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# Effects of the Food-to-Microorganism (F/M) Ratio on $N_2O$ Emissions in Aerobic Granular Sludge Sequencing Batch Airlift Reactors

**Ning Guo [1], Jian Zhang [1], Hui-Jun Xie [2], Lin-Rui Tan [1], Jie-Ning Luo [1], Ze-Yu Tao [3] and Shu-Guang Wang [1,*]**

1 Shandong Provincial Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, 27 Shanda Nanlu, Jinan 250100, China; guoning@mail.sdu.edu.cn (N.G.); zhangjian00@sdu.edu.cn (J.Z.); tanlinrui@126.com (L.-R.T.); ljn13884519252@163.com (J.-N.L.)

2 Environmental Research Institute, Shandong University, 27 Shanda Nanlu, Jinan 250100, China; xiehuij@sdu.edu.cn

3 School of Municipal and Environmental Engineering, Shandong Jianzhu University, Fengming Licheng District Lingang Development Zone, Jinan 250101, China; zeyutao@sina.cn

* Correspondence: wsg@sdu.edu.cn

Received: 30 April 2017; Accepted: 22 June 2017; Published: 29 June 2017

**Abstract:** The present study investigated the effect of the food-to-microorganism (F/M) ratio on nitrous oxide ($N_2O$) emissions in aerobic granular sludge sequencing batch airlift reactors. Three identical sequencing batch airlift reactors were fed with sodium acetate-based wastewater at different chemical oxygen demand (COD) concentrations, resulting in F/M ratios from 0.2 to 0.67 g COD/g SS. The results indicated that $N_2O$ emissions increased with an increase of the F/M ratio. $N_2O$ emissions at the high F/M ratio of 0.67 g COD/g SS were the highest (4.4 $\pm$ 0.94 mg/d). The main source of the high $N_2O$ emissions at the F/M ratio of 0.67 g COD/g SS was nitrifier denitrification, rather than heterotrophic denitrification, confirmed by the qPCR (quantitative real-time PCR) results. The heterotrophic denitrification was destroyed by the DO (dissolved oxygen) diffusing into the sludge particles with porous structures. This study offers theoretical support to study the $N_2O$ emissions in aerobic granular sludge, and can provide guidance for conducting risk assessment and enhancing our ability to predict $N_2O$ production in aerobic granular sludge at different F/M ratios.

**Keywords:** aerobic granular sludge; nitrous oxide emission; F/M ratio; *nirK* gene

## 1. Introduction

Aerobic granular sludge technology is a promising new environmental biotechnological process [1–3]. Aerobic granules have the advantages of excellent settling abilities, a dense granule structure, and a high biomass retention, compared to conventional activated sludge. Therefore, aerobic granular sludge technology is increasingly drawing the interest of researchers committed to biological wastewater treatment technology.

It is generally accepted that the biological treatment process of wastewater occupies an important position among the main sources of nitrous oxide ($N_2O$) [4,5]. $N_2O$ is one kind of greenhouse gas, and its half-life is as long as 114 years in the atmosphere. The global warming potential of $N_2O$ is 310 times higher than for carbon dioxide, and the doubling volume of $N_2O$ in the atmosphere will make the average global temperature rise by 0.4 °C [6]. It is therefore of great importance to study the mechanism of $N_2O$ emissions in wastewater treatment processes.

It has been reported that simultaneous nitrification and denitrification (SND) can be achieved in granular sludge reactors because of the distribution of autotrophic nitrifying bacteria and heterotrophic

denitrifying bacteria. Denitrification of both nitrifying bacteria in the aerobic process and denitrifying bacteria in the anaerobic process are the sources of $N_2O$ [7]. Therefore, the emission of $N_2O$ as an intermediate or end product in the metabolism of microorganisms in aerobic granules cannot be neglected.

The organic loading is one of the most critical parameters for the cultivation of aerobic granular sludge [8,9], as the substrate concentration will influence the granulation process by optimizing and enriching different microbe species [10]. Many efforts have been made to evaluate the effects of different organic loadings on aerobic granular sludge [11]. A relatively low organic loading (0.5–2 kg COD/($m^3$·d); COD—chemical oxygen demand) results in a slow microbial growth rate, while a high organic loading (21.3 kg COD/$m^3$·d) contributes to disintegrated granules and unstable reaction systems [12]. Furthermore, the organic loading could affect the performance of SND. A low organic loading will result in the limiting substrate for denitrification [13]. The limited performance of denitrification will further influence $N_2O$ emissions. In addition, a high organic loading will consume more dissolved oxygen (DO), resulting in a low DO concentration. It has been acknowledged that a low DO concentration can promote $N_2O$ emissions in SND [14,15]. However, the effect of the organic loading on $N_2O$ emissions in aerobic granular sludge has not been well studied.

This study aimed to investigate the mechanism of effects of F/M ratios on the $N_2O$ emission in aerobic granules. The morphology of the granules was determined by scanning electron microscopy (SEM), and the key components of extracellular polymeric substances (EPS) were also measured. A quantitative real-time PCR (qPCR) method was used to determine the abundances of functional genes, including *amoA*, *nirK*, and *nosZ* genes. This work could be helpful to offer a theoretical basis for $N_2O$ reduction in aerobic granular sludge technology.

## 2. Materials and Methods

### *2.1. Aerobic Granular Sludge Bioreactor Setup and Operation*

This study was conducted with three identical sequencing batch airlift reactors (SBARs). Aerobic granular sludge cultivated earlier with synthetic wastewater at the COD of 600 mg/L was used as the seed sludge for the reactors [16]. The seed sludge was well-mixed before it was loaded into the three reactors, to make sure that the three reactors had the same initial mixed liquor suspended solids (MLSS) concentration of 3000 mg/L. A 6 h working cycle was applied over the entire experiment, which was composed of 10 min of feeding, a 320 min aerobic stage, 5 min of settling, 5 min of discharging and 20 min of idling. The hydraulic retention time (HRT) was 12 h, the air supply was provided by virtue of an aeration pump at the bottom of the reactor at an aeration rate of around 3 L/min during the aeration stage, and the DO concentration in the reactors was measured throughout the 6 h cycle. The SBARs were operated at room temperature (20 ± 2 °C).

The operating conditions were the same for the three reactors, R1, R2 and R3, except for the loading substrate concentration. The carbon source in the synthetic wastewater was sodium acetate. The COD concentrations of the influent to the three SBARs were controlled at 600, 1025, and 2000 mg/L by adjusting the sodium acetate concentrations, and this resulted in F/M ratios of 0.2, 0.34 and 0.67 g COD/g SS for R1, R2 and R3, respectively. Correspondingly, the organic loading rates were 1, 1.71 and 3.33 kg COD/($m^3$·d). Other components of the synthetic wastewater were 240 mg of $NH_4Cl$, 58 mg of $K_2HPO_4$, 24 mg of $KH_2PO_4$, 67 mg of $CaCl_2$, 42 mg of $MgSO_4{\cdot}7H_2O$, 42 mg of EDTA, 250 mg of $NaHCO_3$, and 1 mL/L of a trace element solution. The composition of the trace element solution was: $FeCl_3{\cdot}6H_2O$ (150 μg·$L^{-1}$), $H_3BO_3$ (150 μg·$L^{-1}$), $CuSO_4{\cdot}5H_2O$ (30 μg·$L^{-1}$), KI (30 μg·$L^{-1}$), $MnCl{\cdot}4H_2O$ (120 μg·$L^{-1}$), $NaMoO_4{\cdot}2H_2O$ (60 μg·$L^{-1}$), $ZnSO_4{\cdot}7H_2O$ (120 μg·$L^{-1}$), and $CoCl_2{\cdot}6H_2O$ (150 μg·$L^{-1}$). $NaHCO_3$ was dosed into the synthetic wastewater to maintain the reactor pH in the range between 7.0 and 7.8.

At the end of the settling phase of every 6 h cycle, a certain amount of the sludge suspension was withdrawn using a siphon; thus the slow-settling flocs in the suspension sludge were removed.

The volume of the daily sludge discharge was adjusted to keep the MLSS concentration at around 3000 mg/L in the three reactors. The batch experiments were started when the MLSS concentrations could be kept constant, which implied that the SBARs had reached a steady state (about 60 days after the reactor started running). Meanwhile, the COD and ammonium removal rates were above 90%. The batch experiments were performed in triplicate consecutively. In the batch experiments, nitrogen compounds including ammonium ($NH_4^+$-N), nitrite ($NO_2^-$-N) and nitrate ($NO_3^-$-N), and the $N_2O$ emissions were measured. During the aeration reaction stage, the emission gas was sampled every 30 min during the 6 h working cycle. Off-gas was collected from the reactor headspace into a plastic bag by a plastic tube [16].

### 2.2. DNA Extraction and qPCR

Sludge samples were taken from the three reactors at the end of three 6 h batch experiments. For every sample, 1 mL of sludge mixed liquor was centrifuged at 10,000 *g* for 5 min at 4 °C. After that, the precipitate (wet weight) was extracted for DNA extraction using a PowerSoil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. DNA was extracted in triplicate for every sample. A Thermo NanoDrop 1000 spectrophotometer was used to measure the DNA concentration and purity. The extracted DNA was stored at −20 °C until it was used in later analyses.

The 16S rRNA gene fragment and *amoA*, *nirK* and *nosZ* gene fragments were amplified using primer sets F338/R518, *amoA*-1*F*/*amoA-2R*, FlaCu/R3Cu, and *nosZ-F*/*nosZ-1622R*, respectively, as shown in Table 1. Standard plasmids carrying the target genes were attained by PCR and molecular cloning [17], followed by extraction using a Plasmid Mini Kit according to the manufacturer's instructions (Omega, San Antonio, TX, USA).

Six-point standard curves were constructed from 10-fold serial dilutions of plasmids ranging from $10^{10}$ to $10^4$ copies $\mu L^{-1}$. The 16S rRNA, *amoA*, *nirK* and *nosZ* genes of microbes were quantified for all samples using a Roche LightCycler 480 system. The 20 μL qPCR reaction mixture consisted of 10 μL of SYBR Premix Ex Taq (Takara, Kusatsu, Shiga, Japan), 0.4 μL of forward primer, 0.4 μL of reverse primer, 2 μL of the template DNA, and 7.2 μL of sterile distilled water. Each qPCR run consisted of 5 min of initial denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, and annealing and extending at 72 °C for 30 s [18]. All sample measurements were performed in triplicate. The average slopes of all qPCR assays were from −3.1 to 3.5 with $R^2 > 0.98$.

**Table 1.** Target genes and primers used in this study.

| Target Primers | Primers | Primer Sequence | Annealing (°C) |
|---|---|---|---|
| 16S | F338/R518 | ACTCCTACGGGAGGCAGCAGATTACCGCGGCTGCTGG | 55 |
| *amoA* | *amoA*-1F/amoA-2*R* | GGGGTTTCTACTGGTGGTCCCCTCKGSAAAGCCTTCTTC | 53 |
| *nirK* | FlaCu/R3Cu | ATCATGGTSCTGCCGCGGCCTCGATCAGRTTGTGGTT | 55 |
| *nosZ* | *nosZ*-F/nos*Z-1622R* | CGYTGTTCMTCGACAGCCAGCGSACCTTSTTGCCSTYGCG | 56 |

### 2.3. EPS Extraction and Chemical Analysis

The extraction of EPS was performed using the cation exchange resin (CER) method [19]. The sludge samples were centrifuged at 2000 *g* for 15 min at 4 °C, and the supernatant was decanted. Using distilled water, the precipitates were diluted and mixed. The mixture was transferred into an extraction flask and the CER was added. The suspension was stirred for 6 h at 4 °C. The EPS extract was obtained by centrifugation at 12,000 *g* for 1 min. After filtration, the supernatant was used to measure the concentration of the EPS. The content of proteins (PNs) and humic acid were determined by the correction Bradford approach [20]. Polysaccharides (PS) were measured using the phenol–sulfuric method using glucose as the standard [21]. The EPS were extracted in triplicate for every sludge sample.

### *2.4. Analytical and Statistic Analysis Methods*

$NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N, MLSS concentrations, and the sludge volume index (SVI) were measured according to the standard methods [22]. The DO and pH were determined using DO and pH probes (HACH HQ40d, Loveland, CO, USA). In addition, the morphology of the granules was examined with SEM (S-520, Hitachi, Tokyo, Japan) according to the sample treatment method described by previous study [23]. The $N_2O$ concentration was measured with gas chromatography (SP-3410, Beijing, China) using an electron capture detector (ECD) and a Poropak Q column. The $N_2O$ emission quantity was calculated as presented by previous study [24]. The Significant Difference ($p$) of EPS, nitrogen concentration and functional genes under different F/M ratios were calculated with a $t$-test. Average values of three samples were reported as the results (including nitrogen concentration, $N_2O$ emission and DO concentration).

## 3. Results

### *3.1. Characterization of Aerobic Granular Sludge under Different F/M Ratios*

#### 3.1.1. The Settling Ability

Figure 1 shows the SVI values under different F/M ratios. The F/M ratios had an important influence on the settling ability of aerobic granular sludge. The SVI of R3 (F/M ratio of 0.67 g COD/g SS) was the highest among the three reactors, indicating that the settling ability of R3 was poor. The SVI values of R1 and R2 were lower than for R3, indicating that the settling ability of R1 and R2 were better than for R3.

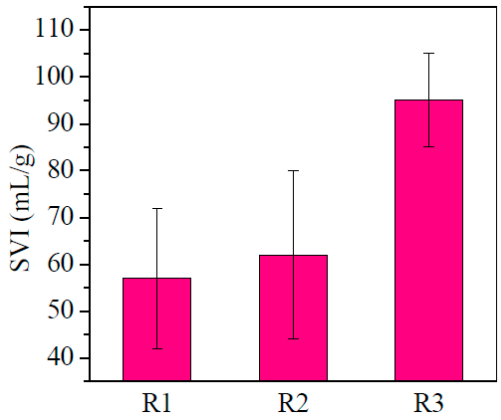


**Figure 1.** The settling ability of aerobic granular sludge of different F/M ratios.

#### 3.1.2. SEM Analysis

SEM was conducted to determine the specific microstructures of aerobic granules. As shown in Figure 2, granules of both R1 and R2 (lower F/M ratios; 0.2 and 0.34 g COD/g SS) had a distinct and dense physical structure; in addition, rod-shaped bacteria were predominant microorganisms, and cells were tightly attached. Aerobic granules of R3 (higher F/M ratio; 0.67 g COD/g SS) exhibited a loose, fluffy morphology (Figure 2c.1), and several granules with crude surfaces and porous structures had disintegrated. Moreover, the dominant bacteria were filamentous bacteria (Figure 2c.2).

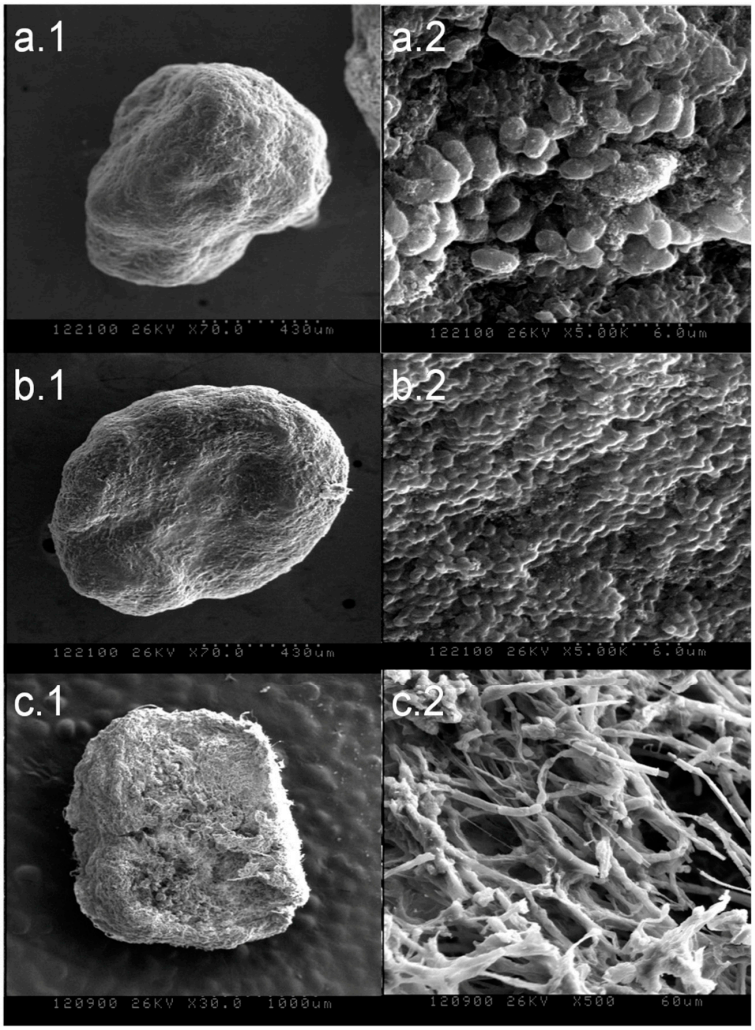


**Figure 2.** SEM (scanning electron microscopy) images of the mature aerobic granular sludge of different F/M ratios. (**a**–**c**) represent 0.2, 0.34 and 0.67 g COD/g SS, respectively, for which (1) and (2) represent different magnifications.

### 3.1.3. The Characteristics of EPS

EPS are viscous substances secreted by microorganisms, which are mainly composed of PS, PN and humic acid [25]. Table 2 shows that the EPS content increased with an increase of the F/M ratio. The EPS of R3 at a high F/M ratio was 20.34 mg/g MLSS, which was the largest among the different F/M ratios. PS was the major constituent of EPS, relative to PN and humic acid. PS of R3 (8.27 mg/g MLSS) was significantly less than that of the other reactors. The content of humic acid in R3 (4.27 ± 0.62 mg/g MLSS) was the most abundant among the three reactors.

**Table 2.** The content of EPS [1] and components of aerobic granular sludge SBARs [2] under different F/M ratios.

| EPS Compostions (mg/g MLSS) | R1 | R2 | R3 |
|---|---|---|---|
| Protein content | 3.86 ± 0.23 * | 5.23 ± 0.28 * | 7.81 ± 0.97 |
| Humic acid content | 1.57 ± 0.16 * | 1.55 ± 0.19 * | 4.27 ± 0.62 |
| Polysaccharide content | 9.21 ± 0.33 * | 11.83 ± 0.46 * | 8.27 ± 0.48 |
| EPS content | 14.64 ± 0.74 * | 18.6 ± 0.96 * | 20.34 ± 1.24 |

Note: An asterisk (*) represents a statistical difference of content in EPS between the samples among R1, R2 and R3 ($p < 0.05$). [1] Extracellular polymeric substances. [2] Sequencing batch airlift reactors.

### *3.2. Performance of Aerobic Granular Sludge SBARs under Different F/M Ratios*

To investigate the degradation process of the pollutants and $N_2O$ emissions, batch experiments were carried out. The performance of aerobic granular sludge SBARs under different F/M ratios is shown in Figure 3. Figure 3 shows that the performance of the three reactors had a similar trend of change during the 6 h cycle. $NH_4^+$-N of all reactors was removed rapidly, and the effluent concentration was maintained under 2 mg/L. The $NO_3^-$-N concentration increased gradually. The $NO_2^-$-N concentration changed in a similar way to $N_2O$ emissions in R3, especially at 240 min, demonstrating that the $NO_2^-$-N concentration played an important role in $N_2O$ emissions.

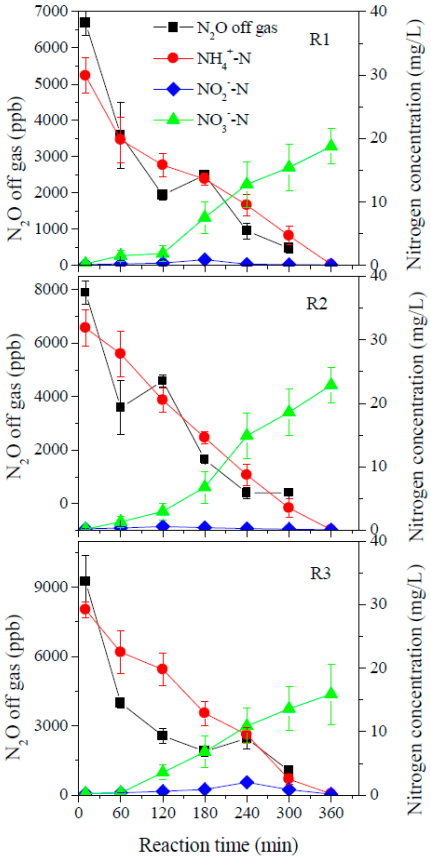


**Figure 3.** Performance of aerobic granular sludge SBARs under different F/M ratios.

The specific $N_2O$ emissions and $N_2O$-N conversion results are shown in Table 3. The emission of $N_2O$ increased with an increased F/M ratio. The $N_2O$ emission in R3 was the highest (4.4 ± 0.94 mg/d) and the $N_2O$-N conversion was 3.79%. Moreover, the results indicated that $NH_4^+$-N and COD removal efficiencies of R1, R2, and R3 were above 90%. It should be noted that the $NH_4^+$-N removal efficiency was the highest. Thus an excellent $NH_4^+$-N and COD removal were obtained in aerobic granular sludge SBARs.

**Table 3.** The $N_2O$ emissions and $NH_4^+$-N and COD removal efficiencies in aerobic granular sludge SBARs under different F/M ratios.

| Removal Efficiency and $N_2O$ Emission | R1 | R2 | R3 |
|---|---|---|---|
| $N_2O$ emission (mg/d); $N_2O$-N conversion (%) | 3.32 ± 0.86 *; 2.77 ± 0.56 * | 3.64 ± 0.38 *; 2.6 ± 0.69 * | 4.4 ± 0.94; 3.79 ± 0.48 |
| $NH_4^+$-N removal (%) | 95.20 ± 3.13 | 96.30 ± 2.98 | 99.30 ± 2.79 |
| COD removal (%) | 93.50 ± 3.4 | 94.5 ± 4.5 | 90.3 ± 6.91 |

Note: An asterisk (*) represents a statistical difference of removal efficiency and $N_2O$ emissions among R1, R2 and R3 ($p < 0.05$).

The DO concentration under different F/M ratios is shown in Figure 4. The DO concentration decreased as the F/M ratio increased. The DO concentration of R3 was much lower than for R1 and R2. This indicated that under the same aeration rate, more DO was consumed by a higher F/M ratio in aerobic granular sludge.

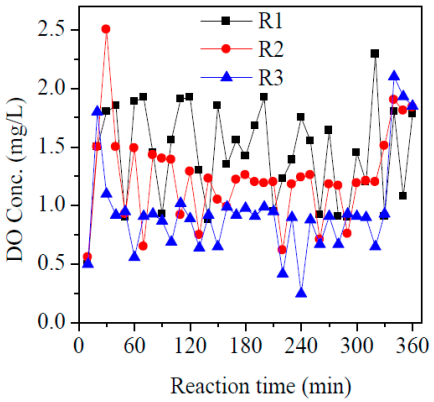


**Figure 4.** DO concentrations during a 6 h cycle in aerobic granular sludge SBARs under different F/M ratios.

### *3.3. The Abundance of Functional Genes under Different F/M Ratios*

The ammonia monooxygenase submit A gene (*amoA*) is an especially useful molecular marker for ammonia-oxidizing bacteria, and the denitrification of ammonia-oxidizing bacteria (AOB) is catalyzed by a copper-containing nitrite reductase (*nirK*). The nitrous oxide reductase enzyme (*nosZ*) represents denitrifiers [14]. The three functional genes were quantified using qPCR to determine the main $N_2O$ emission sources in the three reactors. Figure 5 shows the abundances of *amoA*, *nirK* and *nosZ* genes in aerobic granular sludge under different F/M ratios. The results indicated that there were big differences in the abundances of *amoA*, *nirK*, and *nosZ* genes in the three reactors. The abundance of *amoA* and *nosZ* in R3 were the lowest; in contrast, the abundance of *nirK* was the highest.

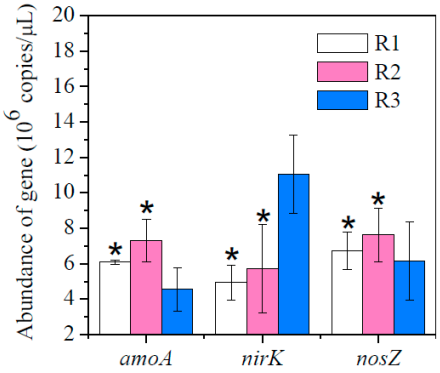


**Figure 5.** Quantitative changes in the abundances of functional genes in aerobic granular sludge SBARs under different F/M ratios.

## 4. Discussion

### *4.1. Characterization of Aerobic Granular Sludge*

#### 4.1.1. The Stability of Aerobic Granular Sludge

We found that the settling ability of R3 was poor, and aerobic granules of R3 exhibited a loose, fluffy morphology. This indicated that the granules were unstable. However, this was inconsistent

with the previous studies finding that glucose-fed aerobic granular sludge had a compact shape under a higher organic loading (15 kg COD/($m^3$·d)), with the same MLSS as for R3 [26]. This was likely due to the organic loading threshold for the instability of aerobic granules depending on the varieties of substrates [27]. The granules could not suffer higher organic loadings when the main carbon source was sodium acetate, however, glucose-fed granules could sustain a higher organic loading.

Furthermore, the dominance of filamentous bacteria resulting in the porous structure of the granules was another contributor to the instability of the aerobic granules in R3. It has been reported that the overgrowth of filamentous microorganisms could result in the disintegration of granules [28]. However, excess filamentous bacteria were generally caused by lower F/M ratios in previous study [28]. This was due to low DO concentrations (<1.1 mg/L) having a strong positive effect on the proliferation of filaments [29]. Therefore, the lowest DO concentration in R3 among the three reactors resulted in the dominance of filamentous bacteria. In contrast, rod-shaped bacteria were the predominant microorganisms in R1 and R2 with higher DO concentrations.

Moreover, PS was also an important factor for the unstable structure of aerobic granules in R3. PS has been reported as a polymeric viscous material that can stick to microbes and enhance the aggregation of microbes. In addition, the filamentous PS among the microbes can connect the dispersive microbes and the communities [30]. Therefore, the fewer PS of R3, relative to R1 and R2, partially resulted in the poor stability of granular sludge.

#### 4.1.2. The Characteristics of EPS

The EPS of R3 at high F/M ratios was the largest among the different F/M ratios. This could be explained by sufficient organic materials sustaining the metabolism of many microbes. In addition, the storages in EPS can serve as substrates for bacterial maintenance in the famine period; the biomass at low F/M ratios would consume more EPS for bacterial maintenance, which would result in less EPS. The content of humic acid in R3 was the most abundant among the three reactors. It has been reported that the sludge retention time affects the content and composition of EPS, and a low sludge retention time could result in a high content of humic acid [31]. Based on the amount of sludge discharge from the three reactors, the sludge retention time was the lowest in R3 among the three reactors. The lowest sludge retention time in R3 may have resulted in the most abundant humic acid among the three reactors.

### *4.2. $N_2O$ Emission of Aerobic Granular Sludge*

Nitrifer denitrification and heterotrophic denitrification are widely acknowledged to be the two main processes responsible for $N_2O$ emissions in aerobic granular sludge [16]. $N_2O$ emissions during nitrifier denitrification and heterotrophic denitrification have been known to be executed by certain bacteria species, mainly AOB and denitrifiers [14,15]. To determine the mechanism of $N_2O$ emission in aerobic granular sludge, the genes *nirK* and *nosZ*, which represented the denitrification of AOB, and denitrifiers were quantified using qPCR method. The results of this study showed that the abundance of *nirK* was the highest, while the abundance of *nosZ* in R3 was the lowest among the three reactors. This indicated that the high $N_2O$ emissions in R3 were mainly from nitrifier denitrification, and heterotrophic denitrification was inhibited.

The inhibited heterotrophic denitrification was due to the anaerobic environment being destroyed by the DO diffusing into the sludge particles of R3 with the porous structure. The poor performance of heterotrophic denitrification resulted in the accumulation of $NO_2^-$-N. However, $NO_3^-$-N was not accumulated at the end of batch experiment. This partially resulted from adsorption by the porous granules of R3. Moreover, as discussed in detail later, the inhibited nitrification was another contributor to the low $NO_3^-$-N concentration. The enhanced nitrifier denitrification might be attributable to the lowest DO concentration and the accumulation of $NO_2^-$-N in R3. It has been reported that oxygen stress was an important factor for nitrifier denitrification, and $N_2O$ yields under oxygen limiting

conditions by nitrifier denitrification could increase [32]. Furthermore, the accumulated $NO_2^-$-N promoted the denitrification of AOB through boosting the expression of the *nirK* gene [33].

The lowest abundance of *amoA* indicated that nitrification in R3 was inhibited. The lower DO concentration in R3 was the major driver for the inhibited nitrification [34]. However, the $NH_4^+$-N removal was not inhibited. This was likely due to adsorption by the porous granules of R3. Nevertheless, the specific adsorbing capacity was not determined in this study because of the complex adsorption process. Therefore, further investigation is needed. In addition, the relatively low sludge retention time may have contributed to the poor nitrification. It has been reported that a low sludge retention time affects the ammonia oxidation [35].

## 5. Conclusions

It was found that the F/M ratio had an important effect on the $N_2O$ emissions in aerobic granular sludge SBARs. $N_2O$ emissions increased with an increased F/M ratio. The ratios of $N_2O$-N to the removed $NH_4^+$-N were 2.77%, 2.94% and 3.79% at F/M ratios of 0.2, 0.34, and 0.67 g COD/g SS, respectively. The high $N_2O$ emission under the F/M ratio of 0.67 g COD/g SS was mainly from nitrifier denitrification rather than heterotrophic denitrification. The heterotrophic denitrification was destroyed by the DO diffusing into the sludge particles at the high F/M ratio of 0.67 g COD/g SS with porous structures. The enhanced nitrifier denitrification may have been attributable to the low DO concentration and the accumulation of $NO_2^-$-N. This study could provide scientific reference for research on $N_2O$ emissions, and can offer guidance for conducting risk assessment and enhancing our ability to predict $N_2O$ production in aerobic granular sludge at different F/M ratios.

**Acknowledgments:** This research was supported by the National Natural Science Foundation of China (51178254, 51208283 and 51508309).

**Author Contributions:** N.G., J.Z. and H.-J.X. conceived and designed the experiments; N.G. and L.-R.T. performed the experiments; N.G., Z.-Y.T. and J.-N.L. analyzed the data; J.Z. and S.-G.W. contributed reagents/materials; N.G. wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

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