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Reduction of N_2O and NO Generation in Anaerobic−Aerobic (Low Dissolved Oxygen) Biological Wastewater Treatment Process by Using Sludge Alkaline Fermentation Liquid

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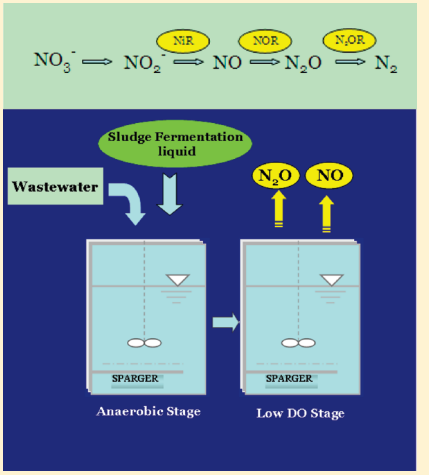
Supporting Information

ABSTRACT: This paper reported an efficient method to significantly reduce nitrous oxide (N_2O) and nitric oxide (NO) generation in anaerobic−aerobic (low dissolved oxygen) processes. It was found that by the use of waste-activated sludge alkaline fermentation liquid as the synthetic wastewater−carbon source, compared with the commonly used carbon source in the literature (e.g., acetic acid), the generation of N_2O and NO was reduced by 68.7% and 50.0%, respectively, but the removal efficiencies of total phosphorus (TP) and total nitrogen (TN) were improved. Both N_2O and NO were produced in the low dissolved oxygen (DO) stage, and the use of sludge fermentation liquid greatly reduced their generation from the denitrification. The presences of Cu^{2+} and propionic acid in fermentation liquid were observed to play an important role in the reduction of N_2O and NO generation. The analysis of the activities of denitrifying enzymes suggested that sludge fermentation liquid caused the significant decrease of both nitrite reductase activity to NO reductase activity ratio and NO reductase activity to N_2O reductase activity ratio, which resulted in the lower generation of NO and N_2O. Fluorescence in situ hybridization analysis indicated that the number of glycogen accumulating bacteria, which was reported to be relevant to nitrous oxide generation, in sludge fermentation liquid reactor was much lower than that in acetic acid reactor. The quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, showed that the use of fermentation liquid increased the number of bacteria capable of reducing N_2O to N_2. The feasibility of using sludge fermentation liquid to reduce NO and N_2O generation in an anaerobic−low DO process was finally confirmed for a municipal wastewater.



INTRODUCTION

Recently, achieving higher nutrient (nitrogen and phosphorus) removal efficiency with less energy consumption (mainly oxygen supply) has become an interesting topic of biological municipal wastewater treatment. Biological nitrification, denitrification, and phosphorus removal using anaerobic−aerobic process with low dissolved oxygen (DO) concentration (0.15−0.5 mg/L), termed as anaerobic−low DO in this study, gained much attention recently because of its lower energy consumption.[1,2] However, it was reported that a significant amount of nitrous oxide (N_2O) was produced in this process.[3−5] In addition, our study showed that nitric oxide (NO) was also generated when wastewater was treated by an anaerobic−low DO process.

N_2O is an important greenhouse gas and can destruct the ozone layer. NO also plays a key role in chemical reactions that cause depletion of the ozone layer.[6] Several researchers studied the control of N_2O emission in biological wastewater treatment processes. Yang et al.[7] reported that the generation of N_2O in an aerobic−anoxic wastewater treatment process was reduced by 50% using the step−feed technique. Fukumoto et al.[8] found that the addition of nitrite-oxidizing bacteria (NOB) to a pig manure treatment process could significantly reduce N_2O emission. It is well-known that carbon source plays an important role in biological wastewater treatment, which is essential for providing electron donor for denitrification and energy for phosphorus uptake.[9] Nevertheless, the strategy to control N_2O and NO generation in the anaerobic−low DO process from the aspect of wastewater−carbon source has never been reported.

In this paper a new method, i.e., using alkaline fermentation liquid of waste-activated sludge as carbon source, for significantly reducing the generation of N_2O and NO in the anaerobic−low DO process was introduced. Acetic acid is the main organic component in municipal wastewater[10,11] and has been widely used in the literature as the carbon source of anaerobic−low DO wastewater treatment process.[3−5] Thus, in this study the effect of fermentation liquid on N_2O and NO generation was compared

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with that of acetic acid first. Then, the mechanisms of sludge fermentation liquid causing lower N_2O and NO generation were investigated. Finally, this new method was tested in real municipal wastewater.

MATERIALS AND METHODS

Sludge Fermentation Liquid Preparation. The main characteristics of waste-activated sludge are shown in the Supporting Information. Sludge fermentation for producing fermentation liquid containing short-chain fatty acids (SCFA) was conducted at pH 10 according to the procedure described previously.[12] After fermentation, the liquid phase was separated, and the released ammonium nitrogen and phosphorus were removed according to the method described in our previous publication.[13] All processes were conducted at 21 ± 1 °C. The fermentation liquid was stored at 4 °C before use. The main characteristics of the supernatant fermentation liquid after nitrogen and phosphorus recovery are as follows (mg COD/L): TCOD (total chemical oxygen demand), 5022 ± 348; SCFA, 2319 ± 252 (including acetic acid, 1234 ± 187; propionic acid, 410 ± 72; *n*-butyric acid, 205 ± 32; isobutyric acid, 159 ± 62; and isovaleric acid, 311 ± 143); soluble carbohydrate, 169 ± 30; and soluble protein, 1033 ± 169. The fermentation liquid had soluble orthophosphorus (SOP, 4.2 ± 0.8 mg/L) and NH_4^+-N (13.2 ± 1.6 mg/L).

Batch Experiments of Nitrification and Denitrification Affecting N_2O and NO Generation in Low DO Stage. As both nitrification and denitrification occurred simultaneously in the low DO stage, the following batch tests were conducted to identify their individual effects on NO and N_2O generation. A total of 0.7 L of activated sludge was taken from parent sequencing batch reactor A (SBR-A) or sequencing batch reactor F (SBR-F) (see the Supporting Information for details) before the end of anaerobic time, centrifuged at 100*g* for 30 s to remove the supernatant, washed three times with 0.9% NaCl solution to remove the soluble compounds, such as NH_4^+-N and SOP, and then resuspended in tap water with a final volume of 0.7 L before being divided equally into two reactors (reactors-1 and -2) with a volume of 1 L each. Before the solution B (Supporting Information) was supplied to two reactors to get a SOP concentration of 70 mg/L each, reactor-1 was sparged with air at a rate of 0.5 L/min for 5 h to exhaust the internal carbon source completely. Then, solution A was added to reactor-1 and KNO_3 (or $NaNO_2$) was supplied to reactor-2 after 0.35 mL of nutrient solution was added to each reactor. Two reactors were stirred under low DO (0.15−0.5 mg/L) condition for 3 h.

Effects of Main Organic Components and Metal Ion (Cu^{2+}) in Sludge Fermentation Liquid on NO and N_2O Generation. SCFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, and isovaleric acids), carbohydrate, and protein were the main organic components of sludge fermentation liquid. In addition, fermentation liquid also contained Cu^{2+}. It has been reported that Cu^{2+} forms the active center of nitrous oxide reductase.[14] To explore their effects on NO and N_2O generation, the following batch experiments were conducted. Five minutes before the end of low DO phase, 2 L of biomass was taken from SBR-A, centrifuged at 100*g* for 30 s to remove the supernatant, washed three times with 0.9% NaCl solution, and then resuspended in tap water with a final volume of 2 L. The aliquot was divided equally into five reactors and then the nutrient solutions, solution A and solution B (Supporting Information), were added to each reactor to achieve final calculated concentrations of NH_4^+-N, SOP, and Cu^{2+} of 30 mg of N/L, 60 mg/L, and 0.00375 mg/L, respectively. The concentration of main organic components in each reactor was (mg COD/L) 300 acetic acid (reactor 1), 200 acetic acid + 100 propionic acid (reactor 2), 160 acetic acid + 80 propionic acid + 60 bovine serum albumin (BSA, model compound of protein) (reactor 3), 160 acetic acid + 80 propionic acid + 30 BSA + 30 glucose (a model compound of carbohydrate) (reactor 4), 160 acetic acid + 80 propionic acid+ 30 BSA + 30 glucose + 0.01 mg/L Cu^{2+} (by the addition of $CuCl_2 \cdot 6H_2O$) (reactor 5). The sludge mixture in all reactors was stirred under anaerobic condition for 120 min, and then aerated for 180 min with DO of 0.15−0.5 mg/L.

Comparison between the Addition of Acetic Acid and Sludge Fermentation Liquid to Municipal Wastewater Affecting NO and N_2O Generation. This investigation was conducted in two SBRs (SBR-MA and SBR-MF), which received municipal wastewater plus acetic acid, and municipal wastewater plus fermentation liquid, respectively. The operation of SBR-MA and SBR-MF was the same as that described in the section "Operation of Parent Sequencing Batch Reactors (SBRs) Fed with Synthetic Wastewater" of the Supporting Information. The municipal wastewater was obtained from the primary sedimentation tank outlet of a wastewater treatment plant in Shanghai, China. Its main characteristics are as follows: TCOD, 170−210 mg COD/L; SCOD (soluble chemical oxygen demand), 132−165 mg COD/L; acetic acid, 15−30 mg COD/L; NH_4^+-N, 20−35 mg/L; TN, 28−38 mg/L; SOP, 2.8−4.8 mg/L; and TP, 3.0−5.5 mg/L; pH 7.4−7.6. The municipal wastewater was supplemented by solution A and solution B (Supporting Information) to get an average initial NH_4^+-N, TN, SOP, and TP of 30, 35.5, 12, and 12.5 mg/L, respectively, in two SBRs. The initial SCOD in two SBRs was maintained at approximately 350 mg/L after the addition of acetic or sludge fermentation liquid. The initial pH of wastewater was adjusted to pH 7.4 ± 0.2 by 4 M HCl or 4 M NaOH.

Analytical Methods. The analyses of COD, NH_4^+-N, NO_3^--N, NO_2^--N, TN, SOP, TP, SCFA, protein, carbohydrate, mixed liquid suspended solid (MLSS), and mixed liquid volatile suspended solid (MLVSS) were conducted in accordance with standard methods.[15] Poly(hydroxyalkanoates) (PHA) [including poly(hydroxyvalerate) (PHV), poly(hydroxybutyrate) (PHB), and poly(hydroxy-2-methylvalerate) (PH2MV)] were conducted according to the method described previously.[16] The N_2O and NO concentrations in both gas and liquid phases were measured by the microsensors (Unisense, Aarhus, Denmark), and their generation rates were calculated according to the reported method.[17] The activities of nitrite, nitric oxide, and nitrous oxide reductases were measured by the consumption of electron acceptor (NO_3^--N, NO_2^--N, NO, and N_2O) with benzyl viologen as synthetic electron donor.[18] Heavy metals were detected by plasma-optical spectrometry (Perkin-Elmer, Optima 2100DU).

The fluorescence in situ hybridization (FISH) technique with 16S rRNA-targeted oligonucleotide probes was employed to monitor the difference of microbial community in the parent SBR-A and SBR-F. Table S1 (Supporting Information) lists the oligonucleotides probes used for FISH in this study.[19,20] PAO 462, PAO 651, and PAO 846 (PAOmix) and EUB 338, EUB 338-II, and EUB 338-III (EUBmix) were applied to characterize the *Candidatus Accumulibacter phosphatis* (phosphorus accumulating organisms, PAO) and domain bacteria. GAO Q431, GAO

Table 1. Comparison of the Generation of NO and N_2O and the Removal Efficiency of NH_4^+-N, TN, and TP in Two SBRs [a]

	SBR-A	SBR-F
N_2O generation (mg N_2O−N/mg N removed) [b]	0.432 ± 0.098	0.135 ± 0.018
NO generation (mg NO-N/mg N removed) [c]	0.024 ± 0.001	0.012 ± 0.001
NH_4^+-N removal efficiency (%)	97.5 ± 2.5	98.8 ± 1.2
TN removal efficiency (%)	65.3 ± 5.3	77.5 ± 6.3
TP removal efficiency (%)	85.5 ± 4.2	98.9 ± 1.1

[a] The data are the averages and their standard deviations of five different measurements. [b] The sum of N_2O generated in gas [0.381 ± 0.086 (SBR-A) and 0.104 ± 0.020 (SBR-F)] and liquid [0.051 ± 0.005 (SBR-A) and 0.031 ± 0.002 (SBR-F)] phases during the entire low DO stage. [c] The sum of NO generated in gas [0.023 ± 0.0009 (SBR-A) and 0.011 ± 0.001 (SBR-F)] and liquid [0.0007 ± 0.00126 (SBR-A) and 0.0006 ± 0.0001 (SBR-F)] phases during the entire low DO stage.

Q989, and GB_G 2 (GAOmix), TFO_DF 218 and TFO_DF 618, and DF 988 and DF 1020 were used for targeting *Candidatus Competibacter phosphatis*, *Defluvicoccus*-related TFO in *Alphaproteobacteria*, and *Defluvicoccus*-related DF in *Alphaproteobacteria*, respectively. These probes were commercially synthesized and 5′-labeled with AMAC, 6-FAM, and TAMRA. The biomass withdrawn from two parent reactors at the end of low DO phase was fixed with 4% freshly prepared paraformaldehyde for 8−10 h at 4 °C. After being rinsed with PBS (pH 7.2), 10 μL samples were immobilized on a gelatin-coated glass slide and then dehydrated by successive ethanol solutions of 50%, 75%, 85%, and 98% each for 3 min before being dried in the air. Hybridizations on the slide glass were performed according to the standard method of FISH[21] with slight modifications. Twenty microliters of hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% SDS (sodium dodecyl sulfate), and 0.2 ng of probes] was hybridized with the fixed samples, and then the slides were incubated in a prewarmed Boekel InSlide Out hybridization oven (Boekel Scientific Inc.) at 46 °C for 2 h. Two negative controls were incubated at the same condition. All hybridization experiments were followed by a washing step at 48 °C for 20 min in a washing buffer containing Tris-HCl (20 mM, pH 7.2), NaCl (70 mM), EDTA (5 mM), and SDS (0.01%), and the NaCl concentration was fixed by experiments. The washing buffer was removed by rinsing the slides with distilled water and the slides were air-dried. The slides were mounted to avoid bleaching the visual pigments and examined with epifluorescence microscope (Nikon, Japan). Within each field, total sludge cell area targeted by each applied probe was expressed as the percentage of the domain cell area targeted by the bacterial probe EUBmix using the functions provided in the image analyzing software (Image-Pro Plus, V6.0, Media Cybernetics), and the signal intensity threshold was determined by negative control.

The quantitative real-time polymerase chain reaction (PCR) was carried out as below. Primers for *nosZ* gene were 5′CGCRACGGCAASAAGGTSMSSGT3′ and 5′CAKRTGCAKSGCRTGGCAGAA3′,[22] which were called nosZ2F and nosZ2R, respectively. Standard curves were constructed for the absolute quantification of *nosZ* gene number using an Applied Biosystems 7500 fast thermal cycler. Genomic DNA extracted from activated sludge was used to amplify *nosZ* gene fragment using primers described above, and the real-time PCR assay was carried out in a volume of 20 μL, which contained 1 μL of 20× SYBR green PCR Master Mix (SYBR Green I Nucleic A, Invitrogen), 0.5 μM of each *nosZ* primer, and 1 μL of template DNA. Thermal cycling conditions for the *nosZ* primers were according to the literature.[22] Then the *nosZ* gene fragment was cloned and verified by sequencing. The copy number of the *nosZ*-containing plasmid was calculated from the concentration and size (base pairs) of the extracted plasmid. A standard curve (Figure S1, Supporting Information) was generated using three replicates of 10-fold serial dilutions of linearized plasmid containing the *nosZ* sequence as a template, and the cycling conditions were described above. Purity of amplified products was checked by the observation of a single melting peak (Figure S2, Supporting Information). Independent quantitative PCR assays were performed for *nosZ* gene from activated sludge acclimated to acetic acid and sludge alkaline fermentation liquid, respectively. Two no-template controls were run for each quantitative PCR assay.

RESULTS AND DISCUSSION

Comparison of NO and N_2O Generation in Two SBRs. After over 4 months of acclimation, two parent SBRs (SBR-A and SBR-F) (see Supporting Information) achieved stable nitrogen and phosphorus removals, and then the generation of N_2O and NO in two SBRs was assayed. During the anaerobic time the generation of N_2O and NO was undetectable in either gas or liquid phase in two SBRs, but remarkable quantities of two gases were generated during the aerobic time (Figure S3, Supporting Information). In addition, the concentrations of NO and N_2O in both liquid and off-gas phases in SBR-A were higher than those in SBR-F. The total N_2O generation in SBR-A and SBR-F was 0.432 and 0.135 mg of N_2O-N/mg N removed, respectively, and the corresponding NO generation was 0.024 and 0.012 mg NO-N/mg N-removed (Table 1). Obviously, the amounts of N_2O and NO generated in SBR-F were respectively reduced by 68.7% and 50.0% compared with those in SBR-A.

It was observed that although SBR-F produced less N_2O and NO than SBR-A, the former had higher TN and TP removal efficiency than the latter and had the same NH_4^+-N removal efficiency as the latter (Table 1). Thus, the low generation of N_2O and NO in SBR-F was not caused by its low NH_4^+-N and TN removal efficiency. In the coming text, the mechanisms for fermentation liquid showing significantly lower NO and N_2O generation than acetic acid were investigated.

Identification of the Source of N_2O and NO Generation. The above study showed that there was no N_2O or NO generated during the anaerobic time. Thus, the much lower generation of N_2O and NO in the anaerobic−low DO process with the use of sludge fermentation liquid was due to their different quantities generated in the low DO stage. According to the literature,[8] NO and N_2O are usually generated in either the nitrification or denitrification process (Figure S4, Supporting Information). As both nitrification and denitrification occurred in the low DO stage, the effects of nitrification and denitrification on N_2O and NO generation in the low DO stage were investigated.

Table 2. Effect of Main Organic Component and Cu^{2+} in Sludge Fermentation Liquid on NO and N_2O Generation in Batch Experiments [a]

carbon source	N_2O generation (mg N/mg N removed)	NO generation (mg N/mg N removed)	TN removal efficiency (%)
acetic acid [b]	0.462 ± 0.058	0.025 ± 0.002	63.1 ± 4.6
acetic acid + propionic acid[b]	0.236 ± 0.091	0.015 ± 0.001	64.4 ± 4.2
acetic acid + propionic acid + protein [b]	0.233 ± 0.042	0.014 ± 0.001	65.5 ± 4.6
acetic acid + propionic acid + protein + carbohydrate [b]	0.210 ± 0.019	0.014 ± 0.001	67.3 ± 3.0
acetic acid + propionic acid + protein + carbohydrate + Cu^{2+}[c]	0.145 ± 0.020	0.012 ± 0.001	68.2 ± 2.3

[a] The data are the averages and their standard deviations in triplicate tests. The data are the sum of N_2O or NO generated in liquid and gas phases. [b] There was Cu^{2+} due to the use of nutrient solution, and its calculated concentration was 0.00375 mg/L. [c] The concentration of added Cu^{2+} was 0.01 mg/L.

According to the batch experiments (Table S2, Supporting Information), it was found that the biomass of SBR-F produced slightly greater N_2O than that of SBR-A during the nitrification process at any ammonium nitrogen concentration investigated. For example, at an NH_4^+-N concentration of 30 mg/L, the amount of N_2O generated from SBR-F was 0.105 mg N/mg N removed, while the N_2O generation in SBR-A was 0.092 mg N/mg N removed. The data in Table S2 (Supporting Information) also showed that two biomasses produced very little NO in the nitrification reaction. However, during the denitrification process the biomass of SBR-A produced much more N_2O and NO than that of SBR-F. By comparing the data of nitrification with those of denitrification (Table S2, Supporting Information), it was obvious that one reason for lower N_2O and NO generation in SBR-F was that the use of sludge fermentation liquid significantly reduced the generation of N_2O and NO in denitrification process of the low DO stage.

Influence of Main Organic Components and Cu^{2+} in Sludge Fermentation Liquid on NO and N_2O Generation. Several factors, such as temperature, sludge retention time (SRT), DO, have been observed in the literature to influence N_2O generation during biological wastewater treatment.[23,24] Nevertheless, in this study these parameters were almost identical in two SBRs (temperature 21 ± 1 °C, DO 0.15−0.50 mg/L, and SRT approximately 22 d). Nitrite and pH have also been reported to affect N_2O generation.[25,26] In the current study, two SBRs had almost the same pH variation during one cycle (Figure S5, Supporting Information). It was also found that nitrite was accumulated in two reactors in the low DO stage, but its concentration in SBR-F was lower than that in SBR-A (Figure S5, Supporting Information). Nitrite was reported to inhibit the reduction of nitrous oxide by forming nitrous acid, which caused the accumulation of nitrous oxide in the denitrification process.[27] Thus, lower N_2O and NO generation was observed in SBR-F.

The denitrification process has been reported to be affected by wastewater−carbon source.[28] In this study, acetic and propionic acids, protein, and carbohydrate were the main organic components of sludge fermentation liquid. The batch tests showed that the presence of propionic acid significantly reduced the generation of both N_2O and NO, but further addition of protein or carbohydrate did not result in more N_2O and NO reduction (Table 2). Most of wastewater−carbon sources (mainly acetic in SBR-A and acetic and propionic acids in SBR-F) were consumed in the anaerobic stage and stored as PHA. As propionic acid synthesized more PHV and less PHB than acetic acid,[29,30] there was greater PHV but lower PHB in SBR-F biomass compared with SBR-A at the end of anaerobic stage (Figure S6, Supporting Information). It has been reported that the use of endogenous carbon source PHB, compared with external acetate, as the electron donor of denitrification can cause nitrous oxide accumulation.[31] Perhaps PHA with higher PHV proportion was a better endogenous carbon source for denitrification than that with lower PHV, which resulted in lower generation of both N_2O and NO in SBR-F than SBR-A.

Nitrous oxide reductase is an enzyme catalyzing the final step of bacterial denitrification (i.e., reducing N_2O to N_2). In its crystal structure there is a catalytic site called Cu_Z, which comprises four copper ions.[14] It was reported that the lack of Cu^{2+} caused the accumulation of N_2O during denitrification.[32] In this study, the initial concentrations of Cu^{2+} in SBR-A and SBR-F were 0.00375 (calculated data) and 0.01 mg/L, respectively. As seen from the batch experiments in Table 2, the presence of 0.01 mg/L Cu^{2+} significantly reduced N_2O generation compared with 0.00375 mg/L Cu^{2+} (0.145 versus 0.21 mg N/mg N removed). Therefore, the presence of a certain amount of Cu^{2+} in fermentation liquid was another important reason for the lower nitrous oxide generation in SBR-F.

Activities of Denitrifying Enzymes in the Low DO Stage of Two Parent SBRs. According to the denitrification pathway (Figure S4, Supporting Information), the generation of N_2O and NO is the net balance of their formation and consumption. Several researchers reported that the decrease of nitrous oxide and nitric oxide reductases caused the accumulation of N_2O and NO.[23,33−35] In this study, the activities of three denitrification enzymes in two SBRs are illustrated in Table 3. The reduction rates of nitric oxide and nitrous oxide were respectively slower than those of nitrite and nitric oxide in both SBR-A and SBR-F, which resulted in the accumulation of N_2O and NO in two SBRs. Furthermore, the levels of NO and N_2O accumulation were reported to be relevant to the ratios of nitrite reduction rate to nitric oxide reduction rate and nitric oxide reduction rate to nitrous oxide reduction rate, respectively.[36,37] The data in Table 3 indicated that SBR-F had lower ratios of nitrite reductase activity to NO reductase activity (N1/N2) and NO reductase activity to N_2O reductase activity (N2/N3) than SBR-A, which was consistent with the lower N_2O and NO generation in SBR-F.

Microbial Community in Two SBRs. The results of FISH analysis (in Figure 1) showed that SBR-F had more PAO than SBR-A (51.4% against 40.6% accounting for the domain bacteria), whereas the former had less glycogen accumulating organisms (GAO) than the latter (6.7% versus 22.7%). Several publications reported that N_2O was the main denitrification product of GAO.[3−5] The number of GAO in SBR-F was only approximately one-third of that in SBR-A, which might be one

Table 3. Comparison of the Activities of Three Key Denitrifying Enzymes in Two Biomasses

biomass	nitrite reductase activity (N1)	NO reductase activity (N2)	N_2O reductase activity (N3)	N1/N2	N2/N3
SBR-A	0.311 ± 0.004	0.242 ± 0.014	0.045 ± 0.004	1.285 ± 0.002	5.387 ± 0.009
SBR-F	0.417 ± 0.015	0.350 ± 0.053	0.090 ± 0.007	1.191 ± 0.013	3.913 ± 0.024

[a] The biomasses were sampled at the end of low DO phase for enzyme assay. [b] The unit of enzyme activity is mg N/g VSS/min. The data are the averages and their standard deviations in triplicate tests.

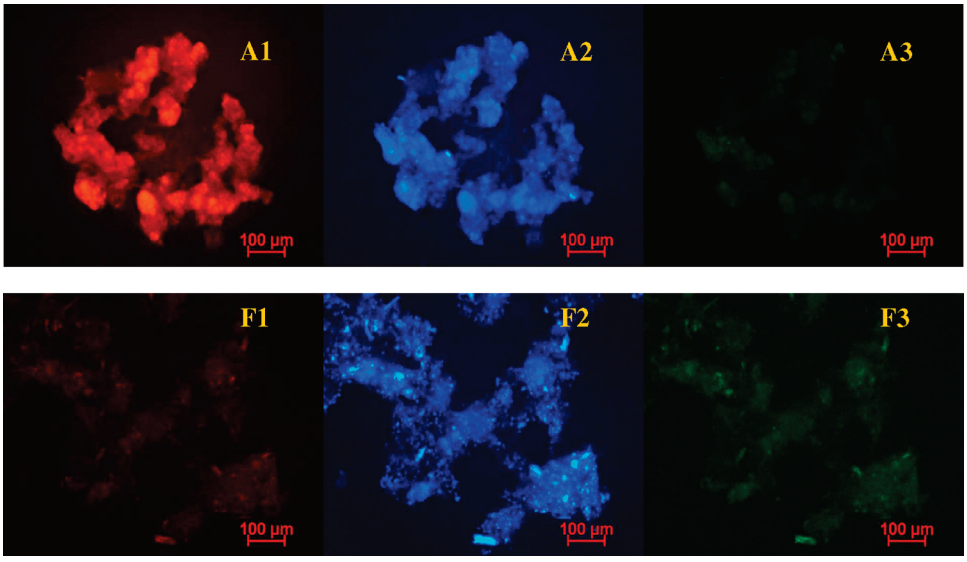

Figure 1. Microscopes of sludge from SBR-A (A1−A3) and SBR-F (F1−F3) at the end of the anaerobic phase as visualized by FISH. Glycogen accumulating organism (GAO) containing *Candidatus Competibacter phosphatis*, *Defluvicoccus*-related TFO in *Alphaproteobacteria*, and *Defluvicoccus*-related DF in *Alphaproteobacteria* was hybridized with TAMARA-labeled GAOMIX, TFO_DF218 and TFO_DF618, and DF988 and DF1020 (red, A1, and F1). *Candidatus Accumulibacter phosphatis* (PAO) was hybridized with AMCA-labeled PAO462, PAO651, and PAO846 (blue, A2, and F2). Probe EUBmix specially stained domain bacteria was labeled with 6-FAM (green, A3, and F3). The images analysis showed that the percentages of PAO and GAO accounting for the domain bacteria were respectively 51.4% and 6.7% in SBR-F and 40.6% and 22.7% in SBR-A.

Table 4. Comparison of the Effects of the Addition of Acetic Acid and Sludge Fermentation Liquid to Municipal Wastewater on NO and N_2O Generation[a]

additional carbon source	acetic acid	sludge fermentation liquid
N_2O generation (mg N_2O-N/mg N removed) [b]	0.507 ± 0.028	0.121 ± 0.016
NO generation (mg NO-N/mg N removed) [c]	0.036 ± 0.003	0.010 ± 0.001
NH_4^+-N removal efficiency (%)	97.5 ± 2.0	97.8 ± 1.2
TN removal efficiency (%)	65.2 ± 4.2	70.8 ± 6.2
TP removal efficiency (%)	95.5 ± 1.1	95.6 ± 1.4

[a] The two SBRs were operated for around 4 months before the TN and TP removal efficiency reached relative stability, and the data are the averages and their standard deviations of six different measurements. [b] The sum of N_2O generated in gas (0.459 ± 0.025 mg N_2O-N/mg N removed in acetic acid SBR and 0.098 ± 0.012 mg N_2O-N/mg N removed in sludge fermentation liquid SBR) and liquid phases (0.048 ± 0.003 mg N_2O−N/mg N removed in acetic acid SBR and 0.023 ± 0.003 mg N_2O−N/mg N removed in sludge fermentation liquid SBR) during the entire low DO stage. [c] The sum of NO generated in gas (0.035 ± 0.0015 mg NO-N/mg N removed in acetic acid SBR and 0.009 ± 0.0009 mg NO-N/mg N removed in sludge fermentation liquid SBR) and liquid phases (0.0008 ± 0.0002 mg NO-N/mg N removed in acetic acid SBR and 0.0006 ± 0.0002 mg NO-N/mg N removed in sludge fermentation liquid SBR) during the entire low DO stage.

main reason for the use of fermentation liquid showing much lower N_2O generation.

It is known that *nosZ* gene encoding nitrous oxide reductase is largely unique to denitrifying bacteria and has recently been used for the detection of denitrifier-specific DNA.[38] In order to quantify the bacteria capable of reducing N_2O to N_2, quantitative real-time PCR assay targeting the *nosZ* gene was conducted. The *nosZ* copies density in SBR-F was 1.16×10^7 copies/g MLVSS, whereas the *nosZ* gene copies density in SBR-A was 6.44×10^6 copies/g MLVSS. The influence of carbon source on *nosZ*-bearing community abundance has also been observed in the literature. For example, Henderson et al.[39] found that the presence of plant residues as carbon source showed higher *nosZ*-bearing community abundance in soil than that of glucose. It was reported that higher density of *nosZ* gene copies was correspondence with more bacteria capable of reducing N_2O to N_2.[22] Therefore the generation of nitrous oxide in SBR-F was much lower than in SBR-A.

Comparison between the Addition of Acetic Acid and Sludge Fermentation Liquid to Municipal Wastewater Affecting NO and N_2O Generation. After the removal efficiency of TN and TP in two municipal wastewater reactors (SBR-MA and SBR-MF) reached relative stability, the generation of NO and N_2O was measured (Table 4). With acetic acid as the additional carbon source, the generated N_2O and NO were 0.507 and 0.036 mg N/mg N removed, respectively. Nevertheless, the generated N_2O and NO were respectively 0.121 and 0.010 mg N/mg N removed when sludge fermentation liquid was used as the supplementary carbon source. Thus, the generation of both NO and N_2O during municipal wastewater treated by anaerobic−low DO process could be significantly reduced by the use of sludge fermentation liquid to replace acetic acid as the additional carbon source, and the nitrogen and phosphorus removal efficiency did not deteriorate.

■ ASSOCIATED CONTENT

Supporting Information. This file contains Tables S1 and S2, Figures S1−S6, and part of the materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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