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Nitrite improved nitrification efficiency and enriched ammonia-oxidizing archaea and bacteria in the simultaneous nitrification and denitrification process

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ABSTRACT

Simultaneous nitrification and denitrification (SND) is effective and energy-saving for wastewater treatment. As an inevitable intermediate product in the SND process, nitrite affects the efficiency of ammonia oxidation and the composition of nitrifiers. To investigate the impact of nitrite on ammonia oxidation efficiency, two reactors performing SND were respectively operated without nitrite (R1 as control) and with 20 mg N/L nitrite addition (R2 as experimental). The total nitrogen removal efficiency was 74.5% in R1 while 99.0% in R2. With nitrite addition (i.e., 20 mg N/L), the ammonia removal rate in R2 increased to 4.5 times of that in R1. The ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) contributed to respective around 46.9% and 41.8% ammonia removal in R2 based on the results of experiments with specific inhibitors. The number of respective AOA and AOB ammonia monooxygenase gene (*amoA*) copies increased by 280 and 30 times due to nitrite addition, according to the qPCR results. The high-throughput sequencing results illustrated the increase of dominant AOB species from 0.40% in R1 to 1.59% in R2 and the phylogenetic tree analysis revealed a close link to *Nitrosospira multiformis*. These results indicated that the ammonia removal efficiency was improved and AOA/AOB were enriched by nitrite addition. The specific nitrite reductases in AOA and AOB boosted the adaptation of nitrite addition. This study demonstrated the positive impacts of nitrite addition on the ammonia removal efficiency and rate in the SND process.

1. Introduction

Nitrogen removal by ammonia/nitrite oxidizing microorganisms is essential in wastewater treatment. The simultaneous nitrification and denitrification (SND) process is claimed to be more efficient and energy-saving than the traditional two-step nitrogen removal process (Hsieh et al., 2007; Kong et al., 2016). The simultaneous presence of numerous microorganisms directly influenced the effectiveness of nitrogen removal. For instance, autotrophic nitrifiers, anoxic denitrifiers along with heterotrophic nitrifiers and aerobic denitrifiers worked cooperatively in the SND process (Li et al., 2019; Tan et al., 2020). Anaerobic ammonia-oxidizing (anammox) microorganisms and pure organisms capable of both heterotrophic nitrification and aerobic denitrification were also reported to be the main communities in the process (Liu et al., 2019; Chen et al., 2012; Fei et al., 2017). The nitrogen removal efficiency is difficult to control in the SND process because various nitrifiers and denitrifiers prefer different conditions.

Ammonia removal could be the restricted step because the ammonia oxidizing microorganisms (AOM) are more sensitive to the environments compared to the heterotrophic denitrifiers. Enhancing AOM abundance and selecting for efficient AOM strains are effective strategies to improve nitrogen removal efficiency. The competition for substrates and intermediates profoundly influenced the distribution of AOM (Fan et al., 2017; Fitzgerald et al., 2015). Notably, nitrite was reported to be effective to reshape AOM populations (Li and Gu 2013; Zhao et al., 2021). Moreover, nitrite affected the ammonia oxidation rates and

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mRNA levels, resulting in varying AOM activities (Cua and Stein 2011). In all, nitrite supplementation presents a promising strategy for reshaping AOM communities to improve the ammonia removal efficiency.

Various environmental conditions were employed to accumulate nitrite in wastewater treatment processes, e.g., shortcut nitrification (Zhou et al., 2011). Nitrite dosage is a common practice to mitigate sewer corrosion (Jiang et al., 2010). These strategies could be integrated into SND process for treating wastewater with excess nitrite. In previous study, 30 mg/L nitrite dosage successfully enhanced the performance of the simultaneous nitrification denitrification and phosphorus removal process (Xie et al., 2021). Nitrite dosage was also employed to enhance nitrogen removal via anammox and nitritation in sequencing batch reactors (Zou et al. 2020). Nevertheless, how nitrite dosage influenced the ammonia removal efficiency and microbial communities in SND process warranting further research.

In this study, nitrite dosage was employed to reshape the AOM and improve the ammonia removal efficiency in SND process. Two intermittent aeration biofilm reactors performing SND were cultivated using different nitrogen resources: one with ammonia and the other with ammonia and nitrite. The total nitrogen (TN) removal efficiency and the ammonia removal rates under different nitrite concentrations were calculated to explore the impact of nitrite on the ammonia removal efficiency. The phylogenetic tree of the dominant ammonia oxidizing bacteria and classic AOB was constructed. Furthermore, chemical inhibitors were applied to explore the contribution of diverse microorganisms. The mechanisms by which nitrite reshaped AOB and AOA in the SND process were proposed. These findings provided implications for using nitrite to improve the ammonia removal efficiency and enrich AOB/AOA in the SND process.

2. Results

2.1. The nitrogen removal performance of both reactors

Fig. 1 showed the nitrogen removal performances in R1 and R2. The biofilm was successfully formed upon the fiber (Fig. S1) and the effluent ammonia concentration in two reactors was 14.50 ± 0.34 mg N/L. Then, 20 mg N/L NO_2^- was added into R2 for analyzing the influence of nitrite on the ammonia removal efficiency on 55 days. From day 73 to day 80, the effluent NH_4^+ concentrations in R1 and R2 were 13.21 ± 2.18 and 0.12 ± 0.06 mg N/L, respectively. Despite the change in influent NO_2^- concentrations in R2, the effluent NO_2^- and NO_3^- concentrations remained negligible. After 80 days, the TN removal efficiency was

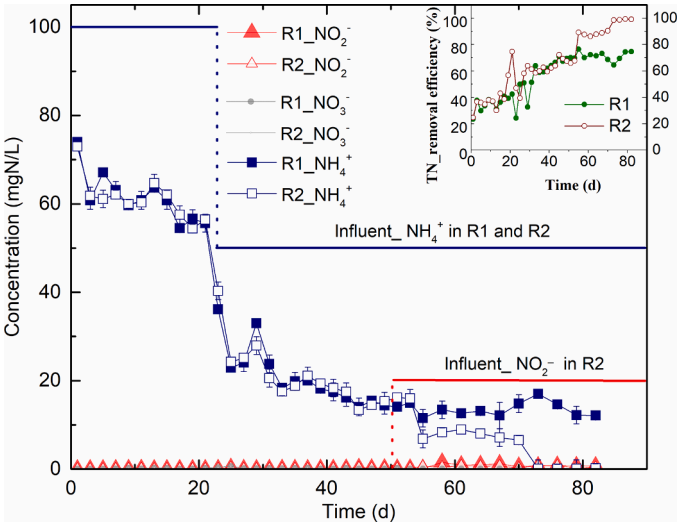

Fig. 1. The nitrogen removal performance in R1 and R2.

74.5% and 99.0% in R1 and R2 respectively, indicating that 20 mg N/L nitrite addition successfully improved the nitrogen removal efficiency in the reactor performing SND.

2.2. The ammonia removal rates in R1 and R2 with different nitrite dosages

The nitrogen removal performance during a cycle showed that both the ammonia removal rate and the TN removal efficiency in R2 surpassed those in R1 (see Figs. 2(a), S2 and S3). The NH_4^+ removal rates at various nitrite concentrations confirmed the results (see Fig. 2(b)). In R2 with 20 mg N/L nitrite, the ammonia removal rate was 3.93 ± 0.50 mg N/(L h), which was 4.5 times more than R1. Moreover, in the batch experiments with 0, and 50 mg N/L nitrite addition, the ammonia removal rate remained as high as that in R2 with 20 mg N/L nitrite dosing. Overall, the ammonia removal rate in R2 surpassed that in R1 regardless of the presence of nitrite, and was unaffected by nitrite concentrations. These results indicated that AOM was reshaped by the nitrite dosage and nitrite was no longer necessary to maintain the improved ammonia removal efficiency.

2.3. The performance of R2 with or without inhibitors

The nitrogen and COD removal performances of R2 in the batch experiments with or without inhibitors were shown in Table 1. When ATU and $NaClO_3$ were added to block ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR) generated by autotrophic nitrifiers and nitrite oxidizing bacteria (NOB) (Batt et al., 2006; Li et al., 2013; Rattier et al., 2014), 7.9% of the ammonia was removed via assimilation, anammox process, and heterotrophic nitrification. In the batch experiment with $NaClO_3$ addition, NOB and AOA were suppressed (Tatari et al., 2017). Therefore, AOA and AOB respective contributed to 41.8% and 46.9% of the ammonia removal. Moreover, the COD removal efficiency only declined by 18.7% when the ammonia removal efficiency dropped by 88.7% with ATU and $NaClO_3$ addition. The high COD

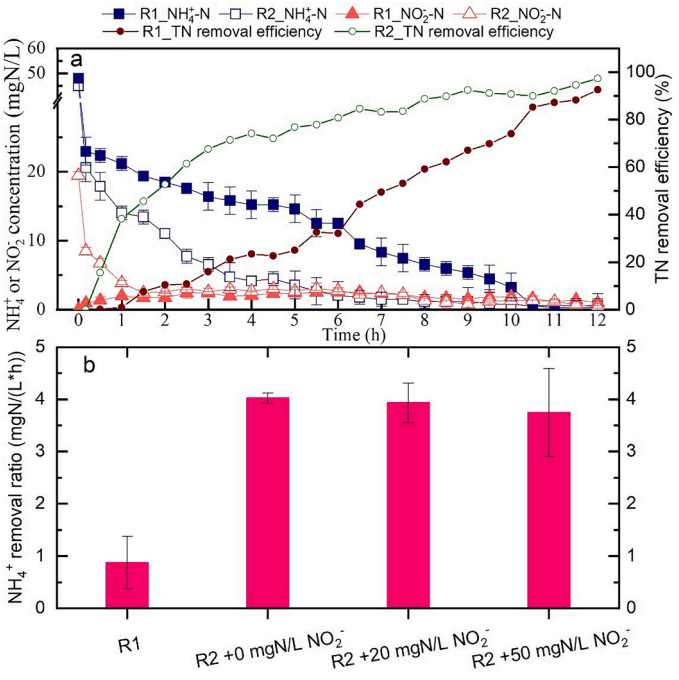

Fig. 2. The nitrogen removal performance and ammonia removal rates of two reactors. (a) The nitrogen removal performance in R1 and R2 throughout a cycle. (b) The ammonia removal rates of two reactors with different nitrite dosages. The ratios were calculated based on the results from the batch experiments and each batch experiment were performed by 2–3 times.

Table 1
The nitrogen and COD removal performance with or without inhibitors.

		None inhibitors	$NaClO_3$	ATU + $NaClO_3$
NH_4^+	Removal efficiency	96.6 ± 0.1% [a]	49.7 ± 2.0% [b]	7.9% ± 0.5 [c]
	Removal rate (mg N/(L h))	4.09 ± 0.04	1.34 ± 0.34	*N*
NO_2^-	Concentration (mg N/L)	*N*	*N*	*N*
NO_3^-	Concentration (mg N/L)	*N*	*N*	*N*
COD	Removal efficiency	93.6 ± 3.1%	85.1 ± 2.5%	74.9 ± 2.9%
Microbes involved in NH_4^+ removal		AOA/AOB/*M*	AOB/*M*	*M*

N: below the detection level. *M*: the microorganisms remove ammonia by assimilation, heterotrophic nitrification, and anammox. *D*: denitrifiers. The contribution of *M*, AOB, and AOA to ammonia removal could be calculated by c, b minus c, and a minus b, respectively.

removal efficiency revealed that the heterotrophic microorganisms including denitrifiers played an important part in the SND process.

2.4. The number of gene copies associated with nitrification

Fig. 3 showed the number of gene copies required for nitrogen removal. The number of AOA and AOB encoding ammonia monooxygenase (*amoA*) gene copies in R2 after NO_2^- addition was 280 and 30 times higher than that in R1, which indicated that ammonia oxidizers were enriched by nitrite dosage. The number of anammox hydrazine synthase genes (*hzsB*) and anammox hydroxylamine oxidoreductase genes (*hao*) was extremely few compared to *amoA* genes which showed that anammox were not the major ammonia removal microorganisms. The number of 16S *Nitrospira* genes indicating the community of *Nitrospira* including Complete Ammonia Oxidizers (comammox) was extremely few in comparison to the *amoA* genes. The results revealed that *Nitrospira* including comammox were not the dominant microorganisms in both reactors. The number of nitrite oxidoreductase genes (*nxrB*) copies in R2 was almost half less than that in R1 indicating better growth of NOB in R1. The heme-containing cytochrome cd1 nitrite reductase genes (*nirS*) and copper-containing nitrite reductase (*nirK)* are required for the nitrite reducing to nitrous oxide process. The number of *nirS* and *nirK* in R2 was 7.7 and 26.2 times higher than those in R1, respectively. These results demonstrated that the nitrite removal by denitrification was strengthened in R2.

2.5. The composition of AOB and the dominant species

The abundance of recognized microorganisms engaged in nitrification as well as the phylogenetic tree of the unclassified *f-Nitrosomonadaceae1* and related AOB were shown in Fig. 4. The nitrifiers including anammox, heterotrophic nitrifiers and autotrophic nitrifiers

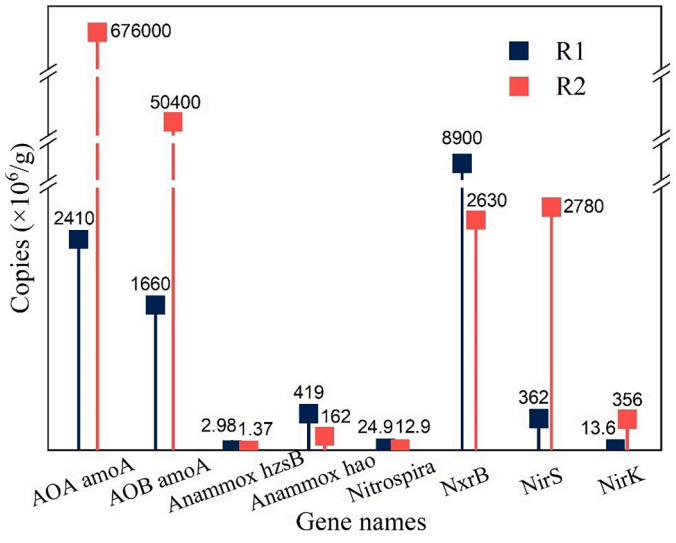

Fig. 3. The number of gene copies involved in nitrogen removal.

were analyzed. Anammox or heterotrophic nitrifiers were not found among the top abundant 100 species, which was inconsistent with previous research that nitrited dosage successfully enriched anammox and AOB in SBR (Zou et al. 2020). The high throughput sequencing analysis illustrated that the most abundant AOB in R1 and R2 were uncultured ones of the *Nitrosomonadaceae* family and named *g-nitrosomona-1* and *f- nitrosomonadaceae-1*, respectively. The abundance of *g-nitrosomona-1* was 1.311% in R1 and 0.003% in R2 while the abundance of the *f- nitrosomonadaceae-1* was observed in the inoculum, R1, and R2 to be 0.398%, 0.362%, and 1.589%, respectively. These results showed that nitrite dosages altered the dominant AOB. The enriched AOB, the *f- nitrosomonadaceae-1*, were closely related to *Nitrosospira multiformis* according to the phylogenetic analysis, demonstrating that the nitrite dosage specifically enriched *Nitrosospira multiformis*-like bacteria.

3. Discussion

3.1. Nitrite addition improved the ammonia oxidizing efficiency and rate

Normally, the ammonia oxidation efficiency was supposed to decrease in the presence of nitrite due to the inhibitory effect of the end-product (Juhler et al., 2009). Nitrite along with proton production during ammonia oxidation, has been reported as a nitrification inhibitor, resulting in a 50% reduction in AOB activity (Zhou et al., 2011). However, in this study, the NH_4^+ removal efficiency was improved from 80% to over 95% after dosing with 20 mg N/L nitrite (see Fig. 1). The reason could be due to the diverse ways in which nitrite affected microorganisms. Enhanced denitrification by nitrite addition caused rapid utilization of organic carbon, subsequently reducing the inhibition of AOM. Nitrite may also reduce specific microorganism by inhibiting the aerobic metabolism (Pijuan et al., 2010) or causing cell death and lysis (Wang et al., 2019) so that AOM grew better due to reduced competition for oxygen. Additionally, the kinetic models for nitrification inhibited by 20 mg N/L nitrite revealed that the difference in the nitrification ratio was insignificant (Carrera et al., 2004). The reconstitution and growth of AOM would be the cause of increased ammonia removal efficiency.

Also, the ammonia removal rate in R2 with nitrite surpassed that in R1 (see Fig. 2). The rate of ammonia removal in R1 was within the range of previously reported AOB (Kits et al., 2017), whereas that in R2 was higher than those except for the comammox. Furthermore, the ammonia removal rate in R2 remained stable despite varying nitrite concentrations, including the absence of nitrite (see Fig. 2), which demonstrated that the AOM community was already reshaped, rendering nitrite unnecessary for ammonia removal. Zhao et al. (2021) also noted that AOB with better nitrite tolerance, such as *Candidatus Nitrosoglobus*, predominated the microbial community after long-term nitrite addition. The results of this work revealed that the AOM with great oxidizing capacity were enriched by nitrite addition.

3.2. Anammox and NOB were decreased in the presence of nitrite

Anammox process could be the primary mechanism for ammonia removal pathway due to the long-term nitrite dosage in the anoxic area

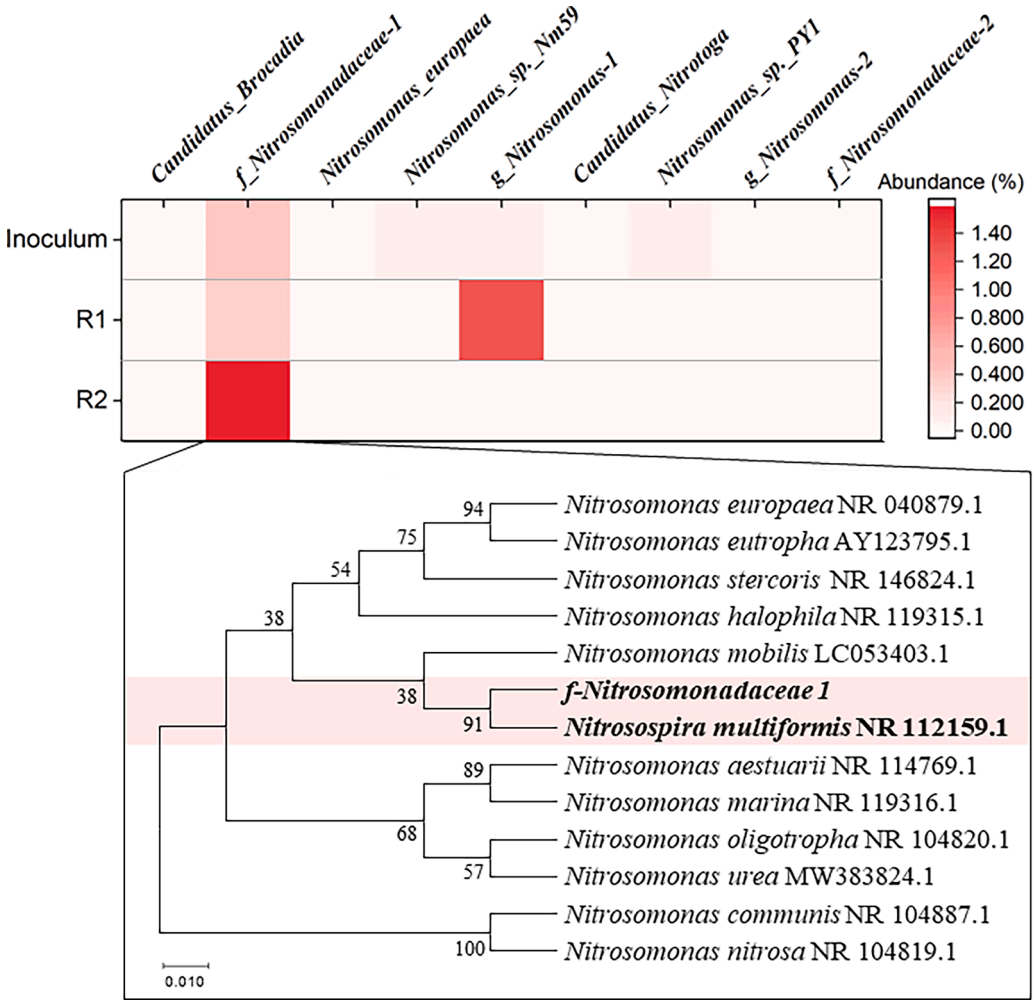

Fig. 4. The composition and phylogenetic tree of AOB.

of the biofilm. However, the low abundance of anammox genus (see Fig. S4), indicator genes (see Fig. 3) and predicted functions (see Fig. S5) indicated that anammox were not the predominant nitrogen removal pathway in this study. The main reason was not the long-term 20 mg N/L nitrite addition, because the inhibition level of nitrite was as high as 400 mg N/L (Lotti et al., 2012). Although anammox species, *Ca. Jettenia*, were reported to be resistant to constant aeration and conduct dissimilatory nitrate reduction to ammonia via nitrite using acetate as electron donors, the organic carbon in the influent and the oxic environment could be the two main causes to limit the growth of anammox (Ali et al., 2015; Gottshall et al., 2021; Long et al., 2013).

Different nitrite dosages were used to enrich specific NOBs, i.e., 20 mg N/L nitrite dosage successfully enriched *Nitrobactor* (Bartosch et al., 2002; Kim and Kim 2006; Tangkitjawisut et al., 2016). However, the decreased *nxrB* gene copy numbers (see Fig. 3) indicated that nitrite addition suppressed NOB in this study. It should be noted that no nitrite accumulation was observed during a cycle (see Fig. 2) or in the batch experiments (see Table 1), even when NXR and the nitrate reduction to nitrite process were both blocked by $NaClO_3$ (Hynes and Knowles 1983). The nitrite produced from ammonia oxidation was probably reduced to nitrogen gas via denitrification immediately. The barely affected COD removal performance with inhibitors (see Table 1) and the increased *nirS* gene copy numbers in R2 suggested the strong nitrite reduction ability via denitrification (Lisa et al., 2017).

3.3. Nitrite enriched AOA and AOB

AOA were not major nitrifiers in activated sludge or biofilm reactors for treating wastewater (Chao et al., 2016; Pjevac et al., 2017; Yu and Zhang 2012). However, the number of AOA *amoA* gene copies significantly increased with nitrite addition and outcompeted AOB amoA gene indicating that nitrite dosages enriched AOA. Although the high *amoA* gene copy numbers were not directly related to the function of ammonia oxidation (Mussmann et al., 2011), the AOA batch experiments results showed that AOA contributed to 46.9% of the ammonia removal efficiency (see Table 1). These findings demonstrated that AOA were important nitrifiers in SND process and that the addition of 20 mg N/L successfully enriched AOA. The mechanisms by which nitrite increased the abundance of AOA were probably linked to genes associated with nitrite reduction. The *nirK* gene observed in AOA demonstrated the ability to use nitrite as a substrate for denitrification by nitrifiers (Francis et al., 2005; Treusch et al., 2010). Moreover, some AOA ecotypes, i.e., *Ca. N. exaquare* tolerated higher nitrite concentrations without ammonia oxidation rate reduction than *N. viennensis* (Sauder et al., 2017). AOA with specific *nirK* genes were enriched at 20 mg N/L nitrite in this study but the insightful mechanisms warrant further research.

Specific beta-AOB communities were enriched by nitrite dosages despite the product inhibition, such as *N. oligotropha, N. europaea* and comammox (Dang et al., 2010; Limpiyakorn et al., 2007; Zhao et al., 2021a). In this study, the *Nitrosospira multiformis*-like AOB were enriched and the mechanism for how they adapted to nitrite addition

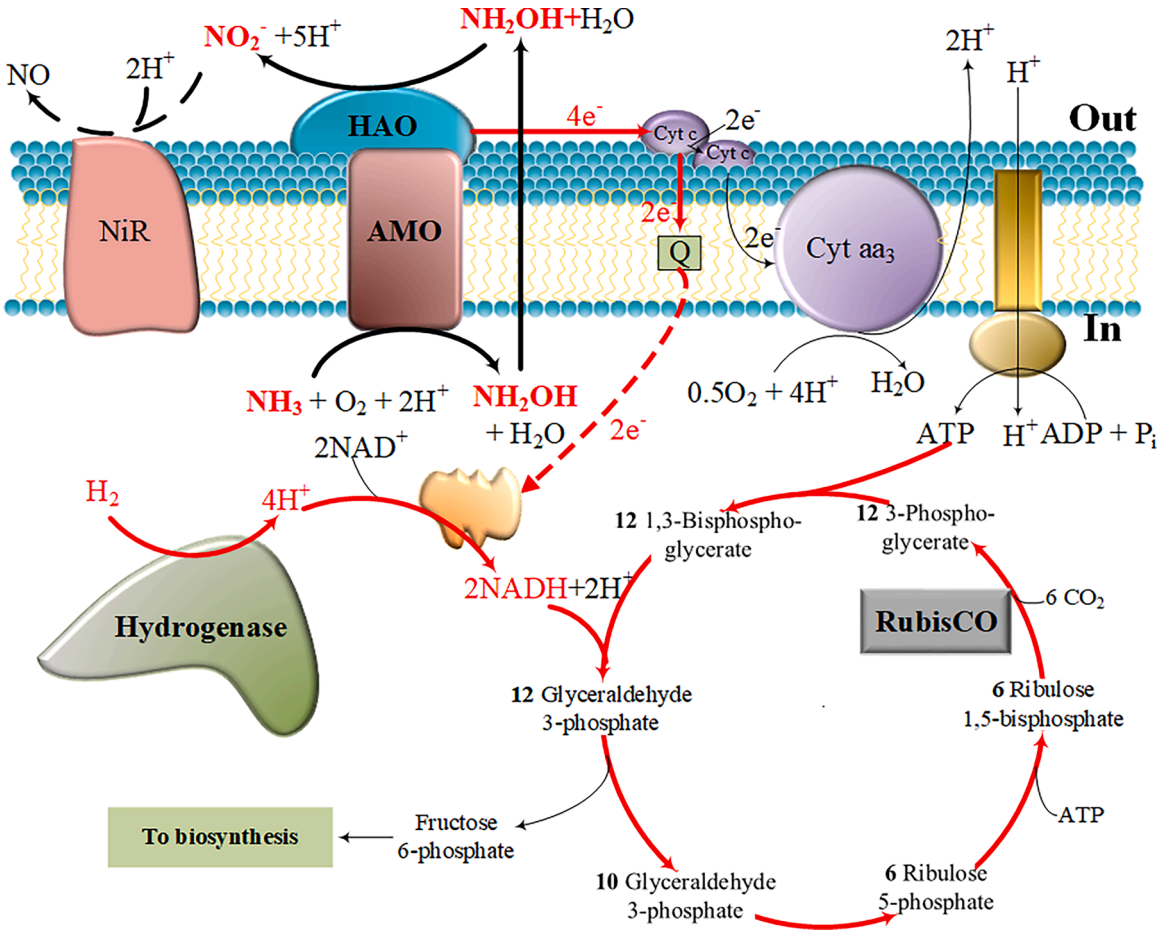

Fig. 5. The proposed mechanism of nitrite-enriched AOB.

was proposed (see Figs. 4 and 5). NO accumulation resulting from nitrite dosing was reported to inhibit NOB while promote AOB (Xie et al., 2021). This phenomenon may be attributed to the capability of AOB, including *Nitrosospira multiformis*, to convert nitrite into NO through the action of nitrite reductase (NIR), a mechanism shared with other AOB (Shaw et al., 2006). The *nirK* gene in *Nitrosospira multiformis* differed from that in *N. europaea* and *N. oceani* in which it has two type 1 Cu ligands rather than just one (Cantera and Stein, 2007), indicating that its *nirK* might perform differently and responded to various environmental signals (Norton et al., 2008), such as high nitrite concentration. Moreover, an ortholog encoding periplasmic nitrosocyanin protein linked with NO or N_2O metabolism in electron transfer was detected in *Nitrosospira multiformis*, whereas it was exclusive to AOB (Basumallick et al., 2005). Moreover, *Nitrosospira multiformis* were the first AOB identified to contain a putative hydrogenase (Norton et al., 2008). The hydrogenase was reported to be linked to membrane-associated H_2 uptake (Hanczar et al., 2002) and couple hydrogen oxidation to NAD reduction for providing reducing power (Schwartz et al., 1998). In *Nitrosospira multiformis*, the hydrogenase served as the donor of reducing power, driving the forward NADH generation chain, which provided energy for the carbon fixation through the Calvin cycle. Therefore, less reverse electron flow was needed during the ammonia oxidation process. The high overall energy produced from ammonia oxidation could represent a niche-specific adaptation to an environment with nitrite supplementation.

4. Conclusions

Ammonia removal efficiency and rate were enhanced by 20 mg N/L nitrite addition in the reactor performing SND. The mechanisms about how nitrite enriched AOA and AOB were proposed. These results showed that nitrite dosage could be an efficient strategy to enrich AOA and AOB with higher ammonia removal efficiency. Nitrite accumulation process (e.g., nitritation) could be combined with SND process to improve the nitrogen removal efficiency in wastewater treatment. The specific conclusions were provided below.

- TN removal efficiency of SND increased from 74.5% to 99.0% after 20 mg N/L nitrite addition.
- Nitrite accelerated the rate of ammonia elimination by 4.5 times. The ammonia removal rate remained stable despite various nitrite dosages (0, 20, and 50 mg N/L).
- AOA and AOB contributed to 46.9% and 41.8% of the ammonia removal.
- The number of AOA and AOB *amoA* gene copies in R2 (with nitrite addition) was 280 and 30 times more than that in R1 (without nitrite addition)
- The enriched AOA and AOB adapted to nitrite possibly because of the different structures of *nirK*.
- *Nitrosospira multiformis*-like bacteria were enriched and became the dominant AOB.

5. Materials and methods

5.1. Culture enrichment and reactor operation

Two 8 L sequencing biofilm batch reactors (SBBR) named as R1 and R2 were filled with 20 pieces of combined polypropylene fibers, similar

to the one reported previously (Chai et al., 2019). The detailed information on the reactors and fibers was shown in the Supplementary materials (see Fig. S6). Air was supplied to the reactors using the air pump (SONGBAO SB-988, China) with a maximum rate of 4 L/min. The temperature was kept at 30 °C by a heater. The pH level was not managed but stayed between 7 and 8.5 in both reactors. The liquid and air were mixed using a magnetic stirrer (DJ-1). The dissolved oxygen (DO) concentrations were around 2 mg O_2/L and 0.01 mg O_2/L in the aeration and anoxic stirring phases, respectively. The cycle contained: 10 min inflow, 170 min aeration, 90 min anoxic stirring, 180 min aeration, 90 min anoxic stirring, 150 min aeration, 15 min settling, 10 min drainage, and 5 min settling. 4 L wastewater was piped out of the reactors at the drainage phase so as to maintain a 24-h hydraulic retention time (HRT).

The inoculum was acquired from the aeration tank of Jiguanshi Wastewater Treatment Plant (WWTP) in Chongqing, China. The scale of the plant is 800,000 m^3/d and Anoxic – Anoxic - Oxic process with a hydraulic retention time (HRT) of 10.5 h is used. The inoculum was the sludge in the aerobic tank with an HRT of 6.8 h. The mixed liquor suspended solids (MLSS) of the inoculum was 7578 mg/L. The 30-minute sludge volume (SV_{30}) was 37%. The ratio of mixed liquor volatile suspended solids (MLVSS) to MLSS was 0.73. Inoculum of 4 L was applied into R1 and R2 respectively so as to attain a MLSS of 3789 mg/L in the reactors.

The influent was synthetic wastewater and it was composed with $CH_3COONa{\cdot}3H_2O$ and NH_4Cl as respective carbon source and nitrogen source, together with 250 mg/L $NaHCO_3$, 44 mg/L KH_2PO_4, 10 mg/L $CaCl_2$, 10 mg/L $MgSO_4{\cdot}7H_2O$, 5 mg/L $FeSO_4{\cdot}7H_2O$ and 1 ml/L trace elements stock according to Van et al. (1996) The influent NH_4^+ and chemical oxygen demand (COD) concentration was respective 100 mg N/L and 400 mg/L in both reactors during the first 23 days, with the NH_4^+ removal efficiency less than 50%. From day 23 on, the influent NH_4^+ concentration of both reactors was reduced to 50 mg N/L to improve the ammonia removal efficiency. After 55 days, 20 mg N/L NO_2^- along with 50 mg N/L NH_4^+ was supplied to R2 to investigate the effect of nitrite.

5.2. Cycle and batch experiments

To explore the nitrogen removal performance, cycle studies were conducted in R1 and R2 on day 80 when the reactors were stable. The procedure, temperature, and influent water quality were the same as those described in Section 2.1. The COD concentrations in two reactors were both 400 mg/L. 50 mg N/L ammonia served as the nitrogen source in R1, while in R2, the nitrogen sources were 20 mg N/L nitrite and 50 mg N/L ammonia. Every 30 min, samples of mixed liquor were taken for COD, NH_4^+, NO_2^- and NO_3^- concentration measurements. Gas samples in the upper phase were collected at the same frequency for N_2O analysis.

Batch experiments were conducted at steady state on day 82. Four batch experiments were performed to explore the impact of various nitrite concentrations on the ammonia removal rates. Each experiment was carried out 2 to 3 times. For the test of R1, 50 mg N/L NH_4^+ was utilized as the nitrogen source. 0, 20, and 50 mg N/L NO_2^- along with 50 mg N/L NH_4^+ were used as nitrogen sources for the R2 test, respectively. In addition, three batch experiments were performed to study the contributions of different ammonia removal pathways. 10 mg/L allylthiourea (ATU) and 1 g/L $NaClO_3$ were used to inhibit the AMO and NXR, respectively (Bedard and Knowles 1989). Detailed information on the COD and nitrogen sources in each batch experiment was shown in Table 2.

Batch experiments were conducted in small-scale SBBRs. 400 ml synthetic wastewater was used, with the same composition as the parent reactors except for the COD and nitrogen sources. One piece of combined fiber with stack biofilm taken from the parent reactor was added in each bottle, attaining the same filling ratio as the parent reactor. A water bath thermostatic oscillator (SHA-B, China) was used to keep the temperature at 30 °C. The procedure was the same as that specified in Section 5.1. The emitted gas mixture was captured by gas collecting bags (E-Switch) for nitrous oxide (N_2O) detection, and the liquor samples were collected for COD, NH_4^+, NO_3^- and NO_2^- concentration measurements hourly.

5.3. Analytical methods

The methods outlined in "Standard Methods for the Examination of Water and Wastewater" were used to measure the concentrations of NH_4^+-N, NO_3^--N, NO_2^--N, TN, and COD (Apha 2005). The ammonia removal efficiency was calculated to show the nitrification performance. TN represents the ammonia, nitrite, nitrate and dissolved organic nitrogen. The TN removal efficiency indicated the overall nitrogen removal efficiency. The emitted N_2O was determined using a gas chromatography with an electron capture detector (ECD). The N_2O capture and calculating methods were according to Hai et al. (2002).

5.4. Microbial analysis

E.Z.N.A.® Soil DNA Kit (omega D5625-01) was used to extract the DNA of the inoculum and microbial samples from R1 and R2 on day 80. The DNA was amplified with the forward primer 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego,USA). Raw FASTQ files were de-multiplexed using an in-house perl script, and then quality-filtered by fastp version 0.19.6 and merged by FLASH version 1.2.7. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA gene database (e g. Silva v138) using confidence threshold of 0.7. The metagenomic function was predicted by PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) based on OTU representative sequences. The overall analysis was performed on the online website of Shanghai MajorBio Biopharm Technology Co., Ltd, China. Detailed information could be found in supplementary material. The raw reads were submitted to BioProject PRJNA612947 at the National Center for Biotechnology Information (NCBI). The potential functions were predicted by Functional Annotation of Prokaryotic Taxa (FAPROTAX) (Louca et al., 2016).

Table 2
The detailed information of batch experiments.

No.	Target	Reactor	COD (mg/L)	Nitrogen source		Remarks
				Type	Concentration (mg N/L)	
1	Nitrification capacity	R1	400	NH_4^+	50	
2		R2	400	NH_4^+	50	
3		R2	400	NH_4^+/NO_2^-	50/20	
4		R2	400	NH_4^+/NO_2^-	50/50	
5	Nitrification pathways	R2	400	NH_4^+	50	No inhibitors
6		R2	400	NH_4^+	50	+$NaClO_3$
7		R2	400	NH_4^+	50	+ATU, $NaClO_3$

The real-time qPCR assays on the samples were conducted by Shanghai Personal Biotechnology Company. 12.4 μL reaction mixture contained 10 μL 2*AceQ U and Probe Master Mix, 0.4 μL forward primer (10 μM) and 0.4 μL reverse primer (10 μM), 0.2 μL of probe, 0.4 μL of 50*ROX Reference Dye 1 and 1 μL template DNA. For all calibration curves, the reaction efficiencies ranged from 94.05 to 100.03 percent, with R^2 values exceeding 0.999. The specific primers were shown in Table S1.

CRediT authorship contribution statement

Yu Xiang: Conceptualization, Formal analysis, Methodology, Writing – original draft. **Tengzhi Zhou:** Formal analysis, Investigation. **Siping Deng:** Data curation, Validation. **Zhiyu Shao:** Methodology, Validation. **Yiwen Liu:** Methodology, Writing – review & editing. **Qiang He:** Project administration, Supervision. **Hongxiang Chai:** Conceptualization, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2023.100204.

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