Microbial community characteristics during simultaneous nitrification-denitrification process: effect of COD/TP ratio

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Abstract

To evaluate the impact of chemical oxygen demand (COD)/total phosphorus (TP) ratio on microbial community characteristics during low-oxygen simultaneous nitrification and denitrification process, three anaerobic-aeration (low-oxygen) sequencing batch reactors, namely R1, R2, and R3, were performed under three different COD/TP ratios of 91.6, 40.8, and 27.6. The community structures of each reactor were analyzed via molecular biological technique. The results showed that the composition of ammonia-oxidizing bacteria (AOB) was affected, indicated by Shannon indexes of the samples from R1, R2, and R3.*Nitrosomonas* was identified to be the dominant AOB in all SBRs. Moreover, the copy numbers of nitrifiers were more than those of denitrifiers, and the phosphorus-accumulating organisms to glycogen-accumulating organisms ratio increased with the decrease of COD/TP ratio.

Keywords

Microbial community COD/TP ratio Simultaneous nitrification and denitrification Molecular biological technique

Introduction

Nutrient (mainly nitrogen and phosphorus) enrichment is acknowledged to be the main cause of eutrophication and is becoming one of the most serious environmental and ecological

concerns worldwide (Dupas et al. 2015). Nitrogen and phosphorus are now required to be more effectively removed during wastewater treatment, which leads to a great need of more efficient biological nutrient removal processes. Thus, simultaneous nitrification and denitrification (SND) process, which has higher nitrogen removal efficiency and low energy consumption, has recently attracted increasing attention for nutrient removal (Holman and Wareham 2005, Castro Daniel et al. 2009, Fu et al. 2009).

In traditional biological treatment processes, nitrogen removal is mainly achieved by separate anaerobic and aerobic phases and is generally carried out in separate bioreactors or by different aeration intervals. However, as it has been reported that some heterotrophic nitrifiers could denitrify nitrite and nitrate aerobically; nitrification and denitrification occurring concurrently in a single reactor under aerobic conditions is often referred as SND process (Chiu et al. 2007). Generally, SND occurs naturally inside microbial biofilms and flocs due to the oxygen gradient established across the biomass (Meyer et al. 2005). Moreover, some heterotrophic bacteria, e.g., *Alcaligenes faecalis* and *Thiosphaera pantotropha*, are capable of performing SND by using organic substrates aerobically as sources of carbon and energy to convert ammonium aerobically into nitrogen gas (Chiu et al. 2007). Meanwhile, *Nitrosomonas*-like ammonia-oxidizing bacteria (AOB) was widely reported to be capable of denitrification (Shrestha et al. 2002). The operating parameters of SND processes, such as oxygen concentration (Hocaoglu et al. 2011), temperature (Zhang et al. 2009), and C/N ratio (Chiu et al. 2007), were found to significantly affect microbial metabolisms, and consequently nutrient removal performance. C/P ratio is reported to significantly influence the nutrients removal and is an essential factor for the optimization of wastewater treatment process, such as anaerobic–anoxic/nitrification sequencing batch reactor (A_2N-SBR) (Wang et al. 2009), UniFed SBR process (Zhao et al. 2008), and SND process (Ge et al. 2010, Jia et al. 2013a). However, to date, special attempts have not yet been devoted to investigating the impact of chemical oxygen demand (COD)/ total phosphorus (TP) ratio on the performance of SND processes in terms of microbial community characteristics.

Additionally, in SND processes, denitrification is driven mainly by intracellular carbon source poly-β-hydroxyalkanoates (PHA), which is produced by phosphorus-accumulating organisms such as phosphate-accumulating organisms (PAOs) during biological phosphorus removal (Zeng et al. 2003, Wang et al. 2011). Under anaerobic conditions, PAOs are able to take up organic substrates and store them as PHA using the

energy obtained from glycogen utilization and hydrolysis of the intracellular stored polyphosphate. Under aerobic or anoxic conditions, PHA was oxidized for phosphorus uptake and denitrification. Hence, it is reasonably hypothesized that the COD/TP ratio, especially the phosphorus load, could influence the enrichment of PAOs as well as the synthesis of PHA, leading to the change of denitrification rate and SND efficiency.

Furthermore, it was reported that the fractions of nitrogen and phosphorus, removed by different biological pathways, strongly depended on C:N:P ratio of the influent (Wang et al. 2009). In particular, the ratio of COD/TP has been shown to significantly affect the microbial community structure and the distribution of PAOs and glycogen-accumulating organisms (GAOs), which were two dominating heterotrophic microbes during biological nutrient removal (Chuang et al. 2011). Many studies have shown that a low COD/TP ratio was favorable to the growth of PAOs instead of GAOs in the enhanced biological phosphorus removal processes (EBPR) (Mino et al. 1998, Thomas et al. 2003). However, in SND process, the microbial community was more complex than that in the EBPR process. A detailed analysis of microbial community characteristics would, therefore, provide valuable information to the better understanding of nutrient removal mechanisms in SND processes under different COD/TP ratios.

The effect of COD/TP ratio on SND performance (i.e., nutrients removal efficiency) has been reported in our previous work (Jia et al. 2013a). As a more fundamental follow-up work, the main effort herein was focused on the microbial community characteristics in order to better understand the underlying mechanisms regarding the impact of COD/TP ratio on SND performance. The community structures of total bacteria (based on 16S rRNA), nitrifiers (AOB based on ammonia monooxygenase submit A gene (*amoA*)), and denitrifiers (based on nitrous oxide reductase gene (*nosZ*)) were analyzed by polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) technique and real-time quantitative PCR (qPCR) detecting system. Besides, the PAOs/GAOs ratio was investigated by fluorescent in situ hybridization (FISH).

Materials and methods

SBR setup and operation

Experiments were performed in three identical lab-scale anaerobic-aeration SBRs (namely R1, R2, and R3), which were operated in parallel under different COD/TP ratios. Each SBR has an effective volume of 5 L. One operating cycle consisted of four stages in series, i.e.,

anaerobic stage (90 min), aeration stage (180 min), settling stage (70 min), and decant stage (20 min). Synthetic wastewater with same COD and ammonium but different TP concentrations were fed into three SBRs, corresponding to the average influent COD/TP ratios of 91.6, 40.8, and 27.6 for R1, R2, and R3, respectively, and theoretical COD/TN ratio of 10.0 for all three reactors. The schematic diagram of the SBR system and detailed composition of synthetic wastewater can be found in our previous work (Jia et al. 2013a).

All SBRs were seeded with the sludge collected from a parent SND SBR, which has been running for more than 1 year in Shandong provincial key Laboratory of water pollution control and resource reuse. The water temperature was maintained at 25 ± 2 °C over the entire experimental period. The solids retention time (SRT) was kept at 20 days for all three reactors to eliminate the influence of operation parameter on biomass diversities, and the mixed liquor suspended solid (MLSS) concentration was approximately in the range of 3,000–3,500 mg/L. An on/off control system was used to control air supply during aeration stage to keep the dissolved oxygen (DO) level between 0.35 and 0.80 mg/L. The DO profiles of three SBRs were shown in Fig. S1 supplementary data.

After running under the selected COD/TP ratios for over 3 months, all SBRs reached their steady state. The concentrations of the effluent were then measured to investigate the nutrient removal efficiency.

DNA extraction and PCR-DGGE

Once the SBRs reached steady state, the sludge samples of each reactor were collected and centrifuged for DNA extraction. The total genomic DNA was extracted from sludge sample using PowerSoil™ DNA Isolation Kit (MO-BIO Laboratories, USA). In order to investigate the total bacteria, nitrifiers and denitrifiers community composition in three SBRs, the bacterial 16S rRNA gene and functional genes *amoA* and *nosZ*were amplified using primer sets 338 F/518R, *amoA*-1 F/*amoA*-2R, and *nosZ*-F/*nosZ*-1622R, respectively (Ibekwe et al. 2003, Enwall et al. 2005, Zhang et al. 2011). A 40-nucleotide GC-clamp was added on the 5′ end of the forward primers to improve the detection of sequence variations in amplified DNA fragments by subsequent DGGE.

For functional genes amplification, PCR reactions were carried out according to the protocol described previously (Hu et al. 2011). The PCR protocol for 16S rRNA amplification was set as follows: 95 °C for 5 min, 16 cycles of 94 °C for 30 s, 65 °C for 30s, and 72 °C for 45 s, with 0.5 °C decrease of the annealing temperature every cycle, followed

by 19 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s, and a final extension cycle at 72 °C for 7 min.

DGGE, cloning, sequencing, and phylogenetic analysis

DGGE analysis was conducted using the Bio-Rad Dcode system (Bio-Rad, USA). Electrophoresis was performed at 120 V for 7.5 h in 1× TAE buffer at a constant temperature of 60 °C. The liner gradients were optimized for efficient separation of bands and were shown as follows: 25–40 % for *amoA*, 30–50 % for *nosZ*gene and 38–52 % for 16S rRNA. To visualize bands, DGGE gels were stained by ethidium bromide and photographed under UV-light after electrophoresis. The species diversity was measured by *Shannon–Wiener*index (*H'*), which was calculated by the following equation:

$$H' = \sum_{i=1}^{s} p_i \log_e p_i$$

where p_i represents the intensity proportion of band *i* in the DGGE profile and *s* is the total number of bands.

Specific bands were excised, washed, and dissolved in sterile water. Then the product was used as template and re-amplified with appropriate primes. The PCR protocol for re-amplification was the same as above described except with free GC-clamp former primers. Following the manufacturer's protocol, the PCR amplicons were purified using the E.Z.N.A® Gel Extraction Kit (OMEGA), and then ligated into the pMD19-T Vector (TaKaRa, Japan) and were further transformed into competent cells DH5α (TaKaRa). White colonies were picked from each cloned sample and cultivated overnight for sequencing (Sangon Biotech., China). The obtained sequences were compared with the other available sequences in the GenBank by BLAST search. Phylogenetic trees were conducted by using MEGA 5, the neighbor-joining method with a bootstrap of 1,000 replications.

Quantitative PCR

Nitrifiers, denitrifiers, and total bacteria genes were quantified by Roche LightCycler®480 on SYBR Green I method. All the samples were investigated with primer sets and protocols as the normal PCR. Reaction mixtures contained 1 μL of template DNA, 10 μL of SYBR® Premix Ex Taq™ (TaKaRa), 0.4 μM primer (without GC-clamp), and of 7 μL ddH_2O. Standard curves for qPCR assays were developed using the plasmids extracted from the pure cultures of correct insert clones of each target gene. The amplification was monitored and

analyzed by the C_t value. Each PCR was performed three times. The specificity of PCR for each target gene was checked using melting curve analysis.

FISH

Sludge sampled from each SBR was firstly fixed in 4 % paraformaldehyde and incubated for 180 min at 4 °C. After fixation, samples were centrifuged for 5 min at 12,000 rpm, then washed twice in 1× phosphate buffer saline (PBS), and re-suspended in volume of 1:1 ethanol/PBS buffer for storage at −20 °C. The fixed samples were dried overnight on a hybridization slide and then incubated in 50, 80, and 100 % ethanol for 3 min per each solution. After dehydration, the tagged hybridization buffer (10 μL) and 25 ng of tagged probe mixture were added to each sample and incubated for 3 h in a humid chamber at 46 °C. The probe mixtures for total bacteria (EubMix) contained equal EUB338, EUB338-II, and EUB338-III. The probe mixtures for PAOs (PAOMix) and GAOs (GAOMix) contained equal PAO462, PAO846 and equal GAOQ431, GAOQ989, respectively. After hybridization, the microscope slides were immersed into 50 mL of tagged washing solution consisting of 1 M Tris (pH 8), 10 % SDS (w/v), and 5 M NaCl for 15 min at 48 °C. The samples were observed with an epifluorescence microscope, and the PAOs/GAOs ratio was estimated using Image-Pro Plus 6.0. The probes and experimental parameters are described in Table S1 in supplementary data.

Results and discussion

Effect of COD/TP ratio on microbial community structure

After running for over 3 months, the pollution removal efficiency and SND efficiency in each reactor were shown in Table S2. In all three SBRs, the SND efficiency was above 80 %, regardless of COD/TP ratio, indicating the occurrence of SND process. Both TP and TN removal efficiencies were enhanced along with the decrease of COD/TP ratio, and R3 gained relatively high nitrogen and phosphorus removal efficiency under low COD/TP ratio. It was mainly achieved by some PAOs capable of denitrification, i.e., denitrifying PAOs (DPAOs), using PHA stored in the anaerobic stage as carbon source and nitrite/nitrate as electron acceptors (Jia et al. 2013a).

In order to get an in-depth understanding regarding the impact of COD/TP ratio on SND performance, the microbial community characteristics were determined by molecular biological technique. The community structure of total bacteria was investigated by PCR-

DGGE and the DGGE patterns based on 16S rRNA gene are shown in Fig. 1a. It can be seen that the DGGE patterns of three sludge samples from SBRs under different COD/TP ratios were similar with no considerable differences. The similarities of different lanes of total bacteria analyses were also calculated based on the DGGE patterns and a dendrogram was constructed using the UPGMA method (Fig. 1b). For calculation of the Shannon diversity index, each band was considered as a unique species. The calculated Shannon–Wiener indexes of total bacteria in R1, R2, and R3 were 3.05, 3.02, and 2.86, respectively.

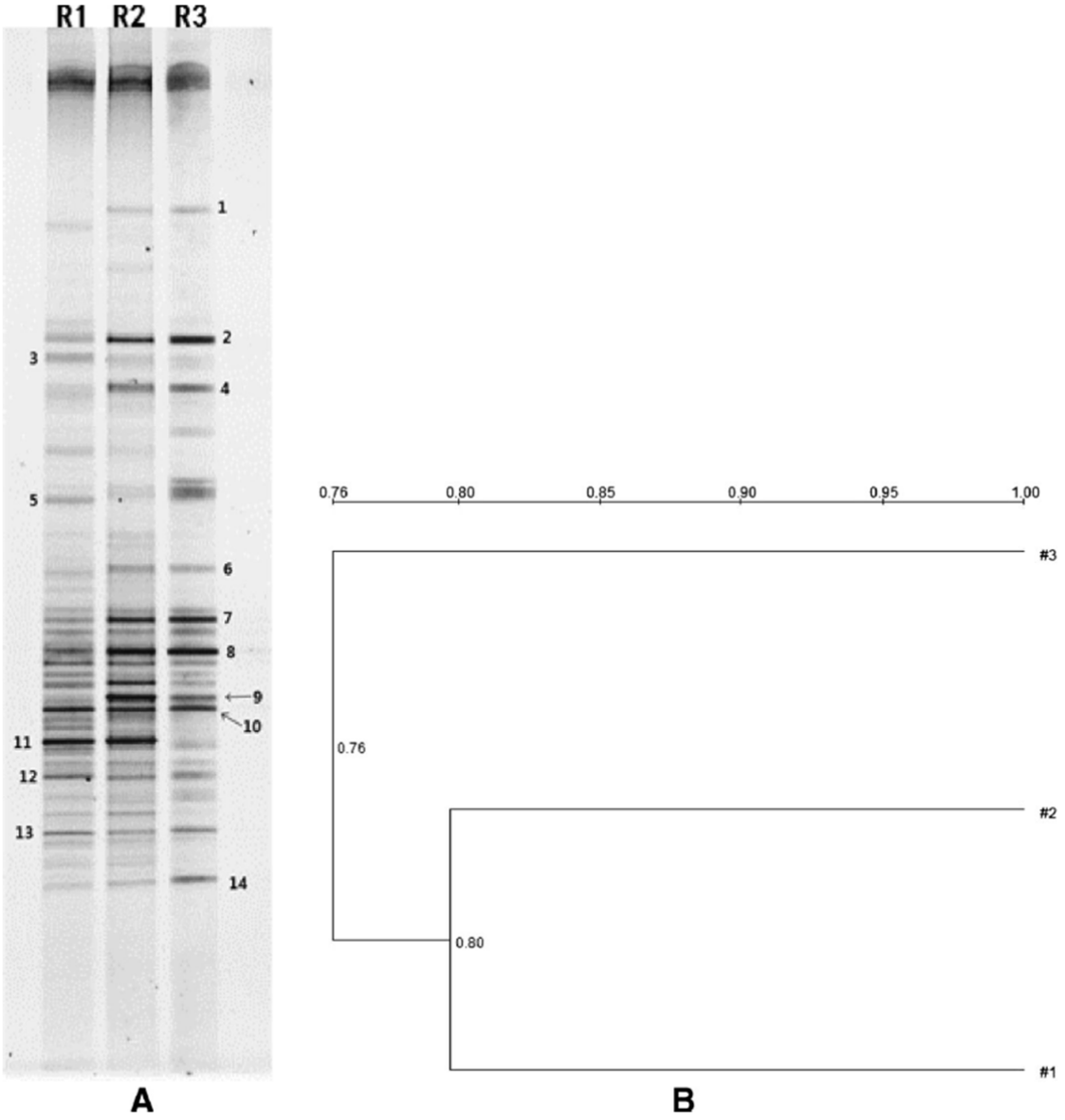

Fig. 1 a DGGE profiles of total bacteria and **b** cluster analysis of sludge samples from SBRs under different COD/TP ratios

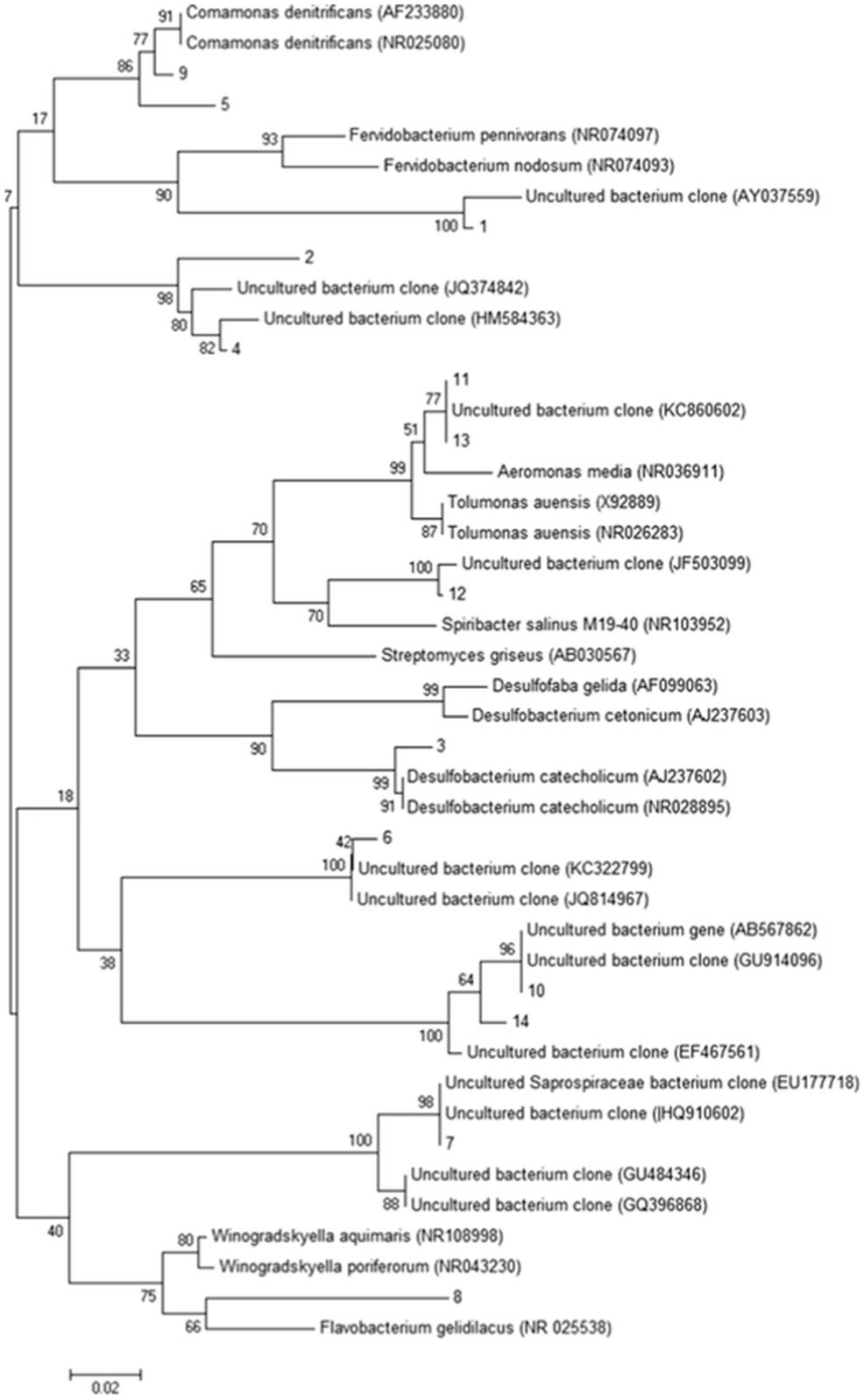

Fig. 2 Neighbor-joining phylogenetic tree of total bacteria in SBRs under different COD/TP ratio, based on partial sequences of 16 s rRNA V3 part. Sequence types correspond with the sequence types presented in Fig. 2a

Some typical sequences were successfully read from the DGGE gels and the NJ phylogenetic tree of total bacteria based on 16 s rRNA gene sequences was constructed (Fig. 2). It was elucidated that bands 12 and 13, which were most identical to *Tolumonas* founded in a freshwater lake, were observed in all samples with similar intensity (Fischer-Romero et al. 1996). Sequence identical to *Desulfobulbaceae* (band 3) was only observed in R1. Sequence identical to *Flavobacterium* founded in lakes was observed intensely in R2 and R3 (van Trappen et al. 2003). The results showed that the whole biomass consisted of β, γ, δ-*Proteobacteria*, *Flavobacteria*, and *Thermotogales*. With the increase of the COD/TP ratio, the population of γ-*Proteobacteria* exhibited a slightly decreasing trend, while *Flavobacteria* and *Thermotogales* seemed to increase. However, in general, no significant differences caused by the change of COD/TP ratio were found in three SBRs.

Effect of COD/TP ratio on nitrifiers and denitrifiers

The DGGE patterns based on *amoA* gene are demonstrated in Fig. 3a. The composition of AOB community was slightly affected by COD/TP ratio. The Shannon–Wiener indexes of three sludge samples from R1, R2, and R3 turned out to be 1.99, 1.35, and 1.51, respectively. The diversity of AOB in R1 was higher than those in R2 and R3. Sequence amoA1 and amoA2 dominated in all samples, whereas other bands were comparatively weak (Fig. 3a). The NJ phylogenetic tree of AOB based on *amoA* gene (Fig. 3b) illustrated that the dominant *amoA* sequences (especially amoA1 and amoA2, which were found in all samples and quite bright) were very similar to the sequences reported in other studies and affiliated to a *Nitrosomonas*-like cluster found in several wastewater treatment plants (WWTPs) and a full-scale submerged membrane bioreactor (Yu et al. 2011, Gao et al. 2013). This suggested that *Nitrosomonas* was the dominant AOB in all SBRs. The capacity of both nitrification and denitrification under low oxygen conditions lead the accumulation of *Nitrosomonas* in SND process (Shrestha et al. 2002). Meanwhile, some unique bands (amoA4 and amoA6) were found in certain SBRs, which were caused by the different COD/TP ratios. Some sequences similar to amoA4 were also discovered in other SND processes (Jia et al. 2013b). However,*Nitrosospira*-like sequences, which was found to be dominated in AOB community in some study (Liu et al.2010), were only found in R3 under the investigated conditions. It seems that the increase of influent phosphorus load could promote the competition of *Nitrosospira* during SND process.

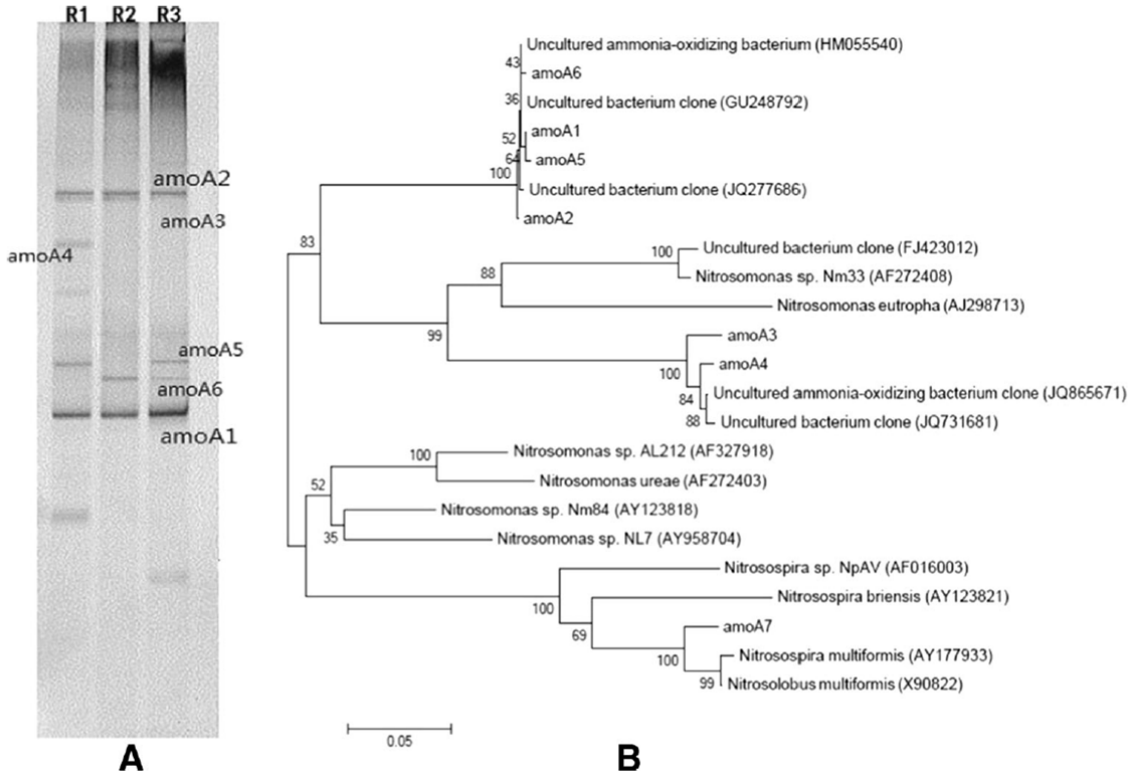

Fig. 3 a AOB patterns based on *amoA* gene and **b** Neighbor-joining phylogenetic tree of AOB based on partial sequences of *amoA* of three SBRs under different COD/TP ratio

In contrast, no significant difference was found in the composition of denitrifier community among the SBRs, according to the DGGE patterns of functional gene *nosZ* (Fig. 4a). It seems that all the denitrifiers, under the investigated conditions, could very well cope with the change of COD/TP ratio. The phylogenetic tree of denitrifiers based on *nosZ* gene showed that all the sequences divided into two parts with high similarity within each part (Fig. 4b). The two parts exhibited high similarities with the uncultured bacterium clones of*nosZ* gene previously found in an intermittently aerated partial nitritation SBR and a highly managed turf grass systems, respectively (Dell et al. 2010, Gabarro et al. 2013). Nevertheless, all sequences showed a low similarity with the known denitrifying bacteria, indicating these sequences may represent new species.

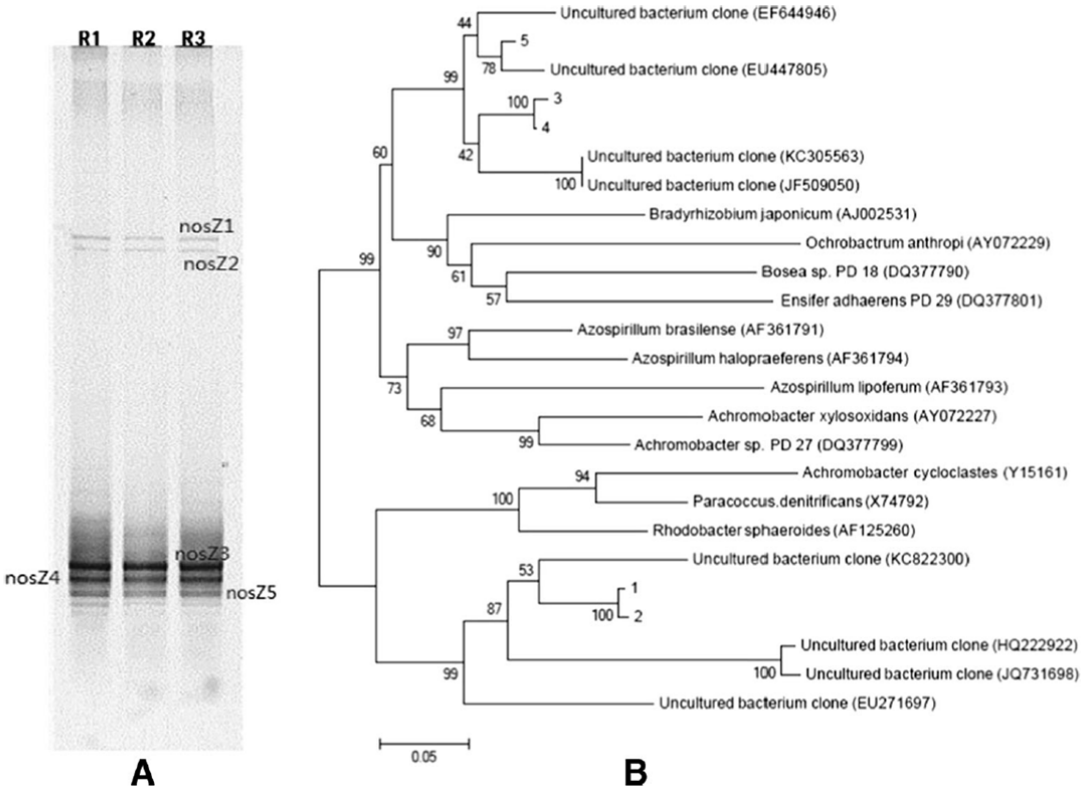

Fig. 4 a Denitrifier patterns based on *nosZ* gene and **b** Neighbor-joining phylogenetic tree of denitrifier based on partial sequences of *nosZ* of three SBRs under different COD/TP ratio

Unlike PCR-DGGE results, the results of real-time qPCR showed very minor difference in the quantity of AOB community (Fig. 5). The ratios of *amoA*/bacterial 16S rRNA gene were almost identical (except from 8.5×10^{-5} to 9.2×10^{-5} on average), which were consistent with another study (Zhang et al. 2010). It meant that the quantity of AOB was not affected by COD/TP ratio under the same influent NH_4^+-N concentration and aeration stage conditions. Similar results were obtained regarding the quantity of denitrifier community. The ratios of *nosZ*/bacterial 16S rRNA gene were 2.8×10^{-5}, 1.7×10^{-5}, and 2.3×10^{-5} in the three SBRs, which was attributed to degrade NO_3^--N/NO_2^--N in liquid by existing nitrate reductase and nitrite reductase, or even the difference of gene expression ratio. The percentages of AOB as well as denitrifiers were quite small in all SBRs. Meanwhile, the copy numbers of *amoA* were about 2~4 times more than that of *nosZ*, probably due to the enough influent NH_4^+-N content and small sludge flocs. The results were different to the previous. Geets et al. (2007) investigated the microbial community structure of the WWTPs and the copy numbers of *nosZ* were much higher than the results presented in this study. In the low DO condition, the denitrification process was partly inhibited, resulting

in the low growth rate of denitrifiers. This may also be the reason for NO_3^--N accumulation in the effluent.

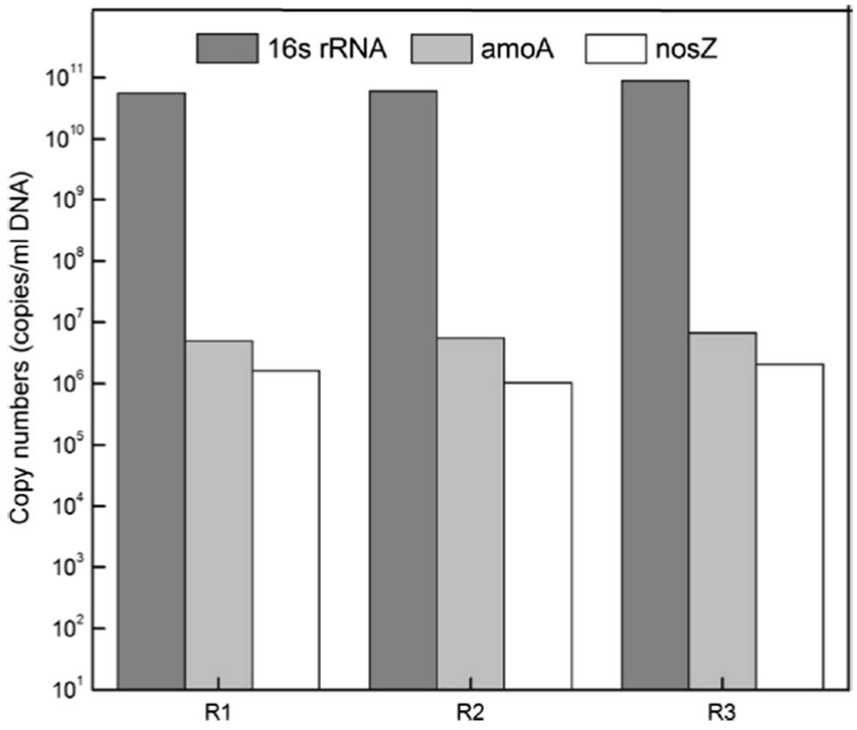

Fig. 5 Copy numbers of 16 s rRNA, *amoA* and *nosZ* genes under different COD/TP ratios (Mean value, $n = 3$)

Effect of COD/TP ratio on PAOs/GAOs ratio

The PAOs and GAOs contents in three SBRs were determined by FISH, and the results are shown in Fig. 6. It can be seen that most microorganisms were gathered in the zooglea, which was helpful to the removal of nutrients. Estimated by using Image-Pro Plus 6.0, the results of FISH showed that based on the total bacteria, the PAOs accounted for 31.88, 31.67, and 26.95 % in R1, R2, and R3, respectively, while the corresponding percentages of GAOs were 34.69, 32.78, and 39.81 %, respectively. The results observed in this study were higher than that reported in previous literatures. Puig et al. (2007) studied the microbial community in a SBR by FISH and found that percentages of PAOs and GAOs were only 17.5 and 3.5 %, respectively. It showed that the PAOs were greatly enriched during the SND process in this study. Although R1 and R2 had almost the same PAOs/GAOs ratio (i.e., around 1.05), R3 presented a much higher PAOs/GAOs ratio of 1.47, which apparently increased with the decrease of influent COD/TP ratio. The results consisted with the previous studies. Mino et al. (1998) found that a low COD/P ratio in influent tends to favor the growth of PAOs instead

of GAOs. The existence of more PAOs indicated that more microorganisms could take up phosphorus and use nitrate and/or nitrite as electron acceptor at the same time during aeration stage in R3. This appeared to be a reasonable microbiological explanation for the better TN and TP removal performance of R3.

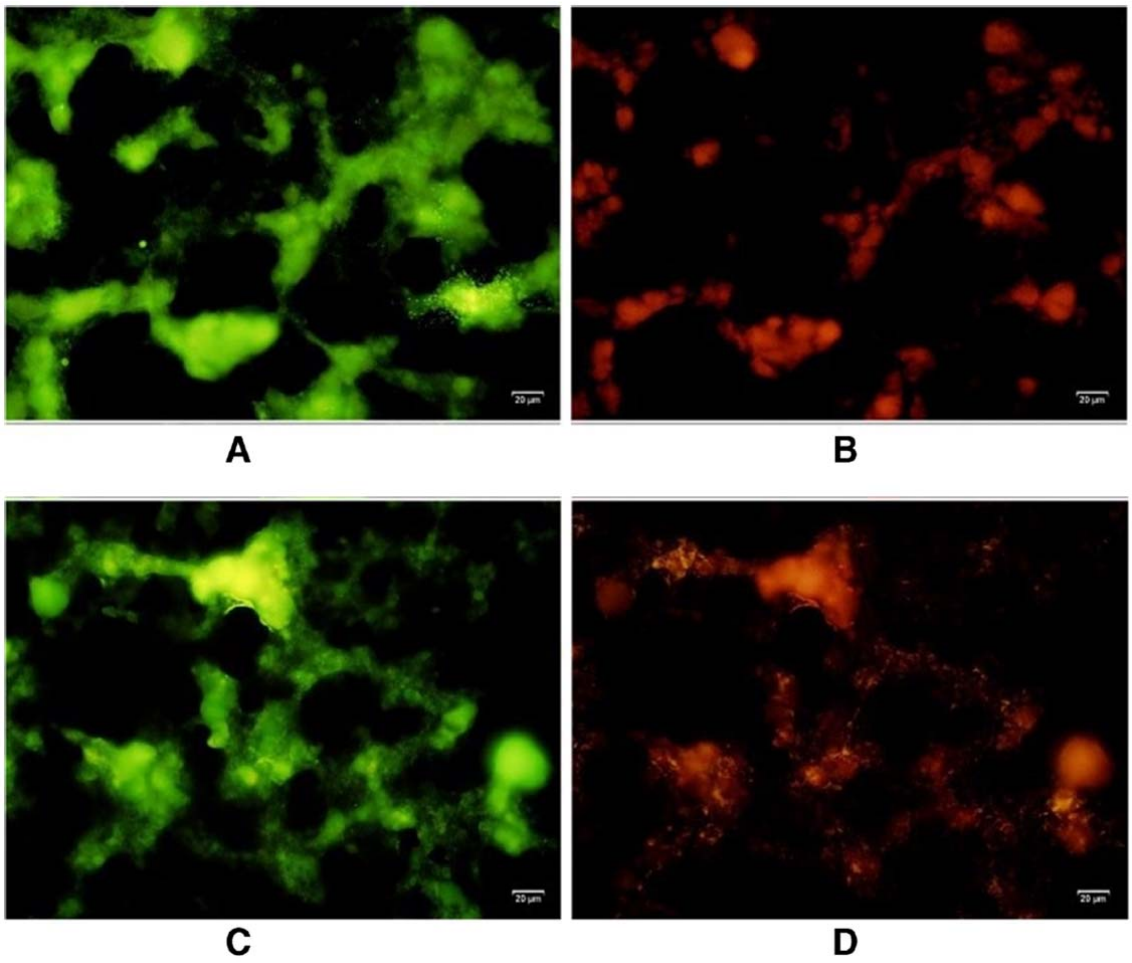

Fig. 6 Detection of PAOs and GAOs by FISH analysis (**a** and **c**: FITC labeled, target for Eubacteria; **b**: Cy3 labeled GAOmix, target for GAO; **d**: Cy3 labeled PAOmix target for PAO)

Conclusions

With the increase of the COD/TP ratio, the population of γ-*Proteobacteria* in the reactors exhibited a slightly decreasing trend. The composition of AOB community was found to be affected by the COD/TP ratio.*Nitrosomonas* capable of both nitrification and denitrification under low oxygen conditions was found to be the dominant AOB in all SBRs. The copy

numbers of *amoA* in all SBRs were more than that of *nosZ*. The PAOs/GAOs ratio showed an upward trend with the decrease of influent COD/TP ratio.

Acknowledgments

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