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Nitrifiers activity and community characteristics under stress conditions in partial nitrification systems treating ammonium-rich wastewater

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ABSTRACT

Long-term exposure of nitrifiers to high concentrations of free ammonia (FA) and free nitrous acid (FNA) may affect nitrifiers activity and nitrous oxide (N₂O) emission. Two sequencing batch reactors (SBRs) were operated at influent ammonium nitrogen (NH₄-N) concentrations of 800 mg/L (SBR_H) and 335 mg/L (SBR_L), respectively. The NH₄-N removal rates in SBR_H and SBR_L were around 2.4 and 1.0 g/L/day with the nitrification efficiencies of 99.3% and 95.7%, respectively. In the simulated SBR cycle, the N₂O emission factors were 1.61% in SBR_H and 2.30% in SBR_L. N₂O emission was affected slightly by FA with the emission factor of 0.22%–0.65%, while N₂O emission increased with increasing FNA concentrations with the emission factor of 0.22%–0.96%. The dominant ammonia oxidizing bacteria (AOB) were *Nitrosomonas* spp. in both reactors, and their relative proportions were 38.89% in SBR_H and 13.36% in SBR_L. Within the AOB genus, a species (i.e., operational taxonomic unit [OTU] 76) that was phylogenetically identical to *Nitrosomonas europaea* accounted for 99.07% and 82.04% in SBR_H and SBR_L, respectively. Additionally, OTU 215, which was related to *Nitrosomonas stercoris*, accounted for 16.77% of the AOB in SBR_L.

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Introduction

Wastewaters such as landfill leachate and anaerobic digester effluent usually contain high concentrations of ammonium nitrogen (NH₄-N). When the conventional nitrification–denitrification process is applied to treat the ammonium-rich wastewater, it requires a high-energy input. Usually, the organic carbon contained cannot satisfy the requirement of denitrification. On the other hand, partial nitrification rather than full nitrification possesses advantages of 25% lower oxygen consumption, 40% lower external organic

carbon requirement, and 63% lower sludge production (Bernet et al., 2001; Fux et al., 2006; Wang and Yang, 2004). Therefore, partial nitrification (nitrification) processes have been developed to treat ammonium-rich wastewater (Lv et al., 2016).

How to achieve high loading rates and how to maintain stable and high nitrification efficiencies are two key aspects for partial nitrification. Maintaining high substance gradients can improve microbial activities efficiently. Therefore, sequencing batch reactor (SBR) systems with the substrate gradients can be applied to achieve high pollutant removal. In the partial

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nitrification system operated at high loading rates, activated sludge would be exposed to high concentrations of free ammonia (FA), free nitrous acid (FNA) and salinity during the long-term operation. Generally, stress conditions such as high salinity may inhibit the activities of microorganisms due to the plasmolysis (She et al., 2016). However, Bassin et al. (2012) found that the proportion of ammonia oxidizing bacteria (AOB) in microbial communities increased with increasing the salt concentration (up to 20 g/L).

The key point for achieving the stable nitrification is to inhibit the activity of nitrite oxidizing bacteria (NOB) while maintaining the activity of AOB. FA and FNA affect nitrite accumulation because their inhibition effects on NOB are stronger than those on AOB. For example, Anthonisen et al. (1976) reported that the inhibition concentrations of FA on AOB were 10–150 mg/L, while those on NOB were 0.1–1.0 mg/L. In addition, AOB can adapt to high concentrations of FA up to 122–124 mg/L after the long-term acclimation, and yet the activity of NOB was mostly inhibited under the same condition (Liang and Liu, 2007). The activity of AOB was still maintained at a relatively high level (the specific $\text{NH}_4\text{-N}$ oxidation rate of 0.9 g/g/day) at the FNA concentration of 4.1 mg N/L (Kinh et al., 2017). However, the activity of NOB was completely inhibited at the FNA concentration of 0.02 mg/L (Vadivelu et al., 2007). Further studies about the inhibition effects of FA and FNA on these nitrifiers should be carried out, especially for those after the long-term acclimation of microbial communities under high concentrations of FA and FNA.

High concentrations of FNA may enhance nitrous oxide (N_2O) emission. N_2O is a strong greenhouse gas, and its greenhouse effect is 300 times higher than that of CO_2 with the life cycle of 114 years (IPCC, 2007). Therefore, even low amounts of N_2O emission influence a lot to the environment. Previous studies about N_2O emission during nitrification treating ammonium-rich wastewater mainly focused on nitrifiers acclimated under relatively low loading rates. For example, Castro-Barros et al. (2016) investigated the effect of nitrite on N_2O emission in a nitrifying lab-scale reactor fed with low-strength ammonium wastewaters and indicated that the N_2O emission factor increased linearly from 0.16% to 1.5% with the step wise increases in the nitrite concentration from 0 to 150 mg/L. Besides, Law et al. (2011) investigated the effect of pH on N_2O emission for activated sludge acclimated under a low loading rate, and found that N_2O emission was influenced little by FA and FNA.

In this study, with a special focus on the nitrification under high ammonium loading rates, the responses of system performance to multiple stressors (i.e., high concentrations of FA, FNA and salinity) and the effects of these stressors on the AOB and NOB activities and N_2O emission were investigated so as to evaluate the sustainable high efficiencies of nitrogen removal.

1. Materials and methods

1.1. Experimental system

Two cylindrical plexiglass SBRs (50 cm in height and 15 cm in diameter) with the working volume of 6 L were used to acclimate nitrifiers at $30 \pm 1^\circ\text{C}$. Each 4-hr SBR cycle consisted of phases of 10-min filling, 180-min aeration, 35-min settling and 15-min

withdrawal/idle. The feeding volume in each SBR cycle was 3 L, and the same volume was discharged, resulting in a hydraulic retention time of 8 hr. The feed and withdrawal of reactors were realized by timer-controlled peristaltic pumps. The inoculated sludge was eluted from backwashed biofilm carriers taken from Xili Reclaimed Water Plant, Shenzhen, China.

The two SBRs were operated at different $\text{NH}_4\text{-N}$ loading rates. The SBR with a high loading rate (SBR_{H}) contained five stages with the $\text{NH}_4\text{-N}$ concentration gradually increased from 100 to, 200, 400, 600 and 800 mg/L, and correspondingly NaHCO_3 was increased from 1800 to 3600, 7200, 10,800 and 14,000 mg/L to adjust the alkalinity. During each stage, when the effluent $\text{NH}_4\text{-N}$ concentration was below 15 mg/L, the influent $\text{NH}_4\text{-N}$ and NaHCO_3 concentrations were increased to the next stage. The influent $\text{NH}_4\text{-N}$ concentration was 335 mg/L for SBR with a low loading rate (SBR_{L}), and accordingly the concentration of NaHCO_3 was 6000 mg/L. Other components of the synthetic wastewater were the same: 50 mg/L Na_2HPO_4 , 70 mg/L CaCl_2 , 400 mg/L MgSO_4 and 0.4 mL/L of trace element solutions. The specific components of trace element solution were prepared according to Smolders et al. (1994).

The sludge retention time (SRT) was controlled at 10 days during the initial acclimation phase, and there was no sludge discharge after three SRTs (30 days) to increase the concentration of the mixed liquor suspended solids (MLSS).

1.2. Batch experiments

Batch experiments were carried out to investigate effects of FA and FNA on the AOB and NOB activities and N_2O emission for the acclimated nitrifiers. The batch reactors with the working volume of 500 mL were made from capped glass flasks, each with four ports on the cap for liquid sampling, gas sampling and aeration. During the batch experiments, the glass bottles were placed into water bath at 30°C and mixed with magnetic stirrers.

For the effects of FA on the AOB and NOB activities and N_2O emission under the dissolved oxygen (DO) concentrations of around 1.0 mg/L, 1500 mL activated sludge was taken from the parent reactors before the end of the aeration phase and then divided into 5 portions with each volume of 300 mL. After centrifugation, supernatant was discharged and the residual sludge was re-suspended with synthetic wastewater to 500 mL to achieve different initial concentrations of $\text{NH}_4\text{-N}$ (100, 200, 400, 800 and 1600 mg/L), and then transferred into batch reactors. Ten-milliliter mixed liquor was taken from each batch reactor to measure MLSS and mixed liquor volatile suspended solids (MLVSS). Finally, batch reactors were aerated to achieve the DO concentrations of 1.0 mg/L. Each batch experiment was lasted for 1 hr, water and gas samples were taken at intervals of 10 min, and simultaneously, pH and DO were measured. After experiments, nitrogen including $\text{NH}_4\text{-N}$, nitrite nitrogen ($\text{NO}_2\text{-N}$), gaseous $\text{N}_2\text{O-N}$ and aqueous $\text{N}_2\text{O-N}$ concentrations in both water and gas samples were analyzed.

Effects of FNA on AOB and NOB activities were also examined under the DO concentrations of around 1.0 mg/L. Experimental methods were similar to those of the experiment of FA effect. During the experiments, the initial $\text{NH}_4\text{-N}$ concentration was fixed at 100 mg/L, while the initial $\text{NO}_2\text{-N}$ concentrations applied were 100, 200, 400, 800 and 1600 mg/L.

1.3. Analytical methods

Mixed liquor was taken to measure MLSS and MLVSS. After the water sample was centrifuged, supernatant was taken for the analyses of $\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$ and nitrate nitrogen ($\text{NO}_3\text{-N}$) (APHA et al., 1999). DO and pH were determined by a DO meter (oxi 315i, WTW, Germany) and a pH meter (pH3110, WTW, Germany), respectively. FA and FNA were calculated according to the methods by Anthonisen et al. (1976).

The activity of AOB (q_{AOB}) was characterized by the specific $\text{NH}_4\text{-N}$ utilization rate (mg N/g MLVSS/hr), whereas the activity of NOB (q_{NOB}) was characterized by the specific $\text{NO}_3\text{-N}$ production rate (mg N/g MLVSS/hr).

N_2O was determined by a gas chromatograph (Agilent 6820, Agilent Technologies, USA) equipped with an electron capture detector (ECD, Agilent Technologies, USA) and a HP-PLOT/Q column (J&W GC Columns, Agilent Technologies, USA) (Shen et al., 2014). The temperatures for detector and oven were 300 and 50°C, respectively. High purity nitrogen gas was used as the carrier gas at a flow rate of 15 mL/min. Pure N_2O gas was used as the standard sample for calibration.

Deoxyribonucleic acid (DNA) was extracted from all activated sludge using a PowerSoil DNA Isolation Kit (Laboratories Inc., CA, USA) according to the manufacturer's instructions. High-throughput Illumina sequencing of 16S rRNA gene amplicons was carried out to analyze microbial communities of activated sludge acclimated in the two reactors as described previously (Aoyagi et al., 2015). Three parallel samples of each reactor were run and analyzed according to Navarro et al. (2015). Briefly, polymerase chain reaction (PCR) primers used were 515f and 806r. The primer sequences were modified to include the Illumina adapter regions, and the reverse primer was encoded with 12-bp barcodes (Caporaso et al., 2012). PCR condition was that as described previously (Navarro et al., 2015). The PCR amplicons were purified firstly with an AMPure XP Kit (Beckman Coulter, USA), and then with a QIAquick gel extraction kit (QIAGEN, USA). The concentration of the purified DNA was determined with a Quant-iT PicoGreen dsDNA reagent and kit (Life Technologies, USA). The purified DNA and an internal control (PhiX Control V3; Illumina) were subjected to high-throughput sequencing with a 300-cycles MiSeq Reagent kit (Illumina) and a MiSeq sequencer. The removal of the PhiX, low-quality ($Q < 30$) and chimeric sequences, and the assembly of the paired-end sequences were described previously (Itoh et al., 2014). The sequences in the libraries were phylogenetically characterized by using the QIIME software (Caporaso et al., 2010). The operational taxonomic units (OTUs) were grouped

by a 97% sequence identity cut-off. Alpha-diversity indices (e.g., Chao1, Shannon, and Simpson reciprocal) were calculated with the QIIME software (Caporaso et al., 2010). Each diversity index was determined based on an equal amount ($n = 39,007$) of sequences subsampled from original libraries ($n = 10$). The DNA sequence data obtained in this study have been deposited in the MG-RAST database under the project title "Nitrifiers physiology and community characteristics under stress conditions in partial nitrification systems treating ammonium-rich wastewater in 2017" with the ID numbers 576810, 576813, 576816, 576819, 576822 and 576825 (6 libraries).

2. Results and discussion

2.1. System performance during the long-term operation

After the long-term operation, SBR_{H} and SBR_{L} achieved high efficiencies of nitrification. In SBR_{H} , the effluent $\text{NH}_4\text{-N}$ concentrations were less than 5 mg/L, and the concentration of $\text{NO}_3\text{-N}$ was 5.99 mg/L. Accordingly, the $\text{NH}_4\text{-N}$ removal efficiency was 99.8%, and the nitrification efficiency ($\text{NO}_2\text{-N}$ to the oxidized nitrogen ($\text{NO}_x\text{-N}$) ratio) was 99.3%. Influent $\text{NH}_4\text{-N}$ concentration of SBR_{L} was around 335 mg/L. After the 28-day operation, SBR_{L} reached steady state, and the effluent $\text{NH}_4\text{-N}$ concentrations were below 1 mg/L; the $\text{NO}_3\text{-N}$ concentrations were less than 15 mg/L. Accordingly, the $\text{NH}_4\text{-N}$ removal efficiency was 99.8%, and the nitrification efficiency was 95.7%. At the steady states, MLVSS of SBR_{H} and SBR_{L} were 3.45 and 2.25 g/L, respectively. Table 1 shows the comparisons of the $\text{NH}_4\text{-N}$ removal loading rates of the present systems treating ammonium-rich wastewaters with some of previously reported systems. The $\text{NH}_4\text{-N}$ removal loading rate obtained in this study was higher than those from most previous studies in Table 1, i.e., less than 1.0 g/L/day. However, Torà et al. (2014) applied continuously feedback control loops treating ammonium-rich wastewater and obtained the $\text{NH}_4\text{-N}$ removal loading rates of 3.6–6.7 g/L/day, which was much higher than the $\text{NH}_4\text{-N}$ removal loading rates obtained in this study. The loading rates in this study could be further increased through (1) improving MLVSS (such as cultivating granule sludge), (2) shortening the duration of the settling phase and (3) increasing DO concentrations during the reaction phase. Further investigations should be carried out to evaluate these effectiveness in this field.

Dynamics of nitrogen concentrations during a typical cycle of SBR_{H} and SBR_{L} are shown in Fig. 1a and b, respectively.

Table 1 – Comparisons on $\text{NH}_4\text{-N}$ removal loadings during ammonium-rich wastewater treatment between previous studies and this study.

System	Influent $\text{NH}_4\text{-N}$ (mg/L)	NLR* (kg N/m ³ /day)	$\text{NH}_4\text{-N}$ removal	Reference
SBR^*	333–1667	0.85–1.2	22%–78%	Ganigué et al., 2009
SBR^*	600 ± 40	0.52	Totally	Antileo et al., 2013
Granular in SBR^*	440–758	1.75	Totally	Torà et al., 2013
MBBR*	600–1200	1.5	83%–91%	Kim et al., 2006
Constant flow	600–800	5.0–9.3	47%–72%	Torà et al., 2014
SBR^*	800 ± 45	2.4	99.8%	This study
SBR^*	335 ± 15	1.0	99.8%	This study

* NLR: nitrogen loading rate; SBR: sequencing batch reactor; MBBR: moving bed biofilm reactor.

Figure 1c shows the dynamics of FA and FNA concentrations in the two reactors. Most of $\text{NH}_4\text{-N}$ was gradually oxidized to $\text{NO}_2\text{-N}$, and only a small proportion was oxidized to $\text{NO}_3\text{-N}$. The specific $\text{NH}_4\text{-N}$ utilization rate and the specific $\text{NO}_3\text{-N}$ production rate were used to characterize the activities of AOB and NOB, respectively. In SBR_{H} , FA concentration decreased from 77.83 to 0.07 mg/L, and FNA concentrations were above 0.045 mg/L during the whole cycle. The AOB and NOB activities in the typical cycle were 43.96 and 0.06 mg N/g MLVSS/hr, respectively. While activity experiments with

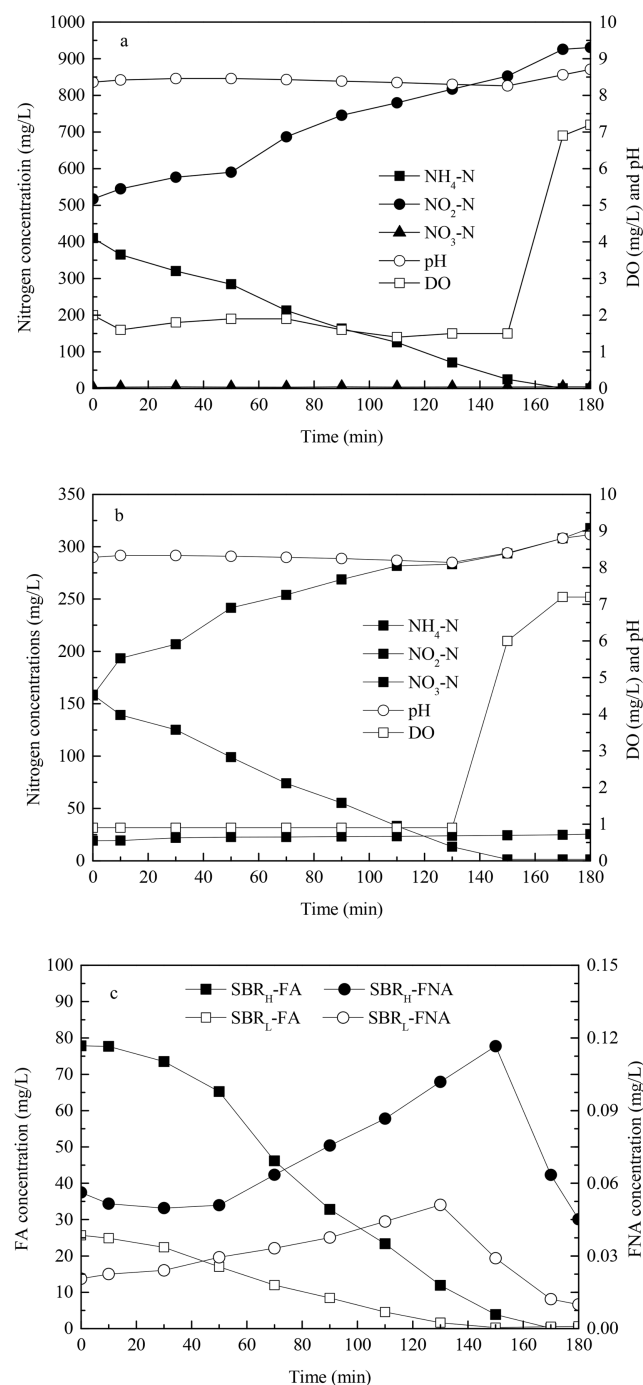


Fig. 1 – Dynamics of nitrogen, DO concentrations and pH during a typical cycle of SBR_{H} (a) and SBR_{L} (b), and dynamics of FA and FNA concentrations during typical cycle in SBR_{H} and SBR_{L} (c).

an initial $\text{NH}_4\text{-N}$ concentration of 100 mg N/L shows that activities of AOB and NOB were 50.67 and 8.43 mg N/g MLVSS/hr under the DO concentration of 4.0 mg/L, respectively (data not shown). In SBR_{L} , FA concentration decreased from 25.69 to 0.61 mg/L, while FNA concentrations increased from 0.021 to 0.051 mg/L (at 130 min) and then decreased to 0.010 mg/L. The AOB and NOB activities in the typical cycle were 28.32 and 0.85 mg N/g MLVSS/hr, respectively. AOB and NOB activities under the DO concentration of around 4.0 mg/L with an initial $\text{NH}_4\text{-N}$ concentration of 100 mg N/L were 65.58 and 13.93 mg N/g MLVSS/hr, respectively (data not shown). NOB activity in both reactors was almost completely inhibited. Although there were high levels of multiple stressors in SBR_{H} , the inhibition of AOB activity was relatively low. It implies that the susceptibility of AOB toward multiple stressors would decrease after the long-term acclimation, while that of NOB changed little. Some of previous studies also showed that AOB was adaptable to high FA concentrations through the acclimation. For example, NOB activity was almost completely inhibited, while AOB activity was inhibited little at the FA concentrations of 122–224 mg/L after the long-term acclimation (Liang and Liu, 2007). It indicates that the high efficiencies of ammonium removal can be achieved at high ammonium loading rates.

The stable and high nitrification efficiencies achieved in the two reactors could be due to the combined effects of DO, FA and FNA. In the two reactors, DO concentrations during the reaction phases were relatively low, and the competition ability of NOB for oxygen was lower than that of AOB, resulting in the inhibited NOB activity. Reportedly, AOB activity would not be inhibited when the DO concentrations were above 0.5 mg/L (Ruiz et al., 2003). In addition, there was a short reaction time after using up the $\text{NH}_4\text{-N}$, leaving only a limited reaction phase for NOB to utilize substrate. This could also contribute to the inhibition of NOB, facilitating nitrite accumulation (Lemaire et al., 2008). However, the effects of FA and FNA on nitrite accumulation were apparently different in the two reactors. Figure 1c shows the dynamics of FA and FNA concentrations during the typical reaction cycles in the two reactors. Furthermore, the effects of the single FA or FNA concentration on the AOB/NOB activity ratios are shown in Appendix A Fig. S1. The AOB/NOB activity ratios of activated sludge in SBR_{H} were overall higher than those in SBR_{L} , which was coincident with the results in the typical cycles. However, the AOB/NOB activity ratios in the typical cycle were much higher than that under the single FA or FNA inhibiting condition. It might be resulted from the fact that the combined effects of FA and FNA on AOB/NOB activity ratios were higher than that of the single FA or FNA effect. This meant that the combined effects of FA and FNA were the key factor for achieving and maintaining the high nitrification efficiencies.

During the simulated typical cycle study (Appendix A Fig. S2), the N_2O emission factor was 1.61% at the DO concentration of 1.0 mg/L in SBR_{H} with the corresponding specific $\text{NH}_4\text{-N}$ oxidation rate of 18.79 mg/g MLVSS/hr. On the other hand, the specific $\text{NH}_4\text{-N}$ oxidation rate was 48.87 mg/g MLVSS/hr in SBR_{L} and the N_2O emission factor was 2.30%. The relatively high N_2O emission factor in SBR_{L} might be caused by the high specific $\text{NH}_4\text{-N}$ oxidation rate. Desloover et al. (2011) obtained the N_2O emission factors of 5.1%–6.6% at the DO concentrations of around 1.0 mg/L in a partial nitrification system. However, Rodriguez-Caballero and Pijuan (2013) reported that

the N_2O emission factor was 0.8% at the DO concentrations of 0.8–1.5 mg/L in a partial nitrification SBR system. It implied that the FA and FNA concentrations in the reactor might influence the N_2O emission. The high FA and FNA concentrations might inhibit the gene expression and reductase activities related to denitrification pathway of AOB, thereby reducing N_2O emission.

2.2. Effects of FA and FNA on the AOB and NOB activities

FA and FNA inhibitions on the AOB and NOB activities are shown in Fig. 2. In SBR_H , when the FA concentrations were around 20 mg/L, AOB activity reached to the highest level. Meanwhile, when the FA concentrations were above 20 mg/L, AOB activity decreased with increasing FA concentrations. When FA concentrations increased from 20 to 110 mg/L, the reduction percentages of AOB activities were around 26.7%. In SBR_L , when the FA concentrations were above 10 mg/L, AOB activity decreased with increasing FA concentrations, the reduction percentages of AOB activities were around 43.6%. In SBR_H , when the FA concentrations were above 10 mg/L, NOB activity decreased with increasing FA concentrations. When FA concentrations further increased from 10 to 112 mg/L, the reduction percentage of NOB activity was 33.2%. In SBR_L , variation trends of NOB activities with increasing FA concentrations were also similar. When FA concentrations were above 10 mg/L, NOB activity decreased with increasing FA concentrations, and the reduction percentage was 62.5%. In the two reactors, the variation trends of AOB activity were different with varying FA concentrations, and the degree of FA inhibition on AOB in SBR_H was lower than that in SBR_L . In the

meantime, the variation trends of NOB activity with increasing FA concentrations were similar, but the degree of FA inhibition on NOB activity in SBR_H was lower than that in SBR_L . These indicated that the susceptibilities of AOB and NOB toward FA inhibition might become low after the long-term acclimation of these nitrifiers to high concentrations of FA.

In SBR_H , the AOB and NOB activities both decreased with increasing FNA concentrations. When FNA concentrations increased from 0 to 0.118 mg/L, the reduction percentage of AOB activity was 27.7%, while that of NOB activity was 54.4%. The AOB and NOB activities along with the increasing FNA concentrations were linear; however, the inhibition degree of NOB was higher than that of AOB at the same FNA concentration. Appendix A Fig. S1 also shows that the AOB/NOB activity ratios increased with increasing FNA concentrations. In SBR_L , the variation trends of the AOB and NOB activities with increasing FNA concentrations were similar. When FNA concentrations were below 0.02 mg/L, the AOB and NOB activities decreased little with increasing FNA concentrations. However, when FNA concentrations increased from 0 to 0.102 mg/L, the reduction percentages of the AOB and NOB activities were 71.9% and 70.0%, respectively. The inhibition degrees of AOB and NOB were similar, and there was little variation of the AOB/NOB activity ratios with increasing FNA concentrations. However, the inhibition degrees of FNA on AOB and NOB activities in SBR_L were higher than those in SBR_H . It meant that FNA inhibition was not the determinant factor for the high nitrification efficiency achieved in SBR_L .

2.3. Effects of FA and FNA on N_2O emission

Effects of FA and FNA concentrations on N_2O emission are shown in Fig. 3. There were no general trends of N_2O emission factor with increasing FA concentrations. In SBR_H , the N_2O emission factors were 0.22%–0.42%. In SBR_L , the N_2O emission factors were 0.34%–0.65%. When FA concentrations were below 40 mg/L, there was little difference of the N_2O emission factors in the two reactors. Meanwhile, when FA concentrations were above 40 mg/L, the N_2O emission factors of SBR_L were significantly higher than those of SBR_H .

In SBR_H , the N_2O emission factors were 0.22%–0.69% during the FNA experiment. When FNA concentrations were below 0.07 mg/L, N_2O emission generally increased with increasing FNA concentrations. On the other hand, when FNA concentrations were above 0.07 mg/L, N_2O emission decreased. In SBR_L , the N_2O emission factors were 0.32%–0.96%, and N_2O emission generally increased with increasing FNA concentrations. The variation trends of N_2O emission with increasing FNA concentrations were different in the two reactors. When FNA concentrations were below 0.07 mg/L, there was little difference in the two reactors. However, when FNA concentrations were above 0.07 mg/L, N_2O emission in SBR_H varied little, while it still increased in SBR_L . The effect of FNA on N_2O emission was higher than that of FA. Generally, the N_2O emission factor increased with increasing FNA concentrations, indicating that high FNA concentrations enhanced N_2O emission. However, N_2O emission in SBR_H varied little at high FNA concentrations (above 0.07 mg/L), which might result from the long-term operation at high concentrations of FNA. Under the high FNA concentrations, N_2O emission might be induced through the operation of denitrification pathway in AOB,

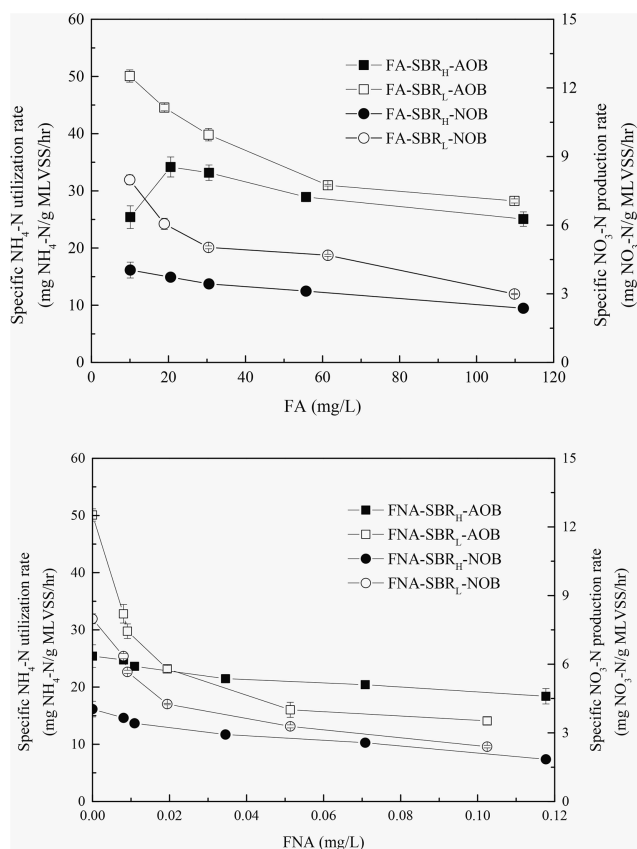


Fig. 2 – FA and FNA inhibition on AOB and NOB activities.

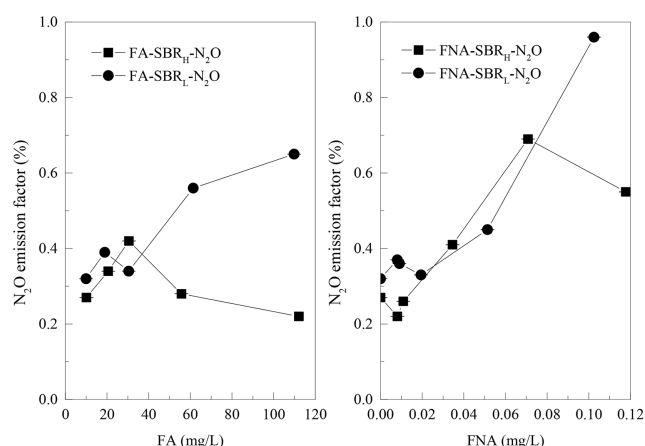


Fig. 3 – Effects of FA and FNA concentrations on N₂O emission.

while FNA had a strong toxic to N₂O reductase (Zhou et al., 2008). Therefore, the mechanism underlying the effect of FNA on N₂O emission is presumed to be as follows: (1) facilitating N₂O emission through AOB denitrification by high NO₂-N concentrations and (2) inhibiting the N₂O reductase activity by high FNA concentrations.

In this study, N₂O emission was strongly influenced by the acclimation conditions. The N₂O emission factor was higher in SBR_L than in SBR_H. It was possible that AOB denitrification might be inhibited when FA or FNA concentration was over the threshold value. Yu et al. (2010) obtained that N₂O generation in *Nitrosomonas europaea* was governed by the enzyme activity and metabolic level. During the long-term operation, the activated sludge microorganisms from SBR_H was acclimated to high FA and FNA concentrations, and the denitrifying activity of AOB was relatively low. In addition, the recovery of related genes transcription and expression might also be influenced by the FA and FNA concentrations. Therefore, N₂O emission increased with the increasing FA or FNA concentration at first, while it decreased when FA or FNA was over certain value. In addition, the dominant pathway of N₂O emission could also be influenced, when the activated sludge was acclimated at different FA and FNA concentrations. For example, NH₂OH oxidation could be the main pathway of N₂O emission, when activated sludge was acclimated at the relatively high NO₂-N (400–1000 mg/L) and high NH₄-N (500 mg/L) concentrations (Law et al., 2013).

2.4. Microbial community analysis

The summary of the Illumina sequencing data is shown in Table 2. The Chao1, Shannon and Simpson reciprocal indices showed that the diversity of microbial communities was higher in SBR_L than in SBR_H. It implied that the concentration of multiple stressors might lower the microbial diversity. Figure 4 shows the phylum- and class-level distribution of microbial community structure based on 16S rRNA gene sequence libraries of SBR_H and SBR_L. The phyla *Proteobacteria* (relative abundance: 54.9%) and *Chloroflexi* (31.0%) were dominant in SBR_H, whereas activated sludge in SBR_L was dominated by *Proteobacteria* (29.9%), *Chloroflexi* (22.1%) and *Bacteroidetes* (16.7%). Generally, *Bacteroidetes* are considered as efficient degraders

Table 2 – Summary of the illumina sequencing data.

Sample	No. of sequences	Diversity indices		
		Chao1	Shannon	1/Simpson
SBR _H	41,254 ± 1302	2667.5 ± 492.7	3.33 ± 0.60	3.98 ± 1.17
SBR _L	46,062 ± 3078	3219.1 ± 790.0	4.60 ± 0.37	9.91 ± 2.33

of organic matters with high molecular weights, such as polysaccharides and proteins (Cottrell and Kirchman, 2000). In the phylum *Proteobacteria*, *Betaproteobacteria* (46.0%) was the dominant class in SBR_H, while *Betaproteobacteria* (14.2%) and *Alphaproteobacteria* (9.2%) were dominant in SBR_L. Wagner et al. (1993) analyzed bacterial community structures in activated sludge from aeration tanks of the two-stage system at high and low loading rates, and indicated that *Proteobacteria* accounted for about 60%–75% of the total. Additionally, *Beta*- and *Gammaproteobacteria* formed the characteristic biota in the high-loading-rate tank, whereas the low-loading-rate tank was dominated by *Alphaproteobacteria*. The ratios of the phylum *Proteobacteria* in our study were relatively low, and the ratio of the class *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria* were also different.

In the two reactors, the main AOB was the genus *Nitrosomonas*, which accounted for 38.89% and 13.36% in SBR_H and SBR_L, respectively. It was consistent with the report by Sui et al. (2014), which found that the AOB proportion declined with decreasing influent ammonia concentrations. Zeng et al. (2013) indicated that higher ammonia concentration referred to lower AOB diversity and the predominant AOB was *Nitrosomonas* genus in partial nitrifying SBR. The relative abundances of OTUs affiliated within *Nitrosomonas* in the two reactors are shown in Table 3, in which the closest cultured relatives of these OTUs are also included. The operational taxonomic unit (OTU) 76 that was phylogenetically identical to *Nitrosomonas europaea* (accession no. NR117649.1; 100% sequence similarity) was dominant, accounting for 99.07% and 82.04% of the AOB genus in SBR_H and SBR_L, respectively. Additionally, OTU 215 that was related to *Nitrosomonas stercoris* (accession no. AB900133.1); 98% sequence

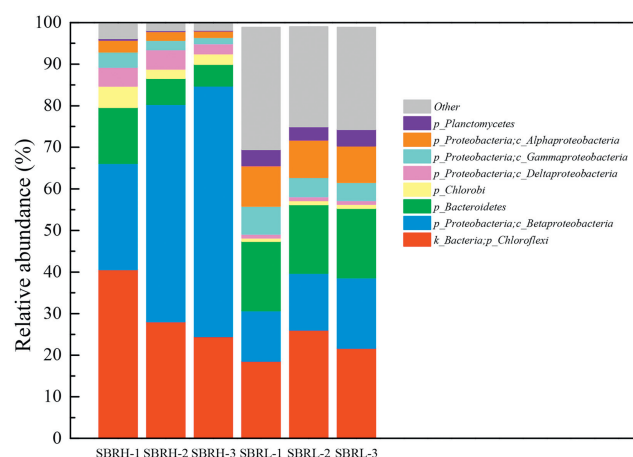


Fig. 4 – Microbial community structure at the phylum- or class-level in the two reactors (three parallel samples for each reactor).

Table 3 – Relative abundance OTUs within the genus *Nitrosomonas* in the two reactors.

OTU	Closest cultured relatives	Accession no.	Similarity (%)	Relative abundance (%)	
				SBR _H	SBR _L
76	<i>Nitrosomonas europaea</i>	NR117649.1	100	0.3853 ± 0.1878	0.1096 ± 0.0323
212251	<i>Nitrosomonas europaea</i>	NR117368.1	98	0.0016 ± 0.0009	0.0005 ± 0.0002
3499	<i>Nitrosomonas nitrosa</i>	NR104819.1	99	0.0004 ± 0.0002	1.39E-05 ± 1.21E-05
213487	<i>Nitrosomonas europaea</i>	NR114606.1	97	0.0003 ± 0.0002	9.69E-05 ± 9.34E-05
187452	<i>Nitrosomonas europaea</i>	NR117649.1	97	0.0002 ± 7.23E-05	6.74E-06 ± 1.17E-05
211370	<i>Nitrosomonas europaea</i>	AL954747.1	97	0.0002 ± 0.0001	6.74E-06 ± 1.17E-05
272911	<i>Nitrosomonas europaea</i>	NR040879.1	99	0.0001 ± 9.34E-05	6.74E-06 ± 1.17E-05
271689	<i>Nitrosomonas europaea</i>	NR117649.1	97	0.0001 ± 3.09E-05	2.23E-05 ± 2.16E-05
148851	<i>Nitrosomonas</i> sp.	KT308520.1	96	0.0001 ± 9.65E-05	2.7E-05 ± 4.67E-05
154630	<i>Nitrosomonas europaea</i>	KF618626.1	97	9.97E-05 ± 4.85E-05	0
152921	<i>Nitrosococcus mobilis</i>	KU860096.1	100	9.76E-05 ± 4.61E-05	0
252492	<i>Nitrosomonas</i> sp.	FN394323.1	98	5.90E-05 ± 5.33E-05	0
32683	<i>Nitrosomonas</i> Al-7K	AF043136.3	97	5.79E-05 ± 6.12E-05	1.39E-05 ± 1.21E-05
96745	<i>Nitrosomonas</i> sp.	KT308520.1	97	5.03E-05 ± 4.36E-05	1.35E-05 ± 2.33E-05
10414	<i>Nitrosomonas</i> sp.	AJ011920.1	98	4.19E-05 ± 3.11E-05	3.67E-05 ± 4.46E-05
268476	<i>Nitrosomonas</i> sp.	KP749218.1	97	4.08E-05 ± 2.80E-05	1.51E-05 ± 1.32E-05
215	<i>Nitrosomonas stercoris</i>	AB900133.1	98	3.29E-05 ± 2.86E-05	0.0224 ± 0.0041
120012	<i>Nitrosomonas europaea</i>	KF618626.1	97	1.73E-05 ± 3.00E-05	0.0001 ± 5.12E-05
9381	<i>Nitrosomonas europaea</i>	NR117649.1	96	0	0.0003 ± 5.29E-05
136651	<i>Nitrosomonas eutropha</i>	M96402.1	98	0	0.0003 ± 2.61E-05
8043	<i>Nitrosomonas</i> sp.	AF363293.1	95	0	5.86E-05 ± 4.49E-05
106001	<i>Nitrosomonas</i> sp.	HF678378.1	97	0	5.68E-05 ± 4.05E-05
118113	<i>Nitrosomonas eutropha</i>	NR027566.1	97	0	4.85E-05 ± 4.28E-05
79647	<i>Nitrosomonas</i> sp.	KT308519.1	97	0	4.54E-05 ± 3.95E-05

similarity accounted for 16.77% of the AOB genus in SBR_L. These indicated that *N. europaea* might have a high NH₄-N tolerance, which could be acclimated under high NH₄-N concentrations. OTU 136651 that was related to *Nitrosomonas eutropha*, which prefer higher NH₄-N concentrations were detected little in this study. It was consistent with the report by Park et al. (2015). Other OTUs which were closely related to *Nitrosococcus mobilis*, *Nitrosomonas stercoris* and *Nitrosomonas* sp. Al-7K existed as minor species in SBR_H, while a small quantity of OTUs which were related to *Nitrosomonas* sp. Al-7K and *Nitrosomonas stercoris* were detected in SBR_L.

3. Conclusions

The NH₄-N removal rates of SBR_H and SBR_L were around 2.4 and 1.0 g N/L/day with nitrification efficiencies of 99.3% and 95.7%, respectively. Furthermore, the N₂O emission factors in SBR_H and SBR_L were 1.61% and 2.30%, respectively. The inhibition of FNA on the AOB and NOB activities was strongly influenced by the acclimation conditions, and the inhibition degrees of FA or FNA on the nitrifiers activity in SBR_H were higher than those in SBR_L. N₂O emission was strongly influenced by FNA, for which the N₂O emission factor was 0.22%–0.96%, while FA has little influence with the N₂O emission factor of 0.22%–0.65%. High-throughput Illumina sequencing showed that the diversity of microbial community was higher in SBR_L than in SBR_H. The main AOB was the genus *Nitrosomonas*, accounting for 38.89% and 13.36% in SBR_H and SBR_L, respectively. Within the genus, an AOB species (i.e., OTU 76) that was phylogenetically identical to *Nitrosomonas europaea* might have a high NH₄-N tolerance, thereby contributing the effective nitrification efficiencies under the high FA stress conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jes.2017.12.020>.

REFERENCES

- Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G., 1976. Inhibition of nitrification by ammonia and nitrous acid. *J. Water Pollut. Control Fed.* 48 (5), 835–852.
- Antileo, C., Medina, H., Bornhardt, C., Muñoz, C., Jaramillo, F., Proal, J., 2013. Actuators monitoring system for real-time control of nitrification–denitrification via nitrite on long term operation. *Chem. Eng. J.* 223, 467–478.
- Aoyagi, T., Hanada, S., Itoh, H., Sato, Y., Ogata, A., Friedrich, M. W., et al., 2015. Ultra-high-sensitivity stable-isotope probing of rRNA by high-throughput sequencing of isopycnic centrifugation gradients. *Environ. Microbiol. Rep.* 7 (2), 282–287.
- APHA, AWWA, WEF, 1999. *Standard Methods for the Examination of Water and Wastewater*. 20th ed. (Washington DC, USA).
- Bassin, J.P., Kleerebezem, R., Muyzer, G., Rosado, A.S., Van Loosdrecht, M.C.M., Dezotti, M., 2012. Effect of different salt adaptation strategies on the microbial diversity, activity, and

- settling of nitrifying sludge in sequencing batch reactors. *Appl. Microbiol. Biotechnol.* 93, 1281–1294.
- Bernet, N., Dangcong, P., Delgenès, J.P., Moletta, R., 2001. Nitrification at low oxygen concentration in biofilm reactor. *J. Environ. Eng.* 127, 266–271.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6 (8), 1621–1624.
- Castro-Barros, C.M., Rodríguez-Caballero, A., Volcke, E.I.P., Pijuan, M., 2016. Effect of nitrite on the N₂O and NO production on the nitrification of low-strength ammonium wastewater. *Chem. Eng. J.* 287, 269–276.
- Cottrell, M.T., Kirchman, D.L., 2000. Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high molecular-weight dissolved organic matter. *Appl. Environ. Microbiol.* 66, 1692–1697.
- Desloover, J., De Clippeleir, H., Boeckx, P., Du Laing, G., Colsen, J., Verstraete, W., et al., 2011. Floc-based sequential partial nitrification and anammox at full scale with contrasting N₂O emissions. *Water Res.* 45 (9), 2811–2821.
- Fux, C., Velten, S., Carozzi, V., Solley, D., Keller, J., 2006. Efficient and stable nitrification and denitrification of ammonium-rich sludge dewatering liquor using an SBR with continuous loading. *Water Res.* 40, 2765–2775.
- Ganigué, R., Gabarró, J., Sánchez-Melsió, A., Rusalleda, M., López, H., Vila, X., et al., 2009. Long-term operation of a partial nitrification pilot plant treating leachate with extremely high ammonium concentration prior to an anammox process. *Bioresour. Technol.* 100 (23), 5624–5632.
- IPCC, 2007. Climate change 2007: synthesis report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change: Geneva, Switzerland.
- Itoh, H., Navarro, R., Takeshita, K., Tago, K., Hayatsu, M., Hori, T., et al., 2014. Bacterial population succession and adaptation affected by insecticide application and soil spraying history. *Front. Microbiol.* 5, 457.
- Kim, D.J., Lee, D.I., Keller, J., 2006. Effect of temperature and free ammonia on nitrification and nitrite accumulation in landfill leachate and analysis of its nitrifying bacterial community by FISH. *Bioresour. Technol.* 97 (3), 459–468.
- Kinh, C.T., Ahn, J., Suenaga, T., Sittivorakulpong, N., Noophan, P., Hori, T., et al., 2017. Free nitrous acid and pH determine the predominant ammonia-oxidizing bacteria and amount of N₂O in a partial nitrifying reactor. *Appl. Microbiol. Biotechnol.* 101 (4), 1673–1683.
- Law, Y., Lant, P., Yuan, Z., 2011. The effect of pH on N₂O production under aerobic conditions in a partial nitrification system. *Water Res.* 45 (18), 5934–5944.
- Law, Y., Lant, P., Yuan, Z., 2013. The confounding effect of nitrite on N₂O production by an enriched ammonia-oxidizing culture. *Environ. Sci. Technol.* 47, 7186–7194.
- Lemaire, R., Marcelino, M., Yuan, Z., 2008. Achieving the nitrite pathway using aeration phase length control and step-feed in an SBR removing nutrients from abattoir wastewater. *Biotechnol. Bioeng.* 100 (6), 1228–1236.
- Liang, Z., Liu, J., 2007. Control factors of partial nitrification for landfill leachate treatment. *J. Environ. Sci.* 19, 523–529.
- Lv, Y., Ju, K., Sun, T., Wang, L., Miao, R., Liu, T., et al., 2016. Effect of the dissolved oxygen concentration on the N₂O emission from an autotrophic partial nitrification reactor treating high-ammonium wastewater. *Int. Biodeter. Biodegrad.* 114, 209–215.
- Navarro, R.R., Aoyagi, T., Kimura, M., Itoh, H., Sato, Y., Kikuchi, Y., et al., 2015. High-resolution dynamics of microbial communities during dissimilatory selenate reduction in anoxic soil. *Environ. Sci. Technol.* 49 (13), 7684–7691.
- Park, H., Sundar, S., Ma, Y., Chandran, K., 2015. Differentiation in the microbial ecology and activity of suspended and attached bacteria in a nitrification-anammox process. *Biotechnol. Bioeng.* 112 (2), 272–279.
- Rodríguez-Caballero, A., Pijuan, M., 2013. N₂O and NO emissions from a partial nitrification sequencing batch reactor, exploring dynamics, sources and minimization mechanisms. *Water Res.* 47, 3131–3140.
- Ruiz, G., Jeison, D., Chamy, R., 2003. Nitrification with high nitrite accumulation for the treatment of wastewater with high ammonia concentration. *Water Res.* 37 (6), 1371–1377.
- She, Z., Zhao, L., Zhang, X., Jin, C., Guo, L., Yang, S., et al., 2016. Partial nitrification and denitrification in a sequencing batch reactor treating high-salinity wastewater. *Chem. Eng. J.* 288, 207–215.
- Shen, L., Guan, Y., Wu, G., Zhan, X., 2014. N₂O emission from a sequencing batch reactor for biological N and P removal. *Front. Environ. Sci. Eng.* 8 (5), 776–783.
- Smolders, G.J.F., van der Meij, J., van Loosdrecht, M.C.M., Heijnen, J.J., 1994. Model of the anaerobic metabolism of the biological phosphorus removal process, stoichiometry and pH influence. *Biotechnol. Bioeng.* 43 (6), 461–470.
- Sui, Q., Liu, C., Dong, H., Zhu, Z., 2014. Effect of ammonium nitrogen concentration on the ammonia-oxidizing bacteria community in a membrane bioreactor for the treatment of anaerobically digested swine wastewater. *J. Biosci. Bioeng.* 118 (3), 277–283.
- Torà, J.A., Moliné, E., Carrera, J., Pérez, J., 2013. Efficient and automated start-up of a pilot reactor for nitrification of reject water, from batch granulation to high rate continuous operation. *Chem. Eng. J.* 281, 319–325.
- Torà, J.A., Lafuente, J., García-Belinchón, C., Bouchy, L., Carrera, J., Baeza, J.A., 2014. High-throughput nitrification of reject water with a novel ammonium control loop: stable effluent generation for anammox or heterotrophic denitrification. *Chem. Eng. J.* 243, 265–271.
- Vadivelu, V.M., Keller, J., Yuan, Z., 2007. Free ammonia and free nitrous acid inhibition on the anabolic and catabolic processes of *Nitrosomonas* and *Nitrobacter*. *Water Sci. Technol.* 56 (7), 89–97.
- Wagner, M., Amann, R., Lemmer, H., Schleifer, K.H., 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* 59 (5), 1520–1525.
- Wang, J., Yang, N., 2004. Partial nitrification under limited dissolved oxygen conditions. *Process Biochem.* 39, 1223–1229.
- Yu, R., Kampschreur, M.J., Van Loosdrecht, M.C.M., Chandran, K., 2010. Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. *Environ. Sci. Technol.* 44 (4), 1313–1319.
- Zeng, T., Li, D., Zeng, H., Zhang, Z., Liu, L., Zhang, X., et al., 2013. Analysis of microbial population dynamics in a partial nitrifying SBR at ambient temperature. *Curr. Microbiol.* 66 (6), 614–620.
- Zhou, Y., Pijuan, M., Zeng, R.J., Yuan, Z., 2008. Free nitrous acid inhibition on nitrous oxide reduction by a denitrifying enhanced biological phosphorus removal sludge. *Environ. Sci. Technol.* 42, 8260–8265.