Long-Term Low DO Enriches and Shifts Nitrifier Community in Activated Sludge

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Long-Term Low DO Enriches and Shifts Nitrifier Community in Activated Sludge

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Ⓢ *Supporting Information*

ABSTRACT: In the activated sludge process, reducing the operational dissolved oxygen (DO) concentration can improve oxygen transfer efficiency, thereby reducing energy use. The low DO, however, may result in incomplete nitrification. This research investigated the long-term effect of low DO on the nitrification performance of activated sludge. Results indicated that, for reactors with 10 and 40 day solids retention times (SRTs), complete nitrification was accomplished after a long-term operation with a DO of 0.37 and 0.16 mg/L, respectively. Under long-term low DO conditions, nitrite oxidizing bacteria (NOB) became a better oxygen competitor than ammonia oxidizing bacteria (AOB) and, as a result, no nitrite accumulated. Real-time PCR assays indicated that the long-term low DO enriched both AOB and NOB in activated sludge, increasing the sludge nitrification capacity and diminishing the adverse effect of low DO on the overall nitrification performance. The increase in the population size of nitrifiers was likely resulted from the reduced nitrifier endogenous decay rate by a low DO. Under long-term low DO conditions, *Nitrosomonas europaea/eutropha* remained as the dominant AOB, whereas the number of *Nitrospira*-like NOB became much greater than *Nitrobacter*-like NOB, especially for the 40 day SRT sludge. The enrichment and shift of the nitrifier community reduced the adverse effect of low DO on nitrification; therefore, low DO operation of a complete nitrification process is feasible.
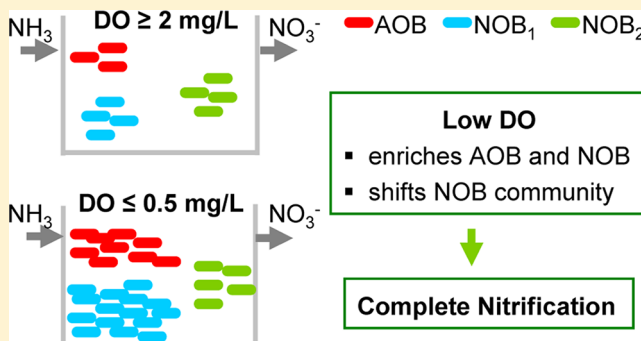
1. INTRODUCTION

With increasing concerns about the fossil fuel energy and climate change, energy conservation is emerging as an essential topic in wastewater treatment. For a typical wastewater treatment plant, the energy used for aeration accounted for approximately 50% of total energy consumption.[1] The aeration need is mainly dependent on the actual oxygen demand and oxygen transfer efficiency. The actual oxygen demand is determined by the amount of contaminants oxidized and biomass produced. The oxygen transfer efficiency is associated with aeration devices, operational dissolved oxygen (DO) concentration, and mixed liquor suspended solids (MLSS) concentration.[2] If the aeration tank is operated with a DO of 0.5 mg/L rather than 2.0 mg/L, the overall oxygen transfer efficiency will increase by 16%,[2]—nearly 10% of total energy saving for the entire treatment plant. Moreover, a low DO concentration can increase sludge production.[3] As a result, the actual oxygen demand may decrease. Besides energy savings, a low operational DO can help promote nitrogen removal and oxygen recovery through simultaneous nitrification and denitrification.

Nitrifiers are less competitive in utilizing DO than heterotrophic bacteria in activated sludge.[2] Therefore, the operational DO for an advanced treatment plant is usually determined by the nitrification needs. When the DO is below 2.0 mg/L, the growth rate of nitrifiers can be inhibited[2,4] and, as a result, incomplete nitrification may occur. On the other hand, a low DO concentration may reduce the endogenous decay rate of nitrifiers. Previously, the effect of DO on nitrification was generally studied using short-term batch tests.[4−6] In these studies, the effect of low DO on nitrifier population change induced by the reduced endogenous decay rate was ignored. Under long-term low DO conditions, however, the endogenous decay could play an important role in the nitrifier population. If a low DO can reduce the endogenous decay rate, the relative nitrifier biomass concentration will increase. A greater nitrifier concentration can reduce the adverse effect of the low DO on overall nitrification performance.

Nitrification is completed in two steps: ammonia oxidized into nitrite by ammonia-oxidizing bacteria (AOB), and nitrite oxidized into nitrate by nitrite-oxidizing bacteria (NOB).[2] Generally, NOB were thought to be more sensitive to low DO concentrations than AOB were.[7−11] In activated sludge, *Nitrosomonas* and *Nitrosospira* were dominant AOB, whereas *Nitrobacter* and *Nitrospira* were dominant NOB.[2,12−15] *Nitro-*

somonas-like AOB and *Nitrobacter*-like NOB were suggested to be "*r*" strategists, whereas *Nitrosospira*-like AOB and *Nitrospira*-like NOB were thought to be "*K*" strategists.[14,16,17] In addition, the sublineages in AOB and NOB groups could have significantly different oxygen affinity.[6] Due to the kinetic diversity, the nitrifying bacteria community in activated sludge may shift as a result of DO change.[4,6] The nitrifiers with a higher oxygen affinity may be selected under long-term low DO conditions.

The objectives of this research were to determine the long-term effect of low DO on the nitrification performance of activated sludge and the nitrifying bacterial community. This information is critical for developing novel control technologies to significantly reduce the operation cost of wastewater treatment plants.

2. MATERIALS AND METHODS

2.1. Reactor Setup, Operation, and Monitoring. Two same-size bench scale complete-mix reactors (31.5 L) were set up and fed continuously with synthetic wastewater to achieve a hydraulic retention time (HRT) of approximately 12 h. The chemical oxygen demand (COD) and the ammonia concentrations in the influent were 180 mg/L and 48 mg-N/L, respectively. The COD was provided with glucose, whereas ammonia was provided with ammonium carbonate. In addition, trace elements were provided in the influent and the details are described in the Supporting Information (SI). Tap water with soluble Ca and Mg concentrations greater than 20 mg/L was used as the solvent. The pH in the aeration tank ranged from 7.0 to 7.5, controlled by a buffer containing K_2HPO_4 and Na_2CO_3. Operational temperature was approximately 20 °C. The two reactors were operated with 10 and 40 day solids retention times (SRTs), respectively. The seed activated sludge was collected from an oxidation ditch in the Southeast Wastewater Treatment Plant in Rolla, Missouri. The nitrification performance in the 10 day SRT reactor was tested with five different DO concentrations (approximately 4, 2, 1, 0.4, and 0.2 mg/L), and the nitrification performance in the 40 day SRT reactor was tested with three different DO concentrations (approximately 4, 0.4, and 0.2 mg/L).

During the experiment, the operational parameters for both reactors, including DO, inflow rate, pH, temperature, aeration intensity, oxidation−reduction potential, and MLSS concentration were monitored regularly. The concentrations of effluent ammonia, nitrite, and nitrate were normally tested three times a week. When the system reached a steady-state with each DO concentration, the effluent biochemical oxygen demand (BOD_5) concentration was measured. The analysis methods for MLSS, BOD_5, pH, DO, ammonia, nitrate, and nitrite are described in the SI.

After operating for at least two SRTs under each condition, the biomass maximum ammonia oxidation rate (AOR_m) and the maximum nitrite oxidation rate (NOR_m) were measured two to three times per week using a respirometer. These measurements lasted for at least one SRT, until the biomass had a consistent AOR_m and NOR_m. Conventional batch kinetics tests were also conducted to determine biomass AOR_m and NOR_m when the system stabilized under each condition to confirm the values determined through the respirometer. The experimental protocols for batch respirometric tests and conventional batch kinetic tests were described in our previous study[18] and also in the SI.

Once reaching the steady-state of each operational condition, a sludge sample (2 mL) was taken and centrifuged at 13 000 rpm for 2 min to separate water and solids. The sludge pellet was persevered under −80 °C for DNA extraction.

2.2. DNA Extraction. Ultraclean soil isolation kits (MoBio Laboratories, Solana Beach, CA) were used to isolate the DNA from sludge pellets by following the manufacturer's protocol. The DNA samples were stored at −20 °C for subsequent assays. We confirmed the intact DNA on 1% agarose gel electrophoresis.

2.3. Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis. T-RFLP was applied to analyze the nitrifying bacterial community of the sludge samples cultivated with various DO concentrations based on the 16S rRNA gene of AOB and NOB.[19] The detailed protocol was described in a previous study.[20] A low concentration of DNA from nitrifiers could result in a low yield of PCR products and then poor T-RFLP electropherograms. Therefore, the DNA from the 16S rRNA gene of AOB and NOB was amplified by nested PCR using universal amplification to increase the initial DNA template concentration, followed by specific amplifications of the 16S rRNA genes of AOB and NOB. The primers used in the universal and specific amplifications and their PCR program were shown in Table S1 and Table S2 (SI), respectively. After specific amplification, the PCR products were purified and digested with MspI restriction endouclease at 37 °C for 3 h (Promega, Madison, WI). The QIAquick purification kit (Qiagen Inc., Valencia, CA) was used for the PCR product purification. Digested PCR products were analyzed using an Applied Biosystem 3130 genetic analyzer and the peak results were analyzed using Peak Scanner software version 1.0 (Applied Biosystems, Foster City, CA). The fragment sizes were compared to the expected standard fragment sizes.[20]

2.4. Real-Time PCR Analysis. Real-time PCR assays were conducted on all DNA samples to determine the changes in the nitrifying bacteria population size as a function of DO concentration. Three independent real-time PCR assays were conducted to quantify total 16S rRNA of AOB, *Nitrobacter*-like NOB, and *Nitrospira*-like NOB in each DNA sample. Each reaction tube was separately loaded with 2 μL of template DNA, 6 pmol of the forward and reverse primers, 10 μL of power SYBR green master mix (No 4367659, Invitrogen, NY), and PCR grade water, for a final volume of 20 μL. Primers with their sequences and real-time PCR programs for each assay were shown in SI Tables S1 and S2, respectively. For AOB 16S rRNA, two forward primers (CTO 189A/B and CTO 189C) were used, as described previously by Hermansson and Lindgren.[21] All real-time PCR assays were performed in triplicate for each sample and included standards and control reactions without a DNA template. The specificity of each PCR assay was confirmed using melting curve analysis and agarose gel electrophoresis. Standards were made using serially diluted plasmid DNA with $10^2−10^8$ copies/μL. The preparation of plasmid DNA followed the protocol made by Dr. Zhiqiang Hu's research group at the University of Missouri−Columbia. All primers and probes were produced by Integrated DNA Technologies, Inc. (Coralville, IA).

Recent findings indicated that ammonia-oxidizing archaea (AOA) might be present in the activated sludge processes.[15,22,23] The primers, Arch-amoAF and Arch-amoAR,[24] were used to amplify the AOA amoA gene in our DNA samples. However, no DNA band at 635 bp was found on 1.5%
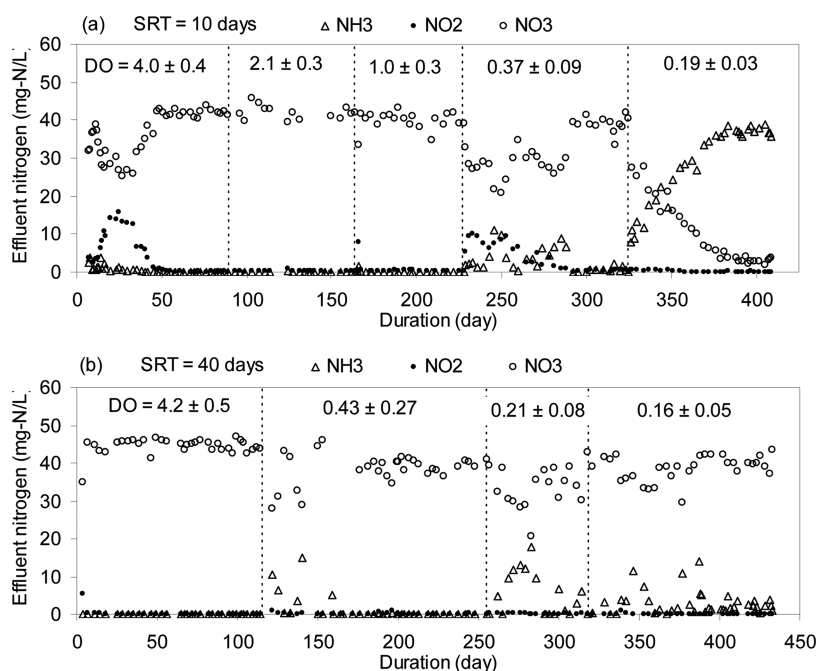
Figure 1. Effluent ammonia, nitrite, and nitrate concentrations under different DO concentrations in the (a) 10 day SRT and (b) 40 day SRT reactors. The unit for the values in the above Figure is mg/L.

agarose gel electrophoresis, indicating that AOA were likely not present in our reactors.

3. RESULTS AND DISCUSSION

3.1. Effluent Quality. Figure 1 shows the nitrification performance for the 10 and 40 day SRT reactors. In the 10 day SRT reactor, the decrease of DO from 4.0 to 2.1 mg/L did not change the effluent quality, and the ammonia and nitrite concentrations maintained at less than 0.1 and 0.2 mg-N/L in the steady-state, respectively (see Figure 1(a)). However, on the second day after decreasing the DO to 1 mg/L, the effluent ammonia and the effluent nitrite concentrations increased to approximately 1.5 and 8 mg-N/L, respectively. Starting from the third day, they were decreased to nearly 0.1 and 1.0 mg-N/L, respectively. Finally, the stabilized effluent ammonia and nitrite concentrations were lower than 0.1 and 0.5 mg-N/L, respectively.

After further reducing the DO to 0.37 mg/L, the effluent ammonia and the effluent nitrite concentrations immediately increased to 1 and 9 mg-N/L, respectively (Figure 1(a)), indicating that incomplete nitrification had occurred. In the first 2 weeks after reducing the DO to 0.37 mg/L, the effluent ammonia concentration increased to approximately 10 mg-N/L, whereas the effluent nitrite concentration gradually decreased. After 2 months (with a DO of 0.37 mg/L), both the effluent ammonia and the effluent nitrite concentrations decreased to below 1 mg-N/L, suggesting that complete nitrification had been recovered. In the steady-state, the average effluent ammonia and nitrite concentrations were 0.85 and 0.46 mg-N/L, respectively. After further reducing the DO to 0.19 mg/L, the effluent ammonia concentration immediately increased to about 8 mg-N/L, and continued to increase (Figure 1(a)). The effluent nitrite, however, did not accumulate. After operating for 50 days at a DO near 0.19 mg/L, the effluent ammonia concentration increased to 37 mg-N/L, with only 10% of ammonia removal. This finding

indicated that the 10 day SRT reactor could not achieve complete nitrification with this low DO.

With a DO ≥ 4.0 mg/L in the 40 day SRT reactor, complete nitrification was quickly achieved (Figure 1(b)). Within the first 3 weeks following the reduction of DO to 0.43 mg/L, the effluent ammonia accumulated. However, complete nitrification was recovered 3 weeks after decreasing the DO to 0.43 mg/L. When the DO was further reduced to 0.21 mg/L, the effluent ammonia concentration increased again. After operating for approximately 4 weeks under a DO of 0.21 mg/L, the effluent ammonia concentration decreased to and maintained at approximately 2 mg-N/L. It was not until the system reached the steady-state at a DO of 0.21 mg/L that the DO was reduced again to 0.16 mg/L to determine the minimum operational DO that could achieve complete nitrification with this particular SRT. In the steady-state with a DO of 0.16 mg/L, the effluent ammonia concentration was approximately 2 mg-N/L, indicating that complete nitrification had almost been achieved. Even with a DO of 0.16 mg/L, the average effluent nitrite concentration was still less than 0.1 mg-N/L.

As expected, the steady-state effluent BOD concentrations in both reactors under various DO concentrations were less than 5 mg/L (data not shown). This indicated that, the experimental low DO concentrations did not impact organic removal significantly.

In reactors with 10 and 40 day SRTs, complete nitrification was almost achieved with a DO level of 0.37 and 0.16 mg/L, respectively. Please note that, for both reactors, incomplete nitrification occurred during the initial period after reducing the DO to below 0.5 mg/L. Complete nitrification, however, eventually recovered after several weeks with the low DO concentration. In these periods, no changes were made to any of the operational conditions, including ammonia loading, temperature, pH, and SRT. There are two possible explanations for the recovery of complete nitrification with a long-term low DO. (a) Both the AOB and the NOB biomass concentrations could have increased, thereby reducing the adverse impact of

low DO on nitrification performance. (b) Nitrifiers could have become less sensitive to low DO concentrations due to a community shift.[6,25]

Previous studies indicated that nitrite oxidation was more sensitive to low DO concentrations than ammonia oxidation[2,7,9,26] and, therefore, when the DO was low, the effluent nitrite would accumulate. In the 10 day SRT reactor, nitrite temporarily accumulated in the initial period when the DO was reduced to 1.0 mg/L, and to 0.37 mg/L. Nitrite accumulation indicated that, in those situations, the nitrite oxidation rate was smaller than the ammonia oxidation rate. These observations supported the statement that nitrite oxidation was more sensitive to low DO concentrations than ammonia oxidation was. After operating for approximately 100 days with a DO of 0.37 mg/L, however, a further reduction of DO (from 0.37 to 0.19 mg/L) resulted in considerable ammonia accumulation but not nitrite accumulation. These findings indicated that nitrite oxidation had become less sensitive to low DO concentrations than ammonia oxidation had.

3.2. Biomass Nitrification Rate.
Figure 2 shows the AOR_m and NOR_m for the sludge cultivated with different SRTs
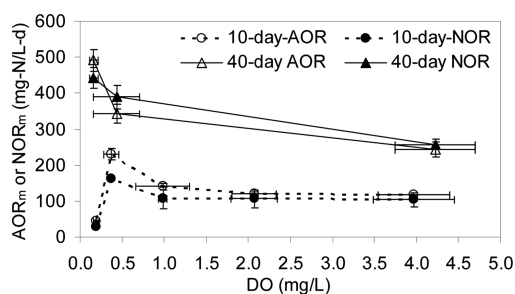

Figure 2. The AOR_m and NOR_m for the activated sludge cultivated with different DO concentrations (mean ± stdev).

and DO levels after reaching steady-state. The low DO had an adverse effect on AOB and NOB growth rates.[4−6] When the reactor was operated under a low DO (≤0.5 mg/L) for a long period, however, both the AOR_m and the NOR_m increased significantly for both sludges (see Figure 2). When the DO was reduced to ≤0.2 mg/L, both the AOR_m and the NOR_m kept increasing for the 40 day SRT sludge, while they dropped dramatically for the 10 day SRT sludge. Please note that, complete nitrification was achieved in the 40 day SRT reactor with a DO of 0.16 mg/L. In the 10 day SRT reactor with a DO of 0.19 mg/L, only nearly 10% of ammonia was removed (assimilated and oxidized). As less ammonia was oxidized, the yield of both AOB and NOB biomass decreased. Therefore, the nitrification capacity for the 10 day SRT sludge, cultivated with a DO of 0.19 mg/L, decreased significantly. For all other conditions, the recovery of complete nitrification with low DO concentrations (Figure 1) was likely due to the sludge having increased its nitrification capacity (Figure 2), which reduced the adverse effect of low DO on the nitrification rate.

The increase in the sludge nitrification capacity under long-term low DO conditions likely resulted from the enrichment of both AOB and NOB. Both Hanaki et al.[27] and Bellucci et al.[28] also found that the sludge, cultivated with a DO ≤ 0.5 mg/L, had a greater ammonia oxidation capacity. They did not report, however, if the nitrite oxidation capacity increased. Bellucci et al.[28] thought that the increase in the ammonia oxidation capacity resulted from a high yield of AOB under low DO

conditions. Moreover, Bellucci et al.[28] indicated that the high yield of AOB could be a result of mixotrophic metabolism. Some AOB species could utilize organic compounds as a carbon source under low DO conditions, which might reduce the energy needed to convert CO_2 to cellular carbon in an autotrophic metabolism system, thereby increasing the cell yield coefficient of AOB. However, these hypotheses were not validated. Indeed, *Nitrosomonas europaea*-like AOB were able to utilize a few organic matters as the carbon source, for example, fructose, pyruvate, and acetate.[29−31] In our experiments, glucose was the sole organic carbon in the influent. Hommes et al.,[30] however, indicated that the glucose could not be utilized as a carbon source in the chemolithoheterotrophic growth of *Nitrosomonas europaea* (ammonia as an energy source, organic as a carbon source, and oxygen as an oxidant). This finding suggested that the chemolithoheterotrophic growth of *Nitrosomonas europaea* could not occur in our reactors. In contrast to the view of Bellucci et al., Hommes et al.[30] also found that *Nitrosomonas europaea*, using either fructose or pyruvate as an organic carbon source, had a smaller growth rate or yield coefficient than using CO_2. In this case, even if *Nitrosomonas europaea* had utilized alternative organics (e.g., metabolism byproducts) as a carbon source, a higher yield for AOB cells could not be observed. Therefore, the increased nitrification capacity for the sludge cultivated under a low DO was not a result of mixotrophic metabolism and additional mechanisms must exist.

The observed yield coefficient of nitrifiers was not only determined by their synthetics yield coefficient but also by the endogenous decay rate and sludge age. The endogenous decay accounts for the loss in cell biomass due to oxidation of internal storage products for energy for cell maintenance, cell death, and predation.[2] In an engineering context, decay should adequately describe the loss of biomass (=loss of activity).[32] The synthetic yield coefficient of nitrifiers was independent of DO.[2] This meant that, when the same amount of ammonia or nitrite was oxidized, the synthetic yield of AOB or NOB would be the same, regardless of DO concentrations. If the sludge age maintained constant, and if the low DO had inhibited the endogenous decay rate of AOB and NOB, then the loss of AOB and NOB due to decay would decrease under low DO conditions. Thus, compared to high DO conditions, the observed yield coefficient of AOB and NOB would increase. In our 10 and 40 day SRT reactors, complete nitrification was almost achieved with a DO of 0.37 and 0.16 mg/L, respectively, suggesting that the synthetic yields of AOB and NOB under both SRTs did not change compared to those with a high DO concentration. The low DO concentrations, however, could reduce the nitrifier biomass loss caused by the endogenous decay. Thus, the biomass concentrations of both AOB and NOB were expected to increase under low DO conditions, thereby increasing sludge nitrification capacity. Munz et al.[33] indicated that a low DO could reduce the endogenous decay rate of AOB biomass under starvation conditions. Please note that the increase in AOR_m and the NOR_m for the sludge cultivated under a low DO could also be caused by a shift of the nitrifier community. For example, a shift from "r" strategist AOB to a "K" strategist AOB could increase AOR_m significantly with the same AOB biomass concentration.

3.3. Short-Term Effect of DO on Nitrification Rate.
Figure 1 indicates, under any tested low DO concentrations, both reactors did not accumulate nitrite (e.g., the effluent nitrite < ammonia). Therefore, we hypothesized that NOB from the

sludge cultivated with a long-term low DO are more competitive than AOB. To confirm this hypothesis, batch tests were conducted to determine the short-term effect of DO on ammonia and nitrite oxidization rates for 40 day SRT sludges cultivated with a high DO and a low DO, shown in Figure 3.
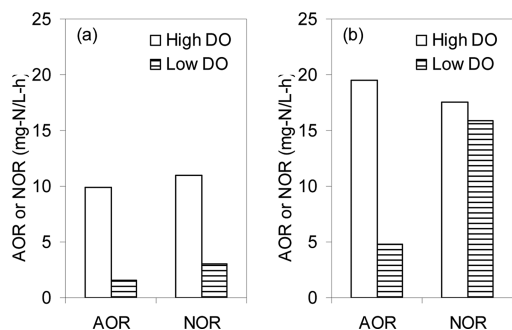

Figure 3. (a) AOR and NOR under a high DO (≥4 mg/L) and a low DO (0.2−0.3 mg/L) for the 40 day SRT sludge cultivated with a DO of 4.2 mg/L; (b) AOR or NOR under a high DO (≥4 mg/L) and a low DO (0.2−0.3 mg/L) for the 40 day SRT sludge cultivated with a DO of 0.16 mg/L.

For the sludge cultivated with a high DO of 4.2 mg/L (Figure 3(a)), a low DO (DO = 0.2−0.3 mg/L) caused an 84% and a 72% inhibition in the AOR and NOR, respectively, indicating that a low DO greatly inhibited both ammonia and nitrite oxidation in this sludge sample. For the sludge cultivated with a DO of 0.16 mg/L (Figure 3(b)), a low DO also greatly inhibited the AOR (inhibition rate = 75%), but only slightly inhibited the NOR (10% reduction). The inhibition of low DO on the NOR in the sludge, cultivated with a low DO, was much smaller than that cultivated with a high DO, indicating that the NOB community had become less sensitive to the low DO concentrations. As a result, no nitrite accumulated when the DO was ≤0.2 mg/L. The sublineages in the group of NOB could have different oxygen affinity and the ones with a high oxygen affinity could be enriched gradually in the low DO systems. After a long-term with a low DO, the entire group of NOB became less sensitive to low DO concentrations, thereby helping nitrite oxidation under low DO conditions. However, the inhibition of low DO on the AOR in the both sludges (cultivated with a high DO and a low DO) was approximately the same, suggesting that long-term low DO might not shift the AOB community significantly.

3.4. Nitrifying Bacterial Communities. The nitrifying bacterial communities in the sludge, cultivated with different SRTs and DO levels, were determined using T-RFLP specifically designed for the identification of AOB and NOB with signature terminal fragment lengths.[19] Figure 4 shows the electropherograms of AOB, *Nitrobacter*-like NOB, and *Nitrospira*-like NOB. As exhibited in Figure 4(a), all samples showed a dominant peak at 161 bp, a signature peak of Group 1 *Nitrosomonas europaea/eutropha* lineage and Group 4 *Nitrosomonas marina* lineage.[20] In our study, the influent was freshwater, so that the marine AOB species in *Nitrosomonas marina* lineage were not relevant. Thus, *Nitrosomonas europaea/eutropha* were dominant under all tested conditions, supporting the AOR result (Figure 3) that the low DO had similar impact on AOR for sludges cultivated with both high DO and low DO concentrations. A small peak at 272 bp was also detected in all

of the samples, which represented the potential presence of Group 1, 2, 3, or 5. We also detected a small peak at 101 bp in some samples, representing the presence of *Nitrosospira*-like AOB.

The DO was an important factor for the AOB community.[4,6,13,34] Previous reports on the DO effect, however, were controversial. Under low DO conditions, Li et al.[13] found that *Nitrosospira* outcompeted *Nitrosomonas*, while other studies[4,34,35] found that *Nitrosomonas* were the dominant AOB. Gieseke et al.[34] reported that *Nitrosomonas oligotropha*-like AOB had a high oxygen affinity. Park and Noguera,[6] however, found that a strain of *Nitrosomonas oligotropha*-like AOB (NL7) had a lower oxygen affinity (K_{DO} = 1.22 mg/L) than *Nitrosomonas europaea*-like AOB (ML1) (K_{DO} = 0.24 mg/L). In our study, *Nitrosomonas europaea/eutropha*-like AOB were the prevalent AOB in all of the samples.

Figure 4(b) presents a prominent peak at 136 bp, which indicates that *Nitrobacter*-like NOB were present in all of the samples. There were some unexpected peaks, which could be a result of an incomplete digestion, uncharacterized *Nitrobacter* species, or imperfectly matched primers.[20] Figure 4(c) illustrates a dominant peak at 272 bp in all of the samples and a high peak at 261 bp in most samples, indicating the presence of *Nitrospira*-like NOB. A high peak at 261 bp occurred in the samples with a DO ≥ 2 mg/L. At a DO ≤ 0.5 mg/L, however, this peak (261 bp) disappeared in the 10 day SRT sludge and its intensity decreased significantly in the 40 day SRT sludge. This change suggests that some sublineages in the group of *Nitrospira* could not survive well under low DO conditions. Unfortunately, T-RFLP assays using the 16S rRNA gene could not differentiate the sublineages in the *Nitrospira* group.

3.5. Real-Time PCR Analysis. As previously discussed, the sludge cultivated with low DO concentrations (≤0.5 mg/L) had both a higher AOR_m and NOR_m (Figure 2), likely due to the enrichment of both AOB and NOB. To confirm this hypothesis, the 16S rRNA gene copies for AOB, *Nitrobacter*-like NOB, and *Nitrospira*-like NOB in all of the sludge samples were quantified using real-time PCR assays, shown in Figure 5.

When the DO was ≥1 mg/L, the average number of AOB was approximately 1.4×10^{11} copy/L-sludge in the 10 day SRT reactor and 1.8×10^{11} in the 40 day SRT reactor (see Figure 5). With a DO of approximately 0.4 mg/L, the average number of AOB increased to 2.8×10^{11} and 3.0×10^{11} copy/L-sludge in the 10 and 40 day SRT reactors, respectively, suggesting that the population size of AOB was almost doubled as a result of low DO. Figure 5 also indicates that both *Nitrobacter* and *Nitrospira* coexisted under all DO concentrations, consistent with the results from T-RFLP assays. When the DO was reduced from 4.0 to 0.37 mg/L in the 10 day SRT reactor, the number of *Nitrobacter* increased from 5.6×10^9 to 2.5×10^{10} copy/L-sludge and the number of *Nitrospira* increased from 3.4×10^{10} to 1.2×10^{11} copy/L-sludge. In the 40 day SRT reactor, when the DO was reduced from 4.2 to 0.43 mg/L, the number of *Nitrobacter* did not change, whereas the number of *Nitrospira* increased from 1.1×10^{11} to 3.6×10^{11} copy/L-sludge. With a further reduction of DO to 0.16 mg/L, the number of *Nitrospira* significantly increased to 1.0×10^{12} copy/L-sludge, whereas the number of *Nitrobacter* increased to a lesser extent.

More AOB and NOB were present in the sludge cultivated with low DO concentrations than that cultivated with high DO levels (Figure 5). This finding supports the hypothesis that the elevated nitrification capacity (Figure 3) for the sludge
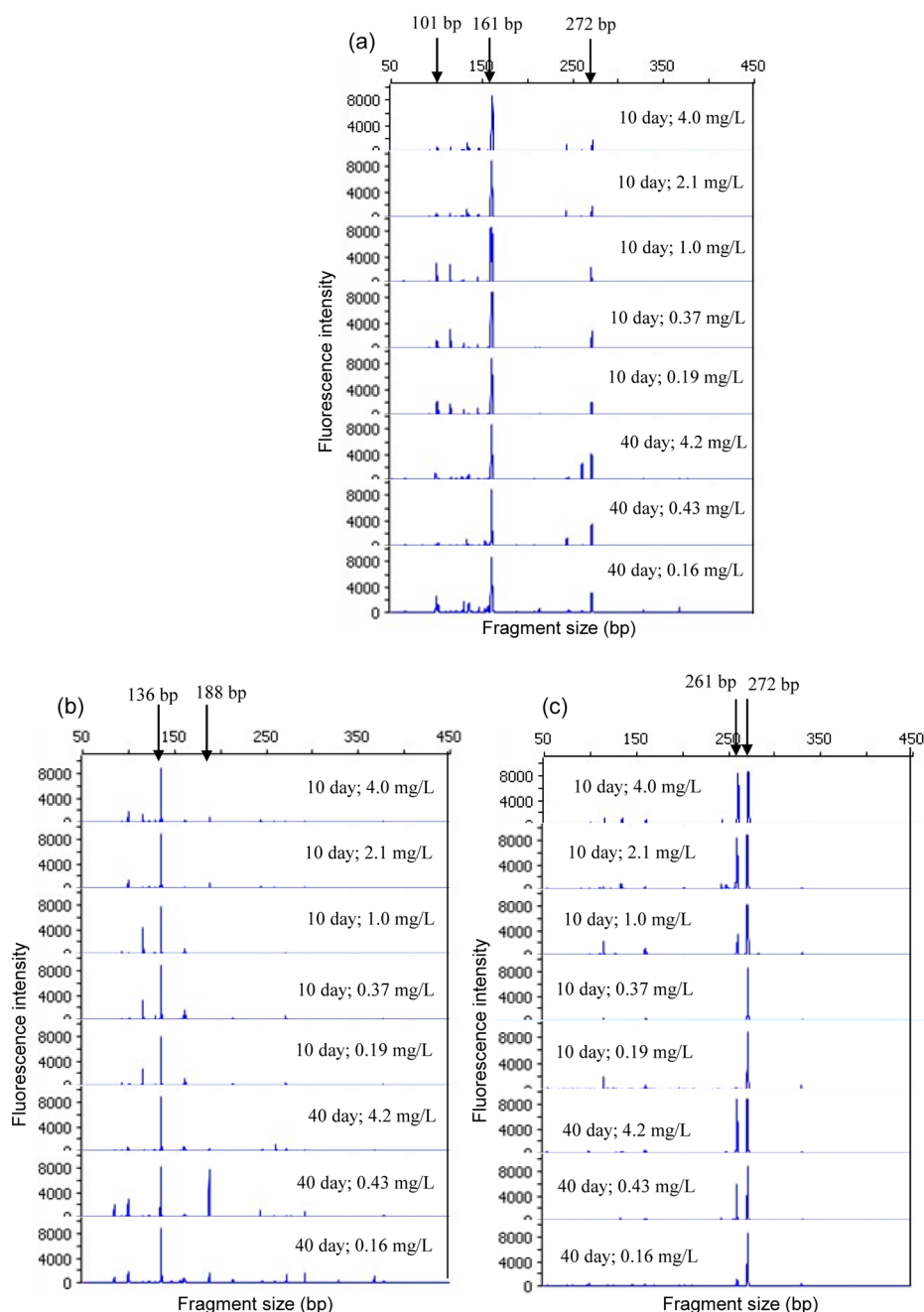
Figure 4. T-RFLP profiles of (a) AOB, (b) *Nitrobacter*-like NOB, and (c) *Nitrospira*-like NOB in the sludge cultivated with different SRTs and different DO levels.

cultivated with low DO concentrations was primarily due to the enrichment of nitrifiers. Furthermore, the increased nitrifier population size confirmed that a low DO had inhibited the endogenous decay rate of nitrifiers. When the endogenous decay rate was slowed down by a low DO, the concentration of nitrifiers in the system would increase gradually if complete nitrification was still achieved. The increased nitrifier population size could reduce the adverse effect of low DO on the overall nitrification performance. As a result, complete nitrification was recovered after operating for a long time with a low DO concentration (Figure 1).

As previously discussed, NOB had become less sensitive to low DO concentrations than AOB after a long-term cultivation with a low DO concentration. The number of *Nitrospira* increased considerably in both reactors when the DO was ≤0.5

mg/L (Figure 5). The number of *Nitrobacter*, however, increased only in the 10 day SRT reactor and changed insignificantly in the 40 day SRT reactor. With a DO ≤ 0.5 mg/L, the number of *Nitrospira* was approximately 3−10 times and 2 orders of magnitude greater than *Nitrobacter* in the 10 and 40 day SRT reactors, respectively. Even assuming that *Nitrobacter* were approximately 10 times more active than *Nitrospira* when DO was unlimited,[17,36] the actual role played by *Nitrospira* in nitrite oxidation in the 40 day SRT reactor was still greater than 90%. Therefore, it seemed that *Nitrospira* were a better oxygen competitor than *Nitrobacter*, which made nitrite oxidation less sensitive to low DO concentrations than ammonia oxidation did under long-term low DO conditions. Blackburne et al.,[36] however, reported that the pure cultures of *Nitrobacter* and *Nitrospira* had similar oxygen affinity (K_{DO} = 0.54 mg/L). In
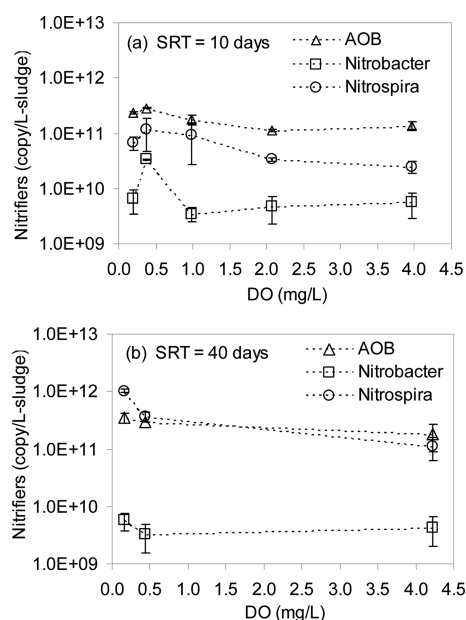
Figure 5. Copies per liter of the16S rRNA gene of AOB, *Nitrobacter*-like NOB and *Nitrospira*-like NOB in the sludge cultivated with different DO concentrations in the (a) 10 day SRT reactor and (b) 40 day SRT reactor (mean ± stdev).

this case, it was hard to explain why NOB became less sensitive to low DO concentrations than AOB. *Nitrospira* consisted of at least four distinct sublineages.[37] Possibly, the sublineages in *Nitrospira* that had a higher oxygen affinity were enriched under long-term low DO conditions. The results of the T-RFLP assay of *Nitrospira* (Figure 4(c)) seemed to support this explanation. As shown in Figure 4(c), the intensity for the peak at 261s decreased significantly in sludge samples cultivated under low DO conditions, indicating that the *Nitrospira* community had shifted. Another possibility was that the same type of *Nitropisra* was enriched under long-term low DO conditions, but their oxygen affinity increased.[25]

Both the long SRTs and the low nitrite concentrations would favor the competition of *Nitrospira*, whereas *Nitrobacter* were more competitive with short SRTs and high nitrite concentrations.[8,17,38] Therefore, beside the low DO concentrations, the long SRT and the low nitrite concentration also helped *Nitrospira* to outcompete *Nitrobacter* in the 40 day SRT with low DO concentrations.

Primer specificity was a potential problem for real-time PCR assays. Closely related non-AOB species might be hybridized in the real-time PCR assays for AOB.[39] In our study, the maximum nitrification rate for the biomass cultivated with a low DO was almost doubled (Figure 2); the population size of AOB was also doubled as a result of low DO (Figure 5); *Nitrosomonas europaea/eutropha*-like AOB were the dominant AOB under high and low DO conditions (Figure 4). All these data were consistent with each other. Therefore, even if some non-AOB species were counted, the increase trend for AOB population size, as indicated by real-time PCR, was still dependable. The primer design for the *Nitrospira* was previously demonstrated to be specific for *Nitrospira*.[40] The real-time PCR design for *Nitrobacter* was first described by Graham et al., 2007[41] and was used in many studies.[42−47] In our study, the detected number of *Nitrobacter* was much lower than that of *Nitrospira*. Therefore, even if some non-*Nitrobacter*

species were detected, the conclusions related to NOB were still reliable.

Under long-term low DO conditions, *Nitrosomonas europaea/eutropha* remained as the dominant AOB (Figure 4(a)); the number of AOB was almost doubled (Figure 5). Therefore, the recovery of complete ammonia oxidation (Figure 1) and the increase in the sludge ammonia oxidation capacity (Figure 2) with a low DO in both reactors were mainly due to AOB enrichment. Similar with AOB, the number of NOB increased significantly as a result of low DO, primarily accounting for the recovery of complete nitrite oxidation and the increase in the sludge nitrite oxidation capacity. The enrichment of both AOB and NOB likely resulted from the inhibition of nitrifier endogenous decay by a low DO, instead of mixotrophic metabolism. Unlike AOB, NOB had increased their oxygen affinity significantly under long-term low DO conditions (Figure 3), which made nitrite oxidation more competitive with oxygen than ammonia oxidation. Consequently, no nitrite accumulated and its effluent concentration was lower than that of ammonia. The increase in the oxygen affinity of NOB was likely due to community shift.

Operating the aeration tank under a low DO could be an effective approach to improve oxygen transfer efficiency therefore reducing the energy consumption for wastewater treatment. It is also the easiest implementable approach for sustainable operation of existing wastewater treatment plants because there is almost no capital investment involved. This research provides evidence and theoretical supports for operating the activated sludge process under low DO conditions without compromising the effluent quality.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed formula for the trace elements added into the influent, experimental protocols for batch respirometric tests and conventional batch kinetic tests, primers and PCR programs for T-RFLP and real-time PCR assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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