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Simultaneous nitrification and denitrification via nitrite in a pilot-scale modified anaerobic-anoxic-oxic reactor

Jiashun Cao[a,b,c], Jianming Cai[a,b,d], Chao Li[a,b,c,*], Zhen Zhang[b], Yusheng Li[b], Minyan Xiao[b]

[a]*Key Laboratory of Integrated Regulation and Resource Development on Shallow Lakes, Ministry of Education, Hohai University, Nanjing 210098, China, emails: caojiashun@163.com (J. Cao), caihhu@126.com (J. Cai), lichao0609@163.com (C. Li)*
[b]*College of Environment, Hohai University, Nanjing 210098, China, emails: 369659097@qq.com (Z. Zhang), 2591832271 @qq.com (Y. Li), 598536907@qq.com (M. Xiao)*
[c]*State Key Engineering Research Center of Efficient Utilization of Water Resources and Engineering Safety, Hohai University, Nanjing 210098, China*
[d]*Zhejiang Dean Technology Company Limited, Ningbo 315040, China*

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ABSTRACT

The performance of simultaneous nitrification and denitrification (SND) via nitrite including partial nitrification and SND was investigated in a pilot-scale-modified anaerobic–anoxic–oxic reactor. Since partial nitrification and SND need a similar requirement on dissolved oxygen (DO) level, a low DO was kept in the operation for achieving SND via nitrite. Results showed that SND via nitrite succeeded between 0.4 and 1.6 mg/L DO concentration. The average total nitrogen (TN) removal efficiency was 67.5%. Four forms of biodenitrification were quantified via the estimation of material balance and calculation of SND. SND via nitrite compared with the other three forms made 32.3% contribution to biodenitrification. The operation of a wastewater treatment plant was continuously studied as a comparison with the pilot reactor. With the same raw water, SND not only saved 82.6% of oxygen consumption as well as 30.4% of carbon source, but also owned a higher effluent quality on TN at 11.2 mg/L. Denaturing gradient gel electrophoresis results showed that after long-term hypoxia acclimation, the dominant denitrifier community changed and the abundance ratio of ammonia-oxidizing bacteria/nitrite-oxidizing bacteria was increased.

Keywords: Partial nitrification; Simultaneous nitrification and denitrification via nitrite; Denitrification pathway; Dissolved oxygen; Denaturing gradient gel electrophoresis

1. Introduction

Partial nitrification, as one cost-effective and sustainable nitrogen removal bioprocess, has gained much attention currently. Partial nitrification has been successfully applied since its discovery in 1975 [1]. Partial nitrification before 2005 was achieved generally via high-ammonia wastewater such as landfill leachate [2], sludge digestion [3], and industrial wastewater [4]. The most classic case of partial nitrification is SHARON [5], which should be carried out at 30–40˚C. Moreover, low-ammonia domestic wastewater and municipal wastewater have also been studied in recent

*Corresponding author.

years [6,7]. Temperature may not be the decisive factor [8,9]. Partial nitrification can also be attained at normal temperature. In fact, partial nitrification was a step limiting reaction velocity. Enriching ammonia-oxidizing bacteria (AOB) and limiting nitrite-oxidizing bacteria (NOB) is the primary point for stably maintaining partial nitrification [10,11], and a relatively lower dissolved oxygen (DO) is a feasible way [12–14]. Compared with conventional nitrification and denitrification, partial nitrification and denitrification via nitrite cannot only reduce the oxygen consumption, but also can save the cost of carbon source.

Simultaneous nitrification and denitrification (SND) has also been reported worldwide [15,16]. Compared with conventional nitrification and denitrification (CND), SND also requires a low DO level similar to partial nitrification. Thus, SND via nitrite as a new nitrogen removal bioprocess with the advantages of the above two processes has been found [17,18].

Numerous studies on partial nitrification and SND have been succeeded in activated sludge process and biofilm process, such as sequencing batch reactor [19,20] and membrane bioreactor [21,22], respectively. Anaerobic–anoxic–oxic (AAO) process occupies 70% of the wastewater treatment capacity in China, but AAO has not been studied much. Moreover, the operation of SND via nitrite has been extensively studied using synthetic wastewater in batch scale [23,24]. The completely biological nitrogen removal process is very complex (Fig. 1). Limited research is available under the condition of municipal wastewater compared with pilot scale, especially the detailed pathway of denitrification and the microbial mechanism remain unclear [32].

Based on the above questions, the objectives of this study are: (1) to achieve SND via nitrite using a pilot-scale-modified AAO reactor and to get higher nitrogen removal efficiency, with energy saving and cost reduction; (2) to reveal the contributions of the four kinds of denitrification ways based on the mass balance calculation; and (3) to compare the pilot reactor with the wastewater treatment plant (WWTP) on their performance and analyze the microbial mechanisms.

2. Materials and methods

2.1. Pilot-scale-modified AAO reactor

This study was conducted in a pilot-scale-modified AAO reactor, at a flow rate of 1.0 m^3/h (Fig. 2). About 70% of the influents flowed into the anaerobic zone and the remaining 30% to the anoxic zone through the distribution zone. It was suitable for reserving carbon resource for denitrification. The oxic zone was designed like an oxidation ditch using the circular flow, and the circular flow ratio was adjusted by a submersible water impeller. A zone named degassing zone such as an anoxic zone was set after the oxic zone and used to further improve the denitrification efficiency. Furthermore, the effects of DO taken by internal recycle to the anoxic zone were avoided utmost. Oxygen was supplied by an air compressor through the air diffuser at the bottom of the oxic zone. Mechanical stirrers were used to provide liquid mixing in the anaerobic and anoxic zones separately. In addition, the pH, oxidation–reduction potential (ORP), and DO concentration were monitored by installing corresponding sensors in the reactor (Fig. 2). The effective depth was 2.19 m and the volume ratio of anaerobic zone:anoxic zone:oxic zone:degassing zone was 1.0:1.2:3.0:0.5, with the total hydraulic retention time of 10 h (2, 2 and 6 h for anaerobic, anoxic, and oxic zone, respectively). The oxic zone was operated under low DO (0.4–1.6 mg/L). The average mixed liquor suspended solids (MLSS) concentration in the reactor was 3,800–7,400 mg/L during the experimental period. The internal recycle ratio was controlled between 50 and 150%, while the sludge recycle ratio between 50 and 100%. The SRT was fixed at 15 d and operation temperature ranged 21–30°C, which agreed to the WWTP.

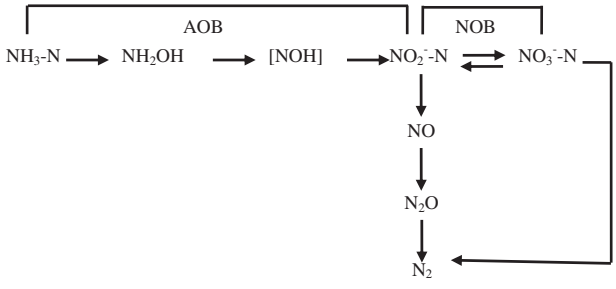

Fig. 1. Biological nitrogen removal process.

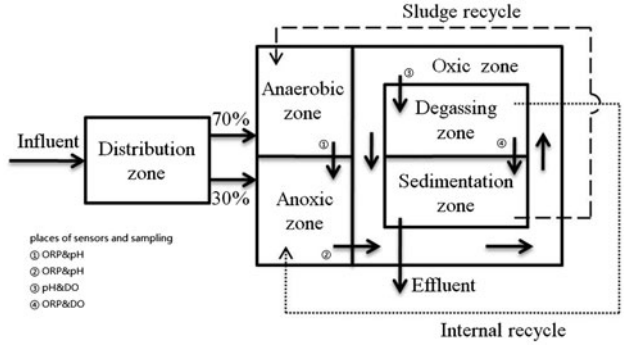

Fig. 2. Schematic diagram of pilot-scale reactor.

2.2. Sources of the wastewater and activated sludge

The activated sludge of the reactor was obtained from the aerobic tank of the WWTP under relatively higher DO level (3.0–6.0 mg/L). Partial nitrification was achieved by limiting the DO level in the oxic zone of the pilot reactor. The influent was the real-time municipal wastewater from the primary sedimentation tank as same as the WWTP. The influent characteristics are described in Table 1 (the average value was obtained from the stable 93 d′ operation). The activated sewage sludge of the pilot reactor was taken from WWTP. After cultivation of the activated sludge, the experiment had lasted for half a year.

2.3. Analytical methods

The temperature, pH, and ORP were detected on line using a HACH P53 pH/ORP analyzer (Hach Company, Colorado, USA). DO level was continuously monitored by a HACH D53 DO analyzer (Hach Company). Total nitrogen (TN), TP, NO_3^--N, and NO_2^--N were measured by an ultraviolet (UV) spectrophotometer. COD, NH_3-N, MLSS, and volatile MLSS (MLVSS) were measured according to Standard Methods [25]. Microscopic examination was performed by a YS100 microscope (Nikon, Japan).

2.4. DNA extraction and polymerase chain reaction (PCR) amplification

Water samples were collected and filtered through 0.2-mm hydrophilic polycarbonate filters. DNA was extracted from the condensed sludge using an UltraClean Soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. 16S rRNA gene fragments were amplified from the extracted total DNA with Taq DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan) using bacterial primer BAC338F (5′-ACT CCT ACG GGA GGC AGC AG-3′.)/BAC518R (5′-ATT ACC GCG GCT GCT GG-3′). BAC338F contained an additional 40 nucleotide GC-rich sequence at 5′ end. The GC-clamp contains the following sequence: 5′-CGCCCGCCGCGCGCGGCGGGCGGGG-CGGGGGCACGGGGGG-3′. The PCR conditions were as follows: initial denaturation at 94℃ for 5 min and 30 cycles of 1 min at 92℃, 1 min annealing at 55℃, and 1 min at 72℃. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

Table 1
Influent characteristics (for the 93 d′ stable operation)

Index	Range
pH	7.22–7.73
COD (mg/L)	61.5–666
TP (mg/L)	1.1–14.4
TN (mg/L)	12.9–79.4
NH_3-N (mg/L)	12.3–49.3
NO_3^--N (mg/L)	0.02–1.54
NO_2^--N (mg/L)	0.01–1.17

2.5. Denatured gradient gel electrophoresis (DGGE) and band sequencing

For DGGE, 100 ng of purified PCR products were separated on polyacrylamide gels (8%, 37.5:1 acrylamide-bisacrylamide) in 0.5 × TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 7.4) at a denatured gradient from 35 to 65% (100% denaturant contains 7 M urea and 40% (vol/vol) formamide) using a D-Code system (Bio-Rad Laboratories) [26]. DGGE was operated at 100 V and 60℃ for 18 h. As previously described [27], the gel was stained with double SYBR Green I (Sigma) and then imaged by UV transillumination after electrophoresis. The gel image was captured by a charge coupled device (CCD) camera and Biocapt (Vilber Lourmat). Images analysis for fragment detection and quantification was conducted on Bio-1D++ (Vilber Lourmat). Densitometric profile for each sample was brought out to define each band's relative contribution to the total signal. Dice index (Cs) of similarity was used to evaluate the similarity of bacterial community among bioreactors [28]. DGGE fingerprints were manually scored by the presence or absence of co-migrating bands without consideration of band intensity, and the cluster analysis was further performed. Individual bands were excised, reamplified, and rerun on a denaturant gradient gel to check their PCR products for sequencing and were purified using a Qiaquick PCR purification kit (QIAGEN).

2.6. Real-time PCR assays

Quantification of AOB was characterized by *amoA* genes targeted real time PCR, with the primers amoA-1F: GGGGTTTCTACTGGTGGT, amoA-2R: CCCCTCK-GSAAAGCCTTCTTC [29]. The experimental condition was: 900 s at 95℃, 40 cycles of 15 s at 95℃, 30 s at 63℃, and 30 s at 72℃ (also for data acquisition step). One last step from 60 to 95℃ with an increase of 0.2 deg/s was added to obtain a specific denaturation curve. The quantification of NOB was referred to the previous literature [30].

Plasmids (pEASY-T1 Cloning Kit, Transtaq) containing cloned these two genes (which were cloned to DH5α before plasmids extracting) were used to draw standard curves ($r^2 > 0.99$ for all, amplification efficiencies was 100.1 and 98.5%, respectively). All the results of abundance were obtained by averaged triplicate samples.

2.7. Calculation of nitrite accumulation ratio (NAR) and SND efficiency

NAR and the SND efficiency [31] were calculated as follows:

$$\text{NAR}\ (\%) = \frac{\rho_{\text{NO}_2-\text{N}}}{\rho_{\text{NO}_2-\text{N}} + \rho_{\text{NO}_3-\text{N}}} \times 100\% \quad (1)$$

$$\text{SND}\ (\%) = \left(1 - \frac{\rho_{\text{NO}_{x^-\text{produced}}}}{\rho_{\text{NH}_3-\text{N}_{\text{removed}}}}\right) \times 100 \quad (2)$$

where $\rho_{\text{NO}_2-\text{N}}$ and $\rho_{\text{NO}_3-\text{N}}$ is the concentration of nitrite and nitrate, $\rho_{\text{NH}_3-\text{N}_{\text{removed}}}$ is the total amount of oxidized NH_3-N after nitrification, and $\rho_{\text{NO}_{x^-\text{produced}}}$ is the concentration of total NO_x^--N (including nitrite and nitrate). Additionally, the material balance method for analyzing the ratio of partial nitrification can refer to our previous study [34].

3. Results and discussion

3.1. Analysis of nitrogen removal

The pilot-scale system achieved stable SND via nitrite after 48-d operation under low DO (average 0.4–1.6 mg/L). To focus on the variation during the stable phase, cycle 1 in Fig. 3 was actually cycle 49 in the study. Because most NOBs would be eliminated after a period of real-time aeration duration control, partial nitrification in the pilot reactor could be directly started up and maintained under a lower DO gradually. Oxygen saturation concentration ranges from 0.2 to 0.4 mg/L in AOBs and between 1.2 and 1.5 mg/L in NOBs. Thus, a large amount of AOBs can be accumulated under low DO due to the higher affinity of DO compared with NOBs [32]. Matrix is limited under the low DO that affects the proliferation rate of both types of nitrifying bacteria [33]. The AOBs are much more active than NOBs, and finally the nitrite accumulation is more stable. Real-time PCR results showed that higher abundance ratio of AOB/NOB was achieved in the pilot-scale system which probably

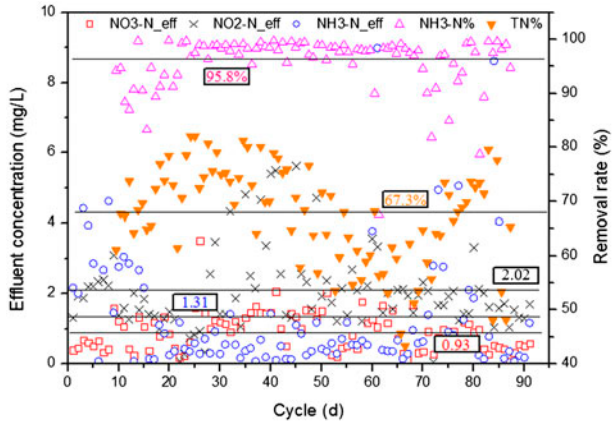

Fig. 3. Nitrogen performance in the pilot reactor (cycle 1 in this figure was actually cycle 49 for the pilot reactor).

responsible for the nitrite accumulation (see in Section 3.3).

The other main reason for the nitrite accumulation in the AAO system under low DO is the synthesis lag of aerobic respiratory enzyme by NOBs. When NOBs enter the aerobic area from the anoxic or anaerobic area, DO is harmful to NOBs, which may be linked to catalase, peroxidase, and superoxide dismutase of NOBs *in vivo* on the synthesis lag. During the respiration, oxygen is converted into short and intermediate products such as peroxides, superoxide, and hydroxyl radicals. The NOB-synthesized catalase, peroxidase, and superoxide dismutase can break down these toxic substances. However, much time is needed to re-synthesize detoxification enzymes because of the flow from the anoxic or the anaerobic areas. To create an environment for the coexistence of anoxic and aerobic areas, low DO should be maintained so as to accelerate the recycle of NOBs in the anoxic and oxic areas for effectually suppressing NOBs.

The system performances including NO_x^--N_{eff}, NH_3-N_{eff}, and TN removal efficiency are shown in Fig. 3.

Clearly, effluent ammonia concentration was maintained at about 1.31 mg/L, with removal efficiency at 95.8% during stable operation (Fig. 3). Meanwhile, the average removal efficiency of TN was 67.3%. The effluent nitrite concentration was 2.02 mg/L, which was significantly higher than nitrate (0.93 mg/L).

3.2. Analysis of denitrification pathway

Fig. 4 shows the variations of NAR and SND which were stable at 68.5 and 66.4%, respectively. Compared with the reduction of ammonia at 7.26 mg/L, the

production of nitrite including nitrate remained at 2.36 mg/L in the oxic zone.

The ammonifiers, nitrobacteria, nitrosobacteria, and denitrifying bacteria are in a symbiotic relationship in the nitrification and denitrification systems. Micro-organisms self-provide nutrient, metabolite or metabolic inhibitor to balance both pH and ORP or remove the accumulated intermediates. An environment suitable for AOBs can be built by controlling DO level as one large difference between AOB and NOB based on microbial growth kinetics, so as to stabilize the nitrite accumulation. Meanwhile, a lower DO in the oxic area was also propitious to SND which can create an anoxic macro-environment as well as an aerobic micro-environment.

Nitrification can be divided into partial nitrification (to nitrite) and full nitrification (to nitrate) depending on whether it is complete or not, or into SND and CND depending on whether nitrification and denitrification occur simultaneously. Thus, four forms of nitrogen bioremoval can be drawn from these two classifications (Fig. 5). The ratio of partial nitrification was calculated not simply using NAR, but also using a material balance method [34]. Clearly, SND via nitrite contributed more to nitrogen removal (about 32.3%), compared with SND via nitrate (12.4%), CND via nitrate (6.3%) and CND via nitrite (16.3%). Consequently, SND via nitrite was the major way of nitrogen bioremoval.

3.3. Comparison with WWTP

In this study, nitrogen removal comparison between the pilot reactor and WWTP was focused, while the whole performance comparison can be seen in Table 2.

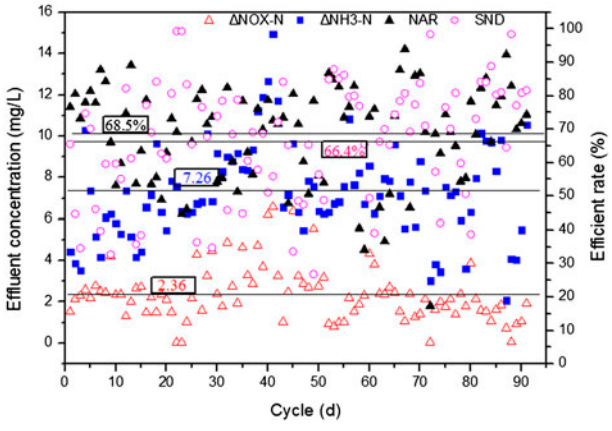

Fig. 4. The variation of NAR and SND.

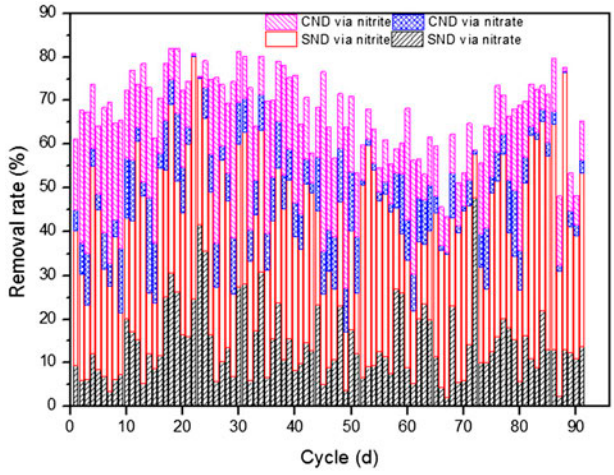

Fig. 5. Four forms of biological nitrogen removal.

Low DO was a critical factor to achieve SND and partial nitrification, but DO level should be above 2 mg/L for nitrification in conventional activated sludge process. Because of this, DO level in many WWTPs is higher than 2 mg/L, and mostly maintains at 4–6 mg/L. According to the WWTP energy analysis, energy consumption costs by fans are more than half of the total electric power consumption. Therefore, the aeration control unit is critical for WWTPs. At present, the aeration systems of most activated sludge-based WWTPs use various methods such as constant control, simple automatic control by control loop or manual local control. However, these methods cannot stably control DO level, which has shortcomings of multivariable control, high correlation, unsteadiness, and large time lag in the wastewater treatment system. Compared with the actual amount, oxygen is too redundant in the system, which is inconclusive to maintain a stable environment for microbial growth.

Table 2
The performance comparison between the pilot plant and WWTP

Index	WWTP	Pilot
COD (mg/L)	56 ± 10	47 ± 8
BOD (mg/L)	9.4 ± 2.1	8.7 ± 2.3
TP (mg/L)	0.89 ± 0.36	0.62 ± 0.28
TN (mg/L)	13.5 ± 1.8	11.2 ± 2.1
NH_3-N (mg/L)	0.89 ± 1.46	1.31 ± 1.67
NO_3^--N (mg/L)	2.86 ± 1.02	0.93 ± 0.56
NO_2^--N (mg/L)	0.32 ± 0.18	2.02 ± 1.11
DO	4.6 ± 0.27	0.80 ± 0.25
MLSS	4,100 ± 725	4,600 ± 443
SVI	82 ± 11	89 ± 8

Fig. 6 shows the nitrogen removal performance and DO level, where DO_W and DO_P are the DO levels of the WWTP and the pilot reactor, respectively; TN_W and TN_P are the TN effluent concentrations of the WWTP and the pilot reactor, respectively. Compared with an average DO at 4.6 mg/L of the WWTP, the pilot reactor was more energy saving with DO at 0.8 mg/L, thus directly saving about 82.6% of oxygen consumption. Moreover, the effluent of TN was lower in the pilot reactor than in the WWTP (11.2 vs. 13.5 mg/L).

Based on the oxygen dynamic adjustment and feedback control theory, an online DO monitor was set at the end of the oxic area. A suitable range of DO was found after commissioning. Oxygen was rationally allocated employing a self-adjusting brake of DO so as to improve the oxygen utilization efficiency and stabilize DO in the oxic area. According to on-line monitoring, typical variations of DO were compared in Fig. 7. DO_P was maintained relatively stable using a frequency-variable DO controller (Siemens).

Energy saving and cost reduction were mainly achieved on TN removal as well as oxygen and carbon source consumption by the pilot reactor. Under the present experimental conditions, the pilot reactor was evidently more efficient in energy saving and cost reduction compared with the WWTP.

An inverter control strategy was employed in the pilot reactor. Air volume that can be saved was calculated as:

$$1 - \mathrm{DO\_P}/\mathrm{DO\_W} = 1 - 0.8/4.6 = 82.6\% \quad (3)$$

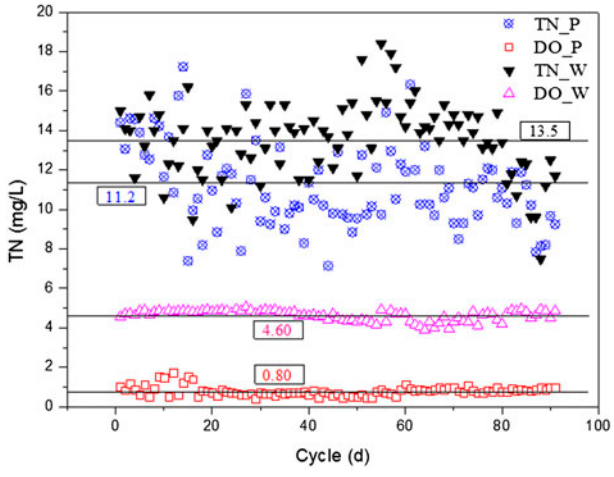

Fig. 6. Nitrogen removal performance and DO level.

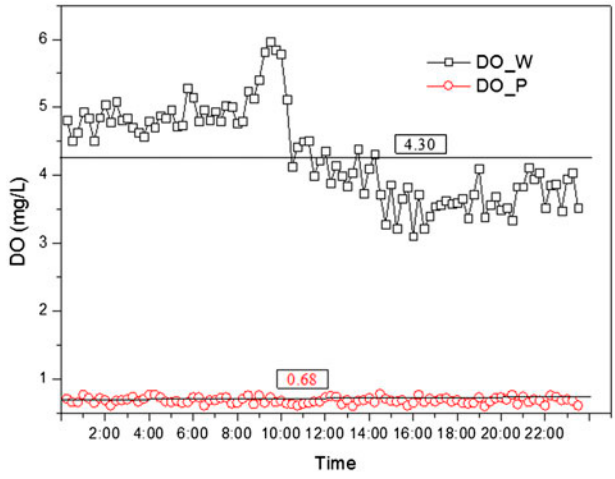

Fig. 7. Typical variations of DO concentration (on day 57).

According to the chemical reaction equation, when denitrification directly began from nitrite, it could save carbon source as well as increase rate of nitrification at 62.5%. In this study, the average economization of carbon source for nitrogen removal is equal to:

$$(\text{SND via nitrite} + \text{CND via nitrite}) \times 62.5\% = (32.3\% + 16.3\%) \times 62.5\% = 30.4\% \quad (4)$$

The denitrification efficiency also increased by 30.4%. Moreover, reactor volume was reduced, and floor space as well as construction cost was saved. As SND was achieved, alkalinity consumed during nitrification and produced during denitrification was neutralized so that the process effectively maintained at stable pH in the oxic area.

Although the seed sludge of the pilot reactor was collected from the WWTP, the two reactors' performances were slightly different after a period under different DO levels.

DGGE fingerprints of activated sludge reflect the diversity of microbial flora, but the DGGE results of bacteria do not necessarily correspond to a certain band [35]. Meanwhile, a DGGE band likewise does not represent a single type of bacteria [36]. By repeatedly tapping the DGGE bands for separation and purification, dominant bacteria could be studied more accurately. Activated sludge of the pilot reactor as well as the WWTP was taken for DGGE analysis which was comprised of diverse bacterial communities (Fig. 8), where W_1, W_2, and W_3 represent the anaerobic, anoxic and oxic areas of the WWTP, respectively; P_1, P_2, and P_3 are the three areas of the pilot reactor, respectively. All the sludge samples were taken at the end of each area.

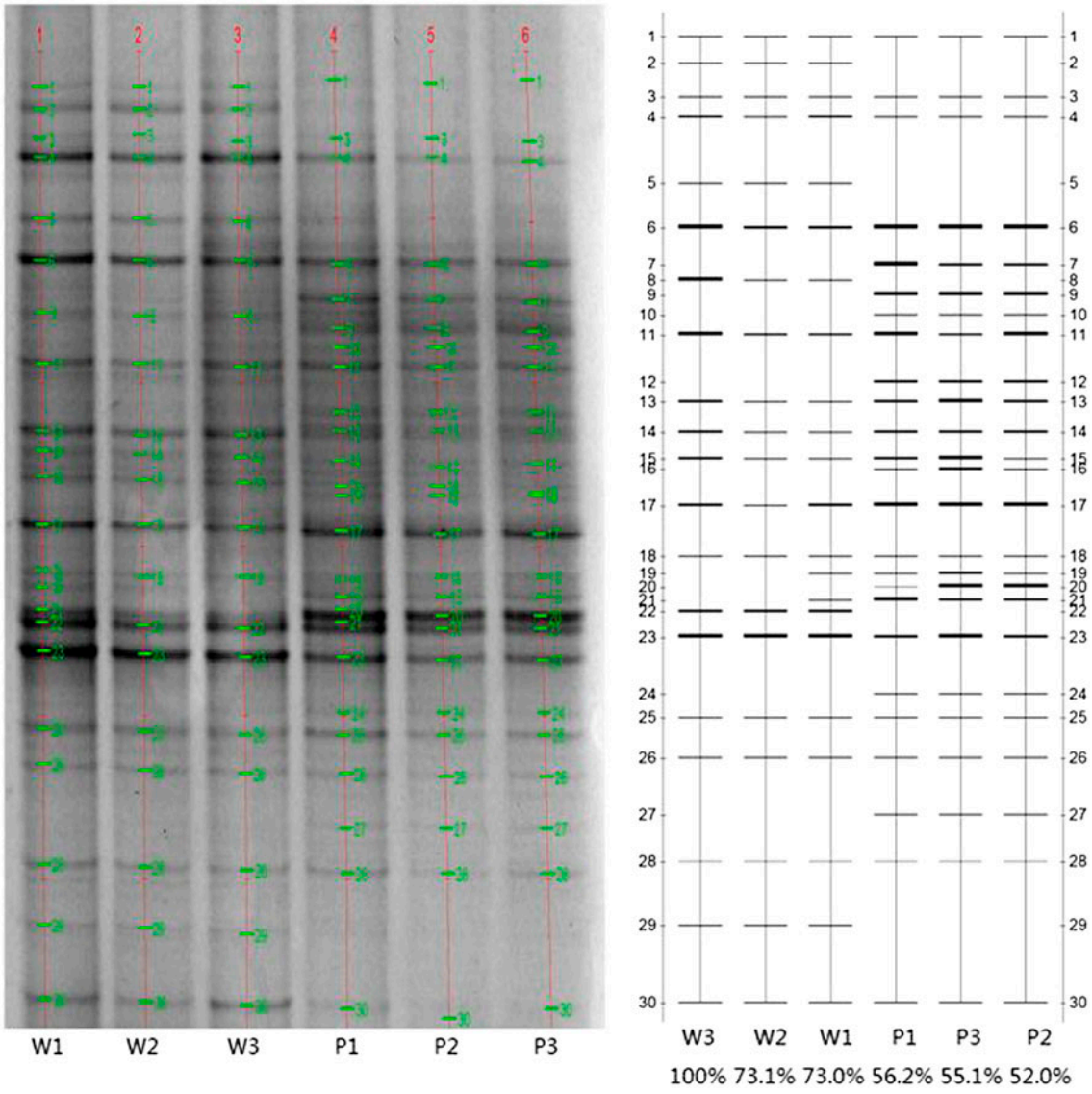

Fig. 8. Schematic representation of the DGGE gel obtained for replicate samples submitted to different sampling areas.

Two operating conditions of each AAO process led to different microbial community structures. The most noticeable difference between the WWTP and the pilot reactor was that some microbial populations were changed after 105-d operation. Some new microorganisms appeared which were favorable for improving the systematic stability and adaptability to confront environmental changes.

Group W and group P owned a highly similar bacterial species owing to the sludge recycle and the internal recycle. However, the quantity of DGGE bands, i.e. the biodiversity in group P was much

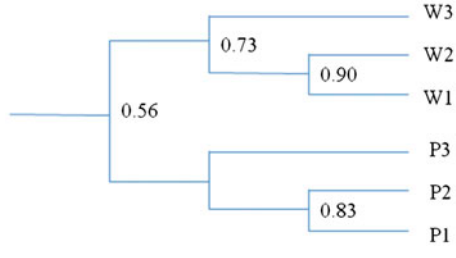

Fig. 9. Clustering analysis of the DGGE patterns.

Table 3
The comparison of the AOB and NOB abundance in pilot reactor and WWTP

	W_1	W_2	W_3	P_1	P_2	P_3
AOB (copies/mg MLSS)	2.03×10^5	6.97×10^5	9.76×10^5	2.46×10^5	3.22×10^6	3.54×10^6
NOB (copies/mg MLSS)	6.53×10^2	3.44×10^3	4.11×10^3	7.86×10^2	4.31×10^3	4.45×10^3

larger comparing with group W, so that the microbial community achieved improvement in the pilot-scale plant, which was advantageous to systematic stability and indicated more diverse potential denitrification pathways comparing to WWTP.

Clustering analysis of the DGGE patterns is shown in Fig. 9. Six samples were divided into two groups. Comparability between ethnic groups was 56%; similarity within groups was 73%. Compared with the oxic areas, the anaerobic and anoxic areas had more similar microbial community structure with 90 and 83% respectively.

Sequencing results showed that the 9-*Brevundiamonas nasdae*, 14-*Pseudomonas denitrificans*, and 20-*Pseudomonas trivialis* were identified as denitrifiers which were predominant in the pilot reactor. The abundances (according to intensity of these bands) of these denitrifying bacteria were comparable in P_2 and P_3, which can be inferred that the oxic area also contained a great amount of denitrifying bacteria. Additionally, some of these denitrifiers, like 14-*Pseudomonas denitrificans* who is characterized by *nirK* gene and mainly performs nitrite reduction [37], ensuring the partial denitrification and agreed with the SND ratio of 66.4% in this study. Especially, 9-*Brevundiamonas nasdae* and 20-*Pseudomonas trivialis* in P_1–P_3 were newly enriched denitrifiers, comparing to W_3, which indicated after long-term hypoxia acclimation, the dominant denitrifier community changed (mainly responsible for the whole microbial community change).

Actually, the existence of internal recycle may lead to comparable community among these sludge samples, but the different living condition (AAO) caused the difference. However, the similarity between P_2 and P_3 cannot be found in W_2 and W_3 (Fig. 8) though they have the same recycle rate, which may be responsible for the better performance in the pilot-scale plant, since P_2 and P_3 together can achieve efficient SND via nitrite with stability.

Table 3 shows the abundance of AOB and NOB based on real-time PCR results for the six samples. Comparing pilot reactor to WWTP, the AOB abundance achieved significant increasing while the NOB abundance changed not that obviously. The highest AOB/NOB ratio was obtained at P_2 and P_3 (Table 2). As is known, the enriching AOB and limiting NOB is the key point for nitrite accumulation and maintaining partial nitrification [11]. Therefore, the high ratio of AOB/NOB in P_2 and P_3 confirmed the present of partial nitrification and SND via nitrite process and the effective denitrification in our pilot reactor, which was due to the suitable DO level controlling.

Partial nitrification and SND via nitrite is a cost-effective way for denitrification, however, it is hard to realize and maintain (parameter controlling). More efforts, rather than this study, should be done to find more key impact factors and reveal the biological mechanisms.

4. Conclusions

SND via nitrite was successfully achieved by the modified pilot-scale AAO reactor under the DO range between 0.4 and 1.6 mg/L. The removal rates of TN, SND, and NAR efficiencies were 67.5, 68.5, and 66.4%, respectively. Based on material balance and the SND efficiency, four forms of biological denitrification pathway were quantified, among of which SND via nitrite made the largest contribution (32.3%). Comparing with the WWTP, the pilot reactor saved 82.6% of oxygen consumption, 30.4% of carbon source and even can increase denitrification rate. Microbial community analysis showed that after long-term hypoxia acclimation, the dominant denitrifier community changed and the abundance ratio of AOB/NOB was increased.

Acknowledgements

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