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EFFECT OF INTERNAL RECYCLE RATIO ON THE DENITRIFICATION PROCESS AND *nirS*-CONTAINING BACTERIA OF AN ANAEROBIC/ANOXIC/OXIC (A^2/O) WASTEWATER TREATMENT PROCESS

Internal recycle (IR) ratio is an important operation parameter for the anaerobic/anoxic/oxic (A^2O) wastewater treatment process. Three laboratory-scale A^2O wastewater treatment processes with IR ratios of 100%, 200%, and 300% were set up to study its influence on the denitrification process and *nirS* gene-containing bacteria. Results showed the removal rate of chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), total nitrogen (TN) and total phosphorus (TP) increased at different levels as the IR rate augmented from 100% to 300%. *NirS* gene numbers were increased from 1.8×10^8 to 3.2×10^8 copies/g MLSS, which was positively correlated with the denitrification rate in anoxic areas. Moreover, similarities were observed in the community structures of denitrifying bacteria that contained the *nirS* gene under different operation modes. These results indicated that increasing the IR rate in the A^2O treatment process could benefit *nirS* gene-containing bacteria and improve denitrification ability observably while maintaining the stability of the community structure of the system.

1. INTRODUCTION

Among multiple types of biological nutrient removal (BNR) processes, generally used in municipal wastewater treatment, anaerobic/anoxic/oxic (A^2O) process with the concurrent function of phosphorous removal is the most common method (accounting for 24 % of all kinds wastewater treatment plants in China [1]). It is a single activated

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sludge growth system composed of main treatment units of anaerobic, anoxic, and oxic areas.

The biological nitrogen removal process includes two steps: nitrification and denitrification [2]. In the A^2O treatment system, nitrification and denitrification bioreactors mainly occur in oxic and anoxic units, respectively. Ammonium-oxidizing bacteria (AOB), ammonium-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB) are three different groups of bacteria which accomplish nitrification process [3]. During this process, ammonia is converted to nitrite by AOB and AOA, then NOB convert nitrite to nitrate. In the denitrification process, nitrate (NO_3^-) and nitrite (NO_2^-) were stepwise converted to gaseous products of nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2) under anoxic or anaerobic conditions [4]. This process is performed by denitrification bacteria, which is physiologically diverse groups of microbes. Commonly, the total nitrogen (TN) removal effect is mainly decided by denitrification reactions that occur in anoxic units.

The parameter of internal recycle (IR) ratio is very significant for the A^2O treatment process, which affects denitrification processes by controlling the amount of IR liquid between oxic and anoxic areas. IR increases the use of available COD by transporting nitrate to the anoxic area, where the COD can be used for denitrification. Although the relationship between IR ratio and nitrogen removal effect in A^2O process has been extensively researched [5], the influence of changes in this rate on the microbial community structure, especially specific function genes closely related with nitrogen removal, has been insufficiently studied. Given the wide use of the A^2O process in municipal sewage treatment, thoroughly understanding the effect of IR ratio could advantage the optimization process to meet the increasing environmental regulations.

In some denitrification researches of microbial communities under anaerobic conditions, a method has been used to recognize all the microbial species in a community, which was based on the taxonomic analysis of the 16S rRNA gene [6]. However, as the groups of bacteria with denitrifying abilities in wastewater treatment systems are phylogenetically diverse, the methods bass on 16S rRNA gene are not capable of exclusively detecting denitrifying bacteria. Lately, researchers found certain functional denitrifying genes with higher evolutionary rates of less conserved functional molecules which can indicate the existence of denitrifying microbe [7]. During the denitrification process, the reduction from nitrite to nitric oxide performed by nitrite reductase (*Nir*) is an important step [8]. In addition, *nirS* and *nirK* genes are recognized the primary enzymes that catalyze the conversion from nitrite to nitric. These two genes translate the same function nitrite reductases but have different structure, that *nirS* gene translates to cytochrome cd1-containing nitrite reductase while the *nirK* gene translates to copper--containing nitrite reductase [6]. The quantification of *nirS* and *nirK* gene has been used to reflecting the abundance of denitrifying bacteria [9]. Of these two types of gene, *nirS* gene is more widely distributed than the other one [10].

This study aims to determine the influence of IR ratio on the denitrification process and *nirS*-containing bacteria in the A^2O treatment system. Three parallel A^2O laboratory-scale wastewater treatment devices were adopted with IR ratios of 100%, 200%, and 300%. The COD, NO_3^--N, NO_2^--N, TN, and total phosphorus (TP) in each unit and effluent were determined. The nitrification rates in oxic and denitrification rates in anoxic and anaerobic areas were calculated via mass balancing, respectively. Moreover, the influence of changes in IR ratio on the *nirS* gene number and microbial community structure based on *nirS* genes of activated sludge were analyzed via quantitative polymerase chain reaction (qPCR) and sequencing analysis.

2. MATERIALS AND METHOD

Reactor setup and operation. Laboratory-scale experiments were performed on the A^2O process, with total effective volumes of 50 dm^3 (Fig. 1), and 5, 10, 30, and 5 dm^3 for anaerobic, anoxic, and oxic areas, and the secondary sedimentation tank, respectively.

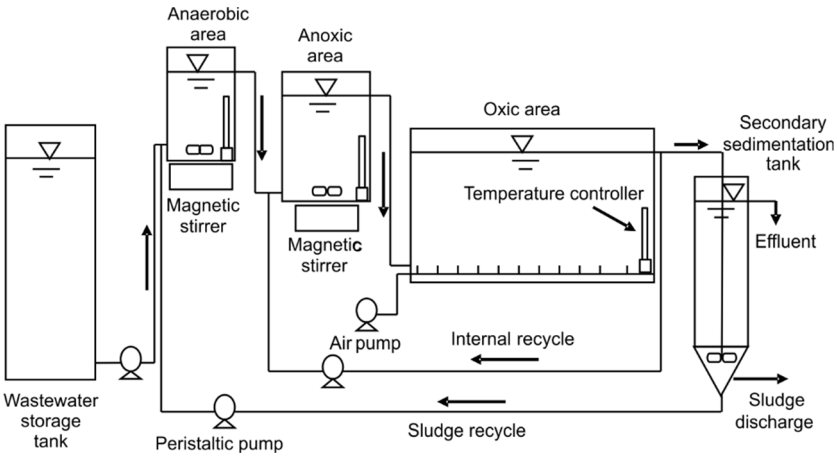

Fig. 1. Schematic diagram of the experimental equipment and process

The seeding sludge for this treatment system was taken from an oxic tank of the Xiaoshangzhuang Wastewater Treatment Plant (Xinxiang, China). The influent wastewater was taken at a flow rate of 85 dm^3/day from a residential area of Henan Normal University. The wastewater quality was as follows: 173.5±42.1 mg/dm^3 COD, 38.1±2.5 mg/dm^3 NH_4^+-N, 1.1±0.7 mg/dm^3 NO_3^--N, 40.3±3.6 mg/dm^3 TN, and 8.4±0.7 mg/dm^3 TP. The mixed liquor suspended solids (MLSS) concentration and sludge retention time (SRT) of the system were kept at 3000±200 mg/dm^3 and 15 days, respectively, while the wastewater temperature was maintained at 25±1 °C by temperature controllers throughout the research period.

Three parallel devices were operated simultaneously with IR ratios of 100%, 200%, and 300%. The sludge recycle ratio was kept at 100% constantly under three operation modes. Both anaerobic and anoxic areas were stirred using a magnetic stirrer (85-2 Sile Shanghai) with 500 rpm rotating speed for the activated sludge to remain suspended. The dissolved oxygen (DO) in the oxic area was maintained at about 2 mg/dm^3 by an air pump with an adjustable airflow meter. A mechanical stirrer with the rotating speed of 30 rpm was installed in the secondary sedimentation tank (US-52 Oteli Beijing). All measurements were taken 8 times in equal time-interval of 4 weeks after over 2 months acclimation under each operating condition.

Calculation methods of nitrification and denitrification rates. In the A^{2}O treatment process, the nitrification rate in the oxic area ($R_{\text{n-oxic}}$), and the denitrification rate in both anaerobic ($R_{\text{dn-anaerobic}}$) and anoxic areas ($R_{\text{dn-anoxic}}$) can be calculated as follows

$$R_{\text{n-oxic}} = \frac{\left(\left[\text{NH}_4^+\text{-N}\right]_{\text{anoxic}} - \left[\text{NH}_4^+\text{-N}\right]_{\text{oxic}}\right)\left(F_{\text{I}} + F_{\text{IR}} + F_{\text{ER}}\right)}{V_{\text{oxic}}\text{VSS}_{\text{oxic}}} \tag{1}$$

where $R_{\text{n-oxic}}$ (g/g VSS·day) is the nitrification rate in the oxic area. $[\text{NH}_4^+\text{-N}]_{\text{anoxic}}$ (mg/dm^3) is the NH_4^+-N concentration in the anoxic area. $[\text{NH}_4^+\text{-N}]_{\text{oxic}}$ (mg/dm^3) is the NH_4^+-N concentration in the oxic area. F_{I} (85 dm^3/day) is the influent flow. F_{IR} (85/170/255 dm^3/day) is the IR flow. F_{ER} (85 dm^3/day) is the sludge recycle flow. V_{oxic} (dm^3) is the volume of the oxic area. VSS_{oxic} (mg/dm^3) is the volatile suspended solids (VSSs) in the oxic area.

$$R_{\text{dn-anaerobic}} = \frac{\left[\text{NO}_x^-\text{-N}\right]_{\text{influent}} F_{\text{I}} + \left[\text{NO}_x^-\text{-N}\right]_{\text{ER}} F_{\text{ER}} \left[\text{NO}_x^-\text{-N}\right]_{\text{anaerobic}} \left(F_{\text{I}} + F_{\text{ER}}\right)}{V_{\text{anaerobic}}\text{VSS}_{\text{anaerobic}}} \tag{2}$$

where $R_{\text{dn-anaerobic}}$ (g/g VSS·day) is the denitrification rate in the anaerobic area, $[\text{NO}_x^-\text{-N}]_{\text{influent}}$ (mg/dm^3) is the sum of the NO_3^--N and NO_2^--N concentrations in the influent, $[\text{NO}_x^-\text{-N}]_{\text{ER}}$ (mg/dm^3) is the sum of the NO_3^--N and NO_2^--N concentrations in the sludge flow, $[\text{NO}_x^-\text{-N}]_{\text{anaerobic}}$ (mg/dm^3) is the sum of the NO_3^--N and NO_2^--N concentrations in the anaerobic area, $V_{\text{anaerobic}}$ (dm^3) is the volume of the anaerobic area, $\text{VSS}_{\text{anaerobic}}$ (mg/dm^3) is the VSS concentration in the anaerobic area.

$$R_{\text{dn-anoxic}} = \frac{\left[\text{NO}_x^-\text{-N}\right]_{\text{anaerobic}} \left(F_{\text{I}} + F_{\text{ER}}\right) + \left[\text{NO}_x^-\text{-N}\right] F_{\text{IR}}}{V_{\text{anoxic}}\text{VSS}_{\text{anoxic}}}$$
$$- \frac{\left[\text{NO}_x^-\text{-N}\right]_{\text{anoxic}} \left(F_{\text{I}} + F_{\text{IR}} + F_{\text{ER}}\right)}{V_{\text{anoxic}}\text{VSS}_{\text{anoxic}}} \tag{3}$$

$R_{dn\text{-}anoxic}$ (g/g VSS·day) is the denitrification rate in the anoxic area, $[NO_x^-\text{-}N]_{oxic}$ (mg/dm^3) is the sum of the NO_3^--N and NO_2^--N concentrations in the oxic area,. $[NO_x^-\text{-}N]_{anoxic}$ (mg/dm^3) is the sum of the NO_3^--N and NO_2^--N concentrations in the anoxic area, V_{anoxic} (dm^3) is the volume of the anoxic area, VSS_{anoxic} (mg/dm^3) is the VSS concentration in the anoxic area.

Wastewater quality analysis. VSS, MLSS, NH_4^+-N, COD, TN, NO_2^--N, NO_3^--N and TP were analyzed by standard methods [11]. The DO in the wastewater was measured using a WTW-Multi 340i (made in Germany).

DNA extraction, polymerase chain reaction amplification, and sequencing. The total DNA was isolated using a Magnetic System-16 (TanBead, Taiwan). *NirS-F* (TACCA CCCSGARCCGCGCGT) and *nirS-R* (GCCGCCGTCRTGVAGGAA) primers were used to amplify the gene segment of the *nirS* gene [12]. Polymerase chain reaction (PCR) amplification was accomplished using the Gene AmpR PCR System (9700, AB, USA) with a final volume of 50 µl and followed by 25 cycles touchdown program: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s. After purification using an agarose gel extraction kit (Dingguo, China), the PCR products were ligated and transformed into *E. coli* DH5α competent cells. Positive clones were selected and cultured on a Luria-Bertani (LB) medium with X-gal, isopropyl β-D-1-thiogalactopyranoside and Amp to submit the clones for sequencing using an ABI 3730DXL DNA sequencer (AB, USA).

Clone library construction and phylogenetic analysis of sequences in clone libraries. The obtained sequences were manually trimmed to exclude vector sequences, then checked for chimaeras by Bellerophon on the Greengenes website[4]. Sequences avoiding chimaeras of each sample were aligned and grouped into universal operational taxonomic units (OTUs) with the threshold of 97% minimum similarity, and individual sequences were also assigned to OTUs to identify the bacterial distribution in sludge. The representative sequence of each OTU in group-specific libraries was aligned with the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). To identify the phylogenetic affiliation of all OTUs, a phylogenetic tree was constructed using the neighbor joining algorithm by MEGA version 6.1, which included representative sequences of each OTU and related sequences from the previous NCBI database.

Real-time quantitative polymerase chain reaction. Denitrifying bacteria counts were determined via qPCR quantification based on the *nirS* gene using the oligonucleotides *nirS-F*: 5-TACCACCCSGAR CCGCGCGT-3 and *nirS-R*: 5-GCCGCC GTCRTGVA GGA A-3 [12]. For *nirS* gene PCR, 2 µl of the extracted DNA was added to a PCR reaction mixture that contained 12.5 µl of SYBR Premix Ex Taq (TaKaRa, Japan), 0.5 µl of *nirS-F* (10 µM each), 0.5 µl of *nirS-R* (10 µM each), and 9.5 µl of dH_2O. A TaKaRa PCR thermal cycler dice

[4]http://greengenes.lbl.gov/

real-time PCR system (TaKaRa Code: TP800) was used for qPCR, and the initial denaturation step was 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s and 65 °C for 30 s. The quantitative software available in the PCR instrument with a standard curve generated by serially diluted PCR products was used for the calculation of the *nirS* gene-containing bacteria from the samples. The sample number of *nirS* was determined by comparing the crossing threshold (CT) cycle against the standard curve CT produced to count copies of the target DNA per sample. A series of tenfold dilutions of the standard DNA for the primer set with the range of 1.0×10^2 (*nirS* gene) copies/cm^3 to 1.0×10^7 (*nirS* gene) copies/cm^3 were adjusted. A linear correlation ($R^2 = 0.999$) was observed in six orders of magnitude with the range of 10^2–10^7 gene copies/cm^3 of standard DNA.

3. RESULTS AND DISCUSSION

3.1. PERFORMANCES OF A^{2}O PROCESS UNDER DIFFERENT IR RATIOS

Table 1 shows the pollutant removal rate in A^{2}O process under IR ratios of 100%, 200%, and 300%. The COD, NH_4^+-N, NO_2^--N, NO_3^--N, TN, and TP concentrations in each unit were investigated (Fig. 2) to deeply understand the pollutant transformation characteristics in these treatment processes.

Table 1

Pollutant removal performances under different IR ratios

	IR = 100%			IR = 200%			IR = 300%		
	Influent [mg/dm^3]	Effluent [mg/dm^3]	Removal rate [%]	Influent [mg/dm^3]	Effluent [mg/dm^3]	Removal rate [%]	Influent [mg/dm^3]	Effluent [mg/dm^3]	Removal rate [%]
COD	172.0±48.7	21.3±3.9	87.6	175.6±30.2	16.1±1.2	90.8	173.0±49.4	15.3±1.8	91.2
NH_4^+-N	37.6±2.5	1.9±1.1	94.9	38.1±3.2	1.9±1.1	95.0	37.9±1.1	1.8±1.8	95.3
NO_3^--N	1.2±0.2	21.6±8.2	–	0.3±0.3	18.0±3.4	–	2.0±0.5	11.9±5.4	–
NO_2^--N	0	0.1±0.1	–	0	0.2±0.2	–	0.1±0.1	0	–
TN	38.8±2.8	21.0±5.1	45.9	39.5±3.3	18.2±3.5	53.9	43.9±3.3	14.1±5.1	67.9
TP	8.6±0.9	1.0±0.1	88.4	8.3±0.5	0.6±0.1	92.8	8.4±0.6	0.5±0.1	94.0

The results reflected that the operating parameter of the IR ratio could influence organic matter oxidations and biological nitrogen removal in the A^{2}O process. High COD and NH_4^+-N removal rates that ranged from 87.6% to 91.2% and from 94.9% to 95.3%, respectively, were observed for three operating modes. As the IR ratio increased from 100% to 300%, the COD, NH_4^+-N, TN, and TP removal rates increased at different levels. The highest increase was observed in the TN removal rate, which increased from

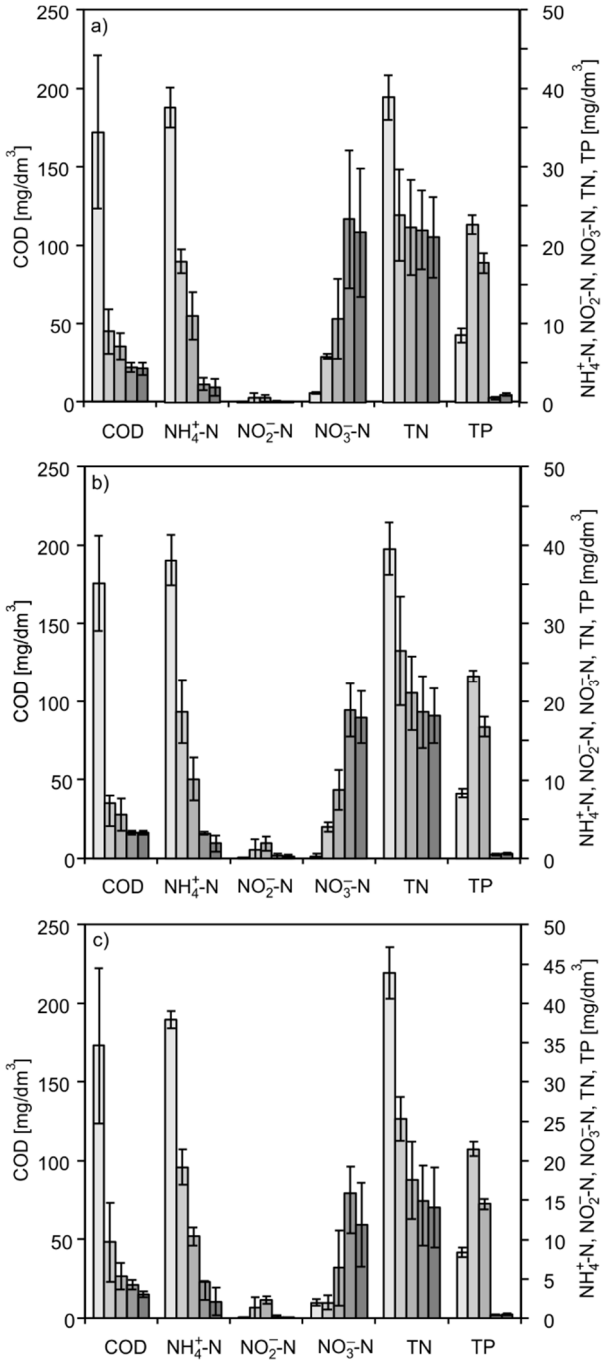

Fig. 2. Pollutant concentrations in each unit under different IR ratios:
a) 100% IR ratio, b) 200% IR ratio, c) 300% IR ratio

45.9% to 67.9% (Table 1). Commonly, TN in wastewater treatment plants (WWTP) in influent contains organic and inorganic nitrogen. The wastewater used in this work was real municipal wastewater rather than synthetic wastewater [13, 14], and the organic nitrogen concentration was observed at a relatively low level in effluent (Fig. 2). As in A^2O process, most organic nitrogen could stepwise biodegrade to inorganic nitrogen under anaerobic treatment process. The NO_3^--N and TN concentrations in the anaerobic, anoxic, and oxic areas all decreased observably as the IR ratio was elevated. This phenomenon revealed that a higher IR was beneficial for denitrification as more NO_x^--N (NO_3^--N and NO_2^--N) denitrification substrates were transported to the anoxic area. However, the energy consumption of the treatment process was positively correlated with the IR ratio, as Baeza et. al. [15] found the economic cost was about five times higher when IR ratio increased from 100% to 500% in an A_2O process. Moreover, the positive effects of raising the IR ratio on the pollutant removal rate were limited [5]. Hence, the experiment with IR ratio higher than 300% was not taken in this study. Fongsatitkul et al. [5] observed a similar phenomenon in an A^2O slaughterhouse wastewater treatment system, where both TN and TP removals moderately increased as the IR ratio doubled from 100% to 200%. However, no significant improvement in either TN or TP removal effect was observed when IR ratio was elevated to 400%. Kim et al. [16] reported that an IR ratio of 300% was the most efficient for a membrane bioreactor combined with a nitrification reactor for piggery wastewater after experimenting with rates of 100%, 300%, and 500%. In this study, an increase in TN removal rate of 22.0% was observed with the IR rate increased from 100% to 300%, as the energy cost of IR is directly related to its flow [15], the economic cost would also be increased to a certain extent. High IR rate was favorable to TN removal, but it would be economically non-profitable if low NH_4^+-N load in influent. In WWTPs, balance should be established between treatment effects and energy cost. As a result, the IR ratio is no need to be maintained at high level invariably and should be adjusted according to the nitrogen loads, from which regular treatment effect and energy-saving consumption could be achieved simultaneously [15].

In addition, denitrification was observed in the sedimentation tank, as NO_3^--N concentration was found slightly lower than that detected in the oxic area (Fig. 2). This was probably because of partial post-denitrification occurred in the secondary sedimentation tank and the available internal COD was used as the carbon source. Phosphorus release was also observed in the second sedimentation tank, but the released TP amount was less compared with some studies [13, 17]. The main reason for this phenomenon was the short hydraulic retention time of the sedimentation tank (1.4 h) in this work.

3.2. NITRIFICATION AND DENITRIFICATION RATE IN THE A^2O PROCESS

The nitrification rate in the oxic area and the denitrification rates in both anaerobic and anoxic areas were accurately calculated according to Eqs. (1)–(3) (Fig. 3) to avoid the influence of dilution effect. The calculation was based on the assumption that all

ammonium decrease was due to nitrification process. Clearly, the real nitrification/denitrification rates were lower than these calculation results as the ammonium/nitrate/nitrite nitrogen used in the cellular maintenance of the bacteria was regarded as the nitrification/denitrification capacity [15]. The DO of each unit was also detected for the three operation modes, as shown by the result in Fig. 4.

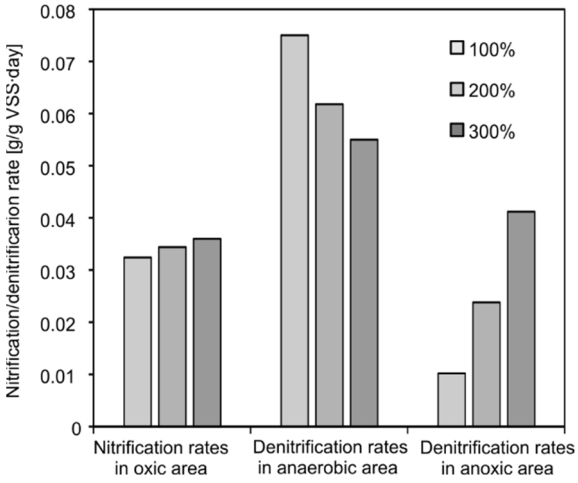

Fig. 3. Nitrification and denitrification rates in the main treatment units

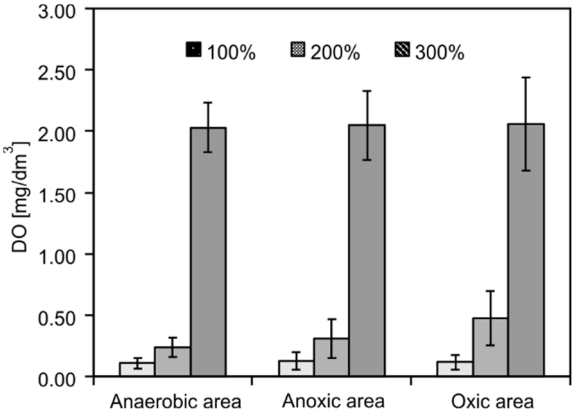

Fig. 4. DO in each unit under different IR ratios

As the IR ratio increased from 100% to 300%, the nitrification rate in the oxic area and the denitrification rate in the anoxic area increased from 3.25×10^{-2} g/g VSS·day to 3.61×10^{-2} g/g VSS·day and from 1.03×10^{-2} g/g VSS·day to 4.12×10^{-2} g/g VSS·day, respectively, whereas the denitrification rate in the anaerobic area decreased from 7.51×10^{-2} g/g VSS·day to 5.50×10^{-2} g/g VSS·day. Unexpectedly, the denitrification rate

in the anaerobic area was higher than that in the anoxic area under the three operation modes. This result was probably because the conditions in the anaerobic area were more appropriate for denitrification reactions than those in the anoxic area for the large amounts of nitrate transported by the sludge recycle liquid and sufficient organic matter from the influent and strictly anaerobic environment. Nevertheless, the implication is not that the TN was mainly removed in the anaerobic area. Besides, the denitrification rate and the hydraulic retention time (HRT) (1.4 h for the anaerobic area, and 2.8 h for the anoxic area) also affected the TN removal amount. With the IR ratios of 100%, 200%, and 300%, 856.8, 705.5, and 627.3 g/day of TN were removed in the anaerobic area, while 234.6, 544.0, and 940.1 g/day of TN were removed in the anoxic area, respectively. Increased IR liquid transported more nitrate from aerobic area to the anoxic area, and the sludge return ratio was kept at 100% under three operation mode, as a result, denitrification reactions were observably enhanced in the anoxic area.

The appropriate DO concentration and sufficient nutrition were two key points for the excellent denitrification effect. In this treatment system, the DO in both anaerobic and anoxic areas were markedly lower than that in the oxic area and increased as the IR was elevated, which was caused by the increased IR liquid that brought more DO from the oxic area. The denitrifying bacteria were evidently sensitive to oxygen because oxygen could suppress the activity of several denitrification catalyzing enzymes. Nitrate respiration can be inhibited by oxygen immediately, and the maximal inhibitory effect was at 0.2% of oxygen saturation [18]. Compared with other denitrification reductases, nitrite reductase showed less inhibited effect. The activity of nitric oxide reductase was about 10 times as high as that of nitrite reductase to prevent the accumulation of NO [19]. The phenomenon of N_2O accumulation easily occurred in oxic treatment section, because nitrous oxide was the most sensitive enzyme to oxygen in the denitrification enzymes [20]. Additionally, compared with nitrite reductase, nitrate reductase was stronger in capturing electron donors, which resulted in a faster reduction rate of nitrate than that of nitrite and caused the accumulation of nitrite [21]; therefore, the NO_2^--N accumulation phenomenon would occur when DO increased, and organic matter was insufficient in denitrification systems (anaerobic and anoxic areas). The NO_2^--N concentrations were relatively low in this treatment process under the three operation modes, but the NO_2^--N accumulation phenomenon occurred in both anaerobic and anoxic areas, and the accumulation concentration increased as the IR ratio was elevated (Fig. 2).

This study indicated that in a limited range (lower than 0.5 mg O_2/dm^3), the variation in DO had a negligible effect on denitrification. However, the influence of nutrition condition seemed more notable than that of DO (within limits) for the denitrification process.

3.3. ANALYSIS OF *nirS*-CONTAINING MICROBIAL COMMUNITIES

The community composition of *nirS*-containing bacteria from the anoxic area was analyzed by clone library under the three operating modes (Fig. 5).

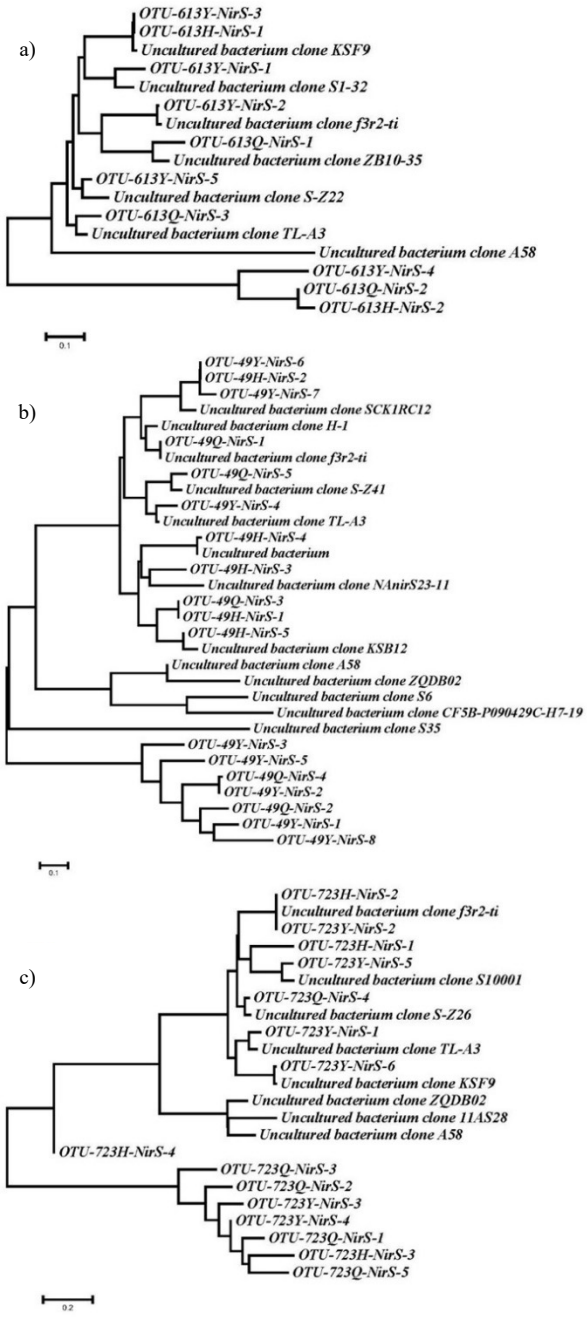

Fig. 5. Neighbor-joining phylogenetic tree based on *nirS* partial sequences: A) 100% IR ratio, b) 200% IR ratio, c) 300% IR ratio

qPCR assay that targeted the *nirS* gene was conducted, as shown by the result in Fig. 6; similar microbial community structure and distinctly different *nirS* number were observed under each operation mode. The *nirS* number increased in the anoxic area as the IR ratio increased, which was positively correlated with the denitrification rate.

Similarities among the denitrifying bacteria that contained the *nirS* gene were observed under different operation modes, and 10, 18, and 14 clones were sequenced from the samples with IR ratios of 100%, 200%, and 300%, respectively (Fig. 5). Uncultured bacterium clones f3r2-ti, TL-A3, and A58 were found in all the samples under the three operation modes. The clone of ZQDB02 was found in both IR ratios of 200% and 300%. The clone of KSF9 was found in both IR ratios of 100% and 300%. In the biological treatment systems, the effect of carbon source on denitrification bacteria population structure was more evident than the influence factors of electron acceptor and C/N ratios [22], because heterotrophic growth was based on the pathways of organic carbon and energy metabolism. As a result, with the permanent influent wastewater and the same seeding sludge, microorganism populations in the A^2O system were slightly impacted by changes of IR ratio.

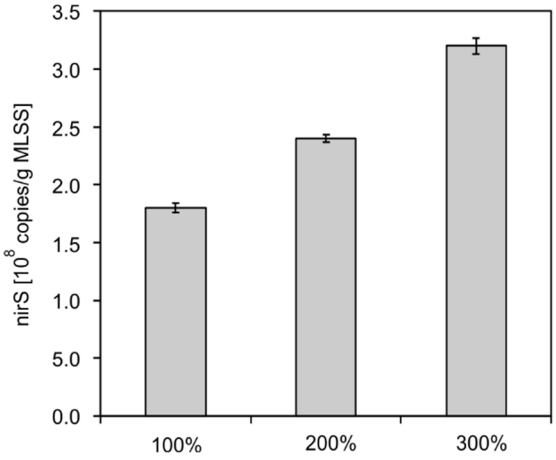

Fig. 6. *NirS* gene copy number in anoxic areas under different IR ratios

The *nirS* number reflected the denitrification ability of NO_2^--N to NO in the treatment system. The *nirS* number in the anoxic area increased as the IR ratio augmented, which revealed that the increase in the denitrification substrate of nitrate benefited the *nirS*-containing bacteria. The anoxic area was the main denitrification unit; therefore, the TN removal rate of the treatment system was positively correlated with the *nirS* gene number. Chon et al. [23] found a similar result in a wastewater-stabilizing constructed wetland, where the diversity and abundance of *nirS* can be affected in terms of the nitrogen removal efficiency. The nitrite reductase appeared less sensitive to O_2 than the nitrate reductase and the

threshold inhibitory concentration was 2.5 mg O_2/dm^3 [24]. The increased DO in the anoxic area did not show any obvious effect on the *nirS*-containing bacteria.

Generally, stable microorganism community structure appeared in functionally stable wastewater treatment systems under steady conditions, but the changes of community structure could occur to adapt in environmental variables. Functional genes that encoded key enzymes involved in various biochemical cycling processes can reveal microbial functional potentials of activated sludge in WWTPs [25]. In this A^2O treatment process, increasing the IR ratio could obviously increase the amount of *nirS*-containing bacteria and enhance treated wastewater quality, and simultaneously keep the denitrification bacteria population structure relatively stable. Therefore, changing the IR ratio was a preferable option to improve the removal effect in the practical treatment process if required, but the economic cost should also be considered.

4. CONCLUSIONS

This work showed the dependency of the denitrification rate on the IR ratio and *nirS*-containing bacteria of an anaerobic/anoxic/oxic WWTP operation. As the IR rate increased from 100% to 300%, the denitrification effect for the whole treatment process improved obviously and the nitrification effect slightly increased. In the A^2O process, the increase in IR rate could enhance COD, NH_4^+-N, TN, and TP removal rates at different levels, but the energy consumption of IR would increase simultaneously. qPCR assay revealed that an increase in IR rate promoted the generation of *nirS*-containing bacteria in the anoxic area. Moreover, the community structure of the denitrifying bacteria that contained the *nirS* gene was less affected by a change in IR rate. These results indicated that raising the IR rate could improve the denitrification ability and simultaneously maintain the stability of the community structure in the system.

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