Efficient Nitrification and Low-Level N₂O Emission in a Weakly Acidic Bioreactor at Low Dissolved-Oxygen Levels Are Due to Comammox

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ABSTRACT Nitrification is an essential process for nutrient removal from wastewater and an important emission source of nitrous oxide (N₂O), which is a powerful greenhouse gas and a dominant ozone-depleting substance. In this study, nitrification and N₂O emissions were tested in two weakly acidic (pH 6.3 to 6.8) reactors: one with dissolved oxygen (DO) at over 2.0 mg/liter and the other with DO at approximately 0.5 mg/liter. Efficient nitrification was achieved in both reactors. Compared to that in the high-DO reactor, N₂O emission in the low-DO reactor decreased slightly, by 20%, and had insignificant correlation with the fluctuations of DO ($P=0.935$) and nitrite ($P=0.713$), indicating that N₂O might not be produced mainly via nitrifier denitrification. Based on quantitative PCR (qPCR), quantitative fluorescent *in situ* hybridization (qFISH), and functional gene amplicon and metagenome sequencing, it was found that complete ammonia oxidizers (comammox), i.e., *Nitrospira* organisms, significantly outnumbered canonical ammonia-oxidizing bacteria (AOB) in both weakly acidic reactors, especially in the low-DO reactor with the comammox/AOB *amoA* gene ratio increasing from 6.6 to 17.1. Therefore, it was speculated that the enriched comammox was the primary cause for the slightly decreased N₂O emission under long-term low DO in the weakly acidic reactor. This study demonstrated that the comammox *Nitrospira* can survive well under the weakly acidic and low-DO conditions, implying that achieving efficient nitrification with low N₂O emission as well as low energy and alkalinity consumption is feasible for wastewater treatment.

IMPORTANCE Nitrification in wastewater treatment is an important process for eutrophication control and an emission source for the greenhouse gas N₂O. The nitrifying process is usually operated at a slightly alkaline pH and high DO (>2 mg/liter) to ensure efficient nitrification. However, it consumes a large amount of energy and chemicals, especially for wastewater without sufficient alkalinity. This paper demonstrates that comammox can adapt well to the weakly acidic and low-DO bioreactors, with a result of efficient nitrification and low N₂O emission. These findings indicate that comammox organisms are significant for sustainable wastewater treatment, which provides an opportunity to achieve efficient nitrification with low N₂O production as well as low energy and chemical consumption simultaneously.

KEYWORDS nitrous oxide, comammox, nitrification, wastewater treatment, weakly acidic condition

To achieve efficient nitrification during wastewater treatment, the nitrifying process is usually operated at a slightly alkaline pH (7.0 to 8.5) and with sufficient dissolved oxygen (DO) (>2 mg/liter) (1–3). The nitrification process is also an important emission source for greenhouse gas nitrous oxide (N₂O) (4). Although operating the reactors under weakly acidic (pH 6.0 to 6.8) and low-DO (<0.5 mg/liter) conditions may reduce

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the operational costs, especially for wastewater without sufficient alkalinity, incomplete nitrification and high N_2O emissions can occur (5, 6). However, if weakly acid-tolerant nitrifiers with high apparent oxygen affinity and low N_2O yield are enriched in nitrifying bioreactors, efficient nitrification, low N_2O emission, and low consumption of energy and chemicals could be achieved simultaneously.

Low pH adversely affects nitrification, mainly because of the limitations of free ammonia (the actual substrate for ammonia oxidizers) and inorganic carbon, inhibition by nitrous acid (HNO_2), and/or the direct effects of high proton concentrations (7–9). The dominant ammonia-oxidizing bacteria (AOB) (e.g., the *Nitrosomonas europaea* lineage) in nitrifying bioreactors are highly sensitive to low pH and show optimum growth at a pH of 7.5 to 8.0 (10, 11). During long-term operation at a low pH, however, acid-tolerant ammonia oxidizers, for example, *Nitrosomonas oligotropha* and *Nitrosospira*-related strains, can be selectively enriched (10, 12). *Nitrosomonas europaea* has an apparent half-saturation constant of ammonia (K_s) of 18 to 70 μM (13), whereas that for the *Nitrosomonas oligotropha* lineage is approximately 3 μM (14). The higher apparent substrate affinity facilitates the adaptation of *Nitrosomonas oligotropha* to a low pH (10). Similarly, ammonia-oxidizing archaea (AOA) are dominant in some acidic environments, which also benefit from their high apparent ammonia affinity of 0.002 to 6.0 μM (15). Notably, the newly discovered complete ammonia oxidizers (comammox) (e.g., *Nitrospira inopinata*) have significantly higher apparent ammonia affinity (0.06 to 0.08 μM) than canonical AOB and most AOA (16–18), which may facilitate their adaptation to weakly acidic environments. A recent study reported that the comammox *Nitrospira* significantly outnumbered canonical AOB in acidic soil environments (19). Moreover, *Nitrospira* species have also been reported to be enriched in some engineered systems under acidic conditions (10, 11). These prior studies, however, did not distinguish comammox from canonical nitrite-oxidizing bacteria (NOB) in the genus *Nitrospira*, and the NOB *Nitrospira* organisms reported in these studies under acidic conditions were likely the comammox *Nitrospira*.

Although low-DO operation can reduce aeration energy, it can inhibit nitrification significantly and result in incomplete nitrification (6, 20). Previous studies, however, found that complete nitrification recovered under long-term low-DO conditions ($\text{DO} < 0.5$ mg/liter), which resulted mainly from the increased nitrifier abundance and the improved apparent oxygen affinity of nitrifiers due to low-DO operation (21–24). For instance, the K_{DO} values for AOB and NOB in activated sludge cultivated with a high DO of 4.2 mg/liter were 0.71 and 0.41 mg/liter, respectively. For activated sludge cultivated under a long-term low DO concentration of 0.16 mg/liter, however, they decreased to 0.39 and 0.04 mg/liter, respectively (25). Although the apparent oxygen affinity of the comammox *Nitrospira* remains to be measured, both the theoretical prediction and genomic studies indicate that they likely adapt to low-DO conditions (26, 27). Moreover, Roots et al. (28), Wang et al. (29), and Beach and Noguera (30) demonstrated that the comammox *Nitrospira* adapted well to low-DO wastewater treatment systems at a slightly alkaline pH and a long solids retention time (SRT). However, it is unknown whether comammox can adapt to low DO in weakly acidic wastewater bioreactors.

N_2O can be biologically generated through the incomplete oxidation of hydroxylamine to nitrite (NO_2^-) by AOB (31). Nitrifier denitrification is another important pathway to produce N_2O , where NO_2^- is subsequently reduced to nitric oxide (NO) and N_2O (32, 33). Nitrifier denitrification is reported to be the primary pathway in most nitrifying systems (34–36). Previous studies based on batch tests indicated that low pH and low DO may significantly influence or promote N_2O production (5, 37–39). A recent study found that the comammox *Nitrospira* lacks nitric oxide reductase (NOR) homologs, indicating that these novel nitrifiers may not produce N_2O via nitrifier denitrification, as with canonical AOB (40). If comammox are

FIG 1 (a and b) Nitrification performance and N₂O emission factor under high-DO acidic conditions (HAC) (a) and low-DO acidic conditions (LAC) (b); (c) boxplot of the effluent NH₄⁺-N, NO₂⁻-N, and N₂O emission factors in both reactors. Ef., effluent.

enriched at low DO in the weakly acidic reactors, N₂O emissions may increase negligibly or even decrease compared to emissions during high-DO operation.

In the present study, we hypothesized that comammox could adapt well to low-DO conditions in the weakly acidic wastewater bioreactors, with the achievement of efficient nitrification and unchanged or even lowered N₂O emissions compared to those produced by high-DO operation. To test this hypothesis, we operated two weakly acidic membrane bioreactors, one at a high DO concentration (>2 mg/liter) and the other at a low DO concentration (approximately 0.5 mg/liter). The nitrification performance and N₂O emission in both reactors were tested over a long period. Additionally, the communities of canonical AOB and comammox under the stabilized nitrification

FIG 2 Effects of pH on the specific ammonia rate (sAOR) or nitrite oxidation rate (sNOR) of biomass cultivated under HAC and LAC. SS, suspended solids.

condition were analyzed to test the hypothesis and explore the correlations of comammox with nitrification efficiency and N_2O production.

RESULTS

Nitrification performance and N_2O emission. As shown in Fig. 1a and b, the two weakly acidic reactors achieved nearly complete nitrification under both high- and low-DO conditions. Ammonia and nitrite did not accumulate in the high-DO acidic condition (HAC) reactor with average effluent concentrations of 0.08 ± 0.12 and 0.05 ± 0.03 mg/liter, respectively. Under weakly acidic conditions with a low DO concentration (approximately 0.5 mg/liter), nitrification performance fluctuated slightly (Fig. 1c), which might have resulted from the fluctuation in operational pH and DO (see Fig. S1 in the supplemental material). The average effluent $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ concentrations were 0.64 ± 0.84 , 0.44 ± 0.46 , and 42.8 ± 2.8 mg/liter, respectively, indicating that efficient nitrification was also achieved under a long-term low DO concentration in the weakly acidic reactor. The sums of effluent $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ in the HAC and low-DO acidic condition (LAC) reactors were 44.3 ± 2.3 and 43.9 ± 2.3 mg/liter, suggesting that denitrification in the low-DO reactor was insignificant ($n \geq 32$, $P = 0.46$).

As shown in Fig. 1, the N_2O emission factor in the HAC reactor ranged from 0.09% to 0.40%, with an average of $0.15\% \pm 0.08\%$ ($n = 28$), while it ranged from 0.04% to 0.34%, with an average of $0.12\% \pm 0.08\%$ ($n = 34$), in the LAC reactor. In contrast to the results of previous studies which reported that low DO induced a higher N_2O emission factor

FIG 3 (a) Numbers of marker or functional gene copies normalized to dry sludge determined based on qPCR (means $\pm$ standard deviations, $n = 9$); (b) ratio of the comammox *Nitrospira* to AOB based on qFISH (the mean values were estimated based on 19 randomly selected fields of view with a concurrency of more than 90% with daime software).

(38, 39), the N₂O emission under long-term low DO did not increase but decreased slightly by 20% ($P=0.171$). These results indicated that efficient nitrification could be achieved under long-term low-DO conditions without increasing N₂O emission in the weakly acidic reactor.

Biomass-specific nitrification rate. The low effluent ammonia and nitrite concentrations, as shown in Fig. 1, indicated the tolerance of nitrifiers to a weakly acidic pH, which was also confirmed by batch experiments (Fig. 2). Although the biomass cultivated with a weakly acidic pH had greater nitrification rates at an alkaline pH, as shown in Fig. 2, they still could oxidize the ammonia efficiently at pH 6.4. For biomass cultivated under a slightly alkaline pH, however, the ammonia oxidation ceased completely at pH 6.4 (Fig. S2). Therefore, the efficient nitrification at pH 6.4 (Fig. 2) again suggested that the weakly acid-tolerant nitrifiers were enriched in both reactors. A one-way analysis of variance (ANOVA) found that the difference in nitrification rate for an HAC biomass between pH 7.6 and 6.4 was significant ($P<0.05$), while it was insignificant for an LAC biomass ($P>0.1$), suggesting that the biomass in the LAC reactor had a stronger tolerance to weakly acidic conditions.

Notably, the weakly acidic biomass cultivated at low DO showed higher specific ammonia and nitrite oxidation rates at all tested pH values (Fig. 2). A t test indicated that the increase was significant ($P<0.05$) at a pH of 6.4.

Nitrifier abundance based on qPCR and qFISH assays. The *amoA* genes of canonical AOB and comammox in both reactors were quantified based on quantitative PCR (qPCR) assays, and the results are shown in Fig. 3a. For NOB, the *Nitrospira nxrB* and *Nitrobacter* 16S rRNA genes were quantified. As shown in Fig. 3a, the comammox *Nitrospira* outcompeted canonical AOB in the weakly acidic reactor regardless of the operational DO concentration, suggesting that comammox can survive well under weakly acidic conditions. In addition, the predominance of the comammox *Nitrospira* over canonical AOB under low-DO conditions was more significant than that at high DO, with comammox/AOB *amoA* gene ratios of 17.1 and 6.6, respectively. The multiple *amoA* copies in canonical AOB genomes and the single *amoA* copy in the known comammox *Nitrospira* genomes consolidated the dominance of the comammox *Nitrospira* over canonical AOB (40, 41). When normalized to the bacterial 16S rRNA gene copies, comammox *Nitrospira amoA* genes had a higher relative abundance (2.01%) in the LAC reactor than that (0.84%) in the HAC reactor. However, the relative abundances of canonical AOB *amoA* genes remained similar, with 0.13% in the HAC reactor and 0.12% in the LAC reactor. These results also suggested that low DO facilitated the competition of comammox over canonical AOB.

The biomasses from HAC and LAC reactors were also analyzed with quantitative fluorescent *in situ* hybridization (qFISH), with results shown in Fig. 3b and Fig. S4. The biomass volume fractions of the target population (canonical AOB and the comammox *Nitrospira*) were individually normalized to the universal bacterial biomass volume with daime software (42). As shown in Fig. 3b, the ratio of the comammox *Nitrospira* to canonical AOB was 2.81 ± 1.11 in the low-DO acidic reactor, which is significantly higher ($P=0.032$) than that in the high-DO acidic reactor (1.98 ± 1.03). Therefore, both qPCR and qFISH determined that the comammox *Nitrospira* surpassed canonical AOB in the weakly acidic reactor, especially with a low DO.

Figure 3a also shows that the abundances of both ammonia oxidizers (canonical AOB plus comammox) and nitrite oxidizers (*Nitrospira* plus *Nitrobacter*) in the low-DO reactor increased significantly, which again indicated that long-term low-DO operation could enrich the nitrifier population size even under acidic conditions. Due to the greater population size, the biomass cultivated under low-DO conditions had a higher nitrification rate, as shown in Fig. 2.

Communities of canonical AOB and the comammox *Nitrospira*. The *amoA* genes of the comammox *Nitrospira* and canonical AOB were amplified and sequenced to further characterize their communities. Operational taxonomic units (OTUs) with a relative abundance greater than 1% were selected for phylogenetic analysis. Additionally, the *amoA* sequences retrieved from metagenome sequencing were also included in the phylogenetic tree construction.

Amplicon sequencing of canonical AOB *amoA* genes revealed that only four OTUs

FIG 4 Phylogenetic tree of *amoA* gene sequences via amplicon sequencing (the 436-bp fragment for comammox *amoA* and the 491-bp fragment for AOB *amoA*) and metagenome sequencing. This tree was rooted by γ -AOB *amoA* genes and the *Methylocystis* sp. *pmoA* gene cluster. The color panels indicate the groups of *amoA* gene sequences. Heatmaps suggest the relative abundances of comammox or AOB *amoA* OTUs based on amplicon sequencing. The bubble plot on the right indicates the abundances of each *amoA* gene recovered from metagenome sequencing.

had a relative abundance greater than 1%. In the HAC reactor, 92.60% of AOB *amoA* reads (OTU 20) were clustered with *Nitrosomonas* sp. strain NP1. In the LAC reactor, 58.41% of AOB *amoA* reads (OTU 20 and OTU 22) were affiliated with *Nitrosomonas* sp. NP1, whereas 34.25% of AOB *amoA* reads (OTU 21 and OTU 23) were clustered with "*Candidatus Nitrosomonas nitrosa*" (Fig. 4). The Shannon index (0.61 for HAC and 2.37 for LAC reactors) obtained from amplicon sequencing suggested that the low-DO canonical AOB community had higher diversity. As shown in Fig. 4, metagenome sequencing also revealed that *Nitrosomonas* sp. NP1 was the most dominant canonical AOB in the HAC reactor. In the LAC reactor, five canonical AOB *amoA* sequences were found based on an *amoA* hidden Markov model (HMM), and their abundances ranged

FIG 5 Abundances of functional genes related to nitrification and denitrification based on metagenome sequencing. *, P value < 0.05 ; **, P value < 0.01 . *amoA*, ammonia monooxygenase subunit A; *amoB*, ammonia monooxygenase subunit B; *amoC*, ammonia monooxygenase subunit C; *hao*, hydroxylamine dehydrogenase; *nxrA*, nitrite oxidoreductase alpha subunit; *nxrB*, nitrite oxidoreductase beta subunit; *narG*, nitrate reductase alpha subunit; *narH*, nitrate reductase beta subunit; *narI*, nitrate reductase gamma subunit; *nirK*, copper-containing nitrite reductase; *nirS*, nitrite reductase; *norB*, nitric oxide reductase subunit B; *norC*, nitric oxide reductase subunit C; *nosZ*, nitrous oxide reductase.

from 1 to 11. Therefore, the results for canonical AOB from both assays were generally consistent. However, an *amoA* gene sequence (i.e., meta AOB *amoA5*), which was indicated as associated with *Nitrospira* by metagenome sequencing, was not detected in the amplicon sequencing.

The sequencing of the functional gene of the comammox *amoA* gene generated 393 OTUs, and the top 8 OTUs with a relative abundance greater than 1% were selected for phylogenetic analysis. Additionally, three comammox *amoA* sequences were found based on an HMM from metagenome sequencing and included in the phylogenetic analysis. Notably, the community of the comammox *Nitrospira* exhibited differences between the two reactors. Based on amplicon sequencing, the eight sequences were clustered within comammox *Nitrospira* clade A. Under the low-DO conditions, comammox *amoA* OTU 1 accounted for 95.2% of comammox *amoA* reads based on amplicon sequencing, which clustered closely with “*Candidatus Nitrospira nitrosa*” and *Nitrospira* sp. strain UW-LDO-01. Based on metagenome sequencing, meta-comammox *amoA1* was the only detected comammox *amoA* sequence with an abundance of 120 in the LAC reactor (Fig. 4). In the HAC reactor, both amplicon and metagenome sequencing showed that the comammox *Nitrospira amoA* sequences were classified mainly into uncultivated strains, such as *Nitrospira* sp. strain SG-bin1, *Nitrospira* sp. strain SG-bin2, and *Nitrospira* sp. strain ST-bin4. The profile of comammox revealed by the *amoA* gene phylogenetic analysis was consistent with the results of taxonomic classification at the species level (Fig. S3).

Functional genes for nitrification, denitrification, and N₂O production. The functional genes encoding key enzymes implicated in nitrification and denitrification were analyzed based on the metagenome (Fig. 5). The relative abundances of *amoA*, -B, and -C were 0.0037% and 0.0212% in the HAC and LAC reactors, respectively. This result indicated that the LAC biomass may have a higher potential for ammonia oxidation, which corresponded to the results from batch experiments (Fig. 2). The relative abundances of nitrite-oxidizing genes (*nxrB*) were higher than those of *amoA* genes in both reactors. The high relative abundance of *nxrB* may have been caused by (i) the high abundance of *Nitrospira* revealed by qPCR and metagenome sequencing and (ii) the existence of multiple copies of the *nxrB* gene in *Nitrospira* genomes (43). Based on metagenome sequencing, we detected that the relative abundances of the genes *nirK*, *norB*, *norC*, and AOB *nirK* were significantly ($P < 0.05$) lower in the LAC reactor than in

FIG 6 Correlations of pH, DO concentration, and $\text{NO}_2\text{-N}$ concentration against the N_2O emission factor in the low-DO acidic reactor.

the HAC reactor, as shown in Fig. 5, which indicated a lower N_2O production potential in the LAC reactor.

DISCUSSION

Efficient nitrification was achieved under long-term low DO in the weakly acidic reactor. Both pH and DO are important factors for nitrification (44–46). Generally, a nitrifying bioreactor is recommended to be operated at a slightly alkaline pH (7.0 to 8.5) and a DO of greater than 2.0 mg/liter. Due to the selection of acid-tolerant nitrifiers or nitrifiers with high apparent oxygen affinity, previous studies demonstrated that complete nitrification could be achieved under weakly acidic plus high DO (10–12, 47) or slightly alkaline plus low DO (1, 21, 22) conditions. The present work further demonstrated that efficient nitrification could be achieved under weakly acidic plus low-DO conditions as well, implying a potential to save both energy and alkalinity for wastewater treatment.

The activities of AOB cultivated under slightly alkaline conditions were inhibited by 80% or even ceased at a pH of ≤ 6.5 (48, 49). However, the biomass in both weakly acidic reactors in the present study performed ammonia oxidation efficiently at pH 6.4 (Fig. 2), suggesting that weakly acid-tolerant nitrifiers were enriched. Moreover, the LAC reactor had higher values of both the specific ammonia oxidation rate (sAOR) and the specific nitrite oxidation rate (sNOR) (Fig. 2), mainly because it contained greater abundances of ammonia and nitrite oxidizers (Fig. 3a). Therefore, the selection and enrichment of weakly acid-tolerant nitrifiers accounted for the efficient nitrification under long-term DO in the weakly acidic reactor.

Comammox adapted to the weakly acidic and low-DO conditions. The discovery of comammox added another group of nitrifiers for ammonia oxidation. Previous studies found that comammox had an oligotrophic lifestyle (16) and could dominate the ammonia oxidizer communities in some biofilms and in an activated sludge bioreactor with an extended SRT. Recently, Roots et al. (28) and Beach and Noguera (30) reported that the comammox *Nitrospira* could adapt well to a low-DO wastewater treatment process with slightly alkaline pH and a long SRT. This study determined that the comammox *Nitrospira* surpassed canonical AOB significantly in both weakly acidic reactors (Fig. 2), suggesting that *Nitrospira* can survive well under weakly acidic conditions. Its high apparent ammonia affinity (0.04 to $0.08\ \mu\text{M}$) may have enabled the comammox *Nitrospira* to grow well under weakly acidic conditions. However, whether *Nitrospira* can adapt to an even lower pH (e.g., pH 3 to 6), like the *Nitrosomonas oligotropha* lineage (47) and *Nitrosococcus*-related AOB (12), is still unknown.

Under weakly acidic conditions, low-DO operation also facilitated the competition of the comammox *Nitrospira* over canonical AOB (Fig. 3). Moreover, the comammox *Nitrospira* communities were distinct between high and low DO concentrations. Based on its *amoA* genes, the comammox *Nitrospira* can be divided into two monophyletic sister clades, i.e., clade A and clade B (50–52). A recent study (53) proposed that

Nitrospira clade A can be further subdivided into clade A.1 (including the cultivable comammox *Nitrospira*) and clade A.2 (containing the uncultivated species). As shown in Fig. 4, the comammox *Nitrospira* detected in both reactors belonged to clade A, which was consistent with many other results found from wastewater treatment processes (28, 50, 54, 55). However, the comammox *Nitrospira* in the LAC and HAC reactors belonged mainly to clade A.1 and clade A.2, respectively, suggesting that DO may be an important impact factor shaping the distribution of clade A.1 and clade A.2.

Low DO did not increase N₂O emission probably due to the predominance of comammox over canonical AOB. The N₂O emission factors varied on a large scale (0% to 16%) during wastewater treatment (56–59). A recent review reported that the average emission factor was 0.27% (60). As shown in Fig. 1, the average N₂O emission factors in the HAC and LAC reactors were 0.15% and 0.12%, respectively. Therefore, the N₂O emissions in the acidic reactors of the present study were low compared to the reference values. Moreover, previous studies suggested that low-DO operation could promote N₂O emission mainly by promoting nitrifier denitrification (37–39, 61). In contrast to the previous results, the long-term low-DO operation in the weakly acidic reactor (LAC reactor) did not increase N₂O emissions compared to those in the HAC reactor (Fig. 1). In addition, the N₂O emission factors in the LAC reactor did not have significant correlations ($P > 0.05$) with the operational pH, DO, or NO₂⁻-N within the experimental ranges (Fig. 6). Figure S5 also shows that these correlations were insignificant in the HAC reactor. The insignificant correlations between N₂O emission factors and DO or nitrite (Fig. 6) implied that the nitrifier denitrification pathway may not be the main reason for N₂O production in both reactors.

Both qPCR and qFISH assays (Fig. 3) determined that comammox *Nitrospira* organisms significantly outnumbered canonical AOB in both weakly acidic reactors, especially under low-DO conditions. The genomic survey, however, showed that the *norB* gene was absent in comammox *Nitrospira* genomes, suggesting a low yield of N₂O by comammox (62). A recent study based on pure culture also confirmed that comammox had a lower yield of N₂O than canonical AOB did (63). Therefore, it was speculated that the domination of the comammox *Nitrospira* was the main reason for the low emission factors under weakly acidic conditions and that the greater predominance of comammox under low-DO conditions was responsible for the slightly decreased N₂O emission.

Although the relative abundances of *nirK* genes were remarkably high, as revealed by metagenome sequencing in both reactors, those for canonical AOB were extremely low (Fig. 5). A large proportion of *nirK* genes belonged to *Nitrospira* in this study (Table S3). Previous studies have documented no activity of the encoded product, copper-containing dissimilatory nitrite reductase, in NOB-like (64) or comammox-like (51) strains. Therefore, the *nirK* gene profile also suggested that nitrifier denitrification may not be the main pathway for N₂O production in our reactors with the *Nitrospira nirK* gene dominating.

Accomplishing efficient nitrification with low N₂O emission with low DO in the weakly acidic reactor suggested a promising prospect in engineering applications. However, more studies are necessary to determine the contributions of different ammonia oxidizers to weakly acidic nitrification and explore the N₂O production mechanisms in the comammox-dominated wastewater treatment systems via transcriptomics and proteomics. Moreover, as *Nitrospira inopinata* is the only available pure-cultivation comammox, it is still unknown whether other members of the comammox *Nitrospira* group produce N₂O enzymatically.

MATERIALS AND METHODS

Membrane bioreactor operation. In this study, two lab-scale membrane bioreactors (working volume = 4.5 liters) were set up and inoculated with biomass from a previous study (65). Both reactors were fed with synthetic domestic wastewater at a constant flow rate of 14.4 liters/day. The chemical oxygen demand (COD) and NH₄⁺-N in the influent were 180 mg/liter and 48 mg/liter, respectively. Other nutrient elements were also added to the influent as previously described (66). The hydraulic retention time was 7.5 h. Both reactors were operated under weakly acidic conditions, with pH ranging from 6.3 to 6.8. With different aeration rates, the DO was controlled at above 2 mg/liter in one reactor (high-DO acidic

condition [HAC]) and at approximately 0.5 mg/liter in the other reactor (low-DO acidic condition [LAC]). During the experiment, no biomass was intentionally discharged to promote comammox growth.

The effluents NH_4^+-N , NO_2^--N , and NO_3^--N were analyzed at least once a week based on Hach test kits and a spectrophotometer (DR3900; Hach, USA). N_2O in the released air from both reactors was also analyzed from the 270th day to the 390th day with stabilized nitrification. The N_2O concentration in the released air was measured using a gas chromatograph (GC-2014 Plus; Shimadzu, Japan) equipped with an electron capture detector. The emission factor of N_2O was calculated using equation 1:

$$\eta_{\text{gas}} = \frac{r_{\text{gas}}}{m_{\text{NH}_4^+-\text{N}}} = \frac{Q_{\text{air}}(C_{\text{N}_2\text{O, gas}} - C_{\text{N}_2\text{O, air}}) \cdot M_{\text{N}_2\text{O}-\text{N}} \cdot P}{R \cdot T \cdot m_{\text{NH}_4^+-\text{N}}} \quad (1)$$

where η_{gas} is the N_2O emission factor, r_{gas} is the gaseous N_2O emission rate (grams of N/day), $m_{\text{NH}_4^+-\text{N}}$ is the NH_4^+-N load for the reactor (grams of N/day), Q_{air} is the air supply rate in the reactor (liters of air/day), $C_{\text{N}_2\text{O, gas}}$ is the measured N_2O concentration in the released air (parts per million by volume), $C_{\text{N}_2\text{O, air}}$ is the background N_2O concentration in the air used for aeration (i.e., 0.31 ppmv), $M_{\text{N}_2\text{O}-\text{N}}$ is the molar mass of N in the N_2O molecule (28 g N/mol), P is the atmospheric pressure (i.e., 1 atm), R is the gas constant (0.082 liters · atm/K · mol), and T is the gas temperature (in kelvins). The dissolved N_2O in the effluent was neglected in the estimation of the emission factor because its contribution was very low, as indicated by previous studies (67). Independent t tests were performed using SPSS 26. The difference in N_2O emission factors between high DO and low DO concentrations in the weakly acidic reactors was analyzed based on independent t test by SPSS 26. In addition, Pearson's correlation coefficients were used to evaluate the correlations between the N_2O emission factors and the nitrite concentration, pH, or DO, which was also performed using SPSS 26.

Determining the biomass nitrification rate. When the nitrification in both reactors was stabilized, batch experiments were performed to determine the biomass-specific ammonia oxidation rate (sAOR) and nitrite oxidation rate (sNOR) at pH values of 7.6, 7.0, and 6.4. A phosphate solution was used to adjust the pH. In the tests, a NH_4Cl or NaNO_2 solution was used to increase the initial ammonia or nitrite concentration to approximately 30 mg/liter, and the decrease in ammonia or nitrite concentration was then measured. During the batch tests, the DO was maintained higher than 4.0 mg/liter and the temperature was maintained at 25°C in a water batch. Additionally, the mixed-liquor suspended solids (MLSS) were measured to calculate sAOR and sNOR.

Sample collection and DNA extraction. Activated sludge samples were collected in triplicate on the 379th day. Each sample was centrifuged to remove the water, and the biomass pellets were stored at -80°C for further analysis. The DNA of each sample was extracted with the FastDNA spin kit for soil (MPBio, CA, USA) according to the manufacturer's instructions. Biomass used for qFISH assay was fixed in 4% paraformaldehyde for 2 h at 4°C . The fixed biomass was stored in 1:1 ethanol (EtOH)–phosphate-buffered saline (PBS) at -20°C until qFISH assays were performed.

qPCR and qFISH assays. qPCR assays targeting the canonical AOB ammonia monooxygenase (*amoA*) via the *amoA*-1F and *amoA*-2R primer sets (68), comammox *amoA* via the *comamoA* F/R primer set (54), *Nitrospira nxrB* via the 169f/638R primer set (43), and total bacterial 16S rRNA genes via the 1055/1392 primer set (69) were performed. All qPCR amplifications were conducted in triplicate on a CFX96 real-time detection system (Bio-Rad, USA). In the qPCR assays, all genes were tested in biological triplicate, and each reaction was performed in technical triplicate. The details of the primers, thermocycling, and reaction conditions can be found in the supporting information and Table S1 in the supplemental material. The specificity of the amplification was tested using a melt curve and agarose gel electrophoresis (see the supplemental material for more details). AOA were also tested using the Arch-*amoA*F/R primer set (70), although no products were detected in the two reactors.

qFISH was performed on the fixed biomass by following previously described methods (71), with modifications. Biomass was stained with probes targeting the comammox *Nitrospira* and β -AOB. A general bacterial probe set was used as the reference. Microscope images were acquired with a confocal laser scanning microscope (LSM800; Zeiss, Germany). Quantification was performed with daime software based on 19 fields of view for each sample (42). More details on qFISH can be found in the supplementary information and Table S4.

Functional gene sequencing. The *amoA* genes for the comammox *Nitrospira* and canonical AOB were amplified using the aforementioned primers. The amplicons of the canonical AOB *amoA* gene were sequenced at Allgene, Inc., in Beijing, China, whereas the comammox *amoA* gene amplicons were sequenced at Genewiz, Inc., in Suzhou, China. All amplicons were sequenced using the MiSeq system (Illumina, San Diego, CA, USA) with Illumina v2 chemistry (2×250 paired-end reads). Both gene amplicons were sequenced from triplicate biological samples as described above. After we filtered and combined the raw data, the sequences were clustered into operational taxonomic units (OTUs) at 97% similarity with QIIME v1.9.1 (72). OTUs with a relative abundance greater than 1% were selected for phylogenetic analysis. The procedures for sequence analysis and phylogenetic inference are detailed in the supplemental material.

Metagenome sequencing and functional analysis. Genomic DNA extracted from samples was used to construct the library for sequencing according to the manufacturer's instructions (Illumina). Sequencing was conducted using a 2×150 paired-end configuration at Genewiz, Inc., in Suzhou, China. The raw sequencing reads were trimmed using cutadapt v1.9.1 (73). The clean reads were assembled *de novo* using MEGAHIT v1.13 (74) to obtain separate assemblies. Prodigal v3.02 (75) was used to predict the genes in each sample. The genes from all samples were clustered using CD-HIT v4.8.1 (76) with default settings. To analyze the relative abundances of UniGenes in each sample, paired-end clean reads were mapped to UniGenes using SOAPaligner v2.2.1 (77) to generate read coverage information. Gene

abundance was calculated based on the number of aligned reads and then normalized to gene length using equation 2:

$$G_k = \frac{r_k}{L_k} \times \frac{1}{\sum_{i=1}^n \frac{r_i}{L_i}} \quad (2)$$

where r represents the number of reads mapped into the genes and L represents the gene's length.

The UniGene sequences were subjected to a BLAST search against a customized database for taxonomy classification. The lowest common ancestor was determined using a metagenome analyzer (MEGAN v6.4.4) (78). The abundance of a species in one sample is equal to the sum of the gene abundances annotated for the species. Diamond v0.8.15.77 (79) was used to search the protein sequences using the KEGG database. The statistical significance threshold of the sequence alignment was set at $1e^{-5}$, and the sequence alignment length was set at no less than 60% of the reference gene protein length. The matched result with the best scores was selected for annotation.

Nitrogen cycle functional genes were identified by searching the KEGG Orthology (KO) numbers (Table S2) against the KEGG annotation table. The identified NAR/NXR genes were then blasted against a curated integrative database (80) to distinguish them from each other. Stamp v2.1.3 (81) was used to assess the statistically significant differential abundances of nitrogen cycle genes between two samples using Fisher's exact test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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