Supplemental Materials

Physiological Characterization of an Anaerobic Ammonium-Oxidizing Bacterium Belonging to the "*Candidatus* Scalindua" group

by

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Supplemental Methods

Biomass samples.

A 7-L membrane bioreactor (MBR) was operated to obtain free-living anammox cells related to "*Candidatus* Scalindua". A biofilm sample (1 g-wet) taken from an up-flow column reactor equipped with a non-woven fabric sheet (1, 2) was gently homogenized and inoculated in the MBR. The MBR was operated at 28°C in the dark with mixing by a propeller at a stirring speed of 200 rpm. A membrane unit composed of a hollow-fibered membrane (polyethylene, Mitsubishi Rayon, Tokyo, Japan) with a 0.4 μm pore size was installed into the MBR. The effective membrane surface area was 800 cm^2. A synthetic marine medium (2.8% salinity) was fed continuously into the MBR at a rate of 1 L day^{-1}. The hydraulic retention time (HRT) in the MBR was set at 7 days. To maintain anoxic conditions and provide buffering capacity, the culture medium was sparged continuously with 95% Ar-5% CO_2. The pH in the MBR was not controlled, but was in the range of 7.3–7.6.

The synthetic marine medium was composed as follows: 28 g L^{-1} of an artificial sea salt SEALIFE (Marine Tech, Tokyo, Japan) supplemented with $(NH_4^+)_2SO_4$ (5 mM), $NaNO_2$ (5 mM), $KHCO_3$ (5 mM), KH_2PO_4 (0.2 mM), yeast extract (1 mg L^{-1}), and 1 mL of trace element solutions I and II (3). Kindaichi et al. (1) reported the detailed composition of SEALIFE. The medium was flushed with N_2 gas for at least 1 h to achieve a concentration of dissolved oxygen (DO) below 0.5 mg L^{-1}. An

anammox enrichment culture dominated by "*Candidatus* Brocadia sinica" (4) was also used to determine the biomass yield (see below).

Fluorescence in situ hybridization (FISH) and aggregation size of anammox bacteria enriched with MBR.

Cells in the MBR were collected after 237 days of operation and fixed in 4% paraformaldehyde. FISH was then conducted according to the procedure described by Okabe et al. (5). A model LSM5 PASCAL confocal laser-scanning microscope equipped with an Ar ion laser (488 nm) and a HeNe laser (543 nm) (Carl Zeiss, Oberkochen, Germany) was used for microscopy. The 16S rRNA-targeted oligonucleotide probes used in this study were a EUBmix comprising EUB338 (6), EUB338II (7), and EUB338III (7) for most bacteria, Amx368 (8) for all anammox bacteria, and Sca1129a and Sca1129b for marine anammox bacteria clone husup-a2 and husup-a7, respectively, enriched in the previous study (1). The probes were labeled with Cy3 or Alexa Flour488 at the 5' end. The average surface area fraction was determined from at least 20 representative laser scanning microscopy projection images using the software LSM5 PASCAL provided by Carl Zeiss (9).

The size distribution of cell aggregates in the MBR at 78 and 328 days of operation was determined with a laser diffraction particle size analyzer, SALD-2000J (Shimadzu, Kyoto, Japan).

Phylogenetic analysis.

Total genomic DNA was extracted from the cells in the MBR after 328 days of operation using the Fast DNA spin kit for soil (MP Biomedicals, Irvine, CA), according to the manufacturer's instructions. To construct the clone library, the regions between the 16S and 23S rRNA genes, including 16S–23S intergenic spacer region (ISR) were amplified using ONE Shot LA PCR MIX kit (Takara Bio, Otsu, Japan) and a *Planctomycetales*-specific primer set (10) and 1037r (11) for 16S–23S rRNA gene. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and

then cloned using a TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The sequencing primers used are shown in Table S1. Phylogenetic trees were constructed by the neighbor-joining (Jukes-Cantor model), maximum parsimony (Phylip DNAPARS), and maximum likelihood (RAxML) methods using the ARB software (12). The 16S rRNA gene sequences showing ≥97% identity were grouped into operational taxonomic units (OTUs). Bootstrap resampling analysis for 1,000 replicates was conducted to estimate the confidence of the tree topologies. The sequence identities among anammox species were calculated using the BLAST searches (13).

Batch experiments.

Batch experiments were performed to determine the following physiological parameters: (a) growth temperature, pH, and salinity range, (b) inhibition by nitrite and ammonium, (c) half-saturation constants (K_s) for nitrite and ammonium, (d) accumulation and consumption of hydrazine after the addition of hydroxylamine, and (e) biomass yield. The biomass from the MBR (aggregate diameter <1.3 μm) after 328 days of operation was washed twice and suspended in an anammox minimal salts medium (3) containing HEPES buffer (final concentration of 2 mM) at a volatile suspended solids (VSS) concentration of 3.5 mg-VSS L^{-1}. To determine the growth temperature, pH, and salinity range, 2.3 mL of biomass suspension was dispensed into 12.5 mL serum vials and sealed with butyl rubber stoppers. Subsequently, ^{15}N-labeled ammonium sulfate (50 mM) (Isotec Inc., Miamisburg, Ohio, USA) and unlabeled sodium nitrite (50 mM) were added into vials at a final concentration of 2 mM using gas-tight syringes and the headspace was replaced by repeatedly vacuuming and purging with helium gas (>99.99995%). The influence of temperature on anammox activity was examined at 5–45°C at pH 7.5 and a salinity of 3.5% (w/v). Activation energy was calculated on the basis of the temperature dependency of the $^{29}N_2$ production rate. The influence of pH was examined at pH 5.0–9.0 at 28°C and a salinity of 3.5% (w/v). The initial pH was adjusted with 0.5 M H_2SO_4 or 1 M NaOH. pH

was not controlled during incubation. The influence of salinity was examined at 0–4.0% (w/v) at 28°C and pH 7.5. The salt concentrations were adjusted by diluting the artificial sea salt SEALIFE. To avoid the lack of minerals in the medium, magnesium sulfate and calcium chloride were added to the synthetic marine medium at final concentrations of 1.2 mM $MgSO_4 \bullet 7H_2O$ and 1.2 mM $CaCl_2 \bullet 2H_2O$, respectively.

Anammox inhibition was evaluated under different concentrations of nitrite and ammonium. The initial concentrations of nitrite and ammonium were 2.5–9.0 mM and 7–16 mM, respectively.

Values of K_s for ammonium and nitrite were determined under nitrite or ammonium-limiting conditions, respectively. The free-living biomass was washed twice with synthetic marine medium and the biomass concentration was set at 37.6 mg-VSS L^{-1}. The aliquots (90 mL) were dispensed into 125 mL serum vials. The initial nitrite and ammonium concentrations were set at 0.5 and 1.2 mM or 1.8 and 0.4 mM, respectively. The K_s values were calculated according to the Michaelis-Menten kinetics.

To confirm the accumulation and subsequent consumption of hydrazine, hydroxylamine and ammonium were supplemented into 20 mL vials containing 15 mL of biomass suspension at final concentrations of 3 mM. The culture was collected at different time intervals, and hydrazine, hydroxylamine, and ammonium concentrations were determined.

Maximum specific growth rate.

Maximum specific growth rate (μ_{max}) was calculated on the basis of the maximum specific anaerobic ammonium oxidation rate (q_{max}) and biomass yield as follows:

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

where μ_{max} is the maximum specific growth rate (h^{-1}), q_{max} is the maximum specific anaerobic ammonium oxidation rate [mol NH_4^+ (mol C)$^{-1}$ day^{-1}], and Y is the biomass yield [Y, mol C (mol NH_4^+)$^{-1}$]. An up-flow glass column reactor equipped with glass filters at the bottom and upper part of the reactor to avoid the biomass washout was used to determine q_{max}. The volume of the up-flow glass

column reactor was 21 mL, and the free-living anammox cells were inoculated as the seed biomass. The initial biomass content was 19.6 mg-VSS. The up-flow glass column reactor was operated at 28°C and pH 7.3–7.6. The synthetic marine medium containing ammonium (5.7 mM) and nitrite (5.7 mM) was continuously fed into the up-flow glass column reactor, and the HRT was set at 0.23 h. The ammonium oxidation rate was calculated on the basis of the ammonium concentrations of the influent and effluent and the HRT. To determine the biomass concentration, all biomass retained in the column reactor was harvested after 50 days of operation, and the VSS was measured. The q_{max} was calculated as described by Oshiki et al. (14).

The biomass yield (Y) was evaluated on the basis of the incorporation rate of [^{14}C]bicarbonate and ammonium oxidation. The free-living biomass was suspended in the synthetic marine medium with various ammonium and nitrite concentrations (1.4 to 7.1 mM each), and [^{14}C]bicarbonate was supplemented at a final concentration of 20 μCi vial^{-1} (740 kBq vial^{-1}). Four milliliters of the culture was dispensed into 5 mL serum vials and incubated at 28°C for 1–3 days. Biomass suspension was collected each day, washed three times with phosphate-buffered saline (PBS), and mixed with scintillation cocktail (Clear-sol I, Nacalai Tesque, Kyoto, Japan). The biomass yield of "*Candidatus* Brocadia sinica" was also determined in the same way, except that it was cultivated in inorganic nutrient medium (14) instead of the synthetic marine medium. Radioactivity was determined with an LSC-5100 liquid scintillation counter (Hitachi-Aloka Medical, Tokyo, Japan). Additional incubation in the absence of [^{14}C]bicarbonate was performed in parallel to determine the ammonium oxidation rate. The biomass yield was calculated as described by Oshiki et al. (14).

Transmission electron microscopy.

Sample preparation for transmission electron microscopy was performed as described by van Niftrik et al. (15) with slight modifications. Briefly, the free-living anammox cells were collected from the MBR reactor after 258 days of operation and cryofixed. Freeze-substitution was carried out with acetone

containing 2% osmium tetroxide. After dehydration with acetone, the cells were embedded in EPON812 resin. Ultrathin sections (80–90 nm) were obtained with an ultramicrotome and then post-stained with uranyl acetate and lead citrate. The sections were observed with an electron microscope (JEM-1200EX, JEOL, Tokyo, Japan) at 80 kV.

Analytical procedures.

Ammonium concentration was determined using the Nessler's method with a UV-visible spectrophotometer (DR-2800, Hach, Loveland, CO) (16). The concentrations of nitrite and nitrate were determined using an ion-exchange chromatography (HPLC 10Avp, Shimadzu, Kyoto, Japan) with a Shodex Asahipak NH2P-50 4D anion column (Showa Denko, Tokyo, Japan) and a UV-VIS detector (SPD-10A, Shimadzu, Kyoto, Japan) after filtration of samples through 0.2-μm pore-size membranes (Advantec, Tokyo, Japan). Nitrite concentration was determined using the diazotization method with a UV-VIS spectrophotometer (UV-2400PC, Shimadzu, Kyoto, Japan) (17) during the determination of the K_s value for nitrite. Hydroxylamine and hydrazine concentrations were determined by the colorimetric method (18, 19). Biomass dry weight was determined as VSS concentration, according to the standard method (17). Protein concentration was determined with a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), as described in the manufacturer's instructions. Biomass protein content was calculated by dividing the protein concentration by the VSS concentration. The production of $^{29}N_2$ and $^{30}N_2$ gases was measured by gas chromatography-mass spectrometry, as described previously (20). Briefly, 50 μl of headspace gas was injected with a split ratio of 1:50 into a gas chromatograph GCMS-QP2010 SE (Shimadzu, Kyoto, Japan) equipped with a CP-Pora BOND Q fused silica capillary column (Agilent technologies, Santa Clara, CA), and the m/z of 28, 29 and 30 were monitored. The standard curve for the quantification of $^{29}N_2$ and $^{30}N_2$ gases was prepared by using $^{30}N_2$ gas (>98 % purity) (Cambridge Isotope Laboratories, Inc., Andover, MA).

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List of supplemental figures

FIG S1 The aggregation size distribution of biomass in the MBR. Squares and circles represent the aggregation size distribution of biomass in the MBR after 78 and 328 days of operation, respectively. Almost all cells (>94.6 %) were suspended as free-living cells (<1.3 μm) at 328 days.

FIG S2 Production of different nitrogen compounds after hydroxylamine and ammonium addition to anammox enrichment cultures. Closed squares, closed circles, and open circles represent ammonium, hydroxylamine, and hydrazine concentrations, respectively.

FIG S3 Evaluation of affinity constants for nitrite (A and B) and ammonium (C and D). Duplicate batch experiments were conducted. Ammonium and nitrite concentrations under nitrite-limiting conditions (A). Open circles and open squares represent ammonium concentrations, whereas closed circles and closed squares represent nitrite concentrations (A). Hanes-Woolf plot of the nitrite concentrations in panel A (B). Ammonium and nitrite concentrations under ammonium-limiting conditions (C). Open circles and open squares represent ammonium concentrations, whereas closed circles and closed squares represent nitrite concentrations (C). Hanes-Woolf plot of ammonium concentrations in panel C (D).

FIG S4 Performance of the up-flow glass column reactor for determination of μ_{max}. The ammonium removal rate gradually increased with time, and a maximum volumetric ammonium removal rate of 4.02 g-N L^{-1} day^{-1} was achieved at 50 days.

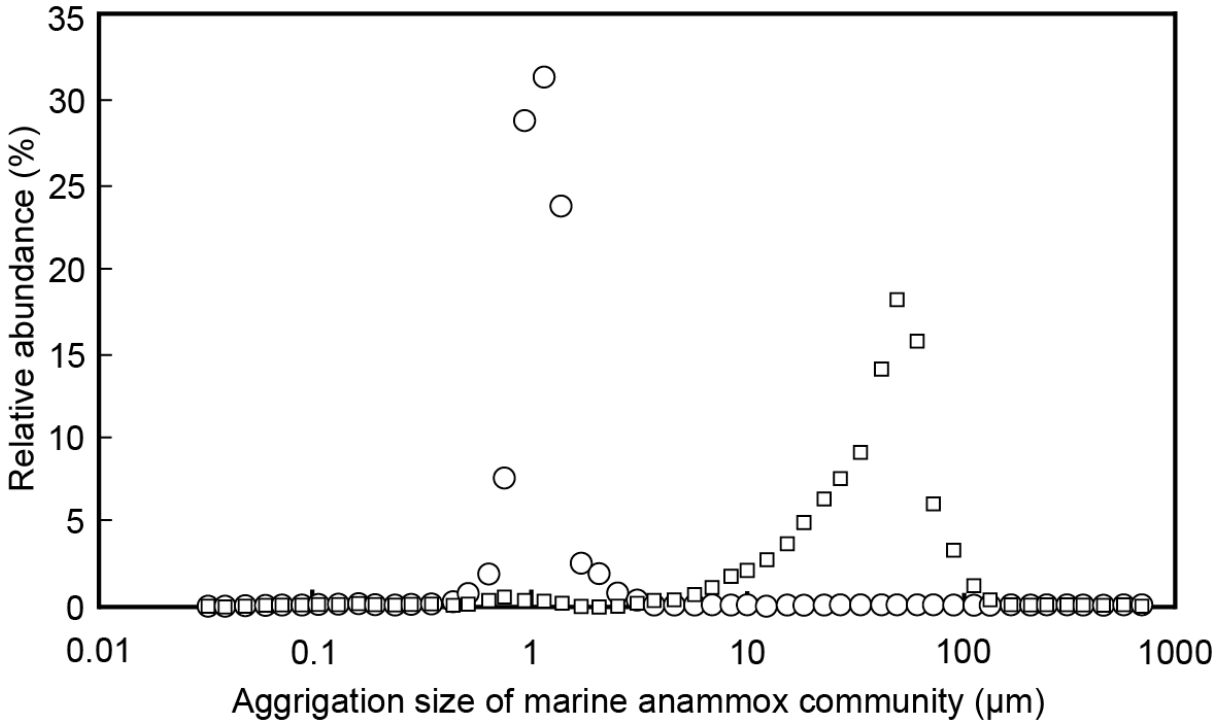
35
30
25
20
15
10
5
0
Relative abundance (%)
0.01
0.1
1
10
100
1000
Aggrigation size of marine anammox community (μm)

FIG S1

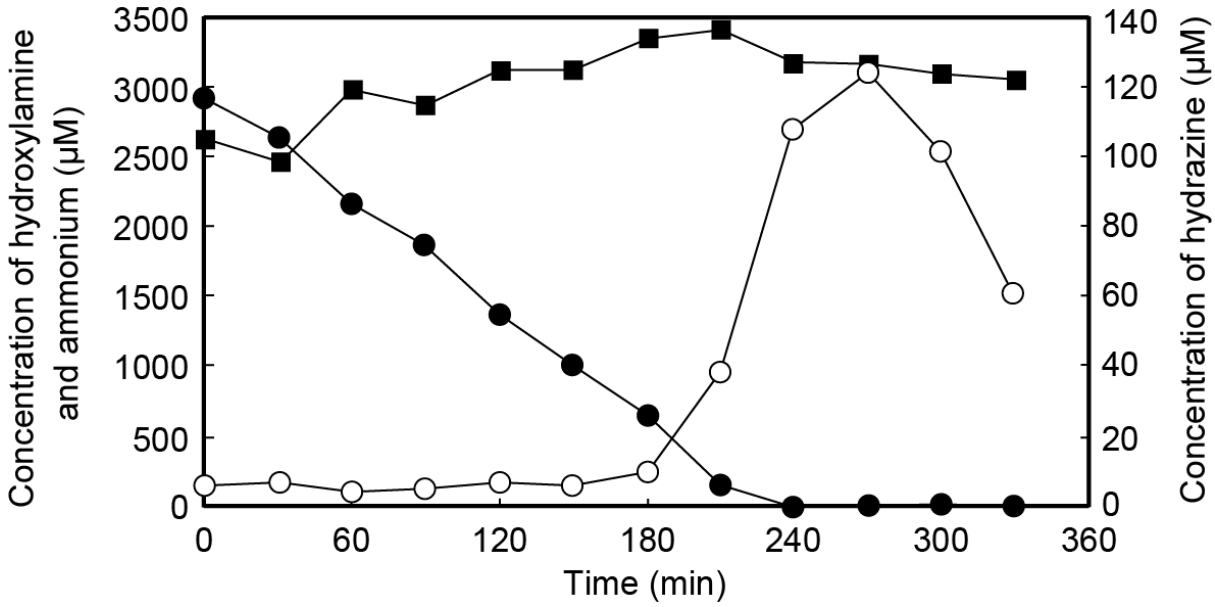
Concentration of hydroxylamine and ammonium (μM)
Concentration of hydrazine (μM)
Time (min)
0
500
1000
1500
2000
2500
3000
3500
0
20
40
60
80
100
120
140
0
60
120
180
240
300
360

FIG S2

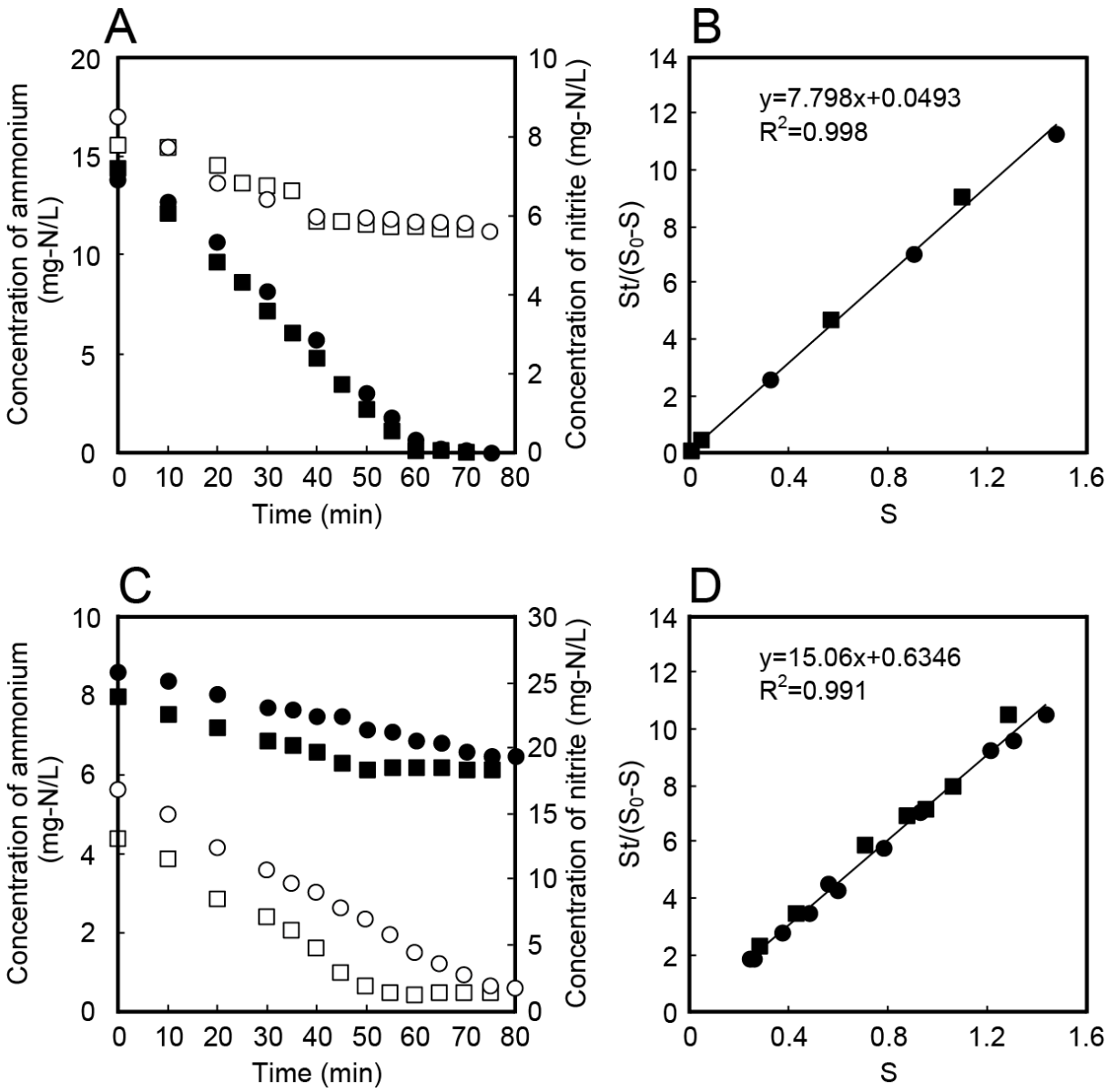
A
Concentration of ammonium (mg-N/L)
Concentration of nitrite (mg-N/L)
Time (min)
B
y=7.798x+0.0493
R2=0.998
St/(S0-S)
S
C
Concentration of ammonium (mg-N/L)
Concentration of nitrite (mg-N/L)
Time (min)
D
y=15.06x+0.6346
R2=0.991
St/(S0-S)
S

FIG S3

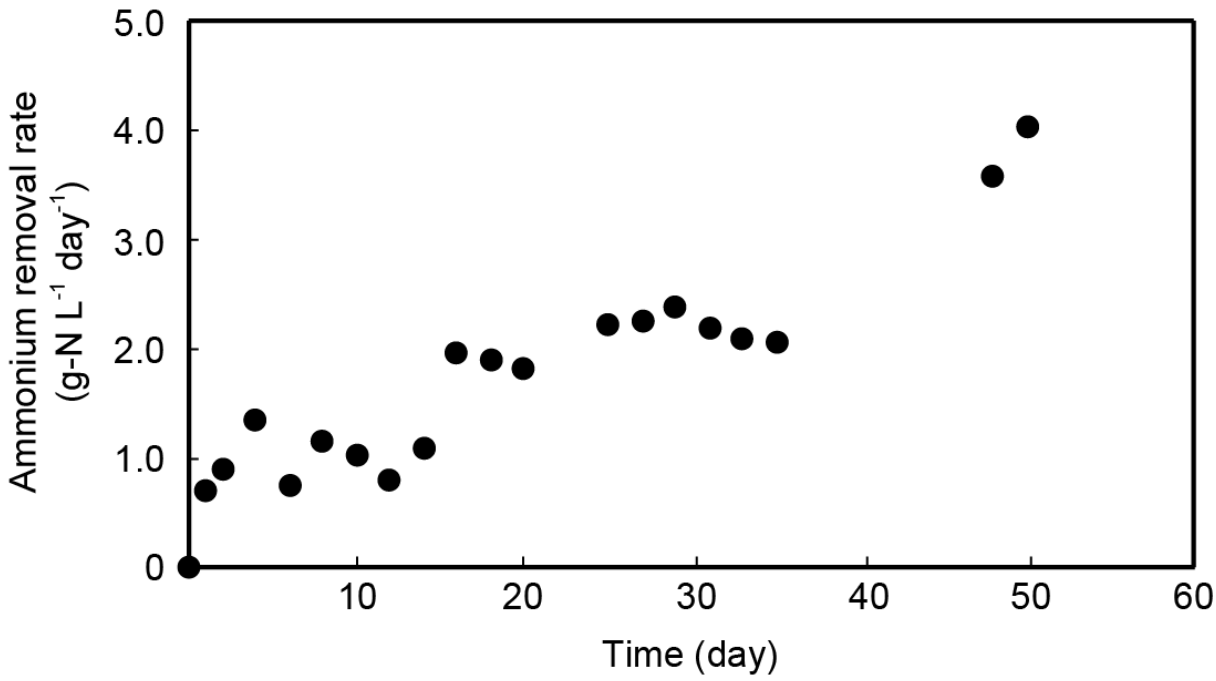
Ammonium removal rate
(g-N L-1 day-1)
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
60
Time (day)

FIG S4

Table S1 Nucleotide sequences of primers used in the present study. Primers other than Pla46f and 1037r were used only for DNA sequencing reaction.

Primer	Sequence (5'-3')	Reference
Forward		
Pla46f	GGATTAGGCATGCAAGTC	10
2891f	AGCGAAAGCGAGTACTAAC	This study
Reverse		
907R	CCGTCAATTCMTTTGAGTTT	21
630R	CAKAAAGGAGGTGATCC	22
1035R	TTCGCTCGCCRCTAC	23
1020R	TCTGGGYTGTTYCCCT	23
1037R	CGACAAGGAATTTCGCTAC	23

Table S2 16S, 16S–23S intergenic spacer region (ISR), and 23S rRNA gene sequence similarity (%) between "*Candidatus* Scalindua sp." and ten anammox species. The following nucleotide sequences were used as representatives of the species: "*Candidatus* Scalindua sp." (AB760234), "*Candidatus* Scalindua wagneri" (EU478692), "*Candidatus* Scalindua brodae"(AY254883), "*Candidatus* Scalindua sorokinii"(AY257181), "*Candidatus* Scalindua marina"(EF602038), "*Candidatus* Scalindua profunda" (2017108002, taxon ID in the IMG/M), "*Candidatus* Jettenia asiatica"(DQ301513), "*Candidatus* Brocadia sinica"(AB565477), "*Candidatus* Brocadia anammoxidans"(AF375994), "*Candidatus* Brocadia fulgida"(EU478693), "*Candidatus* Kuenenia stuttgartiensis"(AF375995), "*Candidatus* Anammoxoglobus propionicus"(EU478694).

	"*Ca.* S. wagneri"			"*Ca.* S. brodae"			"*Ca.* S. sorokinii"			"*Ca.* S. marina"			"*Ca.* S. profunda"			"*Ca.* J. asiatica"			"*Ca.* B. sinica"			"*Ca.* B. anammoxidans"			"*Ca.* B. fulgida"			"*Ca.* K. stuttgartiensis"			"*Ca.* A. propionicus"		
	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S	16S	ISR	23S
"*Ca.* Scalindua sp."	97.0	87.8	ND	92.6	ND	ND	92.5	ND	ND	92.4	ND	ND	91.8	ND	87.7	89.4	87.3	78.9	89.3	85.5	80.2	88.6	85.4	79.8	90.8	ND	ND	89.8	85.4	80.0	88.4	ND	ND
"*Ca.* S. wagneri"				93.3	ND	ND	93.3	ND	ND	92.8	ND	ND	92.4	ND	ND	89.4	83.2	ND	89.4	85.1	ND	88.8	85.1	ND	90.8	ND	ND	90.1	84.6	ND	88.4	ND	ND
"*Ca.* S. brodae"							98.3	ND	ND	96.7	ND	ND	97.3	ND	ND	89.5	ND	ND	90.3	ND	ND	89.7	ND	ND	91.0	ND	ND	90.4	ND	ND	88.7	ND	ND
"*Ca.* S. sorokinii"										96.4	ND	ND	96.5	ND	ND	89.3	ND	ND	90.7	ND	ND	89.9	ND	ND	90.9	ND	ND	90.6	ND	ND	88.9	ND	ND
"*Ca.* S. marina"													95.5	ND	ND	89.5	ND	ND	90.0	ND	ND	89.6	ND	ND	90.5	ND	ND	90.5	ND	ND	88.7	ND	ND
"*Ca.* S. profunda"																89.8	ND	79.3	90.3	ND	79.6	89.8	ND	79.5	90.4	ND	ND	90.7	ND	80.3	88.8	ND	ND
"*Ca.* J. asiatica"																			92.4	84.5	91.3	92.3	83.6	90.3	92.8	82.8	ND	90.6	87.2	88.1	93.6	ND	ND
"*Ca.* B. sinica"																						96.2	88.6	97.1	94.3	88.2	ND	91.2	88.9	89.8	91.1	91.2	ND
"*Ca.* B. anammoxidans"																									94.3	87.1	ND	91.0	88.9	89.3	91.4	91.2	ND
"*Ca.* B. fulgida"																												91.6	89.8	ND	91.8	89.2	ND
"*Ca.* K. stuttgartiensis"																															90.0	89.2	ND

ND; not determined.

The nucleotide sequence corresponding to the ISR of *Ca.* S. profunda is not available because the region is not covered by the contig sequences.