B−50 Population Dynamics and *in situ* Distribution of three Anammox Species Immobilized in Alginate Gel Beads

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1. Introduction:

As one of the important physiological characteristics, the specific growth rate of anammox bacteria has been determined by several molecular biological methods, mainly including real-time quantitative PCR, fluorescence in situ hybridization, and also traditional batch experiments (Tsushima, et al., 2007; Oshiki, et al., 2011). Due to the infeasibility of pure culture, it's rather difficult to determine the growth rate of specific species by using batch experiments. Furthermore, the application of molecular biological methods is feasible only when an exponential growth phase could be achieved for the anammox species (Kartal, et al., 2007), in other words, the engineers have to bear the long-term start-up period before they could calculate the growth rate.

Here we established a repaid and reliable method for the study of specific growth rate of anammox species immobilized in the alginate gel beads determined by RT-qPCR. Along with that, the *in situ* distribution of three anammox species was demonstrated by using FISH with specific 16S rRNA targeting probes.

2. Materials and methods:

2.1. Upflow column reactor: One lab-scale upflow column reactor (V=53.6mL) was established and operated for around three months at T=37.0℃, pH=7.50 ±0.5. The reactors were seeded with alginate gel beads which contained biomass of three candidate species: *Candidate* K. stuttgartiensis, *Candidate* B. sinica and Planctomycetales KSU-1. The packing size of biomass was around 70%. The reactors were fed with synthetic wastewater which contained $(NH_4)_2SO_4$ and $NaNO_2$ as the form of ammonia and nitrite. The concentration varied from 30±10 mg/L to 60±10 mg/L, respectively.

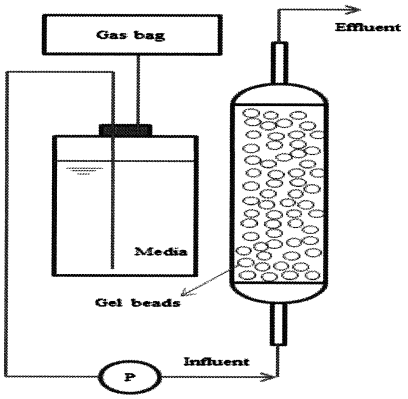

Fig. 1 Column reactor seeded with gel beads

2.2. Primer design for real time PCR: Three new specific primer sets and two TaqMan® probes were designed for the quantification of 16S rRNA of three species as table 1&2 shows.

Table 1- List of 16S rRNA targeted-oligonucleotide primer sets used in this study

Target	Forward primer (5'-3')	Reverse primer (5'-3')
K.stuttgartiensis	GCAGGTGCGTTAATAGCGCAC	TCAAGCCCTGTAGTATCAGAT
B. sinica	GATGGGAACAACAACGTTCCA	TTCTTTGACTGCCGACACCA
KSU-1	GTAAGGGGGTGAATAGCCCTC	TCCAGCCCTATAGTATCAACT

Table 2- List of 16S rRNA targeted- TaqMan® probes

Target	sequence
***Candidate* B.sinica**	5'-FAM-CCGAAAGGGTTGCTAATTCTCA-MGB-3'
Common probe for Other two species	5'-FAM-CAGCAGCCGCGGTAATACAGA-MGB-3'

3. Results and discussion:

3.1. Nitrogen removal performance of upflow column reactor: As figure 2 shows, obvious nitrogen removal could be observed soon after 20 day's operation. This mainly contributed to denitrification process in the reactor because the concentration of nitrate in the effluent is approaching zero and the copy numbers of anammox species were still maintain at a low level. After 20 days, the nitrogen removal efficiency was improving and maintained at around 70%, along with that, the color of biomass turned from almost transparent to light red, which is the symbol of anammox activity. Meanwhile, after 33days, there is an increase of nitrate in the effluent. During the whole operation period, the concentration of nitrite in the effluent always near undetectable level, which is consistent with the previous report that nitrite as the limiting substrate for anammox species (Meyer, et al., 2005).

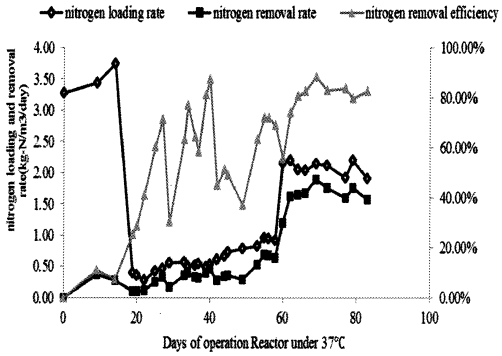

Fig. 2 Performance of reactor

3.2. Population dynamics and specific growth rate: Population dynamics of three anammox species in the column reactors were examined by RT-qPCR as figure 3 shows. Except *Candidate* K. stuttgartiensis species, both *Candidate* B. sinica and planctomycetes KSU-1 exhibited an exponential growth between 27 and 61 days of operation. This well corresponded to the reactor performance and apparent look of the gel beads.

Van der Star, et al., 2007 discussed the population shift of anammox bacteria in the first full-scale anammox reactor in Rotterdam. There they suggested the enrichment of anammox species was determined not by inoculations but by the difference of niches or other environmental parameters. In their reactors, the dominance of species under genera of Brocadia was successfully observed. This hypothesis was also proposed by another research group (Park, et al., 2010). Thus, the result of population dynamics in our reactor may contribute to the environmental parameters. Furthermore, van der Star, et al., 2008 were able to monitor the shift of dominant species in their membrane bioreactor from Brocadia to Kuenenia at a relatively low nitrogen loading rate. Based on that, they suggested the affinity for nitrite be the deciding factor of niche differentiation for Brocadia and Kuenenia species. In addition to that, they considered Kuenenia as the affinity strategist while Brocadia as the growth rate strategist.

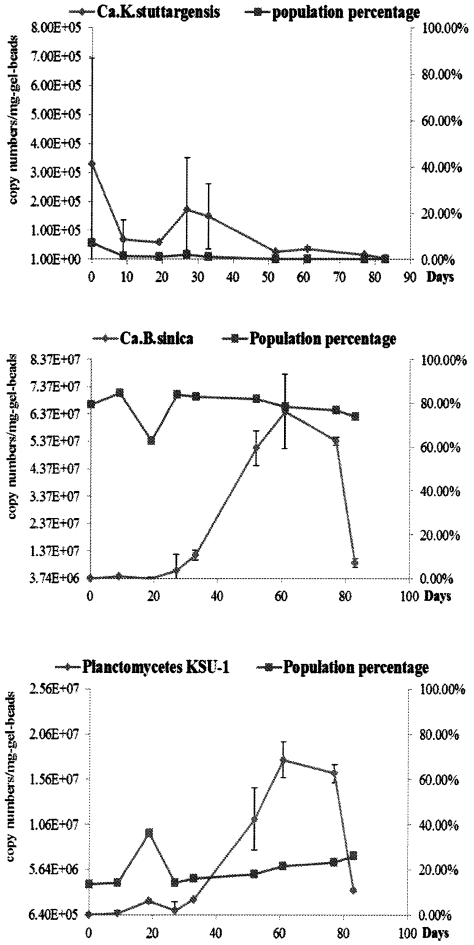

Fig. 3 Populaton dynamics of three species

Now when we look at *Candidate* K. stuttgartiensis species, which displayed an opposite result against our expectation that they reached their maximum growth rate according to the hypothesis made by Star, et al., 2008 that *Candidate* K. stuttgartiensis as the affinity strategists,

we guess its failure was due to the water environment of the medium prepared by ground water. It's highly possible that some certain heavy metal or cations, anions brought a negative effect on the growth of *Candidate* K. stuttgartiensis species. This will be further confirmed by performing reproducibility test.

Based on the logarithmic growth periods, specific growth rates of Brocadia and KSU-1 were calculated as shown in Table 3. The growth rate of *Candidate* B. sinica was little lower than the maximum growth rate reported before (Oshiki, et al., 2011). One possible reason could be contributed to the substrate diffusion within the gel beads. Another reason we guess was consistent with the result of Star, et al., 2008's study, that Brocadia is a growth rate strategist, which means they could not reach their maximum growth rate under such low nitrite concentration employed in our experiment.

Table 3-Calculation of growth rate of KSU-1 and B.sinica at 37℃

Day	27-33	33-52	52-61
species		KSU-1	
growth rate/h	0.0038	0.0034	0.0021
average growth rate/h		0.0031	
species		B.sinica	
growth rate/h	0.0031	0.0032	0.0011
average growth rate/h		0.0025	

Since there is still very limited information regarding the physiological characteristics of planctomycetes KSU-1, we would like to make a hypothesis based on the result of growth rate in table 3 that planctomycetes KSU-1 determined as the affinity strategist since they demonstrated a relatively higher apparent growth rate compared with Candidate B. sinica under low substrate concentration.

3.3. Fluorescence *in situ* hybridization: The percentage (calculated as area) and *in situ* distribution of three species within the gel beads was also examined by FISH. According to figure 4, the percentage of each species is consistent with the result of RT-qPCR, which confirmed its reliability.

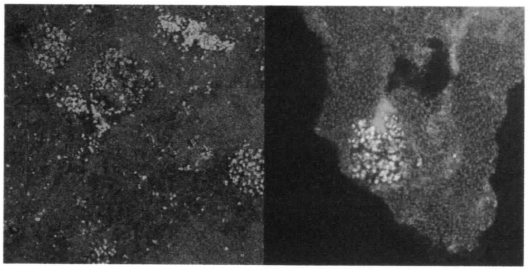

Fig.4 FISH image of gel beads at day 67 employed with specific probes (left: K. stuttgartiensis (red) and KSU-1 (green), right: AMX820 (red) and KSU-1 (green)).

4. Conclusion

1) Real-time qPCR and FISH analysis enable to follow population dynamics of anammox bacteria and to estimate their specific growth rates reliably.

2) The specific growth rate of "*Ca.* Brocadia sinica" in the column reactor operated at 37℃ was little lower than their maximum growth rate ($0.0025h^{-1}$ and $0.0041h^{-1}$, respectively) at low nitrite concentration. Interestingly, planctomycetes KSU-1 proliferated in the column reactor with even higher extent of specific growth rate ($0.0032h^{-1}$).

5. Reference

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