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Original Research

Analysis of Microbial Community Structure in the Start-up Phase of SNAD Process for Domestic Sewage by PCR-DGGE Technique

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Abstract

In order to reveal the biological diversity and the evolution of microbial community structure in the process of sludge cultivation and acclimation at the start-up stage of simultaneous partial nitrification and denitrification (SNAD) process, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) was used to analyze the microbial community structure in different operating periods. And molecular cloning was used for species identification and phylogenetic analysis. The 16S rDNA sequence analysis of the characteristic bands of the DGGE profile of total bacteria showed that the bacterial populations in the sludge were rich in diversity, and β- proteobacteria were slightly more abundant and maintained a relatively stable dominant position. With the operation of the reactor, the secondary populations were strengthened and became new dominant communities, such as Firmicutes, γ- proteobacteria, Bacteroides and so on. The 16S rDNA sequence analysis of the characteristic bands of the DGGE profile of ammonia-oxidizing bacteria showed that the strains obtained by sequencing basically belonged to *Nitrosomonas* of β-proteobacteria. The fast growth rate of this genus made it the dominant ammonia-oxidizing bacteria (AOB) in the activated sludge systems.

Keywords: SNAD, PCR-DGGE, sludge acclimation, microbial community structure, ammonia-oxidizing bacteria

Introduction

The traditional biological nitrogen removal process for domestic sewage treatment has a large area, high energy consumption, and insufficient additional carbon sources, which not only increases the operation cost but also increases the difficulty of operation and management. In recent years, with the in-depth study of microbiology and nitrogen removal process operation, some new biological nitrogen removal processes with high efficiency and low consumption have emerged [1-2].

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Among them, anaerobic ammonia oxidation technology has attracted extensive attention for its advantages such as small space occupation and low operation cost [3-4]. The CANNON (complete autotrophic ammonium removal over nitrite) process can achieve total nitrogen removal through the mutual cooperation of AOB and anammox bacteria in a single reactor, which is mainly used for the treatment of high ammonia nitrogen wastewater [5-6]. Recent research shows that CANNON process can also be implemented in low ammonia nitrogen wastewater [7]. The high concentration of organics can inhibit the growth of anaerobic ammonia oxidizing bacteria to a certain extent [8], but when the concentrations of organics are low, nitrite-oxidizing bacteria, anaerobic ammonia oxidation bacteria and denitrifying bacteria can coexist in a single reactor. Due to the slow growth rate of AOB and anammox [9], the combined process of granular sludge and SBR is helpful to improve the flow holding capacity of the reactor to microorganisms, thus improving the stability of the system. Therefore, the goal of the present study was to run the SBR reactor for combining the process of nitrification, anaerobic ammonia oxidation and denitrification, and finally formed the SNAD process of simultaneous partial nitrification anammox and denitrification, while achieving the high removal effects of COD and ammonia nitrogen.

In 1979, Fisher and Lerman first proposed an electrophoretic technique for detecting point mutations in DNA fragments-DGGE technique [10-11]. In 1993, Muyzer et al. [12] first applied this technology to the ecological research of microorganisms. In recent years, PCR-DGGE technique has been widely used to analyze the community diversity and dynamics of complex microorganisms, and there have been many reports on the research of microorganisms in wastewater activated sludge [13-14]. PCR-DGGE technology has the following advantages. Firstly, multiple samples can be detected simultaneously, and the samples are repeatable; Secondly, total DNA can be extracted directly from the sample without isolation and culture, which overcomes the limitation of traditional methods requiring culture; Furthermore, PCR-DGGE technology can detect microorganisms that only account for 1% of the total population, with very low limitations. Although PCR-DGG technique has many advantages, there are still some limitations of this technique. For instance, if the conditions for electrophoresis are not suitable, it cannot be guaranteed that the DNA fragments with certain sequence differences will be completely separated, and the phenomenon of different DNA sequences migrating to the same location will occur; the number of bands in DGGE cannot accurately reflect the number of different sequences in the analyzed mixture. In this research, PCR-DGGE technique, combined with the 16S rDNA homology comparative analysis, was used to experimentally study the microbial diversity and bacterial population characteristics in different operation periods during the start-up, cultivation and aclimatization of SNAD process for domestic wastewater, which revealed its succession rules and dominant flora, and provided theoretical support to maintain the stable operation of SNAD process.

Materials and Methods

Test Device and Sample Collection

Test Device

The SBR reactor with cylindrical structure was used in the test. The material was organic glass. Its total volume was 90 L. Its effective volume was 70.3 L. The reactor was 62 cm high and 38 cm in diameter. The aeration discs were set at the bottom of the reactor, with blast aeration and a rotameter to regulate the aeration volume. A stirrer (speed 200 r/min) was installed in the reactor for mixing to enhance mass transfer. The temperature sensor was used to monitor and control the water temperature in the reactor online. The drainage outlet was set 20 cm above the bottom, and the drainage ratio was 67.7%. The pH was maintained between 7.5 and 8.0. The SBR device was shown in Fig. 1.

Sample Collection

The test raw water was domestic sewage, and the main water quality indexes in the test phase were

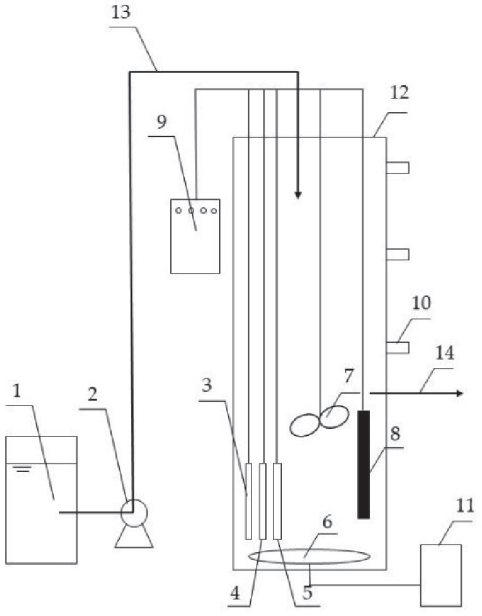

Fig. 1. The schematic diagram of SBR reactor.
1- Water inlet tank; 2- Intake pump; 3- Dissolved oxygen probe; 4- pH Probes; 5- Temperature Probes; 6- Aeration tray; 7- Stirring device; 8- Heating Rod; 9- Control System; 10- Sampling port; 11- Aeration pump; 12- SBR reactor; 13- Inlet pipe; 14- Drainpipe

Table 1. The quality characteristics of domestic sewage (mg/L).

Items	COD_{cr}	NH_4^+-N	NO_2^--N	NO_3^--N	TN	Alkalinity
Scope	200~300	60~80	<1	50~60	100~140	300~400

shown in Table 1. The inoculated sludge was taken from anaerobic ammonia oxidation granular sludge in the lower part of the anaerobic ammonia oxidation fixed bed reactor in the laboratory. The volume of the anaerobic ammonium oxidation fixed bed was 50 L. The water was artificially distributed with an influent NH_4^+-N of 40-50 mg/L and NO_2^--N of 50-60 mg/L. The temperature was 24~26ºC, HRT = 3 h, and the average removal load of total nitrogen was 0.5 kg/ (m^3·d). The first sample was taken from the inoculated sludge on the 1st day. The second sample was taken when nitrosation or ammonification appeared on the 25th day. The third sample was taken when nitrification appeared on the 55th day. After entering the stable period, the sludge matured gradually, and samples were taken approximately once every 12-22 days. The six samples were taken during the operation period, and the sampling numbers were C1-C6 in order.

Text Methods

General Indicators

Carbon and nitrogen compounds in wastewater were determined by the following methods:

NH_4^+-N: Nessler reagent spectrophotometry; NO_2^--N: N-(1-naphthyl)-ethylenediamine spectrophotometry; NO_3^--N: Thymol spectrophotometry; COD_{cr}: Measured according to standard methods issued by the National Environmental Protection Administration of China and the U.S. Environmental Protection Agency. In order to study the change of nitrogen during the period, the sampling analysis along the way was carried out, and TN and TOC were measured by vairo TOC tester.

DNA Extraction

The sludge samples were sedimented in a 1.5 mL centrifuge tube for 10min, and then the supernatant was removed. After high-speed centrifugation at 15,000 rpm for 5min, the supernatant was removed. The 0.3 g of sludge sample was taken for total DNA extraction, and the remaining sludge was frozen for standby. After extraction by Ezup Column Genomic DNA Extraction Kit (Sangon Biotech), 5 μL extract was taken and detected by 1.2% agarose gel. The rest was stored at -20ºC for standby.

PCR Amplification of 16S rDNA

The DNA extracted in the previous step was used as the template, and the primers F341-GC (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGC CCCCGCCCGCCTACGGGAGGCAGCAG -3') and EU500 (5'- GTATTACCGCGGCTGCTGG -3') with specificity for V3 region of 16S rDNA gene of most bacteria were used for PCR amplification. The PCR reaction system (50 μL) included 10×PCR buffer 5 μL, dNTP (each 2.5 $mmolL^{-1}$) 4 μL, F341-GC (20 $\mu molL^{-1}$) 1 μL, EU500 (20 $\mu molL^{-1}$) 1 μL, Taq DNA polymerase (5U) 0.5 μL, DNA template 2 μL, and ddH_20 36.5 μL, and the final volume of the reaction was 50 μL. The PCR adopted the landing amplification procedure, and the specific reaction conditions were as follows: pre-denaturation at 94ºC for 5 min; denaturation at 94ºC for 1min, annealing at 65ºC for 1 min, extension at 72ºC for 1 min, 20 cycles in total, each cycle reduced by 0.5ºC; denaturation at 94ºC for 1 min, annealing at 55ºC for 1min, extension at 72ºC for 1 min, 3 cycles in total; extension at 72ºC for 10 min.

Nested PCR Amplification of Ammonia-Oxidizing Bacteria

The nested PCR technique was used for ammonia-oxidizing bacteria. The first round of PCR used primer pairs specific for ammonia-oxidizing bacteria. The upstream primers were an equimolar mixture of CTO189fA (5'-GGAGAAAGCAGGGGATCG-3'), CTO189fB (5'-GGAGGAAGCAGGGGATCG-3') and CTO189fC (5'-CTAGCYTTGTAGTTTCAAACGC-3'). The downstream primer was CTO654r: 5'-CTAGCYTTGTAGTTTCAAACGC-3', merging base Y (C, T). The PCR reaction system (50 μL) included 10×PCR buffer 5 μL, dNTP (each 2.5 $mmolL^{-1}$) 2 μL, CTO189fA/B/C (20 $\mu molL^{-1}$) 0.5 μL, CTO654r (20 $\mu molL^{-1}$) 0.5 μL, Taq DNA polymerase (5U) 0.5 μL, DNA template 1 μL, and ddH_20 36.5 μL, and the final volume of the reaction was 50 μL. The conditions for PCR were as follows: pre-denaturation at 94ºC for 5 min; each cycle: denaturation at 94ºC for 45 s, annealing at 57ºC for 45s, extension at 72ºC for 90s, 35 cycles in total; after the end of the cycle, then extension at 72ºC for 10 min. In the second round of PCR, the products of the first round of PCR were used as templates, and primers F341-GC and EU500 were used for amplification. The reaction conditions and procedures were the same as section 2.2.3.

DGGE Gel Electrophoresis

The PCR amplification products were separated by Bio Rad DCodeTM DGGE system. The gradient mixing device was used to prepare 8% polyacrylamide gels. The denaturation range of polypropylene gel was 35%-55% (100% of the denaturant was a mixture

of 7 mol·L^{-1} urea and 40% formamide). The concentration of denaturant and acrylamide increased sequentially from the top to the bottom of the gel. After the polypropylene gel was completely solidified, the gel plate was put into an electrophoresis tank with electrophoresis buffer. The 20 μL of PCR samples and 10μL of 6×loading buffer were mixed and added to the upper sample wells, and then electrophoresis was performed in 1×TAE electrophoresis buffer for 8h (60ºC, 130 V). After electrophoresis, the gel was stained with Gel Red nucleic acid gel dye for 30 min and photographed in a gel imaging system (Gel Doc TMXR+, Bio-Rad).

Cloning and Sequencing

The main bands on the gel were cut by a sterilized knife, and they were placed in a 1.5 mL centrifuge tube. The 50 μL sterilized deionized water was added to the tube. Then they were placed at 4ºC overnight to allow the DNA fragments to slowly diffuse out of the gel, which was used as a template for PCR amplification using the F341 and EU500 primers without GC-clamp structure. The amplification procedure was the same as in section 2.2.3. The PCR products were detected and purified by 1.2% agarose gel electrophoresis. The purified DNA fragment was connected to PM18-T Vector (TaKaRa, Japan), and transformed into JM109 competent cells. Then blue and white spots were screened on LB plate containing X-gal, IPTG and ampicillin (Amp). White spots were selected for colony PCR validation, and PCR products with correct fragment size were sent to Shanghai Biotech for sequencing.

Results and Discussion

Analysis of Reactor Operation

The changes in the influent and effluent concentrations and removal rates of NH_4^+-N, NO_3^--N, NO_2^--N and COD during the start-up acclimatization of the SNAD granular sludge process for municipal domestic wastewater are shown in Fig. 2 and Fig. 3 respectively. From 1 to 20 days, the nitrification effect of granular sludge was gradually enhanced. On the 20th day, the effluent ammonia nitrogen concentration decreased to 3 mg/L, the effluent nitrite concentration increased to 58 mg/L, the ammonia nitrogen removal rate increased to 95%, and the nitrite accumulation rate reached 95%. From 21 to 30 days, the nitrification effect of granular sludge remained stable, and the average ammonia nitrogen removal rate and nitrite accumulation rate were 96% and 95% respectively. 31-46d, the nitrogen removal performance of the reactor was gradually enhanced, the effluent ammonia nitrogen was gradually reduced from 45 mg/L to below 10 mg/L, and the total nitrogen removal rate increased from 34% to 74%. From 47 to 60 days, the reactor maintained a high total nitrogen removal capacity. The effluent ammonia nitrogen concentration was below 5 mg/L, and the effluent nitrate nitrogen concentration and nitrous nitrogen concentration remained stable. The average values were 3 mg/L and 4 mg/L respectively. The average total nitrogen removal rate was 85%.

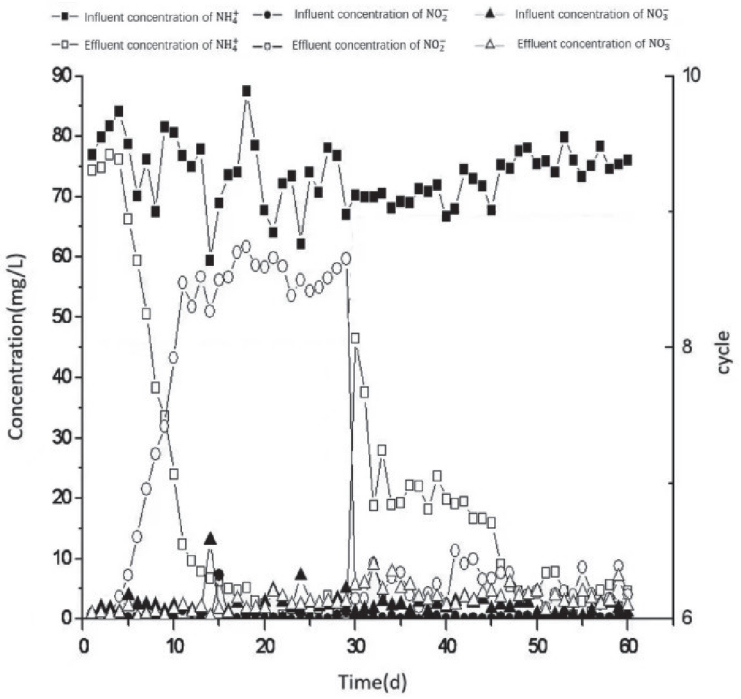

Fig. 2. The nitrogen removal characteristics in SBR reactor.

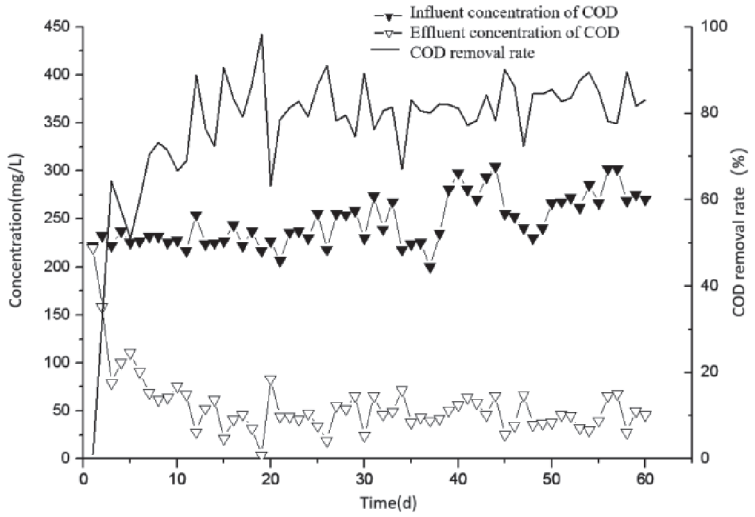

Fig. 3. The COD removal characteristics in SBR reactor.

DGGE Profiling of Total Bacteria

Based on the DGGE profiles of sludge samples C1-C6 during the entire culture acclimatization of SNAD granular sludge, the microorganisms represented by the characteristic bands in Fig. 4 can be broadly classified into four types. The first type of microorganism had always existed and occupied a dominant position in the process of acclimatization, such as the strains represented by band L5, band L10, band L11, band L16 and band L21. Although sometimes the strength of the bands varied sightly, the number of bands were basically stable. It can be concluded that these microorganisms have a strong ability to adapt to changes in the environment and play a large role in the stable operation of the reactor. The second type of microorganism was gradually eliminated with the passage of time, changes in environmental conditions and operating conditions, such as the strains represented by band L19, band L22 and band L24 in Fig. 4. The third type of microorganism represented by band L1, band L2, band L7, band L9, band L14, band L15, band L17 and band L20 basically did not exist at the initial stage of inoculation. While after wastewater acclimatization, especially band L1, band L7, band L14 and band L15 gradually evolved into dominant species under stable operating conditions. These microorganisms cultivated and acclimatization in SBR reactor may be the unique microorganisms in the SNAD system, or these microorganisms are suitable for domestic sewage treatment. The fourth type of microorganism represented by band L3, band L4, band L6, band L8, band L12, band L13, band L18 and band L23 changed unstably during the whole process of cultivation and acclimation, which may not have a great impact on nitrogen removal of the system.

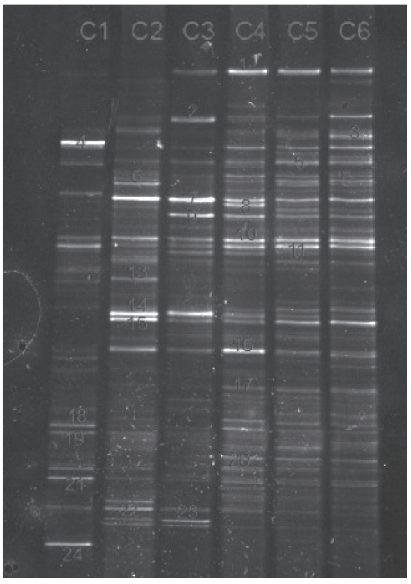

Fig. 4. The DGGE profile of the total bacteria.

Similarity Analysis in DGGE Profiling of Sludge Samples

As shown in Fig. 5, the sequence distribution of the similarity of each lane when sludge sample C6 is used as the standard. As can be seen from the figure, the similarity values between each lane and lane 6 were basically in accordance with the process of culture acclimatization. The closer to lane 6, the better the similarity. The findings indicate that the cultivation and acclimatization of sludge is a gradual and orderly process, and the microorganism gradually evolves into the normal operation state.

According to the analysis results of the similarity coefficient of the total bacterial population of the sludge samples at each stage in Table 2, the similarity coefficient Cs of lane1 and lane 2 was only 12.2%, and the diversity of the main microbial population in the sludge (sample C6) under the stable operation condition after the

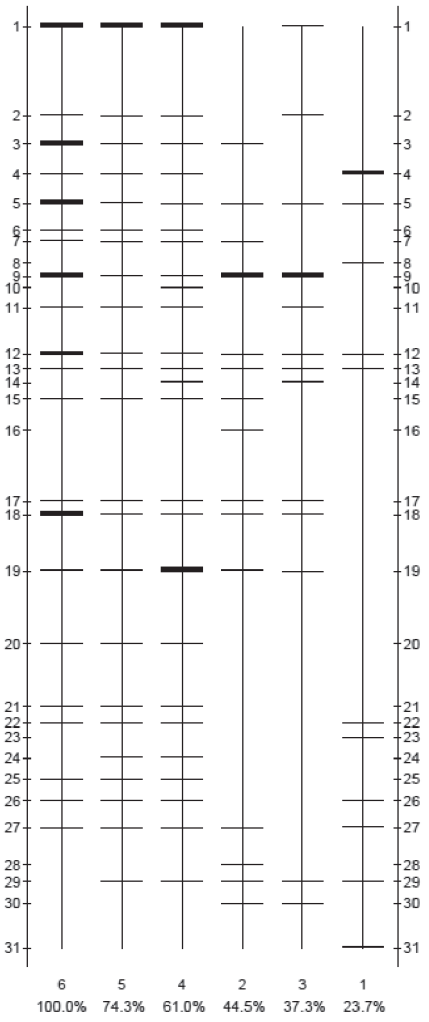

Fig. 5. The similarity index of the total bacteria from C1 to C6.

cultivation and acclimatization of SNAD granular sludge process was only 23.7% compared with that of the sludge C1 just inoculated (Fig. 5). It shows that after anaerobic ammonia oxidation sludge is inoculated into the SBR reactor, the microbial community structure has changed greatly with the cultivation and acclimatization. On the one hand, the environmental conditions and operating conditions of ANAMMOX process and SNAD granular sludge process are quite different. On the other hand, this is also related to the acclimatization of new sewage water quality, and the microbial communities that treat different water quality will certainly be different. After the sewage was acclimatization for a period of time, the microorganisms adapted to the sewage gradually grew up, and the similarity between lane 2 and lane 3 recovered to 57.8%. With the change of environmental conditions, the nitrate bacteria were gradually eliminated by heterotrophic bacteria, which led to the decrease of the similarity between lane 3 and lane 4 (38.7%). The sewage acclimation entered a stable stage for a period of time, the similarity between lane 4 and lane 5 recovered to 73.0%, and the similarity between lane 5 and lane 6 reached 74.3%. The three lanes were both in a stable period and have good similarity.

Similarity Analysis in DGGE Profiling of Sludge Samples

Based on the analysis of DGGE profiles, the representative dominant bands were cut, cloned and sequenced, and the gene sequences obtained from the sequencing were input to the NCBI website. The BLAST program was used to compare and analyze the existing sequences in the database. The main comparison results are shown in Table 3. As shown in Table 3, most of the dominant species in sludge were Uncultured bacterium, Bacteroidetes, β-proteobacteria, γ-proteobacteria, and Firmicutes.

Band L5 had a high homology (99%) with *Pseudomonas* of β-proteobacteria. This bacterium exists widely in nature. It is an important part of water and soil microecosystem, and also an important part of carbon and nitrogen cycle in nature [15]. Band L10 had 97% homology with the Uncultured bate proteobacteria bacterium, belonging to β-proteobacteria. The homology of band L16 with *Simplicispira* sp. YIM219 of β-proteobacteria was 98%. Band L21 had the highest homology with *Aeromonas hydrophila* of γ- proteobacteria. This bacterium is a common parasitic fungus of rot, which is widely distributed in water. Band L11 failed to be sequenced. The first type of microorganisms, namely dominant bacteria, were mostly β-proteobacteria. A large number of studies on microorganisms in sewage treatment systems also show that most nitrifying bacteria and many ordinary

Table 2. The similarities analysis of total bacterial DGGE profiles (Cs).

Sample	C1	C2	C3	C4	C5	C6
C1	100.0	12.2	10.7	30.9	23.8	23.7
C2	12.2	100.0	57.8	34.8	48.2	44.5
C3	10.7	57.8	100.0	38.7	35.9	37.3
C4	30.9	34.8	38.7	100.0	73.0	61.0
C5	23.8	48.2	35.9	73.0	100.0	74.3
C6	23.7	44.5	37.3	61.0	74.3	100.0

Table 3. Similarity sequence of predominant DGGE band.

Band number	Length (bp)	Maximum similarity bacteria in GenBank	Login Number	Similarity (%)	Classification
L1	193	Uncultured Bacteroidetes bacterium	EU283401.1	96	Bacteroidetes
L2	164	Uncultured bacterium	JN397933.1	97	Uncultured bacterium
L3	165	Bacteroidetes bacterium SCGC AAA043-M05	HQ663407.1	97	Bacteroidetes
L4	197	*Nitrosomonas europaea* ATCC 19718	NR_074774.1	99	β-Proteobacteria
L5	170	*Pseudomonas putida* 16S rRNA gene	FJ755909	98	γ-Proteobacteria
L6	173	Uncultured candidate division TM7 bacterium	EU870431.1	95	Firmicutes
L7	173	TM7 phylum sp.	JN713415.1	94	Firmicutes
L9	169	*Acidovorax* sp. Ama-11	HE649223.1	99	β-Proteobacteria
L10	168	Uncultured beta proteobacteria bacterium 16S rRNA	CU926935	97	β-Proteobacteria
L14	198	Enterobacteriaceae bacterium HGH0216	JX520108.1	99	γ-Proteobacteria
L15	178	Uncultured bacterium isolate DGGE gel band WA17 16S rRNA	FJ973478	99	Uncultured bacterium
L16	168	*Simplicispira* sp. YIM219	KJ513864.1	98	β-Proteobacteria
L21	173	*Aeromonas hydrophila*	EF681114	96	γ-Proteobacteria
L22	193	Cytophagaceae bacterium R098	KC252932.1	100	γ-Proteobacteria

heterotrophic bacteria that degrade organic carbon are species in the β-Proteobacteria [16].

Two bands (L19 and L24) in the second type of microorganisms failed to be sequenced. The homology of band L22 and *Cytophagaceae bacterium* R098 of γ-Proteobacteria was up to 100%.

Band L1 had the best homology (96%) with the Uncultured Bacteroides bacterium, which was classified into Bacteroides. Band L2 and band L15 were Uncultured bacterium. Band L7 had a high homology (94%) with TM7 phylum sp. This bacterium is classified into Firmicutes. Hugenholtz et al. [17] had studied TM7 bacteria and confirmed it causes sludge bulking. Chun-Xue Yang et al. confirmed TM7 bacteria had good performance of WAS fermentation under alkaline conditions of certain strength [18]. The homology of band L9 and *Acidovorax* sp. of β- proteobacteria was 99%. *Acidovorax* sp. can use methanol, acetic acid, etc. to provide electron donors for denitrification process [19]. The homology of band L14 and Enterobacteriaceae bacterium HGH0216 of γ- proteobacteria was 99%. Band L17 and band L20 failed to be sequenced. The third type of microorganism was distributed in different classes or genera, with a large evolutionary distance.Band L3 had the best homology (97%) with Bacteroidetes bacteria SCGC AAA043-M05. Band L4 and *Nitrosomonas europaea* of β-proteobacteria had 99% homology. *Nitrosomonas* spp. is mainly *Nitrosomonas europaea* ATCC 19718, an autotrophic ammonia-oxidizing bacterium with the function of converting NH_4^+-N to NO_2^--N, which is the main species of short-range nitrification [20]. Band L6 has a high homology (95%) with the Uncultured candidate division TM7 bacterium. Other bands in the fourth type of microorganisms failed to be sequenced.

DGGE Profiles of Ammonia-Oxidizing Bacteria

Based on the DGGE electropherograms of ammonia-oxidizing bacteria during the entire culture acclimatization of the SNAD process, the species of ammonia-oxidizing bacteria represented by the bands in Fig. 6 could be broadly classified into three types.

Certain genera of ammonia-oxidizing bacteria from the inoculated conventional activated sludge process were present as the top dominant species during the acclimatization of the SNAD granular sludge process culture and remained dominant until the final entry into normal operation, as shown in band L2, band L3, band L4, and band L5 in Fig. 6. It can be clearly known that these species have good adaptability to the new environment. They are in a dominant position both in anaerobic ammonia oxidation sludge and SNAD process, and should play a greater role in the conversion of ammonia nitrogen to nitrite in the reactor. In addition, under different operating conditions, the dominant position of these dominant ammonia-oxidizing bacteria has not changed basically in both the nitrite and nitrification periods, and they can survive well. If these strains can be screened and enriched for large-scale culture acclimatization, it may have a certain effect on improving the removal rate of ammonia nitrogen.

The species represented by band L1 only existed in the inoculated sludge. With the process of cultivation and acclimatization, they were eliminated in the

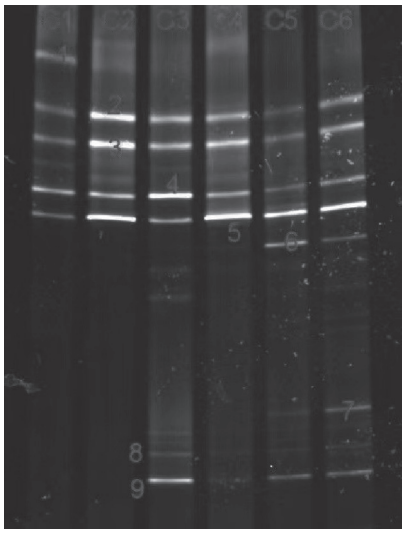

Fig. 6. DGGE profile of the ammonia-oxidizing bacteria.

nitrification stage. It shows that they are not suitable for the new environment and water quality of SNAD.

The ammonia-oxidizing bacteria represented by the band L6, band L7, band L8 and band L9 in the DGGE profile didn't exist during the inoculation period, but gradually emerged through cultivation and acclimatization under the new environment of SNAD process. It shows that the bands of these strains are not as bright as those of the first type of dominant bacteria in the DGGE profile. It may be due to the long generation cycle of ammonia-oxidizing bacteria. It generally takes 30 days or even longer to culture. In addition, these strains were cultivated and acclimatization in the SNAD process environment, which may be representative or unique ammonia-oxidizing bacteria in the SNAD process.

Similarity Analysis in DGGE Profiles of Ammonia-Oxidizing Bacteria

Fig. 7 shows that the sequence distribution of the similarity between each lane and the sludge sample 6 as a standard. According to the distribution of bands and similarity values in the Fig. 7, it can be seen that the community structure of the inoculated anaerobic ammonia oxidation sludge cultured and acclimatization ammonia oxidizing bacteria in the SBR reactor had changed greatly, and the similarity was only 57.4%. It is obvious that there should be a big difference between the ammonia-oxidizing bacteria in the anaerobic ammonia oxidation process and the ammonia-oxidizing bacteria in the SBR reactor process. Some ammonia-oxidizing bacteria suitable for the anaerobic ammonia oxidation process are not suitable for the SBR reactor environment. However, another possibility is that the elimination of some ammonia-oxidizing bacteria is due to acclimatization in the new sewage quality.

Table 4 shows the analysis results of the similarity coefficient of the ammonia-oxidizing bacteria

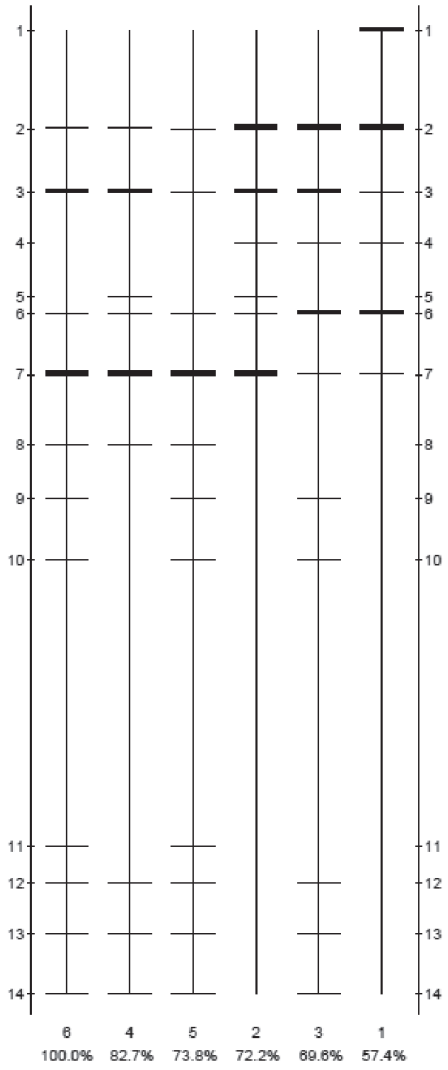

Fig. 7. The similarity index of the ammonia-oxidizing bacteria from C1 to C6.

populations in sludge samples at each stage. The similarity coefficient (Cs) of sample 1 and sample 2 was only 63.3%, which was similar to the situation of the total bacteria. After the anaerobic ammonia oxidation sludge was inoculated to the SBR reactor, the ammonia-oxidizing bacteria population had changed greatly. The similarity coefficients (Cs) between sample 1 and sample 2, sample 2 and sample 3, sample 3 and sample 4, sample 4 and sample 5 were only more than 60%. It indicates that the population of ammonia-oxidizing bacteria will change greatly after a period of cultivation and acclimatization, and then gradually recover. The similarity of sample 5 and sample 6 reached 73.8%, and they basically entered a stable state.

Recovery and Sequencing of Characteristic Bands of Ammonia-Oxidizing Bacteria

The PCR products of DNA recovered by gum-cutting from the bands of DGGE profiles were sent to Shanghai

Table 4. The similarity analysis of ammonia-oxidizing bacteria DGGE profiles (Cs).

Sample	1	2	3	4	5	6
1	100.0	63.3	77.5	60.0	35.7	57.4
2	63.3	100.0	66.4	87.3	49.7	72.2
3	77.5	66.4	100.0	63.6	43.8	69.6
4	60.0	87.3	63.6	100.0	64.2	82.7
5	35.7	49.7	43.8	64.2	100.0	73.8
6	57.4	72.2	69.6	82.7	73.8	100.0

Table 5. Similarity sequence of predominant DGGE band.

Band number	Length (bp)	Maximum similarity bacteria in GenBank	Login Number	Similarity (%)	Classification
L1	169	*Nitrosomonas* sp. JL21	AB000700.1	93	β-Proteobacteria
L2	169	*Nitrosomonas europaea* ATCC 19718	NR_074774.1	98	β-Proteobacteria
L3	169	*Nitrosomonas* sp. HP8	HF678378.1	98	β-Proteobacteria
L4	167	Uncultured *Nitrosomonas* sp.	GQ245688.1	98	β-Proteobacteria
L5	169	*Nitrosomonas eutro*pH*a*	JX545090.1	98	β-Proteobacteria
L6	176	*Nitrosomonas europaea*	NR_117649.1	96	β-Proteobacteria
L7	170	*Azoarcus* sp.	X85434.1	94	β-Proteobacteria
L8	174	*Dechloromonas agitata*	KM262801.1	93	β-Proteobacteria
L9	171	Uncultured bacterium	KC991229.1	100	Uncultured bacterium

Biotech for sequencing. All nine DNA samples were sequenced successfully. The gene sequences obtained from the sequencing were input to the NCBI website and compared with the existing sequences in the database using BLAST program. The main comparison results are shown in Table 5.

As shown in Table 5, the similarity between band L1 and *Nitrosomonas* sp. JL21 (AB000700.1) was 93%. The research showed that *Nitrosomonas* sp. JL21 is the dominant ammonia oxidizing bacteria in activated sludge [21]. The similarity between band L2 and *Nitrosomonas europaea* ATCC 19718 (NR_074774.1) was 98%. *Nitrosomonas europaea* ATCC 19718 is a chemoautotrophic gram-negative bacterium, and it obtains energy and reducing agent needed for its growth from ammonia oxidation to nitrite [22]. The similarity between band L3 and *Nitrosomonas* sp. HP8 (HF678378.1) was 98%. This bacterium is AOB found in Para grass root-based soils growing under normal saline conditions. The similarity between band L4 and Uncultured *Nitrosomonas* sp. (GQ245,688.1) was 98%. This bacterium is an ammonia-oxidizing bacteria found in a large landfill leachate treatment plant. The similarity between band L7 and *Azoarcus* sp. (X85434.1) was 94%. This bacterium is a nitrogen-fixing bacterium that degrades toluene [23]. It doesn't belong to AOB, and may be the test error caused by improper operation. The homology between band L9 and Uncultured bacteria was as high as 100%, which needed further identification. The strains obtained by sequencing basically belong to *Nitrosomonas* of β-Proteobacteria, and *Nitrosomonas* as the dominant bacteria group in the shortcut nitrification system has been reported for many times [24-25].

In natural habitats, AOB sequences acquired in marine systems belong to the γ subclass, while those in non-marine systems are basically listed in the β subclass [26]. In the study of freshwater lake sediments, *Nitrosopira* was found to be the dominant flora and the species distribution of AOB was correlated with sediment depth [27]. AOB, which was the dominant position in the soil system, was also shown to belong to *Nitrosospira*, especially in alkaline and neutral soils [28]. In brackish water environment, attached AOB were dominated by *Nitrosomonas* of β subclass, while planktonic AOB were dominated by *Nitrosospira* of β subclass [29]. In artificial habitats, *Nitrosomonas* and *Nitrosospira* were the maximum specific growth coefficients values for the microorganisms the dominant bacterial groups in wastewater treatment systems [24]. In the SNAD granular sludge process, the dominant AOB belong to *Nitrosomonas*. Compared with *Nitrosospira*, *Nitrosomonas* is more likely to become the dominant AOB in activated sludge system for sewage treatment.

Nitrosomonas often appears in the wastewater biological denitrification system, and they can survive in the low dissolved oxygen environment. Wang et al. [30] found that the major AOB in the encapsulated filler was *Nitrosomonas* in the CANON process for domestic wastewater with encapsulated anaerobic ammonia oxidation granular sludge. Yang et al. found that *Nitrosomonas* was the dominant AOB in Bali WWTP, *Nitrosomonas* was also the dominant AOB in the anaerobic tank of Linkou WWTP [31]. The above researches show that AOB will play a positive role in maintaining the stability of anaerobic ammonia oxidation process. AOB can consume trace dissolved oxygen in the influent to create an anaerobic microenvironment for ANAMMOX bacteria, which is conducive to its enrichment on the particles, so as to exert the effect of anaerobic ammonia oxidation denitrification.

It is worth noting that the bands appearing in the DGGE profile of AOB do not appear in the DGGE profile of total bacteria. The reason may be the AOB functional gene (a*moA*) is selected for PCR-DGGE, and the length of PCR amplified fragments is longer and contain more information. In addition, DGGE analysis of functional genes has a higher resolution of population genetic differences [32].

Conclusions

(1) In the SBR reactor, when anammox granular sludge was inoculated, SNAD granular sludge was successfully cultivated under low aeration rate. The average total nitrogen removal rate was 85% in stable period.

(2) The total bacterial DGGE profiles and sequencing results showed that the dominant bacteria were mainly β-proteobacteria, γ-proteobacteria, Firmicutes, Bacteroidetes and Uncultured bacteria. Microorganisms were widely distributed, and the main dominant species were distributed in different class. Among the detected species, β-Proteobacteria were slightly more and maintained a more stable dominant position. While with the operation of the reactor, secondary populations, such as Firmicutes, γ- Proteobacteria and Bacteroidetes, were strengthened and became the new dominant communities. It provides a certain theoretical basis for improving the acclimatization efficiency of SNAD granular sludge culture and optimizing the operation parameters.

(3) The 16S rDNA sequences of nine ammonia-oxidizing bacteria were obtained by sequencing. The obtained species basically belonged to the *Nitrosomonas* of β-Proteobacteria. *Nitrosomonas* has a fast growth rate, making it the dominant AOB in activated sludge systems.

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Conflict of Interest

The authors declare no conflict of interest.

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