Efficient Nitrification and Low-Level N$_2$O Emission in a Weakly Acidic Bioreactor at Low Dissolved-Oxygen Levels Are Due to Comammox

Deyong Li,$^{a,b}$ Fang Fang,$^c$ Guoqiang Liu$^{a,b}$

$^a$School of the Environment, Guangdong Engineering Research Center of Water Treatment Processes and Materials, Jinan University, Guangzhou, China
$^b$School of the Environment, Guangdong Key Laboratory of Environmental Pollution and Health, Jinan University, Guangzhou, China
$^c$College of the Environment and Ecology, Chongqing University, Chongqing, China

ABSTRACT Nitrification is an essential process for nutrient removal from wastewater and an important emission source of nitrous oxide (N$_2$O), which is a powerful greenhouse gas and a dominant ozone-depleting substance. In this study, nitrification and N$_2$O 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, N$_2$O 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 N$_2$O 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 N$_2$O 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 N$_2$O 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 N$_2$O. 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) 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 N$_2$O emission. These findings indicate that comammox organisms are significant for sustainable wastewater treatment, which provides an opportunity to achieve efficient nitrification with low N$_2$O 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 (N$_2$O) (4). Although operating the reactors under weakly acidic (pH 6.0 to 6.8) and low-DO (<0.5 mg/liter) conditions may reduce
the operational costs, especially for wastewater without sufficient alkalinity, incomplete nitrification and high N₂O emissions can occur (5, 6). However, if weakly acid-tolerant nitrifiers with high apparent oxygen affinity and low N₂O yield are enriched in nitrifying bioreactors, efficient nitrification, low N₂O 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 (HNO₂), 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 (Kₘ) 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 (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 Kₘ 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.

N₂O can be biologically generated through the incomplete oxidation of hydroxylamine to nitrite (NO₂⁻) by AOB (31). Nitrifier denitrification is another important pathway to produce N₂O, where NO₂⁻ is subsequently reduced to nitric oxide (NO) and N₂O (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 N₂O 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 N₂O via nitrifier denitrification, as with canonical AOB (40). If comammox are...
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...
condition were analyzed to test the hypothesis and explore the correlations of comam- 
mox with nitrification efficiency and \( \text{N}_2\text{O} \) production.

**RESULTS**

**Nitrification performance and \( \text{N}_2\text{O} \) emission.** As shown in Fig. 1a and b, the two strongly 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 \((n = 32, \ P = 0.46)\).

As shown in Fig. 1, the \( \text{N}_2\text{O} \) emission factor in the HAC reactor ranged from 0.09% to 0.40%, with an average of 0.15% ± 0.08% \((n = 28)\), while it ranged from 0.04% to 0.34%, with an average of 0.12% ± 0.08% \((n = 34)\), in the LAC reactor. In contrast to the results of previous studies which reported that low DO induced a higher \( \text{N}_2\text{O} \) emission factor.
(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 qualitative 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
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 from 1.0% to 5.0%.
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 *Nitrosospira* 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, 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, amoB, and amoC 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
the HAC reactor, as shown in Fig. 5, which indicated a lower N₂O 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 μM) 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

![Correlations of pH, DO concentration, and NO₂⁻-N concentration against the N₂O emission factor in the low-DO acidic reactor.](image)
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 NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N were analyzed at least once a week based on Hach test kits and a spectrophotometer (DR3900; Hach, USA). N₂O in the released air from both reactors was also analyzed from the 270th day to the 390th day with stabilized nitrification. The N₂O 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 N₂O was calculated using equation 1:

$$\eta_{\text{gas}} = \frac{Q_{\text{air}} (C_{N_{2}O, \text{gas}} - C_{N_{2}O, \text{air}}) \cdot M_{N_{2}O} \cdot \Delta P}{R \cdot T \cdot m_{NH_{4}^{+} \text{-N}} \cdot \Delta H}$$

where $\eta_{\text{gas}}$ is the N₂O emission factor, $Q_{\text{air}}$ is the gaseous N₂O emission rate (grams of N/day), $m_{NH_{4}^{+} \text{-N}}$ is the NH₄⁺-N load for the reactor (grams of N/day), $Q_{\text{air}}$ is the air supply rate in the reactor (liters of air/day), $C_{N_{2}O, \text{gas}}$ is the measured N₂O concentration in the released air (parts per million by volume), $C_{N_{2}O, \text{air}}$ is the background N₂O concentration in the air used for aeration (i.e., 0.31 ppmv), $M_{N_{2}O}$ is the molar mass of N in the N₂O 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 N₂O 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 N₂O 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 N₂O 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 NH₄Cl or NaNO₂ 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 bath. Additionally, the mixed-liquid 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 −20°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 monoxygenase (amoA) via the amoA-1F and amoA-2R primer sets (68), comammox amoA via the comamAO 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 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 daimie 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 QIME 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:

$$G_i = \frac{r_i}{L_i} \times \frac{1}{\sum_{i=1}^n L_i}$$

(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.

ACKNOWLEDGMENTS

This research was supported by the Chinese National Natural Science Foundation (grant 51608230) and grants from the Science and Technology Program of Guangdong Province (2018A050506042) and the Special Support Plan of Guangdong Province (2019TQ05L560).

REFERENCES


