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Anammox bacteria compete for nitrite

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**Microbial competition among anammox bacteria in nitrite-limited
bioreactors**

By

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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*”.

Keywords

Niche differentiation; population dynamics; “*Ca. Brocadia sinica*”; “*Ca. Jettenia caeni*”; “*Ca. Kuenenia stuttgartiensis*”.

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 about 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, 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 summarized 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 could be

the primary factors in determining the dominance of anammox bacteria. However, driving force(s) for the 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 calculated using the Monod model to explain the competition results. The niche differentiation among “*Ca. Brocadia*”, “*Ca. Jettenia*”, and “*Ca. Kuenenia*” was further discussed.

2. Materials and Methods

2.1. Monod growth curve

Specific growth rates (μ) of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. K. stuttgartiensis*” were calculated 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}); $\mu_{\max}$ 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).

2.2. Anammox enrichment cultures

Planktonic free-living cells of “*Ca. B. sinica*” (>95%) 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: CO₂ = 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): FeSO₄·7H₂O (9.0), EDTA·2Na (5.0), NaCl (1.0), KCl (1.4), CaCl₂·2H₂O (1.4), MgSO₄·7H₂O (1.0), NaHCO₃ (84), KH₂PO₄ (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.

2.3. 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 (NH₄)₂SO₄ and NaNO₂ 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 NH₄⁺ and NO₂⁻ 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 NH₄⁺ and NO₂⁻ were supplied at a fixed HRT of 15.8 h. In MBR M4, influent NH₄⁺ and NO₂⁻ 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 NH₄⁺ and NO₂⁻ concentration at 10 mM.

2.3. 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 .

2.4. 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, Qiagen, 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 $\times$ 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 $\times$ 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.

2.5. Fluorescence *in situ* hybridization

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.

3. Results

3.1. Monod growth curves

The classical Monod growth curves of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. K. stuttgartiensis*” were calculated using the previously reported μ_{max} and K_s for nitrite 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 K_s), 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.

3.2. 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 ($\text{NLR} = 0.5 \text{ kg-N d}^{-1} \text{ m}^{-3}$) (**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 ($\text{NLR} = 0.7 \text{ kg-N d}^{-1} \text{ m}^{-3}$) (**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 ($\text{NLR} = 2.7 \text{ kg-N d}^{-1} \text{ m}^{-3}$) (**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).

3.3. Competition in MBRs

In MBR M1 ($\text{NLR} = 0.07 \text{ kg-N d}^{-1} \text{ m}^{-3}$ and $\text{NO}_2^- = 0.0 - 0.36 \text{ mM}$, **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 ($\text{NLR} = 0.10 \text{ kg-N d}^{-1} \text{ m}^{-3}$ and $\text{NO}_2^- = 0.0 - 3.88 \text{ mM}$, **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 ($\text{NLR} = 0.14 \text{ kg-N d}^{-1} \text{ m}^{-3}$ and $\text{NO}_2^- = 0.0 - 0.43 \text{ mM}$, **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 ($\text{NLR} = 0.17 - 0.42 \text{ kg-N d}^{-1} \text{ m}^{-3}$ and $\text{NO}_2^- = 0.0 \text{ to } 0.7 \text{ mM}$, **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**).

3.4. 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 ($\text{NLR} = 4.3 - 8.6 \text{ kg-N d}^{-1} \text{ m}^{-3}$ and $\text{NO}_2^- = 9 - 11 \text{ mM}$ 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.

3.5. 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.

4. 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). These evidences clearly indicate 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 $\text{NH}_4^+ : \text{NO}_2^-$ 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. Although the NLR per se doesn't directly influence the competition based on the classical Monod model (the specific growth rate is solely determined by a limiting substrate concentration), it is linked to the apparent growth rate of anammox bacteria as described below.

The Monod growth curves of the three anammox species were calculated using the currently available kinetic parameters K_s and μ_{max} (**Fig. 1** and **Table 1**). Although the Monod growth curve do not describe the population dynamics of each anammox species in the different reactors, the specific growth rates are the vital parameters in the competition. Based on the Monod growth curves, “*Ca. B. sinica*” would outcompete “*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 K_s , because the K_s values of both species were virtually the same (**Table 1**). The K_s 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). 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. sinica*” and “*Ca. K. stuttgartiensis*” varied widely (**Table 1**).

To check whether or not this discrepancy may be due to inaccuracy of the K_s value, the sensitivity of different K_s values for “*Ca. J. caeni*” (i.e., 0.1, 5 and 35.6 (original) μM) on the specific growth rate (μ) was analyzed using a fixed μ_{max} value (**Table 1**), and the outcomes were compared with “*Ca. B. sinica*” whose K_s ranges from 13 – 55 μM (**Fig. S1**). The μ of “*Ca. J. caeni*” became higher than that of “*Ca. B. sinica*”, when the K_s value for “*Ca. J.*

caeni” was reduced to 5 μM at the corresponding nitrite concentration of $< 4 \mu\text{M}$. Since the nitrite concentrations in the M1 reactor during the initial period were mostly below the detection limit (1 μM), “*Ca. J. caeni*” would have a chance to outcompete “*Ca. B. sinica*” in the competition experiments. Taken together, the sensitive analysis indicated that K_s for nitrite plays a vital role in the competition at low NLR. Therefore, the K_s value must be reevaluated using planktonic free-living enriched cells.

Kinetic parameters such as μ_{max} and K_s 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 K_s , the discrepancy between the classical Monod model prediction and competition results 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 (Fuchslin et al., 2012; Müller and Babel, 1996; Tros et al., 1996; van Bodegom, 2007). For example, “*Ca. B. sinica*” was outcompeted in M1 reactor but not in M2 reactor, which may suggest that “*Ca. B. sinica*” requires a higher maintenance rate.

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).

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

where r_g is biomass growth rate (g-biomass $\text{m}^{-3} \text{d}^{-1}$), Y is true growth yield (g-biomass formed g-substrate consumed $^{-1}$), r_s is substrate utilization rate (g-substrate $\text{m}^{-3} \text{d}^{-1}$), μ_{max} is true maximum specific growth rate (d^{-1}), S is a limiting substrate concentration (g m^{-3}), K_s 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 substrate concentration at which the net bacterial growth is zero (S_{min}) can be related to m and expressed as follows;

$$S_{min} = m K_s / (Y q_{max} - m) \quad \text{eq. (2)}$$

where, q_{max} is maximum specific substrate utilization rate (g-substrate g-biomass $^{-1} \text{d}^{-1}$). 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 competition studies 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 be 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 μ M), 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.

5. 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 K_s for nitrite and missing information of maintenance rate (m). Further (re)evaluation of these kinetic parameters is required.

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Figure caption

Fig. 1 The Monod growth curves of “*Ca. B. sinica*”, “*Ca. J. caeni*” and “*Ca. K. stuttgartiensis*”. The specific growth rate (μ) was calculated as a function of a limiting substrate (nitrite) concentration by using the Monod equation. It should be noted that since the K_s 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 all species.

Fig. 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.

Fig. 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.

Fig. 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.

Fig. 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).

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1 **Table 1.** Physiological characteristics of “*Ca. Jettenia caeni*”, “*Ca. Brocadia sinica*” and “*Ca. Kuenenia stuttgartiensis*”.

Parameter ^a	“ <i>Ca. Jettenia caeni</i> ”	“ <i>Ca. Brocadia sinica</i> ”	“ <i>Ca. Kuenenia stuttgartiensis</i> ”
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	<i>n.d</i> ^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	<i>n.d</i>
NO ₂ ⁻ (μM)	35.6 ± 0.92	34 ± 21	0.2 - 3
Maintenance rate, m (h ⁻¹)	<i>n.d</i>	0.0029 – 0.0081	<i>n.d</i>
Oxidation rate (μmol g-protein ⁻¹ min ⁻¹)			
Formate	6.7 ± 0.6	<i>n.d</i>	5.8 ± 0.6
Acetate	0.79 ± 0.07	<i>n.d</i>	0.31 ± 0.03
Propionate	0.64 ± 0.05	<i>n.d</i>	0.12 ± 0.01
Tolerance			
NO ₂ ⁻ (mM)	11	<16	13, 25
NH ₄ ⁺ (mM)	>20	<i>n.d</i>	<i>n.d</i>
Sulfide (μM)	540	<i>n.d</i>	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: K_s for ammonium and nitrite were determined based on the Monod kinetics;

d: *n.d*; not determined.

Fig. 1 Zhang *et al.* (2017)

Fig. 2 Zhang *et al.* (2017)

Fig. 3 Zhang *et al.* (2017)

Fig. 3 (cont.) Zhang et al. (2017)

Fig. 4 Zhang *et al.* (2017)

Fig. 5 Zhang *et al.* (2017)

Highlights

- Competition for nitrite among three enriched anammox bacteria was systematically investigated.
- “*Ca. B. sinica*” dominated at high NLRs, while “*Ca. J. caeni*” dominated at low NLRs.
- Monod model could not predict the dominance of “*Ca. J. caeni*” at low NLRs.
- NLR was a factor determining ecological niche differentiation of anammox bacteria.