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Relation	



# Nitrogen removal using an anammox membrane bioreactor at low temperature

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## Abstract

Membrane bioreactors (MBRs) have the ability to completely retain biomass and are thus suitable for slowly growing anammox bacteria. In the present study, an anammox MBR was operated to investigate whether the anammox activity would remain stable at low temperature, without anammox biomass washout. The maximum nitrogen removal rates were 6.7 and 1.1 g-N L<sup>-1</sup> day<sup>-1</sup> at 35 and 15°C, respectively. Fluorescence in situ hybridization and 16S rRNA-based phylogenetic analysis revealed no change in the predominant anammox species with temperature because of the complete retention of anammox biomass in the MBR. These results indicate that the predominant anammox bacteria in the MBR cannot adapt to a low temperature during short-term operation. Conversely, anammox activity recovered rapidly after restoring the temperature from the lower value to the optimal temperature (35°C). **The rapid recovery of anammox activity is a distinct advantage of using an MBR anammox reactor.**

**Keywords:** Anammox; membrane bioreactor; low temperature

## INTRODUCTION

Nitrogen removal during wastewater treatment is essential for the balanced development of society through environmental sustainability. Anaerobic ammonium oxidation (anammox) is a biological process mediated by anammox bacteria belonging to the phylum *Planctomycete*-like bacteria (Strous et al., 1999a). The anammox process is promising as a cost-effective method for nitrogen removal from wastewater because it requires no aeration and no added carbon source. However, anammox bacteria are known for their very slow growth so the anammox process requires long start-up periods, meaning that sufficient biomass must be maintained in the system to sustain effective operation.

Many researchers have described the physiological characteristics of anammox bacteria. The optimum temperature range of “*Candidatus Brocadia anammoxidans*”, “*Candidatus Kuenenia stuttgartiensis*”, and “*Candidatus Brocadia sinica*” were reported as 20–43°C (Strous et al., 1999b), 25–37°C (Egli et al., 2001), and 25–45°C (Oshiki et al., 2011), respectively. For the application of the anammox process to real wastewater treatment, several researchers have referred to the influence of lower temperatures on nitrogen removal performance (Sánchez Guillén et al., 2014; Lancker et al., 2015). However, different conclusions were reached regarding the adaptation of anammox bacteria to lower temperatures. **For example, several studies** reported that anammox bacteria could adapt to a low temperature (Hu et al., 2013; Taotao et al., 2015). Conversely, Dosta et al. reported that a low temperature caused irreversible inhibition of anammox activity though inhibition caused

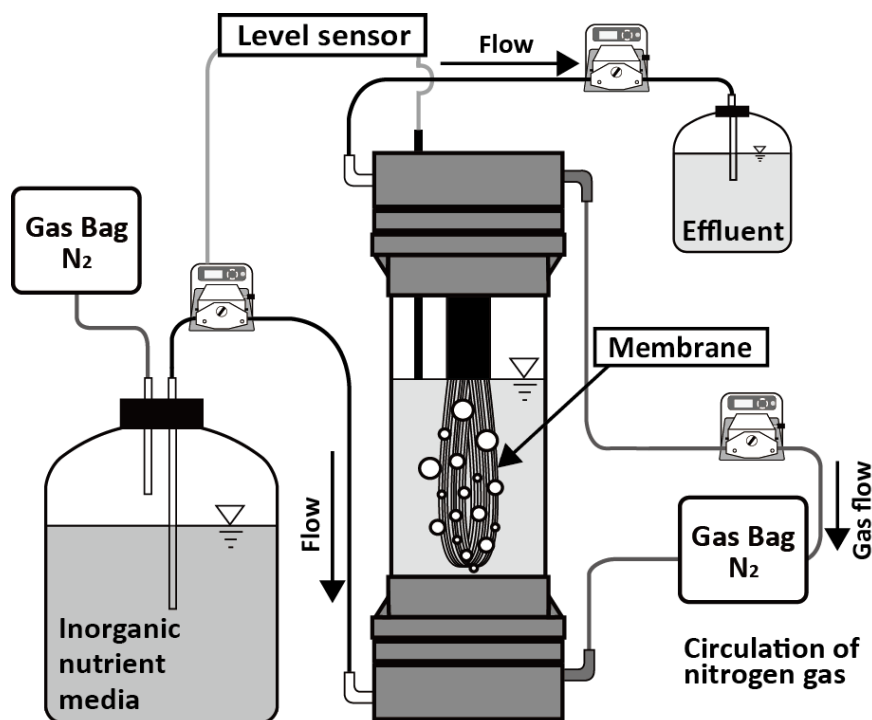
by the accumulation of nitrite resulting from the lower activity at the lower temperature (Dosta et al., 2008). Ma et al. also reported that the nitrogen removal rate decreased when the temperature decreased, although sufficient nitrogen removal performance was maintained (Ma et al., 2013). Results from marine anammox species have also been reported, in which each marine anammox bacterial species had an intrinsic optimal temperature range (Awata et al., 2012). Notably, marine anammox bacteria tend to favour lower temperatures than freshwater species (van de Vossenberg et al., 2008; Awata et al., 2013).

When anammox biomass is washed out because of lower or fluctuating temperature, a long operation period would be needed to recover the nitrogen removal performance. We therefore hypothesized that membrane bioreactors (MBRs) would have benefits for the recovery of nitrogen removal performance because they completely retain biomass within the reactor. Anammox MBRs were successfully operated to investigate the characteristics of anammox bacteria (van der Star et al., 2008; Oshiki et al., 2013). However, fundamental knowledge about the operation of anammox MBRs at different temperatures is currently missing. In this study, an anammox MBR was operated at different temperatures to confirm whether reactor performance could remain stable at lower temperatures, without anammox biomass washout.

## MATERIALS AND METHODS

### Membrane bioreactor

An MBR was operated as shown in **Figure 1**. The working volume of the MBR was 0.64 L. A hollow-fibre membrane unit composed of polyethylene tubes (pore size 0.03  $\mu\text{m}$ , total surface area 0.18  $\text{m}^2$ , Mitsubishi Rayon, Tokyo, Japan) was submerged in the reactor and the membrane flux rate was set at 0.05  $\text{m}^3 \text{day}^{-1}$ . The corresponding influent flow rate and hydraulic retention time were 9.0  $\text{L day}^{-1}$  and 1.70 h, respectively. The membrane was washed with 1% sodium hypochlorite solution after wiping the surface of the membrane, then washed with pure water once every month to maintain membrane flux rate. The inoculum for the MBR was taken from an up-flow column reactor, and was dominated by a freshwater anammox bacterium, "*Candidatus Brocadia sinica*", as reported in a previous study (Tsushima et al., 2007). The concentration of mixed liquor suspended solids (MLSS) was maintained at 8000  $\text{mg-SS L}^{-1}$  by withdrawing biomass once every 2 weeks. The solid retention time (SRT) was calculated to be 88 days. The MBR was operated under six phases at 35°C or 15°C as shown in **Table 1**. The concentration of nitrite in the influent was decreased when the reactor was operated at 15°C to prevent nitrite inhibition of the anammox activity. The nitrogen gas was flowed at 600  $\text{mL min}^{-1}$  into the bottom of the MBR and circulated by a pump.



**Figure 1** Schematic of the anammox MBR configuration used in this study.

**Table 1** Operating conditions.

Period (day)	Temperature (°C)	Influent $\text{NH}_4^+$ (mg-N $\text{L}^{-1}$ )	Influent $\text{NO}_2^-$ (mg-N $\text{L}^{-1}$ )
0-28	35	130-260	170-340
29-84	35-15	50-260	70-340
85-94	35	70-150	100-200
95-114	15	50	70
115-129	35	50-150	70-220
130-146	15	50	70

### Synthetic wastewater

A synthetic nutrient medium was used, containing 3.5–18 mM  $(\text{NH}_4)_2\text{SO}_4$ , 5–24 mM  $\text{NaNO}_2$ , 1.0 mM  $\text{KHCO}_3$ , 0.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.2 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 1 mL of trace element solutions I and II, as described by van de Graaf et al. (1996). The pH was adjusted to 6.5–7.5 using HCl or NaOH. The medium was flushed with  $\text{N}_2$  gas for at least 1 h before adding the nutrients to achieve a dissolved oxygen (DO) concentration below 0.5 mg/L.

### Analytical methods

The concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  were determined using ion-exchange chromatography (HPLC 20A, Shimadzu Co., Kyoto, Japan) with a Shim-pack IC-C4 (Shimadzu) or a Shodex Asahipak NH2P-50 4D anion column (Showa Denko, Tokyo, Japan) and a conductivity detector (CDD-10Avp, Shimadzu) or UV-VIS detector (SPD-10A, Shimadzu) after filtration of the samples through 0.2- $\mu\text{m}$  pore-size cellulose acetate membranes (Advantec Co., Tokyo, Japan) (Awata et al., 2015). The total nitrogen loading and removal rates were calculated based on the concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ , and the hydraulic retention time.

### Phylogenetic analysis

Total DNA was extracted from the MBR at the end of Phase I (day 28) and Phase II (day 84) using the Fast DNA spin kit for soil (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. To construct the clone libraries (Library-35°C and Library-15°C), 16S rRNA gene fragments were amplified using the *Planctomycetales*-specific primer sets Pla46f (Neef et al., 1998) and 1390r (Zheng et al., 1996). The PCR conditions were as follows: 4 min of initial denaturation at 94°C, followed by 30 cycles of 45s at 94°C, 50s at 58°C, and 3 min at 72°C. The final extension was performed for 10 min at 72°C. PCR products were confirmed using a 1% (w/v) agarose gel and were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified PCR products were ligated into a pCR-XL-TOPO vector and transformed into One Shot *Escherichia coli* cells, following the manufacturer's instructions (TOPO XL PCR cloning kit; Invitrogen, Carlsbad, CA, USA). Sequences with 97% or greater similarity were grouped into operational taxonomic units (OTUs) using the ARB software (Ludwig et al., 2004). The phylogenetic tree was constructed using neighbour-joining (distant matrix) with Jukes-Cantor correction, maximum parsimony (Phylip DNAPARS), and maximum likelihood (RAxML) with GTR Gamma model methods in the ARB software, with a database SSU Ref NR release 119 (Pruesse et al., 2007). Bootstrap resampling analysis of 1000 replicates was performed to estimate the confidence of tree topologies. The sequence data of the partial 16S rRNA gene obtained from the MBR was deposited in the GenBank/EMBL/DBJ databases under accession number LC053452 and LC053453.

### FISH analysis

Biomass samples were obtained from Phase I (day 28) and Phase II (day 84) to compare the microbial community composition of anammox bacteria, and fixed in a 4% paraformaldehyde solution for 8 h at 4°C. *In situ* hybridization was conducted according to the procedure described by Okabe et al. (1999), and a model Axioimager M1 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) was used for the observation. The 16S rRNA-targeted oligonucleotide probes used in this study were EUBmix, which was composed of EUB338 (Amann et al., 1990), EUB338II, and EUB338III (Daims et al., 1999) and Amx820 (Shmid et al., 2001). The probes were labelled with Cy3 or Alexa Fluor 488 at the 5' end. For the quantitative determination of microbial composition in the granules, the surface fraction of the specific probe-hybridized cell area and EUBmix probe-hybridized cell area were determined after gentle homogenization (Kindaichi et al., 2004). The average fraction was determined from 16 representative fluorescent images using ImageJ software (Collins, 2007).

## RESULTS AND DISCUSSION

### Effects of temperature on nitrogen removal

The MBR was operated at different temperatures to clarify the influence of temperature on nitrogen removal by the anammox process. **Figure 2** shows the results of time courses for the reactor performance. In Phase I (0–28 days), the MBR was stably operated with an 80% average nitrogen removal efficiency at 35°C and a maximum nitrogen removal rate of 6.7 g-N L<sup>-1</sup> day<sup>-1</sup>. Temperature decreased **rapidly** from Phase I to Phase II. In Phase II (29–84 days), the nitrogen removal rate rapidly dropped when operating at 15°C. After 2 weeks of operation at 15°C, there was no recovery of nitrogen removal but nitrite accumulation **up to 300 mg L<sup>-1</sup>, which is an inhibitory concentration**, was observed. Thereafter, the nitrogen loading rate was gradually decreased to prevent the accumulation of nitrite and to determine whether or not the anammox activity had recovered. The anammox activity recovered slightly when the influent ammonium and nitrite levels were 50 and 70 mg-N L<sup>-1</sup>, respectively,

although the nitrogen removal performance was unstable. The maximum nitrogen removal rate in Phase II was  $1.1 \text{ g-N L}^{-1} \text{ day}^{-1}$ , corresponding to an 84% activity loss. Ma et al. reported that the nitrogen removal rate in an anammox UASB reactor was  $2.28 \text{ g-N L}^{-1} \text{ day}^{-1}$  at  $16^\circ\text{C}$ , which was a 60% activity loss compared with that at  $30^\circ\text{C}$  (Ma et al., 2013). Oshiki et al. also reported that the anammox activity of “*Candidatus Brocadia sinica*” at  $15^\circ\text{C}$  decreased to 25% of the maximum activity obtained at  $35^\circ\text{C}$  in batch experiments (Oshiki et al. 2011). The anammox activity loss in the present study shows that it is possible that the dominant anammox bacteria in the MBR could not adapt to the lower temperature during short-term exposure. The nitrogen removal efficiency increased to 60% when the nitrogen loading rate decreased. The fluctuation in nitrogen removal efficiency would be affected by the slight increase in nitrogen loading rate we imposed to offset the decrease in nitrite concentration. The decreasing nitrogen loading rate is necessary to prevent the inhibition by high concentration of nitrite ( $<16 \text{ mM}$ ) (Oshiki et al., 2011). During Phase III (85–94 days), the MBR was operated at  $35^\circ\text{C}$  to recover the anammox activity and to increase the nitrogen removal efficiency. The nitrogen removal efficiency increased rapidly to 100%, which indicates that the decrease in anammox activity at  $15^\circ\text{C}$  was a reversible inhibition. During Phase IV (95–114 days), the temperature was decreased again to test the possibility of adaptation by the anammox bacteria to the lower temperature at a lower nitrogen loading rate (i.e.,  $1.5\text{--}1.9 \text{ g-N L}^{-1} \text{ day}^{-1}$ ). The nitrogen removal rate and efficiency dropped rapidly in Phase II and also no recovery of activity was observed. In Phase V (115–129 days,  $35^\circ\text{C}$ ) and Phase VI (130–146 days,  $15^\circ\text{C}$ ), the MBR was operated to duplicate Phases III and IV, in which no recovery of anammox activity was observed. From this repeated observation of anammox activity loss, we speculate that the anammox bacteria used in this study could not rapidly adapt to the lower temperature. This result is not consistent with the adaptation of anammox bacteria to a lower temperature observed earlier by Hu et al. (2013). One possible explanation is that the adaptation of anammox bacteria to lower temperature needs a long-term incubation (operation) or a change in the dominant species in the reactor. This hypothesis should be investigated in the future.

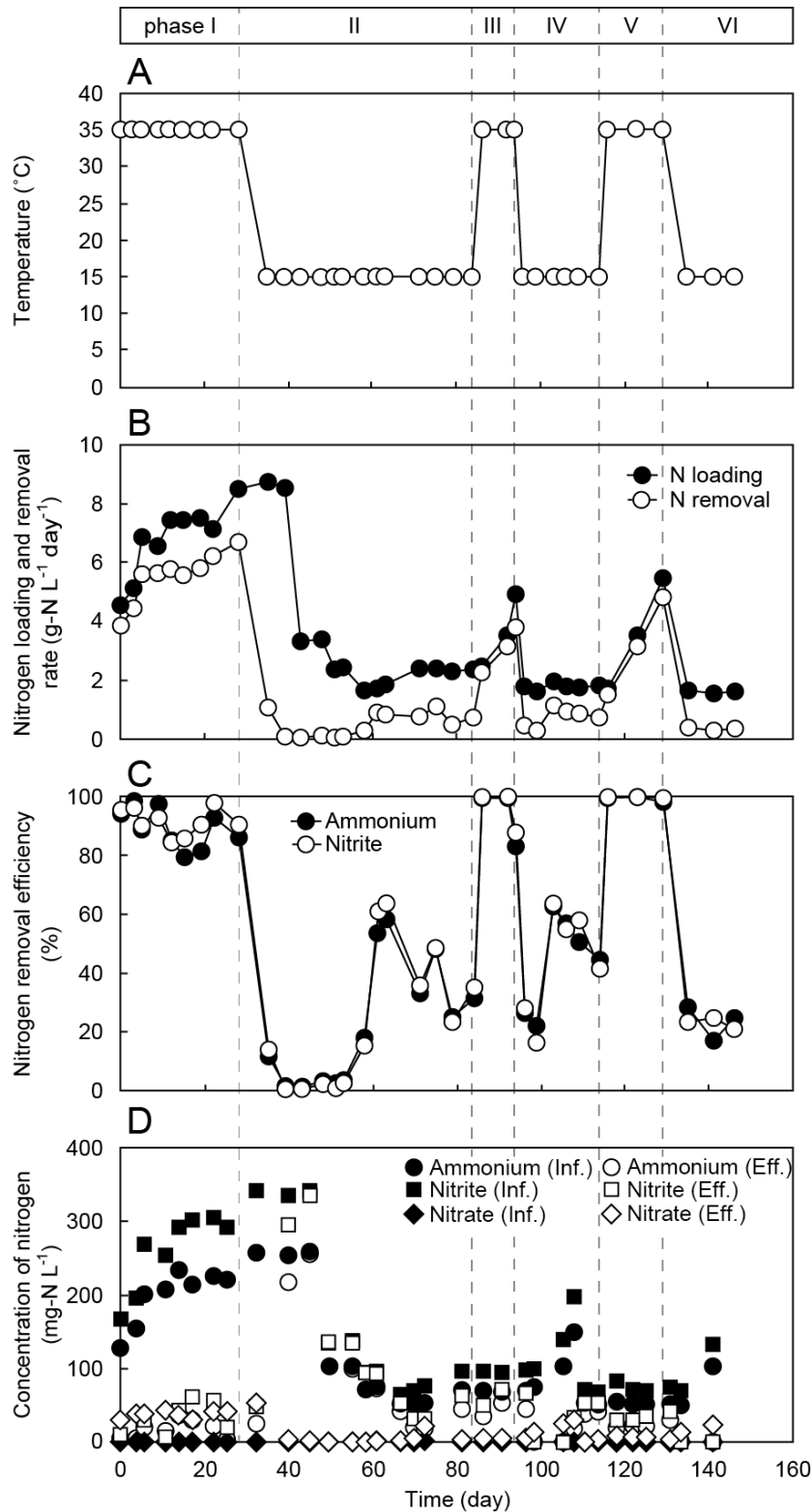
### Microbial community structure

The predominant species at the end of Phases I and II were confirmed, based on the 16S rRNA gene sequences. From phylogenetic analysis, we obtained 37 and 48 clones from Library- $35^\circ\text{C}$  and Library- $15^\circ\text{C}$ , respectively. One OTU was identified from each library on the basis of more than 97% sequence identity. Anammox bacteria closely related to “*Candidatus Brocadia sinica*” with 99.5% sequence identity was predominant in the both biomass samples (Figure 3). The sequence identity of both sequences was 100%, which indicates that the predominant species did not change with temperature. A quantitative FISH analysis was conducted to determine the microbial community composition of the anammox bacteria at the different temperatures. The composition of anammox bacteria in the inoculum was determined as  $76.3 \pm 7.9\%$  of the total bacteria. The anammox bacterial composition was  $68.3 \pm 12.7\%$  (Fig. 4A) and  $65.0 \pm 10.3\%$  (Fig. 4B) at 35 and  $15^\circ\text{C}$ , respectively, which also supports the conclusion that the community composition of the anammox bacteria did not change significantly over the short-term.

### CONCLUSIONS

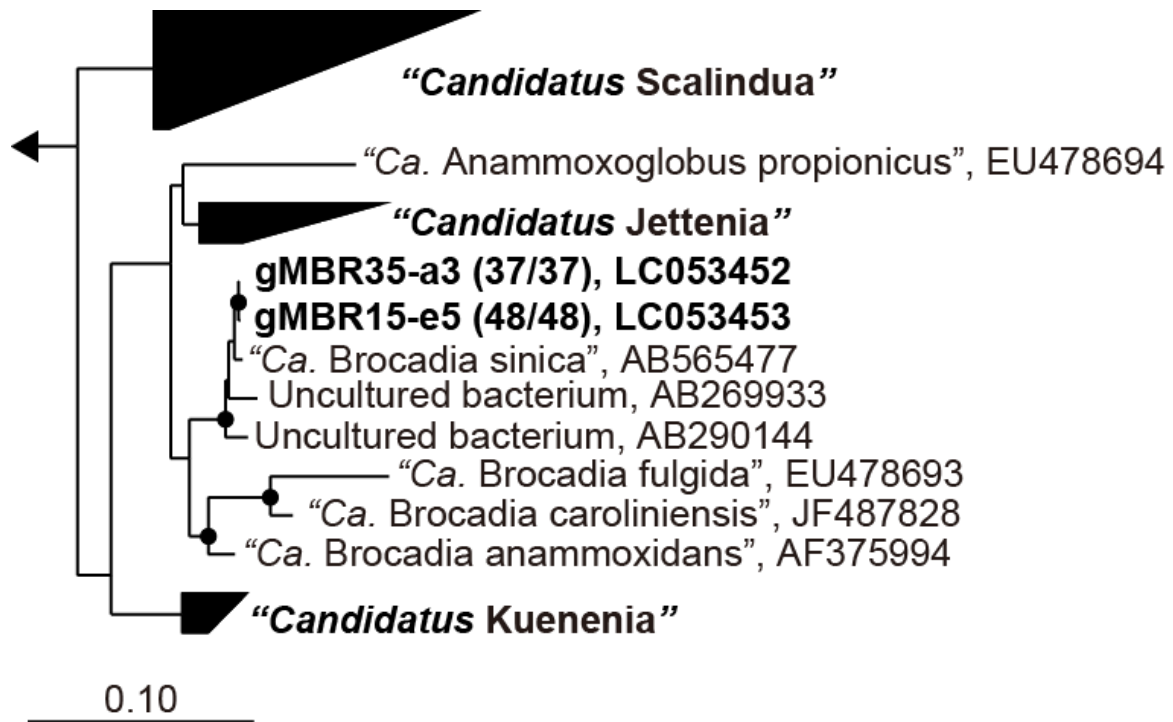
An anammox MBR was operated to investigate the influence of temperature on nitrogen removal performance and the microbial community structure of anammox MBR. The nitrogen removal rate decreased with decreasing operational temperature, but the community composition and predominant species of anammox bacteria did not change significantly. No adaptation of anammox bacteria to the lower temperature was observed during short-term

operation in the anammox MBR. However, the nitrogen removal performance of the MBR rapidly recovered with increasing temperature, which is the one of the advantages of using the MBR. In future work, we are interested in confirming whether the population and microbial community of anammox bacteria in an MBR may change during long-term operation at lower temperatures.

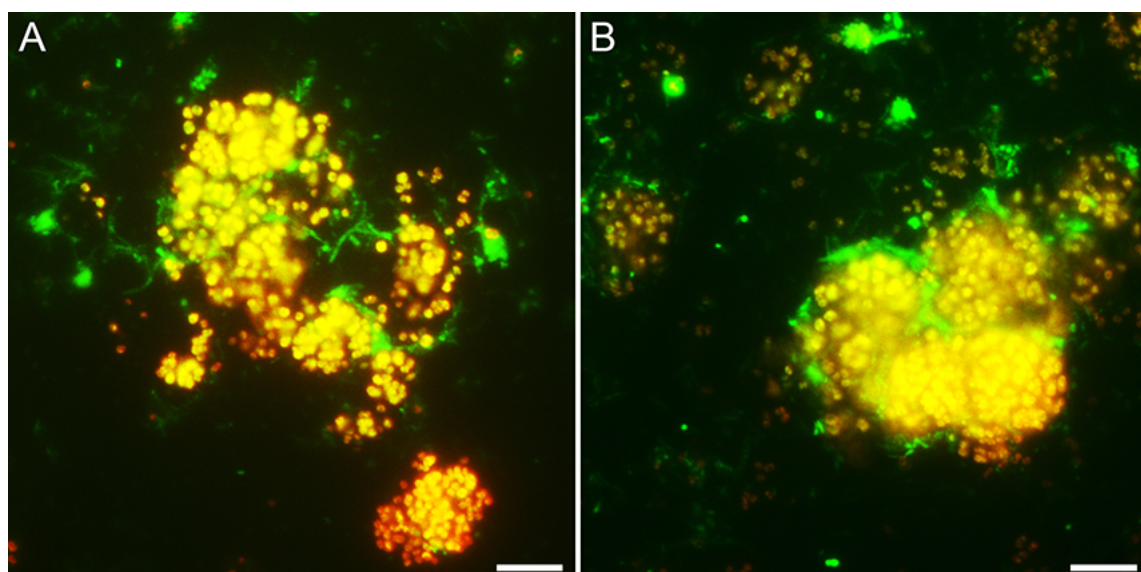




**Figure 2** Nitrogen removal performances of the anammox MBR. Temperature (A), nitrogen loading and removal rates (B), nitrogen removal efficiencies (C), and concentration of nitrogen (D) in each phase.



**Figure 3** Maximum likelihood phylogenetic tree of dominant anammox bacteria in the anammox MBR under different temperatures. gMBR35 and gMBR15 represent the OTU obtained from Phases I and II, respectively. The *Thermotoga* spp. sequence served as the outgroup to rooting the tree. The numbers in parentheses indicate the frequencies of the identical clones analysed. The scale bar represents the number of nucleotide changes per sequence position. Reproducible nodes are indicated as a closed circle (>90% bootstrap support for three inferences used in this study: neighbour joining, maximum parsimony, and maximum likelihood methods with 1000 replicates). The accession numbers of the OTU obtained in this study and the sequences from public data are also indicated.





**Figure 4** FISH images of anammox biomass in the anammox MBR at 35°C (A) and 15°C (B). The bar represents 10 µm. (The full colour version of this figure is available in the online version of this paper, at <http://www.iwaponline.com/wst/toc.htm>)

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