

ARTICLE



Maintenance power requirements of anammox bacteria "*Candidatus Brocadia sinica*" and "*Candidatus Scalindua sp.*"

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Little is known about the cell physiology of anammox bacteria growing at extremely low growth rates. Here, "*Candidatus Brocadia sinica*" and "*Candidatus Scalindua sp.*" were grown in continuous anaerobic membrane bioreactors (MBRs) with complete biomass retention to determine maintenance energy (i.e., power) requirements at near-zero growth rates. After prolonged retentostat cultivations, the specific growth rates (μ) of "*Ca. B. sinica*" and "*Ca. Scalindua sp.*" decreased to 0.000023 h^{-1} (doubling time of 1255 days) and 0.000157 h^{-1} (184 days), respectively. Under these near-zero growth conditions, substrate was continuously utilized to meet maintenance energy demands (m_e) of 6.7 ± 0.7 and $4.3 \pm 0.7 \text{ kJ mole of biomass-C}^{-1} \text{ h}^{-1}$ for "*Ca. B. sinica*" and "*Ca. Scalindua sp.*", which accorded with the theoretically predicted values of all anaerobic microorganisms (9.7 and 4.4 kJ mole of biomass-C⁻¹ h⁻¹ at 37 °C and 28 °C, respectively). These m_e values correspond to 13.4×10^{-15} and 8.6×10^{-15} watts cell⁻¹ for "*Ca. B. sinica*" and "*Ca. Scalindua sp.*", which were five orders of magnitude higher than the basal power limit for natural settings (1.9×10^{-19} watts cells⁻¹). Furthermore, the minimum substrate concentrations required for growth (S_{min}) were calculated to be 3.69 ± 0.21 and $0.09 \pm 0.05 \mu\text{M NO}_2^-$ for "*Ca. B. sinica*" and "*Ca. Scalindua sp.*", respectively. These results match the evidence that "*Ca. Scalindua sp.*" with lower maintenance power requirement and S_{min} are better adapted to energy-limited natural environments than "*Ca. B. sinica*", suggesting the importance of these parameters on ecological niche differentiation in natural environments.

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INTRODUCTION

The anaerobic ammonium-oxidation (anammox) reaction has been utilized as an alternative energy-saving biological process for nitrogen removal from various wastewaters due to lower oxygen requirement, no external carbon source demand, and less sludge production. At present, more than 100 full-scale treatment plants have been in operation around the world [1, 2]. Anammox-based treatment processes are generally suitable for NH_4^+ -rich wastewaters with low C/N ratio (<3.0) such as digester liquor and high-strength industrial wastewaters [3, 4]. Therefore, studies on microbial physiology and growth kinetics of anammox bacteria have been exclusively performed in batch and/or continuous cultures with ample substrates (NH_4^+ and NO_2^-) supply. Under these nutrient-rich conditions, the potential growth abilities of anammox bacteria like maximum specific growth rates (μ_{max}) and maximum specific substrate utilization rates (q_{max}) have been widely reported [5, 6].

In contrast, little is known about cell physiology of anammox bacteria in oligotrophic natural environments, although anammox bacteria have been universally detected in natural freshwater [7, 8], marine [9–12], and terrestrial [13, 14] environments and known to be playing an important role in the global nitrogen cycle [15, 16]. In such natural habitats, the growth rates are expected to be extremely low due to severe substrate limitation. Studying cell physiology at such extremely low (near-zero) growth rates is important to understand their survival (maintenance of viability) strategies in natural environments. For successful survival,

microorganisms must preferably possess high affinity to the limiting substrate (lower K_m values), fast growth rates (higher μ_{max}), and efficient substrate utilization rates. More importantly, maintenance energy (i.e., power) requirement and/or decay rate should be as low as possible [17, 18]. The maintenance energy is generally defined as the energy required for non-growth related processes to sustain the viability of cells (e.g., cell motility, osmoregulation, turnover of macromolecules such as enzymes and RNA, repair of proteins, molecular transport, and so on) other than new biomass synthesis [17, 19]. Therefore, it can be assumed that a relatively large fraction of energy is diverted to maintenance-related cellular processes at near-zero growth rates in energy-limited natural environments. Although a few studies have reported the decay rates of anammox activity (which is not endogenous decay) in batch starvation experiments [20–23], the maintenance power requirements of anammox bacteria have never been determined yet. The endogenous decay is defined as a reduction of active biomass directly coupled to a consumption of electron acceptor under starved conditions [23, 24], and thus is clearly different from the maintenance power requirement.

Most of the laboratory studies for the determination of maintenance power have been performed using chemostats. A retentostat with complete biomass retention was used to quantify the maintenance power requirements for autotrophic *Nitrosomonas europaea* and *Nitrobacter winogradski* [25]. Under the prolonged retentostat cultivation with a constant substrate supply, biomass accumulates to a certain degree, which gradually

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decreases the substrate availability per cell to a minimum power requirement only for maintenance [17, 26]. Under this condition, microorganisms can no longer grow but consume substrate to maintain themselves, implying the establishment of near-zero growth rates. Therefore, more environmentally relevant maintenance power requirements can be determined from the retentostat cultivation.

Anaerobic membrane bioreactors (MBRs) with the same reactor concept as the retentostat have been used to enrich slow-growing anammox bacteria and determine their physiology and kinetic parameters [27–30], because the MBR allows the cultivation of free-living planktonic cells at environmentally relevant low substrate concentrations [31–33]. However, anaerobic MBRs have never been used to measure the maintenance power requirements of anammox bacteria.

In the present study, “*Ca. Brocadia sinica*” (commonly found in the substrate-rich wastewater treatment processes) [29] and “*Ca. Scalindua sp.*” (commonly found in substrate-limiting oceanic environments) [34] were continuously cultivated in anaerobic MBRs with a constant substrate loading rate for long periods to determine the maintenance power requirements at near-zero growth rates. The results revealed that “*Ca. B. sinica*” exhibited 1.6 times higher maintenance power requirement than “*Ca. Scalindua sp.*”, which can explain their ecological niche differentiation.

MATERIALS AND METHODS

Anaerobic membrane bioreactor (MBR)

Two identical anaerobic membrane bioreactors (MBRs) were constructed and operated for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively as previously described [30, 33] (Fig. 1A). Briefly, a 2-L wide mouth reagent bottle (Sanyo, Japan) was used as the culture vessel (a working volume of 1.5 L). To completely retain biomass, a membrane module composed of 300 polyethylene hollow fiber membranes (pore size, 0.1 μm ; tube

diameter, 1 mm; length, 70 mm) was installed in the bottle. The membrane module was directly connected to a peristaltic pump (MP-1000, EYELA, Tokyo, Japan) which was actuated by a water level sensor (HL-S1A, ASONE, Japan) to maintain a constant water level in the MBR.

MBR cultivation

Planktonic “*Ca. B. sinica*” and “*Ca. Scalindua sp.*” biomass (initial concentration; 0.02–0.04 g-protein L^{-1}), which have been maintained in our laboratory [33–35], were inoculated into respective MBRs. The mineral medium was continuously fed into the MBRs at a hydraulic retention time (HRT) of 1 d. The mineral medium for “*Ca. B. sinica*” contained (mg L^{-1}): $(\text{NH}_4)_2\text{SO}_4$ (variable), NaNO_2 (variable), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (9), $\text{EDTA} \cdot 2\text{Na}$ (5.0), NaCl (1.0), KCl (1.4), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), NaHCO_3 (84), KH_2PO_4 (54) and 0.5 mL L^{-1} trace elements solution II [36]. The inorganic nutrient medium for “*Ca. Scalindua sp.*” contained (mg L^{-1}): $(\text{NH}_4)_2\text{SO}_4$ (variable), NaNO_2 (variable), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (114), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (99), KHCO_3 (500), KH_2PO_4 (24.4), 0.5 mL L^{-1} trace element solution I & II [36] and 25 g L^{-1} of SEALIFE (Marine Tech, Tokyo, Japan) as an artificial sea salt [37].

To promote the biomass growth, the concentrations of NH_4^+ and NO_2^- were gradually increased from 1.86 mM to 18.6 mM, which corresponds to nitrogen loading rates (NLRs) of 0.18–1.78 $\text{mmol} (\text{NH}_4^+ + \text{NO}_2^-) \text{L}^{-1} \text{h}^{-1}$. In order to attain the near-zero growth rate, the NLR was fixed at the point when enough active biomass was obtained, which was around 20 days after the beginning of operation. This time is defined as the start of retentostat cultivation. Retentostat cultivation experiment was performed in duplicate to confirm the reproducibility for each anammox bacterium. Since equimolar amounts of NH_4^+ and NO_2^- were supplied, NO_2^- was a limiting substrate in all experiments. The MBR culture was continuously stirred with a magnetic stirrer to maintain homogeneous planktonic biomass. The culture was continuously purged with a mixed Ar and CO_2 (95:5) gas to prevent oxygen contamination. The MBRs of “*Ca. B. sinica*” and “*Ca. Scalindua sp.*” were operated at their optimal temperature (37 °C and 28 °C, respectively). Sludge retention time (SRT) was not controlled. pH was also not controlled, but it was relatively stable throughout the cultivation ranging 7.6–7.8 for “*Ca. B. sinica*” culture and 7.8–8.0 for “*Ca. Scalindua sp.*” culture, respectively.

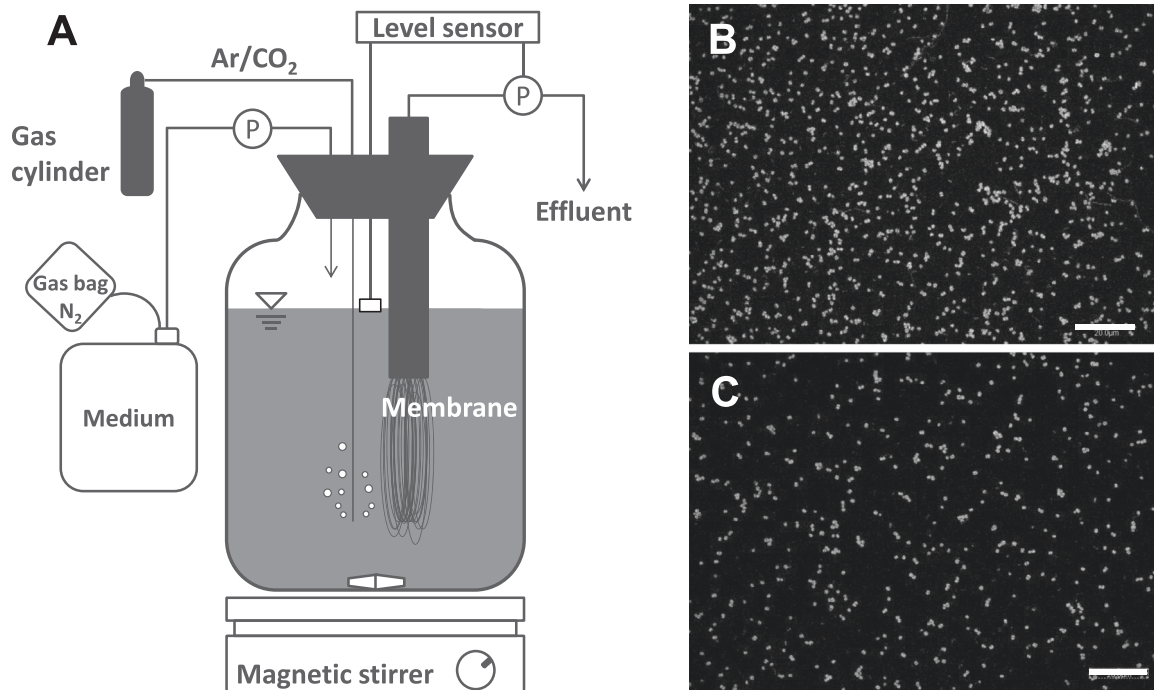


Fig. 1 Schematic illustration of an anaerobic membrane bioreactor (MBR) system and free-living planktonic anammox bacterial cells. Biomass was completely retained in the reactor by polyethylene hollow fiber membranes (pore size = 0.1 μm) (A). A N_2 purged gas bag was connected to the inorganic medium tank (10 L) to avoid O_2 contamination. Confocal laser scanning microscope images of FISH analysis for “*Ca. B. sinica*” (B) and “*Ca. S. japonica*” (C), showing highly enriched and well-dispersed planktonic form of cultures. FISH analysis was performed with FITC-labeled EUB mix probe composed of equimolar EUB338, EUB338II, and EUB338III (green) for most members of *Eubacteria* and TRITC-labeled amx820 probe (red) for anammox bacteria. Anammox bacteria were shown in yellow. Scale bars represent 20 μm .

Chemical analyses

Influent and effluent (permeate) samples were collected from MBRs to determine NH_4^+ , NO_2^- and NO_3^- concentrations. NH_4^+ and NO_2^- concentrations were determined by the colorimetric method with the indophenol and hypochlorous acid [38] and the *N*-(1-Naphthyl)ethylene diamine [39], respectively, after the samples were filtered through 0.2 μm pore membranes (Advantec, Tokyo, Japan). NO_3^- concentration was analyzed using ion-exchange chromatography (IC-2010, TOSOH, Tokyo, Japan) with an TSKgel IC-Anion HS column (TOSOH) [40].

For measurement of biomass concentration ($C_{x,\text{total}}$), MBR culture (2 mL) was withdrawn and centrifuged at 12,000 rpm for 5 min. Harvested cell pellet was mixed with 1% (w/v) SDS solution, and total biomass protein was extracted at 99 °C for 15 min. Extracted protein was collected as a supernatant after centrifugation at 16,400 rpm for 15 min and stored at -80 °C until measurement. Protein concentration was measured by the DC protein assay kit (Bio-Rad, USA) using the bovine serum albumin (BSA) as the protein standard.

Cell viability of anammox bacteria

The viability of anammox bacteria in MBR cultures was determined based on the live/dead staining using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit assay (L-7012, Invitrogen™, USA). MBR cell cultures (2 mL) were collected, washed twice with the N_2 purged mineral medium without ammonium and nitrite, and mixed with 3 μL of stain solution containing equal volumes of SYTO® 9 dye (green) and propidium iodide (PI, red) solution. The stained cell samples were analyzed by an epi-fluorescence microscope (Axiovert 200, Carl Zeiss Microscopy) equipped with the Filter Set 09 (Excitation BP 450-490, Beam Splitter FT510, Emission LP515, Carl Zeiss Microscopy). The numbers of green and red stained cells were manually counted for more than ten samples, and the average ratios (viability) were calculated. The percentage of the anammox bacteria in the MBR culture (total bacteria) was determined based on fluorescence in situ hybridization (FISH) analysis [41]. FISH analysis was performed with FITC-labeled EUB mix probe composed of equimolar EUB338, EUB338II, and EUB338III (green) for most members of *Eubacteria* and TRITC-labeled AMX820 probe (red) for anammox bacteria as previously described [42]. The hybridized cells were observed with a confocal laser scanning microscope (TCS SP8, Leica, Japan) equipped with Ar ion and He-Ne laser. The both probes-hybridized anammox bacterial cells (yellow) and other bacterial cells (green) were directly counted at least 500 cells in randomly chosen 10 microscopic fields of slides prepared for each sample, and the average percentages of anammox bacteria were calculated. The measurements of 16S rRNA gene copy numbers with quantitative PCR confirmed that "*Ca. B. sinica*" and "*Ca. Scalindua sp.*" accounted for more than 99% of the anammox population in respective MBR cultures [35]. Therefore, the measured biomass concentration ($C_{x,\text{total}}$, measured as protein concentration; g-protein L^{-1}) was converted to the concentrations of viable anammox bacteria ($C_{\text{AMX,viable}}$) by multiplying biomass concentration ($C_{x,\text{total}}$), the viability (%) determined from the LIVE/DEAD assay, and the percentage of anammox bacteria from the FISH analysis:

$$C_{\text{AMX,viable}} = C_{x,\text{total}} \times \text{Viability}(\%) \times \text{Percentage of anammox bacteria}(\%) \quad (1)$$

Hereafter, for convenience of explanation, biomass concentration (C_x) refers to the concentrations of viable anammox bacteria ($C_{\text{AMX,viable}}$).

Maintenance coefficient (m_s)

The substrate is practically utilized for both growth and maintenance process. Microbial maintenance has been described in different ways with different assumptions, which was critically reviewed by van Bodegom [17]. When the substrate utilization rate for maintenance process is assumed to be constant and independent of the growth rate, it is denoted as maintenance coefficient (m_s) and can be described as follows [43, 44]:

$$q_s = \frac{\mu}{Y_{\text{max}}} + m_s \quad (2)$$

where q_s is the specific substrate consumption rate (mmol NH_4^+ g-protein $^{-1}$ h $^{-1}$), μ is the specific growth rate (h $^{-1}$), m_s is maintenance coefficient (mmol NH_4^+ g-protein $^{-1}$ h $^{-1}$) and Y_{max} is the maximum biomass growth yield (g-protein (mol NH_4^+) $^{-1}$). According to Eq. (2), m_s and Y_{max} can be determined by plotting the values of q_s against μ , whose relation is linear [44]. Thus, the y-axis intercept determines m_s , and the reciprocal of the slope of the regression line represents Y_{max} . If μ were zero,

then q_s becomes equal to m_s . Thus, m_s can be also directly determined from the MBR retentostat cultivation experiments in which the zero growth rate is achieved. The following relationship between Y_{max} and m_s can be obtained from Eq. (2).

$$\frac{1}{Y} = \frac{1}{Y_{\text{max}}} + \frac{m_s}{\mu} \quad (3)$$

Y is the apparent (observed) growth yield (g-protein (mol NH_4^+) $^{-1}$) with consideration of substrate consumption for both growth and maintenance. This relationship indicates that Y becomes equal to Y_{max} when μ is zero.

Specific maintenance rate (a)

When it is assumed that microbial maintenance directly influences the growth rate, Eq. (S1) can be modified as follows:

$$\frac{dC_x}{dt} = \mu \cdot C_x - a \cdot C_x \quad (4)$$

$$\frac{dC_x}{dt} = -Y \frac{dC_s}{dt} \quad (5)$$

where C_x is the biomass concentration (g-protein L^{-1}), C_s is the residual ammonium or nitrite concentration in MBR (mM), a is the specific maintenance rate (h $^{-1}$), which was originally termed as "endogenous metabolism" [45]. Thus, this is analogous to the decay rate (expressed as a negative growth rate) and can be linked to m_s as follows:[17]

$$a = m_s \cdot Y_{\text{max}} \quad (6)$$

The a actually includes relative decay (death) rate when it is experimentally determined from biomass and substrate mass balances [17].

Minimum substrate concentration (S_{min})

Furthermore, a minimum substrate concentration reflecting maintenance metabolism (S_{min}), at which the net bacterial growth is zero, can be proposed as follows: [17]

$$S_{\text{min}} = K_s \frac{a}{(\mu_{\text{max}} - a)} \text{ or } a = \mu_{\text{max}} \frac{S_{\text{min}}}{K_s + S_{\text{min}}} \quad (7)$$

According to Eq. (6), and (7), the values of a and S_{min} can be calculated by substituting the values of m_s and Y_{max} , which were determined in the MBR retentostat cultivation experiments in this study. K_s is a half-saturation constant of limiting substrate (μM).

Estimation of specific growth rate in MBR cultivation

For steady state conditions (i.e., $dC_s/dt = 0$), the biomass accumulation during retentostat cultivation can be simulated using the following van Verseveld equation (46), which is derived from Eq. (S1–S3 in the Supplemental Information) and Eq. (2).

$$C_x(t) = \left(C_{x,0} - \frac{D(C_{s,\text{in}} - C_s)}{m_s} \right) \cdot e^{-m_s \cdot Y_{\text{max}} \cdot t} + \frac{D(C_{s,\text{in}} - C_s)}{m_s} \quad (8)$$

It should be noted that C_x (g-protein L^{-1}) refers to the concentration of viable anammox bacteria ($C_{\text{AMX,viable}}$) as mentioned above (Eq. (1)). In order to determine the specific growth rate (μ) using Eq. (S1), C_x was fitted with the van Verseveld equation to determine the derivative (dC_x/dt) of Eq. (S1) [46]. Actually, the fitting was done with $C_x = A \cdot e^{B \cdot t} + C$, which is the same shape as Eq (8), using the R software (R Foundation for Statistical Computing, Vienna) and by minimizing the sum of squares of errors by varying A, B, and C [19].

RESULTS AND DISCUSSION

MBR (retentostat) cultivation

Free-living planktonic cells of enriched "*Ca. B. sinica*" and "*Ca. Scalindua sp.*" were continuously cultivated in the MBRs (retentostats) with complete biomass retention to quantify the maintenance coefficients (m_s) (Fig. 1A–C). Two independent MBR retentostat cultivation experiments were performed for 64 and 168 days (34 and 144 days in the retentostat mode with a constant nitrogen ($\text{NH}_4^+ + \text{NO}_2^-$) loading rate (NLR)) for "*Ca. B.*

sinica" and 83 and 120 days (62 and 100 days) for "*Ca. Scalindua* sp.", respectively to confirm the reproducibility. In the second experiments, the wider ranges of specific growth rates were examined by extending the length of retentostat cultivations. Thus, the results of the second runs were shown in the main text, whereas ones of the first runs can be seen in the Supplemental Information (Fig. S1A, B).

Nitrogen loading rate (NLR) was gradually increased by increasing the influent NH_4^+ and NO_2^- concentrations to promote the growth of "*Ca. B. sinica*" and "*Ca. Scalindua* sp." in MBRs (Fig. 2A, B). After about 20 days, the NLRs were fixed at ca. $1.2\text{--}1.6 \text{ mmol } (\text{NH}_4^+ + \text{NO}_2^-) \text{ L}^{-1} \text{ h}^{-1}$, and then the MBRs were further operated for 144 days for "*Ca. B. sinica*" and 100 days for "*Ca. Scalindua* sp.", respectively. Nitrogen removal ratios of both MBRs were $87.6 \pm 1.8\%$ for "*Ca. B. sinica*" and $86.7 \pm 1.8\%$ for "*Ca. Scalindua* sp.", respectively. The permeate NO_2^- concentrations were always close to zero μM , except for abnormally high values caused by oxygen inhibition due to the shortage of Ar/CO_2 gas supply.

In the "*Ca. B. sinica*" MBR cultures, the stoichiometric ratio of consumed nitrite and ammonium ($\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$) was 1.10 ± 0.07 for the first run and 1.20 ± 0.08 for the second run, respectively (Fig. S2A). In addition, the ratio of produced nitrate and consumed ammonium ($\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$) was 0.16 ± 0.02 for the first run and 0.14 ± 0.02 for the second run, respectively. In the "*Ca. Scalindua* sp." MBR cultures, the $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ ratio was 1.13 ± 0.03 for the first run and 1.19 ± 0.08 for the second run, respectively (Fig. S2B).

Furthermore, the $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$ ratio was 0.12 ± 0.02 for the first run and 0.11 ± 0.03 for the second run, respectively. These values are very close to the proposed stoichiometric ratios of anammox process (1.15 and 0.16, respectively) [47], which indicates that nitrogen conversions observed in both MBRs were highly attributed to anammox bacteria.

Biomass accumulation in MBRs

"*Ca. B. sinica*" biomass progressively accumulated in the MBR and gradually levelled off after NLR was fixed at ca. $1.2\text{--}1.6 \text{ mmol } (\text{NH}_4^+ + \text{NO}_2^-) \text{ L}^{-1} \text{ h}^{-1}$ (Fig. 3A), whereas "*Ca. Scalindua* sp." biomass steadily increased even after 100 days (Fig. 3B). It is expected that the complete biomass retention in MBR resulted in accumulation of non-viable cells upon prolonged retentostat cultivation, which do not consume substrate either for growth and maintenance. Therefore, the viability of cultured biomass was determined with the LIVE/DEAD™ BacLight™ Bacterial Viability Kit. The results revealed that the viabilities of both species were stable and high throughout the retentostat cultivations. The average viabilities of "*Ca. B. sinica*" cultures were $90.3 \pm 5.7\%$ for the first run and $88.6 \pm 5.7\%$ for the second run, respectively. Similarly, the average viabilities of "*Ca. Scalindua* sp." cultures were $84.0 \pm 7.2\%$ for the first run and $83.3 \pm 7.9\%$ for the second run, respectively. The slightly lower viability of "*Ca. Scalindua* sp." cultures might indicate that non-viable biomass accumulated to some extent. This partly explains the continuous increase of "*Ca. Scalindua* sp." biomass even after 100 days of cultivation (Fig. 3B). Furthermore,

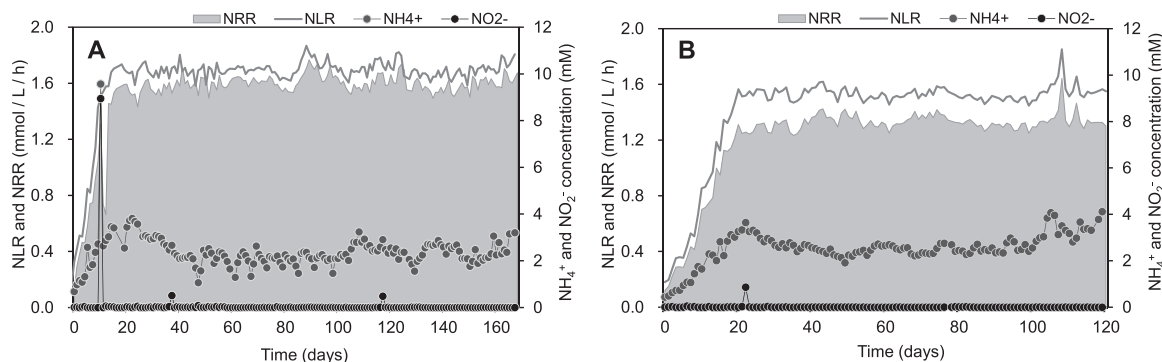


Fig. 2 Long term operation of anaerobic MBRs. Performance of "*Ca. B. sinica*" MBR (A) and "*Ca. Scalindua* sp." MBR (B) during 168 days and 120 days continuous operation, respectively. Orange lines, blue areas, red dots, and black dots represent the nitrogen (NH_4^+ and NO_2^-) loading rate (NLR), nitrogen (NH_4^+ and NO_2^-) removal rate (NRR), NH_4^+ concentration and NO_2^- concentration in the MBRs, respectively. The unexpected oxygen contamination resulted in sudden increases in nitrite concentration. Data of the second experiment (Run-2) were shown.

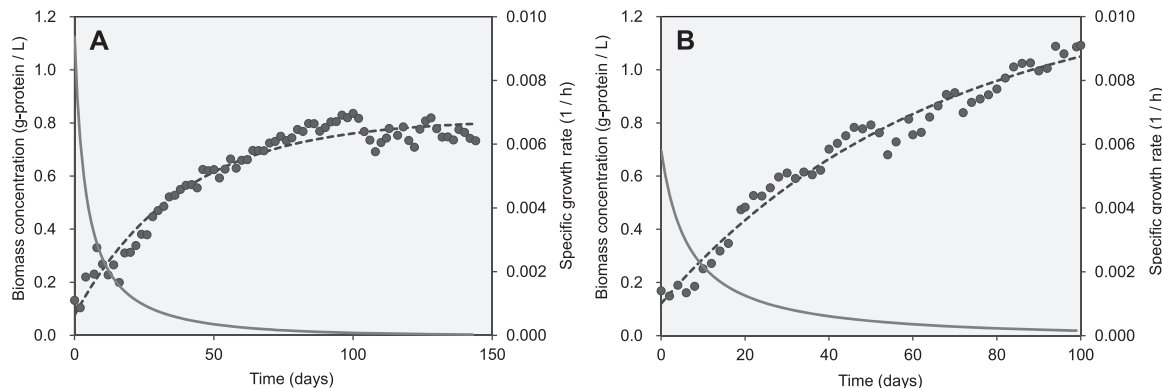


Fig. 3 Biomass accumulation in anaerobic MBRs. Biomass accumulations of "*Ca. B. sinica*" (A) and "*Ca. Scalindua* sp." (B) in MBRs after the NLR was fixed at ca. $1.2\text{--}1.6 \text{ mmol } (\text{NH}_4^+ + \text{NO}_2^-) \text{ L}^{-1} \text{ h}^{-1}$ (operation day 24 and 20 for "*Ca. B. sinica*" and "*Ca. Scalindua* sp." in Fig. 2 were defined as time zero in this Figure). Black dots indicate the measured biomass concentration (g-protein L^{-1}). The broken lines are biomass concentrations fitted with the van Verseveld equation (Eq. (8)). Specific growth rate, μ (h^{-1}), was calculated from the fitted biomass concentration (green line). At the end of retentostat cultivation, μ of "*Ca. B. sinica*" and "*Ca. Scalindua* sp." approached near-zero.

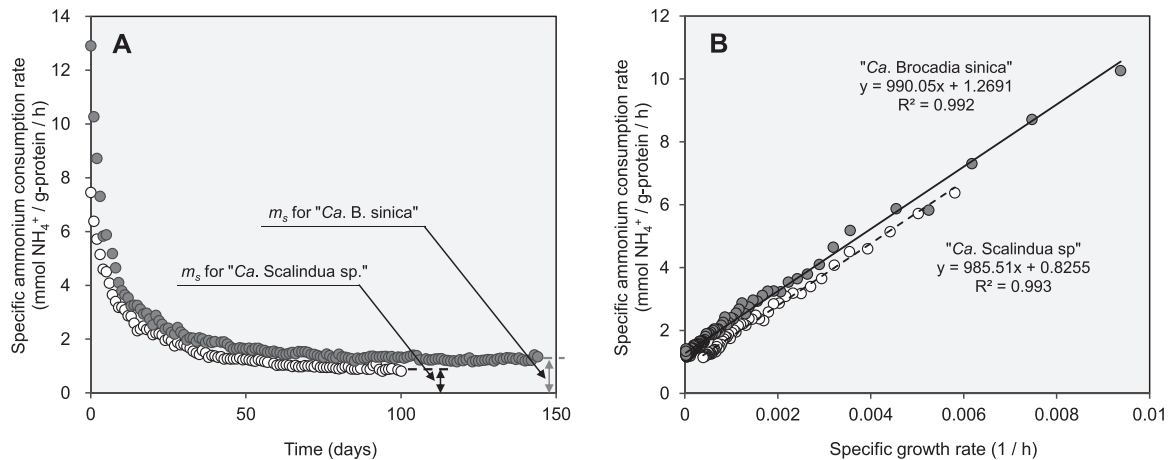


Fig. 4 Determination of maintenance coefficients (m_s). Specific NH_4^+ consumption rates (q_s) in MBR culture of “*Ca. B. sinica*” (filled circle) and “*Ca. Scalindua sp.*” (open circle), which asymptotically approached the maintenance coefficient (m_s) during prolonged retentostat cultivation (A). Relationship between specific NH_4^+ consumption rate (q_s) and specific growth rate (μ) of “*Ca. B. sinica*” (filled circle) and “*Ca. Scalindua sp.*” (open circle), respectively (B). Y-axis intercept of the linear regression line determines the maintenance rate (m_s). A reciprocal of the slope of the regression line is the maximum biomass yield (Y_{\max}).

FISH analyses revealed that “*Ca. B. sinica*” made up $87.5 \pm 4.0\%$ and $86.8 \pm 6.8\%$ of total biomass in the first and second run, respectively. Similarly, “*Ca. Scalindua sp.*” made up $85.0 \pm 3.8\%$ and $87.1 \pm 1.9\%$ of total biomass in the first and second run, respectively. Then viable biomass concentrations of respective anammox bacteria were calculated using Eq. (1). Hereafter, the biomass denotes the viable biomass concentrations of respective anammox bacteria.

The specific growth rates (μ) were estimated based on the fitted biomass accumulation curves (see “Materials and methods”) (Fig. 3A, B). They asymptotically decreased and approached near-zero. At the end of the retentostat cultivation, μ was estimated to be 0.000023 h^{-1} at 144 day for “*Ca. B. sinica*” and 0.000157 h^{-1} at 100 day for “*Ca. Scalindua sp.*”, corresponding to a doubling time of 1255 days and 184 days, respectively. Apparently, this implies that the near-zero growing cultures (defined as μ is less than 0.00019 h^{-1} , corresponding the doubling time of more than 150 days in this study) were obtained by decreasing the substrate availability per amount of biomass. However, it should be noted that these bacterial cultures were not starved, because the substrates were continuously supplied and consumed. The similar results as those shown in Fig. 3 were observed in the first MBR runs (Fig. S3A, B).

Maintenance coefficient (m_s)

The specific NH_4^+ consumption rates (q_s) of “*Ca. B. sinica*” and “*Ca. Scalindua sp.*” are plotted against the cultivation time (t) (Fig. 4A, Fig. S4A, B). Both q_s values decreased and approached asymptotes at near-zero growth rates, which are specific substrate (NH_4^+) utilization rates for only maintenance-related processes and known as the maintenance coefficient (m_s ; $\text{mmol-NH}_4^+ \text{ g-protein}^{-1} \text{ h}^{-1}$) [17, 19, 43]. The average asymptotic q_s value, that is, m_s was directly determined at the near-zero growth rates (the doubling time was > 150 days) to be $1.26 \pm 0.06 \text{ mmol-NH}_4^+ \text{ g-protein}^{-1} \text{ h}^{-1}$ for “*Ca. B. sinica*” and $0.93 \pm 0.11 \text{ mmol-NH}_4^+ \text{ g-protein}^{-1} \text{ h}^{-1}$ for “*Ca. Scalindua sp.*”, respectively (Fig. 4A). According to Eq. (2), the maintenance coefficient (m_s) can be also extrapolated by plotting the q_s against the estimated specific growth rate (μ) (Fig. 4B). Linear regression lines with an R^2 of > 0.99 and > 0.99 were obtained for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively. The Y-intercept gives m_s ($q_s = m_s$ at $\mu = 0$), which was found to be 1.27 and $0.83 \text{ mmol-NH}_4^+ \text{ g-protein}^{-1} \text{ h}^{-1}$ for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively (Table 1). These values correspond well to those directly determined from the $q_s - t$

Table 1. Summary of the maintenance coefficient (m_s), maximum biomass growth yield (Y_{\max}), and specific maintenance (decay) rate (a) of “*Ca. B. sinica*” and “*Ca. Scalindua sp.*” grown in anaerobic MBRs.

Microorganism		m_s [mmol NH_4^+ g-protein $^{-1}$ h $^{-1}$]	Y_{\max} [g-protein (mol NH_4^+) $^{-1}$]	a [h $^{-1}$]
“ <i>Ca. B. sinica</i> ”	Run-1	1.48	0.960	0.0014
	Run-2	1.27	1.010	0.0013
	Average	1.38	0.985	0.0014
	SD	0.15	0.035	0.0001
“ <i>Ca. Scalindua sp.</i> ”	Run-1	0.98	1.574	0.0015
	Run-2	0.83	1.015	0.0008
	Average	0.91	1.295	0.0012
	SD	0.11	0.395	0.0005

Two independent experiments were carried out for each strain. The m_s and Y_{\max} values were deduced from the $q_s - \mu$ linear relationships (Fig. 4B) for each run. The a values were determined as a product of m_s and Y_{\max} , according to Eq. (6). SD is standard deviation. SD standard deviation.

relationship (Fig. 4A). To the best of our knowledge, this is the first experimental data on the maintenance coefficients for anammox bacteria.

Maximum biomass growth yield (Y_{\max})

The Y_{\max} represented by $1/\text{slope}$ of the regression lines (Eq. 2) were found to be 1.01 and $1.02 \text{ g-protein (mol-NH}_4^+)^{-1}$ for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively (Fig. 4B) (Table 1). According to Eq. (3), Y_{\max} becomes equal to the apparent (observed) growth yield (Y) with consideration of substrate consumption for both growth and maintenance when specific growth rate (μ) is zero. Assuming the protein content of biomass is $0.60 \text{ g-protein (g-biomass dry weight)}^{-1}$ [29] and 1 mole of biomass-C equals to $24.6 \text{ g dry biomass}$ [48], the extrapolated Y_{\max} can be converted to $0.068 \text{ mol-biomass-C (mol of NH}_4^+)^{-1}$ for “*Ca. B. sinica*” and $0.069 \text{ mol-biomass-C (mol of NH}_4^+)^{-1}$ for “*Ca. Scalindua sp.*”, respectively (Table 1). The independent duplicate MBR runs showed a reproducible result for “*Ca. B. sinica*” but slightly higher Y_{\max} for “*Ca. Scalindua sp.*” probably due to the

accumulation of non-viable biomass to some extent (Fig. S5A, B). These Y_{max} values were very close to the reported Y values for “*Ca. B. sinica*” (0.063 mol-biomass-C (mol of NH_4^+) $^{-1}$) [29], one for “*Ca. Jettienia caeni*” (0.056 mol-biomass-C (mol of NH_4^+) $^{-1}$) [27], and one for “*Ca. Kuenenia stuttgartiensis*” (0.066 mol-biomass-C (mol of NH_4^+) $^{-1}$) [49], but higher than one for “*Ca. Scalindua sp.*” (0.030 mol-biomass-C (mol of NH_4^+) $^{-1}$ at 28 °C and 2.8% salinity) [34, 50]. The Y values of “*Ca. Scalindua sp.*” were highly dependent on salinity and in the range 0.010–0.019 mol-biomass-C (mol of NH_4^+) $^{-1}$ under low salinity conditions ($\leq 1\%$) [50]. The maximum biomass yields Y_{max} of anammox bacteria obtained in this study are also similar to the observed Y values for chemolithotrophic growth of aerobic ammonium-oxidizing bacteria (AOB) (0.034–0.082 mol-biomass-C (mol of NH_4^+) $^{-1}$) and nitrite-oxidizing bacteria (NOB) (0.020 mol-biomass-C (mol of NO_2^-) $^{-1}$) [25, 51, 52].

Specific maintenance (decay) rate (a)

Based on Eq. (6) with the obtained m_s and Y_{max} , the specific maintenance rates (a) were calculated to be $0.0014 \pm 0.0001 \text{ h}^{-1}$ and $0.0012 \pm 0.0005 \text{ h}^{-1}$ for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively (Table 1). These values were one order of magnitude higher than the death rate ($0.00046 \pm 0.00004 \text{ h}^{-1}$, defined as a decrease rate of amount of viable bacteria under anaerobic starvation conditions) reported by Wang et al. [23].

Retentostat vs. chemostat cultures

In general, the maintenance coefficients (m_s) determined in chemostat cultures were higher than those obtained from the retentostat cultures [25, 53, 54]. This is because m_s is conventionally determined by mathematically extrapolating the Y -intercept of the $q_s - \mu$ linear relationships like Fig. 4B. In chemostat studies, minimum growth is required to compensate the continuous overflow of biomass; thus a q_s value cannot be reliably deduced at near-zero growth rate [55]. In addition, chemostat operation at dilution rates of $< 0.05 \text{ h}^{-1}$ causes heterogeneous distribution of nutrient and biomass culture in space and time such as biomass flocculation and internal wall growth [53, 56]. Thus, the maintenance coefficients derived from chemostat cultures generally reflect the maintenance energy requirement at relatively high metabolic (growth) rates, which differ widely from physiological state in energy-limited natural ecosystems [53, 55]. Many cellular functions are dependent on metabolic (growth) rates [30, 57]. In general, the maintenance energy requirements for growing bacteria were higher than those for non-growing bacteria [17].

In retentostat cultures, near-zero growth can be achieved (Fig. 3) and thus only the most essential functions are maintained such as the repair of proteins and RNA components [30, 57]. Thus, the retentostat cultivation enables more accurate assessment of the growth rate independent maintenance energy requirement. In the present retentostat study, the m_s values deduced from the $q_s - \mu$ linear relationships (Fig. 4B) were almost identical with those directly determined from the $q_s - t$ relationship (Fig. 4A). This is because only q_s values at $\mu < 0.01 \text{ h}^{-1}$ for “*Ca. B. sinica*” and $\mu < 0.005 \text{ h}^{-1}$ for “*Ca. Scalindua sp.*” were used to extrapolate the q_s values at $\mu = 0$ (Fig. 3A, B), which are low enough to assume to be “zero growth”. Thus, the retentostat study provides insights into more realistic cell physiology at near-zero growth rates (i.e., natural environments).

Gibbs energy-based maintenance energy requirements (m_e)

The maintenance coefficients (m_s) can be converted to Gibbs energy equivalent as follows [48]:

$$m_e = m_s \Delta G_r \quad (9)$$

m_e is the biomass specific Gibbs free energy consumption for maintenance (kJ mole of biomass-C $^{-1} \text{ h}^{-1}$) and ΔG_r is the available

Gibbs free energy for anammox reaction ($\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + \text{H}_2\text{O}$) in a MBR system. In this study, the values of ΔG_r in the MBR system was calculated from the following Eqs. (10) and (11)

$$\Delta G_r = \Delta G_r^0 + RT \ln \frac{a_{\text{N}_2}}{a_{\text{NH}_4^+} a_{\text{NO}_2^-}} \quad (10)$$

$$a_i = \frac{C_i}{C_i^0} \gamma_i \quad (11)$$

where ΔG_r^0 is the standard Gibbs free energy of the anammox reaction at respective cultivation temperatures (i.e., 37 °C for “*Ca. B. sinica*” or 28 °C for “*Ca. Scalindua sp.*”, respectively), which are calculated from the standard Gibbs energies of every reactants and products [58–60]. R is the gas constant, T is temperature (K), and a is activity [59]. The activity of species i (a_i) can be related to the measured concentration of i (C_i) as expressed by Eq. (11), where C_i^0 represents the standard state concentration (1 M), and γ_i is the activity coefficient (unitless), which is a function of ionic strength and temperature [59]. The ionic strength of culture medium was $\sim 0.1 \text{ M}$ for “*Ca. B. sinica*” and 0.7 M for “*Ca. Scalindua sp.*”, respectively. The actually measured concentrations of NH_4^+ , NO_2^- and N_2 during near-zero growth (the doubling time was > 150 days) were used to determine the respective activities. Since the MBR culture was continuously purged with a mixed Ar and CO_2 (95:5%) gas to prevent oxygen contamination, P_{N_2} was expected to be negligibly low (assuming $< 10^{-4} \text{ Pa}$). To determine m_e , the obtained m_s , in mmole of NH_4^+ per g-protein per hour, was first converted to m_D in mole of NH_4^+ per mole of biomass-C per hour (Table 2). Assuming the protein content of biomass is $0.6 \text{ g-protein (g-dry biomass)}^{-1}$ [29] and 1 mole of biomass-C = $24.6 \text{ g dry biomass}$ [48], m_D was calculated to be 0.020 and 0.013 mole of NH_4^+ mole of biomass-C $^{-1} \text{ h}^{-1}$ for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively. Thus, the m_e can be calculated to be 6.7 and $4.3 \text{ kJ mole of biomass-C}^{-1} \text{ h}^{-1}$ for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively (Table 2). “*Ca. B. sinica*” was cultured at 37 °C and $\approx 0\%$ salinity, whereas “*Ca. Scalindua sp.*” was cultured at 28 °C and 2.5% salinity. The higher m_e value for “*Ca. B. sinica*” could be attributed to the higher culture temperature even though the higher salinity medium was used for “*Ca. Scalindua sp.*”, which increases the maintenance energy requirement due to osmoregulation and repair of proteins [17].

The estimated m_e values were in good agreement with the ones theoretically predicted for all anaerobic microorganisms grown at 28 °C and 37 °C, i.e., 4.4 and $9.7 \text{ kJ mole of biomass-C}^{-1} \text{ h}^{-1}$, respectively [48]. The m_e values obtained for anammox bacteria were also in the same range as the ones for aerobic nitrifying bacteria, *Nitrosomonas europaea* and *Nitrobacter winogradskyi*, which were determined using retentostats at 20–25 °C. (Table 2). However, the maintenance energy requirements for anammox bacteria were higher than that ones for *Syntrophobacter fumaroxidans* syntrophically grown with *Methanospirillum hungatei* in a chemostat fed with propionic acid at 37 °C (0.14 – $0.20 \text{ kJ mole of biomass-C}^{-1} \text{ h}^{-1}$) and *Pelobacter acetylenicus* syntrophically grown with different H_2 oxidizers in chemostats at 28 °C (1.27 – $2.12 \text{ kJ mole of biomass-C}^{-1} \text{ h}^{-1}$) [61]. This suggests that syntrophic bacteria generally require much less energy to survive in methanogenic environments. It should be noted that maintenance energy requirements depend on the actual growth rates [17, 62], chemostat-based estimates of maintenance energy tend to exhibit higher values due to higher growth rates as mentioned above.

For better comparison with the maintenance energy requirements of other microorganisms, these m_e values were further converted into the unit of cell-specific maintenance power requirement (P_{cs} , J s^{-1} or watts (W) per cell) with assuming 86 fg-C per cell [63], resulting in 13.4×10^{-15} and $8.6 \times 10^{-15} \text{ W cell}^{-1}$ for “*Ca. B. sinica*” and “*Ca. Scalindua sp.*”, respectively (Table 2).

Table 2. The biomass specific Gibbs energy consumption for maintenance (m_e) and cell-specific maintenance power requirements (P_{cs}) for anammox bacteria and other selected microorganisms.

Microorganisms	Temp. (°C)	Cultivation ^a	Substrate (e-donor)	ΔGr (kJ mole of substrate ⁻¹)	m_D (mole of substrate mole of biomass-C ⁻¹ h ⁻¹)	m_e^b (kJ mole of biomass-C ⁻¹ h ⁻¹)	P_{cs}^c ($\times 10^{-15}$ W cell ⁻¹)	Ref
" <i>Ca. Borcadia sinica</i> "	37	R	NH ₄ ⁺	335.8	0.020 ± 0.002	6.7 ± 0.7	13.4	This study
" <i>Ca. Scalindua sp.</i> "	28	R	NH ₄ ⁺	332.9	0.013 ± 0.002	4.3 ± 0.7	8.6	This study
<i>Nitrosomonas marina</i>	20	R	NH ₄ ⁺	275	0.008	2.2	4.4	[48]
<i>Nitrosomonas europaea</i>	25	R	NH ₄ ⁺	275	0.023	6.33	12.6	[25]
<i>Nitrosomonas europaea</i>	25	R	NH ₄ ⁺	275	0.035	9.63	19.2	[53]
<i>Nitrobacter winogradskyi</i>	25	R	NO ₂ ⁻	74	0.009	0.67	1.3	[25]
<i>Syntrophobacter fumaroxidans</i>	37	C	Propionate	8.7	0.023	0.14–0.20	0.3–0.4	[61]
<i>Pelobacter acetylenicus</i> ^d	28	C	Ethanol	21.4–33.4	0.038–0.10	1.27–2.12	2.5–4.2	[61]
<i>Thiobacillus ferroxydans</i>	30	C	S ₂ O ₃ ²⁻	796	0.024	19.1	38.2	[48]
<i>Methanobacterium thermoautotrophicum</i>	65	C	H ₂	22.8	3.41	115	228.9	[48]
<i>Methanococcus thermolithotrophicus</i>	65	C	H ₂	22.8	3.82	129	256.8	[48]

^aMicroorganisms were cultivated in the retentostat (R) or in chemostat (C).^b $m_e = \Delta G_r \times m_D$, assuming protein content of biomass is 0.6 g-protein (g dry biomass weight)⁻¹ [29, 49] and 1 mole of biomass-C = 24.6 g dry biomass [48].^cAssuming 86×10^{-15} g biomass-C per cell [63], $1 \text{ W} = 1 \text{ J s}^{-1}$ ^dSyntrophically grown with different H₂ oxidizers [61].

The cell-specific maintenance power requirements (P_{cs}) for a variety of microbial metabolisms determined in the laboratories were summarized by LaRowe and Amend (2015), in which P_{cs} range over more than 5 orders of magnitude (10^{-12} – 10^{-17} W cell⁻¹). The obtained maintenance power requirements of anammox bacteria were similar to ones of ammonium-oxidizing bacteria (28×10^{-15} W cell⁻¹) and ones of anaerobes (0.36 – 8.6×10^{-15} W cell⁻¹) but 1–2 orders of magnitude lower than ones of aerobic heterotrophs (49 – 4700×10^{-15} W cell⁻¹) [63]. The lowest P_{cs} value determined in the laboratory measurement was 1.9×10^{-17} W cell⁻¹ for a green sulfur bacterium (an anoxygenic phototroph) [63, 64].

It has been suggested that the maintenance power requirements in natural environments can be 3–6 order of magnitude lower than those obtained from laboratory settings like chemostat and even retentostat culture studies [63, 65–67, 68]. Accordingly, the maintenance energy requirement of anammox bacteria in natural environments could be much lower than the ones obtained in the present laboratory study. For example, the cell-specific metabolic rates of anammox bacteria (dominated by "*Ca. Scalindua sediminis*") in ~80,000-y-old subsurface sediment were reported to be 10^{-16} – 10^{-18} mol NH₄ cell⁻¹ d⁻¹ [68], which are at least 2–4 orders of magnitude lower than those measured in the present study (2.7 – 4.1×10^{-14} mol of NH₄ cell⁻¹ d⁻¹). Furthermore, LaRowe and Amend reported the much lower cell-specific maintenance power requirement of 1.9×10^{-19} W cell⁻¹ in the marine sediments in the South Pacific Gyre [67], which was 2 orders of magnitude lower than the lowest value determined in the laboratory [63, 64]. Therefore, further efforts such as transcriptomics and proteomics should be made to bridge the current gap of cell physiology at extremely slow growth rates and maintenance power requirements between the laboratory settings and the natural settings.

Minimum substrate concentration required for growth (S_{min})

S_{min} becomes important at near-zero growth rate. Since NO₂⁻ is usually less abundant than NH₄⁺ in natural environments [6, 69], S_{min} for nitrite, above which net biomass growth occurs, were calculated for "*Ca. B. sinica*" and "*Ca. Scalindua sp.*", respectively, based on Eq. (7). (Table 3). For this calculation, the specific maintenance rate (a) was derived in advance from Eq. (6). using the above-mentioned values of maintenance coefficient (m_s) and maximum biomass yield (Y_{max}). S_{min} can be introduced in the Monod equation as follows to assess its impact on the specific growth rate (μ) as a function of nitrite concentration [17, 70] (Fig. 5):

$$\mu = \mu_{max} \times \frac{(S - S_{min})}{(S - S_{min}) + K_s} \quad (12)$$

The values of half-saturation constant (K_s) for nitrite and maximum specific growth rate (μ_{max}) for "*Ca. B. sinica*" and "*Ca. Scalindua sp.*" used for simulation were taken from the literature [6] and listed in Table 3. Average residual NO₂⁻ concentrations in MBRs at near-zero growth rates (reflecting "actually measured S_{min} ") were $1.93 \pm 2.07 \mu\text{M}$ NO₂⁻ for "*Ca. B. sinica*" and $0.50 \pm 0.86 \mu\text{M}$ NO₂⁻ for "*Ca. Scalindua sp.*", respectively. These measured values were comparable to those calculated S_{min} from Eq. (7). (Table 3). The calculated S_{min} for "*Ca. Scalindua sp.*" was significantly lower ($S_{min} = 0.09 \pm 0.05 \mu\text{M}$ NO₂⁻) than one for "*Ca. B. sinica*" ($S_{min} = 3.69 \pm 0.21 \mu\text{M}$ NO₂⁻) due to lower K_s values. Thus, "*Ca. Scalindua sp.*" with lower S_{min} seems to be able to uptake nitrite more efficiently to satisfy its maintenance power requirement and thus to survive better in the natural environments such as energy-limited oceanic oxygen minimum zones (OMZs) where typical NO₂⁻ concentrations were < 3.0 μM [71]. In contrast, "*Ca. B. sinica*" with higher S_{min} had difficulty to cope with low NO₂⁻ concentrations, and thus has been commonly found in the substrate-rich wastewater treatment processes [16, 35, 40].

Table 3. Model parameters used for growth simulation by the extended Monod equation.

	" <i>Ca. B. sinica</i> "	" <i>Ca. Scalindua sp.</i> "	Reference
a [1/h]	0.0014 ± 0.0001	0.0012 ± 0.0005	This study
S_{\min} for NO_2^- [μM]	3.69 ± 0.21	0.09 ± 0.05	This study
S_{\min} for NH_4^+ [μM]	3.04 ± 0.18	0.59 ± 0.30	This study
K_s for NO_2^- [μM]	34	0.45	[29] [34]
K_s for NH_4^+ [μM]	28	3	[29] [34]
μ_{\max} [h^{-1}]	0.0138	0.0071	[35] [34]

a is the specific maintenance (decay) rate, which was calculated in Table 1.

S_{\min} is the minimum substrate concentration, which was calculated from Eq. (7) with a , K_s , and μ_{\max} .

K_s is the half-saturation constant, and μ_{\max} is the maximum specific growth rate.

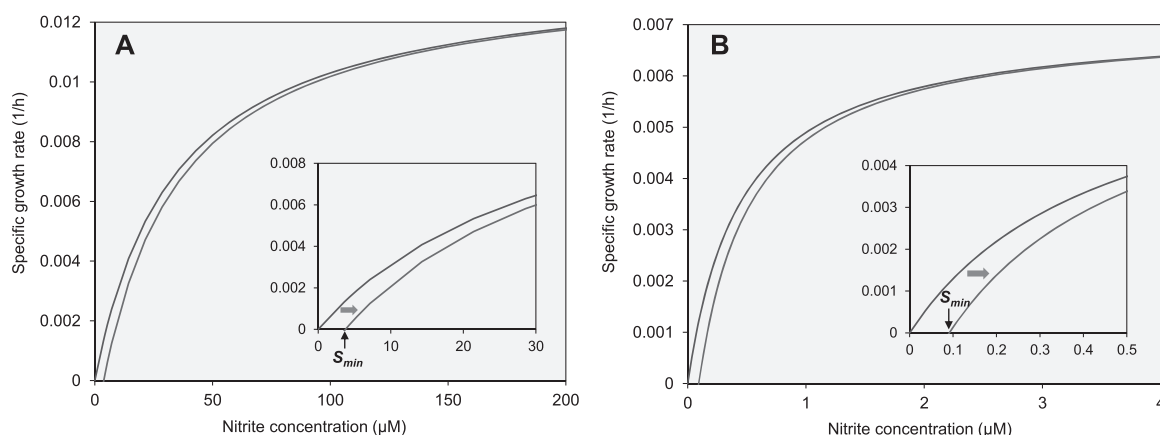


Fig. 5 Impacts of minimum substrate concentration required for growth (S_{\min}) on the specific growth rate (μ). The specific growth rate (μ) and limiting substrate (nitrite) concentration relationship of "*Ca. B. sinica*" (A) and "*Ca. Scalindua sp.*" (B), respectively. The μ were simulated using the original Monod model (red line) and Monod model extended with S_{\min} (blue line), respectively. Inserted figures are magnified views of lower nitrite concentration ranges.

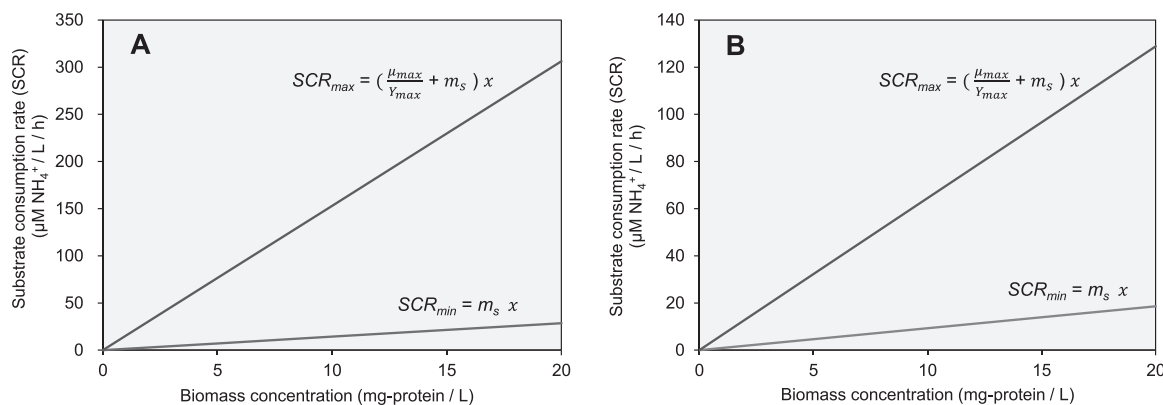


Fig. 6 Minimum substrate input rate required to sustain biomass. Relationships between substrate consumption rate (SCR) and biomass concentration (x) of "*Ca. B. sinica*" (A) and "*Ca. Scalindua sp.*" (B). Red lines represent the maximum substrate consumption rates (SCR_{\max}) as a function of biomass concentration, above which the substrate remains in the culture media (i.e., MBR effluent). SCR_{\max} were determined based on m_s , Y_{\max} , and μ_{\max} . Blue lines represent the minimum substrate consumption rates (SCR_{\min}) or "input rates" to sustain the biomass at zero growth rates ($\mu = 0$), below which the biomass concentration decreases due to biomass decay.

Minimum substrate input rate to sustain biomass

Substrate is consumed for both biomass growth and maintenance process as described as Eq. (2). ($q_s = \mu/Y_{\max} + m_s$). According to this linear relationship, the minimum substrate consumption rate or "input rate" required to sustain the biomass can be defined as $\text{SCR}_{\min} = q_s x = m_s x$, assuming zero growth rate ($\mu = 0$) (blue lines in Fig. 6), below which the biomass decreases due to biomass

decay. For example, to maintain the biomass of $20 \text{ mg-protein L}^{-1}$, SCR_{\min} were 28.6 and $18.6 \mu\text{M NH}_4^+ \text{ L}^{-1} \text{ h}^{-1}$ for "*Ca. B. sinica*" and "*Ca. Scalindua sp.*", respectively, showing "*Ca. B. sinica*" requires 1.5 times more substrate input to sustain the same amount of biomass due to higher m_s . In contrast, the maximum substrate consumption rate (SCR_{\max}) can be calculated from Eq. (2). ($\text{SCR}_{\max} = q_s x = (\mu_{\max}/Y_{\max} + m_s) x$), assuming the maximum

specific growth rate (red lines in Fig. 6), above which substrate remains in the culture medium (i.e., MBR effluent). Thus, wastewater treatment plants should be operated below the SCR_{max} to achieve superior effluent water quality. The SCR_{max} of “*Ca. B. sinica*” was 2.4 times higher than that of “*Ca. Scalindua sp.*” due to higher maximum specific growth rate (μ_{max}) and maintenance coefficient (m_s), implying that “*Ca. B. sinica*” could be a better anammox species for wastewater treatment.

In conclusion, anammox bacteria in natural environments must have physiological mechanisms that allow them to survive and maintain their activity and biomass under extremely low substrate concentrations (energy fluxes). Minimizing the maintenance power requirement could be one of the survival strategies. “*Ca. Scalindua sp.*”, which has been commonly found in energy-limiting oceanic environments, exhibited less maintenance power requirement than “*Ca. B. sinica*”, which has been commonly found in substrate-rich wastewater treatment processes. This indicates the importance of maintenance power requirement on ecological niche differentiation in natural environments.

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COMPETING INTERESTS

The authors declare no competing interests.

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