Multidimensional Reveal of Nitrogen Regulation on Comammox

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Research

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Abstract

Background

The discovery of complete ammonia oxidizer (comammox) was groundbreaking. Comammox can use ammonia as the sole nitrogen source and turn it to nitrate. Moreover, genomic data indicated that comammox contained genes which can metabolize urea and nitrite. However, the feasibility of enriching comammox with urea and nitrite in long term has not been proved. This study enriched comammox's culture by using nitrite in reactor SA and urea in reactor SB.

Results

The nitrification rate of reactor SB (1.29 mg N·g -1 biofilm · d -1) was higher than that in reactor SA (0.6 mg N · g -1 biofilm · d -1) at the 390 th day. Comammox outnumbered ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in both reactor SA (9.04 × 10 9 copies / g biofilm) and reactor SB (5.34×10 10 copies/ g biofilm). In reactor SA, comammox's amoA accounted for 92% of the total amoA, which was higher than that in reactor SB (85%). However, the percentage of comammox (4%) in total bacteria was much lower than reactor SB (14%). The results of metagenomic sequencing showed that all the pathways of nitrogen cycle including nitrification, nitrogen fixation, denitrification, assimilation nitrate reduction, and dissimilation nitrate reduction can be detected in both reactor SA and reactor SB except the anammox pathway. The genes related to nitrite oxidation and nitrate reduction in reactor SA (TPM = 5099; TPM = 3329) was higher than that of in reactor SB (TPM = 4071; TPM = 2984), presumably due to the demand of turning nitrite to nitrate and turning nitrate to ammonia. While genes related to ammonia oxidation and urea metabolism in reactor SB (TPM = 3915; TPM = 3638) was higher than that in reactor SA (TPM = 2708; TPM = 3002).

Conclusion

Nitrite and urea can regulate the enrichment culture of comammox by converting its metabolic pathway. Using nitrite as sole nitrogen source can improve the proportion comammox's amoA in total amoA while using urea as the sole nitrogen source may increase comammox's proportion in total bacteria. These results can accelerate the enrichment of comammox and facilitate the promotion of comammox's engineering operation.

Background

Nitrification is a significant microbial process and is a key step to the nitrogen cycle [1]. Since 1890, nitrification has been regarded as a two-step process, namely ammonia oxidation and nitrite oxidation. In the first step, AOB (ammonia-oxidizing bacteria) [2] and AOA (ammonia-oxidizing archaea) [3] were involved and in the second step, NOB was usually the key contributor [4]. In 2006, Costa et al. claimed that the complete nitrification would generate more energy than either step alone and proposed that there might be a microorganism that could convert ammonia into nitrate directly in a cell [5]. In 2015, two

teams overturned the conventional theory of two-step nitrification by announcing the existence of comammox and obtaining the enriched cultures of Ca. Nitrospira inopinata, Ca. Nitrospira nitrosa, and Ca. Nitrospira nitrificans [6, 7].

Comammox was later found in many natural habitats. The high abundance and frequency of comammox in the water treatment plant [8–10] revealed that comammox might play an essential role in the water treatment process [11]. Palomo et al. and Gonzalez et al. analyzed the comammox genome data derived from water treatment plants [12, 13], and predicted that comammox might have abundance metabolization capability. Pinto et al. detected urt and ure on the comammox genome from a biological filter [10]. Metagenomic data showed that three typical comammox enrichments (pure cultures) contained amo, nxr, urt and ure [13, 14]. urt and ure were only observed in several AOA and AOB [15–17], so fewer number of AOA and AOB could hydrolyze urea in the cell [18, 19]. In addition, comammox contained urtABCDE, a high-affinity urea transporter, which indicated that it can grow at low urea concentration [14, 20]. The urtABCDE may also cause the niche differentiation of comammox, AOA, and AOB, which indicated that urea could be used as a nitrogen source to regulate the ammonia-oxidizing community.

Nitrite is formed by ammonia oxidation and has certain inhibitory effects on microorganisms [21]. AOA and AOB lack nxr and couldn't use nitrite as the sole nitrogen source. Moreover, the activity of AOA and AOB might be inhibited by nitrite [22]. In contrast, comammox contains nxr and its affinity for nitrite resembles that of Nitrobacter hamburgensis [7]. Comammox has the greatest nitrite tolerance in ammonia-oxidizing microorganisms [23]. Therefore, enriched by nitrite as sole nitrogen source can inhibit the growth of AOA and AOB and regulate the ammonia-oxidizing community [22].

The potential metabolic capability of comammox's on nitrite and urea indicates that different nitrogen sources may have different effects on the enrichment of comammox. Hence, understanding the role of different nitrogen sources in the enrichment of comammox can accelerate its enrichment efficiency and maximize its value in application. In this study, the enrichment of comammox was carried out in SBR for 390 days with nitrite and urea as nitrogen sources, respectively. During the enrichment period, methods including qPCR, high-throughput sequencing, and metagenomic sequencing were adopted to analyse the differences between nitrite and urea in regulating the enrichment of comammox.

Materials And Methods

Inoculum and medium

The biofilm which was taken from the surface of a quick sand filter in drinking water plant was used as the inoculum. The medium (per liter) was composed of 50 mg KH_2PO_4, 75 mg KCl, 50 mg $MgSO_4{\cdot}7H_2O$, 584 mg NaCl, 4 g $CaCO_3$, 1 ml TES and 1 ml SWS. 6 mg/l Nitrite and urea were used as nitrogen source in reactor SA and SB respectively. The TES (per liter) consisted of 34.4 mg $MnSO_4{\cdot}1H_2O$, 50 mg H_3BO_3, 70

mg $ZnCl_2$, 72.6 mg $Na_2MoO_4 \cdot 2H_2O$, 20 mg $CuCl_2 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 80 mg $CoCl_2 \cdot 6H_2O$, 1 g $FeSO_4 \cdot 7H_2O$. The SWS (per liter) comprised of: 0.5 g NaOH□3 mg $Na_2SeO_3 \cdot 5H_2O$□4 mg $Na_2WO_4 \cdot 2H_2O$ [6].

Reactor operation

Two sequencing batch reactors (SBRs) with a working volume of 3L were used in this study (Fig. 1). Both SBRs were operated under the same conditions. The operating temperature was 24 °C and the exchange volumetric rate was 50% . The SBRs were operated consecutively for 390 days on three different operating phases. On Phase I (1 d-80 d), HRT was maintained at 96h, followed by 48h on Phase II (81 d-210 d). From Phase III (211 d-390 d) onwards, the HRT was maintained at 24h. Influent nitrite and urea were kept constantly in each phase. The concentrations of ammonia, nitrite and nitrate were estimated according to the standard method of APHA [24].

Nitrification rate determination

0.1 g sludge and 50 mL medium were added into a 100 mL Erlenmeyer flask for the nitrification rate determination in triplicate. The medium contained 0.5 mM ammonia chloride and 1 mM potassium phosphate buffer (pH 7.2) [6]. The flasks were shaken at 120 rpm on a rotary shaker in the dark at 20°C. Samples were collected to determine the concentration of NO_3^--N and NO_2^--N and to calculate the nitrification rate on 0d, 1d, and 2d [25].

DNA extraction and qPCR

The DNA sample was extracted by Power Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA). It was then used for concentration determination by NanoDrop photometer (ND-1000, Isogen Life Science, the Netherlands) [26]. The qPCR amplification was carried out in triplicate by using iCycler iQ5 (Bio-Rad, California, U.S.), to quantify the copy number of total bacteria, AOA, AOB, and comammox. The specific primers were shown in Table S1. The qPCR reaction system was as previously reported [27, 28]. The error bars were calculated using Origin 7.0 (Microcal Software, Northampton, MA) [29].

High-throughput sequencing

The 16S rRNA gene of the bacteria was amplified by 338F-806R primers and sequenced by Illumina MiSeq high-throughput sequencing (Magigene Biological Technology Co. Ltd Guangzhou, China) [30]. The primers were shown in Table S1.

Metagenomic sequencing

The sequencing library was sequenced on the Illumina-Hiseq X-ten (MAGIGENE Biological Technology Co. Ltd Guangzhou, China) platform after being constructed and quality test. All the Scaftigs (≥500 bp) assembled were predicted by MetaGeneMark (Version 3.38) and length information which shorter than 90 nt from the predicted result was filtered. Using CD-HIT (Version: 4.7) to remove redundancy and obtain the unique initial gene catalogue which was clustered by identity 95%, coverage 90%, and chose the longest one from the representative sequences. The unigenes were extracted from NCBI Non-Redundant Protein (NR) database [31].

Results

Reactor operation

The influent and effluent concentrations of ammonia and nitrite as well as their removal rate were shown in Fig. 2. On Phase I, the minimum removal rate of reactor SA was 95%, and became stable at 80d. On Phase II and Phase III, the removal rate of nitrite was kept above 99% without any fluctuation (Fig. 2a). As to reactor SB, 0.02-0.03mg/l ammonia could be detected in the effluent and the minimum removal rate was 89% on Phase I. On Phase II and Phase III, the removal rate of urea was stable. Its ammonia removal rate showed no evident fluctuation when the loading rate increased.

Although different nitrogen sources were used in reactor SA and reactor SB, the general trend were similar. On Phase I, the removal rate of ammonia and nitrite showed fluctuations. On Phase II and Phase III, there was little fluctuation and only nitrite could be detected in the effluent.

Nitrification rate determination

Different nitrogen sources had different effects on the nitrification rate in reactors, which was shown in Fig. 3. On Phase I, two reactors have similar nitrification rate at around 0.19 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$. At the end of Phase II, the nitrification rate in reactor SA was 0.4 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$, 1.48 times higher than that of at the beginning of Phase II (0.27 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$). The nitrification rate in reactor SA was 0.6 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$ at the end of Phase III, which was 3.16 times higher than Phase I, reaching 0.6 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$. The nitrification rate in reactor SB grew faster than that of in reactor SA. At the end of Phase II, the nitrification rate in reactor SB was 0.62 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$,1.72 times higher than that at the beginning of Phase II (0.36 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$). At the end of Phase III, it reached 1.29 mg $N \cdot g^{-1}$ biofilm $\cdot d^{-1}$, 6.79 times higher than that of on Phase I.

The nitrification rates in reactor SB was higher than that in reactor SA on three Phase during the experiment. At the end of Phase III, the nitrification rates in reactor SB was 2.15 times higher than that of in reactor SA.

The abundance of ammonia-oxidizing microorganisms in reactors

Nitrite and urea had a great impact on the enrichment of ammonia-oxidizing microorganism communities. The abundances of AOA, AOB, comammox and the relative abundance of ammonia-oxidizing microorganisms were shown in Fig. 4. From Phase I to the end of the Phase III, the *amoA* gene copy number of AOA in reactor SA increased from 4.46×10^{8}copies/ (g biofilm) to 5.33×10^{8}copies/ (g biofilm) (Fig. 4a); the *amoA* gene copy number of AOB increased from 1.56×10^{7} copies/ (g biofilm) to 2.86×10^{8} copies/ (g biofilm) (Fig. 4j); and the *amoA* gene copy number of comammox increased from 5.81×10^{8}copies/ (g biofilm) to 9.04×10^{9} copies/ (g biofilm) (Fig. 4g). In reactor SB, the *amoA* gene copy number of AOA was 5.12×10^{8} copies/ (g biofilm) on Phase I and increased to 9.52×10^{9} copies/ (g biofilm) on Phase III (Fig. 4b); the *amoA* gene copy number of AOB increased from 1.09×10^{7} copies/ (g biofilm) to 2.54×10^{8} copies/ (g biofilm) (Fig. 4f); the *amoA* gene copy number of comammox increased from 5.32×10^{8} copies/ (g biofilm) to 5.34×10^{10} copies/ (g biofilm) (Fig. 4h).

In reactor SA, the initial proportion of comammox to the total *amoA* was 56%. It accounted for 67% at the 150^{th} day and increased to 92% at the 390^{th} day (Fig. 4b). In reactor SB, the initial proportion of comammox's *amoA* to total *amoA* was 50% and increased to 85% at the 390^{th} day. The abundance of comammox in reactor SB (5.34×10^{10} copies/ g biofilm) was higher than that in reactor SA (9.04×10^{9} copies/ g biofilm). The proportion of comammox to the total ammonia oxidation microorganisms in reactor SA (92%) was higher than that in reactor SB (85%).

Structural differences in microbial communities

Different nitrogen sources had different effects on the structure of bacterial communities (Fig. 5). On Phase I, the results of high-throughput sequencing in reactor SA and reactor SB were similar. At the beginning of Phase II (the 90^{th} day), the relative abundance of *Nitrospirae* in reactor SA increased from 0.94% to 5.3% and to 9.70% at the end of Phase II. The relative abundance of *Nitrospirae* on Phase III (20.60%) was 21.91 times higher than Phase I (0.94%) (Fig. 5a). Reactor SB also showed the same trend. The relative abundance of *Nitrospirae* increased from 0.89% on Phase I to 17.21% on Phase II. On Phase III, it reached 22.40%, 25.20 times higher than that on Phase I (Fig. 5b).

In addition to *Nitrospirae*, the relative abundance of *Proteobacteria* in reactor SA and SB decreased by 6.1% and 10.9%, respectively on Phase III. The relative abundance of *Acidobacteria* reduced from 29.8% to 8.48% in reactor SA, and 29.21% to 6.13% in reactor SB.

The Pathways of microbial metabolism

Different nitrogen sources would affect the nitrogen metabolism of microorganisms' genes, and thus their TPM value. After enrichment with nitrite and urea in reactors for 390 days, the TPM of function genes had changed. Based on the annotated metagenomic sequencing by KEGG database, the metabolic pathways of the nitrogen cycle were shown in Fig. 6. Nitrification, nitrogen fixation, denitrification, assimilation nitrate reduction, and dissimilation nitrate reduction pathways could be found in both reactors. However, neither reactor SA nor SB contained *hzs* that could convert ammonia into hydrozine or *hdh* that could convert hydrozine into nitrogen, that means the TPM values of genes (*nar*, *nap*, *nr*, *nas*, *nir*, *nrf*, *nit-6*) related to nitrate reduction and nitrite reduction in reactor SA were higher than those in reactor SB. The TPM values of both *amo* and *hao* in reactor SB were higher than those in reactor SA, whereas the *nxr* in reactor SA was higher than that in reactor SB. The genes with higher TPM in reactors SA and SB showed comparatively high similarity. Due to different nitrogen sources, *nxr* in reactor SA (TPM = 5099) outnumbered that in reactor SB (TPM = 4071) during the nitrification process, and the *amo* (TPM = 3915) (Fig. 6), *ure* (TPM = 3638) and *urt* (TPM = 4106) (Table S2) in reactor SB had higher TPM values than *amo* (TPM = 2707), *ure* (TPM = 3002) and *urt* (TPM = 3505) in reactor SA.

Discussion

Currently, there were few studies on the culture of comammox with different nitrogen sources. Van Kessel et al. operated reactors for 12 months and found that Nitrospira accounted for 15% of the total bacteria [6, 7]; Yu et al. conducted the enrichment of comammox in 4L continuous flow bioreactor and Nitrospira, accounting for 16%to total bacteria [32]; Roots et al. found that comammox represented 94% of the total ammonia-oxidizing microorganisms and 4% of the total bacteria after 407-day operation of SBR [33]. While the regulation of different nitrogen sources on the enrichment of comammox remains unclear. Daims et al determined that comammox can metabolize urea with gradient dilution method [6] and Camejo et al found the binning as Nitrospira defluvii in the reactor enriched by nitrite [14]. In this study, the regulation of different nitrogen sources on the enrichment of comammox was shown. We enriched comammox with nitrite, and found out that comammox accounted for 92□of amoA. The result was aligned with Roots et al's observation which was enriched by ammonia and low dissolve oxygen [33]. When using urea as sole nitrogen sources, the ratio of comammox to total bacteria was 14.2□, which was higher than that obtained by van Kessel et al [7] and Yu et al [32].

In a neutral environment, part of the urea will be hydrolyzed into ammonia and transported into the medium [34]. Ammonia is ideal for ammonia-oxidizing microorganisms to oxidize. It can passively transport on the cell membrane and be oxidized by amo [35]. Urea can also be hydrolyzed into ammonia in cells by urt and ure [17] which exist in all comammox [14] and some AOA and AOB [14, 15]. In this study, the metagenomic data showed that the ure contained in reactor SB (TPM = 3638) and urtABCDE (TPM = 4106) were higher than ure (TPM = 3002) and urtABCDE (TPM = 3505) (Table S2) in reactor SA, which showed that microorganism enrichment could use urea as the nitrogen source. The pH was maintained at 7.0, so the ammonia transported into the medium during the hydrolyzation can still be used by the microorganisms. Due to comammox's high affinity for ammonia [23], it can also absorb the ammonia hydrolysised by urea [17].

In the previous studies on the kinetics of N. inopinata, Kits et al found that N. inopinata could not grow with NO_2^- as the sole nitrogen source [23]. Palomo et al. found that comammox lacked gene nirA encoding nitrite reductase, and it is impossible for comammox to grow and metabolize with nitrite as the sole nitrogen source [23]. However, Camejo et al. found a similar binning in the reactor with nitrite as the sole electron acceptor to Ca. Nitrospira nitros [14]. Therefore, it indicated that nitrate produced ammonia through nitrate reduction for comammox to absorb [36]. However, Camejo et al. did not conduct a long-term investigation of the changes in the ammonia-oxidizing community and did not clarify the comammox response to nitrite input [14]. In this study, the abundance of AOA and comammox in reactor SA with nitrite as the sole nitrogen source was growing, but comammox accounted for 92% of ammonia-oxidizing microorganisms, much higher than AOA (8%). According to the nitrogen cycle pathway at 390th day, it can be infered that the ammonia sources in the reactors were related to dissimilatory nitrate reduction and assimilation nitrate reduction and genes related to the two pathways showed a higher TPM values in reactor SA (TPM = 9250; TPM = 3252). Nitrate reduction converted a part of nitrite and nitrate into ammonia [37], which was subsequently used by AOA and comammox with higher affinities for ammonia. Compared with most of AOA, comammox had better nitrite tolerance and higher affinities for ammonia ($K_{m(app)}$ = 63 ± 10 nM) [23]. Therefore, comammox played a dominant role in ammonia-oxidizing microorganisms when nitrite was used as the so le nitrogen source (Fig. 4b).

Urea was utilized as the only nitrogen source to enrich comammox, and the culture efficiency was proven to be improved by 1.5-2 times compared with the previous studies [7, 14, 20]. However, when comammox was enriched with nitrite as the nitrogen source, the enrichment rate was relatively lower than in previous studies [7, 14, 20]. Nevertheless, the percentage of comammox to amoA in reaction system increased significantly. The result is similar to Roots's conclusion who obtained 94% of comammox amoA out of total amoA through the optimization of process conditions [33]. This study demonstrates that comammox can be enriched without reducing nitrification rate through the domestication by nitrite or urea. After being enriched by nitrite, reactors could enter the stable phase faster and the ratio of comammox to ammonia-oxidizing microorganism would increase. Being solely enriched by urea, comammox accounted for a large proportion of total bacteria and presented high nitrification rate. Therefore, the study proposed two nitrogen sources to increase the comammox abundance and the ratio of comammox to ammonia-oxidizing microorganisms, thus promoting the engineering operation for comammox.

Conclusion

Overall, this study presented effects of different nitrogen sources on the ammonia-oxidizing community. Future work will involve promoting the ratio of comammox to total bacteria and using metagenomic to obtain good binning to reveal the physiology and biochemistry of comammox enriched by different nitrogen sources. The main conclusion of this study is:

Different nitrogen inputs regulate the enrichment of comammox. Using nitrite as sole nitrogen source may increase the number of nitrite-oxidizing bacteria but it can also improve proportion of comammox's

amoA to total amoA. Using urea as the sole nitrogen source may increase comammox's proportion in total bacteria but AOA will also be enriched and may reduce the proportion of comammox in amoA.

Abbreviations

Comammox: Complete ammonia oxidizer; AOB: Ammonia-oxidizing bacteria; AOA: Ammonia-oxidizing archaea; TPM: Times Per Million; SBR: Sequencing Batch Reactor; HRT: Hydraulic retention time

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets obtained and/or analyzed in current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YZ was responsible for reactor operation and was a major contributor in writing the manuscript. JH was responsible for the analysis of high-throughput and metagenomic data. WY was responsible for the analysis of qPCR data. JW was a contributor in writing the manuscript. JZ was a contributor in analysing data and writing the manuscript. PZ was responsible for the design of experiment and was a contributor in writing the manuscript. BH was responsible for the design of experiment and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

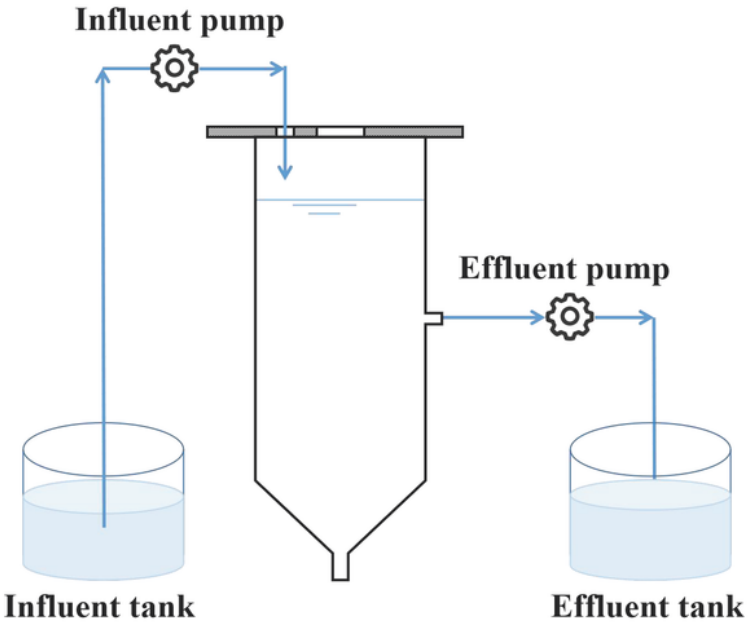

Figure 1

Reactor configuration.

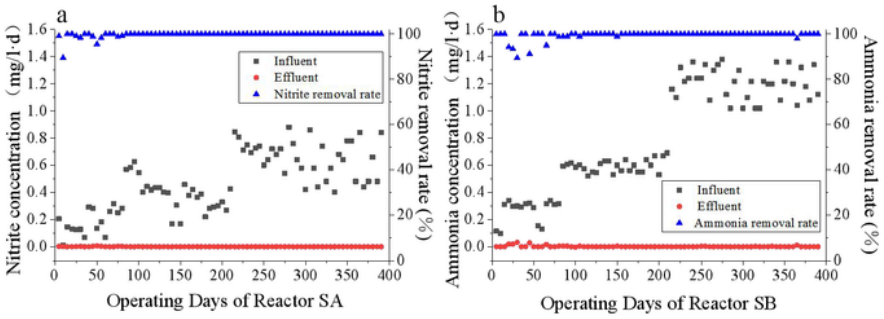

Figure 2

Ammonia and nitrite concentrations and removal rates of influent and effluent. a Reactor SA. b Reactor SB.

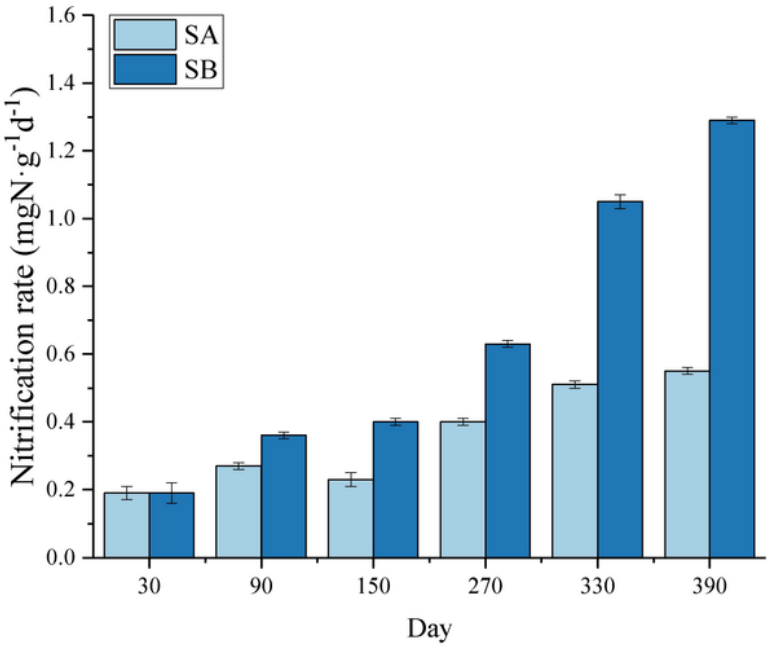

Figure 3

Influence of Different Nitrogen Sources on the Nitrification Rate.

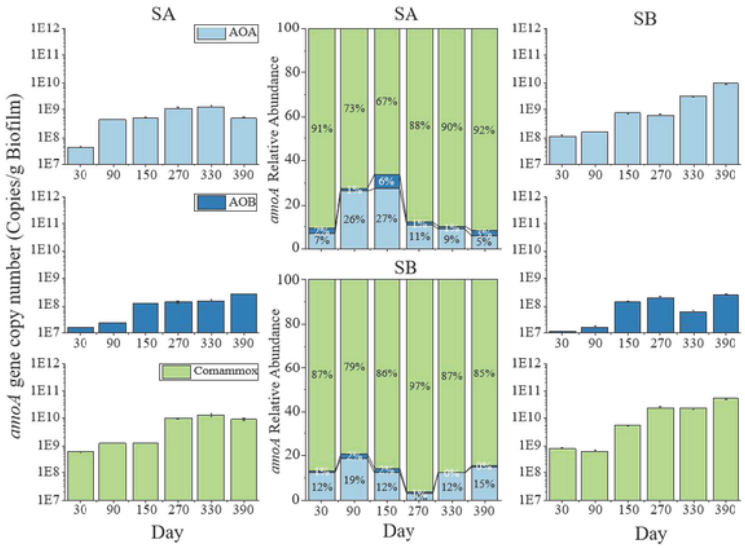

Figure 4

Abundance of Ammonia-oxidizing Microorganisms in different reactors. a amoA gene copies of AOA in reactor SA. b Relative abundance of ammonia-oxidizing microorganisms in reactor SA. c amoA gene copies of AOA in reactor SB. d amoA gene copies of AOB in reactor SA. e Relative abundance of ammonia-oxidizing microorganisms in reactor SB. f amoA gene copies of AOB in reactor SB. g amoA gene copies of comammox in reactor SA. h amoA gene copies of comammox in reactor SB.

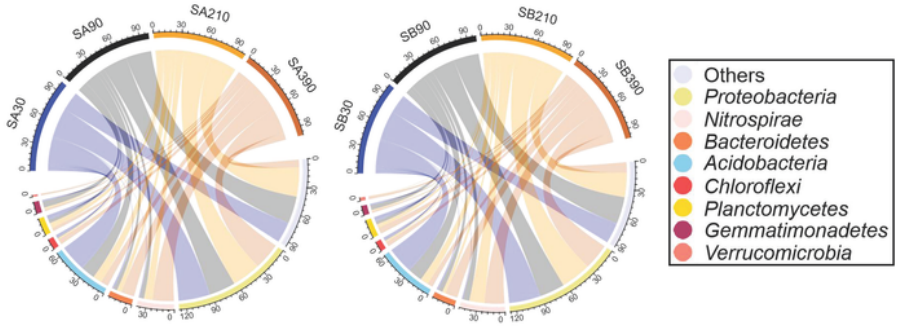

Figure 5

Phylum-level Communities in Reactors. a Reactor SA. b Reactor SB.

Figure 6

Nitrogen Metabolic Pathway and Gene Abundance in Reactors. The red box indicating the existing gene. The black box indicating the nonexistent gene.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx