B1 – B4:** Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" in MBRs. **C1 – C4:** Relative abundance of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis", which were calculated based on the 16S rRNA gene copy numbers.

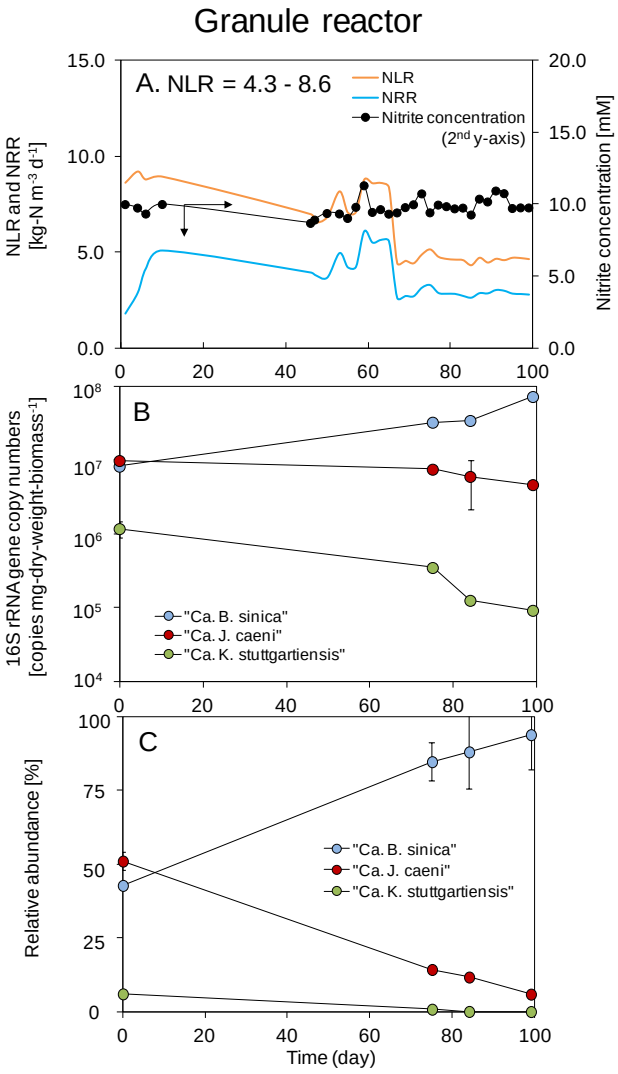


Figure 5.4 Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* Kuenenia stuttgartiensis" in self-aggregated granules in an up-flow column reactor at 37°C. **A:** Nitrogen ($NH_4^+$and NO_2^-) loading rate (NLR) and removal rate (NRR) and nitrite concentration at the bottom of the column reactor. **B:** Population dynamics of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" in the column reactor. **C:** Relative abundance of "*Ca.* B. sinica", "*Ca.* J. caeni" and "*Ca.* K. stuttgartiensis" in granules, which were calculated based on the 16S rRNA gene copy numbers.

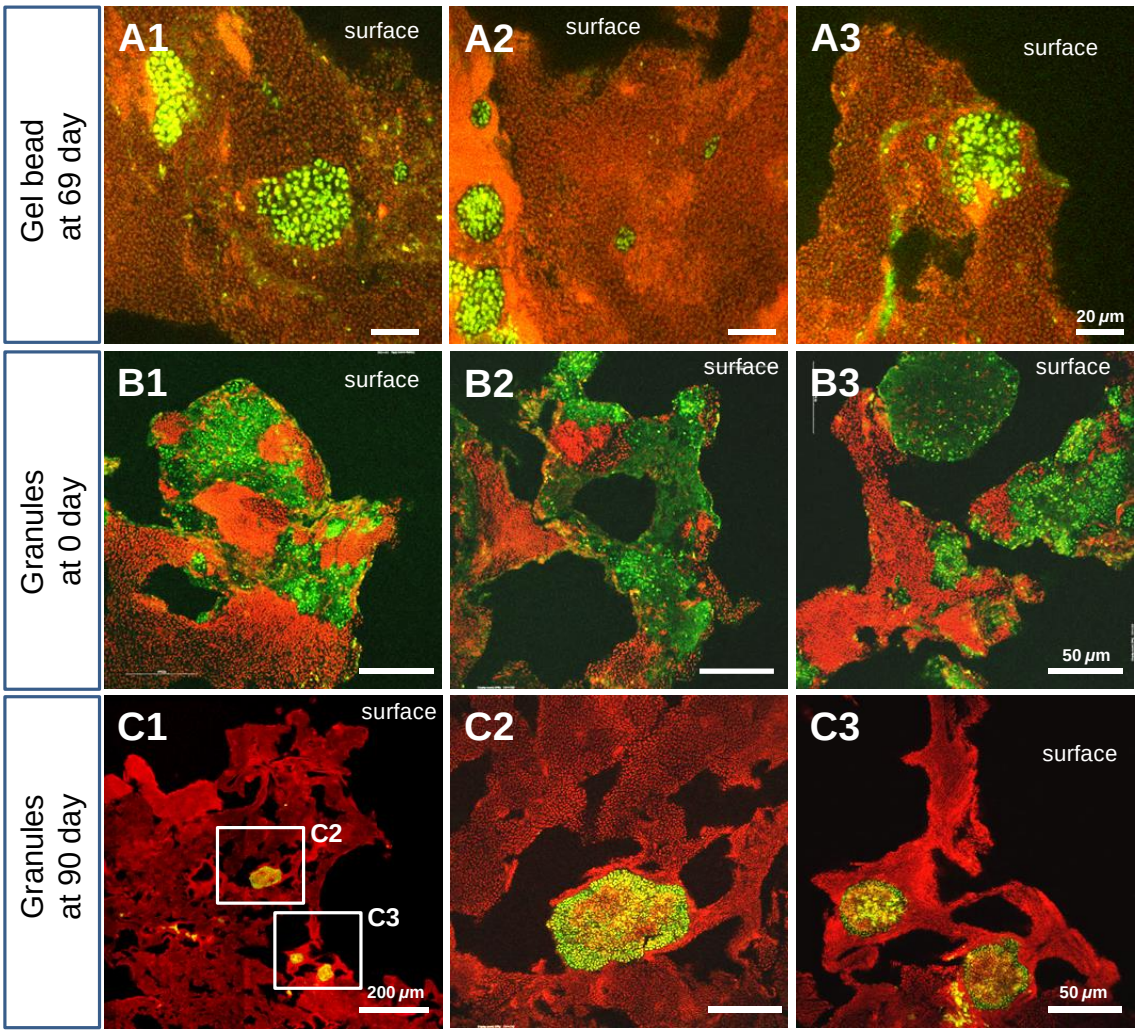


Figure 5.5 Fluorescence *in situ* hybridization (FISH) images of the cross sections of gel beads and self-aggregated granules. The gel beads were taken from G1 reactor on day 69, and the granules were taken on day 0 and 90, respectively. *In situ* hybridization was performed with tetramethylrhodamine-5-isothiocyanate (TRITC) labeled BRS211 probe (red) for "*Ca.* B. sinica" and fluoresceine isothiocyanate (FITC) labeled JEC152 probe (green) for "*Ca.* J. caeni" for all samples. **A1 – A3:** gel beads at day 69, **B1 – B3**: self-aggregated granules at day 0, and **C1 – C3**: self-aggregated granules at day 90. "*Ca*. K. stuttgartiensis" could not be detected in all experiments due to low abundance. "Surface" means the surface of gel beads and granules. Scale bars indicate 20 μm (**A**), 50 μm (**B**), and 50 μm (**C,** except for **C1**, 200 μm). (For color figures, the reader is referred to the web version of this article).

Table 5.1 Physiological characteristics of "*Ca.* Jettenia caeni", "*Ca*. Brocadia sinica" and "*Ca*. Kuenenia stuttgartiensis".

Parameter[a]	"*Ca.* Jettenia caeni"	"*Ca.* Brocadia sinica"	"*Ca.* Kuenenia stuttgartiensis"
Optimal Temp. (°C)	37	37	37
Growth pH	6.5 - 8.5	7.0 - 8.8	6.5 - 9.0
Biomass yield (mmol-C [mmol-NH_4^+]$^{-1}$)	0.056	0.063	n.d[d]
μ_{max} (h^{-1})[b]	0.0075	0.014	0.0096
Affinity constant (K_s)[c]			
NH_4^+ (µM)	17.1 ± 4.3	28 ± 4	n.d
NO_2^- (µM)	35.6 ± 0.92	34 ± 21	0.2 - 3
Maintenance rate, m (h^{-1})	n.d	0.0029 – 0.0081	n.d
Oxidiation rate (µmol g-protein^{-1} min^{-1})			
Formate	6.7 ± 0.6	n.d	5.8 ± 0.6
Acetate	0.79 ± 0.07	n.d	0.31 ± 0.03
Propionate	0.64 ± 0.05	n.d	0.12 ± 0.01
Tolerance			
NO_2^- (mM)	11	<16	13, 25
NH_4^+ (mM)	>20	n.d	n.d
Sulfide (µM)	540	n.d	10 - 300
Salinity (mM)	68 mM	<513 mM	50 - 200 mM
Reference	Ali et al., 2015; Zhang et al., 2017	Oshiki et al., 2011, 2013; Zhang et al., 2017	Egli et al., 2001; Strous et al., 2006; Dapean-Mora et al., 2007; van der Star et al., 2008; Kartal et al., 2007

a: The form of biomass used for characterizing "*Ca.* B. sinica" and "*Ca.* K. stuttgartiensis" were suspended planktonic cells, for "*Ca*. J. caeni", it was in flocculated biomass with an average particle diameter at 150 μm;

b: μ_{max} of "*Ca*. J. caeni" and "*Ca.* B. sinica" were determined based on temporal increase in 16S rRNA gene copy numbers as determined by qPCR, for "*Ca.* K. stuttgartiensis", the μ_{max} was determined based on sludge retention time (SRT) control;

c: *Ks* for ammonium and nitrite were determined based on Michaelis–Menten kinetics;

d: *n.d*; not determined.

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6 TREHALOSE ASSOCIATED OSMOSTRESS RESPONSE IN "*CA*. BROCADIA SINICA" AND "*CA*. SCALINDUA JAPONICA"

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6.1 Abstract

Anaerobic ammonium oxidizing (anammox) bacteria is the newly discovered central contributor to global nitrogen cycle. They have a ubiquitous distribution cross a variety of natural and man-made eco-systems. However, ecological niche differentiation of anammox bacteria remains a black box despite its ecological and practical importance. In this study, we systematically investigated the role of salinity in the niche partitioning between “*Ca*. Brocadia sinica” and “*Ca*. Scalindua japonica”. Individual adaptation and interspecific competition demonstrated the salinity as one important differentiating factor. In addition, we found “*Ca*. B. sinica” synthesize up to $3.9 \times 10^{-2} \pm 6.0 \times 10^{-3}$ μmole mg-BSA^{-1} trehalose upon salinity increase, while “*Ca*. S. japonica” significant incorporate K^+ with limited production of trehalose. These findings lead to a better understanding of niche partitioning from bioenergetic aspects and was able to provide alternative views on natural distribution of anammox bacteria. Lastly, phylogenetic analysis on trehalose biosynthesis (trehalose 6 phosphatase/trehalose 6 phosphate synthase pathways) implied the distinct distance between “*Ca*. B. sinica” and “*Ca*. S. japonica”, raises the probability that this function be acquired from horizonal gene transfer or faded during evolution.

6.2 INTRODUCTION

Standing on 21,752 16S rRNA gene sequences compiled from 111 studies of diverse physical environmental conditions, the major determinant of microbial community composition is found to be salinity rather than temperature, pH, or other physical and chemical factors (Lozupone and Knight, 2007). Although a recent study demonstrated nitrogen loading rate as a factor in the niche differentiation of two freshwater anaerobic ammonium oxidizing (anammox) bacterial species ("Ca. B. sinica" and "Ca. J. caeni") (Zhang et al., 2017a), a statistical study looking at over 6000 anammox 16S rRNA gene sequences from the public database, also indicated salinity as the most important factor governing anammox bacterial distributions, with "Ca. Scalindua" dominated in saline environments (mostly marine) while "Ca. Brocadia" were mostly found in freshwater environments (Sonthiphand et al., 2014). However, verification of salinity as a niche factor for anammox bacteria, especially between so-called marine and freshwater anammox species, has never been systematically investigated. Detailed understanding on the extent of the effect of salinity on adaptation, genetic basis and ecological significance of anammox bacteria are all poorly understood at this moment, despite its importance in understanding the contribution of anammox bacteria to global nitrogen cycle. In fact, both "Ca. Brocadia" and "Ca. Scalindua" have been identified in estuary area with a moderate salinity (Sonthiphand et al., 2014). One study used a mixed population of "Ca. K. stuttgartiensis" and "Ca. S. wagneri" adapting to 3% salinity, after up to one year's operation still 70% of the anammox population belongs to "Ca. K. stuttgartiensis" (Kartal et al., 2006a). These contradictory observations stress the necessity for more precise physiological investigations to reveal the role of salinity in niche partitioning of anammox bacteria.

Bacteria's ability to adapt to fluctuations in the osmolarity of the growth environment is of fundamental importance for their survival (Welsh et al., 1991). To grow in high salinity environments, an intracellular osmolarity that is equal to or higher than that of the surrounding environment is required (Oren, 1999). In general, high intracellular osmotic pressure could be achieved either by uptake and accumulation of inorganic ions such as K+ (salting-in strategy) or by in vivo synthesis and/or uptake of compatible organic solutes (Nieto et al., 1998). It is acknowledged that the strategy of organic osmotic solutes biosynthesis is energetically very expensive, the more so with larger organic molecules such as disaccharides (12 carbon atoms) (Oren, 1999). Therefore, the amount of energy needed to produce the osmotic solutes may greatly

exceed the energy requirement for the biosynthesis of cell components including proteins, nucleic acid, cell walls, etc. In fact, the biomass yield efficiency of “Ca. S. japonica” has been found to be salinity dependent (Awata et al., 2015), might suggest the energy requirement of osmoadaptation. However, details regarding osmostress response of anammox bacteria remains poorly understood at this moment and whether and how this characteristic interferes with the niche partitioning in between either remains to be investigated in more detail.

Here, two anammox species (“Ca. B. sinica” and “Ca. S. japonica”) were focused on. Aiming to evaluate respective response to salinity between freshwater and marine anammox species. We conducted several physiological characterizations including continuous adaptation and interspecific competition combined with physiological measurements and phylogenetic identification. From the results obtained, we demonstrated that salinity is one key factor in niche partitioning between “Ca. B. sinica” and “Ca. S. japonica”. Based on information from genomes and batch tests, we found “Ca. B. sinica” synthesize trehalose, an extremely energy-expensive organic compatible solute under salinity fluctuation. While “Ca. S. japonica” uptook K+ and produce very limited trehalose under salinity change. Taken together, results obtained here suggest that the trehalose associated osmostress response in “Ca. B. sinica” and “Ca. S. japonica” could be one of the factor shaping the distinct niche upon salinity in between.

6.3 MATERIALS AND MEHODS

6.3.1 Anammox bacteria

Planktonic cells of “*Ca*. B. sinica” (> 90%) and “*Ca*. S. japonica” (> 90%) were enriched and cultivated using 2-liter MBRs (Mamoru Oshiki et al., 2013; Zhang and Okabe, 2017) and operated at room temperature. In brief, MBR was equipped with a hollow-fiber membrane unit composed of 300 polyethylene tubes (0.1 μm-pore, 1-mm diam., 70-mm long tubes). A peristaltic pump MP-1000 (EYELA, Tokyo, Japan) was directly connected to the membrane. Water level inside the reactor was controlled using a water level sensor (HL-S1A, ASONE, Japan). The culture medium inside the MBR

was kept mixed using a magnetic stirrer working at 200 rpm. To avoid the contamination of oxygen, mixed gas (Ar: CO_2 = 95:5) was purged into the cell culture at a flowrate of 10 ml min^{-1}. Throughout the cultivation, pH was not controlled but it was always maintained within the range of 7.0 - 8.0. The inorganic nutrient medium was supplied containing (mg L^{-1}): $FeSO_4 \cdot 7H_2O$ (9.0), EDTA·4Na (5.0), NaCl (1.0), KCl (1.4), $CaCl_2 \cdot 2H_2O$ (1.4), $MgSO_4 \cdot 7H_2O$ (1.0), $NaHCO_3$ (84), KH_2PO_4 (54). For "*Ca*. S. japonica", the inorganic nutrient medium was supplied with 20 g L^{-1} of SEALIFE (Marine Tech, Tokyo, Japan), an artificial sea salt, $CaCl_2 \cdot 2H_2O$ (114 mg L-1), $MgSO_4 \cdot 7H_2O$ (99 mg L^{-1}), $KHCO_3$ (500 mg L^{-1}), KH_2PO_4 (24.4 mg L^{-1}), 0.5 mL L^{-1} trace element solution I & II (van de Graaf et al., 1996). The detailed information of the artificial mineral medium of SEALIFE was described previously (Kindaichi et al., 2011; Mamoru Oshiki et al., 2013).

6.3.2 Specific anammox activity (SAA) tests

SAA was measured according to previous description (Oshiki et al., 2011). In brief, Biomass were collected and washed with mineral medium without NH_4^+ and NO_2^- for three times and 5 mL of biomass were dispensed into 12.5 mL glass serum vials sealed with butyl rubber stoppers and aluminum caps. Gas in the headspace of the vials was exchanged with highly pure Helium gas (>99.9999%) by vacuuming and purging using a gas exchange equipment (model IP-8, SANSHIN, Yokohama, Japan). Vials were incubated at room temperature for "*Ca*. S. japonica" and 37 °C for "*Ca.* B. sinica" overnight to consume the residual ammonium and nitrite. Before batch tests, ^{14}N-NH_4Cl, ^{15}N-$NaNO_2$ were added from anoxic stock solutions to each vial using gas tight syringe (VICI, Baton Rouge, LA, USA). Salinity was adjusted by changing the concentration of SEALIFE. Incubation was continued for 4 h. Fifty microliter of headspace gas was collected at a frequency of one hour and subjected 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. Concentration of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ gas were determined by gas chromatography mass spectrometry for quantitative analysis according to previous description (Amano et al., 2007; Waki et al., 2010). The specific ammonium oxidation rate was determined by measuring $^{29}N_2$ production rate. Calculation was based on a standard curve prepared with $^{30}N_2$ standard gas (>98% purity, Cambridge Isotope

laboratories, Tewksbury, MA, USA) (M Oshiki et al., 2013). Biomass concentration subjected to batch analysis was determined as protein concentration and converted to g-VSS L^{-1} as described before (Oshiki et al., 2011). In brief, collected biomass was subjected to centrifugation (18,200×g for 10 min) and the biomass pellet was resuspended in 1 mL 10% (w/v) sodium dodecyl sulfate (SDS) solution. Protein concentration in the supernatant was determined using Lowry method (LOWRY et al., 1951) with DC protein Assay Kit (Bio-Rad) following manufacturer’s instruction. All the anaerobic activity tests were carried out as duplicate.

6.3.3 Adaptation and competition in MBR

Before conducting competition experiment, culture of “*Ca*. B. sinica” and “*Ca*. S. japonica” were first adapted to 1% salinity (increase/decrease salinity stepwisely). For “*Ca*. B. sinica”, ammonium and nitrite concentration was started with 10 mM and reduced to 5.0 mM upon increase of salinity, repectively. Nitrite concentration in bulk phase was carefully monitored to avoid nitrite inhibition when increase the salinity. For “*Ca*. S. japonica”, ammonium and nitrite concentration was kept at 10 mM along with decrease of salinity.

After successful adaptation, two cultures were mixed as equal 16S rRNA gene copy numbers. In the competition experiment, the inorganic nutrient medium was supplied containing: 10 g L^{-1} of SEALIFE (Marine Tech, Tokyo, Japan) as 1% salinity, $CaCl_2 \cdot 2H_2O$ (114 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (99 mg L^{-1}), $KHCO_3$ (500 mg L^{-1}), KH_2PO_4 (24.4 mg L^{-1}), 0.5 mL/L trace element solution I&II (van de Graaf et al., 1996). The ammonium and nitrite were started with 2 mM and increased to 8 mM stepwisely.

6.3.4 Chemical analysis

The water samples from bulk phase of the MBRs were collected, filtered through 0.2-μm-pore membranes (Advantec, Tokyo, Japan), and analyzed for concentration of NH_4^+, NO_2^- and NO_3^- using ion-exchange chromatography (DX-100, DIONEX, Sunnyvale, CA) with an Ionpac CS3 cation column and IonPac AS9 anion column (Okabe et al., 2011). In addition, the NO_2^- concentrations from MBR were also

measured colorimetrically according to the Standard Methods (Eaton et al., 2005). Water quality of "*Ca*. S. japonica" culture and competition containing salinity was measured only using colorimetric method according to standard methods (Eaton et al., 2005) due to the high ionic strength of the medium. Nitrogen loading rate (NLR) and nitrogen removal rate (NRR) were calculated as described previously (Tsushima et al., 2007).

6.3.5 DNA extraction and quantitative PCR

Suspended biomass (2 ml) in MBR were collected routinely to following the population dynamics in competition study. The biomass samples were centrifuged at 14000 rpm for 10 min and biomass pellets were preserved at -80°C till DNA extraction. The DNA extraction was performed using Fast DNA spin kit (BIO101, Qbiogene, Carlsbad, CA) per manufacturer's instructions.

TaqMan qPCR assays were applied to target the 16S rRNA genes of "*Ca.* B. sinica" and "*Ca*. S. japonica" to follow their population dynamics (Zhang et al., 2017b). Possible contamination between two cultures were also confirmed using qPCR, where no cross-contamination was observed (data not shown). The qPCR mixture (20 μL) contained 10 μL of 2× Premix ExTaq (Takara Bio), 0.4 μL each forward and reverse primers (10 μM), 0.8 μL TaqMan probe (10 μM), 0.4 μL of the 50× ROX Reference Dye II (Takara Bio), and 2 μL template DNA. The qPCR was run in MicroAmp Optical 96-well reaction plates (Applied Biosystems) and ABI prism 7500 sequence detection system (Applied Biosystems) with the following reaction conditions. 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 34 sec at 60°C. The standard curves for quantification were prepared by the 10-fold dilutions of plasmid DNA containing the target gene molecules ranging from 10^7 to 10^3 copies/μl. The amplification efficiencies were calculated as described previously (Ishii et al., 2013). "*Ca*. B. sinica" and "*Ca*. S. japonica" possesses one *rrn* operon in their genomes, respectively (Oshiki et al., 2017, 2015).

6.3.6 Fluorescence in situ hybridization

Purity of the adapted enrichment culture of “*Ca.* B. sinica” and “*Ca.* S. japonica” were examined using fluorescence *in situ* hybridization (FISH) analysis. Oligonucleotide probe AMX820 was used to detect 16S rRNA gene of “*Ca.* B. sinica” (Schmid et al., 2000, 2005). Probe Sca1129b was used to target 16S rRNA gene of “*Ca.* S. japonica” (Kindaichi et al., 2011). The AMX820 and Sca1129b probes were synthesized and labeled at the 5’ end with the tetramethylrhodamine (TRITC) or fluorescein isothiocyanate (FITC) by TakaraBio. Biomass samples were collected from the reactors and fixed in 4% paraformaldehyde solution as described previously (Pernthaler et al., 2001). The fixed cells were washed three times with phosphate buffered saline solution (pH 7.2) using filter column with 0.2-μm-pore black polycarbonate membrane filters (25 mm-diam; Adventec). Hybridization was performed according to the protocol described previously (Okabe et al., 1999). Cells were observed under confocal laser scanning microscope (Fluoview FV 300, Olympus, Tokyo, Japan), equipped with multi Ar laser (458nm, 488nm, 514nm), HeNe laser (543nm) and HeNe laser (633nm). The image was analyzed by the software package (ver. 2.0) provided with the microscope.

6.3.7 Genomic acquisition and phylogenetic analysis

Presence and absence of functional proteins related to ion transportation (Na^+:H^+ antiporter, a multisubunit Na^+:H^+ antiporter similar to MnhABCDEFG and K^+ uptake) and organic compatible compounds biosynthesis (glycerol, glycine betaine, ectoine, glucosylglycerol, sucrose and trehalose) (Oren, 1999) were examined in reported genomes of anammox species using blast protein search.

Protein sequences of trehalose 6 phosphatase/trehalose 6 phosphate synthase from publicly available “*Ca.* S. japonica” genome were blasted using NCBI protein blast. Protein sequences of other bacterial and archaeal combined with ones from anammox species were aligned and used to construct a maximum likelihood tree in Mega (Tamura et al., 2011).

6.3.8 Quantification of Na^+, K^+ and trehalose measurements.

Biomass in the measurement of SAA was used for determining Intracellular concentration of Na^+, K^+ and trehalose. Briefly, intracellular concentration of Na^+ and K^+ was determined using inductively coupled plasma emission spectrometer (ICPE-9000, Shimadzu, Japan). Extraction of intracellular Na^+, K^+ followed previous reports (Youssef et al., 2014). In brief, 5 mL biomass suspension was collected and subjected to centrifuge at 10 000 g and 10 min. cell pellets were washed in sterile isotonic NaCl solution. After removal of all traces of medium, the pellet was frozen at -20ºC overnight followed by thawing in 1 ml solution of 10% perchloric acid (PCA). Thawed pellets were vortexed for 5 min, followed by incubation at room temperature for 2 hours during which cells lysed and proteins precipitated. Extracts were then centrifuged at 10 000 g for 15 min. Protein concentration was determined according to Lowry method. The supernatant, containing the cell-free, protein-free, extract was used for quantification of intracellular ions.

Intracellular trehalose concentration was determined after the SAA tests and procedure was performed according to previous reports with little modification (Parrou and François, 1997). Firstly, 5 mL biomass suspension was collected and subjected to centrifuge (10 min at 10 000 g at 4 °C). Cell pellets were carefully drained to remove the culture medium, resuspended in 0.25 mL of 0.25 M Na_2CO_3 using pipetting of Eppendorf tubes, and incubated at 95 °C for 4 h. The mixture was brought to pH 5.2 by addition of 0.15 mL of 1 M acetic acid and 0.6 mL of 0.2 M Na-acetate, pH 5.2. After mixing, mixture was centrifuged at 14000 rpm for 10 min to recover the supernatant. Concentration of trehalose in the supernatant was directly determined enzymatically (Neves et al., 1994) using a commercially available trehalose assay kit (Megazyme, Wicklow, Ireland). Protein concentration was determined using Lowery method.

6.4 RESULTS

6.4.1 Adaptation of “*Ca*. B. sinica” and “*Ca*. S. japonica” culture

Whether salinity is the factor of niche partitioning was illustrated by individual adaptation and interspecific competition. Cultures of “*Ca.* B. sinica” and “*Ca.* S. japonica” were firstly adapted to 1% salinity under room temperature (**Fig. 1)**. In culture of “*Ca.* B. sinica”, nitrogen loading rate was increased from 0.3 to 1.2 kg-N d^{-1} m^{-3} and reduced to 0.3 kg-N d^{-1} m^{-3} when salinity started to increase till 1% (**Fig. 1, A1**). For “*Ca.* B. sinica”, the whole adaptation process took up to 90 days before stable performance could be obtained. Similarly, nitrogen loading rate in the culture of “*Ca.* S. japonica” was maintained at 0.5 kg-N d^{-1} m^{-3} while salinity was gradually decreased from 2% to 1% (**Fig. 1, B1**). Compared with “*Ca.* B. sinica”, this adaptation process for “*Ca.* S. japonica” was considerably short (15 days). During the whole operation, nitrite concentration in bulk phase was always maintained close to 0 mg L^{-1} to avoid nitrite inhibition. The stoichiometry of nitrite to ammonium consumption was 1.25 ± 0.33 in “*Ca.* B. sinica” culture, 1.23 ± 0.12 in “*Ca.* S. japonica” culture, respectively (**Fig. 1, A2&B2**). The purity of both culture after successful adaptation was examined using FISH as illustrated in **Fig. 1, A3&B3**. More than 95% purity for both cultures were obtained.

6.4.2 Competition between “*Ca.* B. sinica” and “*Ca.* S. japonica”

Competition of adapted “*Ca.* B. sinica” and “*Ca.* S. japonica” at 1% salinity was started by directly mixing equal quantity as 16S rRNA gene copy numbers (**Fig. 2**). Nitrogen loading rate was increased from 0.08 to 0.51 kg-N d^{-1} m^{-3} (**Fig. 2, A1**). At day 20, we observed a sharp increase in the nitrite concentration up to 37 mg-N L^{-1} in bulk phase (**Fig. 2, A2**), therefore nitrogen loading rate was reduced to 0.24 kg-N d^{-1} m^{-3} and nitrite accumulation disappeared accordingly. Population dynamics corresponded closely with the reactor performance. During the initial phase, we observed a constant level of “*Ca.* S. japonica” 16S rRNA gene copy numbers with a dramatical decrease of “*Ca.* B. sinica”. Thereafter, from day 16, “*Ca.* S. japonica” started growing

exponentially and completely outcompeted "*Ca.* B. sinica" (relative abundance of "*Ca.* S. japonica" increased from 45.9% at day 0 to 96.8% at day 33) (**Fig. 2, A3**).

6.4.3 Osmoadaptive capabilities inferred from genomes.

We queried the "*Ca*. B. sinica" and "*Ca*. S. japonica" genomes (Oshiki et al., 2017, 2015) for genes putatively involved in osmoadaptation. For ion transportation, at least two Na^+ extrusion mechanisms employing the proton electrochemical gradient as the driving force for exporting Na^+ exist in the genome of "*Ca*. S. japonica": A Na^+: H^+ antiporter of the NhaA, NhaD family, and a multisubunit Na^+: H^+ antiporter similar to MnhABCDEFG. However, Na^+: H^+ antiporter of the NhaA, NhaD family was missing in the genome of "*Ca*. B. sinica". As for potassium uptake, 2 clusters similar to the ATP-dependent low affinity K^+ transporter (Trk) exist in both genomes, each consisting of the membrane component TrkH and the NAD-binding component TrkA. A voltage-gated K^+ channel of the Kef-type, most probably functioning as a facilitated diffusion K^+ uniporter driven by the membrane potential (Youssef et al., 2014), was also identified in both genomes.

On the other hand, genes responsible for compatibles organic solutes were also examined in both genome. As shown in **Table 1**, we examined both genomes for genes responsible for possible organic compatible solutes, including glycerol, glycine betaine, ectoine, glucosylglycerol, sucrose and trehalose (Oren, 1999). Genes for glycerol, glycine betaine, ectoine and sucrose are missing in both genomes. Genome of "*Ca*. B. sinica" contained only one copy of glucosylglycerol-phosphate synthase (EC number: 2.4.1.213) part of the synthesizing system while "*Ca*. S. japonica" genome didn't possess related genes. More importantly, both genomes contain genes necessary for the synthesis of trehalose (**Table 2**). Out of five mechanisms known for trehalose biosynthesis (Avonce et al., 2006), three were identified in the genome of "*Ca*. B. sinica" with one being found in the genome of "*Ca*. S. japonica". In "*Ca*. S. japonica", only genes encoding TPS/TPP pathway were identified: the nucleotide triphosphate diphosphatease (EC number: 3.6.1.9, KO number: K04765) responsible for converting a-D-glucose-1P to UDP-glucose, trehalose 6-Phosphate synthase (EC number: 2.4.1.15, KO number: K00697) responsible for converting UDP-Glucose to Trehalose 6-phosphate, trehalose 6-phosphate phosphatase (EC number: 3.1.3.12, KO number: K01087) responsible for converting trehalose 6-phosphate to trehalose. In the genome

of "*Ca*. B. sinica", in addition to above TPS/TPP pathway, another two pathways were identified. TreT pathway: Trehalose glucosyltransferase (EC number: 2.4.1.245, KO number: K13057) responsible for converting ADP-glucose to trehalose. The third pathway could be separated into two pathways using glycogen as the source with different intermediates (maltooliogosyltrehalose or maltose). TreY/TreZ pathway: maltooligosyl-trehalose synthase (TreY, EC number: 5.499.15, KO number: K06044) responsible for converting glycogen into maltooligosyltrehalose and Maltooligosyl-Trehalose Trehalohydrolase (TreZ, EC number: 3.2.1.141, KO number: K01236) responsible for converting maltooligosyltrehalose to trehalose. TS pathway: maltose alpha-D-glucosyltransferase / alpha-amylase (EC number: 3.2.1.1, KO number: K05343) responsible for converting glycogen into maltose, maltose alpha-D-glucosyltransferase / alpha-amylase (EC number: 5.499.16, KO number: K05343) responsible for converting maltose to trehalose.

6.4.4 Specific anammox activity, trehalose biosynthesis and Na^+, K^+ uptake.

To experimentally identify and verify the osmoadaptative mechanisms adopted by "*Ca.* B. sinica" and "*Ca.* S. japonica", batch tests with measurement of specific anammox activity (SAA), intracellular trehalose and K^+ concentration were conducted (**Fig. 3 & 4**). At 0% salinity, biomass of "*Ca.* B. sinica" had a SAA of 1816.1 ± 183.4 μmole g-VSS^{-1} h^{-1}. At 0.5% salinity, the SAA maintained but reduced to 100.5 ± 36.2 μmole g-VSS^{-1} h^{-1} at 3% salinity. No SAA could be detected at 4%, 5% salinity and negative control without substrates (**Fig. 3A**). Intracellular trehalose concentrations in "*Ca.* B. sinica" cells were highest when grown at low salinities (0.5% and 1%), yielding $3.8 \times 10^{-2} \pm 2.6 \times 10^{-3}$ and $3.9 \times 10^{-2} \pm 6.0 \times 10^{-3}$ μmole mg-BSA^{-1}, respectively (**Fig. 3B**). At 0% salinity, only $2.4 \times 10^{-3} \pm 1.5 \times 10^{-4}$ μmole mg-BSA^{-1} trehalose was produced. Interestingly, concentration of trehalose was rather constant at salinities higher than 1%, yielding $1.7 \times 10^{-2} \pm 3.0 \times 10^{-4}$ to $2.4 \times 10^{-2} \pm 9.9 \times 10^{-4}$ μmole mg-BSA^{-1}. Intracellular Na^+ concentration gradually increased from 1.2 ± 0.1 to 18.1 ± 4.3 μmole mg-BSA^{-1} from 0% to 4% salinity, but only slightly increased at 5% salinity, yielding 19.5 ± 1.0 μmole mg-BSA^{-1}. Intracellular K^+ levels in "*Ca.* B. sinica" were

relatively stable at all salinities examined yielding at around 0.2 μmole mg-BSA^{-1} (**Fig. 3C**).

In "*Ca.* S. japonica", highest SAAs were observed from 0.5% to 2% salinities, from 843.9 ± 89.3 to 889.3 ± 182.7 μmole g-VSS^{-1} h^{-1} (**Fig. 4A**). SAA was gradually decreased by increasing salinity further to 5% as 527.9 ± 153.1 μmole g-VSS^{-1} h^{-1}. No SAA could be observed at 0% salinity and negative control. Salinity dependent production of intracellular trehalose was also observed, but quite limited compared with "*Ca.* B. sinica" (**Fig. 4B**). Trehalose concentrations increased from $1.4 \times 10^{-3} \pm 1.0 \times 10^{-3}$ (0.5%) to $5.2 \times 10^{-3} \pm 2.9 \times 10^{-4}$ (5%) μmole mg-BSA^{-1}. It's interesting that though with no SAA could be detected, trehalose level was higher at 0% salinity than 0.5% salinity. Similar with "*Ca.* B. sinica", Na^+ gradually increased from 1.1 ± 0.1 to 24.1 ± 2.0 μmole mg-BSA^{-1} from 0% to 5% salinity. Interestingly, significant increase of intracellular K^+ concentration was observed for "*Ca.* S. japonica" (**Fig. 4C**). It increased from 0.27 ± 0.01 to 0.78 ± 0.02 μmole mg-BSA^{-1} corresponding to 0.5% to 5% salinity.

6.4.5 Phylogenetic analysis of trehalose 6 phosphatase/trehalose 6 phosphate synthase system

Phylogenetic trees rooted by the trehalose 6 phosphatase/trehalose 6 phosphate synthase protein sequences from "*Ca.* S. japonica" were constructed to illustrate the evolutionary context of this osmoadaptative mechanism. For trehalose 6 phosphatase and trehalose 6 phosphate synthase, online blast using protein sequences from "*Ca.* B. sinica" and "*Ca.* S. japonica" only yields 21% and 30% similarity, respectively (data not shown). Further, "*Ca.* B. sinica" proteins together with other freshwater anammox species (*i.e.* "*Ca*. B. fulgida", "*Ca*. B. sapporoensis", "*Ca*. J. caeni" and "Ca. B. caroliniensis") formed a coherent cluster that was distantly away from "*Ca*. S. japonica" and other bacterial and archaeal phyla (**Fig. 5**).

6.5 DISCUSSION:

Anammox species “Ca. B. sinica” and “Ca. S. japonica” in this study can apparently carve out separate niches due to their differential responses to salinity. We demonstrate that the following: (1) longer period is required for “Ca. B. sinica” to adapt to saline condition. (2) weaker competency of adapted “Ca. B. sinica” compared with “Ca. S. japonica” under saline condition. (3) species specific distribution of trehalose biosynthetic and ion transportation capabilities in anammox bacteria. (4) Synthesis of trehalose and K+ uptake in “Ca. B. sinica” and “Ca. S. japonica” responding to osmostress, respectively and (5) a phylogenetic distinction exists between genus “Ca. S. japonica” and other anammox species regarding trehalose 6 phosphatase/trehalose 6 phosphate synthase system.

Niche differentiation from bioenergetic aspects

Our study is for the first time be able to directly compare the physiological response of two phylogenetically distinct anammox species (i.e. “Ca. B. sinica” and “Ca. S. japonica”) to salinity fluctuation using highly enriched cultures. It took considerably longer time for “Ca. B. sinica” (~ 3 months) to adapt to 1% salinity compared with “Ca. S. japonica” (from 2% to 1%, 15 days). In mixed culture at 1% salinity, though adapted, “Ca. B. sinica” was completely outcompeted by “Ca. S. japonica”. Salinity is therefore a key factor in the niche partitioning between “Ca. B. sinica” and “Ca. S. japonica”. Indeed, results of individual adaptation and mixed competition argues for the need to employ considerations from bioenergetic aspects. Quantification of intracellular trehalose and K+ concentration demonstrates that “Ca. B. sinica” synthesize trehalose (up to 3.9 × 10-2 ± 6.0 × 10-3 μmole mg-BSA-1) to respond to salinity change. Their concentrations are highest at lower media salinities. Alternatively, “Ca. S. japonica” did not synthesize much trehalose (up to 5.2 × 10-3 μmol mg-BSA-1) but uptaking K+ significantly upon salinity increase, highest concentration at higher salinities. Previous studies has demonstrated the importance of trehalose as compatible solutes at lower salinities (Regev et al., 1990). In fact, trehalose-accumulating cyanobacteria are mostly filamentous strains isolated from terrestrial habitats or from freshwater and coastal areas with low salinities, resulting in a mostly a rather low degree of salt resistance (Hagemann, 2011). Besides osmolarity balancing, trehalose has also been found to protect membrane and act as a stress response (Hershkovitz et al., 1991; Hincha and

Hagemann, 2004). Role of trehalose is therefore could not be fully justified. The concentration of K+ in "Ca. S. japonica" was generally lower compared with other halophiles like Halobacteriales due to a lower concentration of salt medium (Awata et al., 2013; Youssef et al., 2014). In current study, we observed a much higher intracellular Na+ concentration than K+ (~ 30 times). Similar phenomenon was also found in halophile Halomonas elongata (Vreeland et al., 1983). In most microorganisms, a minimum concentration of Na+ is essential for growth. However, in case of high intracellular Na+ concentration, all intracellular systems should be adapted to such condition. Proteins in halophilic archaea have been found to highly adapted and magnificently engineered to maintain proper functions in medium containing 2 to 5 M inorganic salts (Dennis and Shimmin, 1997). This might be the reason "Ca. S. japonica", a halophile, could maintain stable SAA at moderate salinity. But for "Ca. B. sinica", high concentration of Na+ flooding into the cell resulted in a significant reduction of SAA at salinity higher than 1%, should be the result of dysfunction of proteins. The plateau of intracellular Na+ increase in Fig. 3C may indicate the self-protection by tightening the cell membrane from "Ca. B. sinica" to avoid further increase in the Na+ concentration. However, molecular mechanism of this specific response to salinity should be investigated further.

Trehalose is an alpha – linked disaccharide composed of 12 carbons. Roughly, to synthesize two molecules trehalose from carbon dioxide by autotrophs, 218 molecules ATP is required to balance the osmolarity that can be compensated by 1 molecule KCl, only 0.50 – 0.67 ATP is required for accumulation (Oren, 1999). Therefore, it could be explained as one of the reasons that energy allocation to trehalose biosynthesis constrained the growth of "Ca. B. sinica", resulting in being outcompeted by "Ca. S. japonica" under 1% saline condition. As for the controversy phenomenon observed before (Kartal et al., 2006b), it is considered that either because aggregated granules were used which delay the selection rate due to diffusion limitation, or species "Ca. Kuenenia stuttgartiensis" adopted ion transportation rather than trehalose synthesis for osmoadaptation. But the latter one requires further verification.

Ecological significance inferred from osmoadaptation

Identification of trehalose associated osmostress response and analysis of its phylogenetic affiliation was able to provide valuable insights into the natural distribution of "Ca. B. sinica" and "Ca. S. japonica". It is recognized that nutrients levels including ammonium and nitrite are lower in marine environments (Beman and

Francis, 2006; Santoro et al., 2008) compared with freshwater environments including man-made ecosystems like wastewater treatment plants (Dragon and Marciniak, 2010; Martens-Habbena et al., 2009; van der Star et al., 2007). Therefore, it could be expected that “Ca. S. japonica” tend to uptaking K+ instead of synthesizing large amount of trehalose. So far, genus “Ca. Scalindua”, though considered as halophile, has never been identified from salt environment higher than marine conditions (Hu et al., 2011; Sonthiphand et al., 2014). Two factors might be involved. Anammox bacteria obtain very limited amounts of energy from dissimilatory metabolism. Its electron donor (ammonium) is relatively oxidized, so that only little energy could be gained. Further, most of the energy generated has to be used to produce NADPH, reducing power required for CO2 fixation (Oren, 1999), resulting a lower biomass yield value (0.063 mmol C [mmol N]-1, 0.03 mmol C [mmol N]-1 for “Ca. B. sinica” and “Ca. S. japonica”, respectively). Therefore, though minor amount, production of trehalose may still be a harsh burden for already “heavy-loaded” “Ca. S. japonica”. Meanwhile, absence of nitrifiers (Oren, 1999) in high salt environment leads to less production of nitrite, one key substrate for anammox bacteria, might further limit growth of anammox bacteria under such condition. For freshwater anammox species from genera “Ca. Brocadia”, “Ca. Jettenia”, “Ca. Kuenenia”, their distributions regarding salinity have been limited to river estuaries with low salinities. This is not only because the not-yet-ready intracellular systems for high salt concentrations, but also because of the huge amounts of energy investment for trehalose biosynthesis if such strategy is the common trait in between. Although adaptation experiment showed “Ca. B. sinica” was able to adapt to saline condition, they could not survive due to interspecific competition, where only robust growth matters. Interestingly, it is revealed from phylogenetic analysis that group of freshwater anammox species was distinctly away from the marine species regarding trehalose metabolisms. It may indicate such function in “Ca. S. japonica” was faded out during evolution, but require further verification.

Engineering implication

Anammox bacteria has received considerable attention in industrial application owing to its economic potential in reducing power and organic inputs for nitrogen removal (Kartal et al., 2010). Saline is always expected in highly concentrated industrial wastewater (landfill leachate, livestock wastewater, etc.) and sometime, municipal wastewater. In Hongkong, sea water is used as a substitute for toilet flushing due to lacking freshwater resource, resulting in an elevated salinity concentration in municipal

wastewater to around 5-6 g/L (Wu et al., 2008) or higher if more sea water is directly utilized (Chen et al., 2003). Previous works has demonstrated several successful adaptation of anammox biomass to high saline environment (30 g/L) (Jin et al., 2011; Kartal et al., 2006b). From the results of current work, it seems more energetically beneficial and timely if "Ca. S. japonica" is used rather than "Ca. B. sinica" for saline wastewater regarding nitrogen removal. Indeed, "Ca. S. japonica" demonstrated a stable activity at high salinity (4 and 5%) where "Ca. B. sinica" completely lost its activity. Such capacity is desired for salinity fluctuation. However, effects of other factors including operating temperature, organic compounds, dissolved oxygen concentrations and substrate concentrations from wastewater should be carefully assessed together for further application.

6.6 CONCLUSION

This study highlights the role of salinity in the niche partitioning between "Ca. B. sinica" and "Ca. S. japonica", and provides ecological and engineering implications to the observed difference in osmostress response.

The complexity and multiplicity of osmostress response in "Ca. B. sinica" and "Ca. S. japonica" reported here, for the first time, partly revealed the physiological response of anammox bacteria to salinity. Yet multiple unanswered questions were raised regarding the origin and motivation of this distinct preference. Detailed biological mechanism of osmostress response was still remained to be investigated. Nevertheless, main conclusions could be withdrawn as follows:

i. It took considerably longer time for "Ca. B. sinica" (~3 months) to adapt to 1% salinity compared with "Ca. S. japonica" (15 days). At 1% salinity, adapted "Ca. B. sinica" could not compete with "Ca. S. japonica" for growth.

ii. "Ca. B. sinica" synthesize trehalose, an extremely energy expensive compatible organics responding to salinity change. Alternatively, "Ca. S. japonica" up taking K+ and produce much less trehalose.

iii. Trehalose associated osmostress response could limited competency of "Ca. B. sinica" with "Ca. S. japonica", one of the reasons shaping the distinct

niche upon salinity in between.

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Table 6.1 Blast hit of genes related to organic compatible solutes regulation in the genomes of "*Ca*. B. sinica" and "*Ca*. S. japonica".

	Organic compatible solutes						
Anammox species	Glycerol	Glycine betaine	Ectoine	Glucosylglycerol	Sucrose	Trehalose	Reference of genome
"*Ca*. B. sinica"	n.d*.	n.d.	n.d.	n.d.	n.d.	Y	(Oshiki et al., 2015)
"*Ca*. S. japonica"	n.d.	n.d.	n.d.	n.d.	n.d.	Y	(Oshiki et al., 2017)

*n.d. not detected.

Table 6.2 Genomic evidence for ion transportation and trehalose biosynthesis in anammox bacteria from sequenced genomes.

		Genomic evidence						
		Ion transportation			Trehalose biosynthesis			
Genus	Species	Na^+:H^+ antiporter	Multisubunit Na^+:H^+ antiporter similar to MnhABCDEFG	K^+ transportation	TPS/TPP	TreT	TreY/TreZ	Reference
"*Ca*. Scalindua"	"*Ca*. S. japonica"	Y	Y	Y	Y	N	N	(Oshiki et al., 2017)
	"*Ca*. S. profunda"	Y	Y	Y	Y	N	N	(van de Vossenberg et al., 2013)
	"*Ca*. S. brodae"	Y	Y	Y	N	Y	N	(Speth et al., 2015)
	"*Ca*. S. rubra"	N	Y	Y	N	Y	N	(Speth et al., 2017)
"*Ca*. Kuenenia"	"*Ca*. K. stuttgartiensis"	N	Y	Y	Y	Y	Y	(Strous et al., 2006)
"Ca. Jettenia"	"*Ca*. J. caeni"	N	Y	Y	Y	Y	Y	(Ali et al., 2015)
"*Ca*. Brocadia"	"*Ca*. B. sinica"	N	Y	Y	Y	Y	Y	(Oshiki et al., 2015)
	"*Ca*. B. sapporoensis"	Y	Y	Y	Y	Y	Y	(Ali et al., 2016; Narita et al., 2017)
	"*Ca*. B. fulgida"	N	Y	N	Y	N	Y	(Gori et al., 2011)

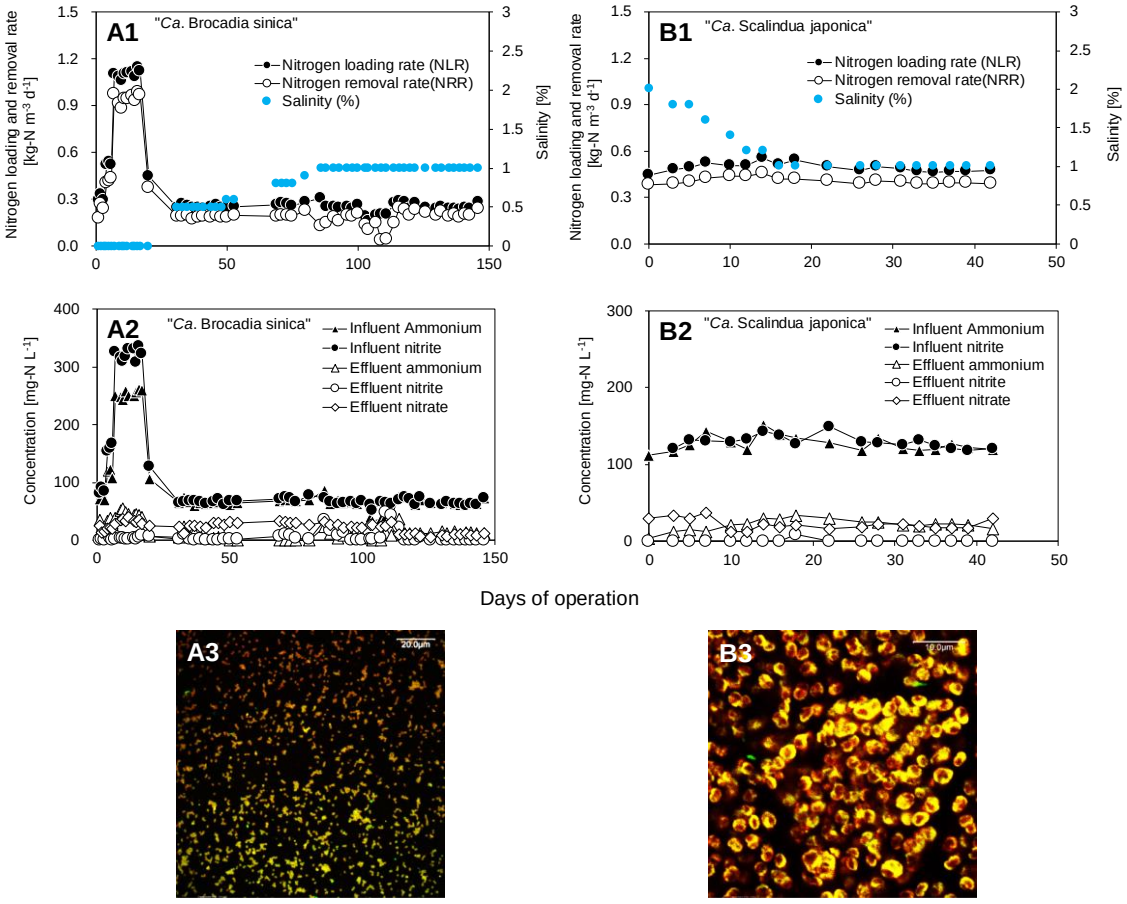

Figure 6.1 Adaptation of "*Ca.* B. sinica" and "*Ca.* S. japonica" to 1% salinity. A1&B1: **Nitrogen loading, removal rates and salinity.** A2&B2: **temporal dynamic of influent ammonium, nitrite and effluent ammonium, nitrite and nitrate concentration.** A3&B3: **Confocal microscopic images of FISH analysis for adapted free-living planktonic cells of "*Ca.* B. sinica" (A3) and "*Ca.* S. japonica" (B3) in MBRs. Cells were hybridized with combination of TRITC-labeled AMX820 probe (red) and FITC-labeled EUB mix probe (green) for "*Ca.* B. sinica" and FITC-labeled EUB338 mix probe (green) and TRITC-labelled probe Sca1129b (red) for "*Ca.* S. japonica". Anammox bacteria were shown in yellow for both panels. White bars indicate 20 and 10 μm.**

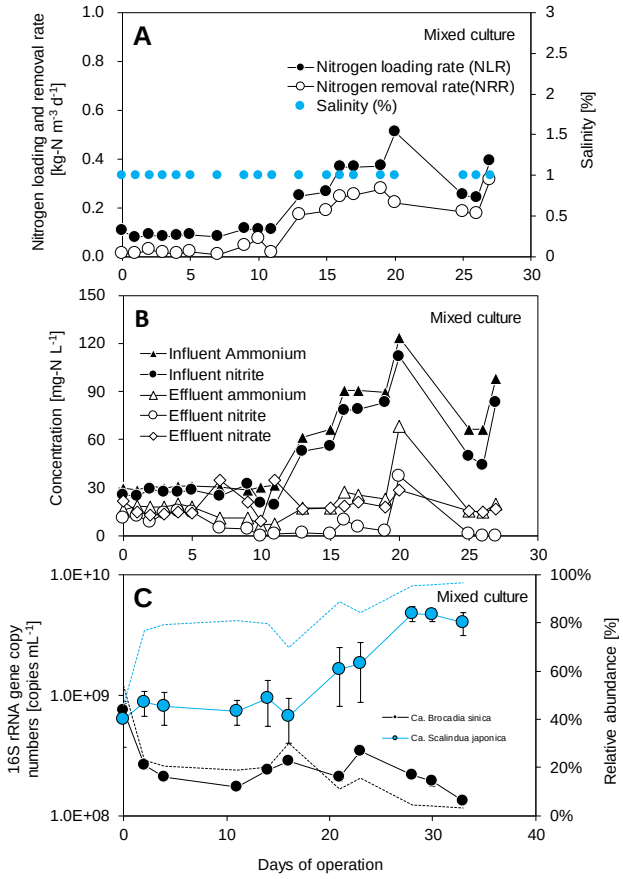

Figure 6.2 Reactor performance and population dynamics of "*Ca.* B. sinica" and "*Ca.* S. japonica" after mixed as equal 16S rRNA gene copy numbers in MBR at 1% salinity under room temperature. A: **Nitrogen ($NH_4^+$and NO_2^-) loading rate and removal rate and salinity in the MBR.** B**: Temporal dynamic of influent ammonium, nitrite and effluent ammonium, nitrite and nitrate concentration.** C: **Absolute population dynamics and relative abundance (dash line) of "*Ca.* B. sinica" and "*Ca.* S. japonica" in the MBR.**

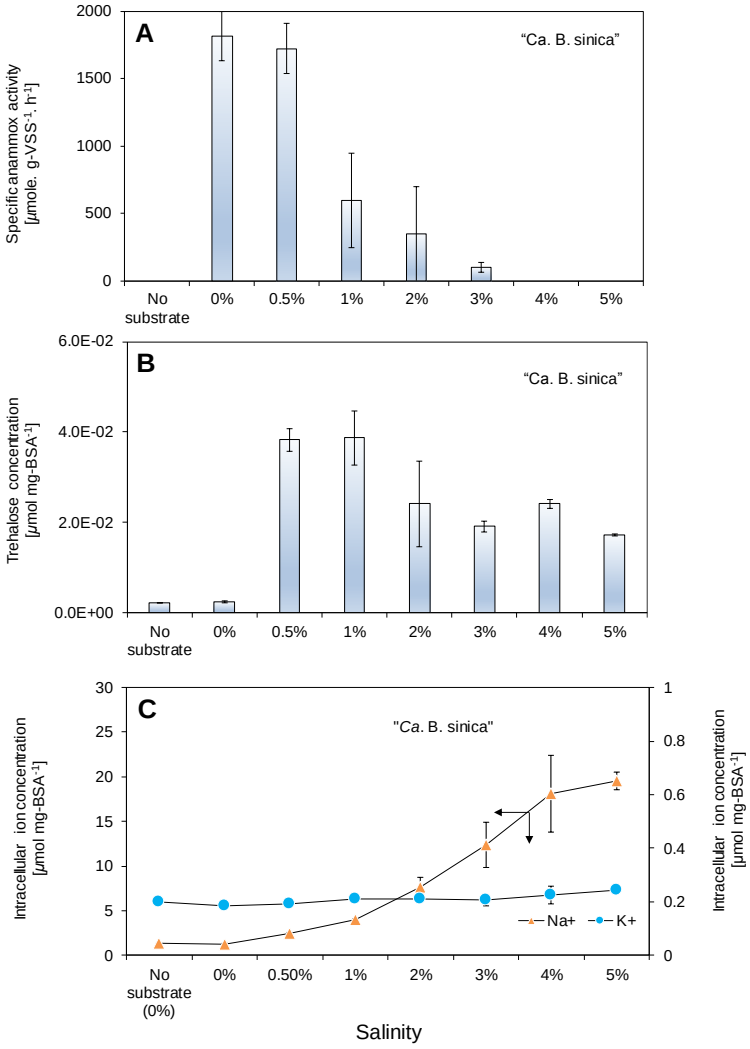

Figure 6.3 Specific anammox activity (SAA), intracellular trehalose and Na^+, K^+ concentration of "*Ca.* B. sinica" at defined salinity. A**: SAA of "*Ca.* B. sinica" indicated by nitrogen gas production rates.** B**: Intracellular trehalose concentration.** C**: Intracellular Na^+ and K^+ concentration. Batch tests were performed as duplicate.**

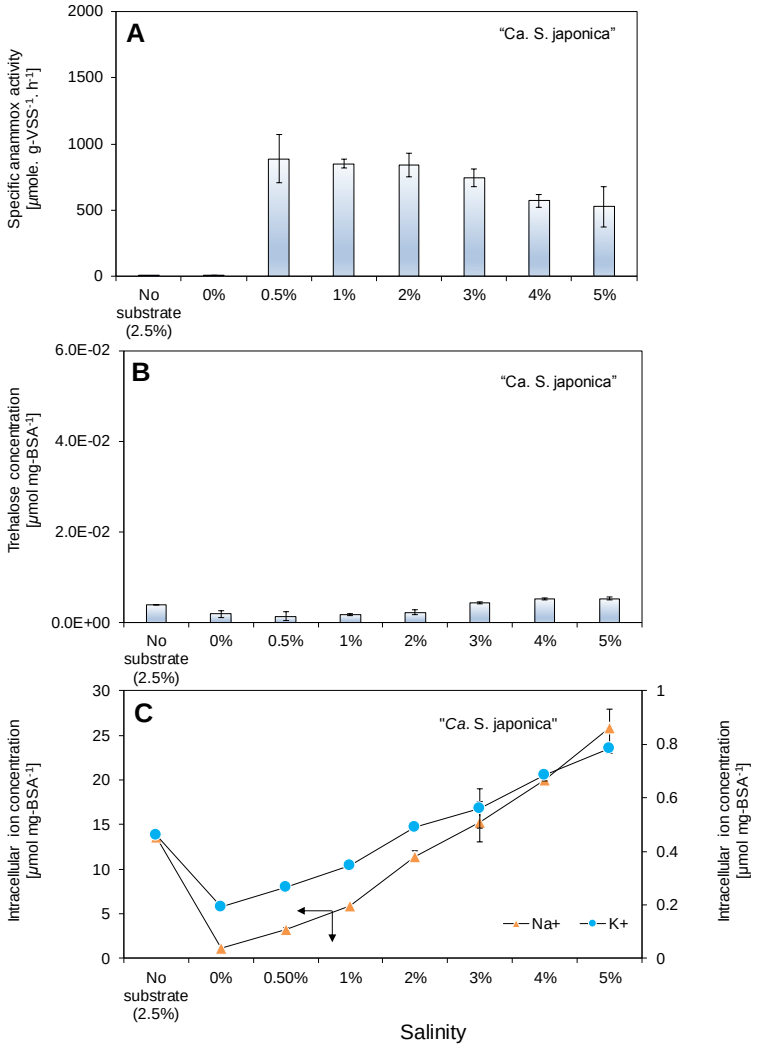

Figure 6.4 Specific anammox activity (SAA), intracellular trehalose and Na^+, K^+ concentration of “*Ca*. S. japonica” at defined salinity. A**: SAA of “*Ca*. S. japonica” indicated by nitrogen gas production rates.** B**: Intracellular trehalose concentration.** C**: Intracellular Na^+ and K^+ concentration. Batch tests were performed as duplicate.**

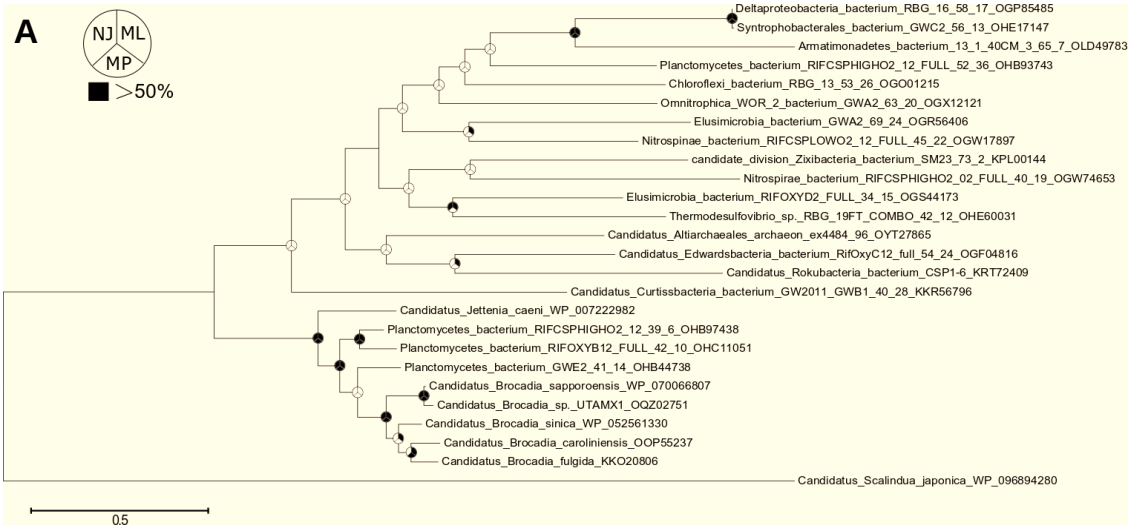

Figure 6.5 Maximum likelihood phylogenetic tree inferred from trehalose-6-phosphate synthase/ trehalose-6-phosphatase protein sequence alignments for members of anammox bacteria as well as other bacterial and archaeal species. **The tree was rooted using trehalose-6-phosphatase (**A**)/trehalose-6-phosphate synthase (**B**) protein sequence of "*Ca.* B. sinica". The scale bar represents the number of nucleotide changes per sequence position. Pie charts at the nodes represent the confidence of the branch topology results, and bootstrap values greater than 50% are filled in black (the neighborjoining method, NJ, for the upper-left sector, the maximum-likelihood method, ML, for the upper-right sector, or the maximum-parsimony method, MP, for the bottom sector). 200, 1000, 200 resamplings for ML, NJ and MP method, respectively.**

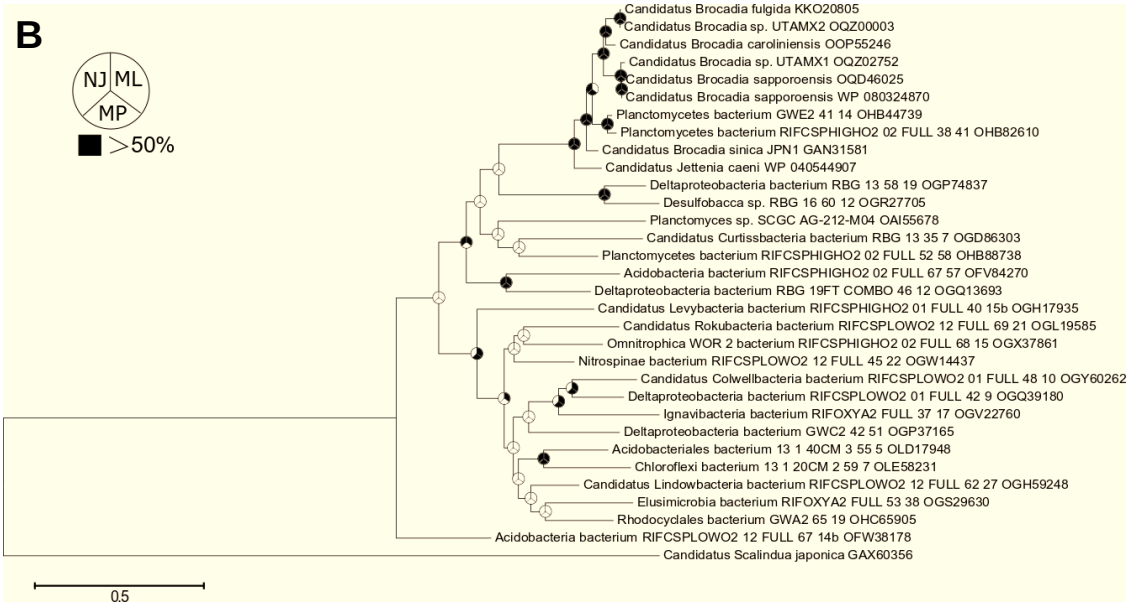

Fig. 5 (continued).

7 INTEGRATION AND PERSPECTIVES

7.1 Integration

The experiments described in current thesis addressed several aspects of the niche differentiation and eco-physiology of the anaerobic ammonium oxidizing (anammox) bacteria. In chapters 3, a method for rapid cultivating anammox planktonic cells was developed. In chapter 4, maximum specific growth rate of three anammox species was re-evaluated. In chapter 5 and 6, factors effecting the niche partitioning of anammox bacteria were systematically studied. In the light of the results obtained in these chapters, several general conclusions and research directions with regard to enrichment, specific growth rates, niche differentiation of anammox bacteria can be made and discussed.

7.1.1 Enrichment

It is still not possible to achieve pure culture of anammox bacteria. Therefore, for ecophysiologycal and biochemical study, enriched culture with high purity and planktonic cells is essential. In this chapter, we developed a reliable method for cultivating planktonic anammox cell with high purity within 35 days. The significance

of this work is to provide much easier access of planktonic anammox culture to researchers working on anammox. Along with the method development, we partly characterized the growth pattern of anammox cells immobilized in gel beads, where we found less aggregation occur compared with natural granules. This observation may further facilitate the development of pure culture of anammox bacteria.

7.1.2 Maximum specific growth rate

Maximum specific growth rate is the essential information, not only in practical application, but also for interpretation of microbial ecology and niche differentiation. Several endevors have been input to this evaluation and measurement, however, due to the lack of appropriate culture and measureing methods, no consistent results could be obtained so far. Here we re-evaluated the maximum specific growth rate of three phylogenetical different anammox species using planktonic cell culture and immobilized gel beads combined with real time quantitative PCR targeting the exponential growth period. As expected, much faster maximum specific growth rates were obtained and the doubling time of anammox bacteria may actually approaching 2 days. Hence, may not be appropriate to be described as slow-growing bacteria.

7.1.3 Niche differentiation

In chapter 5 and chapter 6, we systematically investigated two factors in the niche differentiation of anammox bacteria. In chapter 5, we operated three types of reactors with different kind of biomass (suspended planktonic cells, biomass immobilized in gel beads and naturally aggregated granules) under different nitrogen loading rates (NLRs). Here we selected NLR because of the much easier operation condition compared with nitrite concentrations, especially in MBR systems. In addition, NLR is one of the parameters can be controlled in real wastewater treatment process, therefore, applicable in the future for microbial manipulation. Population dynamics of three anammox species from genera "*Ca*. Brocadia", "*Ca*. Kuenenia" and "*Ca*. Jettenia" were followed quantitatively using qPCR. Unexpectedly, "*Ca*. J. caeni" could outcompete "*Ca*. B. sinica" at low NLR while at high NLR, "*Ca*. J. caeni" was completely outcompeted by "*Ca*. B. sinica". In addition, "*Ca*. K. stuttgatiensis" could not grow under all NLRs supplied, argues for other more fundamental factors restricting

the growth of this specific species. Although NLR was the controlling parameter, as the limiting substrate, nitrite is the sole nutrient that anamox speciss are competeing for. The over grow of "*Ca*. J. caeni" could not be simply explained by the Monod curves since "*Ca*. B. sinica" possesses higher maximum specific growth rate and higher affinity than "*Ca*. J. caeni" according to reported values. However, the accuracy if Ks from "*Ca*. J. caeni" is arguable. It was measured using a culture with flocculated biomass (average particle size at ~150 μm). Therefore, a much lower *K*s is expected and should be able to provide better understanding of the population dynamics. Another factor is the maintenance rate of both species. According to the results obtained, it is speculated that "*Ca*. B. sinica" possess higher maintenance rate than "*Ca*. J. caeni", therefore could not grow under limited NLR.

So far genus "*Ca*. scalinuda" were mostly detected in marine environments requiring salinity. However, one study tried to adapt a mix culture containing "*Ca*. Kuenenia" and "*Ca*. Scalindua" to high salinity condition, but after one year's operation, still 70% of biomass belongs to "*Ca*. Kuenenia". Based on that, the author argued that salinity is not a niche differencing factor for "*Ca*. Kuenenia" and "*Ca*. Scalindua". However, both survey from so far detected 16S rRNA gene sequences and environmental detections suggested that salinity is the factor involved in "*Ca*. Scalindua" and other genera. In chapter 6, we therefore systematically investigated whether salinity is the differentiating factor between "*Ca*. Brocadia sinica" and "*Ca*. Scalindua japonica". Individual adaptation and interspecific competition demonstrated the salinity as one important differentiating factor. In addition, we identified the osmoadaptive strategy employed by "*Ca*. B. sinica" as trehalose synthesis and K^+ uptake for "*Ca*. S. japonica", respectively. These findings lead to the understanding of niche partitioning from bioenergetic aspects and was able to provide alternative views on natural distribution of anammox bacteria. Lastly, phylogenetic analysis on trehalose biosynthesis (trehalose 6 phosphatase/trehalose 6 phosphate synthase pathways) implied the distinct distance between "*Ca*. B. sinica" and "*Ca*. S. japonica", raises the probability that this function be acquired from horizonal gene transfer rather than an ancestral trait. As for the phenomenon observed by Kartal et al., 2006, it is considered that either because aggregated granules were used which delay the selection rate due to diffusion limitation, or species "*Ca*. Kuenenia stuttgartiensis" adopted ion transportation rather than trehalose synthesis for osmoadaptation. But the latter one requires further verification.

7.1.4 Engineering implication.

At present, application of anammox process to real wastewater is attracting great attention. Although the method developed in chapter 3 is mainly used for physiological and biochemical experiments, accurate kinetic information could also be obtained and is of importance for mathematical modelling and plant design. This is the case of maximum specific growth rate determined in chapter 4. Information in chapter 5 and chapter 6 provided important and useful information for species selection for specific waterwater (either with different NLR or different salinity). It is recommended that high NLR influent be treated with "*Ca*. Brocadia sinica" while high salinity ones be treated with "*Ca*. Scalindua japonica".

7.2 Perspectives

In this endeavour, free-living planktonic anammox cell culture could be cultivated rapidly. The maximum specific growth rate of three anammox species were re-evaluated. And niche differentiation between anammox species were illustrated. Still there is a lot of room in this research area that requires further attention from the researchers.

Although single cell and high purity could be obtained for anammox bacteria at this moment, pure culture is still necessary and essential to obtain more precise and accurate information from anammox bacteria, particularly the growth kinetics. In addition, with the development of genomic technology, single cell genomics may applicable for anammox bacteria and more accurate description from genome and transcription could be achieved.

Ecophysiology of anammox bacteria is still essential knowledge for understanding their ecological significance and role in global nitrogen cycle. Definitely, more planktonic cell culture from other anammox species are in a great demand to explore the physiological diversity of anammox bacteria. And to interpret their natural distribution. Indeed, combined with genomic information, more physiological trait could be revealed and more insight could be provided.

Characteristics of "*Ca*. Sclindua" were far from enough despite their huge contribution to global nitrogen cycle. Further efforts were required to study this unique genus.

7.3 Curriculum Vitae and List of publications:

Lei ZHANG was born in Shanghai, China in 1989. After attending the Tongji university, he obtained his bachelor diploma as degree of engineering in the field of water and wastewater treatment in 2012. In Hokkaido university, he studied environmental engineering and microbiology and finished his studies with a Master's thesis on the microbial ecological competition regarding anaerobic ammonium oxidizing (anammox) bacteria in 2014. Right after the Master course, he decided to proceed to PhD course in the same laboratory and continued work on the eco-physiology of anammox bacteria, which result in current thesis.

1. Rapid cultivation of free-living planktonic anammox cells
   Zhang, L and Okabe, S. Water Research, 2017, 127, 204-210.
2. Microbial competition among anammox bacteria in nitrite-limited bioreactors
   Zhang, L., Narita, Y., Gao, L., Ali, M., Oshiki, M., Ishii, S., Okabe, S. Water Research, 2017, 125, 249-258.
3. Maximum specific growth rate of anammox bacteria revisited
   Zhang, L., Narita, Y., Gao, L., Ali, M., Oshiki, M., Okabe S. Water Research, 2017, 116, 296-303.
4. Enrichment and physiological characterization of an anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sapporoensis'
   Narita, Y., Zhang, L., Kimura, Z., Ali, M., Fujii, T., Okabe, S. Systematic and Applied Microbiology, accepted (in press).
5. Draft genome sequence of the anaerobic ammonium-oxidizing bacterium "Candidatus Brocadia sp. 40"
   Ali, M, Haroon, M.F., Narita, Y., Zhang, L., Rangel Shaw, D., Okabe, S., Saikaly, P.E. Genome Announcements, 2016, 4(6), e01377-16.
6. Source identification of nitrous oxide emission pathways from a single-stage nitritation-anammox granular reactor
   Ali, M., Rathnayake, R.M., Zhang, L., Ishii, S., Kindaichi, T., Satoh, H., Toyoda, S., Yoshida, N., Okabe, S. Water Research, 2016, 102, 147-157
7. Enrichment, growth kinetics and niche differentiation of anammox bacteria. Zhang, L, Narita, Y and Okabe, S. (In preparation)

8. Trehalose associated osmostress response in "*Ca*. Brocadia sinica" and "*Ca*. Scalindua japonica". Zhang, L, Oshiki, M and Okabe, S. (In preparation)