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C2/C3 fatty acid stress on anammox consortia dominated by *Candidatus Jettenia asiatica*

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HIGHLIGHTS

- Anammox activity decreases in response to an increasing acetate/propionate content.
- The competition from heterotrophic denitrifiers hinders the anammox activity.
- Anammox bacteria can grow at the present of low-concentration acetate/propionate.
- The growth of "Jettenia" has no superiority under acetate/propionate conditions.

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is a promising technology for autotrophic removal of ammonium and nitrite. In order to expand its industrial application niche, effects of organic matter need more specific investigation. In this study, the effects of C2/C3 fatty acids stress on anaerobic ammonium oxidation (anammox) process were evaluated in batch experiments. BLAST search analysis of 16S rRNA sequences showed that the homology of the highly enriched anammox consortia used here and *Candidatus Jettenia asiatica* (*J. asiatica*) reached 99%. Results showed that low acetate (⩽30 mg/L) and propionate (⩽50 mg/L) concentration does not significantly influence ammonium oxidation. Higher acetate/propionate concentrations caused decrease of ammonium removal. A level of acetate no more than 240 mg/L caused the decrease of ammonium consumption rate by 33%, and 29% for propionate with (<400 mg/L). *J. asiatica* showed higher adaptability to propionate stress than acetate. Real-time quantitative PCR (qPCR) results reveal that anammox bacteria *J. asiatica* are capable of growing at the present of low-concentration acetate (⩽120 mg/L)/propionate (⩽200 mg/L). The anammox *hzo* gene concentrations reached to round 0.5×10^8–1.0×10^8 copies/ml after 36 days cultivation with C2/C3 fatty acid. However, there is no superiority for *J. asiatica* consortia to grow under organotrophic conditions compared to autotrophic ones.

1. Introduction

Anammox (anaerobic ammonium-oxidizing) process, as a key microbial pathway in the biological nitrogen cycle [1], has been successfully applied in treating ammonium-rich wastewater for a low demand of energy and cost [2–4].Currently there are two major drawbacks of anammox process: (i) the doubling time of anammox bacteria (about 8–11 days) is long [5] and (ii) anammox bacteria are sensitive to many environmental factors such as dissolved oxygen (DO) [6], substrate type and concentration [7,8], trace elements [9,10], antibiotics [11], intermediates [12] and organic components [13]. Inherent characteristics of slow-growth and environment-sensitiveness limited the further application of anammox bacteria. Anammox bacteria are autotrophic organisms using bicarbonate as sole carbon source for synthesis of cell biomass (Eq. (1)) [5,14]. Such autotrophic metabolism is attributed as a reason for their slow growth. Organic compounds, especially acetate and propionate, were common contaminants in many kinds of wastewater, including ammonium-rich wastewater. People have tried to investigate the effect of organic matter on anammox process, but they eventually act as inhibitors to anammox bacteria [15]. However, recent studies bring insights into the possibility of organotrophic anammox process. For example, anammox species *Candidatus Ananimoxoglobus propionicus* (*A. propionicus*) [16], *Candidatus Kuenenia stuttgartiensis* (*K. stuttgartiensis*) [17] and *Candidatus Brocadia fulgida* (*B. fulgida*) are all proven to be capable of co-metabolizing fatty acids (C1–C3) [18,19]. They can

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use the fatty acids as electron donor with nitrite/nitrate as the electron acceptor. These all provide new insights into the metabolic variety of anammox bacteria. Hence, investigating the growth and adaptability of anammox bacteria in present of fatty acids is a matter of great interest.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

To our knowledge, none ever reported *Candidatus Jettenia asiatica* (*J. asiatica*)'s ability of organic utilization. Since the genetic relationship between *J. asiatica* and *A. propionicus*is very close to each other [20], it is reasonable to make a hypothesis that *J. asiatica* can also behave organotrophic anammox process. In this study, the effects of C2/C3 fatty acids stress on the anammox activity and acceleration of the total nitrogen (TN) removal rate were evaluated using batch experiments. The adaptation of *J. asiatica* to acetate and propionate at higher level was evaluated. The substrate utilization under different concentrations of acetate/propionate was precisely examined to investigate the feasibility of *J. asiatica* utilized C2/C3 fatty acids. Meanwhile, the growth possibility of highly enriched *J. asiatica* consortia in present of C2/C3 fatty acids was studied by real-time qPCR based on anammox functional gene *hzo*.

2. Materials and methods

2.1. Biomass

The biomass used for batch tests were collected from a lab-scale anammox expanded granular sludge bed (EGSB). The EGSB has been operated more than 9 months with a continuous supply of synthetic wastewater containing mainly nitrite and ammonia. Macro nutrients and trace elements were added as it was described previously [21]. The temperature was controlled at 34 ± 1.0 °C using a thermostatic jacket. The dissolved oxygen and pH in the influent were controlled at 0.2 ± 0.05 and 7.2 ± 0.3 mg/L, respectively. The predominant bacterial species was identified as *J. asiatica* by 16S rRNA sequencing and classification by BLAST [KJ002641].

2.2. Organic compounds addition batch tests

The *J. asiatica* consortia were firstly centrifuged at 8000 rpm for 5 min under 25 °C, and then washed twice by a nitrogen-free mineral medium (pH 7.2–7.5). Then the biomass was mixed with mineral medium. The addition of re-suspension and organic compounds were all carried out in an anaerobic glove box (BACTRONI-2, SHELLAB, USA). The mineral medium was prepared by purging N_2 gas (99.99%) for 30 min and leaving the medium in an anaerobic glove box. Prior to the addition of organic compounds, stock solutions of the substrates were prepared with anaerobic medium. In case of short-term experiment, biomass suspension (10 ml) was dispensed into 100 ml serum glass vials sealed with butyl rubber stoppers and aluminum caps. Then 4 ml ammonium (280 mgN/L) and 5 ml nitrite (280 mgN/L) were added from a stock solution into the vials with a syringe yielding certain final influent concentration. Sodium acetate and sodium propionate (1.63, 3.25, 6.5 and 13 ml) were added to the vials from stock solution by a syringe. Then the nitrogen-free mineral medium was added to 65 ml. The final concentrations of acetate and propionate were 30, 60, 120 and 240 mg/L and 50, 100, 200 and 400 mg/L, respectively (Table 1). For a long-term batch experiment, 20 ml biomass suspension was dispensed into 130 mlliquidin 300 ml serum glass vials. The mixed medium (pH 7.2–7.5) with ammonium, nitrite and different carbon resource (sodium acetate, sodium propionate and potassium bicarbonate) were added into the vials, leading to final influent concentrations round 140, 168 mgN/L and 2 Mm (120 mgN/L acetate, 200 mgN/L propionate and 120 mgN/L bicarbonate), respectively. The details about the medium were shown in Table 1. The vials were sealed with butyl rubber stoppers and screw top, followed by a subsequent incubation at 35 ± 0.5 °C, while the vials were shaken at 150 rpm in order to keep the biomass aggregates in solution. The medium was removed and replaced by a fresh medium, containing ammonium, nitrite and different carbon resources every 48 h (see above).The medium and biomass samples for further analysis were taken from the vials at appropriate time intervals.

Table 2
Oligonucleotides used in this research.

Name	Probe sequence 5′–3′	Hybridization	Reference
hzocl1F1	TGYAAGACYTGYCAYTGG	Anammox bacteria	[23,35]
hzocl1R2	ACTCCAGATRTGCTGACC	Anammox bacteria	[23,35]

2.3. Chemical analysis

Medium samples taken from the vials were filtered to remove the biomass and analyzed immediately after pretreatment. Ammonium was analyzed by the Nessler spectrophotometric method. Nitrite and nitrate concentrations were measured by ion chromatography (Dionex 2010 i, USA). DO and pH were measured by a portable multi-parameter test set (WTW pH/Oxi 340i, Germany). Volatile suspended solids (VSS) were measured according to standard methods [22]. Acetate and propionate concentrations were measured by a GC-D4890 gas chromatograph (Agilent, USA) with a flame ionization detector. The high-purity nitrogen gas (99.999%) was used as a carrier gas, and hydrogen and air were used as detector gas. All the samples were analyzed in triplicate.

2.4. qPCR

Long-term experiment samples were taken every 7–10 days for qPCR of partial 16S rRNA genes and stored at −80 °C until DNA extraction. Nucleic acids were extracted using a bacterial DNA

Table 1
Tests medium and parameters.

Parameter	Average concentration(mg/L)								
	Short-term batch tests medium					Long-term batch tests medium			
	I	II	IIII	IV	V	I	II	III	IV
Bicarbonate	–	–	–	–	–	–	120	–	–
Acetate	–	30	60	120	240	–	–	120	–
Propionate	–	50	100	200	400	–	–	–	200
NH_4^+–N			17.7 ± 0.9				140.9 ± 4.3		
NO_2^-–N			21.5 ± 0.6				187.3 ± 3.4		
pH					7.2–7.5				
Temperature					35 ± 0.5 °C				
Trace elements					1.25 mL/L [21]				
Mineral medium					[21]				

extraction kit (Tianzd, Inc, Beijing, China) according to the manufacturer's protocol. The DNA was detected by agarosegel electrophoresis. The qPCR mixture composed of 10 μl of 2× SGExcel Fast SYBR Mixture (with ROX) (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. China), 3 μl of the template DNA, 1 μl of forward and reverse primers (10 mM). The qPCR was performed using an ABI7500 instrument (Foster City, CA, USA). The details of primer and probe used in this study are shown in Table 2. The primer pair (hzocl1F1–hzocl1R2) [23] targeting *hzo* gene of anammox bacteria was used for this study. The program for the qPCR assay consisted of 30 min of denaturation at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 94 °C, annealing (1 min at 55 °C)and elongation (1 min at 72 °C) [23]. Real time-detection was performed at annealing stage. Melt curve analysis was performed for confirming the bind specificity of primer pair. Each sample was run in triplicate wells. Plasmid DNA (Beijing Microread Gene Technology Co., Ltd.) was diluted seven times yielding a series of final concentrations (from 1.9×10^1 to 1.9×10^9 copies/ml) for constructing a standard curves.

2.5. Statistical analysis

Statistical analyses were carried out using Microsoft Excel 2007 Analysis ToolPak. A statistical One-Way ANOVA was applied to the results and the 0.05 level of significance was used.

The research was carried out in the state key laboratory of water resources and water environment in Harbin Institute of Technology, China (2013.01–07).

3. Results and discussion

3.1. The effects of C2/C3 fatty acids with various levels on nitrogen removal

The responses of the anammox biomass to acetate/propionate stress under the condition of a fixed initial substrate level were performed by batch test. Low acetate (⩽30 mg/L) and propionate concentration (⩽50 mg/L) does not significantly influence ammonium oxidation (Fig. 1a and b, $P > 0.05$). For higher acetate/propionate concentration (⩾60/100 mg/L), there were significant differences in the effect of ammonium removal ($P < 0.001$). A level of acetate no more than 240 mg/L caused the decrease of ammonium consumption rate by 33%, and 29% for propionate with (⩽400 mg/L).This is in accordance to some previous studies that anammox bacteria activity could be affected by redundant organic compounds. Chamchoi et al. found a gradual reduction of anammox activity with an increase of COD concentration in a range of 100–400 mg/L [24]. Ni et al. also found high organic lording could suppress anammox activity and 3.1 was the threshold COD to N

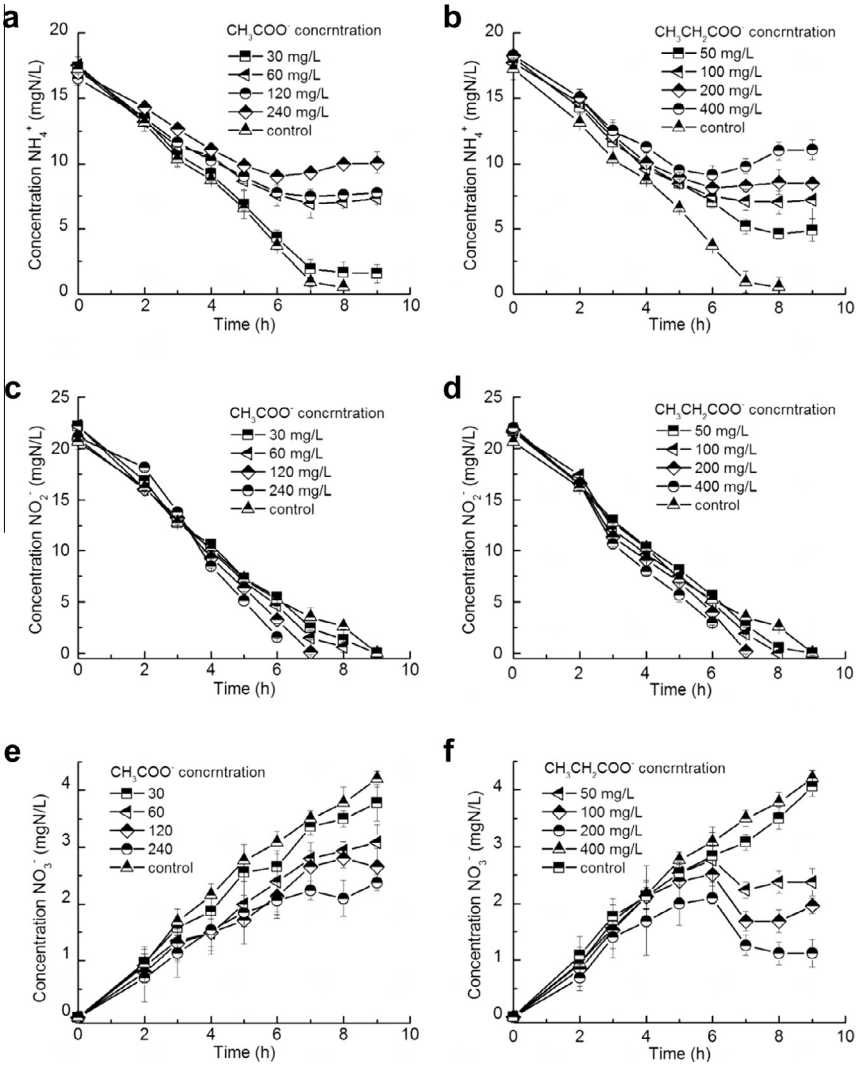

Fig. 1. Time courses of ammonium, nitrite and nitrate concentrations at different acetate/propionate concentrations. (a, c and e) Changes of NH_4^+-N, NO_2^--N and NO_3^--N concentrations in present of acetate; (b, d and f) changes of NH_4^+-N, NO_2^--N and NO_3^--N concentrations in present of propionate.

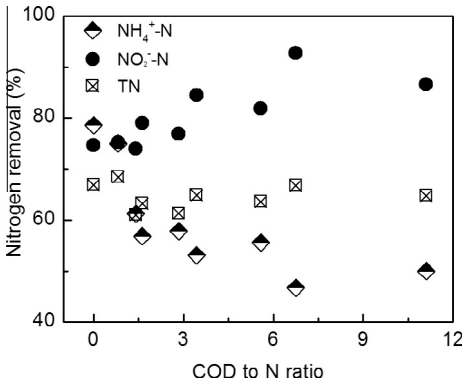

Fig. 2. Changes in the nitrogen removal efficiency with different COD–N ratio.

ratios [25]. There are two possible reasons explaining the negative response in anammox activity from high organic concentration: (1) organic compounds inhibit anammox activity and (2) organic matters introduce microbial competition for substrate(s). It is more likely that heterotrophic denitrifiers' competition plays an important role in this study. Metabolic versatility showed that organic matters could serve as alternative electron donors in anammox process [26]. Miyoko et al. revealed that anammox activity was not significant changed in $^{15}NO_2/^{14}NH_4^+$ medium with swine wastewater (BOD 20 mg/L) or acetate (6 mg/L) [27]. Meanwhile, acetate/propionate addition does not lead to significant variation on nitrite conversion (Fig. 1c and d). But the nitrate production decreased with increasing acetate/propionate (Fig. 1e and f), which lead to a stable total nitrogen (TN) conversion. The specific NRR was 17.3 mgN/g VSS h^{-1} at 240 mgN/L acetate, and 19.5 mgN/g VSS h^{-1} at 400 mgN/L propionate, respectively. The growth rate of the heterotrophic denitrifiers which have much shorter doubling times (2–16 h) was higher than anammox bacteria (8–12 days) [28]. The heterotrophic denitrifiers are a strong competitor to anammox bacteria by competing for electron acceptors (nitrite/nitrate), which could cause the increased nitrite conversion and decreased nitrate production in an organic group.

In order to further evaluate the effect of C2/C3 fatty acids concentration on nitrogen removal more precisely, the relationship between COD and N ratio and N-removal efficiency was shown in Fig. 2. The COD–N ratio that can be cultivated based on the acetate/propionate and substrates concentration. To convert moles of acetate/propionate to the COD (mg COD/L), a conversion factor of 1.07 g COD/g acetate and 1.5 g COD/g propionate was used. At low COD:N ratios (⩽1.5), anammox microorganisms could obtain high ammonia conversion which mean they can compete with denitrifying bacteria. The high COD–N ratio resulted in lower ammonium removal, but could keep higher TN removal. Ammonium removal was dropped to around 50% when the ratio was over 6.0. It can be clearly observed that the anammox microorganisms showed the capacity of high tolerance to propionate stress. When the COD–N ratio was nearly 11.0, the ammonium removal was able to reach 50%.

3.2. Consumption of C2/C3 fatty acids at different levels

The C2/C3 fatty acids were measured in order to investigate the consumption of organic matters by bacteria in batch experiment (Fig. 3). Along with the declining of ammonia and nitrite concentration, acetate/propionate was consumed simultaneously by highly enriched *J. asiatica* consortia. The conversion rates of acetate at the four concentrations at 6 h were 70.2%, 61.9%, 42.4%, 49.7%, and 82.3%, 42.7%, 45.9%, 49.1% for propionate accordingly. The missing acetate and propionate were probably (1) utilized as electron donors by *J. asiatica* or (2) consumed by heterotrophs as cell carbon source and electron donors. Previous studies demonstrated that anammox bacteria are sometimes versatile in metabolism [17,29]. The organic compounds served as electron donors for anammox bacteria could participate in ammonium and nitrate metabolism and the process of 'disguised denitrification' by the reduction of nitrate to N_2 [30]. The genetic relationship of *J. asiatica* was closed to *A. propionicus* [20], which has been shown to be capable of co-metabolizing the fatty acids, especially the propionate. "*Jettenia*" probably possess same capability. Interestingly, at low acetate (⩽60 mg/L) and propionate (⩽75 mg/L) concentrations, the consumption rates of acetate/propionate was lower than high level. It was mean that anammox microorganisms could compete with denitrifying bacteria and keep well activity at low C2/C3 fatty acids levels. Most of the heterotrophic denitrifiers can use organic substances as electron donors and carbon resources (e. g methanol for *Hyphomicrobium*) [31]. When the carbon source is sufficient, the growth of heterotrophic denitrifiers was inevitable and rapid proliferation. It was embodied in the consumption rate of acetate/propionate after 4 h. The rate was enhanced accompanied with decrease of nitrite/nitrate concentration (Fig. 1).Thus the most likely reason will be that those heterotrophic denitrifiers consumed residual most of C2/C3 fatty acids in organic groups.

3.3. Effect of C2/C3 fatty acids on growth of J. asiatica consortia

Long-term batch tests were performed to investigate the adaptation of possible growth of *J. asiatica* in the present of acetate/propionate without inorganic carbon. The anammox biomass was cultivated for 36 days with different carbon resources. After 36 days, the number of anammox *hzo* gene copies of control group (without any carbon resource) decreased from 3.2×10^7 copies/ml to 6.8×10^6 copies/ml (Fig. 4a). In addition, the removal efficiency for ammonium and nitrite also decreased. Cultivated with bicarbonate, the anammox *hzo* gene concentration increased to 8.6×10^8 copies/ml and the ammonium and nitrite removal efficiencies were stable at 83.2% and 83.7%, respectively (Fig. 4b). Compared with the cells grow on inorganic carbon, the growth of *J. asiatica* cells under acetate/propionate shows no superiority.

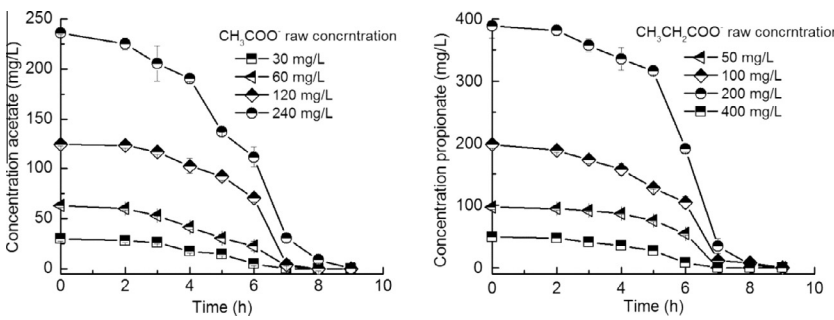

Fig. 3. Time courses of acetate and propionate concentrations.

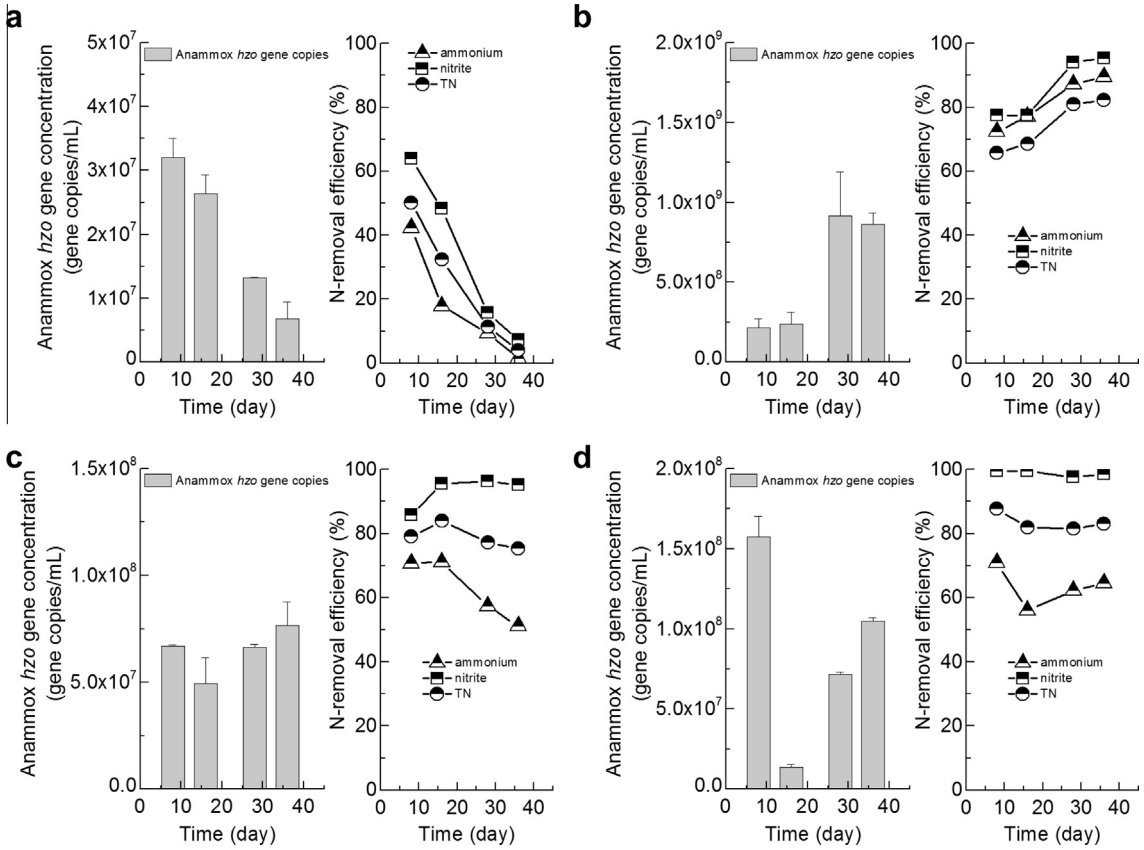

Fig. 4. Copy numbers of *hzo* gene of anammox bacteria and N-removal efficiencies in different stages with different carbon sources. (a) Without carbon resource; (b) bicarbonate; (c) acetate; (d) propionate.

After 16 days cultivation in the present of acetate/propionate, the anammox *hzo* gene concentration firstly decreased to 4.9×10^7/1.4×10^7 copies/ml and then increased to 7.7×10^7/1.0×10^8 copies/ml after 36 days (Fig. 4c and d).The number of anammox *hzo* gene copies was lower in organic substrates compared with the medium contained bicarbonate. Undesirable growth of heterotrophic denitrifiers played a key role in this process, which competed with anammox bacteria for substrates. The anammox activity was limited which resulted in a lower ammonia removal (50–60% in the acetate group and 60–70% in the propionate group). However, the nitrite removal was significantly higher than the bicarbonate group which caused high TN removal efficiencies.

The results clearly indicated that *J. asiatica* cells are capable of growing at the present of low-concentration of C2/C3 fatty acids. Several anammox species had been proven that it could utilize organic substrates as an energy source. The organic acids serve as electron donors for the reduction of nitrate to ammonium (via nitrite). The generated CO_2, nitrite and ammonium could be utilized by anammox bacteria. In this study, we measured the CO_2 concentration in the headspace of the short- and long-term batch experiments and found that the CO_2 contents were always below the detection limit. This may be because of the too slow CO_2 production rate [18,29,32]. While, a few obligate chemoautotrophs have been proven that can grow under either autotrophic or heterotrophic condition [33,34]. Anammox bacteria, including *J. asiatica*, may also possess this unusual capacity, using organic compound for both carbon and energy source. In addition, the anammox bacteria cultivated with propionate was shown higher activity than in acetate. Combined with the results in Section 3.1, it is supposed that anammox bacteria have different utilization pathway between acetate and propionate.

4. Conclusions

The effect of acetate/propionate on anammox activity and the possibility of growth with acetate/propionate were explored on highly enriched anammox consortia in batch experiments. Low acetate (⩽30 mg/L) and propionate (⩽50 mg/L) concentration does not significantly influence ammonium oxidation. Acetate at 240 mg/L and propionate 400 mg/L could cause the decrease of ammonium consumption rate by 33% and 29%. Anammox microorganisms showed higher adaptability to propionate stress than acetate. Molecular results clearly indicate that anammox bacteria *J. asiatica* are capable of growing at the present of low-concentration acetate (⩽120 mg/L)/propionate (⩽200 mg/L) with a low growth rate. However, the growth of *J. asiatica* cells under acetate and propionate has no superiority compared with the growth on bicarbonate.

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